Characterization of Cucurbit Yellow Stunting Disorder Virus, a *Bemisia tabaci*-Transmitted Closterovirus

Ana Célix, Anabel López-Sesé, Nuria Almarza, María Luisa Gómez-Guillamón, and Emilio Rodríguez-Cerezo

First, third, and fifth authors: Centro Nacional de Biotecnología-CSIC, Cantoblanco, 28049 Madrid, Spain; and second and fourth authors: Estación Experimental "La Mayora"-CSIC, 29750 Algarrobo-Málaga, Spain.
We thank CICYT for their support to E. Rodríguez-Cerezo (grant BIO93 1180) and to M. L. Gómez-Guillamón (grant AGF92 1064).
Accepted for publication 3 September 1996.

ABSTRACT

Célix, A., López-Sesé, A., Almarza, N., Gómez-Guillamón, M. L., and Rodríguez-Cerezo, E. 1996. Characterization of cucurbit yellow stunting disorder virus, a *Bemisia tabaci*-transmitted closterovirus. Phytopathology 86:1370-1376.

Symptoms of interveinal chlorosis (yellowing) are commonly observed in melon or cucumber plants grown in greenhouses in the southeastern coast of Spain. The agent of the disease was determined to be cucurbit yellow stunting disorder virus (CYSDV). CYSDV was shown to be transmitted specifically by the tobacco whitefly (*Bemisia tabaci*), was retained by the vector for at least 7 days, and had an experimental host range restricted to members of the family Cucurbitaceae. Filamentous, flexuous virus particles typical of the closteroviruses were observed in infected plants. The length distribution of the virus particles showed two peaks at 825 to 850 nm and 875 to 900 nm. Analysis of double-stranded (ds) RNA extracts revealed two major dsRNA species of approximately 8 and 9 kbp. Random cDNA cloning of viral dsRNA was performed, and a virus-specific cDNA clone (p410) of 557 nucleotides that hybridized with the smaller of the two viral dsRNA species was identified. Computer-assisted analysis showed that the deduced amino acid sequence of p410 was significantly similar to the HSP70 homologs of the closteroviruses and showed greater similarity to the HSP70 homolog of the *B. tabaci*-transmitted lettuce infectious yellows closterovirus (LIYV) than to the HSP70 homologs of the aphid-transmitted closteroviruses. The data suggest that CYSDV is a member of a newly recognized subgroup of closteroviruses with bipartite genomes exemplified by LIYV.

Yellowing diseases of greenhouse- and open field-grown cucurbits caused by whitefly-transmitted clostero- and clostero-like viruses are economically important in many areas of the world. Since the late 1970s, yellowing diseases caused by viruses transmitted by the greenhouse whitefly (*Trialeurodes vaporariorum* (Westwood)) have been reported to occur in greenhouse-grown cucurbits worldwide, including southeastern Spain (13,18,21,24, 27). These viruses have received different names, but they have not been fully characterized and, therefore, their relationships have yet not been determined. However, they all share biological properties with beet pseudo-yellows virus (BPYV) (7), a virus with a virion morphology (17) typical of the closteroviruses. Furthermore, a virus from southern France termed cucumber chlorotic spot virus has been partially characterized, and its genetic organization appears to be similar to that of the closteroviruses (26). Recently, a number of virus isolates from different countries, as well as the original BPYV, were shown to cross-hybridize (5).

A second whitefly-transmitted closterovirus, lettuce infectious yellows virus (LIYV), was first reported in 1981 causing yellowing in open field-grown cucurbits in North America (3,9) and, so far, has not been reported in other geographical areas. In contrast to BPYV, LIYV is transmitted specifically by the tobacco whitefly (*Bemisia tabaci* (Gennadius)) and is able to infect many crop plants apart from cucurbits. The genetic organization and complete nucleotide sequence of the LIYV genome have been reported (15,16). LIYV has a bipartite RNA genome and has been proposed as the type member of a new subgroup of closteroviruses having two genomic RNAs (6).

In Spain, the out-of-season cultivation of melon (*Cucumis melo* L.) and cucumber (*Cucumis sativus* L.) is of great economic importance, reaching 16,000 ha of plastic greenhouses throughout the southeastern coast. These greenhouse-grown crops have been seriously affected since 1982 by a yellowing virus disease associated with the *T. vaporariorum*-transmitted BPYV-like virus (13, 21). Since the early 1990s, *B. tabaci* has progressively displaced *T. vaporariorum* as the prevalent whitefly found in the greenhouses of southeastern Spain. However, yellowing symptoms in melon and cucumber crops continue to be widespread, even though in many of the greenhouses only *B. tabaci* is found (A. Célix, A. López-Sesé, N. Almarza, M. L. Gómez-Guillamón, and E. Rodríguez-Cerezo, *unpublished data*). The symptoms observed in plants grown in *B. tabaci*-infested greenhouses are indistinguishable from those induced by the BPYV-like virus, i.e., at the early stages, plants show chlorotic leaf spots that coalesce until the complete yellowing of the leaf lamina, where only veins remain green.

This paper reports that the new yellowing disease of melon and cucumber reported in Spain is caused by a closterovirus transmitted specifically by *B. tabaci* and not by *T. vaporariorum*. We describe the transmission parameters and host range of the virus, as well as the virion particle morphology and double-stranded (ds) RNA species found in infected tissue. Based on the above studies and on partial nucleotide sequence analysis, this virus appears to be a member of a subgroup of closteroviruses with bipartite RNA genomes. We also describe a reverse transcription-polymerase chain reaction (RT-PCR) assay for the presence of the virus. Based on the positive results obtained with the RT-PCR assay (see below) on samples of plants affected by the formerly described "yellow stunting disorder" of cucurbits (8,12), we use the name

Corresponding author: E. Rodríguez-Cerezo
E-mail address: patologia@samba.cnb.uam.es

Publication no. P-1996-1016-01R

"cucurbit yellow stunting disorder virus" (CYSDV) to refer to this whitefly-transmitted closterovirus.

MATERIALS AND METHODS

Biological materials. The CYSDV isolate used in this study was originally obtained from a melon plant collected near Málaga, Spain, in 1992 and maintained in melon (cv. Piel de Sapo) plants by transfers with the vector *B. tabaci*. A Spanish isolate of a BPYV-like *T. vaporariorum*-transmitted closterovirus (21) was maintained in melon (cv. Piel de Sapo) plants by vector transmission. Nonviruliferous *T. vaporariorum* and *B. tabaci* colonies originally obtained near Málaga, Spain, were maintained on melon plants.

Virus transmission and virus-vector relationships. Mechanical transmission of CYSDV from diseased to healthy melon or cucumber plants was tested by standard techniques using 0.1 M phosphate buffers (pH 7.0), with or without Na-diethyldithiocarbamate, and Carborundum as abrasive.

Transmission tests with whiteflies were done by the leaf cage method using melon (cv. Piel de Sapo) plants. In experiments to determine transmission by different whitefly species (*B. tabaci* or *T. vaporariorum*), nonviruliferous whiteflies were given a 48-h acquisition period on symptomatic leaves and then placed in groups of 40 insects on each of 20 healthy plants at the two-leaf stage for a 72-h inoculation period. The experiment was replicated twice. Plants were observed in all experiments for symptom appearance during a 60-day period.

To determine the transmission efficiency of CYSDV by *B. tabaci*, whiteflies were given an acquisition period of 48 h and placed for 72 h in groups of 1, 5, 10, 20, 30, 40, or 60 insects on each of 10 melon plants. Three replications of the experiment were performed. The acquisition threshold was studied by placing *B. tabaci* (12 groups of 60 whiteflies per time point studied) on symptomatic leaves and transferring them after 2, 6, 18, 24, 48, or 54 h to each of 12 healthy plants for a 72-h inoculation period, with three replications. To determine the inoculation threshold, whiteflies were allowed a 48-h acquisition and then placed in groups of 60 for 2, 6, 12, 24, 48, or 72 h on healthy plants. Three experiments with 12 plants per treatment were done. The persistence of CYSDV in *B. tabaci* was studied by transferring insects (60 per plant) after a 48-h acquisition period to healthy plants and performing subsequent daily transfers (up to seven) to healthy plants. Three replications of the experiment were performed with 12 plants per transfer.

Host range studies. The experimental host range of CYSDV was studied by placing groups of 60 *B. tabaci* after a 48-h acquisition period on 10 seedlings of each plant species tested, where they were allowed a 72-h inoculation period. Plants were scored for symptom appearance for 60 days, and each plant was individually tested for the presence of CYSDV by back-inoculation with *B. tabaci* to melon (cv. Piel de Sapo) plants. Three replications of each experiment were done.

Purification of virus particles and electron microscopy. CYSDV virions were purified from infected leaves of melon and cucumber plants by three different methods described for LIYV (3,9,15). Twenty microliters of the preparations were floated onto copper grids, negative-stained with 2% phosphotungstic acid (pH 7.0), and observed by transmission electron microscopy to ascertain the presence of viral particles. Vein dips were done by hand-sectioning infected tissue along a vein and dipping the section on a drop of 2% phosphotungstic acid, pH 7.0.

Extraction and analysis of dsRNA. dsRNAs were extracted from 8 g of CYSDV-infected melon or cucumber leaves as described (23) without doing a second step of CF-11 cellulose purification. The dsRNAs were concentrated by ethanol precipitation, dissolved in H_2O, and analyzed by electrophoresis in nondenaturing 0.7% agarose gels in Tris-acetate-EDTA (TAE; 40 mM Tris-HCl [pH 7.9], 2.5 mM sodium acetate, and 0.5 mM EDTA). The ds nature of the RNAs was confirmed by digestion with ribonuclease A (10 μg/ml) in 0.2 M NaCl or in H_2O.

Synthesis and cloning of cDNA and sequence analysis. First-strand cDNA was obtained using as a template about 500 ng of CYSDV dsRNA as described (4). Second-strand cDNA was synthesized by the method of Gubler and Hoffman (11), blunt-ended with T4 DNA polymerase, purified in a Centricon-100 concentrator (Amicon, Beverly, MA), ligated to *Eco*RV-digested, dephosphorylated pBluescript (Stratagene Inc., La Jolla, CA), and transformed into competent *Escherichia coli* strain DH5α cells by standard methods (20). Recombinant plasmids were digested with *Eco*RI-*Hin*dIII and analyzed by electrophoresis in agarose gels. Clones containing inserts larger than 500 bp were used to prepare probes for Northern blot experiments. Plasmid dsDNA of clone p410, which was found to hybridize with CYSDV dsRNA, was sequenced by the dideoxynucleotide chain termination method using Sequenase (Amersham International, Bucks, United Kingdom). Nucleotide and deduced amino acid sequences were compared and aligned using the BESTFIT program of the Genetics Computer Group (GCG) package (University of Wisconsin, Madison).

Northern blot analysis of CYSDV dsRNA. Northern blot analysis was carried out following electrophoresis of CYSDV dsRNA extracts in 0.7% agarose gels in TAE buffer. The dsRNA was transferred to a nylon membrane (Zeta Probe; Bio-Rad Laboratories, Richmond, CA) in 250 mM NaOH for 1.5 h. The membrane was soaked in 50 mM NaOH and 10 mM NaCl for 5 min, rinsed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min, and exposed to a UV source to cross-link the RNA to the membrane. The ^{32}P-labeled probes were prepared using gel-purified plasmid p410 cDNA insert, released by *Eco*RI-*Hin*dIII digestion, as described (10). Prehybridization, hybridization, and washing (20) were carried out at 68°C, and the blots were exposed at –80°C using an intensifying screen (E. I. du Pont de Nemours, Wilminton, DE).

Total RNA extraction and RT-PCR procedures. Total RNA was extracted from 0.2 g of leaf tissue ground in liquid nitrogen with 2 volumes of 2% sodium dodecyl sulfate (SDS), 100 mM Tris-HCl (pH 8.0), and 10 mM EDTA. After vortexing, samples were extracted with 1 volume of phenol-chloroform, and the aqueous phase was adjusted to 2 M LiCl. After overnight incubation at 4°C, samples were centrifuged at 12,000 × *g* for 15 min, and the pellets were dissolved in sterile H_2O (100 μl). About 2 μg of total RNA (5 to 10 μl) was heated with 100 ng of oligonucleotide 410L (5′-TTGGGCATGTGACAT-3′) at 65°C for 5 min, chilled on ice, and used in 20-μl reactions as the template for first-strand cDNA synthesis with reverse transcriptase (Seikagaku America, Rockville, MD) following standard protocols (20). Aliquots (5 μl) of the RT reaction were used in 25-μl PCR reactions with 100 ng each of primers 410L and 410U (5′-AGAGACG-GTAAGTAT-3′). The PCR reactions consisted of 40 cycles each of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The amplified products were analyzed in 1.2% agarose gels in TAE buffer, and Southern blot hybridization analysis was performed as described (20) using p410 specific 32P-labeled probes synthesized as described above.

RESULTS

Transmission of CYSDV. The two whitefly species found in the greenhouses of southeastern Spain were tested. CYSDV could not be transmitted from melon to melon plants by *T. vaporariorum* (0 out of 40 plants infected), but was readily transmitted by *B. tabaci* (27 out of 40 plants infected). Attempts to transmit CYSDV mechanically were unsuccessful. The efficiency of transmission of CYSDV by *B. tabaci* was studied, and the data obtained are shown in Table 1. The probability of a single whitefly

transmitting CYSDV can be estimated from these data (22) and gives a value of 0.032 ± 0.013. Long acquisition and inoculation periods were required by *B. tabaci* to transmit CYSDV efficiently (Tables 2 and 3). The ability of *B. tabaci* to serially transmit CYSDV was determined in daily serial transfers on susceptible host (melon) plants. The results (Table 4) show that CYSDV persists in the vector for a long time, at least 7 days. No specific assay was done to determine the existence of a latent period of CYSDV in the vector.

Host range and symptoms. In experimental transmission tests with *B. tabaci*, CYSDV induced in melon (cv. Piel de Sapo) plants initial symptoms of chlorotic spots along the leaf veins between 14 and 20 days postinoculation. Sometimes the initial symptoms also consisted of prominent yellowing of sectors of a leaf. Symptoms evolved to complete yellowing of the leaf lamina (except the veins) and rolling and brittleness of the leaves. Several crop species cultivated in the greenhouse area of southeastern Spain, as well as some weed species, were included in a partial host range study of CYSDV. The results (Table 5) show that CYSDV was able to infect only members of the family Cucurbitaceae, inducing yellowing symptoms similar to those described above for melon infections.

Virion purification and particle morphology. Very flexuous virus particles were consistently found in very low amounts in vein dips from yellowing cucumber and melon plants (data not shown). The procedures described by Brown and Nelson (3), Duffus et al. (9), and Klaassen et al. (15) were used in an attempt to purify the virus particles. Yields were always very low, and we were unable to quantify the preparations. Electron microscopy was used to monitor the virion purification steps, showing that considerable amounts of virus particles were lost in low-speed centrifugation steps (data not shown). This loss was particularly important when polyethylene glycol was used in the purification procedure (3). Also, tubular structures from the host plant (observed in control preparation from healthy melon or cucumber plants) frequently copurified with virus particles.

Upon centrifugation through a sucrose gradient (15), flexuous virus particles showing the cross-banding and open helix pattern characteristic of the closteroviruses were observed (Fig. 1A), and their length distribution was examined (Fig. 1B). The distribution of particle sizes of CYSDV appeared to have two modal lengths at about 825 to 850 nm and 875 to 900 nm (Fig. 2B).

Analysis and cloning of dsRNA. Attempts to extract RNA from CYSDV virion preparations by standard methods did not yield detectable amounts of CYSDV-specific RNA (data not shown). Alternatively, we extracted dsRNAs from healthy and CYSDV-infected plants. Electrophoretic analysis of dsRNAs isolated from CYSDV-infected melon or cucumber plants consistently revealed the presence of two major dsRNAs (dsRNA1 and dsRNA2) (Fig. 2, lane 3) not present in healthy control plants (Fig. 2, lane 2). Similar amounts of contaminating ribosomal RNA were present in dsRNA preparations from healthy or CYSDV-infected samples (Fig. 2, lanes 2 and 3), thus serving as a loading control. The ds nature of dsRNAs 1 and 2 was confirmed by ribonuclease digestion in high salt (data not shown). Based on their electrophoretic mobilities, the approximate sizes of dsRNAs 1 and 2 are between 8 and 9 kbp. The use of CYSDV-infected cucumber plants was preferred to CYSDV-infected melon plants for viral dsRNA extraction, because of the frequent presence of endogenous dsRNA species in commercial melon cultivars (data not shown).

TABLE 1. Efficiency of transmission of cucurbit yellow stunting disorder virus by *Bemisia tabaci*[a]

	Insects per plant						
	1	5	10	20	30	40	60
Transmission rate (%)[b]	3	24	24	44	47	68	85

[a] Acquisition and inoculation periods were fixed (48 and 72 h, respectively). Group tests were performed on 10 melon plants.
[b] All transmission rates are averages of three replications of each experiment.

TABLE 2. Acquisition threshold of transmission of cucurbit yellow stunting disorder virus by *Bemisia tabaci*[a]

	Acquisition access (h)				
	2	6	18	24	48
Transmission rate (%)[b]	3	22	80	89	85

[a] Inoculation period was fixed (72 h). Twelve melon plants (60 whiteflies per plant) were inoculated per time point.
[b] All transmission rates are averages of three replications of each experiment.

TABLE 3. Inoculation threshold of transmission of cucurbit yellow stunting disorder virus by *Bemisia tabaci*[a]

	Inoculation access (h)					
	2	6	12	24	48	72
Transmission rate (%)[b]	8	25	67	83	92	97

[a] Acquisition period was fixed (48 h). Twelve melon plants (60 whiteflies per plant) were inoculated per time point.
[b] All transmission rates are averages of three replications of each experiment.

TABLE 4. Persistence of transmission of cucurbit yellow stunting disorder by *Bemisia tabaci*[a]

	Serial transfer (day)						
	1	2	3	4	5	6	7
Transmission rate (%)[b]	89	72	67	61	53	44	39

[a] Acquisition period was fixed (48 h). Twelve melon plants (60 whiteflies per plant) were used for the daily transfers.
[b] All transmission rates are averages of three replications of each experiment.

TABLE 5. Experimental host range of cucurbit yellow stunting disorder virus (CYSDV)[a]

Family *Species*	Symptomatic plants[b]/ inoculated plants
Cucurbitaceae	
Cucumis melo L. cv. Piel de Sapo	30/30
Cucumis sativus L. cv. Bellpuig	30/30
Cucurbita maxima Duchesne	26/30
Citrullus lanatus (Thunb) Matsum. & Nakai cv. Sugar Baby	30/30
Cucurbita pepo L. cv. Negro Belleza	19/20
Solanaceae	
Lycopersicon esculentum Mill. cv. Daniella	0/30
Solanum melongena L. cv. Redonda Negra Lisa	0/30
Capsicum annuum L. cv. Italico	0/30
Nicotiana tabacum L. cv. Xanthi	0/10
Nicotiana benthamiana L.	0/10
Compositae	
Lactuca sativa L. cv. Summer Bibb & Romana	0/30
Sonchus oleraceus L.	0/30
Leguminosae	
Phaseolus vulgaris L.	0/30
Pisum sativum L. cv. Alderman (Australia de Enrame)	0/20
Brassicaceae	
Capsella bursa-pastoris (L.) Medicus	0/30
Malvaceae	
Malva parviflora L.	0/30
Portulacaceae	
Portulaca oleracea L.	0/30
Chenopodiaceae	
Chenopodium album L.	0/30

[a] Each plant was inoculated with 60 *Bemisia tabaci* for 72 h. Insects were previously allowed to feed on CYSDV-infected melon plants for 48 h.
[b] Symptomatic plants were confirmed to be CYSDV-infected by back-inoculation to indicator plants.

The dsRNA preparations from CYSDV-infected plants were digested with ribonuclease A in high salt and used as the template for random-primed synthesis of cDNA and subsequent cloning into plasmid vectors. Only 10 clones with inserts larger than 0.5 kb were obtained, and these were used as probes in CYSDV dsRNA Northern blots. Only one of the clones (p410) was of viral origin. Clone p410 hybridized specifically with CYSDV-dsRNA2 (Fig. 3, lane 2) and not with CYSDV-dsRNA1. Longer exposures (Fig. 3, lane 3) revealed the presence of minor amounts of smaller dsRNAs: one distinct dsRNA was about 5 kbp, and a number of dsRNAs between 2.5 and 5 kbp were also detected.

Nucleotide sequence of p410 and sequence comparisons. The nucleotide sequence of p410 revealed a cDNA insert of 557 nucleotides (Fig. 4A). Computer-assisted translation of the p410 sequence resulted in a deduced amino acid sequence that could be aligned with a region of the protein encoded by the closteroviruses that has significant similarity with cellular HSP70 heat shock proteins (Fig. 4B). The region covered by p410 comprises the highly conserved segments D to G of the HSP70-related family of proteins (1). The closterovirus HSP70 homolog sequence is available for two aphid-transmitted closteroviruses, citrus tristeza virus (CTV) (14) and beet yellows virus (BYV) (2), and for one whitefly-transmitted closterovirus, LIYV (16). The comparison of the partial CYSDV HSP70 homolog amino acid sequence with those of CTV and BYV showed 28% identity (45% similarity when chemically similar amino acids are considered identical), and this value rose to 48% identity (64% similarity) when the comparison was made with the same region of the LIYV HSP70 homolog. Thus, in the region studied, CYSDV was more similar to whitefly-transmitted LIYV than to aphid-transmitted CTV or BYV. However, the level of identity between the two whitefly-transmitted closteroviruses in the region compared indicates that they are different viruses, rather than strains of the same virus.

Detection of CYSDV by RT-PCR in infected tissue. The availability of the p410 (Fig. 4A) nucleotide sequence and comparison of its deduced amino acid sequence with equivalent regions of other closterovirus-encoded HSP70 homologs (Fig. 4B) was used to locate dissimilar regions that could serve to design oligonucleotide primers specific for the detection of CYSDV. Two oligonucleotide primers, 410U and 410L (Fig. 4A, underlined), were designed and used in RT-PCR experiments using total RNA extracts of plants as the template. The results (Fig. 5A, lanes 5 and 6) show that the chosen oligonucleotides produced a PCR product of the expected size (465 bp) from total RNA extracts of CYSDV-infected tissues. The amount of total RNA used was equivalent to about 10 mg of fresh CYSDV-infected tissue. In contrast, no detectable PCR product was produced when the total RNA extracts derived from noninfected plants (Fig. 5A, lanes 2 and 3) or from plants infected with the Spanish BPYV-like virus (Fig. 5A, lane 4) were used as the template.

The specificity of the amplified PCR products was analyzed by Southern blot hybridization with a p410 specific probe. The results (Fig. 5B) show that the PCR product amplified from CYSDV-infected plant extracts was not an artifact and also showed the absence of hybridization for samples from noninfected plants or those infected by the BPYV-like virus.

DISCUSSION

A whitefly-transmitted virus was found to be the causal agent of a yellowing disease of protected crops of melon (*Cucumis melo*) and cucumber (*Cucumis sativus*) in the horticultural area of southeastern Spain. The virus is a member of the closteroviruses, as suggested by the results described here on the mode of transmission, particle morphology, host range, and nucleotide sequence of part of the viral HSP70 homolog gene. This closterovirus can be specifically detected by RT-PCR with the oligonucleotide primers described in Figure 4A. The RT-PCR assay has been applied to samples of plants infected with the virus causing "yellow stunting disorder" of cucurbits, which have reached epidemic proportions in Middle East countries since 1985 (8,12). The results show that a PCR product was detected and its nucleotide sequence was identical to that reported in this paper (B. W. Falk, *personal communication*). Therefore the closterovirus isolated in Spain and the agent causing the "yellow stunting disorder" of cucurbits (8,12) seem to be the same. The name "cucurbit yellow stunting disorder virus" has been adopted (8), and the characteristics of this widespread and important virus are described herein.

CYSDV has flexuous particles typical of the closteroviruses, with a bimodal length of about 825 to 850 nm and 875 to 900 nm (Fig. 1). It is transmitted specifically by the tobacco whitefly (*B.*

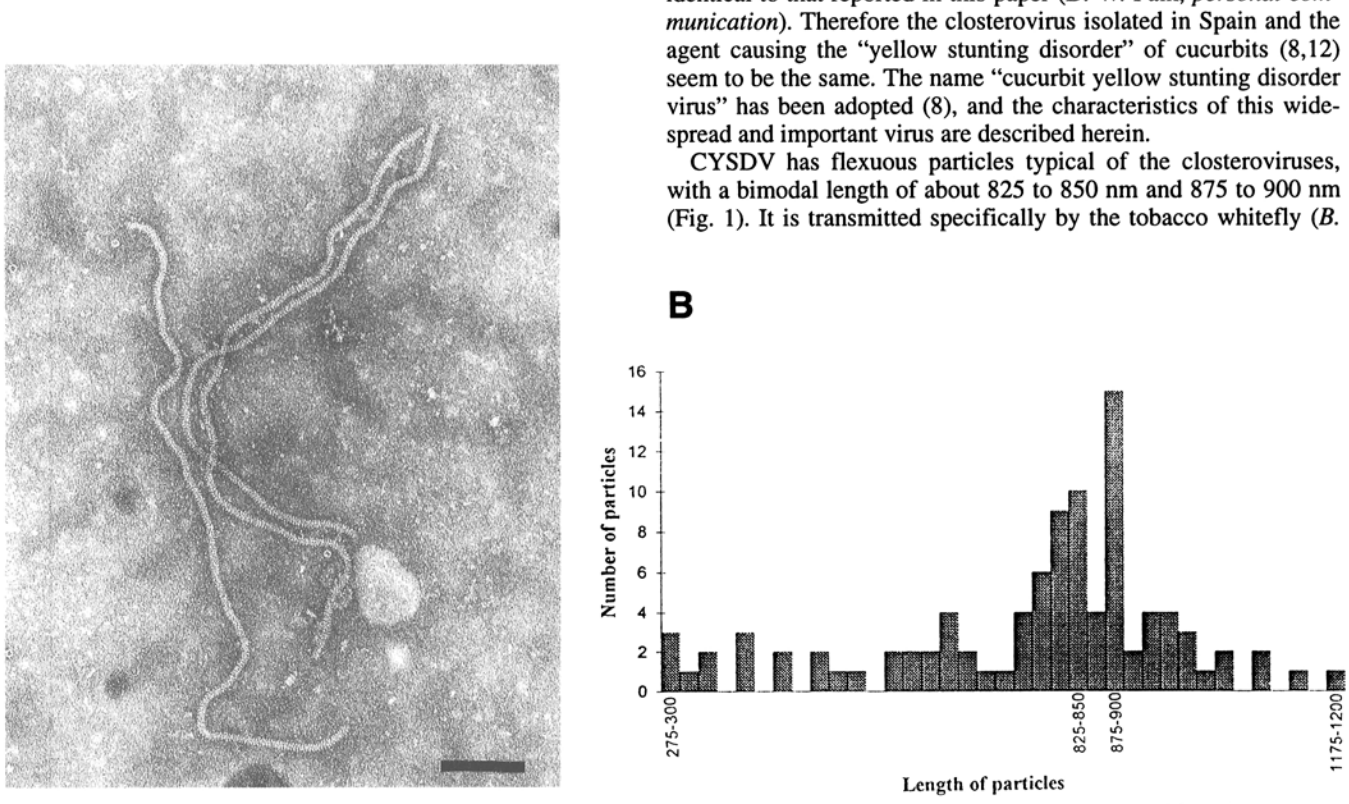

Fig. 1. A, Electron micrograph of purified cucurbit yellow stunting disorder virus (CYSDV) particles stained with 2% phosphotungstic acid (PTA). Bar represents 100 nm. **B,** Distribution of lengths (nm) of CYSDV virus particles purified from melon plants and negatively stained with 2% PTA.

tabaci), and seems to have a narrow experimental host range restricted to the family Cucurbitaceae. CYSDV can be retained at least 7 days by the whitefly vector and can be transmitted by Spanish populations of *B. tabaci* of both B (*B. argentifolii*) and non-B biotypes (A. Célix, F. Beitia, J. L. Cenis, and E. Rodríguez-Cerezo, *unpublished data*). Although watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) and zucchini squash (*Cucurbita pepo* L.) are cultivated in the greenhouse area of southeastern Spain and have been shown to be experimental hosts for CYSDV (Tables 1–4), we have not, so far, observed yellowing symptoms or isolated CYSDV from these cucurbits under natural growing conditions.

The isolation of CYSDV in Spain parallels the spread of its vector, *B. tabaci*, in the greenhouses of the southeastern coast. The displacement of *T. vaporariorum* by *B. tabaci* as the whitefly most commonly found in greenhouses in southeastern Spain began in the late 1980s, probably because of a combination of a lack of effectiveness against *B. tabaci* of the methods used to control *T. vaporariorum* and the arrival to the area of populations of *B. tabaci* more aggressive than the preexisting Spanish populations. CYSDV induces symptoms in melon and cucumber that cannot be easily differentiated from those caused by the BPYV-like closterovirus that has been detected in the area since 1982 (13,21). However, the two cucurbit-yellowing viruses found in Spain differ in the specificity of whitefly transmission (*B. tabaci* or *T. vaporariorum*) and in the transmission parameters. CYSDV seems to be less efficiently transmitted by *B. tabaci* than the BPYV-like virus is by *T. vaporariorum*. The probability of a single whitefly transmitting CYSDV estimated (22) from data presented in Table 1 is 0.032 ± 0.013, while the same calculation applied to the BPYV-like virus (21) gives a probability of 0.087 ± 0.039. Acquisition and inoculation thresholds, as well as persistence in the vector, are longer for CYSDV (Tables 2–4) than for the BPYV-like virus (21). The viruses also differ in their host ranges; that of BPYV (21) appears to be wider than for CYSDV. Oligonucleotide primers designed to amplify CYSDV genetic material from infected tissue by RT-PCR failed to amplify a product with samples infected with the Spanish BPYV-like virus, suggesting genetic differences between CYSDV and BPYV. Therefore, CYSDV and BPYV seem to be different viruses causing a very similar disease.

The yellowing disease of cucurbits caused by CYSDV in Spain and in the Middle East countries is also similar to that caused by the *B. tabaci*-transmitted closterovirus LIYV in the United States (9). Our results show that CYSDV is related to LIYV, but should be considered a different virus. The comparative analysis of the partial (557 nucleotides [nt]) nucleotide sequence obtained from CYSDV (Fig. 4) shows higher identity at the amino acid level (48%) to LIYV than to the aphid-transmitted closteroviruses (28%), indicating that CYSDV and LIYV are two related, but different, *B. tabaci*-transmitted closteroviruses. Also, the host range of LIYV (9) is much wider than that of CYSDV and includes hosts in at least 14 botanical families apart from the family Cucurbitaceae. For the species tested for both viruses, LIYV could infect plants in the genera *Lactuca*, *Sonchus*, *Capsella*, and *Malva* (9), while CYSDV could not (Table 5). Even taking into account the inherent variations encountered in studies performed with insect vectors, the transmission properties of LIYV and CYSDV seem to be different: CYSDV (Tables 1–4) has longer acquisition and inoculation thresholds and is retained longer by the vector than LIYV (9). Based on data reported by Duffus et al. (9), the probability of a single *B. tabaci* whitefly transmitting LIYV is 0.152 ± 0.049, higher than the value estimated for CYSDV (0.032 ± 0.013). This could also be because of the *B. tabaci* population used in our study being less efficient as a vector than the *B. tabaci* populations used in other studies.

A property that may be shared by CYSDV and LIYV is the possession of a bipartite RNA genome. The genome of LIYV consists of two single-stranded RNA molecules of 8,118 nt and 7,193 nt (15,16). Based on the dsRNA pattern obtained from CYSDV-infected plants, the genome of CYSDV may also consist of two RNA molecules of a size similar to those of LIYV. This pattern

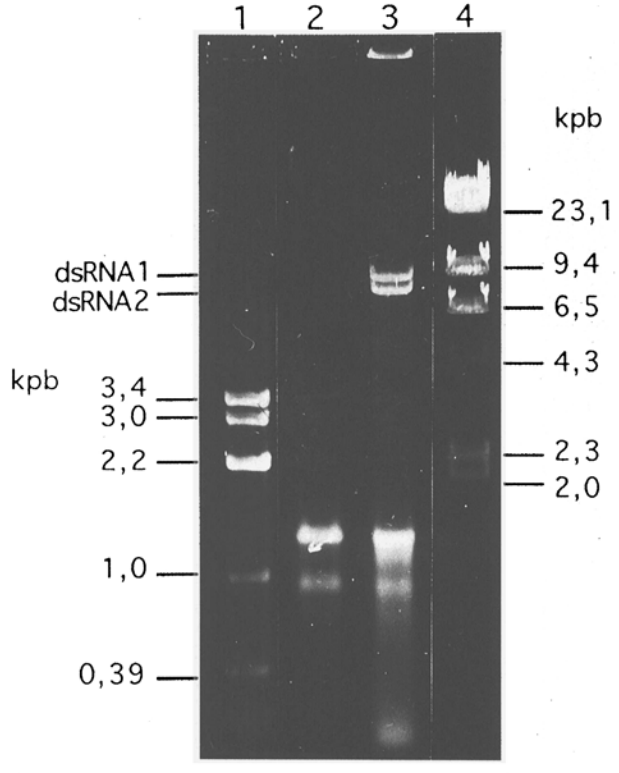

Fig. 2. Agarose gel electrophoresis of double-stranded (ds) RNA preparations from uninfected or cucurbit yellow stunting disorder virus (CYSDV)-infected cucumber plants. Molecular markers are dsRNAs from cucumber mosaic cucumovirus plus satellite RNA-infected tobacco plants (lane 1) and *Hin*dIII-digested dsDNA of phage lambda (lane 4). Lane 2, dsRNA extract from 8 g of uninfected cucumber plant. Lane 3, dsRNA extract from 8 g of CYSDV-infected cucumber plant. Two major dsRNAs specific of CYSDV-infected plants (dsRNA1 and dsRNA2) are observed in lane 3 and absent in lane 2. Ribosomal RNA is present contaminating the dsRNA preparations loaded in lanes 2 and 3 and serves as a loading control. Numbers indicate sizes (kbp) of molecular markers.

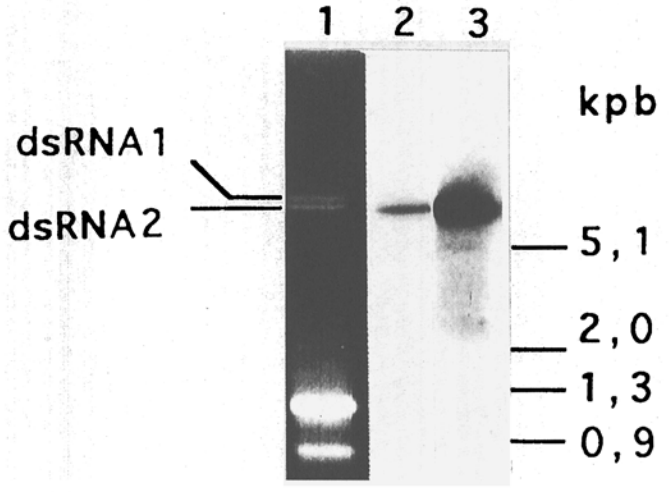

Fig. 3. Lane 1, Agarose gel electrophoresis of double-stranded (ds) RNA extracted from cucurbit yellow stunting disorder virus (CYSDV)-infected cucumber plant and Northern blot hybridization with the p410-specific cDNA probe. Autoradiograms after lane 2, 3-days or lane 3, 7-days exposure times. dsDNA molecular weight markers (indicated to the right) correspond to fragments generated by *Eco*RI-*Hin*dIII digestion of phage lambda DNA. The position of CYSDV dsRNAs 1 and 2 is indicated in the dsRNA preparation loaded in lane 1, which also contains contaminating ribosomal RNA.

consists of two major dsRNAs (dsRNA1 and dsRNA2) of about 8 to 9 kbp each. It appears that CYSDV-dsRNA2 is not a ds form of a subgenomic RNA as suggested by the fact that clone p410 hybridizes specifically with the dsRNA2 and not with dsRNA1. This result is consistent with the hypothesis of CYSDV and LIYV having a similar genomic organization: in LIYV the HSP70 homolog gene is located in the smaller (RNA2) of the two genomic RNAs (15,16). The finding of a bimodal distribution of lengths among CYSDV particles (Fig. 1) would also be consistent with the hypothesis of CYSDV having a bipartite genome in which each RNA is encapsidated separately. The modal length initially reported for LIYV virions (1,800 to 2,000 nm) (9) differs widely from the results reported here for CYSDV, but recent reexamination of LIYV purified virion preparations have shown results similar to those reported here for CYSDV (25). The name *Biclovirus* (for *bi*partite *clo*sterovirus) has been proposed for a new genus exemplified by LIYV that would belong to the family Closteroviridae (6). Our data suggest that CYSDV could also be a member of the proposed *Biclovirus* genus. Two whitefly-transmitted clostero-like viruses of tomato (25) and sweet potato (19) have recently been shown to have bipartite genomes, suggesting that they also could belong to the proposed *Biclovirus* genus and further support the transmission by whiteflies as a characteristic of this new group of plant viruses.

The fact that CYSDV and BPYV are found in the same greenhouse-growing area causing diseases of almost identical symptomatology raises the question of the relative incidence of each of these closteroviruses in southeastern Spain. This aspect is important for controlling the yellowing diseases as the resistance genes found in melon germ plasm for CYSDV do not confer resistance to BPYV (M. L. Guillamón, *unpublished data*). Although *T. vaporariorum* has been displaced in many greenhouses by *B. tabaci*, it has not disappeared from the area, and it may be found coexisting in certain greenhouses with *B. tabaci* (A. Célix, A. López-Sesé, N. Almarza, M. L. Gómez-Guillamón, and E. Rodríguez-Cerezo, *unpublished data*). The RT-PCR assay described in this work could be used to specifically detect CYSDV in infected leaf samples and, therefore, to study CYSDV incidence. The development of a similar assay for the Spanish BPYV-like virus will allow the study of the frequency of mixed infections and relative incidences of CYSDV and BPYV. The RT-PCR assay described here will also serve to test for the presence of CYSDV in other areas of the world where there is a need to identify the various whitefly-transmitted closteroviruses that cause yellowing diseases in vegetable crops.

A

```
  1 CTCTGACTATTAGAGACGGTAAGTATGTCACAGTGGCTGATACTGAAG
                oligo 410U
 49 GTGATTCGTTTTTGGGAGGTAGAGACATTGATAATGCTATCGGTGACT
 97 ATATAACAACGACGTATGGTATGAAAGGTGGTTTGTCCGCTGACGTAC
145 TGGCGTCTATAAAGGAGGATTGTAATTCTAAGGGTAGAGAGAATTTCA
193 ATGTTATAGATTCATCAGGCAAACTTCATAATGTGAAATTCACAAGAC
241 AAGATCTGAGTCGTTGCATTGAACCTTTCTCTAAGAAGAGCATAGCAC
289 TGCTTGATAATATGGTAGTGCGTAACATAACAAAAGATTCCGTTGTGT
306 TTATGGTTGGAGGTTCATCATTGTTGAAGAAAGTTCAACATGATGTGA
385 TGAATTACTGTGCTAGAACGAAACTAGAATGCATCATTGATAAAGACC
432 TTAGATCAGCAGTGTCATTTGGTTGCTCTATGTCACATGCCCAAGAAG
                                   oligo 410L
481 ACACAAAAAATATGATATACATCGATTGTAATTCACATCCCTTAATGG
529 ACATATCATATTTTTGTTCTCCAAAGTTA
```

B

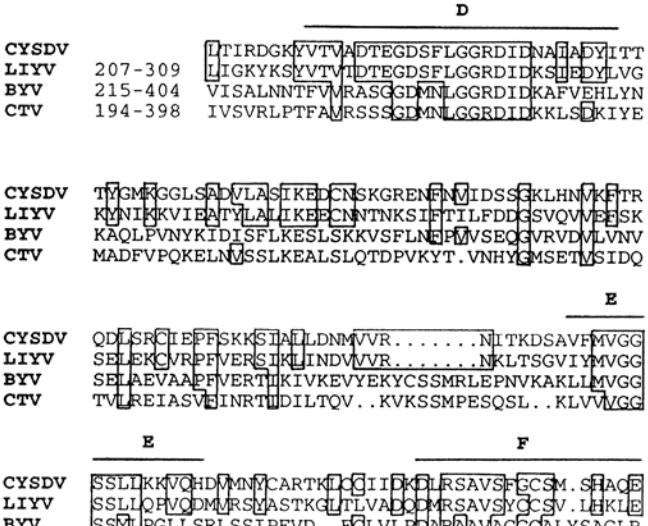

Fig. 4. A, The nucleotide sequence of cucurbit yellow stunting disorder virus (CYSDV) cDNA clone p410 (GenBank accession U67170). The sequences of oligonucleotides 410U and 410L, used to amplify CYSDV RNA by reverse transcription-polymerase chain reaction (RT-PCR), are underlined. **B,** Alignment of the deduced amino acid sequence of clone p410 (CYSDV) with a region of the HSP70 homologs of a whitefly-transmitted closterovirus (lettuce infectious yellows closterovirus [LIYV]) and two aphid-transmitted closteroviruses (beet yellows virus [BYV] and citrus tristeza virus [CTV]). Numbers to the left indicate the HSP70 homolog amino acid residues used in the alignment. Residues identical to those deduced for CYSDV are boxed. The conserved motifs D to G of the HSP70-related family of proteins (1) are indicated. Sequences of LIYV (16), BYV (2), and CTV (14) have been published.

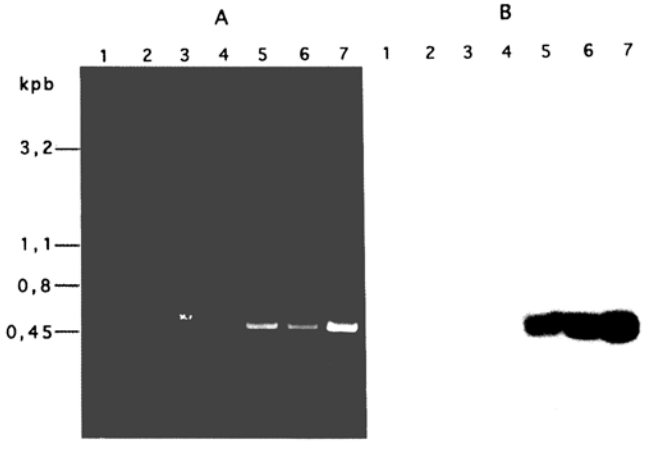

Fig. 5. Detection of cucurbit yellow stunting disorder virus (CYSDV) in infected melon and cucumber plants by reverse transcription-polymerase chain reaction (RT-PCR) with oligonucleotides 410U and 410L. **A,** Agarose gel electrophoresis of RT-PCR products obtained from total RNA extracts from noninoculated lane 2, melon or lane 3, cucumber plants, from a lane 4, cucumber plant infected with the Spanish BPYV-like closterovirus, and from CYSDV-infected lane 5, melon or lane 6, cucumber plants. Lane 1 is a negative control of PCR without cDNA added and lane 7 is a positive control of PCR using p410 DNA as the template. Molecular size markers (kbp) are indicated to the left. **B,** Southern blot of gel shown in **A** probed with a ^{32}P-labeled *Eco*RI-*Hin*dIII fragment of p410.

LITERATURE CITED

1. Agranovsky, A. A., Boyko, V. P., Karasev, A. V., Koonin, E. V., and Dolja, V. V. 1991. Putative 65 kDa protein of beet yellows closterovirus is a homologue of HSP70 heat shock proteins. J. Mol. Biol. 217:603-610.
2. Agranovsky, A. A., Koonin, E. V., Boyko, V. P., Maiss, E., Frotschl, R., Lunina, N. A., and Atabekov, J. G. 1994. Beet yellows closterovirus—Complete genome structure and identification of a leader papain-like thiol protease. Virology 198:311-324.
3. Brown, J. K., and Nelson, M. R. 1986. Whitefly-borne viruses of melons and lettuce in Arizona. Phytopathology 76:236-239.
4. Coffin, R. S., and Coutts, R. H. A. 1992. dsRNA cloning and diagnosis of beet pseudo-yellows virus by PCR and nucleic acid hybridization. Intervirology 33:197-203.
5. Coffin, R. S., and Coutts, R. H. A. 1995. Relationships among *Trialeurodes vaporariorum*-transmitted yellowing viruses for Europe and North America. J. Phytopathol. 143:375-380.
6. Dolja, V. V., Karasev, A. V., and Koonin, E. V. 1994. Molecular biology and evolution of closteroviruses: Sophisticated build-up of large RNA genomes. Annu. Rev. Phytopathol. 32:261-285.
7. Duffus, J. E. 1965. Beet pseudo-yellows virus, transmitted by the greenhouse whitefly (*Trialeurodes vaporariorum*). Phytopathology 55:450-453.
8. Duffus, J. E. 1995. Whitefly transmitted yellowing viruses of the cucurbitaceae. Cucurbitaceae 94:12-16.
9. Duffus, J. E., Larsen, R. C., and Liu, H. Y. 1986. Lettuce infectious yellows virus—A new type of whitefly-transmitted virus. Phytopathology 76:97-100.
10. Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.
11. Gubler, U., and Hoffman, B. J. 1983. A simple and very efficient method for generating cDNA libraries. Gene 25:263-268.
12. Hassan, A. A., and Duffus, J. E. 1991. A review of a yellowing and stunting disorder of cucurbits in the United Arab Emirates. Emir. J. Agric. Sci. 2:1-16.
13. Jordá-Gutiérrez, C., Gómez-Guillamón, M. L., Juárez, M., and Alfaro-García, A. 1993. Clostero-like particles associated with a yellows disease of melons in south-eastern Spain. Plant Pathol. 42:722-727.
14. Karasev, A. V., Boyko, V. P., Gowda, S., Nikolaeva, O. V., Hilf, M. E., Koonin, E. V., Niblett, C. L., Cline, K., Gumpf, D. J., Lee, R. F., Garnsey, S. M., Lewandowski, D. J., and Dawson, W. O. 1995. Complete sequence of the citrus tristeza virus RNA genome. Virology 208:511-520.
15. Klaassen, V. A., Boeshore, M., Dolja, V. V., and Falk, B. W. 1994. Partial characterization of the lettuce infectious yellows virus genomic RNAs, identification of the coat protein gene and comparison of its amino acid sequence with those of other filamentous RNA plant viruses. J. Gen. Virol. 75:1525-1533.
16. Klaassen, V. A., Boeshore, M. L., Koonin, E. V., Tian, T. Y., and Falk, B. W. 1995. Genome structure and phylogenetic analysis of lettuce infectious yellows virus, a whitefly-transmitted, bipartite closterovirus. Virology 208:99-110.
17. Liu, H.-Y., and Duffus, J. E. 1990. Beet pseudo-yellows virus: Purification and serology. Phytopathology 80:866-869.
18. Lot, H., Delecolle, B., and Lecoq, H. 1982. A whitefly transmitted virus causing muskmelon yellows in France. Acta Hortic. 127:175-182.
19. Pio-Ribeiro, G., Winter, S., Jarret, R. L., Demski, J. W., and Hamilton, R. I. 1996. Detection of sweet potato virus disease-associated closterovirus in a sweet potato accession in the United States. Plant Dis. 80:551-554.
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
21. Soria, C., Gómez-Guillamón, M. L., and Duffus, J. E. 1991. Transmission of the agent causing a melon yellowing disease by the greenhouse whitefly *Trialeurodes vaporariorum* in southeast Spain. Neth. J. Plant Pathol. 97:289-296.
22. Swallow, W. H. 1985. Group testing for estimating infection rates and probabilities of disease transmission. Phytopathology 75:882-889.
23. Valverde, R. A., Nameth, S. T., and Jordan, R. L. 1990. Analysis of double-stranded RNA for plant virus diagnosis. Plant Dis. 74:255-258.
24. van Dorst, H. J. M., Huijberts, N., and Bos, L. 1980. A whitefly-transmitted disease of glasshouse vegetables, a novelty for Europe. Neth. J. Plant Pathol. 86:311-313.
25. Wisler, G. C., Liu, H.-Y., Klaasen, V. A., Duffus, J. E., and Falk, B. W. 1996. Tomato infectious chlorosis virus has a bipartite genome and induces phloem-limited inclusions characteristic of the closteroviruses. Phytopathology 86:622-626.
26. Woudt, L. P., de Rover, A. P., de Haan, P. T., and van Grinsven, M. Q. J. M. 1993. Sequence analysis of the RNA genome of cucumber chlorotic spot virus (CCSV), a whitefly transmitted closterovirus. (Abstr.) Page 326 in: International Congress of Virology, IX, Glasgow, Scotland.
27. Yamashita, S., Doi, Y., Yora, K., and Yoshino, M. 1979. Cucumber yellows virus: Its transmission by the greenhouse whitefly *Trialeurodes vaporariorum* Westwood, and the yellowing disease of cucumber and muskmelon caused by the virus. Ann. Phytopathol. Soc. Jpn. 45:484-496.