Etiology of Planta Macho, a Viroid Disease of Tomato

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We thank Anne C. Jackson and Steven D. Smith for technical assistance.

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Accepted for publication 30 April 1980.

ABSTRACT

Galindo A., J., Smith, D. R., and Diener, T. O. 1982. Etiology of Planta Macho, a viroid disease of tomato. Phytopathology 72:49-54.

A disease of tomato, locally known as "Planta Macho" (male plant) (PM) disease, occurs in the Mexican states of Morelos and Mexico. Symptoms resemble those induced in tomato by the potato spindle tuber viroid (PSTV), but are more severe; nucleic acid extracts from infected, but not from healthy, plants contained a low-molecular-weight RNA with electrophoretic mobility identical to that of PSTV; and infectivity distribution after electrophoresis in polyacrylamide gels coincides with the position in the gel of the disease-specific RNA. This RNA, therefore, is the etiological agent of the disease. The RNA might be a severe strain of PSTV, but preinoculation of plants with a mild strain of PSTV affords little, if any, protection against symptom expression of the PM agent. Also, several plant species that are susceptible to PSTV are resistant to the PM agent; and several others that are susceptible to both agents display symptoms when infected with PSTV but remain symptomless, when infected with the PM agent. We, therefore, consider the PM agent, not as a severe PSTV strain, but as a distinct viroid for which we propose the term Tomato Planta Macho Viroid (TPMV).

Additional key words: cross-protection, RNA, gel electrophoresis.

In Mexico, a disease of tomato (*Lycopersicon esculentum* Mill.) has been observed in the Mexican states of Morelos and Mexico, and particularly in the tomato-growing area of Cuahutla, Mor. The disease is locally known as "Planta Macho" (male plant) (hereafter abbreviated PM), because affected plants do not produce marketable fruit. In some years, the disease causes severe damage and, occasionally, some farmers suffer complete crop loss.

Infected plants are severely stunted and exhibit strong epinasty of leaves and leaflets. Old leaves turn yellow and dry out. The laminae of leaflets become crinkled and brittle. Under optimal conditions for symptom formation, a veinal and stem necrosis also may develop. Infected plants produce more flowers and fruits than healthy ones, but the fruits remain small (about the size of marbles) and have no commercial value.

The disease was first described by Belalcazar and Galindo in 1974 (1). Results of their investigation showed that it is caused by an infectious agent that is readily transmissible mechanically, but not by aphids or via seed. In the field, the disease is presumably spread by contact of diseased foliage with healthy plants or contaminated tools. When expressed sap from infected plants was subjected to sucrose density gradient centrifugation, a prominent light-scattering band was formed, whereas in tubes containing expressed sap from healthy plants, no such band was discernible. Analysis of this band revealed infectivity, an antigenic compound, and very small particles, as well as protein and nucleic acid. On the basis of these results, the disease was considered to be caused by a virus (1).

Later work, however, raised doubts about this conclusion. Galindo and Rodriguez (6) examined the small particles found in the light-scattering band by electron microscopy and concluded that they were not virus particles. When they prepared nucleic acid from the light-scattering band and fractionated the nucleic acid by treatment with lithium chloride (LiCl), they detected a highly infectious component in the LiCl-soluble fraction. Presumably, because this nucleic acid was of relatively low molecular weight, it appeared possible that a viroid, and not a virus, could be the causal agent of the disease (3). Viroid etiology of the disease might also be suspected on the basis of its symptoms, which resemble those of the tomato bunchy top disease (8) and those induced in tomato by the potato spindle tuber (12) and citrus exocortis (14) viroids.

We report here that the PM disease is indeed a viroid disease and describe some properties of the causative viroid.

MATERIALS AND METHODS

Materials. Ribonuclease A (RNase) (three times crystallized) and deoxyribonuclease I (DNase) (electrophoretically purified) were obtained from Worthington Biochemical Corporation,

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Freehold, NJ 07728. All reagents used were of analytical grade. Materials for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA 94804. Ribonuclease-free sucrose was obtained from the Schwarz/Mann Division of Becton, Dickinson and Co., Orangeburg, NY 10962.

Pathogen propagation. The Tomato Planta Macho viroid (TPMV) was propagated in tomato cultivar Rutgers plants that were inoculated mechanically at the cotyledonary stage. Plants were kept in a greenhouse at about 28 C. Aboveground portions of plants were harvested after symptoms were well developed (15–21 days after inoculation) and were immediately frozen at −20 C. Shoots of healthy plants of equal age were similarly harvested and frozen as controls.

Infectivity assays. Rutgers tomato plants at the cotyledonary stage were inoculated by lightly rubbing an inoculum-soaked cotton applicator over corundum (22-μm, 600-mesh)-dusted cotyledons and terminals, immediately followed by rinsing with water. Three seedlings were used with each of four dilutions (undiluted, 1/10, 1/100, 1/1,000) of the inoculum. The plants were kept in a greenhouse at about 28 C and were observed daily. As soon as symptoms started to appear, numbers of symptom-bearing plants in each group were recorded at 2-day intervals. When in three consecutive readings a change in the number of plants with symptoms was no longer noted, readings were discontinued. An infectivity index was then calculated for each inoculum as described elsewhere (11).

Preparation of subcellular fractions. Ten grams of TPMV-infected tomato leaves were ground in 30 ml of a solution containing 0.5 M sucrose, 0.02 M tris (hydroxymethyl)-aminomethane (tris)-HCl, pH 7.5, 0.002 M $MgCl_2$, 0.02 M KCl, and 0.04 M mercaptoethanol. The slurry was expressed through Miracloth to remove fibers and large tissue pieces. The extract was then fractionated by centrifugation for 10 min at 250 *g*. The resulting pellet was resuspended in 1 ml of 0.02 M phosphate buffer, pH 7, and bioassayed. The supernatant was centrifuged for 10 min at 1,000 *g* and the resulting pellet resuspended and bioassayed as above. This process was continued with centrifugation for 10 min at 10,000 *g*, then for 30 min at 80,000 *g*, and finally for 2 hr at 100,000 *g*. Pellets resulting from each centrifugation were resuspended and, together with the final supernatant, bioassayed in tomato seedlings.

TABLE 1. Infectivity distribution of TPMV after LiCl fractionation[a]

Fraction and log dilution	Days postinoculation 19	21	24	26	28	31	33	35	Infectivity index[c]
LiCl supernatant									
0	0[b]	2	2	3	3	3	3	3	
−1	0	2	3	3	3	3	3	3	
−2	1	2	2	3	3	3	3	3	
−3	0	1	1	1	1	1	2	2	
−4	1	1	1	1	1	1	1	1	195
LiCl pellet									
0	0	0	0	0	1	1	2	2	
−1	0	0	0	0	0	0	0	0	
−2	0	0	0	0	0	0	0	0	
−3	0	0	0	0	0	0	0	0	
−4	0	0	0	0	0	0	0	0	6

[a] Fractionation with 2 M LiCl; see Materials and Methods.
[b] Number of plants with symptoms of three plants inoculated.
[c] See reference 11.

TABLE 2. Nuclease sensitivity of Tomato Planta Macho Viroid (TPMV)

Treatment[a]	Infectivity index[b]
None	64
DNase	84
RNase	0

[a] Portions (10-μl) of a LiCl supernatant fraction from TPMV-infected tissue, containing 200 μg nucleic acid in TKM buffer, were mixed with 1.0 μl of either water, a 0.1 mg/ml solution of RNase, or a 0.1 mg/ml solution of DNase and were incubated for 1 hr at 25 C. Tenfold dilutions (10^{-1} to 10^{-6}) of each preparation were then assayed for infectivity.
[b] Determined as described in reference 11.

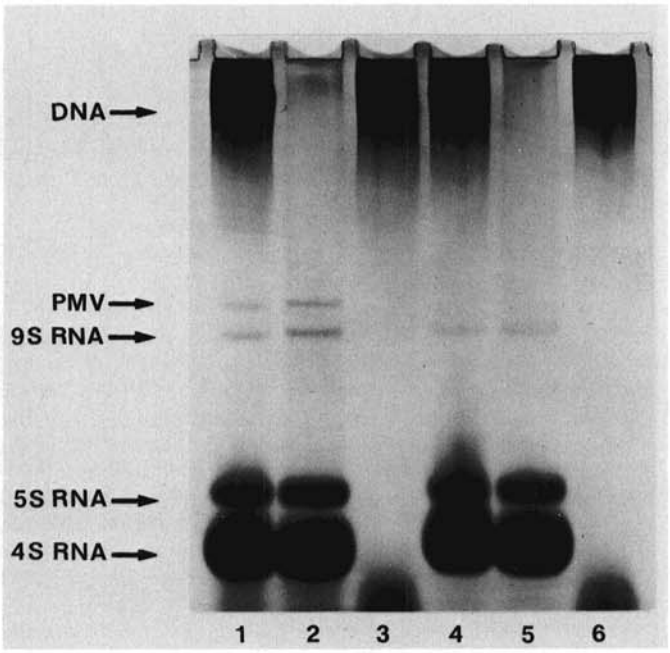

Fig. 1. Polyacrylamide gel electrophoresis of nucleic acid extracts from healthy and TPMV-infected tomato leaves and effect of nuclease treatment on nucleic acids in the latter extract. Nucleic acids were extracted and analyzed in a 5% slab gel as described in Materials and Methods. Lanes 1–3, extract from TPMV-infected leaves; 4–6, extract from healthy leaves; 1 and 4, not treated with nucleases; 2 and 5, treated with DNase; 3 and 6, treated with RNase.

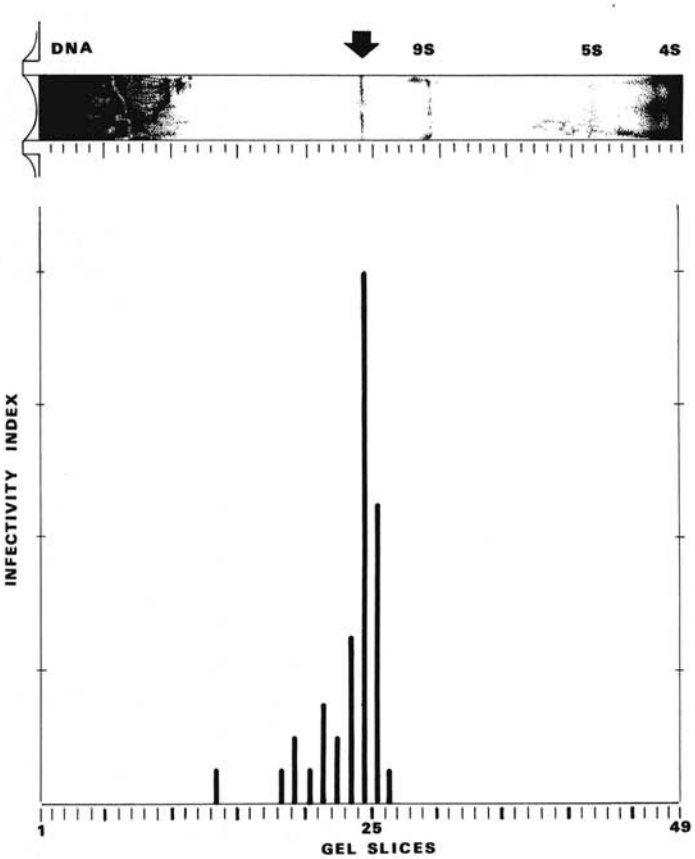

Fig. 2. Polyacrylamide gel electrophoresis of nucleic acid extract from TPMV-infected tomato leaves (upper) and infectivity distribution in the gel (lower). Note coincidence between disease-specific RNA (arrow) and maximal infectivity in the gel.

Nucleic acid extraction. Nucleic acids were isolated from frozen healthy and infected tomato tissue by the method of Morris and Wright (9). Nucleic acids in the supernatant after lithium chloride treatment (ie, LiCl supernatant) were precipitated with ethanol, redissolved in TKM buffer (0.1 M tris-HCl, 0.01 M KCl, 0.1 mM $MgCl_2$, pH 7.4), and dialyzed against TKM buffer for 16 hr at 4 C. Nucleic acids precipitated by lithium chloride (ie, LiCl pellet) were also redissolved in TKM buffer and dialyzed against TKM buffer for 16 hr at 4 C. The final volumes of both fractions were identical (3 ml). These fractions were used for bioassay and analysis in polyacrylamide gels.

Polyacrylamide gel electrophoresis. Nucleic acid samples to be analyzed were mixed with one-tenth volume of a 50% (w/w) aqueous solution of sucrose containing 10% (w/v) phenol blue. Electrophoresis was carried out in 5% polyacrylamide (2.5% *bis*-acrylamide cross-linked) gels in a running buffer composed of 0.04 M tris-HCl, 0.001 M sodium EDTA, and 0.012 M sodium acetate, pH 7.2. For the first 30 min, a potential of 20 V was applied; for the next 60 min, 50 V; and for the remainder of each run, 100 V. Electrophoresis was terminated when the dye had reached the lower edge of the gel. Nucleic acids were stained by a 30-min immersion, with gentle stirring, in 0.4 M sodium acetate, 0.4 M acetic acid, 0.02% methylene blue, followed by destaining in water.

Determination of infectivity distribution in gels. Samples of a LiCl supernatant fraction from infected plants were electrophoresed in adjoining tracks of a 5% polyacrylamide slab gel. After electrophoresis, the two tracks were separated and nucleic acids in one track were visualized by staining, as described above. The other, unstained, track was cut into 1-mm-wide slices, each slice was homogenized in water, and 10-fold dilutions were assayed for infectivity.

Partial purification of TPMV. Nucleic acids were extracted by the direct phenol extraction method (4) from tomato leaves infected with the PM agent and, for comparison, from leaves infected with the potato spindle tuber viroid (PSTV). After gel filtration in Sephadex G-100 columns (4), viroid-containing fractions were reconcentrated by ethanol precipitation and resuspension in 0.5–1.5 ml of water. The resulting preparations were then subjected to electrophoresis in 5% polyacrylamide slab gels (4). The viroid-containing bands were excised and the viroids were eluted and reconcentrated (2). Analysis of final preparations on 20% polyacrylamide gels revealed, aside from the prominent viroid bands, two or three faint bands, corresponding to minor cellular RNA species of low molecular weight (Fig. 3).

RESULTS

Infectivity of nucleic acid fractions. Bioassays of the LiCl supernatant and pellet fractions derived from nucleic acid extracts of plants infected with TPMV revealed that essentially all infectivity was located in the supernatant fraction (Table 1).

Similarly prepared fractions from healthy plants were never infectious in the tomato assay.

Nuclease sensitivity of the infectious agent. To determine whether TPMV is RNA or DNA, LiCl supernatant fractions were incubated with RNase or DNase. As shown in Table 2, incubation with RNase led to complete loss of infectivity, whereas incubation with DNase had no effect. Evidently, TPMV is composed of RNA, not DNA.

Gel electrophoresis of LiCl supernatant fractions from healthy and TPMV-infected plants. Localization of the PM agent in the LiCl supernatant fraction suggested that the agent was a low-molecular-weight RNA; that is, a viroid. To obtain more definitive evidence, preparations from healthy and infected plants were subjected to electrophoresis in polyacrylamide gels. Fig. 1 shows that LiCl supernatants from both healthy and PM-infected plants contain DNA, 4S, 5S, and 9S RNA, as well as traces of at least one minor nucleic acid constituent and that another prominent band, close to the middle of the gel, is present in the preparation from infected, but not in that from healthy plants. Fig. 1 also shows that this band is absent in preparations that had been treated with RNase, but is not affected by treatment with DNase.

Infectivity distribution in gels. To investigate whether the disease-specific RNA represents the infectious RNA, distribution of infectivity in polyacrylamide gels was determined as described in Materials and Methods. As shown in Fig. 2, infectivity was detected in only 10 of the 49 slices; highest levels of infectivity were present in slices 23–25. Alignment with the stained gel tract run simultaneously (upper portion of figure) indicates that the slice with the highest level of infectivity (No. 24) coincides with the position in the gel of the disease-specific RNA.

Comparison of the electrophoretic mobility of the disease-specific RNA with that of PSTV. Electrophoresis of partially purified preparations of the disease-specific RNA and of PSTV in adjoining tracts of a 20% polyacrylamide gel showed that both migrate at the same rate (Fig. 3).

Comparison of some biological properties of TPMV with those of PSTV. Results so far discussed indicate that the PM agent has viroidlike properties but, in view of the observation that its electrophoretic mobility is identical with that of PSTV, the question arose whether it is a strain of PSTV or an unrelated viroid. Although chemical information, such as RNA fingerprints or nucleotide sequence data, are required to definitively answer this question, comparison of biological properties of the PM agent with those of PSTV would be useful. This would be the case if the two agents could be clearly distinguished from one another on the basis of biological properties, which would indicate that the two are distinct viroids. If, on the other hand, the two agents could not be distinguished from one another in this fashion, the results would be ambiguous.

Comparative host ranges. One such biological property is the

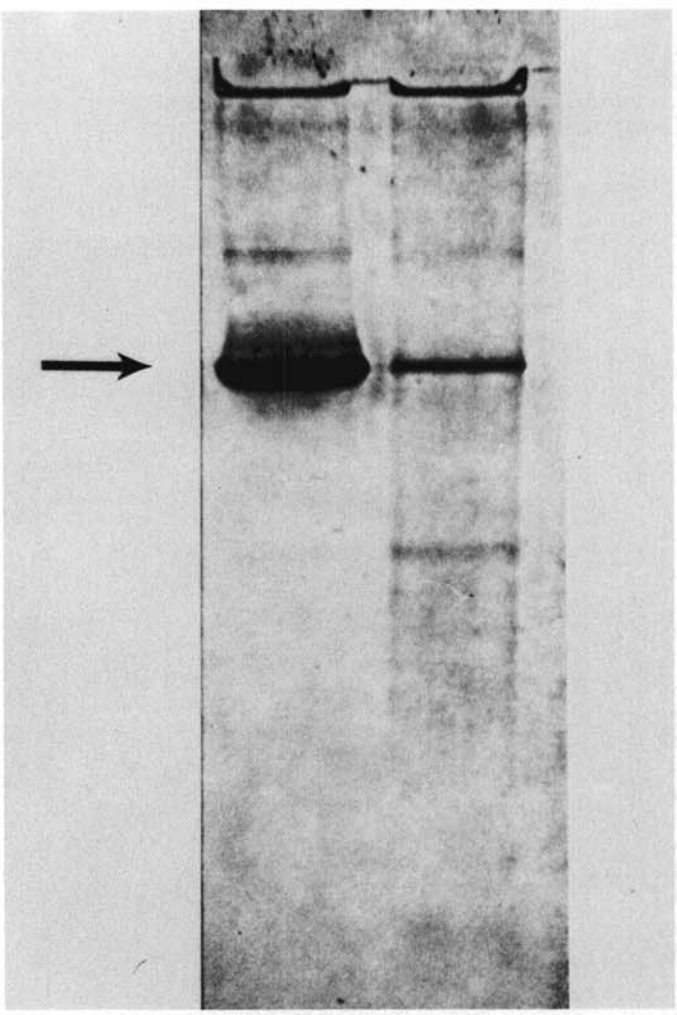

Fig. 3. Electrophoresis in a 20% polyacrylamide slab gel of partially purified PSTV (left) and TPMV (right). Note equal rate of migration of the two viroids (arrow).

host range of TPMV as compared with that of PSTV.

Earlier work (1) had already revealed several plant species resistant to TPMV but susceptible to PSTV and other species in which both viroids are able to replicate but in which infection with PSTV, but not with TPMV leads to visible symptoms.

In the present work, another distinguishing species has been detected, *Gynura aurantiaca* DC. Both PSTV (16) and the citrus exocortis viroid (CEV) (17), are known to replicate and induce characteristic symptoms in this species. Inoculation of *G. aurantiaca* plants with TPMV resulted in its replication, as evidenced by our ability to recover the RNA from uninoculated portions of the plants 3 wk after inoculation. In contrast to PSTV- and CEV-infected *G. aurantiaca*, however, plants infected with TPMV remained symptomless.

Although TPMV and PSTV share many host species, significant differences, both with respect to susceptibility and symptom expression exist. Thus, *Gomphrena globosa*, *Datura stramonium*, and *Nicotiana tabacum* support PSTV, but not TPMV, replication. *Nicotiana glutinosa*, *Solanum melongena*, and *S. tuberosum* are hosts of both viroids, but only infection with PSTV results in symptom development.

Distribution of TPMV within infected plants. Table 3 shows that TPMV was detected in all assayed parts of tomato plants. Mature symptomless leaves were consistently found to contain smaller amounts than young symptom-bearing leaves. Largest amounts of TPMV were found in stems in one experiment and in roots in

TABLE 3. Relative concentration of TPMV in different parts of infected tomato plants[a]

Plant part	Infectivity index	
	Exp. 1[b]	Exp. 2[c]
Root	44	59
Stem	65	48
First and second leaves (symptomless)	40	45
Third and fourth leaves (symptoms)	53	58

[a]Plants were inoculated at the cotyledonary stage with an extract from TPMV-infected plants. Plants were kept in a greenhouse until they reached the four-leaf stage. Extracts were then prepared from various parts of the infected plants by triturating tissue in 0.02 M phosphate buffer, pH 7 (1:1, w/v).

[b]In this experiment, greenhouse temperatures were relatively low and symptoms were mild. Each extract was assayed undiluted and diluted 1/10, 1/100, and 1/1,000 (in 0.02 M phosphate buffer, pH 7) on three plants each.

[c]In this experiment, greenhouse temperatures averaged 29 C and symptoms were severe. Each extract was assayed undiluted and diluted 1/10 and 1/100 on five plants each.

TABLE 4. Subcellular distribution of TPMV in tomato leaf and stem tissue

Fraction[a]	Major component	Infectivity index	
		Stems	Leaves
250 *g* pellet	Nuclei	6	26
1,000 *g* pellet	Chloroplasts	14	18
10,000 *g* pellet	Mitochondria	19	9
80,000 *g* pellet	Membranes	18	24
100,000 *g* pellet	Ribosomes	12	9
100,000 *g* supernatant	Postribosomal supernatant	0	0

[a]See Materials and Methods.

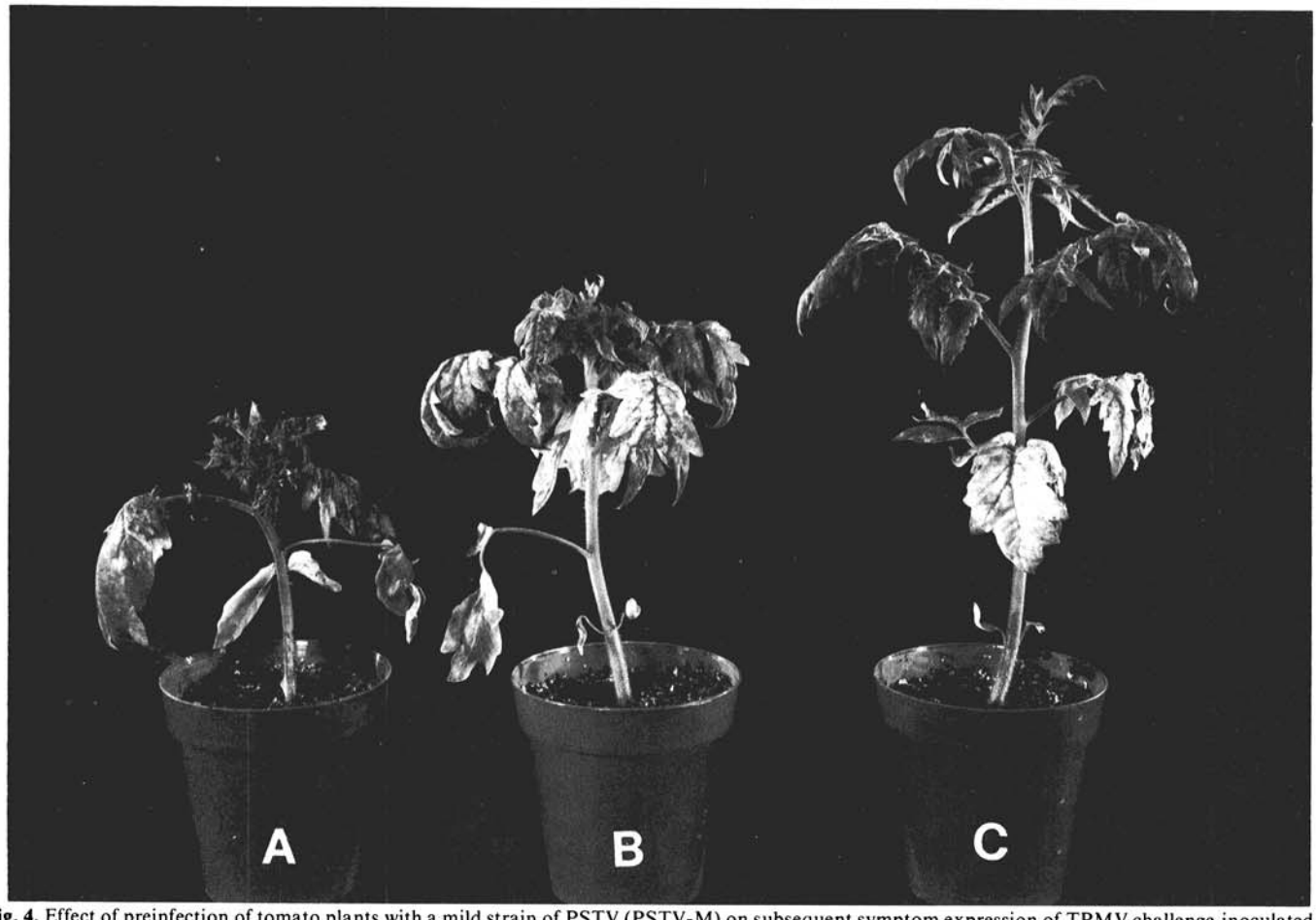

Fig. 4. Effect of preinfection of tomato plants with a mild strain of PSTV (PSTV-M) on subsequent symptom expression of TPMV challenge-inoculated 5 days after inoculation with PSTV-M. Plants B and C were inoculated with PSTV-M on day 0, and plants A and B with TPMV on day 5. Note slightly reduced stunting and somewhat less severe symptoms of TPMV in the doubly infected plant B than in the singly infected plant A.

another (Table 3).

Subcellular distribution of TPMV. As shown in Table 4, some infectivity was detected in all subcellular fractions, except the postribosomal supernatant. In leaf cells, highest levels of infectivity were present in the fraction consisting primarily of cell nuclei and in the 80,000 *g* pellet which was considered to be largely composed of membranes and membrane fragments.

In stem cells, on the other hand, relatively little infectivity was associated with the nuclear fraction, most infectivity being divided among the "mitochondrial" and "membrane" fractions, with lower levels present in the "chloroplast" and "ribosomal" fractions.

Cross-protection. Infection with a mild strain of PSTV is known to protect tomato plants against the expression of symptoms due to superinfection with a severe PSTV strain (5) or with CEV (10). With viruses, cross-protection usually is believed to indicate relationship between the protecting and the superinfecting viruses (7). It was, therefore, of interest to determine whether infection of tomato plants with a mild strain of PSTV would protect against symptom expression of TPMV inoculated at a later date.

Three groups of four tomato seedlings each were inoculated at day 0 with a mild strain of PSTV (PSTV-M) and were challenge-inoculated with TPMV at days 5, 11, and 17, respectively. As controls, three groups of four plants each were inoculated with TPMV alone at days 5, 11, and 17, respectively. Also, to compare symptom formation and growth effects of each viroid, three tomato seedlings each were singly inoculated with either TPMV or PSTV-M.

Table 5 shows that, as expected, infection of plants with PSTV-M alone had little effect on their growth and resulted in mild symptoms, whereas infection with TPMV alone strongly reduced the height attained by plants and caused severe symptoms.

When plants were challenge-inoculated with TPMV 5 days after inoculation with PSTV-M, the stunting observed in plants infected with TPMV alone was slightly reduced and, 16–40 days after the first inoculation, symptoms were somewhat less severe (Table 5 and Fig. 4).

When plants were challenge-inoculated 11 or 17 days after inoculation with PSTV-M, no significant differences in growth were noted between the doubly infected plants and those inoculated solely with TPMV on the day of the challenge inoculation. Because by the time of inoculation of either group with TPMV, the plants had become much larger, effects on plant growth were much reduced as compared with the test in which the challenge-inoculation was made on day 5. As compared with the controls, a slight reduction in symptom severity was again noted in doubly infected plants. This was noticeable 9 and 40 days after the first inoculation in the test with challenge on day 11; but only 40 days after the first inoculation in the test with challenge on day 17 (Table 5).

Thus, all tests indicate that, regardless of the time interval between inoculation of plants with PSTV-M and challenge-inoculation with TPMV, a slight interference with the full expression of the PM disease in tomato occurred in doubly infected plants. This interference, however, was so slight that it cannot be regarded as a true cross-protection phenomenon. This conclusion is strengthened by the observation that, in doubly infected plants, symptoms of PM disease consistently appeared earlier and were initially more severe than in previously uninfected control plants that were inoculated with TPMV at the same time (Table 5). Although the cause of this phenomenon is unknown, it suggests a synergistic, rather than antagonistic, relationship between the two pathogens.

TABLE 5. Effects of infection of tomato plants with a mild strain of PSTV (PSTV-M) on symptom expression by TPMV

Inoculation protocol[a]	Height of plants in cm at day[b]			Symptom intensity at day[c]			
	16	29	40	13	16	29	40
Inoculation at day 0							
PSTV-M/···	16	27	33	+	++	++	++
TPMV/···	10	12	13	+++	+++	++++	++++
Challenge at day 5							
PSTV-M/TPMV	13	14	16	++	++	+++	+++
···/TPMV	11	11	13	+	+++	++++	++++
Challenge at day 11							
PSTV-M/TPMV	16	23	26	−	++	++	++
···/TPMV	18	24	26	−	−	+++	+++
Challenge at day 17							
PSTV-M/TPMV	17	28	31			++	++
···/TPMV	18	30	30			−	+++

[a] Numerator: first inoculation at day 0. Denominator: challenge inoculation at day specified.
[b] Average of four plants each.
[c] Average of four plants each. − = no symptoms, + = mild, ++ = moderate, +++ = severe, ++++ = very severe symptoms.

DISCUSSION

The results presented leave little doubt that the cause of the PM disease of tomato is infection by a viroid. Nucleic acid extracts from infected plants are highly infectious, and when high-molecular-weight RNAs are separated from low-molecular-weight RNAs, essentially all of the infectious agent is found in the latter fraction. The agent is readily inactivated by RNase, but is not affected by treatment with DNase. When nucleic acid preparations are subjected to polyacrylamide gel electrophoresis, one low-molecular-weight RNA band is discernible in extracts from infected, but not in extracts from healthy plants. Finally, infectivity distribution in gels coincides with the position in the gel of the disease-specific RNA.

Because the symptoms of PM disease resemble those induced in tomato by PSTV and because the electrophoretic mobility of TPMV is identical with that of PSTV, TPMV could be a severe strain of PSTV. Four sets of observations, however, indicate that this is not the case:

(i) Significant differences in the respective host ranges of PSTV and TPMV were found. Three species that have been reported earlier to support PSTV replication proved to be resistant to the PM agent; three additional species that have been reported to react with visible symptoms upon infection with PSTV also supported replication of TPMV, but without expression of visible symptoms.

(ii) The same mild strain of PSTV, that had been shown earlier to protect tomato plants against symptom expression due to superinfection with a severe PSTV strain (5,10), was largely ineffective in protecting against symptom expression due to superinfection with TPMV.

(iii) The large amount of TPMV found in stems of infected plants contrasts with the small amounts found in stems of PSTV-infected tomato plants (9). Also, distribution of the infectious RNA among subcellular fractions differs from that of PSTV, the great majority of which (at least in leaves) is located in the nuclei of infected cells (3). The subcellular distribution of TPMV in leaf cells resembles that reported for CEV (13) but, as compared with CEV, TPMV occurs in stem tissue at far higher concentration and, contrary to CEV, TPMV is symptomless in *G. aurantiaca*.

(iv) In contrast to PSTV, which has been shown to be transmissible through the seed of infected plants (15), seed transmission of TPMV could not be demonstrated (1).

We conclude that the PM agent is a viroid and, because some of its biological properties are readily distinguishable from those of PSTV, that it is not a PSTV strain, but a distinct viroid, for which we propose the name Tomato Planta Macho Viroid (TPMV).

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