The Beet Leafhopper-Transmitted Virescence Agent Causes Tomato Big Bud Disease in California

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ABSTRACT

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Mycoplasmalike organisms (MLOs) are associated with big bud disease of tomatoes (*Lycopersicon esculentum*) in many parts of the world. In only a few cases, however, have the MLOs associated with the disease been characterized. We used biological and genetic data to establish that the causal agent of tomato big bud (TBB) disease in California is the beet leafhopper-transmitted virescence agent (BLTVA) MLO. Healthy *Circulifer tenellus* leafhoppers acquired the BLTVA MLO from field-collected, symptomatic tomato plants and transmitted it to healthy tomato plants, which developed typical big bud symptoms. Healthy tomatoes inoculated with the BLTVA type line (FC-83-13) also developed the floral gigantism and virescence characteristic of the disease. A California TBB MLO isolate caused symptoms typical of those caused by the BLTVA, including induction of premature flowering, on a standard plant host range. Southern blot analysis of DNA from field plants and from greenhouse tomato plants inoculated with a California TBB MLO isolate showed that all samples possessed plasmids that hybridized with a cloned BLTVA MLO plasmid. *Macrosteles fascifrons* did not transmit a virescence agent from symptomatic, field-collected tomatoes, and tomato plants infected with western aster yellows MLO failed to develop floral gigantism or virescence.

Tomato big bud (TBB) disease was named for the prominent symptom of swollen, virescent buds that never develop into normal flowers (Fig. 1). It was first described in Australia (35) and has since been reported in many other tomato-growing areas, including Europe (33,45), India (46), Israel (47), and the United States (5–7,17). In Australia, symptoms include purple veins, thickened stems, dwarfed leaves, and shoot proliferation (35). In Arkansas and New York, infected plants had similar symptoms, including a stiff, erect growth habit (6,17). In contrast, diseased plants collected in California had only minor dwarfing and shoot proliferation (M. E. Shaw, *personal observation*).

Electron microscopy studies have shown that TBB is associated with a mycoplasmalike organism (MLO) (3,6,17). Because MLOs have not been successfully cultured in vitro (31), they have been characterized mainly by biological properties such as host range, symptoms, and vector specificity, which require large expenditures of time, effort, and greenhouse space to determine.

Recently, MLOs have been detected and differentiated with polyclonal antisera (4,19,21,29,42,43), monoclonal antibodies (28), and DNA hybridization assays (8,9,20,21,25,27,37). These techniques have been used to partially characterize the MLOs that cause TBB in several regions. In Australia, where the disease causes serious economic losses, the TBB MLO is transmitted by the leafhopper *Orosius argentatus* (Evans), which also transmits several other MLO-associated diseases, including legume little leaf, lucerne witches'-broom, and possibly purple top of potato (2). The

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genetic relationships among the causal agents of these diseases have not been reported, although the biological data suggest that they are similar but not identical MLOs (2). European stolbur disease causes big bud symptoms on tomatoes and is transmitted primarily by the planthopper *Hyalesthes obsoletus* Signoret. Many strains of stolbur have been identified on the basis of host and vector relationships (45). Other diseases, such as potato witches'-broom, are also reported to cause big bud symptoms on tomatoes (33), but relationships among the various MLOs have not been determined. Results of DNA hybridization studies indicate that TBB in the eastern United States is caused by an MLO of the eastern aster yellows (AY) group (1,27). The etiology of TBB in California has not been established.

At least two distinct groups of MLOs appear to cause virescence in herbaceous hosts in California (40). The western AY MLO is transmitted by the aster leafhopper, *Macrosteles fascifrons* (Stål), and some other leafhopper species (38,44). The more recently described beet leafhopper-transmitted virescence agent (BLTVA) MLO is transmitted by the beet leafhopper, *Circulifer tenellus* (Baker) (14). These MLOs have wide, overlapping host and geographic ranges (16) and cause similar symptoms of virescence and phyllody in many plant hosts. They differ, however, in vector specificity: *C. tenellus* transmits the BLTVA but not the AY MLO, while *M. fascifrons* transmits the AY but not the BLTVA MLO (22,38; D. A. Golino, *unpublished*). In addition, the BLTVA MLO induces premature flowering (the host induction response [HIR]) in certain plants that flower in response to exogenous application of gibberellic acid, but the AY MLO does not (13,15).

We present biological and genetic data that indicate that the BLTVA MLO is the primary causal agent of TBB in California.

MATERIALS AND METHODS

Isolation of DNA from field-collected and experimentally inoculated plants. Tomato plants with the enlarged calyx characteristic of TBB were collected near San Diego, CA, in early fall of 1986 and 1989. Other tomato plants with various symptoms were collected from other locations in the western United States from 1987 through 1991 (Table 1). Extracts of plant samples were enriched for MLOs as previously described (21), and DNA was extracted from the MLO-enriched preparation by the method of Dellaporta et al (10) except that sodium dodecyl sulfate (SDS) incubations were done at room temperature rather than at 65 C.

Leafhopper transmission. Groups of approximately 500 healthy *M. fascifrons* or *C. tenellus* leafhoppers were fed on bouquets of symptomatic tissue from four tomato plants. Tomato is not a good feeding host for either leafhopper species, and high mortality occurs with long feeding times. Therefore, after a 24-hr feeding period on diseased tomato, a

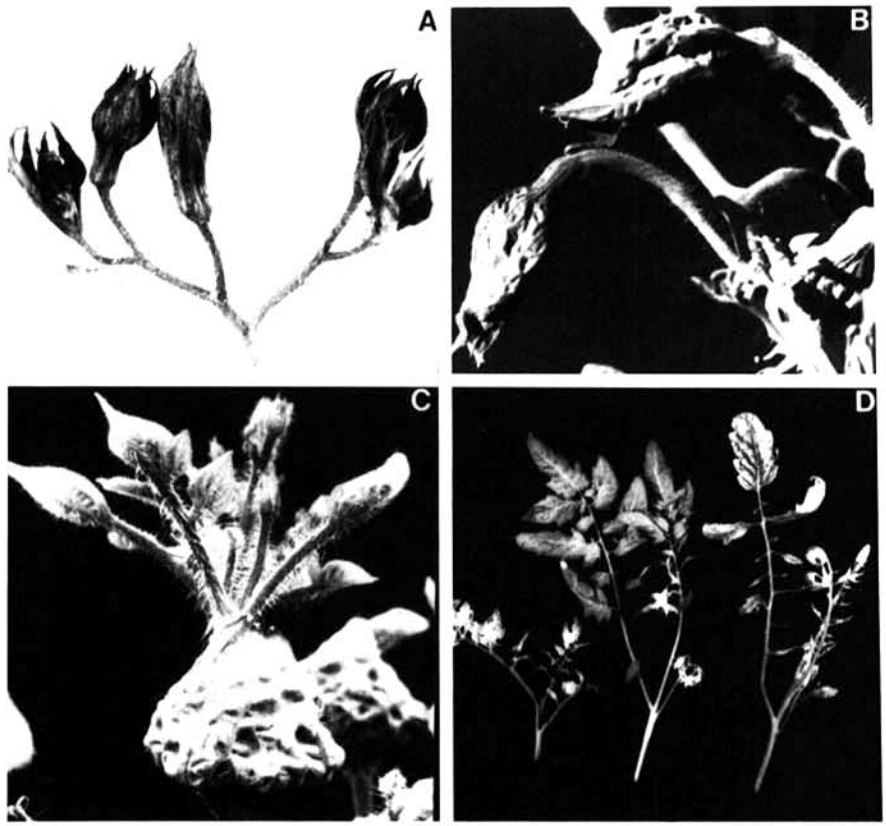

Fig. 1. Tomatoes with symptoms typical of California tomato big bud disease: **(A)** Typical field-collected plant. **(B)** Plant inoculated with field isolate 70C. **(C)** Plant inoculated with the beet leafhopper-transmitted virescence agent mycoplasmalike organism (MLO) type line FC-83-13. **(D)** A plant inoculated with the Tulelake aster yellows MLO (left), one fed on by healthy *Circulifer tenellus* (center), and one inoculated with field isolate 70C via infectious *C. tenellus* (right).

Table 1. Characteristics of field-collected tomato samples

Collection location	Date	Accession numbers[a]	Number tested	Symptoms[b]	Hybridization[c]
Prosser, WA	April 1991	119	1	V,P,FG	BL+
Sacramento, CA	August 1990	116	1	V,P	BL+
Las Cruces, NM	April 1990	96,96	2	PV,S,LD	Neg
San Diego, CA	October 1989	80A,B,C; 81A,B; 82A,B; 83; 84A,B	10	FG,FP,V	BL+
San Diego, CA	September 1989	70A,B,C	3	FG,V	BL+
Oceanside, CA	November 1988	54A	1	FG,FP,V	NT
		54B,C	2	FG,FP,V	BL+
Sacramento, CA	September 1988	34	1	All stem	Neg
Fresno, CA	August 1987	2	1	All stem	Neg
San Diego, CA	September 1986	TBB 86	1	FG,V	NT

[a] Accession numbers refer to fields where samples were collected; letters following accession numbers refer to individual plants from the field.
[b] FG = floral gigantism, FP = floral proliferation, LD = leaf deformation, P = phyllody, PV = proliferation of vegetative buds, S = stunting, V = virescence, all stem = no leaves or flowers on plants.
[c] BL+ = beet leafhopper-transmitted virescence agent (BLTVA) mycoplasmalike organism (MLO) type plasmids present as determined by hybridization with cloned BLTVA MLO plasmid DNA and Southern blot analysis of DNA extracted from field samples; Neg = no hybridization between the BLTVA MLO probe and the sample DNA; NT = not tested.

daikon radish plant (*Raphanus sativus* L. 'Summer Cross Hybrid') was added to cages of *C. tenellus* and a plantain plant (*Plantago major* L.) to cages of *M. fascifrons* to support the insects.

After a 3-wk latent period, surviving leafhoppers were caged in groups of five or 10 on healthy periwinkle (*Catharanthus roseus* (L.) G. Don) as previously described (15). Inoculated plants were observed for 3 mo for symptom development. Periwinkle plants that developed virescence were grafted to healthy periwinkle plants that were then used as source plants for MLO acquisition by additional groups of about 500 healthy *C. tenellus* or *M. fascifrons* leafhoppers. After a 3-wk latent period, leafhoppers in groups of 10 were caged on six daikon plants (*C. tenellus*) or six plantain plants (*M. fascifrons*). If the daikon or plantain developed symptoms, approximately 750 healthy leafhoppers of the appropriate species were fed on the symptomatic plant for 3 wk, and these infectious vectors were then used to inoculate plants for the host range studies.

Host range plants. Five to 10 inoculative leafhoppers were caged on each of six plants of tobacco (*Nicotiana rustica* L.), celery (*Apium graveolens* L.), daikon radish, plantain, and tomato for the 1989 isolate 70C. Twenty daikon or Chinese cabbage (*Brassica pekinensis* (Lour.) Rupr. 'Michihli') plants were used to test the 1986 isolate TBB 86. Healthy *C. tenellus* and *M. fascifrons* leafhoppers were fed on a similar group of host range plants that were used as negative controls. After a 1-wk inoculation access period, plants were fumigated with DDVP (15) and observed for 3 mo for symptom development.

Inoculations with MLO type lines. Tomato plants were inoculated with type lines of the BLTVA MLO and western AY MLO strains, and symptoms that developed were compared with symptoms observed on field-collected plants and on plants inoculated with TBB MLO. Healthy *M. fascifrons* leafhoppers, healthy or BLTVA MLO-infected *C. tenellus* leafhoppers, and plants were raised as previously described (15).

Table 2. Symptoms of host range plants infected with isolate 70C of the tomato big bud mycoplasmalike organism

Host	Symptoms[a]	No. infected/ no. inoculated
Celery	HIR,Y	6/12
Daikon	HIR,Y,FP,FG	14/24
Periwinkle	V,P	3/8
Plantain	P,FP	6/15
Tobacco	Y,P,FP	6/12
Tomato	FG,V,P,PV	4/12

[a] FG = floral gigantism, FP = floral proliferation, HIR = host induction response, P = phyllody, PV = proliferation of vegetative buds, V = virescence, Y = yellow leaves.

Healthy *M. fascifrons* leafhoppers were descendants of H. H. P. Severin's shortwing culture, which has been maintained since its inception at the University of California, Berkeley (39,44).

Four plants of each of the host range species were leafhopper-inoculated with the BLTVA MLO type line FC-83-13 (14). Six tomato plants inoculated with each of three laboratory lines of AY MLO (severe [SAY MLO], dwarf [DAY MLO], and Tulelake [TLAY MLO]) were provided by A. H. Purcell (Department of Entomology, University of California, Berkeley). These AY MLO lines were originally described by J. H. Freitag (12) and have been maintained in the laboratory at Berkeley for more than 30 yr by transmission with *M. fascifrons*. The infectivity of the *M. fascifrons* leafhoppers used in this test was verified by removing them from the AY MLO-infected acquisition plant (aster) in groups of 10 and placing them sequentially on healthy asters, tomatoes, and asters. All aster and tomato plants were held and observed for symptom development for 3 mo.

DNA was extracted from healthy tomato plants and from periwinkle plants infected with the BLTVA MLO or AY MLO type lines as described in the previous section for the field isolates. Infected periwinkle plants were used rather than tomatoes because the DNA hybridization experiments were done before the insect inoculations were completed, so type line-inoculated tomato plants were not available.

Southern blot hybridizations. To determine the genetic relationships between the TBB MLO field isolates and BLTVA and SAY MLOs, we extracted DNA from the field isolates, electrophoresed it on 1% agarose gels in Tris borate-EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 20 mM ethylenediamine tetraacetic acid [EDTA]), and transferred it to nylon membranes (Nytran; Schleicher & Schuell, Keene, NH) following the method of Southern as described by Maniatis et al (30). Membranes were hybridized with a cloned 11-kb BLTVA MLO plasmid (40) that was radioactively labeled with ^{32}P-dATP using random oligoprimers (Multiprime Kit; Amersham Corp., Arlington Heights, IL). Probe DNA was stripped from the membranes by boiling the membranes in 0.01× saline sodium citrate (SSC) (1× SSC is 150 mM NaCl, 15 mM sodium citrate), 0.5% SDS. The blots were then hybridized with a ^{32}P-labeled recombinant plasmid containing a 4.1-kb fragment of the SAY MLO chromosome (pAYC4) (26). Membranes were then stripped again and hybridized with a ^{32}P-labeled, cloned 4.9-kb fragment of an SAY MLO plasmid (pPSA45) (25).

Cloned MLO plasmid DNAs that were used as hybridization probes were gel-purified as follows: ethidium bromide/cesium chloride (EtBr/CsCl)-purified recombinant plasmid DNAs were digested with the appropriate restriction enzymes, the digested DNAs were electrophoresed in a 1% agarose gel using 1× TAE buffer (40 mM Tris acetate, 1 mM EDTA), and the insert DNA bands were excised from the gels. The excised DNA was recovered from the gel with either an Elutrap (Schleicher & Schuell) or Gene Clean Kit (Bio 101, La Jolla, CA). The EtBr/CsCl-purified recombinant plasmid containing the SAY MLO chromosomal DNA (pAYC4) was linearized with *Eco*RI, and both insert and vector DNA were ^{32}P-labeled as previously described.

Hybridizations were performed in 50% formamide solutions at 42 C as previously reported (21). Initial posthybridization washes (30 min each) were of moderate stringency: two times in 2× SSPE (1× SSPE is 180 mM NaCl, 10 mM Na_2PO_4, 1 mM EDTA; pH 7.7), 0.1% SDS at 37 C and two times in 0.2× SSPE, 0.1% SDS, the first at 55 C and the last at 37 C. Because a small amount of cross-hybridization was observed on some samples when the membranes were probed with the cloned SAY MLO plasmid fragment, the wash stringency for these blots was increased by lowering the salt concentration to 0.1× SSPE, 0.1% SDS and raising the temperature to 65 C.

RESULTS

Insect transmission of field isolates and host plant responses. In three of the four transmission tests, healthy *C. tenellus* leafhoppers transmitted a virescence-inducing agent from symptomatic, field-collected tomato plants to periwinkle. No transmission occurred in the fourth test.

One of the three field isolates (70C) was transmitted by *C. tenellus* from infected periwinkle to the series of host plants, where it produced symptoms characteristic of those induced by the BLTVA, including floral gigantism of tomatoes (Fig. 1B), premature flowering (the HIR) of celery and daikon, and virescence of other species (Table 2). In 1986 tests, field isolate TBB 86 was transmitted by *C. tenellus* to daikon (10/20) and Chinese cabbage (18/20), and it caused the HIR in these hosts.

M. fascifrons did not transmit any virescence agents from either field-collected TBB MLO-infected tomatoes (0/6) or experimentally inoculated periwinkle (0/4).

MLO type line inoculations. Tomatoes inoculated with BLTVA line FC-83-13 developed the floral gigantism characteristic of TBB (Fig. 1C). Tomato plants infected with the DAY MLO (2/6) and TLAY MLO (4/6) lines showed chlorosis and severe stunting, and their leaves were red and distorted; however, none of the AY MLO-infected tomato plants developed either enlarged calyxes or virescent flowers (Fig. 1D). The AY MLO-infected

tomatoes eventually died, while the BLTVA MLO-infected plants remained alive in the greenhouse for the duration of the experiment. No tomato plants inoculated with the SAY MLO developed symptoms, even though aster test plants fed upon by all groups of SAY MLO inoculative leafhoppers, both before and after the inoculation access period on the tomato plants, developed typical SAY symptoms, indicating that the SAY MLO leafhoppers were inoculative.

Southern blot analysis. Autoradiographs of Southern blots revealed numerous bands of plasmid (extrachromosomal) DNA when DNA from symptomatic, field-collected tomato plants was hybridized with ^{32}P-labeled, cloned BLTVA MLO plasmid DNA (Fig. 2A). No extrachromosomal DNAs were detected in most of the samples when the membranes were hybridized with ^{32}P-labeled, cloned fragments of SAY MLO plasmids. However, five samples had extrachromosomal DNAs that hybridized with the SAY MLO plasmid probe even after the membranes were washed at the higher stringency (Fig. 2C). In two of these field isolates (70B and 54B), some of the plasmids that hybridized with the cloned SAY and BLTVA MLO plasmids had the same electrophoretic mobilities (Fig. 2B and C), which suggests that these MLO plasmids had some homology with both SAY and BLTVA MLO type plasmids. In other samples, such as 70A and 70C, the plasmids that hybridized with the cloned SAY MLO plasmid probe had different mobilities than those that hybridized to the cloned BLTVA MLO plasmid probe, indicating that the cross-hybridizing plasmids are probably not the same in these samples. Except for sample 54B (Fig. 2C), those plasmids that cross-hybridized (samples 70A, B, and C) hybridized more strongly with the cloned BLTVA MLO plasmid probe than with the cloned SAY MLO plasmid probe. None of the samples that cross-hybridized with BLTVA and AY MLO plasmid probes hybridized with the cloned fragment of SAY MLO chromosomal DNA (*data not shown*).

Neither of the cloned SAY MLO plasmid or chromosomal probes hybridized with the BLTVA MLO type line, which agrees with previous reports (25,26). Similarly, the cloned BLTVA MLO plasmid probe did not hybridize with the AY MLO type lines (40). No hybridization occurred between any MLO probe and DNA from healthy tomato or periwinkle.

One of the field isolates with cross-hybridizing plasmids (70C) was transmitted by *C. tenellus* from symptomatic, field-collected tomato to periwinkle and from the experimentally inoculated periwinkle back to healthy tomatoes and the host range plants (Table 2). This

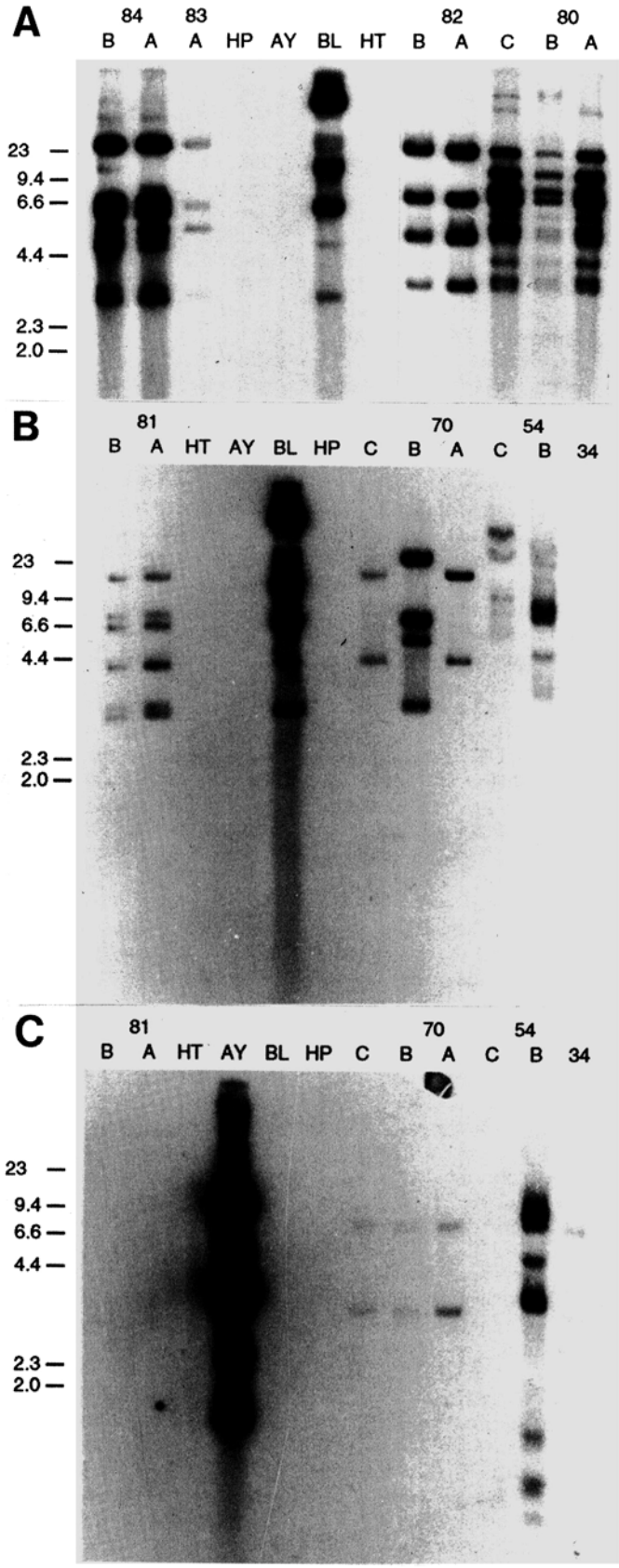

Fig. 2. Southern blot analysis of extrachromosomal DNAs in field-collected tomato plants with typical big bud symptoms. DNA was extracted from the samples, electrophoresed in 1% agarose gels, transferred to nylon membranes, and hybridized with a ^{32}P-labeled, cloned beet leafhopper-transmitted virescence agent (BLTVA) mycoplasmalike organism (MLO) plasmid **(A and B)** or a cloned fragment of aster yellows MLO plasmid **(C).** Membranes were washed under stringent conditions following hybridization. Numbers 34, 54, 70, and 80–84 are the accession numbers presented in Table 1. Letters A–C refer to individual plants taken from the same location. BL = tomato inoculated with BLTVA MLO type line FC-83-13; AY = periwinkle inoculated with severe aster yellows MLO; HP = healthy periwinkle; HT = healthy tomato. Molecular size markers are λ *Hin*dIII fragments.

isolate was not transmitted by *M. fascifrons*. Isolate 70C caused symptoms characteristic of BLTVA MLO infection on the host range plants and did not produce any symptoms characteristic of AY MLO infection.

DISCUSSION

On the basis of biological and genetic evidence, we conclude that the BLTVA MLO is the primary causal agent of TBB in California. MLOs in field-collected plants with symptoms of TBB were transmitted by *C. tenellus* and not by *M. fascifrons*. Field-collected TBB isolates also caused the HIR, a biological property associated with the BLTVA MLO but not with the AY MLO (13). Thus, these two biological properties of TBB isolates are indicative of the BLTVA MLO but not the AY MLO. In addition, Southern blot hybridizations showed strong homology between TBB MLO plasmids and a cloned plasmid from the BLTVA MLO type line. Weak or no hybridization occurred between DNA from TBB field plants and a cloned fragment of SAY MLO plasmid DNA, and no hybridization was observed between TBB sample DNAs and a cloned fragment of SAY MLO chromosomal DNA. In addition, restriction fragment length polymorphism (RFLP) patterns, using cloned MLO 16S rRNA genes as probes, of field-collected TBB plants and BLTVA MLO type lines were identical and were distinct from the RFLP patterns of western AY MLO isolates (40).

TBB MLO plasmids that cross-hybridized with the cloned fragment of SAY MLO plasmid DNA used as a hybridization probe may be present in low titer, or they may have weak homology with the cloned SAY MLO plasmid fragment. Cloned fragments of SAY MLO plasmids used in this study hybridize with extrachromosomal DNAs from virescence-inducing MLO isolates obtained from diverse hosts and geographic locations (25). Western AY MLO lines and the maize bushy stunt (MBS) MLO have plasmids that cross-hybridize even though the MLOs are transmitted by mutually exclusive insect vectors with different native plant host and geographic ranges (25). Therefore, it is unlikely that the plasmids from the AY MLO and the MBS MLO were recently exchanged in dually infected plants or insects. However, because tomato can be a host of both the BLTVA and AY MLOs, it is theoretically possible that plasmids could be transferred between these two MLOs in planta. Although transfer of plasmids between MLOs has not been demonstrated, interspecies plasmid transfer is not uncommon among phytopathogenic bacteria (32). The cloned AY MLO plasmid fragment did not hybridize with healthy tomato plant DNA, so the cross-hybridization is unlikely to be of plant origin. Alternatively, the field-collected TBB plants could have been dually infected with another uncharacterized MLO; however, no atypical 16S rRNA RFLP patterns were observed in the TBB plants (40).

Sample 34 had plasmid DNA that hybridized weakly to cloned SAY MLO plasmid DNA but did not hybridize to cloned BLTVA MLO plasmid fragments or the cloned AY MLO chromosomal fragment. This plant had extremely small leaves, no flowers, and considerable stem proliferation, which made it appear very bushy, rather than typical TBB symptoms. This sample may have been infected with an AY MLO but at a concentration so low that the chromosomal DNA, which is present in lower concentration than the plasmid DNA, was not detected (8,18,24,37). Because the symptoms of sample 34 were distinctly different from those caused by either BLTVA or western AY MLO type lines, it is also possible that this field-collected plant was infected with an uncharacterized MLO.

Freitag (11) demonstrated that *Macrosteles* sp. transmitted AY MLO from field-grown tomato plants with symptoms of stunting, yellowing, and thickened, stiff foliage to plantain. The inoculated plantains developed symptoms typical of AY MLO infection. Tomatoes inoculated with these same AY MLO type lines in the laboratory developed the same symptoms of stunting and chlorotic leaves with purple veins that Freitag originally described. No symptoms resembling TBB were observed in any of the 224 plants of 12 cultivars tested by Freitag. In our experiments, symptoms of tomato plants infected with DAY or TLAY MLO were similar to those reported by Freitag. No flowers were observed on the infected plants. Raymer and Milbrath (34) reported that *Macrosteles* sp. leafhoppers transmitted the MLO that causes potato late-breaking disease to tomato and that there was no enlargement of the calyx as seen in TBB. Kunkel (23) reported that graft transmission of AY MLO to tomatoes caused a witches'-broom, but he did not describe the effect on flower production. Our plants were inoculated when they were very small, and they were subsequently kept in a greenhouse. It is possible that under field conditions, or if more mature plants had been inoculated, some flowering would have occurred. However, Freitag (11) did not mention the occurrence of flowers (normal or abnormal) on the naturally field-infected plants, and his attempts to inoculate plants in the field failed.

Infection by the BLTVA MLO has also caused losses in crops other than tomato. For example, the BLTVA MLO causes a premature flowering of carrots that results in a woody, unmarketable taproot (41). At present, the overall incidence of BLTVA MLO in California is low. This may be due in part to control of *C. tenellus* populations as a result of the state's curly top virus control program. If leafhopper control programs were reduced or discontinued, vector populations could increase and economic damage to tomato and other crops could result. Leafhopper populations apparently reached high levels in eastern Washington and Oregon in the fall of 1990, and much of the daikon and red radish seed crop was lost in fields because of high levels of BLTVA MLO infection (36).

The BLTVA MLO appears to be the primary causal agent of TBB in California. Further studies are necessary to identify the MLOs that cause TBB in other regions of the world.

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