Accumulation and Translocation of Tomato Yellow Leaf Curl Virus (TYLCV) in a Lycopersicon esculentum Breeding Line Containing the L. chilense TYLCV Tolerance Gene Ty-1

Ilana Michelson, Dani Zamir, and Henrek Czosnek

Department of Field and Vegetable Crops and the Otto Warburg Center for Biotechnology in Agriculture, Faculty of Agriculture, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel.
Corresponding author: H. Czosnek; telephone: 972 8 481249; fax: 972 8 468265; e-mail: czosnek@hujiagri.ac.il
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ABSTRACT


The major tomato yellow leaf curl virus (TYLCV) tolerance gene, Ty-1, of the wild tomato species Lycopersicon chilense was mapped to chromosome 6 by using restriction fragment length polymorphism (RFLP) markers and introgressed into the domesticated tomato L. esculentum. Two nearly isogenic breeding lines were obtained. In infected fields, plants from line 52, which contained the Ty-1 allele, remained symptomless (tolerant line); plants from line 50, which did not contain the Ty-1 allele, were all symptomatic (susceptible line). The effect of the Ty-1 gene on TYLCV DNA accumulation and translocation was investigated by hybridizing plant DNA extracts with a viral DNA probe. After inoculation with a small number of viruliferous whiteflies (three insects per plant), plants of the tolerant line 52 had barely detectable levels of TYLCV DNA compared with plants of the susceptible line 50. Agroinoculation of whole plants showed that similar amounts of TYLCV DNA accumulated in both lines. The long-distance movement of TYLCV was impaired in the tolerant plants. After inoculation of the youngest leaf on each plant with 50-70 whiteflies, TYLCV DNA accumulated in the inoculated leaves of plants from both lines, although at a slower rate in plants of line 52. In plants from susceptible line 50, the viral DNA moved from the site of inoculation to the neighboring leaves and to the roots, in contrast to plants from the tolerant line, where it remained confined to the inoculated leaf. Western blot analysis of proteins from the inoculated plants with an antiserum against TYLCV confirmed that the movement of the capsid protein was associated with that of the viral DNA. Removal of all leaves except the inoculated youngest leaf hastened the transport of virus towards the roots of the susceptible plants but not of the tolerant plants. Plants of line 50 developed symptoms in all experiments, while plants of line 52 always remained symptomless. Therefore, the Ty-1 gene is associated with inhibition of disease symptoms; and at low titer inoculum, viral accumulation is significantly reduced in inoculated tissue. At high titer inoculum, viral long-distance translocation is limited.

Additional keywords: virus movement, virus replication, virus resistance.

Tomato yellow leaf curl virus (TYLCV) is a whitefly-transmitted monopartite geminivirus (5,19). It infects tomato cultivars in fields and greenhouses, causing up to 100% losses in crop production in many countries in the Mediterranean Basin, Africa, and Southeast Asia (7). There has been considerable effort toward the breeding of resistant cultivars. Some accessions of wild tomato species have served as sources for the introgression of resistance into the domesticated tomato (Lycopersicon esculentum Mill.). Depending on the plant source, resistance was reported to be controlled by one to five genes, either recessive or dominant (29). The first commercial tolerant cultivar, TY20, resulting from the introgression of resistance from L. peruvianum into L. esculentum (22), showed delayed symptoms and accumulation of viral DNA (25).

Characterization of host resistance genes can provide important insight into the mechanisms of plant-virus recognition, the signaling of host response, and the molecular basis of viral disease. To understand the genetics and the molecular basis of resistance to TYLCV, we mapped the main resistance gene (Ty-1) of the immune wild tomato species L. chilense LA969 by using restriction fragment length polymorphism (RFLP) markers and introgressed it into a tomato cultivar (30). Two nearly isogenic breeding lines that differ only in the L. chilense chromosome segment associated with resistance to TYLCV were developed by RFLP-assisted selection. Line 52, which is homozygous for the L. chilense Ty-1 allele, rarely developed symptoms upon whitefly-mediated inoculation in the field and in the laboratory. When present, symptoms were very mild and much delayed. Line 50, which does not contain the L. chilense Ty-1 locus, was fully symptomatic.

The accumulation and long-distance movement of TYLCV in the tolerant and susceptible tomato lines were compared in order to understand the effect of a single locus on resistance of plants to viruses. Our experiments show that the presence of the Ty-1 allele is associated with a dramatic reduction in virus accumulation and long-distance movement.

MATERIALS AND METHODS

Nearly isogenic tomato lines tolerant (line 52) and susceptible (line 50) to TYLCV. L. esculentum (cultivar M82-1-8) was crossed as a female parent with L. chilense LA969, and the interspecific hybrid was backcrossed as a male parent to L. esculentum. Offspring were grown in TYLCV-infected fields, and symptomless plants were selected. The presence of the L. chilense chromosomal segment(s) was verified by RFLP analysis during the breeding process. Selfing and backcrossing together with mapping analysis were employed to produce two nearly isogenic BC3S3 lines, 50 and 52. Line 52 contains a single detectable L. chilense chromosome segment of about 15 centimorgans mapped to chromosome 6 and bearing the Ty-1 locus. Field tests showed that this is the only segment necessary to confer tolerance to TYLCV (30). Line 50, which does not contain this allele, was the susceptible control. In TYLCV-infected fields, plants of line 52 remained symptomless (tolerant line), while plants from line 50 developed typical symptoms (susceptible line).

Whitefly-mediated inoculation of tomato plants. Virus cultures were maintained in tomato plants (L. esculentum ‘FA144’).
Whiteflies were maintained on cotton plants (Gossypium hirsutum ‘Akala’) grown in wooden cages held in an insect-proof growth chamber. Virus was acquired by the whitefly vector after an access period of 24 h on a TYLCV-infected tomato plant (31). Inoculation of tomato plants at the four-leaf stage was achieved either by exposing the whole plant to the insects in a closed cage or by exposing the youngest true leaf (leaf 1, 1 cm long or longer) of each plant to the insects in a leaf cage. After 48 h of feeding, the plants were grown in an insect-proof greenhouse and sprayed biweekly with serprenatrin (Smash).

Agroinoculation of tomato plants. Agrobacterium tumefaciens At:pTY4 containing a tandem repeat of the TYLCV genome (19) was used for agroinoculation. Tomato plants were agroinoculated at the four-leaf stage by making a 1 cm-long longitudinal incision between the cotyledons and the oldest (fourth) leaf and applying 100 μl of an Agrobacterium culture (at various concentrations) on the wound.

Analysis of plant tissues for the presence of TYLCV DNA. DNA extracts prepared from tomato leaves were submitted to gel electrophoresis and blotted onto nylon-based membranes as described (6). Leaves and whole plants were squashed onto the membranes with a glass rod; stems were either longitudinally cut or serially sliced from the apex to the roots and impregnated on the membrane (18). Membranes were hybridized (19) with a radiolabeled full-length TYLCV DNA and washed twice for 30 min in 1X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C before autoradiographic exposure. Autoradiograms were quantified with a densitometer and the ImageQuant software, version 3.22 (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis of TYLCV capsid protein. Polyclonal antiserum raised in rabbits against a purified preparation of TYLCV was the gift of B. Gronenborn (C.N.R.S., Gif sur Yvette, France). The antiserum was cross-absorbed with acetone-washed powder of noninfected tomato leaves before use. Young leaf tissue (250 mg) was triturated in 250 μl of protein loading buffer (10 mM Tris HCl [pH 6.8], 2% [w/v] sodium dodecyl sulfate [SDS], 10% [v/v] glycerol, 0.001% [w/v] bromophenol blue, and 4% [v/v] 2-mercaptoethanol) with a micropestle in an Eppendorf tube. Twenty-five microliters from the cleared extract was boiled for 5 min and applied to a 10% SDS-polyacrylamide gel (15). After electrophoresis, proteins were transferred (26) onto wetted Hybond C membranes (Amersham, Buckinghamshire, England) in semidry transfer buffer (39 mM glycine, 48 mM Tris, 0.004% SDS, and 20% methanol) with a semidy transfer cell (Hoefer, San Francisco, CA). The membranes were blocked with phosphate-buffered saline containing 10% (w/v) dried skimmed milk (PBS-M), washed, and incubated in PBS-M with the TYLCV antiserum diluted 1:1,000. After the membranes were washed, antigen-antibody complexes were detected with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins by the enhanced chemiluminescence procedure (ECL Western blotting, Amersham).

RESULTS

Rate of TYLCV DNA accumulation in sensitive and tolerant tomato plants after whitefly-mediated inoculation. Plants were caged with viruliferous insects, and the rate of TYLCV DNA accumulations in plants from the susceptible (line 50) and tolerant (line 52) lines after whitefly-mediated inoculation were compared. The differential responses of the two lines were seen when plants were inoculated with a low number of viruliferous whiteflies, which still ensured 100% infection of sensitive plants. Ten plants of line 50 and 10 plants of line 52 were caged for 48 h with three viruliferous whiteflies per plant. Symptoms were monitored every week during an 8-wk period. Presence of viral DNA was assayed 3, 5, and 8 wk after inoculation by hybridizing DNA from the youngest leaf with a viral DNA probe.

Viral DNA was barely detectable in plants of line 52 during the 8-wk experiment. Five of the plants of line 50 showed significant amounts of viral DNA 3 wk after inoculation. Eight weeks after inoculation, TYLCV DNA was barely discernible in only three plants of line 52, while all the plants of line 50 were infected (Fig. 1). All plants of line 52 remained symptomless, while all plants of line 50 showed heavy symptoms 8 wk after inoculation.

TYLCV DNA accumulation in agroinoculated tomato plants. The slow virus accumulation observed in plants of line 52 might have been caused by the inhibition of virus replication at the site of inoculation or by the inability of the virus to move out from the site of inoculation to the neighboring cells and to the vascular tissue. In order to investigate whether tissues of plants of line 52 could support viral replication, plants from line 52 where agroinoculated with various concentrations of Agrobacterium At:pTY4. Plants were injected in the stem with At:pTY4 at one of four concentrations: 10, 1, 0.1, or 0.01 OD540/ml. For each Agrobacterium concentration, five plants from each line were agroinoculated; five untreated plants from each line were used as a control. For a period of 4 wk after inoculation, symptoms were monitored, and TYLCV was probed every 7 days by hybridizing DNA from the youngest leaf with a viral DNA probe (Fig. 2).

Viral DNA was barely detectable 1 wk after agroinoculation. Two weeks after agroinoculation, large amounts of viral DNA accumulated in about half of the agroinoculated plants of line 50, independent of the concentration of Agrobacterium injected. At 2 wk, TYLCV DNA was detected only in those plants of line 52 that had been agroinoculated with 1 and 10 OD540 of At:pTY4 per milliliter. Three weeks after agroinoculation, most of the plants of line 50 showed strong hybridization signals at all concentrations of Agrobacterium inoculum, while plants of line 52 showed weaker hybridization signals. Four weeks after agroinoculation, plants of lines 50 and 52 showed similar amounts

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Fig. 1. Autoradiographic detection of tomato yellow leaf curl virus (TYLCV) DNA in tomato plants from the susceptible (50) and tolerant (52) lines after whitefly-mediated inoculation. Three, 5, and 8 wk after inoculation, total DNA was extracted from the first leaf of each of 10 plants (numbered 1 to 10) from both lines, blotet, and hybridized with a viral DNA probe. Each lane represents a different plant. OC, SC, and SS = open circular, supercoiled, and single-stranded DNA forms, respectively, of the TYLCV genome.

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of viral DNA, whether they were inoculated with a low or a high concentration of At: pTY4 inoculum (Fig. 2).

Plants of line 50 began to develop symptoms 10 days after agroinoculation. All plants of line 50 showed heavy symptoms 4 wk after agroinoculation. Plants of line 52 did not show any symptoms for the 4-wk period, independent of the concentration of the Agrobacterium inoculum. Therefore, TYLCV DNA can accumulate in the tolerant line at levels similar to those seen in plants of the susceptible line after a short period of inhibition.

Translocation of virus in whole tomato plants after inoculation of the youngest leaf with whiteflies. After inoculating the youngest leaf (leaf 1) of each plant with viruliferous whiteflies, the long-distance movement of TYLCV in plants of the susceptible and tolerant lines was monitored for 16 days. A large number of whiteflies were used to overcome the inhibition of virus accumulation observed when plants of the tolerant line 52 were inoculated with a small number of whiteflies (Fig. 1). Leaf 1 of 10 plants of each line was caged for 24 h with 50–70 viruliferous whiteflies. Two plants of each line were sampled 2, 4, 7, 9, 12, and 16 days after inoculation. The presence of TYLCV DNA was assayed by hybridizing tissue squashes with a virus-specific probe. The shoot apex, leaves 1–6, the upper stem (between leaf 1 and leaf 2), the middle stem (between leaf 4 and leaf 5), the lower stem (1–2 cm below leaf 6), and the roots were analyzed (Fig. 3).

Viral DNA was undetectable in plants from either line 2 days after inoculation. Four days after inoculation, small amounts of viral DNA were detected in the upper leaves of plants of lines 50 and 52. At that time, viral DNA was also detected in the roots of plants of line 50 but not of line 52. Seven days after inoculation, plants of line 50 showed strong hybridization signals in the shoot apexes, young leaves, and upper stems and weaker signals in the lower stems and roots. Weak hybridization signals were seen in young leaves and the upper stems of plants of line 52. Ten days after inoculation, strong hybridization signals were

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**Fig. 2.** Autoradiographic detection of tomato yellow curl virus (TYLCV) DNA in tomato plants from the susceptible (50) and tolerant (52) lines after agroinoculation. Plants were injected with one of four concentrations of Agrobacterium At::pTY4: 10, 1, 0.1, or 0.01 OD600/ml. For each concentration, four plants of line 50 and four plants of line 52 (numbered 1 to 4) were used. One, 2, 3, and 4 wk after agroinoculation, total DNA was extracted from each plant's first leaf, blotted, and hybridized with a viral DNA probe. Each lane represents a different plant. Markers on the right represent the mobility of the TYLCV DNA forms, from top to bottom: open circular, superflocculent, and single-stranded.

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**Fig. 3.** Autoradiographic detection of tomato yellow curl virus DNA in tissues of tomato plants from the susceptible (50) and tolerant (52) lines after inoculation of the first leaf. Two, 4, 7, 10, 12, and 16 days after whitefly-mediated inoculation, tissues of two plants of the susceptible line (designated 50-1 and 50-2) and two plants of the tolerant line (designated 52-1 and 52-2) were squashed onto a membrane with a 0.6-mm-diameter dot matrix (to ensure that squashes from the same organ contained equivalent amounts of tissue). The squashes were hybridized with a viral DNA probe. S = squashes of shoot apex; L1-L6 = squashes of leaf 1 to leaf 6; US, MS, and LS = squashes of cross sections of upper, middle, and lower parts of the stem, respectively; and R = squashes of roots.
observed in the apices and young leaves of plants of both lines but were detected in the stems and roots of plants of line 50 only (one plant of line 50 apparently escaped inoculation). Twelve days after inoculation, large amounts of viral DNA were detected in all the sampled tissues of plants from line 50, except leaf 6. At this time, the viral DNA was still confined to the upper leaves and upper stems of plants from line 52. The distribution of the viral DNA in the tissues of plants from the two lines did not change over time (Fig. 3). The quantitative analysis of these data (Fig. 4) confirmed that in the tolerant plants, the virus DNA was confined to the inoculated leaf (leaf 1) and to the upper part of the plant (shoot apex, upper stem, and leaf 2), while in the susceptible plants, it had spread to the older leaves, the lower part of the stems, and the roots. In all tissues tested, the concentration of virus DNA in the tolerant plants was generally lower than that in the susceptible ones.

Translocation of the TYLCV capsid protein in whole tomato plants was followed after the youngest leaf (leaf 1) was inoculated with viruliferous whiteflies as described above. The long-distance movement of the capsid protein in plants of the susceptible and tolerant lines was monitored by Western blotting of tomato tissues with an antiserum against a preparation of virions (Fig. 5). Three weeks after inoculation, virus capsid protein was detected in all the tissues sampled from plants of the susceptible line 50; large amounts were found in the young leaves, stems, and roots. In plants of the tolerant line 52, the capsid protein was confined to the inoculated leaf and to a lesser extent to the shoot apex. These results indicated that the TYLCV capsid protein was associated with the viral DNA in both susceptible and tolerant plants.

TYLCV movement along the stems of tomato plants after inoculation of the youngest leaf with whiteflies. In order to investigate whether leaves act as a barrier to the transport of the virus to the roots of plants of line 52, the translocation of the virus was monitored from the inoculated youngest leaf along the stem to the roots after all other leaves were removed. The youngest leaf of each of 12 plants of line 52 and six plants of line 50 was caged with 50–70 viruliferous whiteflies. All the leaves, except the inoculated one, were removed immediately after inoculation. Plants were monitored for the location of virus 3, 6, 9, 12, 17, and 21 days after inoculation. At each of these days, two plants of line 52 and one plant of line 50 were cut longitudinally, and both halves were squashed onto a nylon membrane. The squashes were hybridized with a virus-specific probe (Fig. 6).

Three days after inoculation, strong hybridization signals could be seen in plants of line 50 from the inoculated leaves along the stem to the roots. Weak hybridization signals were detected in the inoculated leaves of plants from line 52. Six days after inoculation, the intensity of hybridization signals was stronger in the inoculated leaves of plants of line 52. The intensity of the hybridization signal continued to increase gradually over time, and the signal spread to the upper portion of the stems in plants of line 52. Twenty-one days after inoculation, plants from line 52 showed strong signals in the inoculated leaves and in the upper portion of the stems, but the hybridization signal was barely

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**Fig. 4.** Quantitation of tomato yellow leaf curl virus (TYLCV) DNA in tissues of tomato plants from the susceptible (50) and tolerant (52) lines after inoculation of the first leaf (Fig. 3). Full bars represent plants from the sensitive line (left bar is plant 56-1, and right bar is plant 50-2). Empty bars represent plants from the tolerant line (left bar is plant 52-1, and right bar is plant 52-2). The height of each bar is proportional to the hybridization signal obtained with the TYLCV DNA probe. S = shoot apex; L1–L6 = leaves; US, MS, and LS = upper, middle, and lower parts of the stem, respectively; and R = roots.

**Fig. 5.** Western blot analysis of tomato yellow leaf curl virus (TYLCV) capsid protein in tissues of tomato plants from the susceptible (50) and tolerant (52) tomato lines probed with an antiserum against TYLCV 21 days after whitefly-mediated inoculation of the first leaf. S = shoot apex; L1–L5 = leaves; US, MS, and LS = upper, middle, and lower parts of the stem, respectively; and R = roots. Markers on the left represent mobility of proteins (in kDa). Arrows indicate the mobility of TYLCV capsid protein.
detectable in the lower portion of the stems and in the roots. The inoculated leaves of plants of line 50 began to show symptoms 2 wk after whitefly-mediated inoculation. Plants from line 52 remained symptomless during the 21-day experiment. These results show that the removal of all but the inoculated leaves accelerated the translocation of the virus along the stems to the roots of plants of line 50 but not of plants of line 52, where the virus was confined to the inoculated leaves and barely spread to the lower part of the stems.

**DISCUSSION**

In recent years, the investigation of plant resistance to viruses has focused on transgenic plants expressing viral coat proteins, satellite RNAs, antisense RNAs, ribozymes, and antibodies (12, 24). Unfortunately, plant resistance to viruses conferred by natural genes is still poorly understood, especially on the molecular level (10,11). The effect of the resistance genes has usually been determined by comparing susceptible and resistant plants, in most cases of unrelated genetic background. In this study, we have compared two nearly isogenic tomato breeding lines that differ in only one L. chilense chromosome segment containing the TYLCV tolerance gene Ty-1 (30). In another study (25), TYLCV DNA accumulation in tomato plants from tolerant breeding lines and from susceptible cultivars was compared. In these lines of various genetic backgrounds, tolerance was introgressed from L. peruvianum, where it is a polygenic trait not mapped onto specific chromosomes. The comparison of two nearly isogenic lines (susceptible and tolerant), differing only in a single mapped chromosomal segment, allows better understanding of the expression of a host viral disease resistance gene. The effect of the Ty-1 gene was observed at two levels: viral accumulation and long-distance translocation.

TYLCV DNA accumulation in plants of the tolerant line 52 was a function of the level of inoculum. When the level of inoculum was low, TYLCV DNA barely accumulated in leaves of plants of the tolerant line; when it was high, significant amounts of viral DNA were detected, but it accumulated at a rate slower than that in plants of the susceptible line 50. This dependence on virus titer inoculum was observed with two methods of virus delivery that differ notably. After whitefly-mediated inoculation, TYLCV replicates in the primary infected cells, moves to neighboring cells, and spreads throughout the plant. In the case of agro-inoculation, Agrobacterium disperses large amounts of double-stranded viral DNA into the plant tissues, bypassing the stages of virus uncoating, synthesis of the replicative form, and long-distance transport (13). Although the number of cells infected initially by agroinoculation is greater than that by whitefly inoculation, in both cases viral DNA may not be distributed evenly to all cells. While cell-to-cell spread may contribute to the increase of virus titer in susceptible plants, it may be inhibited in plants of the tolerant line, causing the apparent delay in accumulation of virus DNA.

In susceptible plants, TYLCV DNA moved from the inoculated youngest leaf to the four or five upper leaves and to the roots, the same route followed by assimilates (2). In contrast, TYLCV DNA translocation in tolerant plants was restricted to the second leaf and to the shoot apex. Reduced rate of cell-to-cell movement in the tolerant line may account for these results. If fewer cells are infected in systemically infected leaves of plants of the line 52, the rate of spread of virus to the root through long-distance transport would also be reduced, possibly to a point where it would not be detectable. The restriction of virus capsid protein to the shoot apex and youngest leaf of plants from the tolerant line confirms that virus long-distance translocation is inhibited in these plants. In sensitive plants, virus movement was accelerated by the removal of all but the inoculated leaf 1, probably because of a source-sink regulation effect (14). The inhibition of virus translocation was not alleviated in leaf-depleted tolerant plants, indicating that the inhibition of virus spread is not caused by an alteration in the stream of metabolites.

Inhibition of virus accumulation and/or virus short- and long-distance translocation are among the most conspicuous mechanisms of plant virus resistance (1). It has been proposed that this type of resistance corresponds to dominant alleles expressed constitutively (10). Although the effect of a single resistance gene

![Fig. 6. Autoradiographic detection of tomato yellow leaf curl virus DNA in tomato plants from the susceptible (50) and tolerant (52) lines after inoculation of the first leaf and removal of the other leaves immediately after inoculation. Three, 6, 9, 12, 17, and 21 days after inoculation, one plant of line 50 and two plants of line 52 were sectioned longitudinally, squashed onto a membrane, and hybridized with a viral DNA probe.](#)
in isogenic lines (sensitive and resistant) has not been reported yet to the best of our knowledge, results similar to ours have been obtained with pepper varieties inoculated with cucumber mosaic virus (20), where resistance depends on a combination of two or three genes (23).

We do not know how the TYLCV resistance gene Ty-I functions to suppress disease symptoms and virus translocation. TYLCV is apparently not modified in its essential virulent functions in tolerant plants of line 52, since it can be transferred by whiteflies from these plants to sensitive test plants to induce the normal disease profile of symptoms and accumulation of viral DNA (not shown). The Ty-I gene product may be targeted at viral factors necessary for TYLCV DNA cell-to-cell movement. When small amounts of viral DNA are inoculated, there are enough Ty-I products to inhibit cell-to-cell movement, resulting in an apparent high titer of viral DNA. When the level of inoculum is high, the Ty-I product is not sufficient to entirely block cell-to-cell transport of viral DNA, which results in a slow but significant spread and accumulation of viral DNA.

Many plant RNA viruses encode proteins that increase the gating capacity of plasmodesmata, allowing virus passage from cell to cell (8,17,28). In two bipartite whitefly-transmitted geminiviruses, African cassava mosaic virus and tomato golden mosaic virus, two proteins encoded by DNA B (BR1 and BLI) control the spread of virus (3,9) and may play a role in the induction of disease symptoms (21,27). It is possible that the Ty-I gene product inhibits the expression or interferes with the function of a similar, yet unidentified, TYLCV movement protein. Alternatively, the Ty-I gene product may be targeted at the host plant, modifying the gates by which the virus spreads within the plant and preventing the virus (or virus nucleoprotein) from reaching the roots and passing into the xylem.

The comparative analysis of the TYLCV-sensitive and TYLCV-tolerant lines will aid in the understanding of the mechanism of plant resistance to viral diseases. This will allow us to understand plant virus interactions at the molecular, physiological, and ultrastructural levels. It may also aid in the understanding of macromolecular trafficking in plants (4,16) and its regulation by using viruses, such as the phloem-restricted TYLCV, as probes.

LITERATURE CITED