Spread of *Tomato apical stunt viroid* (TASVd) in Greenhouse Tomato Crops Is Associated with Seed Transmission and Bumble Bee Activity

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ABSTRACT

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Tomato apical stunt viroid (TASVd) has been reported as a devastating pathogen of greenhouse tomato in Israel. This isolate shares 92 and 99% identity with the Ivory Coast type strain and an Indonesian strain, respectively. No information is available regarding the epidemiology of this viroid complex. The present study indicates that TASVd is not transmitted by the aphid *Myzus persicae* or the whitefly *Bemisia tabaci*, nor through root infection in infested soil. However, the results indicate that the viroid may be able to invade the embryonic tissues of the seed and transmission rates through seed may reach 80%. Moreover, it was confirmed that bumble bees (*Bombus terrestris*) can transmit the viroid from infected tomato source plants to healthy plants. Based on these findings, it is suggested that the primary spread of the viroid in greenhouse tomato plants is by seed transmission, and secondary distribution occurs by the pollination activity of bumble bees.

Viroids are the smallest known agents of infectious diseases of plants. The genomes of most viroids range in size from 246 to 399 nucleotides and consist of highly structured, single-stranded, circular RNA molecules that lack a capsid protein and detectable messenger RNA activity. Taxonomically, viroids are grouped into two families, the larger of which is the *Pospiviroidae*. This family includes several viroids that were found to cause disease in field- and greenhouse-cultivated tomato plants (4). Natural infections with the following viroids have been reported: *Tomato planta macho viroid* (TPMVd; 5), *Tomato apical stunt viroid* (TASVd; 1,21), *Potato spindle tuber viroid* (PSTVd; 3,19), a strain of *Citrus exocortis viroid* (CEVd; 10,19), *Tomato chlorotic dwarf viroid* (TCDVd; 17,19), and *Columnnea latent viroid* (CLVd; 19). The host symptoms of all these viroid diseases are similar, and include stunting, apical proliferation, apical leaf narrowing and yellowing, leaf crinkling, tissue brittleness, and necrosis (16).

TASVd first was found in Ivory Coast in tomato plants grown in small gardens (21). Based on its nucleotide sequence, it was classified as a member of the family *Pospiviroidae* (7). An Indonesian strain of TASVd that elicited severe symptoms in

tomato was sequenced by Candresse et al. (2) and was found to share 91.5% identity with the Ivory Coast strain of TASVd. A third distinct strain of TASVd was isolated from the ornamental plant *Solanum pseudocapsicum* and was found to have 92.1% sequence identity with TASVd-Ivory Coast. Although this viroid (designated TASVd-S) is considered to be a strain of TASVd and does infect tomato experimentally, it has not been found in tomato plants in nature (18). Another isolate, TASVd-Is, shared 92, 99, and 87% identity with the Ivory Coast type strain, the Indonesian strain, and TASVd-S, respectively. Since 1999, TASVd-Is has been found consistently in tomato greenhouses in Israel, where it causes a devastating disease in this crop (1). The lack of information regarding the epidemiology of TASVd is a limiting factor in developing controls against the pathogen. The present study was intended to characterize the epidemiological factors and mechanisms that affect the spread of TASVd in tomato greenhouses.

MATERIALS AND METHODS

Inoculum source. A laboratory culture of TASVd-Is was maintained on tomato (*Lycopersicon esculentum*) plants held in an insect-proof greenhouse. Transfers were performed by mechanical inoculation or by grafting.

Plant maintenance. All plants used in the experiments were grown in an insect-proof, air-conditioned greenhouse at temperatures ranging from 22°C at night to a maximum of 35°C during the day. Insecticides were applied once a week to prevent insect transmissions.

Identification of inoculum sources in nature. Various botanically identified weeds were collected from the open field, planted in plastic pots, sprayed against insects, and kept in an insect-proof greenhouse. Each plant was inoculated mechanically with crude sap extracted from TASVd-infected tomato plants and rubbed on carborundum-dusted plants. Two weeks later, the same plants were reinoculated to ensure infection. Two months post inoculation, the plants were screened for infection, visually and by Northern blot hybridization, with a TASVd-specific molecular probe as described below.

Tests of transmission via whiteflies and aphids. Virus-free whiteflies (*Bemisia tabaci*) from a laboratory culture were reared on cotton in an insect-proof, air-conditioned greenhouse at temperatures ranging between 25 and 35°C (night and day, respectively). Insects were introduced to TASVd-infected *Nicotiana rustica*, *Physalis floridensis*, and tomato source plants for 48 h of acquisition access. Then, groups of 40 insects were transferred to each of five individually caged healthy tomato plants for 48 h of inoculation access. Following inoculation, test plants were sprayed with an insecticide and kept in an insect-proof greenhouse pending the appearance of symptoms. Infection of the inoculated plants was tested by Northern blot hybridization.

Aphids (*Myzus persicae*) were maintained on Chinese cabbage in an air-conditioned growth chamber at a constant temperature of 25°C. The above-described insect transmission and viroid detection procedures also were employed for the aphid transmission tests.

Transmission via bumble bees. The ability of bumble bees (*Bombus terrestris*) to transmit TASVd was tested in a 50-mesh screenhouse (25 by 10 m). Tomato plants (var. Hazera 189) at the four-leaf stage were planted individually in 10-liter plastic containers and placed in three rows of 17 plants each. To prevent transmission by mechanical contact, the plants were separated from one another by 1.5 m, and pruning activity was minimized. In cases where pruning was required, tools were disinfected with 3% sodium hypochlorite solution before dealing with the next plant.

Two weeks after planting, the two plants at the opposite ends of each row were mechanically inoculated with TASVd. Three weeks later, all plants were sampled for

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viroid infection and were found to be negative, except those that were mechanically inoculated.

A commercial bumble bee hive harboring 50 insects (Bio-Bee, Biological Control Industries, Sedei Elyahu, Israel) was introduced into the screen house, and the plants were tested for viroid infection at 3-week intervals, starting 1 month after the introduction of bumble bees. To compensate for the relatively small forage area, the bumble bees were fed artificially from time to time by introduction of pollen into the hive.

Transmission via the soil. This aspect was examined in two ways. Tuff was collected from points close to the root systems of infected tomato plants growing in a commercial greenhouse, and 20 tomato seedlings (at the two-true-leaf stage) were planted into the tuff in plastic pots. The plants were held in an insect-proof greenhouse and were tested for the presence of the viroid by Northern blot hybridization 30 days after planting. Alternatively, a perlite growing medium was infested with an inoculum prepared by homogenization of 400 g of viroid-infected tomato leaves in 800 ml of 0.01 M phosphate buffer, pH

7. The inoculum was mixed with 8 liters of perlite before being packed in plastic pots. Twenty tomato seedlings were transplanted into the infested medium and kept in the greenhouse for 30 days before being tested for viroid infection.

Seed disinfestation. Tomato seed were harvested from naturally TASVd-infected plants or from plants that had been infected in the laboratory. To eliminate external contamination with the viroid, seed collected from fruit harvested from TASVd-infected plants were stirred for 30 min in glass beakers containing 40 ml of 10% trisodium phosphate (TSP) or 2% sodium hypochlorite. The seed then were washed in distilled water and dried on Whatman filter paper, and their total RNA was extracted and analyzed as described below.

Viroid detection in plant tissues and seed. Tomato plants at the four-leaf stage were inoculated mechanically with crude viroid preparations applied to carborundum-dusted leaves. One month post inoculation, samples of 0.1 g were collected from individual leaves along the tomato stem, as well as from the root system extraction of total RNA. To determine the

presence of the viroid in the flower organs, flowers from TASVd-infected and noninfected control plants were dissected and the various organs were separated and pooled in groups of five, prior to extraction of total-RNA. Groups of 10 seed each from infected and healthy tomato plants were used for RNA extraction.

The EZ-RNA Kit (Biological Industries, Beit Haemek, Israel) was used for the extraction of total RNA from leaves, flowers, and seed according to the manufacturer's instructions. Quantitative determination of total RNA in samples was done by a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

RNA separation on denaturing gels. Aliquots (20 μ l) of equal amounts of total RNA and 6 μ l of ethidium bromide were mixed with an equal volume of denaturing buffer containing 25 $\times$ 3(*N*-morpholino)-propanesulfonic acid (MOPS) (4%), di-formamide (50%), and di-formaldehyde (5%). Denaturation was carried out by incubation of samples at 65°C for 15 min, followed by incubation on ice for 5 min. Samples of total RNA were separated electrophoretically on a denaturing 1% agarose gel containing 1 $\times$ MOPS and 1.9% di-formaldehyde. Gels were run in 1 $\times$ MOPS buffer at 75 V for 3 h.

Northern blots and viroid detection. The separated RNA was blotted onto nitrocellulose membranes (Schleicher & Schuell, Germany) in the presence of 10 $\times$ SSC and kept overnight before hybridization with a TASVd-specific riboprobe prepared from a full-length TASVd-Is clone, as described previously (1). Prehybridization and hybridization were carried out as described before (1).

RESULTS

Screening for natural hosts of TASVd.

The following weeds were inoculated mechanically with crude TASVd inoculum: an *Amaranthus* sp., *Anagallis coerulea*, *Achusa aggregata*, *Beta vulgaris*, *Calendula arvensis*, *Chenopodium murale*, *Chrysanthemum coronarium*, *Crepis sancta*, *Erigeron crispus*, *Erodium cicutarium*, *Fumaria densiflora*, *Malva nicaeensis*, *Oxalis pes-caprae*, *Senecio vernalis*, *Sinapis arvensis*, *Sonchus oleraceus*, *Trifolium repens*, *Vicia angustifolia*, and *V. sativa*. None of these weeds were found to be hosts of TASVd.

Insect transmission of TASVd. Aphids and whiteflies are common pests of greenhouses and serve as vectors for many important viral diseases; therefore, it was important to determine whether they also were involved in the spread of TASVd. In all the experiments, both aphids (*Myzus persicae*) and whiteflies (*Bemisia tabaci*) failed to transmit the viroid (data not presented).

Transmission by bumble bees. Pollination by bumble bees is an integral part of greenhouse cultivation, and previous find-

Fig. 1. Northern blot analysis of total RNA extracted from tomato plants exposed to pollination activity of bumble bees in the presence of *Tomato apical stunt viroid*-infected source plants. Results of the first sampling date, 35 days after the introduction of the hive. Lane 1 = healthy tomato plant, lane 2 = infected tomato plant, lanes 3–15 = tomato plants exposed to pollination activity of bumble bees, and M = marker DNA.

Fig. 2. Northern blot analysis of total RNA extracted from tomato leaves and flowers to monitor the distribution of *Tomato apical stunt viroid* in mechanically inoculated tomato. Lane 1 = infected control tomato plant; lane 2 = healthy control tomato plant; lanes 3–10 = leaves from the lower to the upper plant parts, respectively; lanes 11–13 = flowers; and M = marker DNA.

ings of PSTVd pollen transmission in potato and tomato with pollinator bees (3,8) raised the possibility that TASVd was transmitted similarly. This option was examined as described in Materials and Methods. The spread level of TASVd from the mechanically inoculated source plants to neighboring healthy tomato plants 35 days after the introduction of the bumble bees is shown in Figure 1. Three months later, the recorded accumulated disease incidence in the screenhouse reached 30% (data not presented).

The spread of TASVd in tomato plant tissues. A set of experiments was performed to characterize the patterns of viroid spread within the tissues of infected tomato plants, including leaves, flowers, and roots.

Northern blot analysis, performed 1 month post inoculation, indicated that the distribution of the viroid among leaves at different positions along the tomato stem was not uniform, and the viroid was absent from lower leaves and from some of the flowers tested (Fig. 2). Variation in the titer of the viroid nucleic acid was noted in the sampled leaves (Fig. 2).

Test results confirmed the presence of the viroid in flower organs, including sepals, petals, ovaries, and stamen sacs of flowers collected from viroid-infected plants (Fig. 3). TASVd also could be detected in roots of infected tomato plants (data not shown).

Transmission through soil. Plants were examined 1 month after exposure of the root system to viroid-infested media. No viroid transmission occurred when the roots were exposed to naturally or artificially infested medium (data not shown).

Transmission by seed. When 30 tomato seedlings that originated from seed that had been collected from fruit of TASVd-infected tomato plants were allowed to grow in the greenhouse, 80% were infected by the viroid (data not presented).

Seed can be infested as a result of external contamination of the integument, as well as through invasion of the viroid into embryonic tissues. Tomato seed were surface disinfested with 10% TSP or 2% sodium hypochlorite as described in Materials and Methods. The viroid was present in untreated seed that had been collected from TASVd-infected tomato plants but not in those from healthy control plants or after a disinfestation treatment (Fig. 4), indicating that external seed contamination is possible. Disinfestation of seed harvested from TASVd-infected plants could not prevent viroid transmission to progeny seedlings (Fig. 5), indicating that the viroid was able to invade the embryonic tissues of the seed.

DISCUSSION

No information was available on the epidemiological factors involved in the spread of TASVd in greenhouses. In the

present study, we tested several hypotheses that had been proposed by other workers to explain the spread of viroids in nature. Galindo et al. (5) reported on the transmission of TPMVd by the aphid *M. persicae*. In a later study (13), it was found that this aphid could transmit PSTVd from potato plants that were doubly infected with this viroid and with *Potato leaf roll virus* (PLRV). In this case, transmission occurred through packaging of the viroid nucleic acid in the PLRV virion. We cannot exclude the possibility that a similar mechanism occurs in mixed infections by TASVd and an isometric plant virus (e.g., *Cucumber mosaic virus*). However, the present study yielded no evidence to support this hypothesis or that of direct transmission of the viroid by *M. persicae* or *B. tabaci*.

The presence of TASVd in the flower parts or in the root system may have an epidemiological significance. It has been suggested that PSTVd, one of the most studied viroids, spreads within tomato plants in a pattern similar to photoassimilate transport through the phloem (12). Zhu et al. (23) demonstrated that PSTVd was absent from leaves that were acting as sources of assimilates at the time of inoculation, and that it was transferred only into leaves acting as sinks, and did not invade the shoot apical meristem. The absence of the viroid from lower leaves (Fig. 2) may result from their functional status as source leaves, thus supporting the notion of a phloem-spreading paradigm.

PSTVd was found in sepals of infected tomato plants but not in other floral parts,

Fig. 3. Northern blot analysis of total RNA extracted from flower organs collected from *Tomato apical stunt viroid*-infected tomato plants. Lane 1 = healthy ovaries, lane 2 = healthy sepals, lane 3 = healthy petals, lanes 4–6 = sepals from flowers of infected plants, lanes 7–8 = ovaries from flowers of infected plants, lanes 9–10 = stamen sacs from flowers of infected plants, lanes 11–13 = petals from flowers of infected plants, and M = marker DNA.

Fig. 4. Northern blot analysis of total RNA from treated and untreated seed extracted from fruit of plants infected with *Tomato apical stunt viroid*. Lanes 1–3 = healthy seed, lanes 4–6 = untreated seed, lanes 7–9 = 10% trisodium phosphate-treated seed, lanes 10–12 = 2% sodium hypochlorite-treated seed, and M = marker DNA.

Fig. 5. Northern blot analysis of total RNA extracted from tomato seedlings germinated from seed extracted from fruit of plants infected with *Tomato apical stunt viroid*. Lane 1 = healthy tomato, lane 2 = TASVd-infected leaves, lanes 3–8 = seedlings originating from untreated seed, lanes 9–18 = seedlings originating from 10% trisodium phosphate-treated seed, and M = marker DNA.

despite the existing vascular connections among floral parts and the plant (23,24). These findings are in sharp contrast to the results presented, which demonstrated the presence of TASVd in all tested flower parts (Fig. 3). These contradictions may be attributable to the less sensitive in situ hybridization protocol that was used by Zhu et al. (23,24).

Seed transmission has been reported for some viroids, and it probably is highly significant in their epidemiology. Seed transmission has been reported for the following viroids: *Avocado sunblotch viroid* (ASVd; 20), *PSTVd* (3,14), *Chrysanthemum stunt viroid* (CSVd), *Hop stunt viroid* (HSVd; 8), *Coconut cadang-cadang viroid* (CCCVd; 11,22), *Apple scar skin viroid* (ASSVd; 6), *Coleus blumei viroid* (CbV; 15), and *Grapevine yellow speckle viroid* (GYSVd; 22).

The results presented demonstrate clearly that TASVd is efficiently transmitted via tomato seed (Fig. 5) and, in some cases, transmission rates via seed from mechanically inoculated tomato plants were close to 80%. This mode of transmission probably is responsible for the primary infection in commercial greenhouses. Disinfestation treatments reduced the signal from infested seed preparations below the detection limits (Fig. 4) but could not prevent its transmission to progeny plants (Fig. 5). The inability to detect the viroid following disinfestation treatment (Fig. 4) probably was due to its low titer in the embryonic tissues.

This study proves, for the first time, that bumble bees are involved in the secondary spread of TASVd in greenhouses (Fig. 1), in addition to the efficient secondary spread via workers' infested hands and tools (16). The transmission by bumble bees may result from the wounding of flowers during visits of the insects, as was reported for the *Pepino mosaic virus* (PepMV; 9), or from the introduction of infected pollen to the stigma, from which the viroid could enter the embryo after the fusion of the sperm cells with the ovules. Pollen transmission of TASVd is still awaiting confirmation.

Based on the results presented here, we assume that the initiation of epidemics within greenhouses starts from plants

originating from infected seed and that secondary spread is facilitated by plant injury by workers activity (16) as well as by flower visits of bumble bees. The presence of a natural host of TASVd is still obscure; however, it is possible that the invasion of TASVd into tomato cultures is facilitated by some pollinating insects that transmit the viroid either by pollen from infected source plants or by inoculum introduction during wounding of visited flowers.

The potential of TASVd to damage tomato crops is very high because of its rapid spread and its devastating effects on the plants (1). The most efficient way to eliminate this threat would be to establish an indexing system that would prevent the use of the TASVd-infested seed batches that serve as initiators of TASVd epidemics.

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