Eur J Plant Pathol (2010) 126:111–115
DOI 10.1007/s10658-009-9515-2

Transmission of *Tomato chlorotic dwarf viroid* by bumblebees (*Bombus ignitus*) in tomato plants

Shohei Matsuura · Yosuke Matsushita · Reiko Kozuka · Sachiko Shimizu · Shinya Tsuda

Received: 12 April 2009 / Accepted: 2 July 2009 / Published online: 15 July 2009

Abstract Quantitative PCR revealed that *Tomato chlorotic dwarf viroid* (TCDVd) was present in substantial amounts in viroid-infected tomato flowers. Healthy tomato plants were arranged in two different glasshouses, and plants were mechanically inoculated with TCDVd. Bumblebees (*Bombus ignitus*) were then introduced into the glasshouses to reveal whether the viroid was transmitted from infected source plants to neighbouring healthy plants. TCDVd infection was found in neighbouring tomato plants more than 1 month after the introduction of the bees, some of which expressed symptoms, in both glasshouses. Thus, bumblebees transmitted TCDVd from tomato to tomato by pollination activities.

S. Matsuura (✉) · S. Shimizu
Hiroshima Prefectural Technology Research Institute,
Agriculture Research Centre,
Higashihiroshima, Hiroshima 739-0151, Japan
e-mail: s-matsuura81314@pref.hiroshima.lg.jp

Y. Matsushita
National Institute of Floricultural Science,
Tsukuba, Ibaraki 305-8519, Japan

R. Kozuka
Chiba Prefectural Agriculture and Forestry Research Centre,
Chiba-shi, Chiba 266-0006, Japan

S. Tsuda
National Agricultural Research Centre,
Tsukuba, Ibaraki 305-8666, Japan

Keywords Anther · Pollination · *Potato spindle tuber viroid* · Quantitative PCR · Rutgers

Viroids are the smallest known pathogens. They consist of small, highly structured, single-stranded RNA molecules that lack a capsid protein and detectable messenger RNA activity (Diener 2001). There are two viroid families: *Pospiviroidae* (five genera) and *Avsunviroidae* (three genera). The genus *Pospiviroid*, in the family *Pospiviroidae*, contains nine species, eight of which infect solanaceous plants. Of these, *Tomato planta macho viroid* (TPMVd; Galindo et al. 1982), *Tomato apical stunt viroid* (TASVd; Antignus et al. 2002), *Potato spindle tuber viroid* (PSTVd; Mumford et al. 2004; Verhoeven et al. 2004), *Citrus exocortis viroid* (CEVd; Verhoeven et al. 2004), *Tomato chlorotic dwarf viroid* (TCDVd; Singh et al. 1999), and *Columnea latent viroid* (CLVd; Verhoeven et al. 2004) naturally infect tomato plants (*Solanum lycopersicum*, formerly *Lycopersicon esculentum*).

TCDVd, which is closely related to PSTVd, infects ornamental plants (James et al. 2008; Matsushita et al. 2009; Singh and Dilworth 2009) and causes economic damage to tomato production, with severe symptoms such as small fruit, dwarfing, leaf chlorosis, and vein necrosis. TCDVd first occurred in Canada in tomato seedlings grown from seed imported from the Netherlands via the USA (Singh et al. 1999). In Japan, TCDVd caused severe damage to tomato plants in commercial greenhouses in Hiroshima and Chiba

prefectures in 2006 (Matsushita et al. 2008). Although TCDVd has since been eradicated in Japan, it is important to assess its transmissibility by bumblebees in tomato greenhouses in order to develop a control strategy and to estimate the risk of its dispersal over long distances, since tomato plants are often pollinated by bumblebees. In this study, we clarified that TCDVd is transmitted by bumblebees (*Bombus ignitus*) from tomato to tomato via pollination.

We first investigated TCDVd levels in each organ of tomato plants, as these influence its transmissibility via insect vectors. Tomato leaves infected with TCDVd (GenBank/EMBL/DDBJ accession no. AB329668) were ground in 0.2 M phosphate buffer (pH 8). The homogenate was inoculated by razor blade into the stems of four tomato plants (cv. Rutgers) at the 3rd to 4th true leaf stage. The plants, growing in plastic pots containing a sandy soil and bark compost mixture, were maintained in a glasshouse at 25 to 35°C under a natural photoperiod. Various organs of the plants were collected 33 days after inoculation. Total RNA was extracted from 20 mg of each organ by RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and dissolved in 100 µl water. Total RNA was quantified by spectrophotometry at 260 nm. Complementary DNA was synthesised from 1 µl template RNA using a ReverTra Ace First Strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). TCDVd was quantified by TaqMan gene expression assay: The sequences of the forward (66F 5′-ATCCC GAGAGAAACAGGGTTTT-3′) and reverse (184R 5′-AGTTGTTTCCACCGGGTAGTTG-3′) primers were based on the sequence of the TCDVd. This primer pair amplifies a 119-bp DNA product. TaqMan probe 96 T (5′-FAM-CTTTCTGATTCGGTTTCC-MGB-3′) was based on a sequence located between the primers. Polymerase chain reaction (PCR) mixtures contained 10 µl TaqMan gene expression master mix, 900 nM each primer (66F and 184R), 250 nM TaqMan probe, and 1 µl cDNA template in a total volume of 20 µl. The quantitative PCR assay was conducted with a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with a thermal cycling programme of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The amount of TCDVd within each organ of the plants was determined by comparison with a standard curve generated using the same primers and varying concentrations of clone with TCDVd sequence.

Tomato plants developed typical symptoms of severe stunting, leaf chlorosis, leaf curl in the upper leaves, and small flowers 33 days after inoculation (2nd flower bud stage). Although a significant difference was observed between stems (around the middle leaf) and anthers, >100 pg of TCDVd μg^{-1} of total RNA was detected in all organs, indicating viroid replication throughout the plants, including floral parts (Fig. 1). This result agrees with a previous report that the closely related PSTVd was present in the floral organs of various plants, including tomato (Singh 2006), and raised the possibility of TCDVd transmission by bumblebees.

To determine the transmissibility of TCDVd by bumblebees, we next investigated whether it was transmitted from infected tomato plants to neighbouring healthy plants in two glasshouses (each 6 m×6 m). Fifteen plastic containers (25 cm×25 cm×37 cm) of a sandy soil and bark compost mixture with IB compound fertiliser (2 g l^{-1}) were arranged in three rows of five at a spacing of 1.0 m×1.8 m (Fig. 2). We used cv. House Momotaro as inoculum source plants instead of highly susceptible Rutgers in order to secure flowering on infected source plants. Five healthy plants of House Momotaro comprised the middle row, and ten healthy plants of Rutgers comprised the two flanking rows. TCDVd was inoculated into the top leaves of all plants

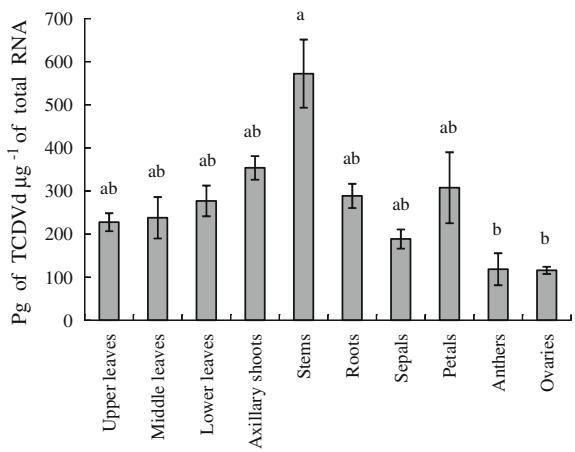

Fig. 1 Quantity of *Tomato chlorotic dwarf viroid* (TCDVd) in each organ of tomato cv. Rutgers. Vertical bars indicate standard errors of four replicates. Means followed by different letters are significantly different by Kruskal-Wallis test ($P<0.05$)

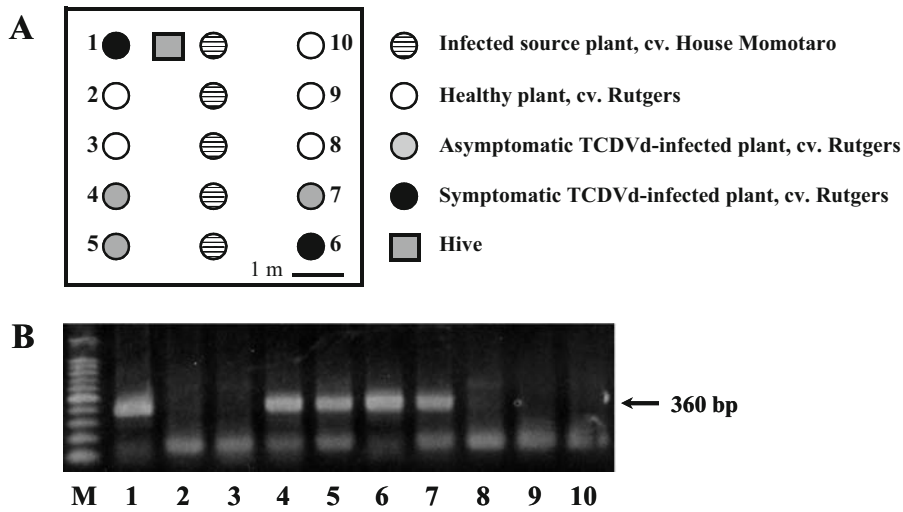

Fig. 2 Transmission of TCDVd from tomato to tomato by bumblebees (*Bombus ignitus*) 52 days after introduction in a glasshouse (6 m×6 m). A: Spatial distribution of TCDVd infection in plants. B: Detection of TCDVd in plants by RT-PCR. Lanes 1–10, plants neighbouring infected source plants. M, 100-bp DNA ladder

of House Momotaro with carborundum at the 1st flower bud stage in mid-October in order to produce sources of infection. A bumblebee (*B. ignitus*) hive (~50 individuals; Arysta LifeScience, Tokyo, Japan) was introduced into one glasshouse 13 days after inoculation, and tomato plants were maintained at 20 to 30°C under natural photoperiod (~11 h light). In the untreated control glasshouse, plants were grown under the same condition except that bumblebees were not introduced. Plants were minimally managed, and aged lower leaves were removed by gloved hand (disposable PVC gloves) to prevent transmission by mechanical contact between leaves. All surviving bumblebee workers were collected 21 days after introduction, when inoculated source plants stopped blooming (4th to 5th flower stage) because of the disease. Thereafter, the upper leaflets of all Rutgers plants were collected every 2 weeks. Total RNA was extracted from leaves and cDNA was synthesised as before. PCR amplification was performed in 30-μl reaction mixtures containing 4 μl template DNA, 200 nM primer pair P3/P4 (Behjatnia et al. 1996), 200 μM dNTPs, and 0.75 units of KOD Dash DNA polymerase (Toyobo, Osaka, Japan) for 35 cycles of 45 s at 98°C, 10 s at 61°C, and 45 s at 74°C. This primer pair amplifies a product of approximately 360 bp. Heads and legs from 20 bumblebees were crushed separately using a ShakeMaster (BioMedical Science Inc., Tokyo, Japan) at low temperature in liquid nitrogen. Total RNA was extracted from insect tissues using TRIzol Reagent with the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA), followed by reverse transcription (RT)-PCR amplification as before.

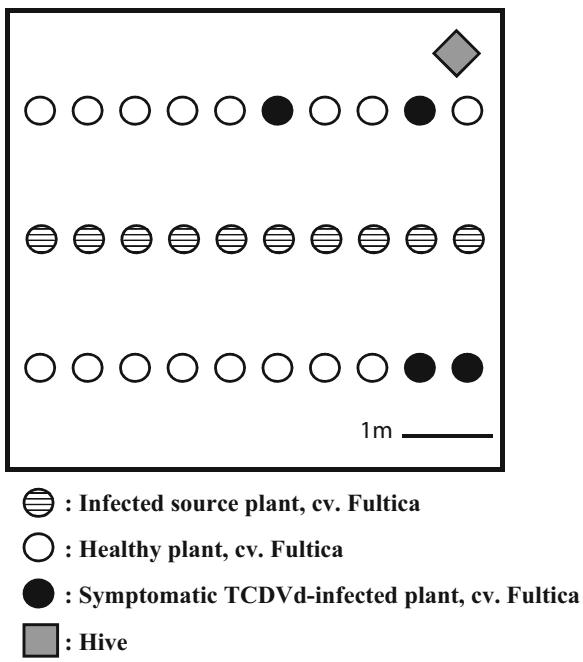

Fig. 3 Spatial distribution of TCDVd infection in tomato plants 4 months after introduction of bumblebees in a glasshouse (5.5 m×6 m)

The incidence of flower bruising (bite marks) ranged between 80% and 90% on all plants, indicating sufficient pollinator visitation to flowers. Two plants of Rutgers were found to be asymptomatically infected with TCDVd 37 days after the introduction of the

bumblebees. At 52 days, these two plants expressed typical symptoms of TCDVd, and viroid infection was confirmed in another three symptomless Rutgers plants (Fig. 2). Infection was not observed in any Rutgers plants in the control glasshouse during the experiment. Four bee heads and one leg were positive for TCDVd.

Additionally, we tested transmission of TCDVd by bumblebees in a different glasshouse. This glasshouse (11 m×6 m) was divided by a screen (0.4 mm mesh) into a bumblebee (treatment) plot and a non-bumblebee (control) plot. Fertiliser was applied at the rate of 75-95-75 kg ha^{-1} of N-P-K. Healthy tomato seedlings (cv. Fultica) were transplanted in three rows of ten plants in late October. To prevent transmission by mechanical contact from infection source plants to neighbouring healthy plants, each row was separated by 1.4 m. TCDVd was mechanically inoculated into the top leaves of all plants in the middle row at 1st flowering stage in early November. A bumblebee hive (~50 individuals) was introduced into the treatment plot 23 days after inoculation, and tomato plants were maintained at 15 to 30°C under natural photoperiod (~10 h light). Plants were carefully managed to avoid contact transmission within a row, and the axillary shoots were nipped by hand with new disposable gloves for each plant to prevent transmission. Plants were examined visually for TCDVd disease symptoms and finally tested for viroid infection by RT-PCR as before.

All infection source plants developed symptoms of stunting, leaf chlorosis, and leaf distortion within 1 month after the hive introduction and were TCDVd-positive by RT-PCR. The incidence of flower bruising peaked (>90%) 2 weeks after introduction of bumblebees and then decreased gradually over the next 2 weeks. Four of the 20 neighbouring (uninoculated) tomato plants expressed symptoms of TCDVd 4 months after introduction of bumblebees, and these symptomatic plants were verified to be infected with TCDVd by RT-PCR. These four symptomatic plants were close to the hive (Fig. 3). All symptomless tomato plants neighbouring infected source plants were negative for viroid infection by RT-PCR at final assessment. Infection was not observed in any neighbouring tomato plants in the control plot without bees.

Several plant viruses are transmitted by bumblebees through pollination (Okada et al. 2000; Shipp et al. 2008). TASVd (genus *Pospiviroid*) also was transmitted by bumblebees (Antignus et al. 2007). Here, bumblebees transmitted TCDVd from viroid-infected tomato plants to healthy plants, some of which developed symptoms. The final disease incidence in the first glasshouse experiment reached 50%, higher than that reported for TASVd (30%; Antignus et al. 2007). The high transmission efficiency may be due to frequent visits to the same flowers because of the limited space and small number of plants. A possible reason for the clustering of symptomatic plants in the second experiment is a higher frequency of flower visiting closer to the hive. The relatively low temperature and short photoperiod in the glasshouse may explain the 4-month lag between the introduction of bumblebees and symptom expression in the second experiment. Bumblebees perform buzz pollination, grasping the anthers with their mandibles and vibrating their bodies vigorously (Velthuis and Doorn 2006). We detected a substantial amount of TCDVd in the anthers of infected plants (Fig. 1), and detected the viroid more frequently in bee heads than in legs. Thus, it seems likely that TCDVd is mechanically transmitted with crude sap via mandibles. However, it is necessary to consider horizontal transmission through viroid-contaminated pollen carried by bumblebees, since tomato plants artificially pollinated with pollen from PSTVd-infected plants became systemically infected (Kryczyński et al. 1988).

TCDVd is mainly transmitted via contaminated fingers and pruners along the row during greenhouse practices such as supporting and harvesting. Therefore, removal of infected plants and disinfection of tools are vital to controlling viroid disease in tomato greenhouses. Considering that the approximate pollination range per hive is 2000 m^2, the efficiency of TCDVd transmission by bumblebees in commercial greenhouses might be expected to be much lower than we observed. However, our results suggest that bumblebees can transmit the viroid long distances. Further studies under commercial-like conditions will be necessary to clarify the efficiency of transmission of TCDVd by bumblebees.

Acknowledgements We are grateful to Dr. Taeko Takeuchi of Chiba Prefectural Agriculture and Forestry Research Centre, Japan for her advice. This work was supported in part by Research Project (No. 1976) for the Utilisation of Advanced Technologies in Agriculture, Forestry and Fisheries, of the Ministry of Agriculture, Forestry and Fisheries of Japan.

References

Antignus, Y., Lachman, O., Pearlsman, M., Gofman, R., & Bar-Joseph, M. (2002). A new disease of greenhouse tomatoes in Israel caused by a distinct strain of *Tomato apical stunt viroid* (TASVd). *Phytoparasitica, 30*, 502–510.

Antignus, Y., Lachman, O., & Pearlsman, M. (2007). Spread of *Tomato apical stunt viroid* (TASVd) in greenhouse tomato crops is associated with seed transmission and bumble bee activity. *Plant Disease, 91*, 47–50.

Behjatnia, S. A. A., Dry, I. B., Krake, L. R., Conde, B. D., Connelly, M. I., Randles, J. W., et al. (1996). New *Potato spindle tuber viroid* and *Tomato leaf curl geminivirus* strains from a wild *Solanum* sp. *Phytopathology, 86*, 880–886.

Diener, T. O. (2001). The viroid: biological oddity or evolutionary fossil? *Advances in Virus Research, 57*, 137–184.

Galindo, J., Smith, D. R., & Diener, T. O. (1982). Etiology of planta macho, a viroid disease of tomato. *Phytopathology, 72*, 49–54.

James, T., Mulholland, V., Jeffries, C., & Chard, J. (2008). First report of *Tomato chlorotic dwarf viroid* infecting commercial petunia stocks in the United Kingdom. *Plant Pathology, 57*, 400.

Kryczyński, S., Paduch-Cichal, E., & Skrzeczkowski, L. J. (1988). Transmission of three viroids through seed and pollen of tomato plants. *Journal of Phytopathology, 121*, 51–57.

Matsushita, Y., Kanda, A., Usugi, T., & Tsuda, S. (2008). First report of a *Tomato chlorotic dwarf viroid* disease on tomato plants in Japan. *Journal of General Plant Pathology, 74*, 182–184.

Matsushita, Y., Usugi, T., & Tsuda, S. (2009). Host range and properties of *Tomato chlorotic dwarf viroid*. *European Journal of Plant Pathology, 124*, 349–352.

Mumford, R. A., Jarvis, B., & Skelton, A. (2004). The first report of *Potato spindle tuber viroid* (PSTVd) in commercial tomatoes in the UK. *Plant Pathology, 53*, 242.

Okada, K., Kusakari, S., Kawaratani, M., Neguro, J., Ohki, S. T., & Osaki, T. (2000). *Tobacco mosaic virus* is transmissible from tomato to tomato by pollinating bumblebees. *Journal of General Plant Pathology, 66*, 71–74.

Shipp, J. L., Buitenhuis, R., Stobbs, L., Wang, K., Kim, W. S., & Ferguson, G. (2008). Vectoring of *Pepino mosaic virus* by bumble-bees in tomato greenhouses. *Annals of Applied Biology, 153*, 149–155.

Singh, R. P., Nie, X., & Singh, M. (1999). *Tomato chlorotic dwarf viroid*: an evolutionary link in the origin of pospiviroids. *Journal of General Virology, 80*, 2823–2828.

Singh, R. P. (2006). Reassessment of the presence of viroid species of the genus *Pospiviroid* in infected floral parts, using reverse transcription-polymerase chain reaction and infectivity assays. *Canadian Journal of Plant Pathology, 28*, 242–249.

Singh, R. P., & Dilworth, A. D. (2009). *Tomato chlorotic dwarf viroid* in the ornamental plant *Vinca minor* and its transmission through tomato seed. *European Journal of Plant Pathology, 123*, 111–116.

Velthuis, H. H. W., & van Doorn, A. (2006). A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie, 37*, 421–451.

Verhoeven, J. Th J., Jansen, C. C. C., Willemen, T. M., Kox, L. F. F., Owens, R. A., & Roenhorst, J. W. (2004). Natural infections of tomato by *Citrus exocortis viroid*, *Columnea latent viroid*, *Potato spindle tuber viroid* and *Tomato chlorotic dwarf viroid*. *European Journal of Plant Pathology, 110*, 823–831.