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Distribution of *tomato chlorotic dwarf viroid* in floral organs of tomato

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Abstract In situ hybridization was used to analyze the distribution pattern of *Tomato chlorotic dwarf viroid* (TCDVd) in floral organs of tomato plants. Following TCDVd invasion of floral organs, it became localized only in sepals at an early developmental stage, then reached other floral organs at the flower opening stage, with the exception of part of the placenta and ovules. When distribution of TCDVd was compared with that of *Potato spindle tuber viroid* (PSTVd), TCDVd was not detected in the outer integument around the embryo sac even though PSTVd was able to invade there, suggesting that such specific distribution might reflect the frequent occurrence of viroid disease on crops caused by PSTVd-seed transmission.

Keywords In situ hybridization · Ovules · Potato spindle tuber viroid · Seed transmission · Shoot apical meristem · Tomato

Viroids are the smallest and simplest of the plant pathogens, consisting of a single-stranded circular naked RNA genome, 246 to 401 nucleotides in length, which lacks any protein coding sequences (Ding 2009; Di Serio and Flores 2008). Approximately 30 viroids have been discovered worldwide and classified into two families: the *Pospiviroidae* and the *Avsunviroidae* (Di Serio and Flores 2008). Members of the family *Pospiviroidae*, type species *Potato spindle tuber viroid* (PSTVd; Diener 1972), which is the most analyzed viroid in the world, have highly conserved regions in their rod-shaped secondary structure, replicate in the nucleus of infected cells, and are considered to lack ribozyme activity. PSTVd moves from cell to cell via plasmodesmata (Ding et al. 1996), and systemic trafficking from organ to organ occurs through the phloem (Zhu et al. 2001). Zhong et al. (2008) illustrated areas of single-cell replication and systemic trafficking on a genomic map of viroid RNA by conducting a genome-wide mutational analysis to reveal the role of PSTVd loops.

Initial occurrences of some viroid diseases in the field are due to seed-borne infection, which is then spread through mechanical contact with other plants, for PSTVd (Hunter et al. 1969; Kryczynski et al. 1988; Singh 1970), *Apple scar skin viroid* (ASSVd; Hadidi et al. 1991), *Avocado sunblotch viroid* (Allen et al. 1981; Wallace and Drake 1962), *Coconut cadang-cadang viroid* (Wah and Symons 1999), *Chrysanthemum stunt viroid* (Chung and Pak 2008), *Coleus blumei viroid* (Singh et al. 1991), *Grapevine yellow speckle viroid* (Wah and Symons 1999) and *Tomato apical stunt viroid* (Antignus et al. 2007). Although PSTVd is known to be transmitted at high

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frequency by contaminated seeds (Fernow et al. 1970), the molecular biology and histochemistry of this mechanism have not yet been fully analyzed. Previously, ASSVd was biochemically detected in the cotyledons, embryos, seed coats and subcoats of infected seeds (Hadidi et al. 1991). This suggests that the viroid molecules in infected seeds originate from viroid replication in flower ovules and/or ovaries. Successful accomplishment of seed transmission by viroids might therefore be required to infect generative organs such as ovules and pollen.

Tomato chlorotic dwarf viroid (TCDVd), belongs to the *Pospiviroid* genus and infects ornamental plants (James et al. 2008; Matsushita et al. 2009; Singh and Dilworth 2009), showing 85-89% sequence identity with PSTVd (Singh et al. 1999), causing economic damage to tomato (*Solanum lycopersicum*) production, with severe symptoms such as small fruit, dwarfing, leaf chlorosis, epinasty and vein necrosis. TCDVd was first discovered in tomatoes cultivated in Canada (Singh et al. 1999), and was subsequently reported in tomatoes (Verhoeven et al. 2004) and petunias (*Petunia* x *hybrida*) in the USA (Verhoeven et al. 2007), in *Verbena* x *hybrida* in India (Singh 2006), in petunias in the UK (James et al. 2008) and in tomato in Japan (Matsushita et al. 2008). In addition, TCDVd is transmitted by bumblebee (*Bombus ignitus*) pollination activities between tomato plants (Matsuura et al. 2010). However, the viroids have many different features, including host plants. *Gomphrena globosa* are hosts for PSTVd but are not susceptible to infection by TCDVd (Matsushita et al. 2009).

Although seed transmission of TCDVd has previously been described (Singh and Dilworth 2009), we could not confirm it in laboratory tests. However, high-frequency seed transmission of PSTVd has been recognized in numerous publications (Benson and Singh 1964; Fernow et al. 1970; Singh 1970). We therefore used in situ hybridization to analyze differences in distribution patterns of TCDVd in shoot apical meristems (SAM) and floral organs at each developmental stage of tomatoes, comparing to those of PSTVd, and then discuss the possibility that different invasion patterns might influence viroid seed transmission.

Two viroids, TCDVd (359nt; GenBank/EMBL/DDBJ accession no. AB329668; Matsushita et al. 2008) isolated from Hiroshima Prefecture, Japan and PSTVd (358nt; Matsushita et al. 2010) isolated from Fukushima Prefecture, Japan, with an identical genome sequence to the Cape gooseberry strain (Accession no. EU862231; Verhoeven et al. 2009), were employed for this study. Nucleotide differences between the two viroids were observed in 37 nucleotides. Tomato (*S. lycopersicum* cv. Rutgers) plants were cultivated in a growth chamber maintained at 27°C day/25°C night temperature regimes and 16/8-h light/dark cycle.

Total RNA was extracted from infected tomato leaves frozen (−20°C) using TRIzol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. To obtain full-length TCDVd cDNA and PSTVd cDNA for the development of an artificial inoculation system based on an infectious cDNA plasmid, reverse-transcription polymerase chain reaction (RT-PCR) was conducted using the reverse primer Posi94-R (5′-GGATCCCTGAAGCGCT-3′) and the forward primer Posi94-F (5′-CCGGGGAAACCTGGAG-3′) according to methods previously described by Matsushita and Kumar (2009). First, amplified cDNAs were inserted into the pGEM-T Easy vector (Promega). For synthesis of TCDVd (+) RNAs containing an 11nt sequence duplication (5′-GGATCCCCGGG-3′) which is required to infect a plant (Owens et al. 1986), fragments containing this sequence synthesized from pGEM-T easy with full-length TCDVd were transferred into pBluescript II SK(−) after digestion with *Spe* I and *Pst* I. Final forms of each plasmid (p94TCV for TCDVd or p94PSV for PSTVd) were linearized with *Xba* I and used as templates for in vitro transcription using the T7 RiboMax Large Scale RNA kit (Promega, Madison, WI) following the manufacturer's protocol. After in vitro transcription, DNA templates were removed by digestion with RNase-free DNase. TCDVd or PSTVd transcripts were inoculated onto Carborundum-dusted cotyledons of tomato seedlings. RNase-free water was rubbed onto tomato plants as a mock-inoculation negative control. Inoculated plants were placed into a growth chamber to allow for further growth. TCDVd or PSTVd infection was determined by RT-PCR of leaflets collected from the uppermost leaf of each plant, as previously described by Matsushita et al. (2010).

Digoxygenin (DIG)-labeled minus-stranded TCDVd and PSTVd riboprobes were prepared by in vitro transcription using *Sal* I-linearized p94TCV or p94PSV as template, respectively, in a DIG-11-UTP NTP mix (Roche Diagnostics GmbH, Mannheim, Germany) and

T7 RNA polymerase (Roche) according to the manufacturer's protocol.

Sampled tissues from the ovule and surrounding placenta were processed using the Sainte-Marie method with some modifications (Sainte-Marie 1962). Apical stems and flower buds 0.5–10 mm in length were fixed in FAA (10% formaldehyde, 50% ethanol, 5% acetic acid) overnight at 4°C. After dehydration, infiltration and replacement with xylene, the samples were embedded in paraffin (Paraplast Plus, Oxford Labware). Paraffin sections were prepared 10–12 μm thick and applied to slide glasses coated with aminopropylytriethoxy silane (APS; Matsunami Glass, Osaka, Japan). After 10–15 min at room temperature, sections became flat, and excess water was removed from the edges using an absorbent tissue. When the sections had dried completely, slides were placed on a warming plate overnight at 42°C.

Sections were de-waxed twice in xylene, 10 min each, and washed twice in 100% ethanol, 5 min each. After hydration through a graded ethanol series (90%, 70%, 50%, 30%) followed by sterile distilled water, slides were treated with 2 μg ml^{-1} proteinase K (in 100 mM Tris, pH 7.5, 50 mM EDTA) for 30 min at 37°C. Slides were washed twice in phosphate-buffered saline (PBS), then dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, 100%) followed by sterile distilled water. Sections were dried in a vacuum using a water aspirator for 1 h and hybridization was carried out overnight in a humidified box at 50°C. The hybridization mixture consisted of 50% deinoized formamide, 10% dextran sulphate, 0.3 M NaCl, 1× TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5), 1× Denhardt's solution, 1 μg μl^{-1} yeast tRNA and DIG-labeled RNA probe. About 180 μl of the hybridization mixture was used per slide. After hybridization, slides were washed with 4× SSC (1× SC; 150 mM NaCl, 15 mM Na citrate, pH 7.0) three times for 15 min at 50°C and incubated in RNase buffer (0.5 M NaCl, 15 mM Tris, 5 mM EDTA, pH 7.5) containing 20 μg ml^{-1} RNase A. Slides were washed twice with 0.5× SSC at room temperature. Next, the sections were incubated with Anti-Digoxigenin-AP, Fab fragments (Roche; 1:1000 dilution in DIG buffer; 0.15 M NaCl, 0.1 M Tris, pH 7.5) for 1 h at room temperature. Following a final wash with DIG buffer, sections were incubated with the color substrate solution (100 μl of NBT/BCIP Liquid; Sigma) in the dark. When colour had sufficiently developed, the sections were mounted and examined under a microscope. Observations were confirmed by examination of serial sections. At least six plants were used for in situ hybridization as samples to make sure its reproducibility.

To determine TCDVd distribution in the apical meristem, longitudinal sections of shoot apices from tomato infected with either viroid 2 months after inoculation were examined by in situ hybridization with strand-specific TCDVd riboprobes (Fig. 1). TCDVd was detected in the leaf primordium but not in the SAM or lateral shoot meristem (data not shown). No hybridization signal was observed in non-infected control plants. Previously, it was shown that $PSTVd^{Int}$ (Accession no. AY937179) did not invade the SAM of infected tomato (Zhu et al. 2001; 2002), and Di Serio and Flores (2008) suggested that RNA silencing may prevent entry of PSTVd RNA into the SAM. Di Serio et al. (2010) also revealed using in situ hybridization that RNA-dependent RNA polymerase 6 (RDR6), which restricts the systemic spread of some RNA viruses and precludes their invasion of the apical

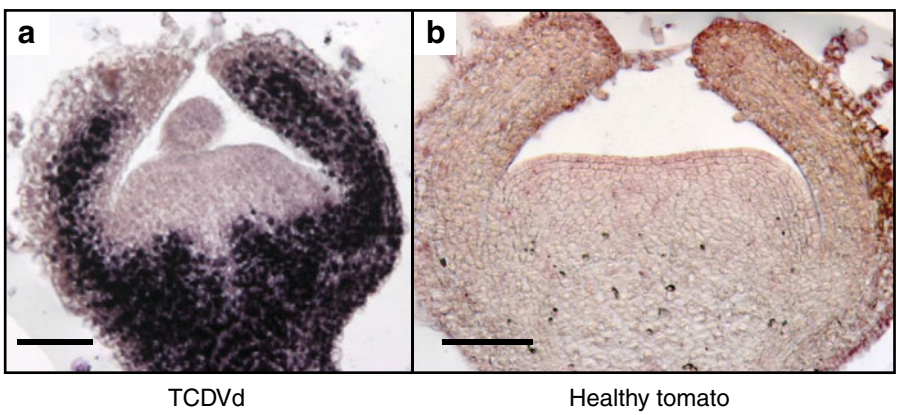

Fig. 1 Distribution of TCDVd in the shoot apical meristem (SAM) of an infected tomato. **a** Longitudinal section of a TCDVd infected SAM. **b** Longitudinal section of a healthy plant SAM. Bar shows 50 μm in each

growing tip, is involved in restricting PSTVd access to floral and vegetative meristems of *N. benthamiana*, thus providing genetic evidence for an antiviroid RNA silencing mechanism.

To investigate the developmental infection process of TCDVd in floral parts, hybridization signals from infected floral organ sections were observed at serial developmental stages. Stages of flower development were defined according to Brukhin et al. (2003). At stages 4–6 of flower development (0.6–1 mm long buds), TCDVd was detected in sepals but not other floral parts such as petal primordia, stamen primordia or carpel (Fig. 2a). TCDVd invaded parts of petal primordia at stage 8 (Fig. 2b), then parts of stamens at stage 9 (Fig. 2c). From stage 4 to 12, TCDVd did not invade ovaries (Fig. 2a to e), but at stage 13 it was localized in the placenta and ovary wall but not the ovules (Fig. 2f). These data suggest that while TCDVd has an ability to invade ovary but not ovule. These data therefore suggest a form of regulation for viroid trafficking in floral organs in each developmental stage. The RNA motif in the PSTVd genome is known to affect cell-to-cell trafficking and, however, this motif is not required for trafficking through the same cellular boundary in mature leaves, indicating developmental control of RNA trafficking (Qi et al. 2004).

To investigate differences in distribution between TCDVd and PSTVd in various ovary parts at the flower opening stage, transverse sections of breaking buds from inoculated tomato plants were prepared. TCDVd was detected in the ovary wall and placenta near the vascular bundle but not in ovules of TCDVd-inoculated tomato plants (Fig. 3a). By contrast, PSTVd was present in ovary walls, the placenta and ovules of PSTVd-inoculated tomato plants (Fig. 3b). Histochemical analysis of PSTVd used in situ hybridization to show that the viroid existed in the sepals but not the petals, stamens or ovary of flowers from infected plants (Zhu et al. 2001). However, later reassessment by RT-PCR showed that PSTVd was in fact distributed in all floral parts (sepals, petals, stamens, and pistils) of tomato (Singh 2006). As shown in Fig. 3, PSTVd invaded the outer integument but not the inner integument around the embryo sac (Fig. 3d), while TCDVd was absent from both integuments (Fig. 3c). Additionally, in more mature flowers of TCDVd-infected tomato plants, TCDVd was also absent from ovules (data not shown). Since TCDVd and PSTVd share 89.6% sequence identity, differences in invasion of floral organs are likely to be associated with differences in genomic structures, especially the pathogenicity domain (P), the variable domain (V) and the right terminal domain (T_R).

An analysis of seed localization of TCDVd revealed that the viroid was absent from ovules of infected tomato seeds (cv. Rutgers) as shown in Figs. 2 and 3, and in contrast to PSTVd. Singh and Dilworth (2009) previously reported that 16 out of 60 seeds obtained from the fruits of a large tomato (cv. Sheyenne) at 11 weeks post-inoculation with a *Vinca minor* isolate of TCDVd (Accession no. EU625577) showed seed-borne viroid disease on germinated seedlings, indicating a seed transmission rate of 26.7%. However, when 120 seeds obtained

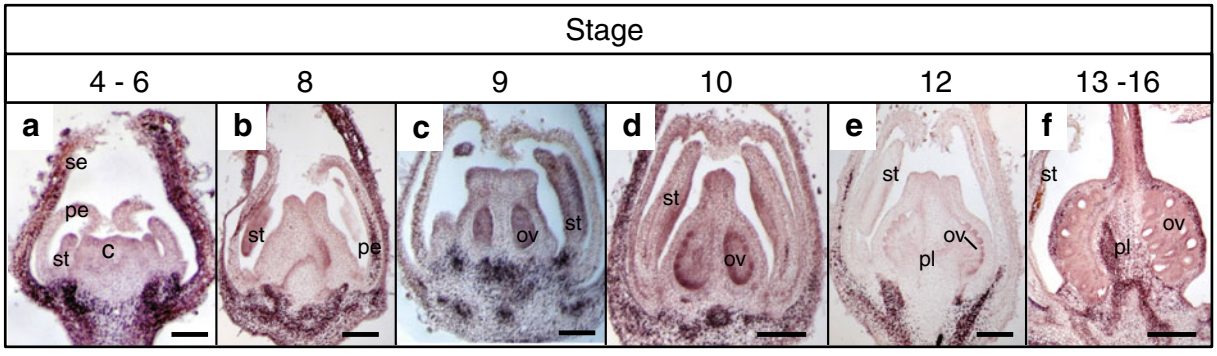

Fig. 2 Changes of distribution of TCDVd in flowers buds ranged from stage 4–12 over developmental stages of tomato. Stages of flower development are defined according to Brukhin et al. (2003). **a**–**f** Longitudinal section of a TCDVd-infected flowers buds. *c* carpel, *ov* ovule, *pl* placenta, *pe* petal, *se* sepal, *st* stamen. *Bars* indicate 100 μm in **a**, 250 μm in **b**, **c** and 500 μm in **d**, **e**, **f**, respectively

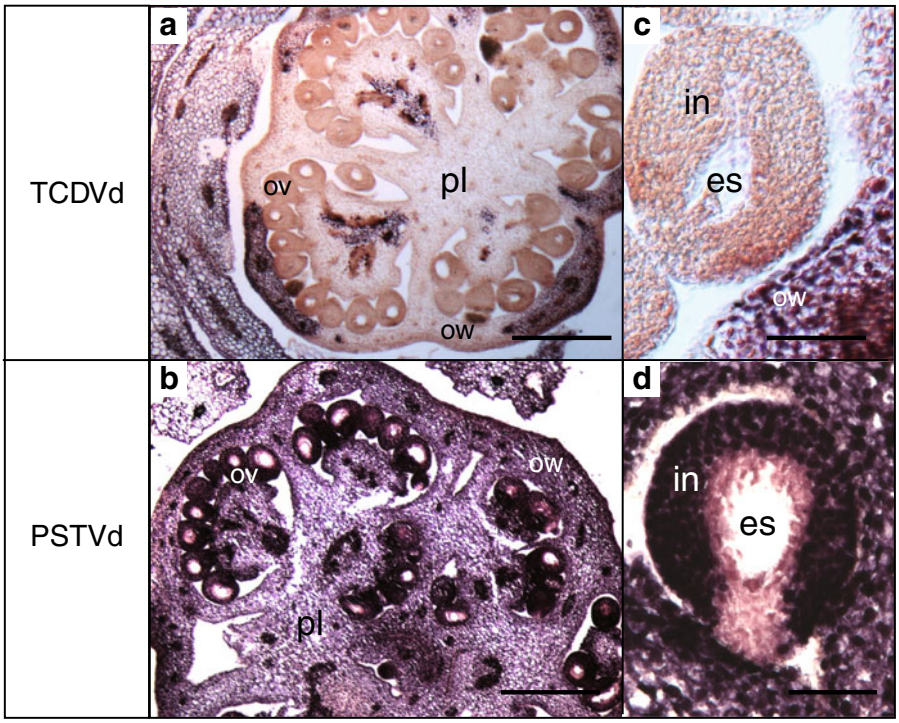

Fig. 3 Distribution of TCDVd and PSTVd in flowers of an infected tomato at stage of flower opening. **a** Transverse section of a TCDVd infected tomato ovary. **b** Transverse section of a PSTVd infected tomato ovary. **c** High magnification of the ovary showing ovules and ovary wall of tomato plants infected with TCDVd. **d** Magnification of the ovary showing ovules and ovary wall of tomato plants infected with PSTVd. *es* embryo sac, *in* integuments, *ov* ovule, *ow* ovary wall, *pl* placenta. *Bars* represent 500 μm in **a**, **b** and 50 μm in **c**, **d**, respectively

from TCDVd (Accession no. AB329668)-infected tomato (cv. Rutgers) were sown in the soil in the present study, no seed transmission was observed in our laboratory (data not shown). Although this is inconsistenct with a previous report (Singh and Dilworth 2009), histochemical data for TCDVd-infected floral organs (Fig. 3) are in agreement with this result, as they show that the viroid was absent from ovules in the early developmental stage of floral organs. It is supposed that the combination of virus/viroid strain and host plant-affects seed transmission rates (Roberts et al. 2003). While an occurrence of seed transmission of TCDVd has been previously reported (Singh and Dilworth 2009) this is likely to be extremely low on tomato because of undershooting to seeds in reproductive organs.

Since TCDVd did not reach ovules on tomato in this study, it is unlikely that it could spread via tomato seed into the fields and, hence, the main transmission of TCDVd could be through use of tools contaminated by other plants that are vegetatively propagated and/or in which TCDVd is transmitted by seed. While seed transmission of *Citrus exocortis viroid* (CEVd) has not been demonstrated in citrus (Duran-Vila and Semancik 2003), it has been reported in *Verbena* × *hybrida*, the symptomless vegetatively-propagated host plant (Singh et al. 2009) that is also an asymptomatic host of TCDVd (Matsushita et al. 2009). In addition, the wide host range of TCDVd is the same as that of CEVd (Duran-Vila and Semancik 2003). To prevent the spread and import of TCDVd-infected plants in international trading, it is therefore necessary to survey symptomless vegetatively-propagated hosts plants, particularly those in which seed transmission of viroids definitely occurs.

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