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Torradoviruses are transmitted in a semi-persistent and stylet-borne manner by three whitefly vectors

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ABSTRACT

Members of the genus *Torradovirus* (family *Secoviridae*, type species *Tomato torrado virus*, ToTV) are spherical plant viruses transmitted by the whitefly species *Trialeurodes vaporariorum* and *Bemisia tabaci*. Knowledge on the mode of vector transmission is lacking for torradoviruses. Here, the mode of transmission was determined for *Tomato marchitez virus* (ToMarV). A minimal acquisition access period (AAP) and inoculation access period (IAP) of approximately 2 h each was required for its transmission by *T. vaporariorum*, while optimal transmission required an AAP and IAP of at least 16 h and 8 h, respectively. Whiteflies could retain the virus under non-feeding conditions for at least 8 h without loss of transmission efficiency, but upon feeding on a non-host plant in between the AAP and IAP they retained the virus for no more than 8 h. Similar conditions supported transmission of isolates of ToTV and Tomato chocolàte virus (ToChV) by *T. vaporariorum* and *B. tabaci*. Additionally, similar experiments revealed the banded-winged whitefly (*Trialeurodes abutilonea*) as a vector for all three virus species. The results are congruent with acquisition and retention periods for semi-persistent virus transmission. RT-PCR detection analysis of ToTV and ToMarV in the vector's body revealed their presence in the stylet, but not in the head where the pharynx of the foregut is located. The results altogether indicate a semi-persistent stylet-borne mode of vector transmission for torradoviruses. Additionally, this is the first group of spherical viruses transmitted by at least three different species of whiteflies.

1. Introduction

The genus *Torradovirus* (family *Secoviridae*) currently harbors the two species *Tomato torrado virus* (ToTV) and *Tomato marchitez virus* (ToMarV). Additionally, two tentative species were assigned to this genus; Tomato chocolate spot virus (ToChSV) and Tomato chocolàte virus (ToChV) (Sanfaçon et al., 2012). All these viruses infect tomato (*Solanum lycopersicum* L.) and are able to cause substantial economic losses in susceptible cultivars due to necrosis on leaves and necrotic rings and patches on fruits (Batuman et al., 2010; Verbeek et al., 2007, 2008, 2010a). A severe necrosis in leaves, stems and fruits may occur, giving the plant a burnt-like appearance. This burnt-like appearance was the origin of the name of torrado disease (torrado means burnt or roasted), given by Spanish farmers, who faced this new disease in their tomato crops at the beginning of this century (Alfaro-Fernández et al., 2010).

Torradoviruses possess small spherical virions, approximately 30 nm in diameter, composed of three coat proteins with molecular masses of approximately 23, 26, and 35 kDa. The genome of torradoviruses is bi-partite and consists of single stranded positive sense RNAs with approximate sizes of 7.2–7.8 kb for RNA1 and 4.9–5.7 kb for RNA2 (Batuman et al., 2010; Verbeek et al., 2007, 2008, 2010a). RNA1 contains one open reading frame (ORF) with motifs for the protease, helicase and RNA-dependent RNA polymerase (RdRp). RNA2 contains two ORFs of which, after comparison to other plant picorna-like virus genomes, the most 5′-located ORF appears unique to torradoviruses. The function of the RNA2–ORF1-encoded protein is still unclear. The second ORF on RNA2 has coding regions for a putative movement protein and the three coat proteins (Verbeek et al., 2007). Torradovirus genomes have remarkably long 3′-untranslated regions (UTRs) on both RNAs which range from approximately 652 nt (ToMarV) to over 1400 nt (ToChV). These 3′-UTRs possess unique species-specific regions which are highly conserved between the two RNAs of the same torradovirus species (Verbeek et al., 2010a).

As vectors interact with all three vertices of the epidemiological triangle (McNew, 1960), the pathogen, the host and the environment, they are of utmost importance to the epidemiology of infectious diseases. Understanding the way how vectors transmit pathogens increases knowledge of their epidemiologies and helps to develop control measures. The observation of large numbers of whiteflies (family *Aleyrodidae*) in greenhouses containing tomato

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crops infected with torrado disease pointed towards a whitefly-borne nature of the causal agent. Until 2005, all plant viruses transmitted by whiteflies belonged to the genera *Begomovirus*, *Crinivirus*, *Ipomovirus* or *Carlavirus* (Jones, 2003; Navas-Castillo et al., 2011). Virions from these viruses have either geminate (begomoviruses) or filamentous (criniviruses, ipomoviruses and carlaviruses) morphologies and are transmitted in a persistent or semi-persistent manner, respectively. However, for the whitefly transmitted *Cowpea mild mottle virus* (family *Flexiviridae*, genus *Carlavirus*, CMMV) both non-persistent and semi-persistent transmission was reported (Brown and Czosnek, 2002; Jeyanandarajah and Brunt, 1993; King et al., 2012; Navas-Castillo et al., 2011). Before then, no spherical viruses were reported to be whitefly transmitted. Early transmission experiments on a virus isolated from Polish greenhouse tomato crops, which only later was identified as an isolate of ToTV, showed that this virus was transmitted by the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood), but not by the green peach aphid, *Myzus persicae* (Sulzer) (Pospieszny, 2005; Pospieszny et al., 2010). Later, Amari et al. (2008) showed that ToTV was also transmitted by the sweetpotato whitefly, *Bemisia tabaci* (Gennadius). Although these studies identified whiteflies as the vectors of the causal agent of torrado disease, the mode of transmission remained unknown.

Unraveling the mode of transmission of torradovirus is important for understanding the epidemiology of these viruses, which is needed for development of disease management and control strategies (Jeger et al., 2004). Here, the length of time that whiteflies need to acquire these viruses (acquisition access period, AAP), the length of time needed to inoculate the virus into a new host (inoculation access period, IAP), and the length of time that the virus is retained and transmitted by the vector (retention period) were determined for two isolates of ToMarV and the vector *T. vaporariorum*. Additional transmission studies, applying the optimal AAP and IAP as determined for ToMarV, were conducted with ToTV and ToChV using *T. vaporariorum* and *B. tabaci*.

As the banded-winged whitefly, *Trialeurodes abutilonea* (Haldeman), was reported as a vector for four other plant viruses – amongst them two viruses which are also transmitted by other whitefly species; *Sweet potato chlorotic stunt virus* (family *Closteroviridae*, genus *Crinivirus*, SPCSV) and *Tomato chlorosis virus* (family *Closteroviridae*, genus *Crinivirus*, ToCV) (Jones, 2003) – this whitefly was tested for its ability to transmit ToTV, ToMarV and ToChV. The results indicate that torradoviruses are transmitted in a semi-persistent manner by the whitefly species *T. vaporariorum*, *T. abutilonea* and *B. tabaci*. Initial localization studies of ToTV and ToMarV in the whitefly suggest that torradoviruses are retained on the stylets and thus likely transmitted in a stylet-borne manner.

2. Materials and methods

2.1. Virus isolates

ToTV, ToMarV and ToChV were isolated from infected tomato leaves and propagated in *Physalis floridana*, *Nicotiana glutinosa* or *N. hesperis* '67A' as described before (Verbeek et al., 2007, 2008, 2010a). The origins of the virus isolates used in this study are listed in Table 1. All virus isolates were maintained in alternative host plants in an insect-free greenhouse at 20 °C and stored as leaf samples in liquid nitrogen.

Table 1
Origin of virus isolates used in this study.

Virus species	Acronym	Isolate	Origin
Tomato torrado virus	ToTV	PRI-ToTV0301	Spain
Tomato marchitez virus	ToMarV	PRI-TMarV0601	Mexico
Tomato marchitez virus	ToMarV	T592	Mexico
Tomato chocolàte virus	ToChV	ToChV-G01	Guatemala

2.2. Insect species

A colony of *T. vaporariorum*, was obtained from J. Klapwijk, Koppert BV, Berkel en Rodenrijs, The Netherlands. This colony was propagated and maintained on *N. tabacum* cv. 'White Burley'. *B. tabaci* (type B), was obtained from the Laboratory of Entomology, Wageningen University, Wageningen, The Netherlands. *B. tabaci* was also maintained on *N. tabacum* cv. 'White Burley'. Adults of *T. abutilonea* were obtained from W.M. Wintermantel, USDA, Salinas, CA, USA. They were the start of a colony of *T. abutilonea* which was maintained on *Physalis acutifolia* (Miers) Sandwith (synonym: *P. wrightii*). A colony of *M. persicae*, biotype Mp2 (Verbeek et al., 2010b) was maintained on Chinese cabbage (*Brassica rapa* var *pekinensis* L.). All colonies were kept in insect rearing cages (BugDorm, Taichung, Taiwan) in a climate chamber at 20 °C (±1 °C) and a day-night regime of 16 h/8 h.

2.3. Transmission studies

After evaluation of several test plants in transmission studies, we selected *N. glutinosa* as source plant and *N. hesperis* '67A' as receptor plant. Both plants were accepted as a feeding source by the three whitefly species (*T. abutilonea* accepted *N. glutinosa* only for a period of 24 h), and were susceptible to all virus isolates used in the experiments. To prevent loss or decrease of transmission efficiency by repeated mechanical inoculation passages, the *N. glutinosa* source plant was always mechanically inoculated with a homogenate from a torradovirus-infected *N. hesperis* '67A' plant inoculated by whiteflies previously. The first successful whitefly-mediated transmission was established following the procedure of Pospieszny et al. (2010) using *T. vaporariorum* as vector. Three time factors are indicative for the mode of transmission of plant viruses: (a) the AAP, (b) the retention period, and (c) the IAP (Nault, 1997). In all transmission experiments, the plants were transferred to an insect-free greenhouse compartment after inoculation and killing the whiteflies. Plants were monitored three weeks for symptom development and in the same period plants and greenhouses were carefully monitored for the presence of insects. In each experiment non-inoculated plants were incorporated as a negative control.

2.3.1. Determination of minimal and optimal acquisition access periods

The majority of the experiments were conducted with ToMarV and *T. vaporariorum*. In each treatment, approximately 275 adult whiteflies were caught in a 250 ml Erlenmeyer flask, using a simple aspirator device. We did not select for males or females or for a certain age, but caught the whiteflies randomly from the colony. When whiteflies were actively feeding and difficult to dislodge, they were left untouched in order to avoid damaging them. The flask was covered with a piece of Parafilm® and the whiteflies were starved for 2 h. Subsequently, they were released in an insect rearing cage containing one *N. glutinosa* source plant. The whiteflies were allowed to probe and feed on the source plant for AAP's varying from 0.5 h to 48 h. After the AAP, four cohorts of 50 adults were caught in small Erlenmeyer flasks (50 ml) and the flasks covered with Parafilm®. Each flask was placed near a *N. hesperis* '67A' test plant under a small cage and the Parafilm® was removed to give whiteflies access to the test plant. For experiments with AAP's shorter than 24 h, the whiteflies which remained on the source plant were allowed to fulfill an AAP of 24 h. Subsequently, 50 whiteflies were collected in the same way as described above and also placed under a cage containing a *N. hesperis* '67A' test plant. This control experiment was performed