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Clathrin-mediated endocytosis is involved in *Tomato yellow leaf curl virus* transport across the midgut barrier of its whitefly vector

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ABSTRACT

Tomato yellow leaf curl virus (TYLCV) is a begomovirus transmitted by the whitefly *Bemisia tabaci*. The circulative translocation of the virus in the insect is known in its broad line. However, transit of TYLCV from the digestive tract into the haemolymph is poorly understood. We studied the involvement of clathrin in this process by disrupting the clathrin-mediated endocytosis and the endosome network using inhibitor feeding, antibody blocking and dsRNA silencing. We monitored the quantities of TYLCV in the whitefly and virus transmission efficiency. Following endocytosis and endosome network disruption, the quantity of virus was higher in the midgut relative to that of the whole insect body, and the quantity of virus in the haemolymph was reduced. The transmission efficiency of TYLCV by the treated insects was also reduced. These findings indicate that clathrin-mediated endocytosis and endosomes play an important role in the transport of TYLCV across the whitefly midgut.

1. Introduction

Plant viral diseases are a major threat to world agriculture (Scholthof et al., 2011). Insect vectors, such as aphids, leafhoppers, mealybugs or whiteflies, are responsible for the spread and epidemic of the majority of plant viruses. Among the plant viruses transmitted by insects, begomoviruses have become serious constraints to the production of a variety of crops in the tropics, subtropics and warm temperate regions in the past three decades (Navas-Castillo et al., 2011). So far, more than 300 species have been described in the genus *Begomovirus* (family *Geminiviridae*), of which Tomato yellow leaf curl virus (TYLCV) is one of most economically important, threatening the production of tomato but also of other crops and ornamentals, worldwide (Navas-Castillo et al., 2011; Czosnek, 2007; Brown et al., 2015; http://www.ictvonline.org/virusTaxonomy.asp as accessed on 26 December 2016). Similarly, the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae), the begomovirus vector, is considered a species complex, including some notorious pests such as the Middle East-Asia Minor (MEAM1) and Mediterranean (MED) species, formerly referred to as the B and Q "biotypes" respectively (De Barro et al., 2011). TYLCV is known to be transmitted exclusively by the *B. tabaci* whitefly complex in a circulative and non-propagative manner (Czosnek et al., 2002; Hogenhout et al., 2008). And recently, a case of transmission via the seeds of infected tomato plants was reported (Kil et al., 2016). Whether TYLCV can replicate in the whitefly vector has been a subject of debate (Pakkianathan et al., 2015; Sánchez-Campos et al., 2016). Much effort has been invested to unravel the complex interactions between begomoviruses and their whitefly vectors. From the virus side, only the coat protein has been found so far to be involved in virus-whitefly interactions (Briddon et al., 1990; Caciagli et al., 2009; Hofer et al., 1997; Wei et al., 2014). From the whitefly side, various factors, such as the autophagy pathway, heat shock protein 70, endosymbionts and a recently reported midgut protein, have been shown to be involved in the begomovirus circulative transmission (Ghanim, 2014; Gotz et al., 2012; Kliot et al., 2014; Luan et al., 2011; Rana et al., 2016; Wang et al., 2016). The *B. tabaci Knottin-1* gene appears to play a role in regulating the quantity of TYLCV ingested and transmitted by the insect (Hariton-Shalev et al., 2016). Despite of these progresses, the proteins involved in the virus circulation within the whitefly body, in particular the precise routes, through which the virions follow to cross the midgut barrier into the haemolymph, are still largely unknown.

Once orally acquired by its whitefly vector, TYLCV follows the sequential path head-midgut-haemolymph-salivary gland (Ghanim et al., 2001). In this process, midgut and salivary glands are the two

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Table 1
Primers used in this study.

Gene	Primer	Sequence (5′–3′)	Reference
TYLCV	TYLCV F	ATCGAAGCCCTGATATCCCCCGTGG	Ghanim et al., 2001
	TYLCV R	CAGAGCAGTTGATCATG	
TYLCV V1	TYV1 RTF	GAAGCGACCAGGCGATATAA	Sinisterra et al., 2005
	TYV1 RTR	GGAACATCAGGGCTTCGATA	
B.tabaci β-Actin	Bt *β-Actin* RTF	TCTTCCAGCCATCCTTCTTG	Sinisterra et al., 2005
	Bt *β-Actin* RTR	CGGTGATTTCCTTCTGCATT	
GFP	*GFP* T7F	TAATACGACTCACTATAGGGAGACCACTGACCCTGAAGTTCATCTGC	Present study
	GFP T7R	TAATACGACTCACTATAGGGAGACCACGTCTTGTAGTTGCCGTCGTC	
Clathrin heavy chain	*CHC* T7F	TAATACGACTCACTATAGGGAGACCACCCTTACGAATGGCAGTGAGA	Present study
	CHC T7R	TAATACGACTCACTATAGGGAGACCACCAAGGATTCGTGTTTGTTCAAT	
Clathrin heavy chain	*CHC* RTF	CTCGGTGGAAAGGAAATGTT	Present study
	CHC RTR	AAGGTTGACAGTTCCGGATT	
rab7	*Rab7* T7F	TAATACGACTCACTATAGGGAGACCACTTGGTGACTCAGGTGTTGGT	Present study
	Rab7 T7R	TAATACGACTCACTATAGGGAGACCACACCCAACGACTGAAACCTCT	
rab7	*Rab7* RTF	TAGATGCATGGCGAGATGA	Present study
	Rab7 RTR	GCTTCGTTGAAACTGCTCTG	

major barriers to be crossed by the virus in order to be successfully transmitted by the whitefly (Hogenhout et al., 2008). Previous work from our laboratory has shown that TYLCV could enter specific cells of *B. tabaci* MED salivary gland, while the close relative *Tomato yellow leaf curl China virus* was unable to do so (Wei et al., 2014). The midgut of the greenhouse whitefly *Trialeurodes vaporariorum*, a TYLCV non-vector, was found to constitute a barrier that prevents the entrance of TYLCV into the insect haemolymph (Czosnek et al., 2002; Ohnishi et al., 2009). Transmission electron microscope analysis showed that TYLCV accumulates in vesicle-like structures in the midgut epithelial cells of the vector whitefly *B. tabaci*, but not in those of the non-vector *T. vaporariorum* (Uchibori et al., 2013). These vesicle-like structures represent the most commonly seen structures in endocytosis, suggesting the involvement of endocytosis in the process of TYLCV transport across the *B. tabaci* midgut barrier.

Endocytosis describes the de novo production of internal membranes from the plasma membrane lipid bilayer (Doherty and McMahon, 2009). Endocytosis is involved extensively in many different biological processes, including synapse storage of neurotransmitters and virus entry pathway. Apart from direct penetration, endocytosis represents the most common path exploited by virus to enter its host or its vector cells, especially clathrin-mediated endocytosis (Mercer et al., 2010). In the process of clathrin-mediated endocytosis, proteins recruit cargo such as virus into developing clathrin-coated pits, which are subsequently processed into clathrin-coated vesicles. Then these vesicles are further processed and clathrin is removed. Finally, the vesicles normally undergo modification through the endosome network, including early and later endosome, and are dispatched to their destination (Doherty and McMahon, 2009; Mercer et al., 2010). Apart from clathrin, which is composed of a heavy and a light chain, many other proteins are found to be involved in clathrin-mediated endocytosis and the ensuing endosome sorting, including dynamin, rab5, rab7 and others (Doherty and McMahon, 2009). Of those proteins, rab5 and rab7 are tightly associated with early endosome and later endosome respectively, and also serve as molecular markers in the search of endosome network function (Huotari and Helenius, 2011; Lee et al., 2013; Cheng et al., 2012).

In the present study, we found that following disruption of the whitefly clathrin-mediated endocytosis, either by inhibitors or by dsRNA, and after a 48 h acquisition access period (AAP) on TYLCV-infected leaves, the quantity of virus in the midgut (relative to that of the whole body) was increased and the quantity of virus in the haemolymph was decreased. Moreover, TYLCV immunofluorescence detection in midgut of whitefly where endocytosis was inhibited before virus acquisition showed that TYLCV accumulated in the midgut. In addition, to explore whether the endosome network plays a role in this process, antibody against rab5 and dsRNA against *rab7* were used. The results showed that the ability of TYLCV to cross the midgut was impaired as indicated by the decrease of the quantity of virus in the haemolymph. Our findings provide the first evidence that clathrin-mediated endocytosis as well as early and late endosome compartment is involved in TYLCV transport across the whitefly midgut barrier.

2. Materials and methods

2.1. Virus, insects and plants

An infectious clone of the TYLCV isolate SH2 (GenBank accession number: AM282874.1) was provided by Professor Xueping Zhou (Institute of Biotechnology, Zhejiang University). It was agroinoculated into tomato (*Solanum lycopersicum* cv. Hezuo903) to obtain TYLCV-infected plants. Whiteflies of the *B. tabaci* MED species (mtCOI GenBank accession code: GQ371165) were reared on tomato (*S. lycopersicum* cv. Hezuo903) in insect proof cages in a climate chamber at 26–28 °C, 14:10 light/dark (light: 6:30–20:30), 60–80% relative humidity. The purity of the whitefly cultures was monitored every three generations using PCR-restriction fragment length polymorphism and mtCOI sequencing (Qin et al., 2013). All tomato plants were planted singly in 1.5 l pot with potting mix (peat moss, vermiculite, organic fertilizer, perlite in a 5:1:1:1 ratio by volume) and kept in insect proof greenhouses under natural lighting supplemented with artificial lighting for 14 h a day from 06:00–20:00 and controlled temperature at 25 ± 3 °C.

2.2. Membrane feeding of inhibitor, antibody and dsRNA, and virus acquisition

Whitefly adults were collected and approximately 180 adults were released into each of the glass tubes with a diameter of 1.5 cm and a length of 10 cm. One opening of the tube was covered with double layers of parafilm filled with a diet solution and the other was covered with gauze. For inhibitors, we chose chlorpromazine (CPZ) (Sigma, USA), the specific inhibitor of clathrin assembly at cell surfaces and disassembly at endosome, and dynasore (DYN) (Sigma, USA), a specific dynamin inhibitor (Wang et al., 1993; Macia et al., 2006). In these experiments, 15% sucrose solution with 400 μM CPZ or DYN containing 0.1% DMSO were used to feed the whiteflies, and 15% sucrose solution containing 0.1% DMSO was used as control. For antibody, anti-rab5 monoclonal antibody (Cell Signaling Technology, USA) was added into 15% sucrose solution with a dilution of 1:50%, and 15%