SCIENCE CHINA
Life Sciences

•RESEARCH PAPER•

October 2018 Vol.61 No.10: 1254–1265
https://doi.org/10.1007/s11427-017-9283-4

Differential efficiency of a begomovirus to cross the midgut of different species of whiteflies results in variation of virus transmission by the vectors

Lilong Pan, Qunfang Chen, Tao Guo, Xinru Wang, Ping Li, Xiaowei Wang & Shusheng Liu*

Ministry of Agriculture Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Institute of Insect Sciences, Zhejiang University, Hangzhou 310058, China

Received January 2, 2018; accepted February 28, 2018; published online May 18, 2018

Begomoviruses are important crop viral disease agents, and they are transmitted by whiteflies of the *Bemisia tabaci* complex. Although the transmission of begomoviruses by whiteflies has been studied for many years, the mechanisms governing differential transmission of begomoviruses by different species of the *Bemisia tabaci* complex remain largely unknown. Here we firstly compared the transmission efficiency of tobacco curly shoot virus (TbCSV) by four species of the *B. tabaci* complex and found that Asia II 1 transmitted this virus with the highest efficiency, whereas MEAM1 transmitted it with the lowest. Next, by performing quantitative analysis of virus and immune-fluorescence detection, we found that the efficiency of TbCSV to cross the midgut wall was higher in Asia II 1 than in MEAM1. Finally, we set the quantities of virions in the haemolymph to the same level in Asia II 1 and MEAM1 via injection and then compared their capacity in TbCSV transmission, and found that the difference in TbCSV transmission between them became smaller. Taken together, our findings suggest that the efficiency of a begomovirus to cross the midgut wall of a whitefly to reach the vector's haemolymph plays a significant role in determining transmission of the virus.

begomovirus, *Bemisia tabaci*, differential transmission, midgut

Citation: Pan, L., Chen, Q., Guo, T., Wang, X., Li, P., Wang, X., and Liu, S. (2018). Differential efficiency of a begomovirus to cross the midgut of different species of whiteflies results in variation of virus transmission by the vectors. Sci China Life Sci 61, 1254–1265. https://doi.org/10.1007/s11427-017-9283-4

INTRODUCTION

Plant diseases caused by viruses are major constraints to the production of crops (Scholthof et al., 2011). In recent decades, viruses belonging to the genus *Begomovirus* (family Geminiviridae) have been shown to cause significant losses to the production of many crops as exemplified by the losses caused by cotton leaf curl disease to the cotton industry in Pakistan or tomato yellow leaf curl disease to the tomato industry in the Mediterranean Basin (Briddon and Markham, 2000; Navas-Castillo et al., 2011). Begomoviruses are transmitted by whiteflies of the *Bemisia tabaci* cryptic species complex (De Barro et al., 2011; Liu et al., 2012; Navas-Castillo et al., 2011). Within this complex, different cryptic species may transmit the same begomovirus with different efficiency (Guo et al., 2015; Li et al., 2010; Wei et al., 2014), and the efficiency of a given whitefly species may vary for transmitting different begomoviruses (Liu et al., 2010; Wei et al., 2014). However, the factors associated with the variations of begomovirus transmission are poorly known.

According to the transmission characteristics, such as the time required by the vector to acquire the virus and the retention time of the virus in the vector, the mode of begomovirus transmission of *B. tabaci* whiteflies has been

*Corresponding author (email: shshliu@zju.edu.cn)

described as persistent circulative (Hogenhout et al., 2008; Rosen et al., 2015). The research on whitefly transmission of begomoviruses has so far been conducted mostly with tomato yellow leaf curl virus (TYLCV) (Czosnek et al., 2002; Czosnek et al., 2017). Following oral acquisition from virus-infected plants by whiteflies, the virus has to move along food canal, reach the esophagus and the midgut, cross the filter chamber and the midgut wall to reach the haemolymph, and then translocate into the primary salivary glands, from where the virus is egested with saliva into new plants for virus inoculation (Ghanim et al., 2001a). In this journey of the virus within the body of a whitefly, the midgut of whitefly represents the first cellular barrier that viruses have to confront following acquisition. The midgut of whitefly is a loop, due to the tight connection between filter chamber, descending midgut and ascending midgut (Ghanim et al., 2001b). The route of virus movement within the midgut was hypothesized to follow along the filter chamber, the caeca, the descending midgut and finally the ascending midgut (Ghanim et al., 2001b; Ghanim et al., 2009). Transmission electron microscopy analysis of whitefly midgut showed the anterior part of the descending midgut was the major entry site for TYLCV to reach the haemolymph (Uchibori et al., 2013). However, the importance of midgut in virus movement and transmission has rarely been examined in detail and quantitatively.

Tobacco curly shoot virus (TbCSV) was firstly characterized in 2001, and later field survey showed that it was one of the major begomoviruses that were associated with tobacco leaf curl disease and tomato leaf curl disease in Yunnan province, China (Li et al., 2002; Liu et al., 2007; Xie et al., 2002). Moreover, TbCSV has been reported to infect pepper, common bean, sunflower and tomato in India (Shilpi et al., 2015). In the laboratory, TbCSV transmission experiments have been done with two species of the *B. tabaci* whitefly complex, i.e., Middle East-Minor Asia 1 (MEAM1) and Asia II 3, and the data indicated that both species of whiteflies could transmit TbCSV only with low levels of efficiency (Jiu et al., 2006). In regions where TbCSV is a disease agent, in addition to MEAM1 and Asia II 3, other species of the *B. tabaci* complex, in particular Asia 1 and Asia II 1, also occur (Hu et al, 2014; Jiu et al., 2006; Liu et al., 2007; Li et al., 2002).

In the present study, we firstly performed TbCSV transmission tests with four species of whiteflies that have been recorded in regions where TbCSV has been observed, and then conducted a series of observations on virus movement and distribution in two whitefly species, one with the highest efficiency of TbCSV transmission and one with the lowest. Our data indicate that, the efficiency of the virus to cross the midgut wall of a whitefly plays a significant role in determining the vector's capacity for transmitting the virus.

RESULTS

TbCSV transmission efficiency among four species of whiteflies

Four species from the *B. tabaci* complex, i.e., MEAM1, MED, Asia1 and Asia II 1, were compared for their efficiency of TbCSV transmission. In the first experiment where one viruliferous adult per plant was used for virus transmission, the percentages of tobacco plants that were found infected with virus DNA were significantly different between the four species of whiteflies in the order of Asia II 1>Asia 1>MED>MEAM1, with the highest (44.4%) by Asia II 1 and the lowest (0.0%) by MEAM1 (Figure 1A). Likewise, the percentages of tobacco plants with virus infection symptom (Figure S1 in Supporting Information) differed between the four whitefly species with the same pattern, with the highest (38.9%) by Asia II 1 and the lowest (0.0%) by MEAM1 (Figure 1B).

In the second experiment where five viruliferous adults per plant were used for virus transmission, the percentages of tobacco plants that were found infected with virus DNA were significantly different between the four species of whiteflies in the order of Asia II 1>MED>Asia 1>MEAM1, with the highest (96.3%) by Asia II 1 and the lowest (0.0%) by MEAM1 (Figure 1C). Likewise, the percentages of tobacco plants with virus infection symptom also differed between the four whitefly species with the same pattern, with the highest (92.6%) by Asia II 1 and the lowest (0.0%) by MEAM1 (Figure 1D).

In the third experiment, ten viruliferous adults per plant were inoculated for virus transmission. Here, only two of the four whitefly species, i.e., Asia II 1 and MEAM1 were compared. The percentage of successful transmission, as indicated by the presence of virus DNA, was significantly higher by Asia II 1 (97.0%) than by MEAM1 (10%; Figure 1E). Likewise, the percentage of successful transmission, as indicated by the presence of disease symptom, was significantly higher by Asia II 1 (94.0%) than by MEAM1 (10%; Figure 1F).

Virus acquisition capacity of Asia II 1 and MEAM1

When adults of Asia II 1 or MEAM1 were allowed to feed for virus acquisition on TbCSV-infected tobacco plants for various periods of time from 6 to 168 h, the relative quantity of viruses in each of the two whitefly species increased with increase of feeding time, and at each of the time intervals where quantities of viruses were compared between the two of whitefly species, the quantity of virus was always significantly higher in Asia II 1 than in MEAM1 (Figure 2A; $P<0.05$ in all cases).

The relative quantity of virus in the honeydew excreted by

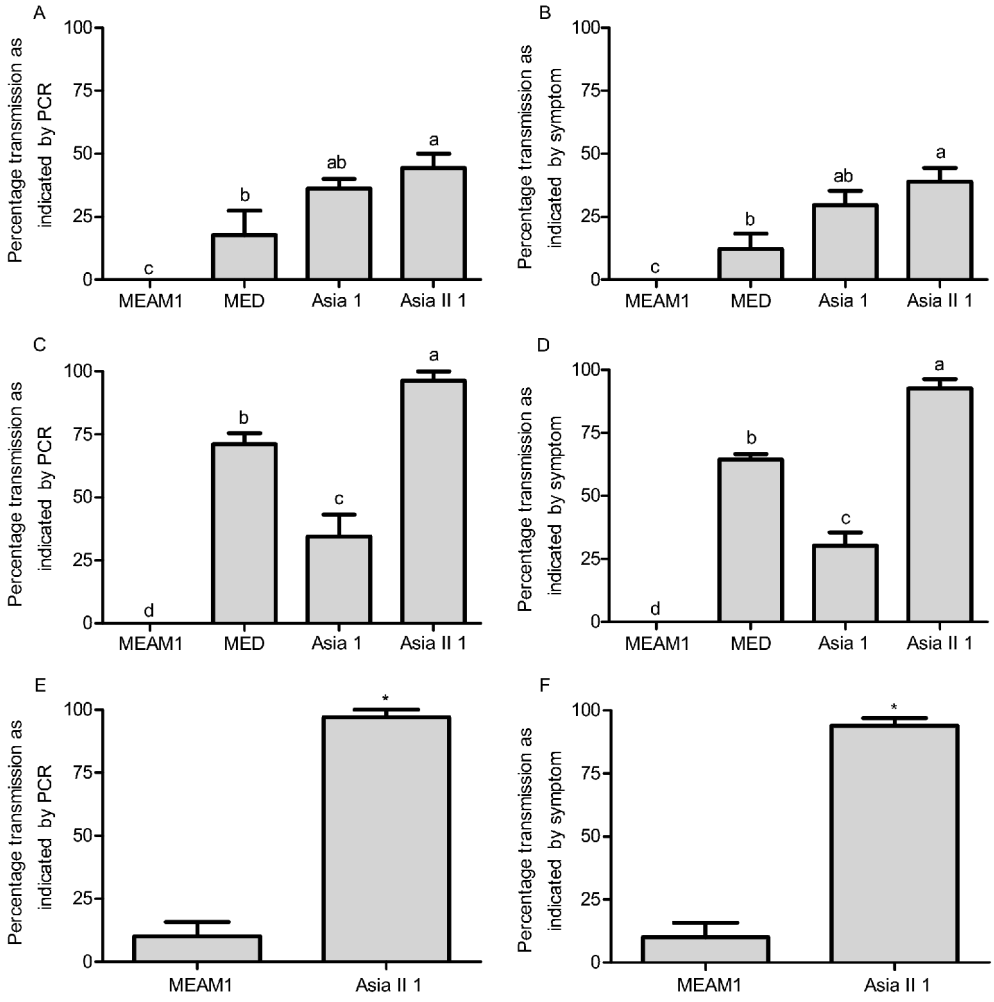

Figure 1 Transmission efficiency of TbCSV by four species of the *B. tabaci* complex. The number of whiteflies per test plant were 1 for A and B, 5 for C and D, 10 for E and F, and in each experiment, three replicates were conducted and 5–11 plants were used in each replicate. Data is presented as the mean± standard errors of mean (mean±SE) of the percentage of PCR positive test plants for A, C and E, and percentage of test plants that showed typical symptom for B, D and F. Different letters above the bars in A, B, C and D, and * above the bars in E and F indicates significant difference (one-way analysis of variance, $P<0.05$ for A–D; independent *t*-test, $P<0.05$ for E and F).

MEAM1 was always significantly higher than that by Asia II 1, in each of the three time intervals, i.e. 24, 48 and 168 h, when comparison was made between the two whitefly species (Figure 2B–D; $P<0.05$ in all cases).

Relative quantity of virus in organs of MEAM1 and Asia II 1

When each of the two whitefly species, MEAM1 and Asia II 1, was given a period of 24 h feeding on TbCSV-infected tobacco plants for virus acquisition, the relative quantities of virus in midgut, haemolymph and primary salivary glands were all significantly higher in Asia II 1 than those in MEAM1 (Figure 3A–C; $P<0.05$ in all cases). Likewise, for whiteflies that were given a period of 48 h for virus acquisition, the virus quantity in each of the three organs was significantly higher in Asia II 1 than that in MEAM1 (Figure 3D–F; $P<0.05$ in all cases).

TbCSV signals in midgut of MEAM1 and Asia II 1

After the adults of MEAM1 and Asia II 1 were given a feeding period varying from 6 to 168 h for virus acquisition, the midguts of each of the two whitefly species were visualized using immune-fluorescence for the presence of TbCSV signals, at each of the six time intervals of 6, 12, 24, 48, 96 and 168 h.

As shown in Figure 4, after 6 h of feeding for virus acquisition, no virus specific signals (red) were observed in midguts of either MEAM1 or Asia II 1. Then, in Asia II 1 the virus signals were observed after 12 h feeding for virus acquisition, and the signals became stronger as the time of feeding for virus acquisition increased, and the signals were firstly observed in the filter chamber, then the caecum, and finally the descending midgut and ascending midgut. In contrast, in MEAM1 no virus specific signals were observed for the entire period of observation from 6 to 168 h.

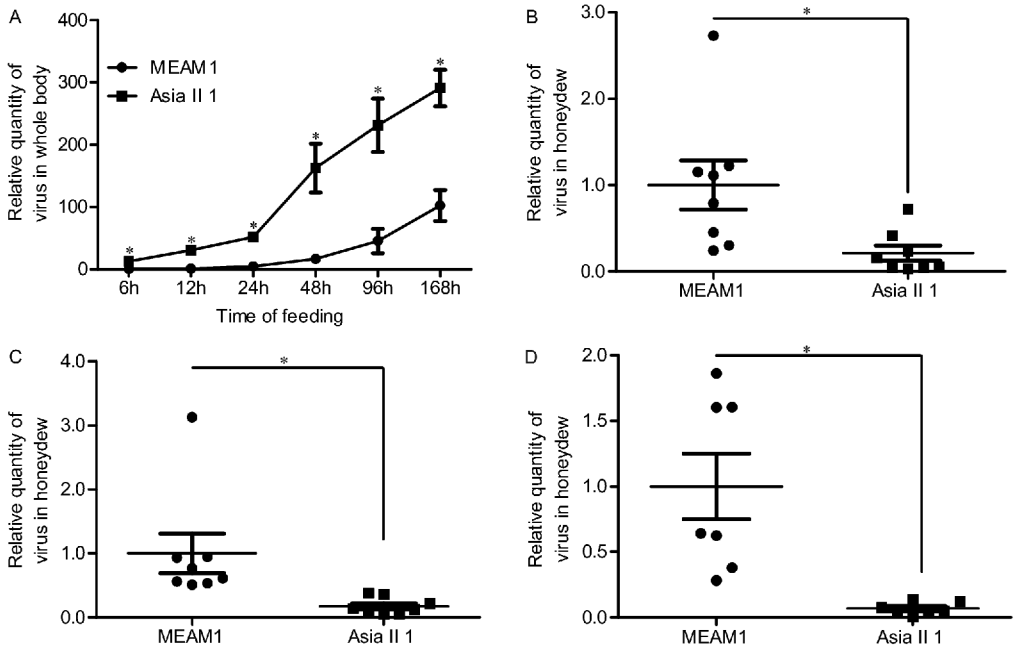

Figure 2 Quantity of virus in the whole body of MEAM1 and Asia II 1 whiteflies after they have fed on TbCSV-infected tobacco for various periods of time (A) and virus in their honeydew at 24 (B), 48 (C) and 168 h (D). Data is presented as the mean±SE of relative quantity of virus and * indicates significant differences (independent *t*-test, $P<0.05$ for A; Mann-Whitney *U* test, $P<0.05$ for B–D).

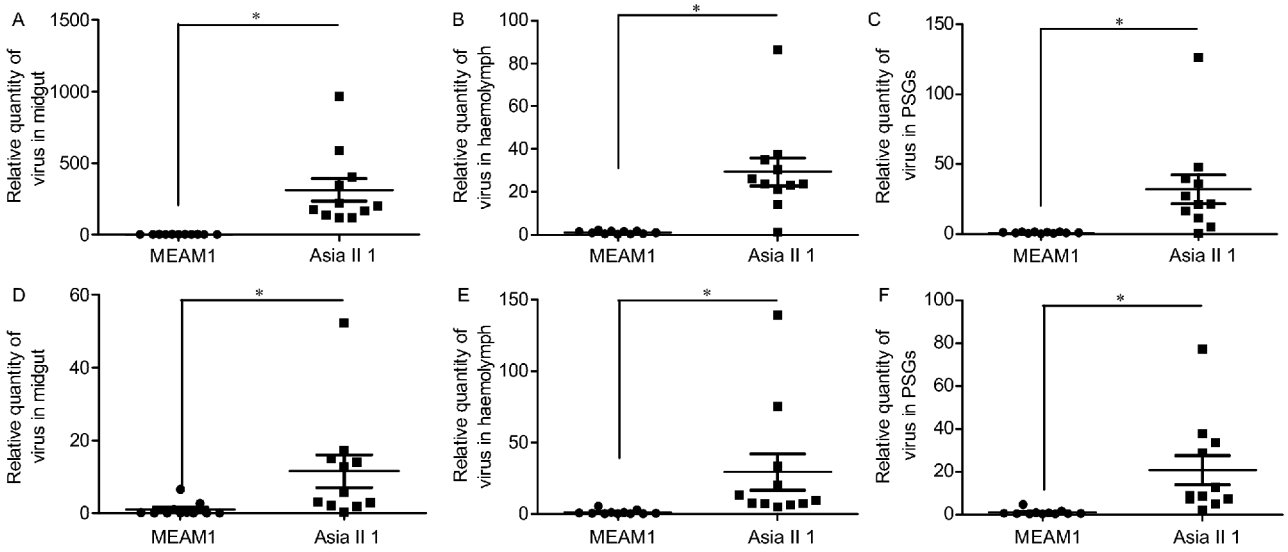

Figure 3 Quantity of virus in different organs/tissue of MEAM1 and Asia II 1 whiteflies when they have fed on TbCSV-infected tobacco plants for 24 and 48 h. The midgut (A), haemolymph (B), and primary salivary gland (C) at 24 h, and the midgut (D), haemolymph (E), and primary salivary gland (F) at 48 h. Data is presented as the mean±SE of relative quantity of virus and * indicates a significant difference (Mann-Whitney *U* test, $P<0.05$).

TbCSV signals in primary salivary glands of MEAM1 and Asia II 1

After 6 and 12 h feeding for virus acquisition, no virus signals (red) were observed in the primary salivary glands of Asia II 1, and then virus signals were observed after 24 h feeding for virus acquisition and the signals became stronger as the feeding time increased from 24 to 168 h. All virus signals were seen in the internal ducts of the central region of the primary salivary glands. In contrast, in MEAM1, no virus specific signals were observed in the primary salivary glands at all-time points checked from 6 to 168 h (Figure 5).

TbCSV transmission and relative quantity in different organs of MEAM1 and Asia II 1 when the whiteflies were reared from TbCSV infected tobacco

Compared to the standard procedure for virus transmission

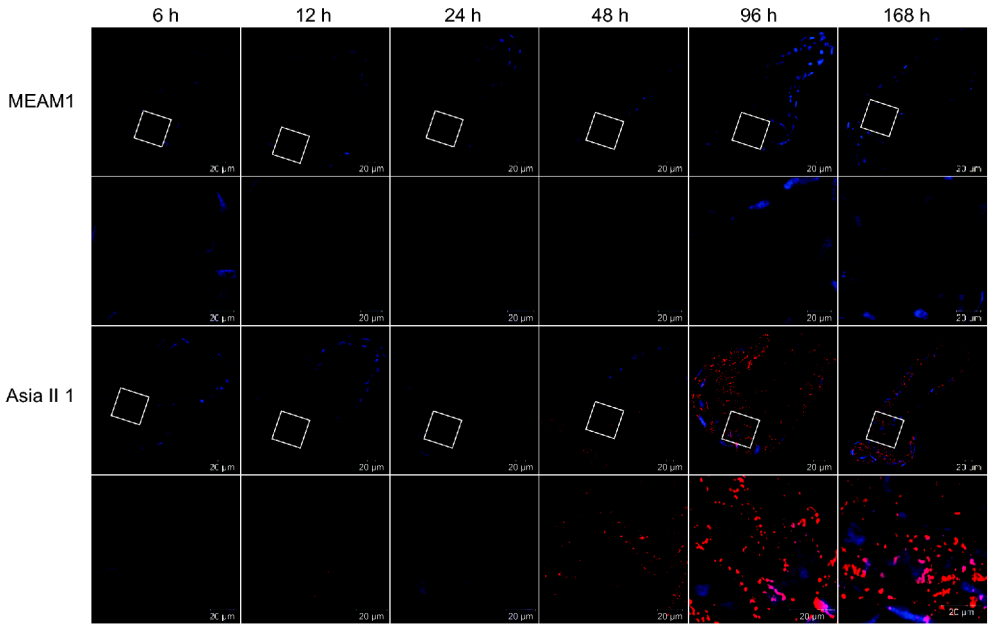

Figure 4 Immune-fluorescence detection of TbCSV in midguts of MEAM1 and Asia II 1 whiteflies after they have fed on TBCSV-infected tobacco plants for various periods of time. TbCSV was detected by a mouse anti-TYLCV antibody and a goat anti-mouse secondary antibody conjugated to FITC (red), and nuclei were stained with DAPI (blue). Images showing the typical virus signal are presented. For each of the two whitefly species at each of the time points, upper panels show a whole view of midguts and lower panels show the enlarged view of the filter chamber (boxed in the upper panels).

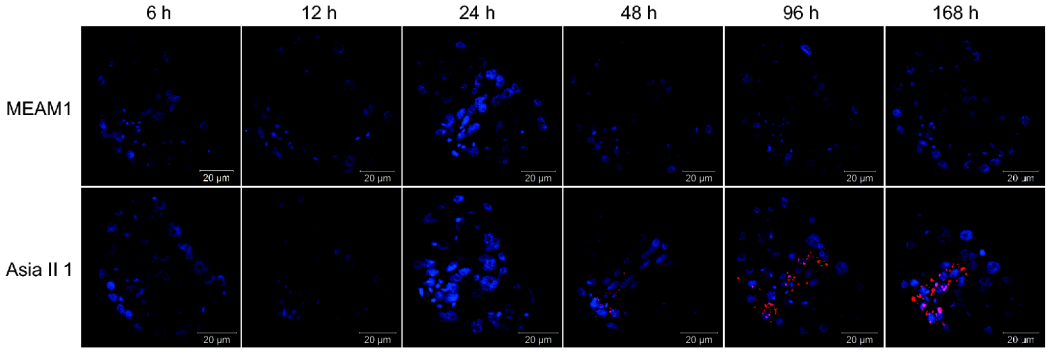

Figure 5 Immune- fluorescence detection of TbCSV in primary salivary glands of MEAM1 and Asia II 1 whiteflies after they have fed on TbCSV-infected tobacco for various periods of time. TbCSV (red) and nuclei (blue). Images showing the typical virus signal are presented.

tests, viruliferous whiteflies used in this part of the study were obtained in a different way. Instead of giving non-viruliferous adults a 48 h feeding on TbCSV-infected plants for virus acquisition, the viruliferous whiteflies used here were obtained by rearing the individuals continuously on TbCSV-infected tobacco from egg to adult, i.e., the individuals were given the opportunity to obtain virus continuously from egg to adult. The procedure for virus inoculation and detection was the same as that of the standard procedure.

The data of virus transmission tests showed that in tobacco plants inoculated by viruliferous adults of Asia II 1, viral DNA was detected in 87.9% of them and typical viral symptom was found in 82.1% of them, whereas in the test plants inoculated by MEAM1, viral DNA and viral symptom were observed in none of them (Figure 6A and B; $P<0.05$ in both cases). Meanwhile, as shown in Figure 6C–E, the quantity of virus in midgut, haemolymph and primary salivary glands of Asia II 1 was significantly higher than that in MEAM1 ($P<0.05$ in all cases). In addition, TbCSV signals were observed by immune-fluorescence in the midguts and primary salivary glands dissected from Asia II 1, whereas no viral signals were observed in either of the two organs from MEAM1 (Figure 6F and G).

TbCSV transmission and presence of virions in primary salivary glands of MEAM1 and Asia II 1 when the whiteflies were injected with TbCSV virions

Compared to the standard procedure for virus transmission

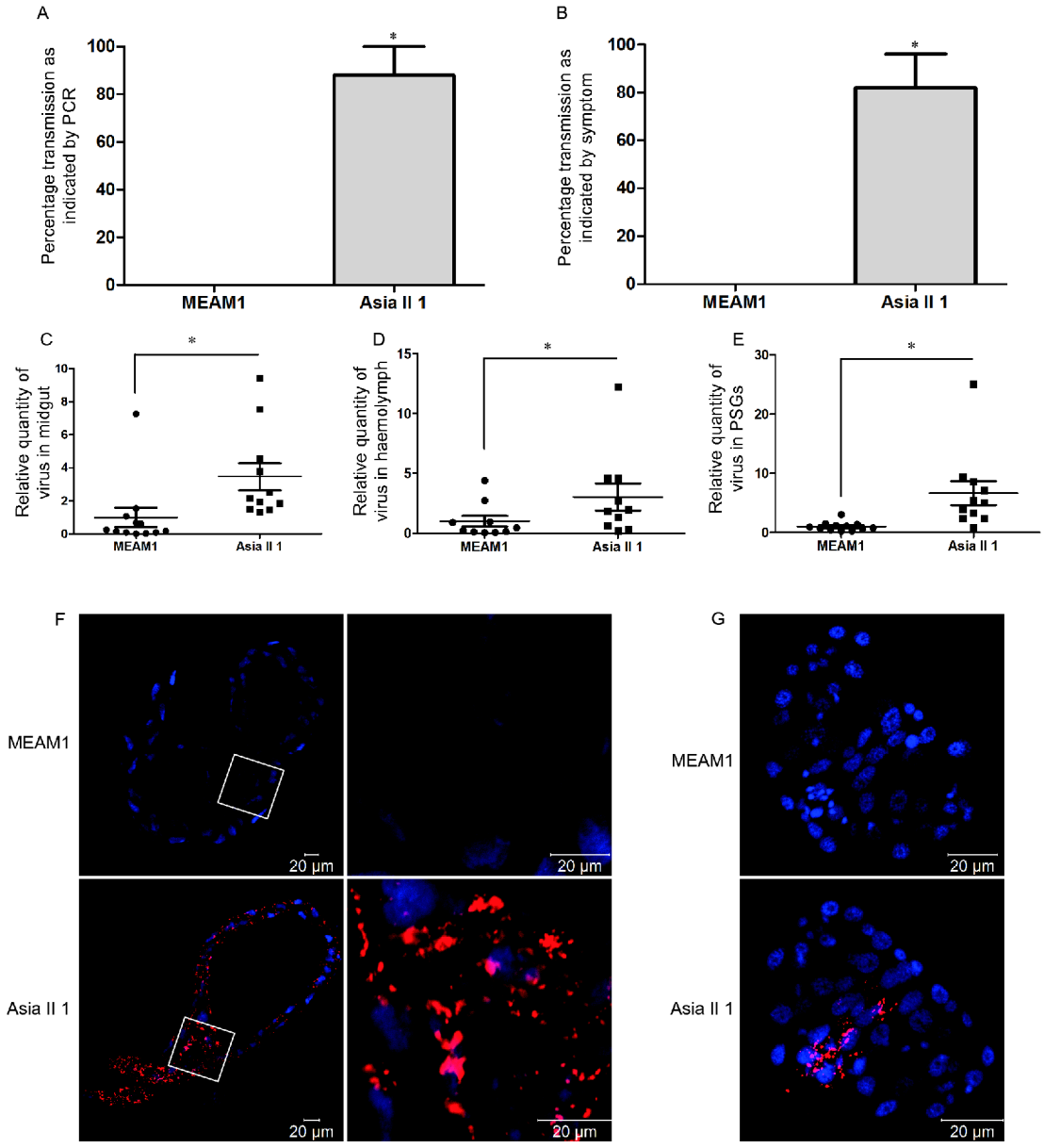

Figure 6 Transmission efficiency, quantity of virus and immune-fluorescence detection of TbCSV in organs of MEAM1 and Asia II 1 whiteflies that were reared from egg to adult on TbCSV-infected tobacco. Efficiency of virus transmission as shown by PCR positive test plants (A) and plants showing typical virus infection symptom (B); relative quantity of virus in midgut (C), haemolymph (D) and primary salivary gland (E); and immune-fluorescence detection of TbCSV (red) and nuclei (blue) in the midgut (F) and salivary gland (G). Data is presented as the mean±SE. * in A–E indicates a significant difference (independent *t*-test, $P<0.05$ for A and B; Mann-Whitney U test, $P<0.05$ for C–E).

tests, viruliferous whiteflies used in this part of the study were also obtained in a different way. Instead of giving non-viruliferous adults a 48 h feeding on TbCSV-infected plants for virus acquisition, the viruliferous adults used here were obtained by injection of TbCSV virions into non-viruliferous adults. After injection of TbCSV virions, the quantity of virions in adults of both MEAM1 and Asia II 1 was analyzed by qPCR, and the results showed that no significant difference was found between the two species of whiteflies (data not shown).

The data of virus transmission tests showed that in tobacco plants inoculated with viruliferous adults of MEAM1 and Asia II 1, 57.9% and 80.6% of them were detected with virus DNA, and 34.7% and 69.0% of them were observed with typical viral symptom (Figure 7A and B; $P<0.05$ in both cases)

In whitefly adults that were not injected with virions, no TbCSV signals were detected in the primary salivary glands by immune-fluorescence in all individuals of either MEAM1 or Asia II 1. In contrast, in whitefly adults that were injected with virions, the primary salivary glands of some individuals in each of the two species were observed with TbCSV signals, at both 48 and 168 h post injection. At each of two time points, relatively more individuals were observed with

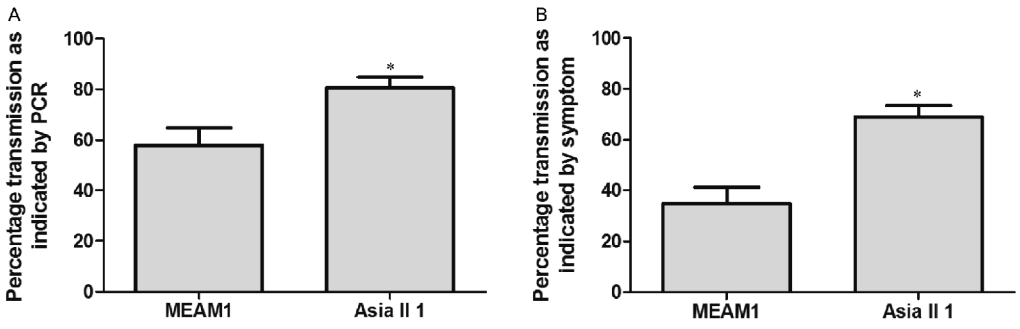

Figure 7 Transmission efficiency of TbCSV of MEAM1 and Asia II 1 whiteflies that were injected with TbCSV virions. Efficiency of virus transmission as shown by PCR positive test plants (A) and plants showing typical virus infection symptom (B). Data is presented as the mean±SE and * above the bars indicates a significant difference between the two species of whitefly (independent *t*-test, $P<0.05$).

TbCSV signals in Asia II 1 than that in MEAM1 (Figure S2 in Supporting Information and Table 1).

DISCUSSION

In the present study, firstly, TbCSV transmission experiments showed that among the four species of whiteflies tested, Asia II 1 was the most efficient transmitter while MEAM1 was the least efficient (Figure 1). Secondly, the analyses of the quantity of virus in whiteflies and their honeydew following continuous virus acquisition illustrated that Asia II 1 was much more efficient in retaining TbCSV than MEAM1, as indicated by the higher quantity of the virus in the honeydew of MEAM1 than that of Asia II 1 (Figure 2). Thirdly, analyses of TbCSV quantities and immune-fluorescence detection of the virus in different organs of whiteflies indicated that, compared to Asia II 1, MEAM1 translocated relatively less TbCSV virions from midgut to haemolymph (Figures 3–6). Finally, when the quantities of TbCSV virions in the haemolymph were set up to the same level in the two species of whiteflies via injection of virions, MEAM1 became significantly more effective in achieving virus transmission than before, and the difference in the levels of virus transmission between the two species of whiteflies likewise became much smaller (Figure 7). These data jointly indicated that TbCSV has higher efficiency of crossing the midgut wall of Asia II 1 than that of MEAM1, and this higher efficiency of Asia II 1 seemed to be partially responsible for its higher capacity in transmitting this virus.

For persistently transmitted viruses, once orally acquired from infected plants, at first, viruses need to translocate from gut lumen to the haemolymph of the insect vector to achieve successful transmission (Hogenhout et al., 2008). For example, when tomato spotted wilt virus is acquired by the adults of the western flower thrips (*Frankliniella occidentalis*), it fails to move from the gut to the haemolymph of adults and, in consequence fails to be transmitted; however, when the virus was acquired by the thrips during the larval stage, the ensuing adults are able to transmit the virus (Ullman et al., 1992). In whiteflies, TYLCV virions can be ingested by the greenhouse whitefly, *Trialeurodes vaporariorum*, but are unable to move from the whitefly's midgut to its haemolymph, and this virus cannot be transmitted by the greenhouse whitefly (Czosnek et al., 2002). And recently, it has been shown that a begomovirus found in China, papaya leaf curl China virus (PaLCuCNV) crosses the midgut wall of MED less efficiently than that of MEAM1, resulting in lower level of PalCuCNV transmission by MED than that by MEAM1 (Guo et al., 2018). Moreover, when a virus possesses only a weak capacity to cross the gut of its vector, the efficiency of virus transmission will be very low; however, when virions are injected to the haemolymph of their vectors the efficiency of virus transmission will be significantly increased. This phenomenon was observed in the transmission of maize Iranian mosaic virus by *Peregrinus maidis* (Ammar et al., 2005). Interestingly, this phenomenon was also observed in the current study on the transmission of TbCSV by whiteflies. However, the physiological and molecular mechanisms underlying the variation of efficiency of viruses to cross the midgut wall of whiteflies are barely known.

In aphids, a group of insects related to whiteflies, the cross of gut by viruses they transmit persistently has long been proposed to be mediated by receptor-mediated endocytosis (Gildow, 1993). And recently, membrane alanyl aminopeptidase N was proposed to be a receptor for pea enation mosaic virus in the pea aphid, *Acyrthosiphon pisum* (Linz et al., 2015). In whiteflies, clathrin mediated endocytosis, which is supposed to be activated by virus-receptor interaction, was recently reported to be involved in TYLCV transport from midgut to the haemolymph (Pan et al., 2017). These reports suggest that whiteflies have specific receptors for begomoviruses. Both begomoviruses and whiteflies of the *B. tabaci* complex are known to be diverse in species and genetic structure (Brown et al., 2015; De Barro et al., 2011; Liu et al., 2012; Zerbini et al., 2017). Our current study suggests the existence of different begomovirus receptors in

Table 1 Examination of TbCSV signals in primary salivary glands (PSGs) of MEAM 1 and Asia II 1 at 48 and 168 h post virion injection in two separate experiments. Typical immune-fluorescence images of the primary salivary glands of un-infected whitefly adults and adults at 48 and 168 h after virion injection was shown in Figure S2 in Supporting Information.

No. of experiments	Time post injection	Whitefly species	No. of PSGs observed	No. of PSGs with virus signal
Exp. 1	48 h	MEAM1	26	4
		Asia II 1	22	8
	168 h	MEAM1	32	1
		Asia II 1	36	15
Exp. 2	48 h	MEAM1	18	1
		Asia II 1	10	4
	168 h	MEAM1	26	7
		Asia II 1	17	12

different species of whiteflies. These receptors may have different features, such as different affinities to a given species of virus, which may play a significant role in determining the differential efficiency of a virus to cross the midgut wall and reach the haemolymph of different species of whitefly. With the growing of biological and genetic information of whitefly-begomoviruses interactions such as the findings achieved in this study and publication of more whitefly genomes as well as the application of novel techniques, receptors of begomoviruses in whiteflies may be expected to be discovered in the near future (Chen et al., 2016; Linz et al., 2015). The discovery of receptors will not only help decipher the nature of whitefly transmission of begomoviruses, but also facilitate the development of novel control techniques against the vectors and viral diseases (Chougule et al., 2013).

In this study, four species of whiteflies, two invasive (MEAM1 and MED) and two indigenous (Asia II 1 and Asia 1), were compared for their efficiency in transmitting TbCSV. In general, the indigenous species of whiteflies exhibit higher efficiency in transmitting this virus than the invasive species. As TbCSV is indigenous to China, the indigenous whiteflies have probably been associated with TbCSV for thousands of years, while the invasive whiteflies have been associated with this virus only in recent decades. During the long-term association between the indigenous whiteflies and TbCSV, the vectors and the virus may have co-evolved towards better compatibility in many aspects of their biology, for example, more efficient receptors for the virus. The higher efficiency of the indigenous whiteflies, compared to invasive whiteflies, in transmitting TbCSV may be related to their much longer time of association with this virus.

MATERIALS AND METHODS

Insect, virus and plants

For insects used in this study, four species of whiteflies were collected from the field between 2009–2012. The mt*COI* GenBank accession codes for each of the species of whiteflies are KM821540 for MEAM1, GQ371165 for MED, KC540757 for Asia 1 and DQ309077 for Asia II 1. These four species of whiteflies were used in the study because each of them occurs naturally at some of the regions where TbCSV had been recorded (Hu et al., 2014; Prasanna et al., 2015). One culture of each species was established and maintained on cotton plants (*Gossypium hirsutum* L. cv. Zhemian 1793) in the laboratory. Every three generations, the purity of each whitefly culture was monitored using PCR-restriction fragment length polymorphism and mt*COI* sequencing (Qin et al., 2013). All insect rearing, as well as all the experiments that used insect rearing, were conducted in insect-proof cages at 26°C±2°C, 16 h L: 8 h D in temperature-controlled rooms.

For viruses used in this study, TbCSV isolate Y35 (GenBank accession code: AJ420318) and its associated betasatellite (GenBank accession code: AJ421484) were agro-inoculated into 3–4 true leaf stage tobacco (*Nicotiana tabacum* L. cv. NC89), and the plants were used approximately 3–4 weeks post virus inoculation. Prior to use in any experiment, the status of TbCSV infection of each plant was determined by symptom inspection and PCR detection. For PCR detection of TbCSV, the first fully expanded leaf of the test plants was collected and DNA extraction was performed using Plant Genomic DNA Kit (Tiangen, Beijing), and then PCR were performed using those DNA samples as template and the primers for TbCSV PCR detection were primer 1 (5′-ACAGAAGTCCTGATGTCCCT-3′) and primer 2 (5′-AGAGCACCAGAACCGTCC-3′). All plants including tobacco and cotton were grown in an insect proof greenhouse under natural lighting and controlled temperature at 25°C±3°C.

Standard procedure for virus transmission tests

For obtaining viruliferous whitefly adults to be used in virus transmission tests, whitefly adults, that developed from cotton and emerged for 1–3 days, were collected. The adults were placed on TbCSV-infected tobacco plants to feed for a

period of 48 h to acquire the virus. These adults were then collected and a given number of adults, as designated in the design of a given experiment, were inoculated to new individual test plants to feed for another period of 48 h for virus transmission. Immediately after the 48 h virus transmission, the whitefly adults were removed and kept for subsequent determination of virus infection status using PCR, and the test plants were sprayed with imidacloprid (20 mg L^{-1}) to kill all the whitefly eggs produced during the 48 h inoculation period and prevent them from further development. The test plants were further cultured for about four weeks and the virus infection status of the test plants was then determined by PCR detection and symptom inspection.

Comparison of virus transmission among four species of whiteflies

All experiments of virus transmission were conducted using the standard procedure, and three transmission experiments were conducted using different number per plant of viruliferous adults enclosed in leaf clip cages for inoculation. The leaf clip cages used in the experiment were described previously (Ruan et al., 2007). For the first experiment, four treatments with all the four species of whiteflies were conducted, and one viruliferous was inoculated for each test plant. For the second experiment, again four treatments with all the four species of whiteflies were conducted but the number of adults inoculated to each plant was five. For the third experiment, only two treatments with MEAM1 and Asia II 1 were conducted and the number of adults inoculated to each plant was ten. For each replicate in an experiment 5–11 plants were used, and for each of the three experiments three replicates were conducted.

The data of the above experiments for comparison of virus transmission showed that, among four species of whiteflies, Asia II 1 was the most efficient transmitter while MEAM1 was the least. For the purpose of this study, in the following experiments to examine virus quantity in different organs of whiteflies and virion injection, only these two species of whiteflies were used.

Analysis of virus quantity in whitefly whole body, honeydew and dissected organs

For analysis of the quantity of virus in the whole body of whiteflies, adults of MEAM1 and Asia II 1 were placed to feed on TbCSV-infected tobacco plants continuously for virus acquisition. Thereafter, whitefly adults were collected in groups of 15 at designated time points following virus acquisition, and DNA extraction was performed using the lysis buffer (50 mmol L^{-1} KCl, 10 mmol L^{-1} Tris, 0.45% Tween 20, 0.2% gelatin, 0.45% NP40, 60 mg mL^{-1} protease K with pH at 8.4). For each of the two whitefly species at a designated time points, 3–4 replicates were conducted.

For analysis of the quantity of the virus in whitefly honeydew, honeydew was first collected using the following methods: two leaf clip cages were placed on the undersurfaces of each leaf of TbCSV infected tobacco plants, one for each of the two species of whiteflies and 20 adults in each leaf clip cage. Eight replicates were conducted. The bottom of the leaf clip cages were covered with aluminum foil so that the honeydew produced by whiteflies feeding on the leaves would fall onto the tinfoil. The honeydew of whiteflies was collected at a designated interval of time of feeding, and viral DNA was extracted using PureLink Viral RNA/DNA Mini Kit (Invitrogen, USA). The honeydew samples were then subjected to real time analysis and the copy number of virus was obtained as normalized to a standard curve. The standard curve was made by real-time quantitative PCR reaction using the serial dilutions of plasmid extracted from the infectious clone of TbCSV as the template.

For analysis of whitefly midguts and primary salivary glands, these organs were dissected and collected, and DNA extraction from these organs were performed using the same lysis buffer as described above after they were washed twice with PBS. For collection of haemolymph, single whitefly was placed into PBS on glass slides, and then the abdomen was cut to release the content, the midgut was removed, and the remaining liquid was collected and the lysis buffer was added. For each of the organs of either MEAM1 or Asia II 1, 10–12 replicates were conducted.

All samples for analysis were incubated at 65°C for 1.5 h followed by 10 min in boiling water. Real time PCR was performed using SYBR Premix Ex Taq II (TaKaRa, Japan) and CFX96™ Real-Time PCR Detection System (Bio-Rad, USA) with the primers for *TbCSV*: primer 1 (5′-GAAGCGTCCAGCAGATATAA-3′) and primer 2 (5′-GGGACATCAGGACTTCTGTA-3′), and primers for whitefly *actin*: primer 1 (5′-TCTTCCAGCCATCCTTCTTG-3′) and primer 2 (5′-CGGTGATTTCCTTCTGCATT-3′).

Immune-fluorescence detection of viruses in whitefly midguts and primary salivary glands

The immune-fluorescence detection of viruses in MEAM1 and Asia II 1 was performed using the methods as described by Wei et al. with some modifications (Wei et al., 2014). Firstly, intact midguts and primary salivary glands were dissected in PBS, and they were incubated in 4% paraformaldehyde (MultiSciences Biotech., Hangzhou) at room temperature for 1 h. Then the samples were washed and incubated in 0.5% Triton X-100 for 30 min and the samples were blocked with 1% BSA for 1 h at room temperature. After that, anti-TYLCV CP monoclonal antibody (mAb)

(provided by Professor Xueping Zhou, Institute of Biotechnology, Zhejiang University) was added with a dilution of 1:400 and the samples were incubated overnight at 4°C. The next day, the samples were washed and blocked using solution with 549-conjuncted secondary antibody (Earthox, Beijing) with a dilution of 1:400 were added. After 2 h incubation at room temperature, all samples were washed and placed on slides for observation under Zeiss LSM710 confocal microscope (Zeiss, Germany). For each experiment, 10 midguts or primary salivary glands were examined for each of the two whitefly species.

Experiments with viruliferous whiteflies that developed from egg to adult on TbCSV-infected tobacco plants

In the experiments presented above, viruliferous adults used for virus transmission tests were obtained by rearing from egg to adult on virus-free cotton and upon emergence they were transferred to feed on TbCSV-infected tobacco plant for 48 h for virus acquisition. In this part of the study, viruliferous adults used in the experiments were obtained by rearing the individuals continuously on TbCSV-infected tobacco plants from egg to adult emergence, and newly emerged (<24 h after emergence) adults were collected for virus transmission tests and analysis of virus quantity in various organs of the whiteflies. For the virus transmission test, five adults were inoculated to each test plant. Two treatments for MEAM1 and Asia II 1 were conducted using the standard procedure described above, and each treatment had three replicates with 10–12 plants in each replicate. In addition, immune-fluorescence detection of virus signals in the midgut and primary salivary glands was conducted with newly emerged adults for both species of whitefly. For each organ of either MEAM1 or Asia II 1, 10 adults were examined.

Purification of TbCSV virions and injection of purified virions into whitefly haemolymph

This aspect of the study was also conducted with only MEAM1 and Asia II 1. Approximately one month prior to virion purification, 3–4 true leaf stage *Nicotiana benthamiana* plants were agro-inoculated with TbCSV isolate Y35 and its associated beta-satellite. One month later, virions were purified from leaves showing typical symptom using method II as described by Czosnek et al. (Czosnek et al., 1988), omitting the step of sucrose gradient (Sánchez-Campos et al., 2016). Pellets containing virions were re-suspended in 0.1 mol L^{-1} phosphate buffer containing 0.2 mmol L^{-1} EDTA with pH at 7.0 and the solution was stored in −70°C fridge after the addition of equal volume of glycerol. The copy number of stored TbCSV virions was 1.475×10^{10}. For injection, the stored solution of virus particles was diluted from 1 to 10 with PBS. The injection was performed with FemtoJet (Eppendorf, Germany), and the area between the mesothorax and the metathorax was chosen to conduct the injection using the methods as described by Ghanim et al. (Ghanim et al., 2007). The injection volume per insect was 8.0 nL on average. Post injection, the whiteflies were allowed to feed on cotton plants for 48 h for recovery, and then virus transmission tests were performed using the standard procedure as described above. Each plant was inoculated with five whitefly adults. Two treatments for MEAM1 and Asia II 1 were conducted, and each treatment had three replicates with 8−9 plants in each replicate. In addition, 48 and 168 h post injection, immune-fluorescence detection of virus signals in primary salivary glands was performed using the method as described above.

Statistical analysis

For analysis of virus transmission efficiency, all percentage data were arcsine square root transformed, and one-way analysis of variance (ANOVA) along with Fisher's least significant difference (LSD) were used in experiments with more than two treatments, and Student's independent *t* test was recruited in experiments with only two treatments. For the comparison of quantity of virus, all real time data were calculated using $2^{-\triangle Ct}$ as normalized to actin, and for the analysis of relative quantity of virus in whitefly whole body between the two whitefly species at each time point, Student's independent *t* test was used, whereas for analysis of quantity of virus in midgut, haemolymph and primary salivary glands, nonparametric *t* test (Mann-Whitney) was used. All data were presented as the mean±SE. The differences between treatments were considered significant when $P<0.05$. All statistical analysis was conducted using SPSS 20.0 Statistics and EXCEL.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

Acknowledgements *This work was supported by the National Natural Science Foundation of China (31390421).*

Ammar, E.D., Gomez-Luengo, R.G., Gordon, D.T., and Hogenhout, S.A. (2005). Characterization of *Maize iranian mosaic virus* and comparison with Hawaiian and other isolates of *Maize mosaic virus* (Rhabdoviridae). J Phytopathol 153, 129–136.

Briddon, R.W., and Markham, P.G. (2000). Cotton leaf curl virus disease. Virus Res 71, 151–159.

Brown, J.K., Zerbini, F.M., Navas-Castillo, J., Moriones, E., Ramos-Sobrinho, R., Silva, J.C.F., Fiallo-Olivé, E., Briddon, R.W., Hernández-Zepeda, C., Idris, A., et al. (2015). Revision of Begomovirus taxonomy based on pairwise sequence comparisons. Arch Virol 160, 1593–1619.

Chen, W., Hasegawa, D.K., Kaur, N., Kliot, A., Pinheiro, P.V., Luan, J., Stensmyr, M.C., Zheng, Y., Liu, W., Sun, H., et al. (2016). The draft genome of whitefly *Bemisia tabaci* MEAM1, a global crop pest, provides novel insights into virus transmission, host adaptation, and inse-

cticide resistance. BMC Biol 14, 110.
Chougule, N.P., Li, H., Liu, S., Linz, L.B., Narva, K.E., Meade, T., and Bonning, B.C. (2013). Retargeting of the *Bacillus thuringiensis* toxin Cyt2Aa against hemipteran insect pests. Proc Natl Acad Sci USA 110, 8465–8470.
Czosnek, H., Ber, R, Antignus, Y, Cohen, S, Navot, N, and Zamir, D (1988). Isolation of *Tomato yellow leaf curl virus*, a geminivirus. Phytopathology 78, 508–512.
Czosnek, H., Ghanim, M., and Ghanim, M. (2002). The circulative pathway of begomoviruses in the whitefly vector *Bemisia tabaci*—insights from studies with *Tomato yellow leaf curl virus*. Ann Appl Biol 140, 215–231.
Czosnek, H., Hariton-Shalev, A., Sobol, I., Gorovits, R., and Ghanim, M. (2017). The incredible journey of Begomoviruses in their whitefly vector. Viruses 9, 273.
De Barro, P.J., Liu, S.S., Boykin, L.M., and Dinsdale, A.B. (2011). *Bemisia tabaci*: a statement of species status. Annu Rev Entomol 56, 1–19.
Ghanim, M., Morin, S., and Czosnek, H. (2001a). Rate of *Tomato yellow leaf curl virus* translocation in the circulative transmission pathway of its vector, the whitefly *Bemisia tabaci*. Phytopathology 91, 188–196.
Ghanim, M., Rosell, R.C., Campbell, L.R., Czosnek, H., Brown, J.K., and Ullman, D.E. (2001b). Digestive, salivary, and reproductive organs of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) B type. J Morphol 248, 22–40.
Ghanim, M., Kontsedalov, S., and Czosnek, H. (2007). Tissue-specific gene silencing by RNA interference in the whitefly *Bemisia tabaci* (Gennadius). Insect Biochem Mol Biol 37, 732–738.
Ghanim, M., Brumin, M., and Popovski, S. (2009). A simple, rapid and inexpensive method for localization of *Tomato yellow leaf curl virus* and *Potato leafroll virus* in plant and insect vectors. J Virol Methods 159, 311–314.
Gildow, F.E. (1993). Evidence for receptor-mediated endocytosis regulating luteovirus acquisition by aphids. Phytopathology 83, 270–277.
Guo, T., Guo, Q., Cui, X.Y., Liu, Y.Q., Hu, J., and Liu, S.S. (2015). Comparison of transmission of *Papaya leaf curl China virus* among four cryptic species of the whitefly *Bemisia tabaci* complex. Sci Rep 5, 15432.
Guo, T., Zhao, J., Pan, L.L., Geng, L., Lei, T., Wang, X.W., and Liu, S.S. (2018). The level of midgut penetration of two begomoviruses affects their acquisition and transmission by two species of *Bemisia tabaci*. Virology 515, 66–73.
Hogenhout, S.A., Ammar, E.D., Whitfield, A.E., and Redinbaugh, M.G. (2008). Insect vector interactions with persistently transmitted viruses. Annu Rev Phytopathol 46, 327–359.
Hu, J., Jiang, Z.L., Nardi, F., Liu, Y.Y., Luo, X.R., Li, H.X., and Zhang, Z. K. (2014). Members of *Bemisia tabaci* (Hemiptera: Aleyrodidae) cryptic species and the status of two invasive alien species in the Yunnan province (China). J Insect Sci 14, 281.
Jiu, M., Zhou, X.P., and Liu, S.S. (2006). Acquisition and transmission of two Begomoviruses by the B and a non-B biotype of *Bemisia tabaci* from Zhejiang, China. J Phytopathol 154, 587–591.
Li, M., Hu, J., Xu, F.C., and Liu, S.S. (2010). Transmission of *Tomato Yellow Leaf Curl Virus* by two invasive biotypes and a Chinese indigenous biotype of the whitefly *Bemisia tabaci*. Int J Pest Manage 56, 275–280.
Li, Z.H., Li, G.X., Xie, Y., Zhang, Z.K., and Zhou, X.P. (2002). Tomato leaf curl disease in Yunnan is caused by *Tobacco curly shoot virus* (in Chinese). Chin J Virol 18, 355–361.
Linz, L.B., Liu, S., Chougule, N.P., and Bonning, B.C. (2015). *In vitro* evidence supports membrane alanyl aminopeptidase N as a receptor for a plant virus in the pea aphid vector. J Virol 89, 11203–11212.
Liu, J., Li, M., Li, J., Huang, C., Zhou, X., Xu, F., and Liu, S. (2010). Viral infection of tobacco plants improves performance of *Bemisia tabaci* but more so for an invasive than for an indigenous biotype of the whitefly. J Zhejiang Univ Sci B 11, 30–40.
Liu, S.S., Colvin, J., and De Barro, P.J. (2012). Species concepts as applied to the whitefly *Bemisia tabaci* systematics: how many species are there? J Integr Agric 11, 176–186.
Liu, Y., Xie, Y., Liao, B.L., Bmugiira, R., and Zhou, X.P. (2007). Occurrence and distribution of geminiviruses in tobacco in Yunnan province of China. Acta Phytopathol Sin 37, 566–571.
Navas-Castillo, J., Fiallo-Olivé, E., and Sánchez-Campos, S. (2011). Emerging virus diseases transmitted by whiteflies. Annu Rev Phytopathol 49, 219–248.
Pan, L.L., Chen, Q.F., Zhao, J.J., Guo, T., Wang, X.W., Hariton-Shalev, A., Czosnek, H., and Liu, S.S. (2017). Clathrin-mediated endocytosis is involved in *Tomato yellow leaf curl virus* transport across the midgut barrier of its whitefly vector. Virology 502, 152–159.
Prasanna, H.C., Kanakala, S., Archana, K., Jyothsna, P., Varma, R.K., and Malathi, V.G. (2015). Cryptic species composition and genetic diversity within *Bemisia tabaci* complex in soybean in India revealed by mt*COI* DNA sequence. J Integr Agricult 14, 1786–1795.
Qin, L., Wang, J., Bing, X.L., and Liu, S.S. (2013). Identification of nine cryptic species of *Bemisia tabaci* (Hemiptera: Aleyrodidae) from China by using the mt*COI* PCR-RFLP technique (in Chinese). Acta Entomol Sin 56, 186–194.
Rosen, R., Kanakala, S., Kliot, A., Cathrin Pakkianathan, B., Farich, B.A., Santana-Magal, N., Elimelech, M., Kontsedalov, S., Lebedev, G., Cilia, M., et al. (2015). Persistent, circulative transmission of begomoviruses by whitefly vectors. Curr Opin Virol 15, 1–8.
Ruan, Y.M., Luan, J.B., Zang, L.S., and Liu, S.S. (2007). Observing and recording copulation events of whiteflies on plants using a video camera. Entomol Exper Applic 124, 229–233.
Sánchez-Campos, S., Rodríguez-Negrete, E.A., Cruzado, L., Grande-Pérez, A., Bejarano, E.R., Navas-Castillo, J., and Moriones, E. (2016). *Tomato yellow leaf curl virus*: no evidence for replication in the insect vector *Bemisia tabaci*. Sci Rep 6, 30942.
Scholthof, K.B.G., Adkins, S., Czosnek, H., Palukaitis, P., Jacquot, E., Hohn, T., Hohn, B., Saunders, K., Candresse, T., Ahlquist, P., et al. (2011). Top 10 plant viruses in molecular plant pathology. Mol Plant Pathol 12, 938–954.
Shilpi, S., Kumar, A., Biswas, S., Roy, A., and Mandal, B. (2015). A recombinant *Tobacco curly shoot virus* causes leaf curl disease in tomato in a north-eastern state of India and has potentiality to trans-replicate a non-cognate betasatellite. Virus Genes 50, 87–96.
Uchibori, M., Hirata, A., Suzuki, M., and Ugaki, M. (2013). *Tomato yellow leaf curl virus* accumulates in vesicle-like structures in descending and ascending midgut epithelial cells of the vector whitefly, *Bemisia tabaci*, but not in those of nonvector whitefly *Trialeurodes vaporariorum*. J Gen Plant Pathol 79, 115–122.
Ullman, D.E., Cho, J.J., Rfl, M., Westcot, D.M., and Custer, D M (1992). A midgut barrier to *Tomato spotted wilt virus* acquisition by adult western flower thrips. Phytopathology 82, 1333–1342.
Wei, J., Zhao, J.J., Zhang, T., Li, F.F., Ghanim, M., Zhou, X.P., Ye, G.Y., Liu, S.S., and Wang, X.W. (2014). Specific cells in the primary salivary glands of the whitefly *Bemisia tabaci* control retention and transmission of Begomoviruses. J Virol 88, 13460–13468.
Xie, Y., Zhou, X., Zhang, Z., and Qi, Y. (2002). *Tobacco curly shoot virus* isolated in Yunnan is a distinct spe-cies of Begomovirus. Chin Sci Bull 47, 197–200.
Zerbini, F.M., Briddon, R.W., Idris, A., Martin, D.P., Moriones, E., Navas-Castillo, J., Rivera-Bustamante, R., Roumagnac, P., Varsani, A., and Ictv Report Consortium, A. (2017). ICTV virus taxonomy profile: *Geminiviridae*. J General Virol 98, 131–133.

SUPPORTING INFORMATION

Figure S1 Tobacco plants showing un-infected and TbCSV infected symptom. As compared to un-infected plants, TbCSV infected plants show dark green color, stunting and leaf curl in the young leaf.

Figure S2 Typical immune-fluorescence images of the primary salivary glands of un-infected whitefly adults (A) and adults at 48 (B) and 168 h (C) after virion injection were presented.

The supporting information is available online at http://life.scichina.com and https://link.springer.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.