Vector development and vitellogenin determine the transovarial transmission of begomoviruses

Jing Wei1,a, Ya-Zhou He1,a, Qi Guo1, Tao Guo1, Yin-Quan Liu1, Xue-Ping Zhou2, Shu-Sheng Liu1, and Xiao-Wei Wang1,b

1Ministry of Agriculture Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Institute of Insect Sciences, Zhejiang University, Hangzhou 310058, China; and 2Institute of Biotechnology, Zhejiang University, Hangzhou 310058, China

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The majority of plant viruses are transmitted by insect vectors between hosts, and transovarial transmission of viruses from vector parents to offspring has great significance to their epidemiology. Begomoviruses are transmitted by the whitefly Bemisia tabaci in a circulative manner and are maintained through a plant–insect–plant cycle. Other routes of begomovirus transmission are not clearly known. Here, we report that transovarial transmission from female whiteflies to offspring often happens for one begomovirus, Tomato yellow leaf curl virus (TYLCV), and may have contributed significantly to its global spread. We found that TYLCV entry of the reproductive organ of its vector mainly depended on the developmental stage of the whitefly ovary, and the transovarial transmission of TYLCV to offspring increased with whitefly adult age. The specific interaction between virus coat protein (CP) and whitefly vitellogenin (Vg) was vital for virus entry into whitefly ovary. When knocking down the expression of Vg, the entry of TYLCV into ovary was inhibited and the transovarial transmission efficiency decreased. In contrast, another begomovirus, Papaya leaf curl China virus (PaLCuCNV), CP did not interact with whitefly Vg, and PaLCuCNV could not be transovarially transmitted by whiteflies. We further showed that TYLCV could be maintained for at least two generations in the absence of virus-infected plants, and the adult progenies were able to infect healthy plants in both the laboratory and field. This study reports the transovarial transmission mechanism of begomoviruses, and it may help to explain the evolution and global spread of some begomoviruses.

begomovirus | transovarial transmission | vector development | vitellogenin | whitefly

Maternal transmission of microbes, including viruses, bacteria, microsporidia, and fungi, by arthropods is a universal phenomenon in nature (1–3). Of the ∼700 known plant viruses, more than 75% are dependent upon arthropod vectors for transmission between hosts, and some of them can be transmitted vertically from mother to offspring in a transovarial manner (4, 5). Because transovarial transmission controls virus dispersal in space and time and thus has great importance to virus epidemiology, it has received constant attention from entomologists and virologists (6, 7). However, despite its importance, transovarial transmission of plant viruses by insects remains uncommon. Depending on the mode of transmission, plant viruses are classified into four categories including nonpersistent, semipersistent, circulative-nonpropagative, and circulative-propagative (4). So far, only circulative-propagative plant viruses, such as ronaviruses, rhabdoviruses, and tenuiviruses, have been confirmed to be transovarially transmitted, because transovarial transmission usually requires the replication of viruses in the vector (8).

There are multiple barriers during the circulative transmission of plant and animal viruses, including midgut infection barrier, dissemination barrier, salivary gland escape barrier, and transovarial transmission barrier (9). Passage of viruses through these barriers requires specific interactions between virus and vector components (10). Identification of putative components could lead to new strategies to combat virus spread. During the past decades, a number of virus and insect proteins involved in this transmission process have been identified in some virus-vector combinations (11–13). However, mechanisms underlying transovarial transmission of viruses have rarely been reported, especially for circulative-nonpropagative viruses, because this group of viruses is generally believed not able to replicate and transovarially transmit in their insect vectors.

Begomoviruses contain the largest known genus of ∼288 species of plant viruses and are generally known to be transmitted by the whitefly Bemisia tabaci in a circulative-nonpropagative manner (14, 15). The whitefly B. tabaci is now recognized as a complex containing at least 35 cryptic species (16, 17). During the past 50 y, the two most predominant and damaging species of the B. tabaci complex, Middle East Asia Minor 1 (MEAMI) and Mediterranean (MED), which have been commonly referred to as biotype B and biotype Q, respectively, have invaded many countries worldwide and displaced some indigenous whitefly species (17). With the global invasion of MEAMI and MED whiteflies, many economic crops are at great risk of infection with begomoviruses (15, 18, 19). Among these viruses, Tomato yellow leaf curl virus (TYLCV) is one of the most devastating viral diseases and has spread to more than 50 countries and regions (20, 21). Interestingly, although most of the studies reported an absence or low frequency of transovarial transmission in begomoviruses, one case reported that TYLCV can be transovarially transmitted at high efficiency (22) (Table S1). Therefore, much remains to be learned about whether and how the begomoviruses are vertically transmitted.

In this study, we used TYLCV and Papaya leaf curl China virus (PaLCuCNV), a newly isolated begomovirus in China

Significance

The majority of plant viruses are transmitted by insect vectors. Transovarial transmission of virus from female vectors to offspring can be very important in maintaining a source of infection and therefore has great epidemiological relevance. Identification of vector and virus components involved in transovarial transmission can lead to new strategies to combat virus spread. Here, we found that the specific interaction between viral coat protein and vector vitellogenin determines transovarial transmissibility of begomoviruses, which have caused great damage to agricultural production and are generally believed not to be transovarially transmitted by insect vectors. Our study gives valuable clues for designing strategies to block begomovirus transmission and provides insights into the evolution and global spread of begomoviruses.


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1J.W. and Y.-Z.H. contributed equally to this work.

2To whom correspondence should be addressed. Email: xwwang@zju.edu.cn.

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Results

TYLCV Entry in Whitefly Ovary at the Mature Stage of Ovarian Development. First, we observed the anatomy of ovary and ovariole at different developmental phases in female adult whiteflies, which has been reported by Guo et al. (25). Briefly, the ovary development could be classified into four stages according to whitefly development: stage I, freshly emerged whitefly; stage II, 1–2 d after eclosion (DAE); stage III, 3–10 DAE; and stage IV, 11–14 DAE (Fig. S1A). The ovariole development could be divided into four phases (phases I, II, III, and IV) based on oocyte yolk accumulation. The yolk content of oocytes gradually increased by phase (Fig. S1A) (25). Then, we investigated the localization of TYLCV and PaLCuCNV in ovaries of viruliferous MEAM1 adult whiteflies at different developmental stages. Interestingly, TYLCV virions were mainly located in ovaries of the more mature stages (stages III and IV), but not in ovaries of the less mature stages (stages I and II) (Fig. 1A), indicating that whitefly development may affect the transovarial transmission of TYLCV. To substantiate this observation, the transovarial transmission of TYLCV to vector offspring was examined using MEAM1 whiteflies at 1 or 11 DAE, respectively. The adult whiteflies at 1 DAE transmitted TYLCV to the eggs and nymphs of their progeny in a low frequency (8–13%), but no viral DNA was found in the adult progeny (Fig. 1B). Notably, the old mother whiteflies at 11 DAE vertically transmitted TYLCV to all developmental stages of their progeny efficiently (Fig. 1B). Virus transmission assays showed that none of the 24 healthy plants were infected by TYLCV when exposed to the adult progenies of 1 DAE viruliferous whiteflies, whereas 5 out of 24 plants were infected after inoculation by adult offspring of 11 DAE viruliferous whiteflies (Fig. 1C). Our data indicate that the age of whitefly is responsible for transovarial transmission efficiency of TYLCV.

Whitefly Ovary Is a Selective Barrier That Blocks the Transovarial Transmission of PaLCuCNV. Interestingly, PaLCuCNV was never detected in the ovary of viruliferous whiteflies (Fig. S1B) or in any developmental stage of the progeny produced by viruliferous MEAM1 whiteflies at 1 or 11 DAE (Table S2). Virus accumulation experiment revealed that, after a 48-h access acquisition period (AAP), the abundance of TYLCV in the whole bodies of adults was comparable to that of PaLCuCNV, at both 1 and 11 DAE (Fig. 1D). However, the abundance of TYLCV in ovaries of whiteflies at 11 DAE was severalfold that of whiteflies at 1 DAE, whereas for PaLCuCNV, the abundance of the virus was low in ovaries of adult whiteflies at both 1 DAE (Fig. 1E). We next performed immunofluorescence staining assays to detect the two viruses in midguts (MGs), primary salivary glands (PSGs), and ovaries of whiteflies at 11 DAE, and found that all MGs were TYLCV-positive or PaLCuCNV-positive; and 96% PSGs were TYLCV-positive, and 88% were PaLCuCNV-positive, indicating that both TYLCV and PaLCuCNV can reach MG and PSG effectively. However, although 79% of tested ovaries were TYLCV-positive, none of the ovaries was PaLCuCNV-positive.

![Image](https://example.com/image.png)
(Fig. 1F and Fig. S1 C–E). These observations clearly imply that the failure of transovarial transmission of PaLCuCNV is due to nonpenetration of ovary rather than to inefficient virus intake.

**Interaction Between Viral CP and Vector Vg Is Vital for Virus Entry into Whitefly Ovary.** The above results indicate that virus entry of whitefly ovary mainly relies on viral type and whitefly developmental stages. As Vg plays a critical role in yolk formation and oogenesis (26–29), we assumed that Vg is involved in the entry of TYLCV into ovary. Interestingly, the expression level of Vg was positively correlated with the transovarial transmission efficiency of TYLCV (Fig. 2A). We then tested whether TYLCV CP or PaLCuCNV CP interacts with Vg in vivo by coimmunoprecipitation. Both anti-TYLCV CP antibody and anti-Vg antibody coimmunoprecipitated the other interacting proteins, whereas neither anti-PaLCuCNV CP antibody nor anti-Vg antibody coimmunoprecipitated the other proteins (Fig. 2 B and C). In vitro pull-down assay also confirmed that whitefly endogenous Vg was coeluted with GST-fused TYLCV CP, but not with GST-fused PaLCuCNV CP (Fig. 2D). These findings suggest that the specific interaction between viral CP and whitefly Vg is vital for virus entry of ovary.

Next, we traced the entry process of Vg and TYLCV into oocytes of viruliferous whitefly at 11 DAE. The ovariole of the whitefly is mainly composed of three parts: the tropharium, segregating oocyte, and vitellarium (30). The oocyte, located in the center of vitellarium, is surrounded by a single layer of follicular cells on its surface and has a nutritive cord connected with the tropharium (30) (Fig. S2A). We did not detect TYLCV virions in phase I ovarioles of viruliferous whiteflies when Vg was not detected in the ovarioles (Fig. 2E and Fig. S2B). TYLCV virions were first detected in the space between follicular cells of phase II ovarioles, when Vg began to accumulate in the space between follicular cells and just started to be absorbed into oocytes (Fig. 2E and Fig. S2B). Then TYLCV virions were detected not only in the space between follicular cells but also in the oocytes of phase III ovarioles, when massive Vg accumulated in the space between follicular cells and was rapidly absorbed into the oocytes (Fig. 2E and Fig. S2B). Moreover, TYLCV and Vg colocalized with each other in both the space between follicular cells and oocytes (Fig. 2E). These results suggest that the entry of TYLCV virions into the oocyte shares the same route of Vg transport into the oocyte.

To verify the role of Vg in transovarial transmission of TYLCV, we knocked down the expression of Vg using RNA interference (RNAi). Compared with whiteflies fed with dsGFP (control), Vg mRNA level decreased by 67% in the group fed with dsVg (Fig. 3A) and Vg protein level also decreased (Fig. S3A). As the expression of Vg was decreased after ingestion of dsVg, the maturation of ovaries dissected from the treated whiteflies was delayed (Fig. 3B). Meanwhile, the number of eggs laid by female whiteflies after dsVg treatment decreased by 29% compared with the control (Fig. 3C). After inoculation on TYLCV-infected plants for 48 h, the whiteflies had similar level of virus in the whole bodies when they had been treated with dsVg or dsGFP (Fig. 3D); however, the abundance of TYLCV DNA decreased by 73% in the ovaries of dsVg-treated whiteflies compared with that of the control (Fig. 3E). Moreover, the overall TYLCV and Vg signals in dsVg-treated ovaries were lower than those of the control (Fig. S3 B–I), suggesting that knockdown of Vg expression reduced yolk accumulation into oocytes and impeded the entry of TYLCV into the ovary of whitefly. Most importantly, the frequency of TYLCV transmission to eggs was reduced by 90% compared with the control group (Fig. 3F). Furthermore, the transmission rate of TYLCV to eggs by the adults decreased by 57% after oral ingestion of anti-Vg antibody, which would bind to Vg and therefore

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**Fig. 2.** Vg is involved in TYLCV entry into whitefly oocyte. (A) The expression patterns of Vg in adult female whiteflies at different developmental stages. Mean ± SEM of three experiments. *P < 0.05* (one-way ANOVA, LSD test). (B and C) Coimmunoprecipitation (CO-IP) of Vg with anti-CP monoclonal antibody (B) and vice versa (C) in whitefly crude extracts. (D) B. tabaci endogenous Vg coeluted with GST-fused TYLCV CP but not with GST-fused PaLCuCNV (PaL) CP. (E) Localization of TYLCV and Vg in follicular cells (upper lane) and oocytes (lower lane) of ovarioles at different developmental phases. Cell nucleus was stained with DAPI (blue). For phases II and III, both Vg (red) and CP (green) are shown. Yellow color indicates the overlay of red and green. CP, follicular cells; O, oocyte.
inhibit CP–Vg interaction, compared with preimmune serum (control)-treated group (Fig. 3G).

To examine the role of viral CP in transovarial transmission, we exchanged partial CP sequence of TYLCV with that of PaLCuCNV (Fig. S4A), and observed the transovarial transmission of the mutated TYLCV (mTYLCV) and mutated PaLCuCNV (mPaL) by whiteflies at 11 DAE. Both mTYLCV and mPaL induced typical disease symptoms after agroinoculation into tomato plants (Fig. S4B). After a 48-h AAP, the whiteflies had a similar amount of mTYLCV and mPaL in their whole bodies (Fig. 3H). However, mTYLCV could not be detected in eggs of viruliferous whiteflies, whereas mPaL was transmitted to the eggs in a high frequency (85%) (Fig. 3I). Immunofluorescence analysis of viruliferous whiteflies at 11 DAE showed that nearly 90% of the tested MGs and 80% of the tested PSGs were mTYLCV- and mPaL-positive. For the ovaries, none of the tested samples was mTYLCV-positive, whereas 71% were mPaL-positive (Fig. S5). Overall, these results demonstrate that specific interactions between viral CP and whitefly Vg determine the transovarial transmission of TYLCV.

Transovarial Transmission of TYLCV and PaLCuCNV by MED Whiteflies.

To examine whether this phenomenon also exists in other whitefly species, we further investigated the transovarial transmission of TYLCV and PaLCuCNV by MED whiteflies, which is another invasive species of the B. tabaci complex (17). MED whiteflies of 1 DAE transmitted TYLCV only to the eggs and nymphs of their progeny; however, whiteflies of 11 DAE transmitted TYLCV to eggs, nymphs, and adults of their progeny efficiently (67–85%) (Fig. 4A). Whereas 8 out of 24 tested plants were infected with TYLCV after inoculation by adult progeny of 11 DAE viruliferous MED whiteflies, no viral DNA was detected after inoculation by progenies of viruliferous MED whiteflies at 1 DAE (Fig. 4B). Similarly, PaLCuCNV was never detected in the progeny of viruliferous MED whiteflies at both 1 and 11 DAE (Table S2). Immunostaining showed that both TYLCV and PaLCuCNV can invade the MG and PSG of MED whiteflies. As for the ovaries, nearly 50% of the tested were TYLCV-positive, whereas none was PaLCuCNV-positive (Fig. S6 A–D). Furthermore, Vg and TYLCV CP colocalized in mature ovarioles of viruliferous MED whiteflies (Fig. S6E), suggesting that Vg of MED whitefly is also involved in the entry of ovary by TYLCV.

TYLCV Can Be Maintained for at Least Two Generations by Whitefly Vector. Next, we examined whether TYLCV could be vertically transmitted from the first generation to the following generations. We conducted transovarial transmission assays with the first-generation adults, which developed from eggs deposited on cotton by viruliferous MEAM1 and MED whiteflies at 11 DAE. We found that the first-generation adults could transmit TYLCV to the second-generation progenies through ovum, with 59–65% for MEAM1 whiteflies and 3–35% for MED whiteflies (Fig. 4C). Moreover, the second-generation adults were able to transmit TYLCV to healthy plants, with 4 out of 30 (13%) test plants being TYLC-positive for MEAM1 whiteflies and 1 out of 30 (3%) test plants being TYLCV-positive for MED whiteflies (Fig. 4D). Additionally, field cage trials and field sampling provided evidence of transovarial transmission of TYLCV by MEAM1 and MED and its relevance to TYLCV epidemiology in the field (Fig. S7 and Table S3).

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Transovarial Transmission of TYLCV and PaLCuCNV by MED Whiteflies.
Discussion
Vertical transmission of viruses from female parent to offspring is an important strategy for viruses to speed up transmission, and is recognized to play a significant role in viral epidemics (2, 3). Transovarial transmission of plant viruses has received constant attention since 1935 (5–7). However, most of the reports were focused on the study of circulative, nonpropagative viruses, such as closteroviruses, rajoviruses, rhadoviruses, or tenuiviruses (31–34). Even so, some research groups have investigated the transovarial transmission of circulative-nonpropagative begomoviruses in the past 50 y. However, whether begomoviruses can be transmitted transovarially is still in great controversy. Although most of the studies reported absence of transovarial transmission (35–39), several studies reported the presence of transovarial transmission, but the efficiencies varied considerably between studies (40–42) (Table S1). Our study proved that TYLCV can be effectively transmitted via ovary of whitefly adults of elder age, but not by white flies of very young age (Fig. 1). Our findings explained why different results were obtained in previous experiments on transovarial transmission of TYLCV in the last half century, as the age of whiteflies used in those experiments was different. The only report that showed a very high rate of TYLCV transovarial transmission actually used 5–8 DAE whiteflies (42), whereas the other two studies with very low frequency used newly emerged whiteflies (40, 41). In addition, not all begomoviruses can be transovarially transmitted (Table S1); therefore, the virus strains used in different experiments may also contribute to the discrepancy.

Fig. S5
Immunoprecipitation was performed using serum 1:1) and each group was used to inoculate one uninfected tomato plant. The fragments of TYLCV and PaLCuCNV CPs were covalently linked to glutathione-Sepharose 4 Fast Flow (catalog no. 17-0405-03; GE Healthcare) and the specific interactions were measured using monoclonal antibodies anti-CP (51), anti-Vg (52), or anti-Actin (catalog no. A07028; Beyotime) as controls. Monoclonal antibodies anti-CP (51), anti-Vg (52), or anti-Actin (catalog no. E021020-02; EarthOx) and their corresponding preimmune sera (catalog no. A07028; Beyotime) as controls were incubated with whitefly protein extracts for 10 h at 4 °C, followed by incubating with protein G-Sepharose beads for 1 h. Then, the bead suspensions were boiled in PAGE buffer for 15 min and then resolved by SDS-PAGE. Each immunodetection was repeated at least three times. Incubation with antibodies was followed by ECL Plus Detection (catalog no. 170-5060; Bio-Rad). Protein G-Sepharose beads bound to anti-Actin antibody or preimmune sera were used as negative controls.

GST Pull-Down Assay. The fragments of TYLCV and PaLCuCNV CPs were amplified and cloned into pGEX-6p-1 for fusion with GST. Primers are listed in Table S4. All recombinant proteins were expressed in Escherichia coli strain Rosetta and purified. The GST-tag–fused protein, TYLCV CP–GST or PaLCuCNV CP–GST, was bound to glutathione Sepharose beads (catalog no. 17-5132-01; GE Healthcare) for 3 h at 4 °C, and the mixtures were centrifuged for 5 min at 100 × g, and the supernatants were discarded. The nonviruliferous whitefly soluble protein extracts or the His-tag fusion proteins were added to the bead suspension and incubated for 1 h at 4 °C. The beads were washed five times with PBS, the beads-bound proteins were eluted by boiling in PAGE buffer for 5 min, and then the proteins were separated by SDS-PAGE gels. Immunoblotting was performed using anti-CP and anti-Vg antibodies.

Gene Silencing by Oral Ingestion of dsRNA. RNA silencing was performed as previously described (53). Briefly, dsRNAs were diluted into 15% (w/v) sucrose solution at the concentration of 300 ng/μl. Approximately 100 adult whiteflies at 2 DAE were released into each feeding chamber. The tube was incubated in an insect-rearing room for 48 h. Subsequently, whiteflies were transferred to TYLCV-infected tomato plants and maintained for 48 h, and transovarially transmit TYLCV to their progeny efficiently, and the virus can be maintained for at least two generations by whiteflies in the absence of a host plant (Figs. 1B and 4). Moreover, the adult progenies that developed on virus nonhost plant can infect healthy plants in the field (Fig. S7). With an almost invisible size, viruliferous eggs laid by virus-infected whiteflies on plants can be transported for long distance with plants by human activities. Thus, the transovarial transmission may also contribute significantly to the global spread of TYLCV as well as the outbreak of TYLCV disease in the field.

Methods
Transmission of Virus via Ovary by Whitefly. Viruliferous MEAM1 and MED adult whiteflies at different developmental stages or under different treatments were collected in groups of 10 each (female/male = 1:1). For each replicate of a treatment, a group of insects was placed on the lower surface of a leaf of cotton plant, a nonhost plant of TYLCV and PaLCuCNV, enclosed in a leaf clip cage. The adults were left on the leaf to feed, mate, and oviposit for 72 h at 26–31 °C, 40–60% relative humidity, and a photoperiod of 14:10 h (light:dark). Then, all adults were removed and five eggs were collected from each replicate using disposable sterilized needles (one needle for each individual) and stored at –20 °C for detection of virus DNA subsequently. The remaining eggs were left on the leaf to develop. Eighteen and 25 d later, five nymphs and five adults were collected from each replicate, using disposable sterilized needles (one needle for each individual) and stored at –20 °C for detection of virus DNA subsequently.

Transmission of TYLCV to Plants by Adult Progeny. For indoor transmission assays, the adult progenies of viruliferous whiteflies were collected in groups of 10 (female/male = 1:1) and each group was used to inoculate one uninfected tomato plant. The inoculation was performed on the top second leaf of the plant at the 3–4 true-leaf stage (~3 wk after sowing) for a 48-h inoculation access period, using a leaf clip cage. Twenty-four uninfected tomato plants were used for the first-generation adults and 30 for the second-generation adults. The plants were then sprayed with imidacloprid at a concentration of 20 mg/L. All of the whiteflies adults and eggs, and maintained until symptoms had developed.

Communoprecipitation Assay. Immunoprecipitation was performed using protein G-Sepharose 4 Fast Flow (catalog no. 17-0405-03; GE Healthcare) according to the manufacturer’s instructions. Whitefly protein extracts were prepared in cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mg/ml leupeptin, 2 mg/ml aprotinin, 1 mM EDTA). Monoclonal antibodies anti-CP (51), anti-Vg (52), or anti-Actin (catalog no. E021020-02; EarthOx) and their corresponding preimmune sera (catalog no. A07028; Beyotime) as controls were incubated with whitefly protein extracts for 10 h at 4 °C, followed by incubating with protein G-Sepharose beads for an additional 2 h at 4 °C. After washing five times with lysis buffer, immunoprecipitated proteins were eluted by boiling in PAGE buffer for 5 min, and then resolved by SDS-PAGE. Each immunodetection was repeated at least three times. Incubation with antibodies was followed by ECL Plus Detection (catalog no. 170-5060; Bio-Rad). Protein G-Sepharose beads bound to anti-Actin antibody or preimmune sera were used as negative controls.

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then RNA was extracted from 20 female individuals to examine the Vg mRNA level, and total proteins were extracted from 30 female individuals to examine the Vg protein level after deRNA treatment. The remaining insects were used for quantitative assays and virus transmission tests. Each set of experiment was repeated three times.

**Immunofluorescence Assay.** For virus localization, ovaries, MGs, and PSGs of female whiteflies at required developmental stages were dissected after a 48-h AAP on virus-infected plants. For Vg antibody staining, ovaries of whiteflies at 11 DAA were dissected and ovarioles were dispersed from ovaries. The specimens were fixed in 4% paraformaldehyde for 1 h at room temperature, and washed in PBST containing 0.1% Triton X-100 three times for 1 h each. Then the specimens were blocked in PBST containing 1% BSA (catalog no. A3828; MultiSciences Biotech) for 2 h at room temperature, followed by incubation with anti-Vg monoclonal antibody (1:500) or anti-Cp monoclonal (1:500) or polyclonal antibody (1:500) in PBST containing 1% BSA overnight at 4 °C and then with goat anti-mouse (1:1500) or goat anti-rabbit (1:500) secondary antibody labeled with DyLight 488 (catalog no. LK-GAM4882; MultiSciences Biotech) or Dylight 549 (catalog no. LK-GAR5492; MultiSciences Biotech) in PBST containing 1% BSA for 1 h at room temperature after extensive washing. The nucleus was stained with 100 nM 4′,6-diamidino-2-phenylindole (DAPI) (catalog no. ab104139; Abcam) in PBST for 2 min at room temperature.

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