Viral pathogens hitchhike with insect sperm for paternal transmission

Qianzhuo Mao\textsuperscript{1,2}, Wei Wu\textsuperscript{1}, Zhenfeng Liao\textsuperscript{1}, Jiajia Li\textsuperscript{1}, Dongsheng Jia\textsuperscript{1,2}, Xiaofeng Zhang\textsuperscript{1}, Qian Chen\textsuperscript{1}, Hongyan Chen\textsuperscript{1}, Jing Wei\textsuperscript{1} & Taiyun Wei\textsuperscript{1,2}

Arthropod-borne viruses (arboviruses) can be maternally transmitted by female insects to their offspring, however, it is unknown whether male sperm can directly interact with the arbovirus and mediate its paternal transmission. Here we report that an important rice arbovirus is paternally transmitted by the male leafhoppers by hitchhiking with the sperm. The virus-sperm binding is mediated by the interaction of viral capsid protein and heparan sulfate proteoglycan on the sperm head surfaces. Mating experiments reveal that paternal virus transmission is more efficient than maternal transmission. Such paternal virus transmission scarcely affects the fitness of adult males or their offspring, and plays a pivotal role in maintenance of viral population during seasons unfavorable for rice hosts in the field. Our findings reveal that a preferred mode of vertical arbovirus transmission has been evolved by hitchhiking with insect sperm without disturbing sperm functioning, facilitating the long-term viral epidemic and persistence in nature.

\textsuperscript{1}Vector-borne Virus Research Center, Fujian Province Key Laboratory of Plant Virology, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China. \textsuperscript{2}State Key Laboratory for Ecological Pest Control of Fujian and Taiwan Crops and College of Life Science, Fujian Agriculture and Forestry University, Fuzhou 350002, China. These authors contributed equally: Qianzhuo Mao, Wei Wu. Correspondence and requests for materials should be addressed to J.W. (email: weijing0306@163.com) or to T.W. (email: weitaiyun@fafu.edu.cn)
Many devastating plant, animal, and human pathogens are vectored by arthropod insects\(^1,^{2}\). For example, *Rice stripe virus* (RSV) transmitted by planthoppers has caused a serious agricultural threat in Asian rice-growing countries\(^3\), and Zika virus transmitted by mosquitoes has caused a recent public health threat in Americas\(^4\). Frequently, arthropod-borne viruses (arboviruses) can be vertically transmitted to the vector progeny population to ensure survival during adverse conditions for horizontal transmission\(^5,^{6}\). Thus, vertical transmission is an important endemic maintenance mechanism for arboviruses in nature. Vertical virus transmission by insect vectors in nature may include maternal or paternal transmission\(^7-^{14}\). Maternal transmission of arboviruses through transovarial passage has been extensively investigated\(^8,^{11-^{14}}\), however, whether sperm-mediated paternal virus transmission occurs remain undetermined. Oocytes accumulate a large quantity of cytoplasm that provide a room for viral infection, whereas sperms discard their cytoplasm during spermatogenesis and transform into a streamlined shape with the small head consisting of condensed nucleus and the slender tail made of microtubule bundles for motility\(^15\). Therefore, if arboviruses can be vertically transmitted via insect sperm, a possible target may be the outer membrane of the sperm. Considering the extremely streamlined sperm structure, viral infection to the sperm head is expected to impair normal functioning of the sperms\(^16-^{18}\). For example, the presence of human immunodeficiency virus in the human sperm and Zika virus in mice sperm would damage sperm normal functioning\(^16-^{18}\), and, thus, sperm-mediated paternal virus transmission may seem unlikely to occur. In this study, however, we demonstrate that a preferred mode of parental virus transmission has been evolved by hitchhiking with the sperm of male insect vectors without disturbing sperm functioning in a leathopper-borne plant reovirus.

More than 75% of plant viruses can be transmitted by aphids, leaffoppers, planthoppers, whiteflies, and other vectors in a persistent, semi-, or non-persistent manner, thereby providing fertile ground for mechanistic studies on vector transmission\(^3,^{7,^{19}}\). The mechanisms for vertical transmission of persistent plant viruses between an infected female and its offspring through transovarial passage have been demonstrated\(^5,^{8,^{11-^{14}}\). For example, we have determined that RSV, a tenuivirus, and *Tomato yellow leaf curl virus*, a begomovirus, exploit the existing oocyte entry paths of vitellogenin to overcome transovarial transmission barriers in planthopper or whitefly vectors\(^11,^{12}\). We also have shown recently that transovarial transmission of *Rice dwarf virus* (RDV), a plant reovirus, is mediated by the specific interaction of the viral capsid protein with the outer membrane protein of an obligate symbiotic bacterium of the vector green rice leaffoppers\(^20\). *Rice gall dwarf virus* (RGDV), also a plant reovirus, causes epidemic outbreaks and extensive rice yield losses in Asian rice-growing countries, and has long been thought to be transmitted by a transovarial mechanism in green rice leaffoppers\(^21-^{24}\). However, we observed recently that the percentage of transovarial transmission (~20%) in the main vector of RGV, the green rice leaffopper *Recilia dorsalis*, is much lower than the overall percentage (~80%) of vertical transmission\(^24\). Unlike RDV, RGV virions encounter strong barriers to enter the oocytes in female vectors for maternal transmission\(^24\). The significant disparity between the percentages for the vertical and maternal transmission suggested that RGDV may have evolved to be paternally transmitted by male insects to the offspring. Here, we report that a high efficiency of sperm-mediated paternal transmission route of RGDV by male *R. dorsalis* occurs without affecting the fitness of male insects or their offspring, which may play a vital role in long-term maintenance and spread of RGDV in the field.

**Results**

**Paternal transmission of RGDV through vector generations.** To explore whether RGDV can be paternally transmitted, we observed vertical transmission of RGDV from viruliferous (V\(^+\)) male (♂) or female (♀) *R. dorsalis* reared under controlled greenhouse conditions (Fig. 1a). In the eggs laid by the individual V\(^+\)♀ leaffoppers that mated with nonviruliferous (V\(^−\))♂ leaffoppers, 22% were positive for RGDV (Fig. 1b), consistent with our earlier observation\(^5\); in contrast, 73% were RGDV-positive of the eggs produced by V\(^−\)♂ leaffoppers that mated with V\(^+\)♀ (Fig. 1b), indicating that paternal transmission is ~3.3 times as efficient as maternal transmission, similar to the efficiency of vertical transmission by field-caught leaffoppers (Fig. 1c). Interestingly, the highest efficiencies of vertical transmission (82%) were observed in both laboratory-reared and field-caught leaffoppers when both parents were viruliferous (Fig. 1b, c).

We then determined the epidemiological significance of paternal virus transmission in the field. Over the past 30 years, viral disease caused by RGDV is always epidemic in the field in Southern China\(^2,^{22,^{24}}\). During its winter months (November to March) in Guangdong, Southern China when rice plants are rarely present, the weed *Alopecurus aequalis* becomes the primary habitat of rice leaffoppers for up to two generations (Fig. 1d). Generally, RGDV infection in the weed *A. aequalis* was never observed in the field during the winter months, though a very low rate of viral infection in *A. aequalis* occurred under laboratory conditions (Supplementary Table 1). Thus, the weed *A. aequalis* was not a suitable reservoir for RGDV in the field. After the late-planted rice is harvested, infected leaffoppers move to grass weeds and overwinter (Fig. 1d). The overwintering generations move to rice and spread the virus in warm areas where rice is planted in early April (Fig. 1d). We surveyed the leaffopper populations in Guangdong for the presence of RGDV in winter for 4 consecutive years. Although the percentage of viruliferous leaffoppers dropped following the transfer to *A. aequalis*, 20–30% of the overwintering leaffoppers carried RGDV in March of all 4 years when spring rice became available (Fig. 1e). Interestingly, significantly higher percentages of male leaffoppers were viruliferous than females (Fig. 1f). Paternal transmission also remained more efficient than maternal transmission when examined for three successive generations (Fig. 1g). These data indicate a pivotal role of paternal transmission in the overwintering of RGDV in the field.

**Paternal transmission does not affect offspring fitness.** We then determined whether the efficient paternal virus transmission affected the fitness of male *R. dorsalis* or their offspring. We found that V\(^+\) males exhibited no significant differences in either mating (Fig. 2a) or survival (Fig. 2b) compared with V\(^−\) males. Generally, one male can mate with around four virgin V\(^−\) females in 3 days (Fig. 2a). Interestingly, paternal transmission of RGDV had no significant deleterious effects on female fecundity, progeny egg development and hatching rates when compared the crossing between V\(^−\) female and V\(^+\) male with that of V\(^−\) female and V\(^−\) male (Fig. 2d). In contrast, V\(^+\) females died earlier than V\(^+\) males and produced eggs with severe developmental defects compared with that of V\(^−\) females (Fig. 2b, d). The surviving offspring from maternal transmission were able to reach adulthood and transmit the virus as efficiently as the ones from paternal transmission (Supplementary Figure 1a). Moreover, neither maternal nor paternal transmission affected the offspring sex ratios (Fig. 2c). Therefore, paternal transmission of RGDV would avoid the deleterious effect of maternal transmission on viruliferous vector population, thus promoting viral transmission. Taken together, the above data suggest that paternal transmission...
Late-planted rice 2015

V+ V– V+ V+

V+ × V+

Maternal V– × 2014

“ means ± SD of three independent experiments of four mating combinations in second generation of overwintering insects collected in late February.

consequently, virus-decorated sperms in the spermatheca moved to the

used immuno

sperm heads in the male reproductive system (Fig. 3a). We then investigated whether the sperm-mediated paternal transmission of RGDV by hitchhiking with the sperm. Our

transmission of RGDV occurred in male R. dorsalis. Our

immunofluorescence and electron microscopy revealed an association of dense RGDV particles with the plasma membrane of sperm heads in the male reproductive system (Fig. 3a–f). We then used immunofluorescence microscopy to observe how RGDV hitchhiked a ride on the sperm to offspring. RGDV was initially detected in the spermatheca of V– females at 3 days post mating with V+ males (Fig. 3g–i and Supplementary Table 2). Subsequently, virus-decorated sperms in the spermatheca moved to the

ovisperm for fertilizing the mature eggs during ovulation (Fig. 3j and Supplementary Table 2). RGDV became detectable in the genital tract but not in the oocytes of females even at 10 days post mating (Fig. 3m–o and Supplementary Table 2). Electron microscopy showed that virus-decorated sperms were present in the dissected spermatheca of V+ females (Fig. 3p), confirming the transfer of virions from V+ males to V– females. Immunofluorescence assays revealed that RGDV circulated within V– females after mating with V+ males, and ~ 63% of tested female intestines were infected at 10 days post mating (Supplementary Table 3). However, the virus was rarely observed in the oocytes, indicating that strong ovarian transmission barrier occurred (Supplementary Table 3). Moreover, V+ females, which acquired virus by mating with V+ males could transmit RGDV to rice plants, and the transmission efficiency increased with the time

of RGDV as a preferred mode of vertical transmission has been evolved during the long-term virus-vector interactions.

Paternal transmission of RGDV by hitchhiking with the sperm.

We then investigated whether the sperm-mediated paternal transmission of RGDV occurred in male R. dorsalis. Our immunofluorescence and electron microscopy revealed an association of dense RGDV particles with the plasma membrane of sperm heads in the male reproductive system (Fig. 3a–f). We then used immunofluorescence microscopy to observe how RGDV hitchhiked a ride on the sperm to offspring. RGDV was initially detected in the spermatheca of V– females at 3 days post mating with V+ males (Fig. 3g–i and Supplementary Table 2). Subsequently, virus-decorated sperms in the spermatheca moved to the

ovisperm for fertilizing the mature eggs during ovulation (Fig. 3j and Supplementary Table 2). RGDV became detectable in the genital tract but not in the oocytes of females even at 10 days post mating (Fig. 3m–o and Supplementary Table 2). Electron microscopy showed that virus-decorated sperms were present in the dissected spermatheca of V+ females (Fig. 3p), confirming the transfer of virions from V+ males to V– females. Immunofluorescence assays revealed that RGDV circulated within V– females after mating with V+ males, and ~ 63% of tested female intestines were infected at 10 days post mating (Supplementary Table 3). However, the virus was rarely observed in the oocytes, indicating that strong ovarian transmission barrier occurred (Supplementary Table 3). Moreover, V+ females, which acquired virus by mating with V+ males could transmit RGDV to rice plants, and the transmission efficiency increased with the time

of RGDV as a preferred mode of vertical transmission has been evolved during the long-term virus-vector interactions.

Paternal transmission of RGDV by hitchhiking with the sperm.

We then investigated whether the sperm-mediated paternal transmission of RGDV occurred in male R. dorsalis. Our immunofluorescence and electron microscopy revealed an association of dense RGDV particles with the plasma membrane of sperm heads in the male reproductive system (Fig. 3a–f). We then used immunofluorescence microscopy to observe how RGDV hitchhiked a ride on the sperm to offspring. RGDV was initially detected in the spermatheca of V– females at 3 days post mating with V+ males (Fig. 3g–i and Supplementary Table 2). Subsequently, virus-decorated sperms in the spermatheca moved to the

ovisperm for fertilizing the mature eggs during ovulation (Fig. 3j and Supplementary Table 2). RGDV became detectable in the genital tract but not in the oocytes of females even at 10 days post mating (Fig. 3m–o and Supplementary Table 2). Electron microscopy showed that virus-decorated sperms were present in the dissected spermatheca of V+ females (Fig. 3p), confirming the transfer of virions from V+ males to V– females. Immunofluorescence assays revealed that RGDV circulated within V– females after mating with V+ males, and ~ 63% of tested female intestines were infected at 10 days post mating (Supplementary Table 3). However, the virus was rarely observed in the oocytes, indicating that strong ovarian transmission barrier occurred (Supplementary Table 3). Moreover, V+ females, which acquired virus by mating with V+ males could transmit RGDV to rice plants, and the transmission efficiency increased with the time
To identify the sperm attachment protein, RGDV P8 fused to glutathione-S-transferase (GST) was generated as bait to screen the sperm proteins extracted from *R. dorsalis* (Supplementary Figure 3a). Mass spectrometry analysis of the pulled down proteins identified 12 peptides with four different types mapped to heparan sulfate proteoglycan (HSPG) (Supplementary Figure 3b), a ubiquitous cell surface protein, which has been exploited by many pathogens such as viruses, bacteria, and parasites for their initial attachment and subsequent cellular entry25,26. Here, we determined that *R. dorsalis* HSPG was 3941 amino acids long and consisted of five domains (GenBank accession number MH060173) (Fig. 4e). The 12 identified peptides all targeted the third domain (domain III) of HSPG (Supplementary Figure 3b). Yeast two-hybrid assay showed that RGDV P8 interacted only with the domain III of HSPG (Supplementary Figure 4a–f). The specific interaction between RGDV P8 and HSPG domain III was also independently verified by GST pull-down assay (Fig. 4g). RT-qPCR and western blot assays revealed an enriched expression of HSPG in the male reproductive system compared with the remaining tissues or the female reproductive system of *R. dorsalis* (Supplementary Figure 5a–e). Specific HSPG antibodies recognition of the sperm head and testis, but not the intestines, was also verified by immunofluorescence microscopy (Supplementary Fig. 3c).

**Interaction of RGDV P8 and heparan sulfate proteoglycan.** We further determined how RGDV virions were associated with the plasma membrane of sperm heads. We detected the initial binding of purified RGDV virions to the head of live sperm dissected from nonviruliferous *R. dorsalis* after 5 min incubation (Fig. 4a). With longer incubation times, more virions accumulated on the sperm heads (Fig. 4a). Similar in vitro binding of RGDV to live sperm was also detectable after incubation with the major outer capsid protein P8, but not with the minor outer capsid protein P2 of RGDV (Fig. 4b, c and Supplementary Figure 2a, b). Furthermore, the specific binding to the head of live sperm was abolished by pretreatment with antibodies against intact virions or P8, but not P2 (Fig. 4d). These results indicate that the major outer capsid protein P8 mediates the direct binding of RGDV virions to the sperm head.

**Post mating (Supplementary Figure 1b).** These observations provide direct evidence for the paternal transmission of RGDV by “hitchhiking” with the sperms in the *R. dorsalis* male reproductive system to the spermatheca of females, from there the sperms move to the oviduct to fertilize the eggs passing through outwards to be deposited (Fig. 3g).

**Fig. 2** Comparison of fitness variables between male and female vectors. a Mean number of V− females that copulated with single V+ or V− male in 24, 48, or 72 h. b Effects of RGDV infection on the longevity of male and female adult *R. dorsalis*. c Sex ratio of offspring produced by parental insects from different mating combinations (V− × V−, V− × V+, V+ × V−, or V+ × V+). d Progeny egg number, size and hatching rate of female adults from different mating combinations (V− × V−, V− × V+, V+ × V−, or V+ × V+). Data are presented as mean ± SE of three independent experiments of four mating combinations. The significance of any differences was tested using Student’s *t* test a–b or Tukey’s HSD test c–d. *P* < 0.05. Different letters after means in the same column a–c or line (d) indicate a significant difference at *P* = 0.05, and the means do not differ significantly if they are indicated with the same letter. V+, viruliferous. V−, nonviruliferous.
accumulation in the spermatheca in vivo expression of HSPG, thereby inhibited the sperm binding the newly emerged viruliferous male.

Importantly, microinjecting dsRNA targeting HSPG mRNA into the spermatheca, then move to the oviduct to fertilize eggs. All immunofluorescence microscopy showing association of RGDV virions (red arrows) with the plasma membrane of sperm heads. Sperms from V. dorsalis females at 10 days post mating. RGDV accumulation in the spermatheca and HSPG on the head of sperm was readily detectable (Fig. 4j) and reduced the accumulation of RGDV in the male reproductive systems (Fig. 4n) and the subsequent paternal transmission of RGDV to offspring (Fig. 4o). It is clear that RGDV infection can trigger the enriched accumulation of HSPG in vector male reproductive systems (Fig. 4n) and the subsequent paternal transmission of RGDV from V. dorsalis to offspring (Fig. 4o). It is clear that RGDV infection can trigger the enriched accumulation of HSPG in vector male reproductive systems (Fig. 4n) and the subsequent paternal transmission of RGDV from V. dorsalis to offspring (Fig. 4o). It is clear that RGDV infection can trigger the enriched accumulation of HSPG in vector male reproductive systems (Fig. 4n) and the subsequent paternal transmission of RGDV from V. dorsalis to offspring (Fig. 4o).
Paternal transmission of RGDV by minor leafhopper vector. In addition, we examined if this phenomenon also occurred in minor vector of RGDV, the rice leafhopper *Nephotettix cincticeps*. We first observed that the viruliferous rates of natural *R. dorsalis* and *N. cincticeps* population in Guangdong were ~58% and 5% during planting seasons, respectively (Fig. 5a). The acquisition efficiency of *N. cincticeps* under experimental conditions was only 13%, whereas that of *R. dorsalis* was over 80% (Fig. 5b). In the eggs laid by the individual V\(^{+}\) female that mated with V\(^{-}\) male of *N. cincticeps*, 18% were positive for RGDV (Fig. 5c); in contrast, 62% were RGDV-positive of the eggs produced by V\(^{-}\) female that mated with V\(^{+}\) male of *N. cincticeps*, indicating that paternal transmission is ~3.4 times as efficient as maternal transmission by *N. cincticeps*, similar to the efficiency of paternal transmission by *R. dorsalis* (Fig. 5c). Immunofluorescence microscopy also showed a close association of RGDV with the head surface of sperms dissected from seminal vesicles of V\(^{+}\) males of *N. cincticeps* (Fig. 5d). However, virus attachment to the sperm of the
Discussion
A handful of studies have described the mechanistic basis of vertical maternal transmission of arboviruses and its role in epidemic persistence of viruses6–8, whereas the occurrence of vertical paternal transmission in nature is undetermined. In this study, we discovered a previously unknown phenomenon: a rice arbovirus can be efficiently transmitted from male insect vectors to offspring through a direct interaction of viral outer protein with the cell surface HSPG of the sperm head without affecting the fitness of male insects or their offspring. More importantly, an infected male can potentially transfer viruses to more offspring because a male can mate repeatedly with females and thus enhance virus spread. By contrast, during maternal transmission, viral propagation in the oocytes of female ovary often causes cytopathologic changes, decreasing the fitness of insect offspring24,25. Thus, males transmit a remarkably higher proportion of viruses to insect offspring through sperm than females transmit through ovaries. Furthermore, viruliferous males survive much longer than viruliferous females, and finally, more males are infected by RGDV than females in the field. More importantly, the paternal transmission rate (~60%) is evidently higher than the maternal rate (~20%) in field-collected R. dorsalis populations. Thus, during the cold seasons unfavorable for virus-infected rice hosts in the field, the maintenance efficiency of RGDV through up to two insect generations tends to decrease. We deduce that such a sperm-mediated paternal transmission is a more powerful type of vertical virus transmission than maternal transmission by insect vectors in many cases, and plays a vital role in the efficient maintenance of RGDV during the cold seasons in the field. Taken together, a preferred mode of vertical arbovirus transmission has been evolved by hitchhiking with insect sperm, thereby explaining a natural long-term endemic pattern of RGDV throughout Southern China for ~30 years.

At present, whether other vector-borne viral pathogens can be carried by insect sperm and then be paternally transmitted with high efficiency in nature is as yet unknown. Interestingly, arboviruses such as La Crosse virus and Zika virus can be paternally transmitted by male mosquitoes with low efficiency, but viral antigens were not observed within sperms28–30. It seems that La Crosse virus and Zika virus in mosquitoes can be veneerally transmitted by male accessory sex gland fluid rather than by sperm, which agrees with the findings for the symbiotic rhabdoviruses in Drosophila31. We cannot rule out the possibility that paternal transmission of RGDV occurs not through sperm but through seminal fluid. However, even though RGDV can enter into female oocytes from the infected male seminal fluid, it would encounter strong ovarian barriers24, and, thus it appears to be of a low level of occurrence and constitute a relatively minor component of the vertical transmission rate. Given the widespread occurrence of long-term epidemic for RGDV in nature, the virus has gained an evolutionary advantage by hitchhiking with the insect sperm for vertical propagation. We anticipate other arboviruses may have also evolved a similar strategy to ensure viral survival during adverse conditions for horizontal transmission.

Our study reveals a mode of vertical transmission of an arbovirus. Lack of virus-infected host plants in cold seasons presents a bottleneck for arbovirus transmission so that sperm-mediated paternal transmission in vector populations may be critical to viral persistence in the field. Our data lay a foundation for further investigation of the mechanisms underlying paternal virus transmission and ecological significance, and provide insights into the development of efficient approaches to attenuate viral epidemic by targeting sperm-mediated paternal transmission mechanism.

Methods
Crossing experiments. Four treatments of crossing were conducted for R. dorsalis adults from a laboratory-reared colony and a field-caught colony, respectively. In each treatment, one newly emerged adult female was crossed with one newly emerged adult male as follows: (i) V− female × V− male (V−2); (ii) V− female (V−q) × V− male (V−2); (iii) V− female (V−q) × V+ male (V+2); and (iv) V+ female (V+q) × V+ male (V+2). The V− and V+ R. dorsalis populations have been established in our laboratory (Supplementary Materials and Methods). Our preliminary test confirmed that >80% of insects contained RGDV in the viruliferous populations27. Thus, for laboratory-reared colony, the 5th instar nymphs were caught from V− or V+ R. dorsalis populations and reared separately in glass tubes until eclosion for crossing. Generally, the percentage of V+ R. dorsalis populations caught from the areas where RGDV incidence was high was above 60%. Thus, for field-caught colony, the individual 3rd or 4th instar nymphs of R. dorsalis were caught directly from the rice fields where RGDV incidence was high or from virus-free rice fields in Guangdong, China and reared separately in glass tubes until eclosion. For the mating procedure, the newly emerged potential V+ adults were chosen for mating one to one with the V− adults in glass tubes containing one rice seedling for 5 days, and the seedling was replaced daily with a new rice seedling to avoid viral acquisition from plant hosts. At the end of the 6th day, the potential V+ males in each of the tubes were collected for virus detection by real-time-polymerase chain reaction (RT-PCR) assay, and the females were left in each of the tubes to lay eggs for another 5 days and the seedling in each tube was renewed daily. The potential V+ females were then collected for virus detection by RT-PCR assay. Among these independent experiments with the laboratory-reared colony, the number of eggs detected in each independent experiment was as follows: V− × V+
## Vertical transmission of RGDV by the vector

**Fig. 5** Paternal transmission of RGDV by the vector, the rice leafhopper *N. cincticeps*. **a** The viruliferous rates of *R. dorsalis* and *N. cincticeps* (25 males and 25 females) collected from the fields in Guangdong, China. **b** The acquisition efficiencies of RGDV by *R. dorsalis* and *N. cincticeps* collected from the fields. **c** Vertical transmission of RGDV by the field-caught V⁺ and V⁻ *N. cincticeps* via mating. Eggs were collected for tracing RGDV. **d** Association of RGDV particles with sperm heads of *N. cincticeps* and the non-vector wheat leafhopper *P. alienus*. The sperms dissected from the male leafhoppers *N. cincticeps* or *P. alienus* were microinjected with RGDV virions, stained with virus-rhodamine (red) and DAPI (blue), and examined by immunofluorescence microscopy. All images are representative of at least three replicates. Bars, 10 μm. **e** Comparison of amino-acid sequences of HSPG DoIII of *R. dorsalis*, *N. cincticeps*, and *P. alienus*. **f** A yeast two-hybrid assay was used to detect the interaction between RGDV P8 and HSPG DoIII from *N. cincticeps*. Immunofluorescence staining. For visualizing viral infection to the male reproductive system, second instar nymphs of *R. dorsalis* were fed on diseased rice plants for 2 days and then transferred to non-infected rice seeds. At different days after eclosion, the reproductive system from 30 males was excised, fixed, immunolabeled with virus-specific IgG conjugated to horseradish peroxidase (HRP) (1:1000 dilution), and actin dye phalloidin-fluorescein isothiocyanate (FITC) (Invitrogen, cat. F432; 1:200), and then processed for immunofluorescence microscopy. For visualizing viral association with sperm, mature sperms were excised from the seminal vesicles of V⁺ males and then smeared on poly-lysine-treated glass slides. The sperms were fixed and immunolabeled with virus-rodamin (0.5 μg/μl), and then stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma, cat. D9542). For visualizing the virus in females after transfer from males, virgin V⁻ females were mated one on one with V⁺ male adults in individual glass tubes for 3 days. Males were then collected and confirmed for RGDV-positive by RT-PCR assay. The reproductive system was excised from each of the 30 females at 3, 6, and 10 days after mating with the V⁺ males, and then smeared on poly-lysine-treated glass slides.
Electron microscopy. The seminal vesicles of V+ male adult R. dorsalis or the spermathea of female adult R. dorsalis at 3 days after crossing with V+ males were excised and fixed with 2% paraformaldehyde and 2% w/v glutaraldehyde in phosphate-buffered saline (PBS) for 2 h at room temperature, and then postfixed with 1% w/v osmium tetroxide in PBS for 1 h at room temperature. The fixed samples were dehydrated in grade series of ethanol up to 100% and embedded in Spurr’s resin (SPI Ltd). Sections were observed with a transmission electron microscope (H-7650, HITACHI).

RGDV-sperm binding in vitro. Mature, live sperms from the seminal vesicles were smeared on poly-lysine-treated glass slides, and then incubated with purified RGDV virions (0.01 μg/μl) for 5, 30, or 90 min For detecting the binding of P2 or P8 of RGDV with sperm in vitro, His-tagged P2 or P8 was expressed in Escherichia coli strain Rosetta, and the proteins were purified using nickel-nitritotriacetic acid resin (Qiagen). The samples were also incubated with purified proteins (0.5 μg/μl) for 60 min and then stained with P2- or P8-specific IgG conjugated to rhodamine, P2-rhodamine (0.5 μg/μl) or P8-rhodamine (0.5 μg/μl), respectively, and then processed for confocal microscopy. Alternatively, purified RGDV virions were pre-incubated with P2 or P8 antibodies (0.5 μg/μl) for 10 min, and then incubated with live sperms. The samples were stained with virus-rhodamine and DAPI, and then processed for confocal microscopy.

Identification of sperm proteins that interact with RGDV P8. RGDV P8 was used as bait to screen the interacting proteins extracted from sperms of R. dorsalis using a GST pull-down assay. In brief, the GST-fused P8 or GST was bound to glutathione-Sepharose beads (Amersham) for 4 h at 4 °C, then washed extensively with washing buffer (300mM NaCl, 10mM Na2HPO3, 2.7 mM KCl and 1.7M KH2PO4). Immunoprecipitated proteins were detected by western blot with His-tagged antibodies (Sigma, cat. SAB4301134, 1:1000) and GST-tagged antibodies (Sigma, cat. SAB4200692, 1:1000) separately.

Knockdown of HSPG expression in R. dorsalis. A DNA fragment spanning a 994bp in-frame targeting HSPG domain III was amplified by PCR using forward primer 5′-ATTCCTGAAAGCTTAACTGACTCATATAGGGGGCTGCGAAC TGGTTGACTA-3′ and reverse primer 5′-ATTCCTGAAGCTTAACTGACTCATATAGGGGGCTGCGAAC TGGTTGACTA-3′ and reverse primer 5′-ATTCCTGAAGCTTAACTGACTCATATAGGGGGCTGCGAAC TGGTTGACTA-3′. The amplicons were sequenced in accordance with the manufacturer’s instructions. Relative levels of gene expression were normalized to a housekeeping gene elongation factor 1alpha gene (EF1, accession number AB836665) and estimated by the 2 -ΔΔCt (cycle threshold) method. A pool of 20 insects was used for each replicate. We performed three replicates for each time point and each concentration, respectively. The data were back-transformed after analysis in the text, and multiple comparisons of the means were conducted using a one-way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference test at the P=0.05 significance level. Comparisons between time points were conducted using Student’s t test. The data were back-transformed after analysis in the test, figures, and tables.

Xenoyt two-hybrid assay. Interaction between HSGP of R. dorsalis, N. cincticeps or P. aliferus and the major capsid protein P8 of RGDV was tested in a yeast two-hybrid assay (Y2H) using a DUALmembrane yeast two-hybrid kit (DUALystems Biotech) according to the manufacturer’s manual. The P8 gene of RGDV was cloned into the bait vector pBT3-STE (PBT-STE-RGDV P8), and the five domain sequences of HSGP of R. dorsalis as well as HSGP domain III of N. cincticeps and P. aliferus were cloned into the prey vector pPr3-N (pPr3-N-DoIII, pPr3-N-DoIII, pPr3-N-DoIV, and pPr3-N-DoV) (Supplementary Table 4). The bait and prey plasmids were co-transformed into the yeast strain NM526. Plasmids pBT3-STE-RGDV and pPr3-N, pBT3-STE and pPr3-N-DoIII were co-transformed to test self-activation. Plasmids pBT3-STE and pGST1-Nub (positive control) or pPr3-N (negative control) were used to control transformant NM526. All transformants were grown on synthetic dropout (SD)-Try-Leu agar plates (SD-2) –SD-4) or 6-3-Ile-Leu-His-Ade agar plates (SD-4) for 4–4 days at 30 °C. To assay the expression of fusion proteins, each yeast extract of transformant strain was assayed for RGDV, and female leafhoppers were left in the tubes for oviposition. The offspring of each cross (dsGFP-treated, n = 136; dsHSPG-treated, n = 111) were tested for RGDV by RT-PCR assay.

References

1. Eigenbrode, S. D., Bosque-Pérez, N. & Davis, T. S. Insect-borne plant pathogens and their vectors: ecology, evolution, and complex interactions. Annu. Rev. Entomol. 63, 169–191 (2018).
2. Mayer, S. V., Tesh, R. B. & Vasilakis, N. The emergence of arthropod-borne viral diseases: a global prospective on dengue, chikungunya and zika fevers. Acta Trop. 166, 153–165 (2017).
3. He, M., Guan, S. Y. & He, C. Q. Evolution of Plasmodium falciparum in the mosquito. Mol. Phylogenet. Evol. 109, 343–350 (2017).
4. Liu, Y. et al. Evolutionary enhancement of Zika virus infectivity in Aedes aegypti mosquitoes. Nature 545, 482–486 (2017).
5. Hogenhout, S. A., Ammar, E. D., Whitfield, A. E. & Redinbaugh, M. G. Insect vector interactions with persistently transmitted viruses. Annu. Rev. Phytopathol. 46, 327–359 (2008).

6. Sebastian, L., Paul, R. E. & Louis, L. Determinants of arborivirus vertical transmission in mosquitoes. PLoS Pathog. 12, e1005548 (2016).

7. Wei, T. & Li, Y. Rice reoviruses in insect vectors. Annu. Rev. Phytopathol. 54, 99–120 (2016).

8. Jia, D. et al. Vector mediated transmission of persistently transmitted plant viruses. Curr. Opin. Virol. 28, 127–132 (2018).

9. Watts, D., Pantuwatana, S., Defoliart, G., Yuill, T. M. & Thompson, W. H. Transovarial transmission of a plant virus is mediated by vitellogenin in its insect vector. PLoS Pathog. 10, e1003949 (2014).

10. Wei, J. et al. Vector development and vitellogenin determine the transovarial transmission of begomoviruses. Proc. Natl Acad. Sci. USA 114, 6746–6751 (2017).

11. Huo, Y. et al. Transovarial transmission of a plant virus is mediated by vitellogenin in its insect vector. PLoS Pathog. 10, e1003949 (2014).

12. Wei, J. et al. Vector development and vitellogenin determine the transovarial transmission of begomoviruses. Proc. Natl Acad. Sci. USA 114, 6746–6751 (2017).

13. Grylls, N. E. Rugose leaf curl—a new virus disease transovarially transmitted by the leafhopper Aulacorthum torridum. Aust. J. Biol. Sci. 7, 47–58 (1954).

14. Sylvester, E. S. Transovarial passage of the whitefly vectored sweet potato yellow virus in the aphid Hyperomyzus lactucae. Virolology 38, 440–446 (1969).

15. Dali, R. & Afekeus, B. A. Characteristics of the sperm structure in Heteroptera (Hemiptera, Insecta). J. Morphol. 164, 301–309 (1980).

16. Garolla, A. et al. Sperm viral infection and male infertility: focus on HBV, HCV, HIV, HPV, HSV, HCMV, and AAV. J. Reprod. Immunol. 100, 20–29 (2013).

17. Ma, W. et al. Zika virus causes testis damage and leads to male infertility in mice. Cell 167, 1311–1324 (2016).

18. Gorov, O. et al. Zika virus infection damages the testes in mice. Nature 540, 438–442 (2016).

19. Ng, J. C. & Falk, B. W. Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. Annu. Rev. Phytopathol. 44, 183–212 (2006).

20. Jia, D. et al. Insect symbiotic bacteria harbour viral pathogens for transovarial transmission. Nat. Microbiol. 2, 17025 (2017).

21. Omura, T. et al. Rice gall dwarf, a new virus disease. Plant Dis. 64, 795–797 (1980).

22. Inoue, H. & Omura, T. Transmission of Rice gall dwarf virus by the green rice leafhopper. Plant Dis. 66, 57–59 (1982).

23. Fan, H. et al. Rice gall dwarf: a new virus disease epidemic in the west of Guangdong province of south China. Acta Phytopathol. Sin. 13, 1–6 (1983).

24. Liao, Z. et al. Virus-induced tubules: a vehicle for spread of viornis into ovary oocyte cells of an insect vector. Front. Microbiol. 8, 475 (2017).

25. Chen, Y., Görtz, M., Liu, J. & Park, P. W. Microbial subversion of heparan sulfate proteoglycans. Mol. Cells 26, 415–426 (2008).

26. Tiwari, V., Maus, E., Sigar, I. M., Ramsey, K. H. & Shukla, D. Role of heparan sulfate in sexually transmitted infections. Glycobiology 22, 1402–1412 (2012).

27. Chen, Y. et al. Adverse effects of rice gall dwarf virus upon its insect vector Recita dorsalis (Hemiptera: Cicadellidae). Plant Dis. 100, 784–790 (2016).

28. Thompson, W. H. & Beaty, B. J. Venereal transmission of La Crosse (California encephalitis) arbovirus in Aedes triseriatus. Sci. Rep. 6, e23 (2017).

29. Li, C. X. et al. Vector competence and transovarial transmission of two Aedes aegypti strains to Zika virus. Emerg. Microbes Infect. 6, e23 (2017).

30. Campos, S. S. et al. Zika virus can be venerealy transmitted between Aedes aegypti mosquitoes. Parasite Vector 10, 605 (2017).

31. Longdon, B., Wilfert, L., Obbard, D. J. & Jiggins, F. M. Rhabdoviruses in two species of Drosophila: vertical transmission and a recent sweep. Genetics 188, 141–150 (2011).

32. Lan, H. et al. Small interfering RNA pathway modulates persistent infection of a plant virus in its insect vector. Sci. Rep. 6, 20699 (2016).

Acknowledgements
We are grateful to Shouwei Ding for helpful discussions and editorial assistance, to Shusheng Liu for comments on the manuscript. We thank Dr. Xufeng Wang from Chinese Academy of Sciences for providing the P. alius colony. This work was supported by grants from National Natural Science Foundation of China (Grants 31730071, 31770166, 31870148, and 31870149) and National Key Research and Development Plan Foundation (2016YFD0300707).

Author contributions
Q.M., W.W. designed all experiments. Q.M., Z.L., J.W. and T.W. analyzed the data. Q.M., W.W. and Z.L. performed the protein interaction and electron microscopy. Q.M., W.W. and Z.L. performed the protein interaction experiments and found the role of HSPG. Q.M., W.W. and J.W. performed the experiments for immunofluorescence staining and electron microscopy. Q.M., W.W. and Z.L. performed the experiments for immunofluorescence staining and electron microscopy. Q.M., W.W. and Z.L. performed the experiments for immunofluorescence staining and electron microscopy. Q.M., W.W. and Z.L. performed the experiments for immunofluorescence staining and electron microscopy. Q.M., W.W. and Z.L. performed the experiments for immunofluorescence staining and electron microscopy.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-08860-4.

Competing interests: The authors declare no competing interests.

Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

Journal peer review information: Nature Communications thanks Grant Hughes, Veronique Brault, and the other anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019