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Transmission of areca palm velarivirus 1 (APV1) by *Pseudococcus cryptus*

Xue Zhao[1†], Ruibai Zhao[1†], Xianmei Cao[1], Yutian Wang[1], Hongxing Wang[1*] and Xi Huang[1*]

Abstract

Betel palm is one of the most economically important crops in Southeast Asia. The occurrence and expansion of yellow leaf disease (YLD) have significantly impacted betel palm plantations. Our previous research demonstrated that areca palm velarivirus 1 (APV1) was associated with YLD and transmitted by *Ferrisia virgata* (striped mealybug), causing YLD in betel palms. This finding provides strong etiological evidence of the role played by APV1 in YLD. Controlling YLD is a pressing issue with significant challenges. One viable approach is to prevent the virus from spreading by disrupting the transmission vectors. Therefore, it is imperative to identify potential vectors of APV1. In this study, we detected APV1 in the stylet, foregut, midgut, and hindgut of *Pseudococcus cryptus* (cryptic mealybug) using immunocapture RT-PCR and in situ immunofluorescence localization. *P. cryptus* transmitted APV1 in a non-circulative, semi-persistent manner. The retention time of APV1 in *P. cryptus* was notably longer than that in *F. virgata*. Additionally, field investigations revealed that three other insects infesting betel palms also carried APV1. In summary, APV1 may also be transmitted by other mealybug species, and the lower specificity of transmission vectors makes it challenging to control the spread of this devastating disease. This work provides timely knowledge for the prevention and management of YLD.

Keywords Yellow leaf disease, Areca palm velarivirus 1, *Pseudococcus cryptus*, Transmission

Background

Yellow leaf disease (YLD) poses a significant threat to betel palms (*Areca catechu* L.) in Southeast Asia. YLD has been historically associated with phytoplasma in India, China, and Sri Lanka, based on electron microscopic observations and RT-PCR amplification of the 16S ribosomal RNA (rRNA) gene (Nayar and Seliskar 1978; Muddumadiah et al. 2014; Nair et al. 2014; Kanatiwela-de Silva et al. 2015). However, conclusive evidence regarding the etiology of YLD has been lacking thus far. The derbid planthopper *Proutista moesta* (Westwood) has been suggested as the vector of phytoplasma in areca palms (Ponnamma et al. 1997), but this proposal awaits solid evidence. Furthermore, phytoplasma has not been detected in many YLD samples collected in India and China (Purushothama et al. 2007; Wang et al. 2020), leaving the etiological agent of YLD in need of further clarification (Khan et al. 2023).

Areca palm velarivirus 1 (APV1) was first identified in YLD leaf samples through small RNA sequencing (Yu et al. 2015). Subsequently, YLD was determined through RNA-seq and digital gene expression analysis, confirming the association between APV1 infection and YLD symptomatic samples using RT-PCR and transcription analysis (Wang et al. 2020; Cao et al. 2021). Based on phylogenetic comparisons and genome structure analyses, APV1 was proposed as a novel member of the genus *Velarivirus*

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within the family *Closteroviridae*. APV1 exhibits typical flexuous, filamentous particles and possesses a positive-sense, single-stranded RNA genome of 17,546 nucleotides (nt), encoding 11 open reading frames (ORFs). To date, 23 APV1 isolates have been identified, demonstrating highly conserved sequences in seven ORFs (> 95% nucleotide identity) at the 3′ terminus and significant genetic diversity in three ORFs (81–87% nucleotide identity) at the 5′ terminus (Wang et al. 2020; Cao et al. 2021).

The classification of the family *Closteroviridae* relies on phylogenetic analyses of viral conserved proteins, including helicase, HSP70, and RNA-dependent RNA polymerase, as well as the specificity of vector transmission (Karasev 2000; Dolja et al. 2006; Al Rwahnih et al. 2012; Jelkmann et al. 2012; Rubio et al. 2013; Naidu et al. 2015). These conserved protein phylogenies closely related with the type of vector transmission. Ampeloviruses are transmitted by mealybugs, while criniviruses and closteroviruses are transmitted by whiteflies and aphids, respectively (Karasev 2000; Dolja et al. 2006). Although velariviruses and criniviruses exhibit a closer genetic relationship in phylogenetic analyses (Martelli 2019; Zhang et al. 2022), their distinct transmission vectors and genome structures prevent their inclusion in a single genus (Jelkmann et al. 2012; Lim et al. 2015; Zhang et al. 2022).

In previous research, APV1 was found to be transmitted by *Ferrisia virgata*, leading to YLD in areca palm seedlings. APV1 is the sole velarivirus known to be transmitted by mealybugs (Zhang et al. 2022). To gain a better understanding of the ecological factors influencing the spread of this mealybug-borne virus, it is essential to identify the vector species and transmission mode for APV1 accurately. Additionally, the correct identification of insect vectors is crucial for the development of effective disease management practices. In this study, we investigated the transmission properties of APV1 by mealybug species *Pseudococcus cryptus*. Several other insects infesting betel palms were found to carry APV1 as well, suggesting that APV1 may be transmitted by other mealybug species, indicating a low vector-virus specificity.

Results

APV1 transmission by *P. cryptus*

To describe symptom development following APV1 transmission by *P. cryptus,* healthy betel palm seedlings were inoculated with *P. cryptus* for a 72 h inoculation access period (IAP). RT-PCR detected APV1 in all 36 inoculated seedlings at 90 days post inoculation (dpi). The initial yellowing symptoms appeared at the tip of the leaflets around 60 dpi. These symptoms then spread along the vascular tissue, leaving the midvein green, creating a distinctive yellow-green border distinguishing the symptoms from physiological yellowing. Typical YLD symptoms, as described previously (Wang et al. 2020; Zhang et al. 2022), were observed at 90 dpi. Seedlings mock-inoculated with APV1-free mealybugs did not display any yellowing symptoms (Fig. 1).

Localization of APV1 in the digestive system of *P. cryptus*

To locate APV1 in *P. cryptus*, female mealybugs that had fed on APV1-infected seedlings were dissected. APV1 was detected by RT-PCR in the stylets, foregut, midgut, and hindgut of all mealybugs but not in the hemolymph and eggs (Fig. 2), suggesting that APV1 is not transmitted from viruliferous females to offspring via the ovaries. The absence of APV1 in the hemolymph indicates that APV1 transmission by *P. cryptus* might occur in a non-circulative mode.

To further visualize APV1 in situ, immunofluorescence localization was performed. The untreated digestive system of *P. cryptus* adults was initially observed under a confocal microscope, revealing strong auto-fluorescence in stylets, while other tissues such as the foregut, midgut, hindgut, and Malpighian tubules exhibited weak auto-fluorescence (Additional file 1: Figure S1). When tissues of *P. cryptus* without APV1 were treated with monoclonal antibodies against APV1-CP and FITC-labeled secondary antibodies, strong fluorescence was observed in the Malpighian tubules, suggesting non-specific labeling (Additional file 1: Figure S1b). In the gut tissue of *P. cryptus* after a 48 h acquisition access period (AAP) on source plants, granular fluorescent signals were detected in the foregut and midgut (Fig. 3a, b). More prominent green fluorescent particles were observed in the foregut, midgut, and hindgut of *P. cryptus* after 72 h AAP and extended AAP durations (Fig. 3c–f). The strength of the granular fluorescent signal was proportional to the AAP duration, indicating that longer AAP periods led to increased APV1 ingestion and retention in the gut tissue.

Effect of different stages of *P. cryptus* on APV1 transmission

To compare the transmission efficiency of different instar nymphs, both first-instar and third-instar nymphs of *P. cryptus* were APV1 positive after a 72 h AAP. However, the inoculation efficiency of third-instar nymphs (83.33%) was significantly higher than that of first-instar nymphs (20%) (Additional file 2: Table S1). Therefore, third-instar nymphs of *P. cryptus* were selected for subsequent inoculation experiments.

Effect of starvation on APV1 ingestion by *P. cryptus*

Third-instar mealybugs starved for 12 h exhibited a significantly higher virus acquisition rate compared to those starved for 0, 6, or 24 h (Fig. 4). Prolonged starvation may

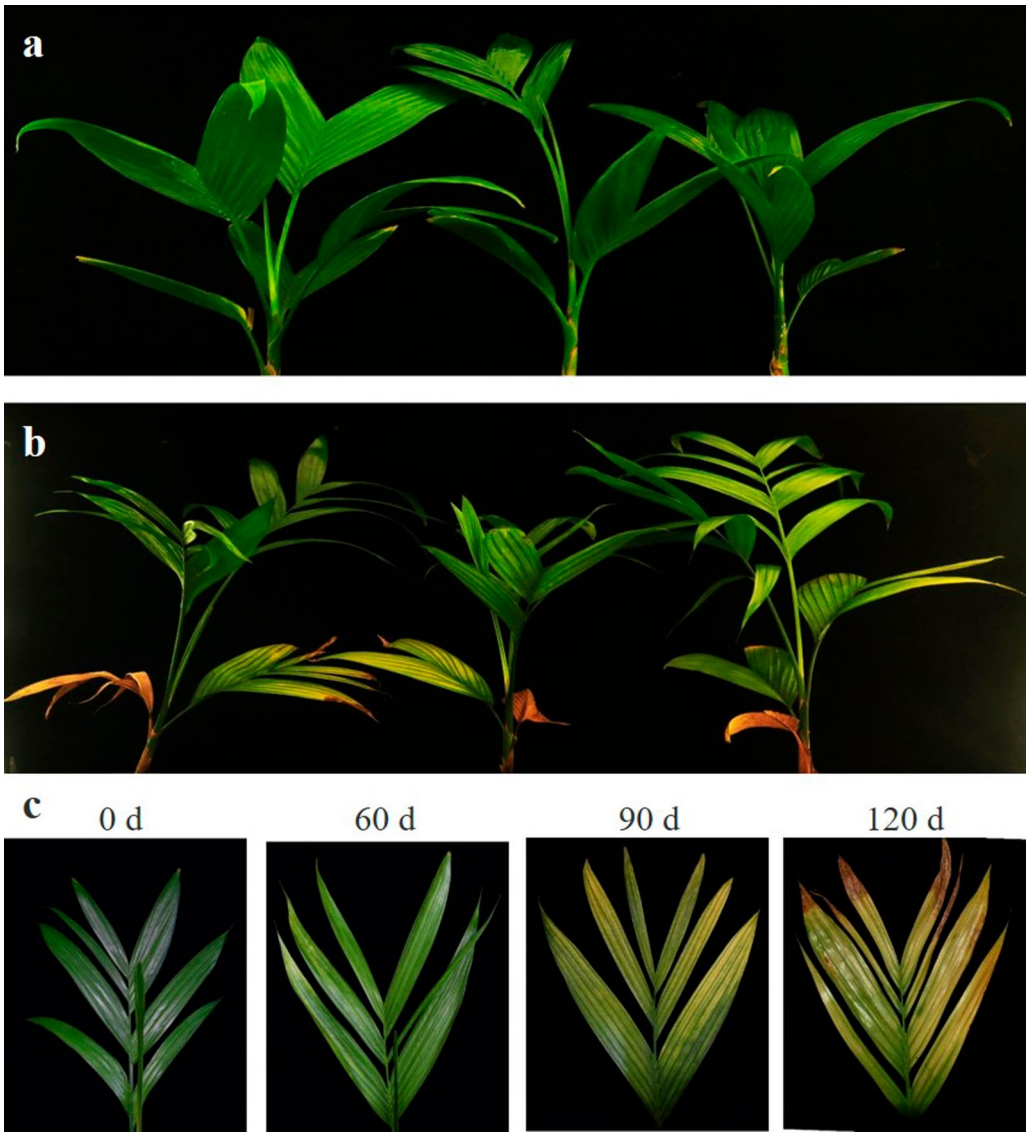

Fig. 1 Inoculation of areca palm velarivirus 1 (APV1) with *P. cryptus* mealybugs caused YLD symptom on betel palm seedlings. The *P. cryptus* with or without APV1 were transferred to healthy areca palm seedlings. After an inoculation access period (IAP) of 72 h, the plants were sprayed with acetamiprid to kill the mealybugs. **a** and **b** Symptoms after mock inoculation with *P. cryptus* carrying no APV1, or inoculation with *P. cryptus* carrying APV1 at 120 dpi. **c** Leaf symptoms development after inoculation with *P. cryptus* carrying APV1 from 0 to 120 dpi

reduce the viability of third-instar nymphs, and those starved for 6 h might not have been hungry enough, resulting in a lower virus acquisition rate. Therefore, third-instar nymphs starved for 12 h were selected for inoculation experiments.

Retention time of APV1 in *P. cryptus*

To determine the retention time of APV1 in *P. cryptus*, third-instar nymphs were transferred to non-host potatoes after acquiring APV1. RT-PCR revealed that the detection rate of APV1 sharply decreased after transfer to non-host plants. However, the maximum retention time of APV1 in *P. cryptus* reached 16 days (Fig. 5), notably longer than that of APV1 in *F. virgata* (8 days) (Zhang et al. 2022). Mealybugs reared on potatoes were then transferred to healthy betel palms to test viral transmission efficiency. Mealybugs reared on potatoes for one day successfully transmitted APV1 to healthy betel palms, but their transmission ability was lost after feeding on potatoes for more than 2 d (Additional file 1: Figure S2), suggesting that APV1 transmission by *P. cryptus* occurs in a semi-persistent mode.

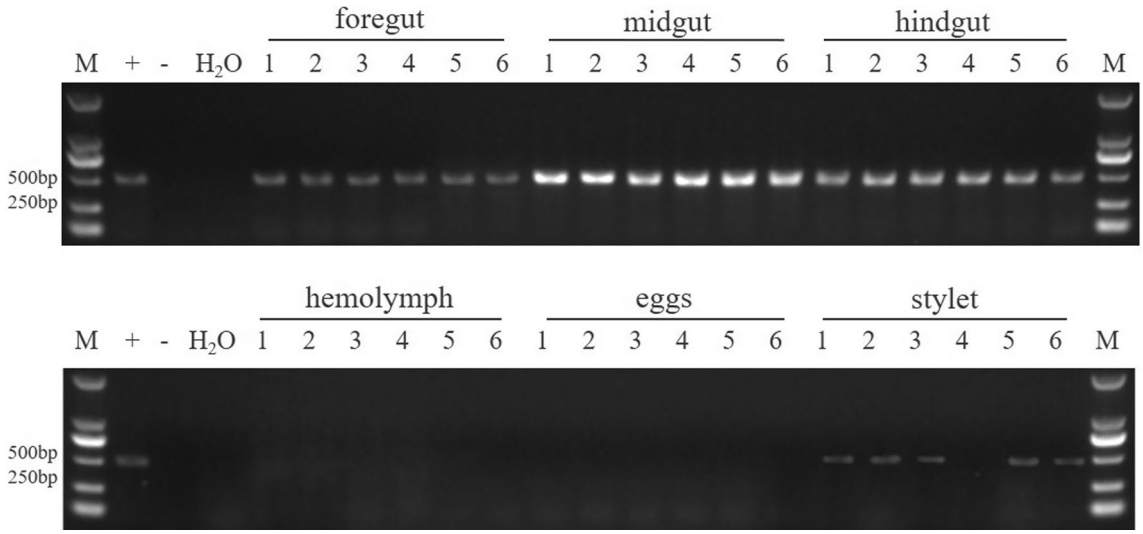

Fig. 2 Detection of APV1 in different tissues of *P. cryptus*. Foregut, midgut, hindgut, hemolymph, stylets, and eggs from adult female of *P. cryptus* after 72 h AAP (10/group, six repeats) were isolated by immunocapture RT-PCR using APV1-specific primers CPrp4-F/CPrp4-R

Effect of AAP and IAP on APV1 transmission efficiency by *P. cryptus*

To assess the effect of AAP on APV1 transmission efficiency by *P. cryptus*, third-instar nymphs were transferred to APV1 source plants for 1–72 h AAP durations and then retransferred to test plants for a 72 h IAP. The APV1 transmission rate increased as the AAP duration lengthened, reaching a maximum of 100% at 72 h AAP (Fig. 6a). A similar trend was observed for the effect of varying IAP after 72 h AAP, with transmission rates closely related to IAP duration and reaching a maximum at 72 h IAP (Fig. 6b). These findings provide important insights for APV1 inoculation via the *P. cryptus* vector.

Identification of unknown APV1 vectors

While APV1 has been known to be transmitted by only two mealybug species thus far, it is still unknown how many vectors can transmit APV1. To screen potential APV1 vectors, different insect species were collected from betel palm orchards in Hainan province and subjected to RT-PCR detection. APV1 was detected in three other species of insects: *Aleurocanthus spiniferus, Icerya seychellarum* (Additional file 1: Figure S3), and *Ceroplastes rusci* (data not shown), i.e. the aleyrodid, the monophlebid, and the coccid. Among these, *I. seychellarum* appears to be a potential vector of APV1.

Discussion

The transmission process is a crucial step in the virus infection cycle, with most plant viruses being transmitted from one host to another through vectors. The primary vectors include aphids, whiteflies, mealybugs, and other hemipterans (Ng and Falk 2006; Whitfield et al. 2015; Whitfield and Rotenberg 2015). Plant virus transmission by hemipteran vectors can be classified into non-persistent, semi-persistent, and persistent modes based on biological properties. This study aimed to comprehensively analyze the transmission parameters of APV1 by *P. cryptus*. APV1 could be transmitted by *P. cryptus* after just a 1 h acquisition access period (AAP) and a 1 h inoculation access period (IAP), with transmission efficiency increasing with the extension of AAP or IAP, up to 72 h. Immunofluorescence localization and immunocapture RT-PCR revealed that APV1 was predominantly retained in the foregut and midgut but not in the salivary glands or eggs. Offspring hatched from viruliferous *F. virgata* did not carry APV1. The maximum retention time of APV1 in *P. cryptus* reached 16 d, which was longer than that of APV1 in *F. virgata* (8 d) (Zhang et al. 2022) and that of ampelovirus grapevine leafroll-associated virus 3 (GLRaV-3) in *Planococcus ficus* (Tsai et al. 2008). However, *F. virgata* lost infectivity 2 d after leaving APV1-infected betel palm seedlings. All these data suggest that APV1 transmission by *P. cryptus* occurs in a semi-persistent mode. Furthermore, APV1 was not detected in hemolymph, suggesting that *F. virgata* transmitted APV1 in a non-circulative manner.

The life stages of mealybugs have a significant effect on the transmission efficiency of plant viruses. A previous study indicated that the transmission efficiency of first-instar nymphs of *F. virgata* was much higher than that of adult mealybugs (Zhang et al. 2022). In the case of the ampelovirus GLRaV-3, first-instar nymphs transmitted the virus, while third-instar nymphs of *Pseudococcus*

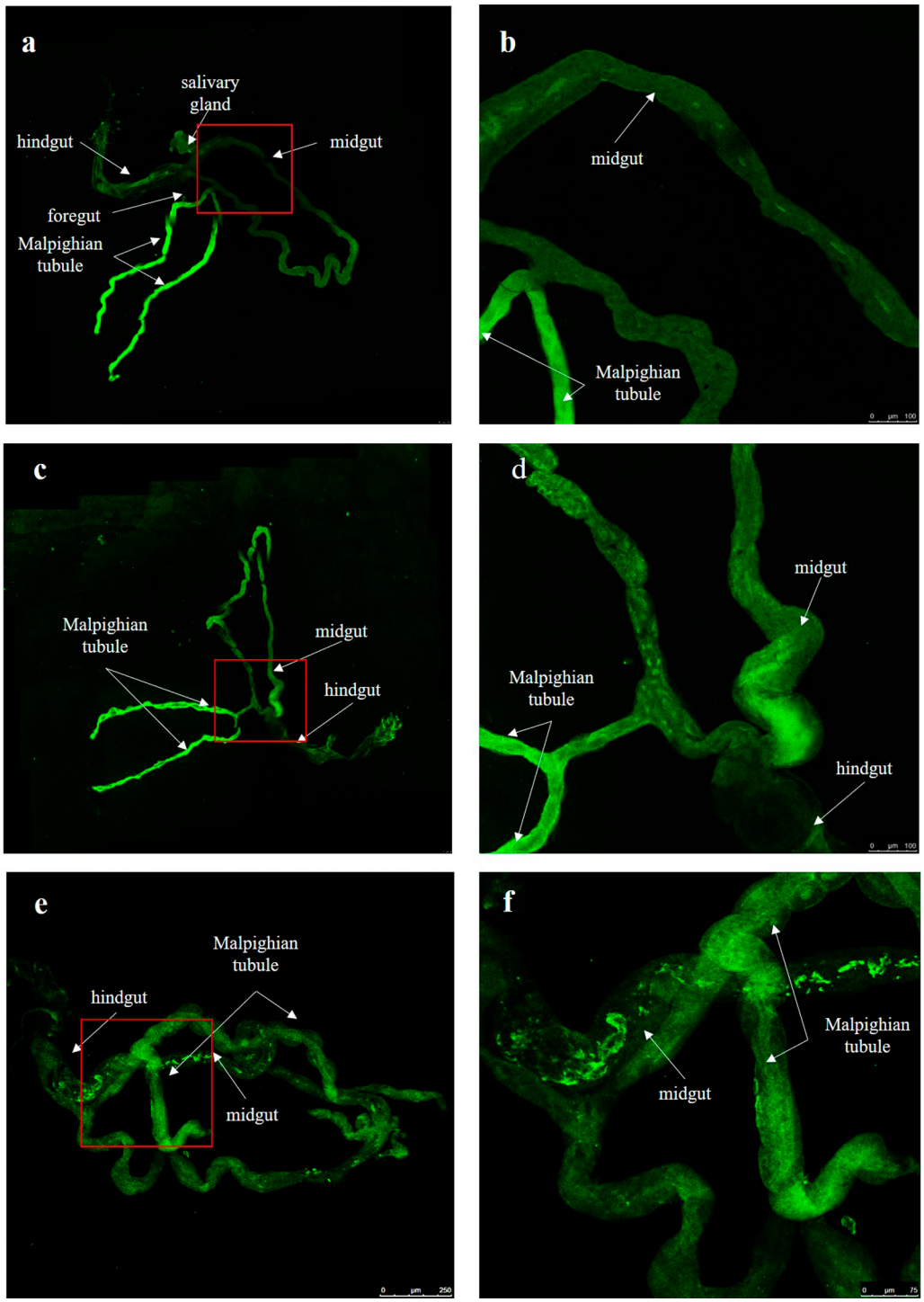

Fig. 3 Localization of APV1 in *P. cryptus* after different acquisition access periods (AAP). **a** and **b** The digestive system was separated from *P. cryptus* after 48 h AAP. **c** and **d** The digestive system was separated from *P. cryptus* after 72 h AAP. **e** and **f** The *P. cryptus* reared long-term on APV1-infected betel palm seedlings. Overviews (left) and partial enlargement (right) of the green fluorescence-marked APV1 virions were photographed under confocal microscopy after immunostaining

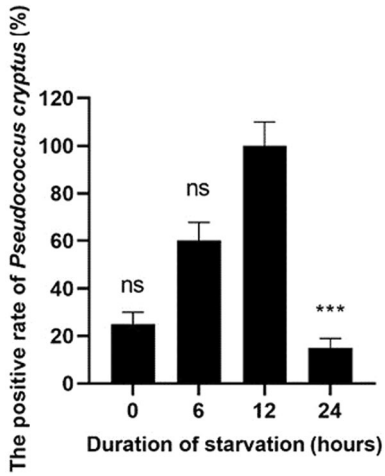

Fig. 4 Effects of starvation affects ingestion of APV1 by *P. cryptus*. After starvation for 6, 12, and 24 h, 3rd instar nymphs of *P. cryptus* were transferred to YLD source plants for 1 h AAP. Ingestion of APV1 was detected by RT-PCR. The significance analysis was performed by Fisher's exact test

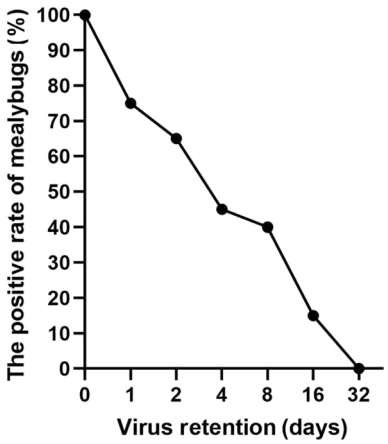

Fig. 5 Retention time of APV1 in *P. cryptus*. The third instar nymphs were transferred to germinating non-host organic potatoes after 72 h AAP of APV1 and removed for RT-PCR detection after 0, 1, 2, 4, 8, 16, and 32 days on the non-hosts, respectively. The experiment was repeated three times and 20 mealybugs were detected each time

calceolariae and *P. longispinus* were non-transmitters (Petersen and Charles 1997). Given that the first-instar nymphs are the most mobile stage of mealybugs, and their dispersal by wind is likely easier than that of other stages under field conditions, it has been proposed that the spread of GLRaV-3 may be largely driven by the first-instar nymphs of mealybugs (Tsai et al. 2008). APV1 was found to be transmitted by both first-instar and third-instar nymphs of *P. cryptus*. However, the transmission efficiency of third-instar nymphs was higher than that of first-instar nymphs, though the exact reasons for this difference require further investigation.

Vector transmission represents a specific event involving complex and specific virus-vector interactions. While ampeloviruses, criniviruses, and closteroviruses are transmitted by mealybugs, whiteflies, and aphids, respectively, the phylogenies and vector types are highly consistent in the classification for these three genera (Karasev 2000; Dolja et al. 2006). However, velariviruses are an exception. Velariviruses and criniviruses have closer genetic distances in phylogenetic analysis (Martelli et al. 2002; Zhang et al. 2022), yet they possess different transmission vectors and distinct genome structures. Both ampeloviruses and velariviruses are transmitted by mealybugs, but they do not show a close genetic relationship in phylogenetic analysis (Jelkmann et al. 2012; Lim et al. 2015; Zhang et al. 2022), suggesting the complexity and specificity of virus-vector interactions in velarivirus transmission.

To date, APV1 is the sole member of velarivirus with known transmission vectors. APV1 is transmitted by at least two species of mealybugs, i.e. *F. virgata* and *P. cryptus*. Field investigations have revealed that *A. spiniferus, I. seychellarum*, and *C. rusci* also ingest APV1 (Additional file 1: Figure S3). Whether they are potential vectors for APV1 transmission still requires further identification. Given that most GLRaVs can be transferred by multiple mealybug species (Tsai et al. 2008; Almeida et al. 2013; Naidu et al. 2015; Wistrom et al. 2016), it is possible that APV1 could be transmitted by additional mealybug species. APV1 exhibits lower vector specificity, and both vector mealybugs have low specificity for natural hosts. This complicates the efforts to control the spread of this devastating disease.

Methods

Plant materials

One-year-old betel palm seedlings were procured from Danzhou City, Hainan Province, China. These seedlings were cultivated under controlled conditions in growth chambers, maintaining a temperature of 28 °C ± 1 °C, relative humidity between 70 and 80%, and a photoperiod of 16 h light to 8 h darkness.

Mealybug collection and rearing

Mealybugs (*P. cryptus*) carrying APV1 were collected from betel palm plantations in Shangen Town, Wanning City, Hainan Province. Mealybugs were reared on pumpkin fruits (*Cucurbita moschata*) within nylon net cages, maintained under room conditions. The first generation hatched to establish a mealybug colony, and the offspring of the third generation were used for experimentation.

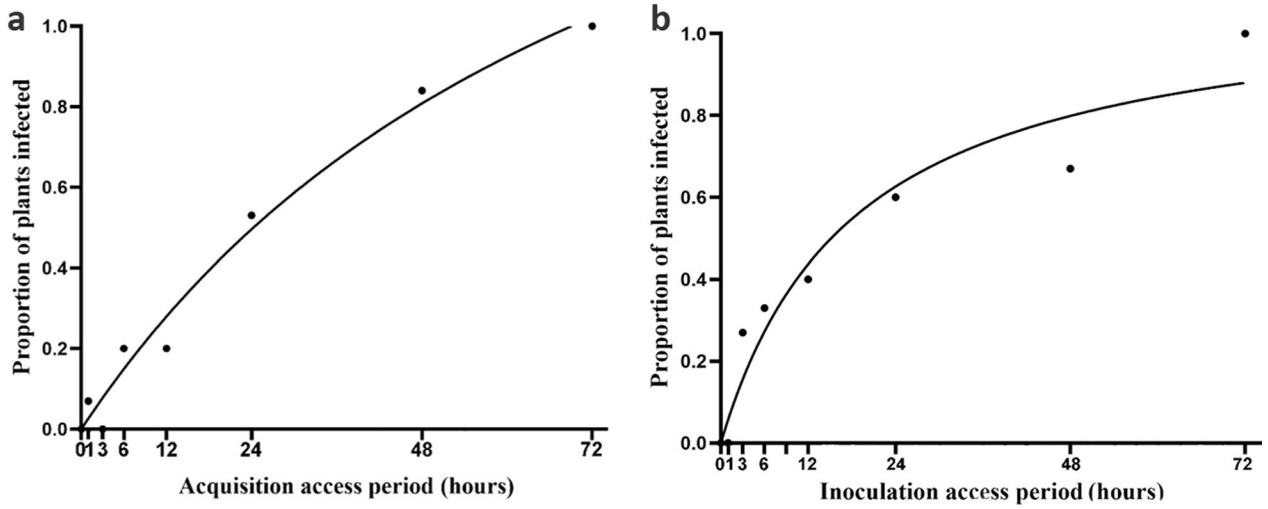

Fig. 6 Effect of acquisition access period (AAP) and inoculation access period (IAP) on transmission efficiency of APV1 by *P. cryptus*. **a** and **b** The transmission rates were determined by RT-PCR following various AAPs and a 72 h IAP or following a 72 h AAP and various IAPs. In both panels, the line indicates the model-predicted probability of transmission, and the points indicated the proportion of positive plants at each time point, and the line indicates the positive rate of plants predicted by the Michaelis–Menten model. Sample size n = 15 for each time point. Each betel palm seedling was inoculated by five 3rd instar *P. cryptus*

APV1 transmission by *P. cryptus*

Fifty 3rd instar nymphs of *P. cryptus* were collected from pumpkin and allowed a 48 h AAP on YLD-infected trees. To confirm the viruliferous status of the mealybugs, RT-PCR was employed to detect APV1. Another fifty *P. cryptus* nymphs reared on pumpkin served as controls. Mealybugs, with or without APV1, were then transferred to healthy areca palm seedlings. After a 72 h IAP, the plants were treated with acetamiprid to eliminate the mealybugs.

Localization of APV1 in *P. cryptus*

P. cryptus adults were dissected under a stereomicroscope, and their entire digestive systems were isolated. Tissues from stylets, foregut, midgut, hindgut, hemolymph, and eggs (20 eggs/tube) were separated and subjected to immunocapture-RT-PCR using APV1-specific primers (Additional file 2: Table S2), following previously described methods (Zhang et al. 2022). Additionally, dissected tissues were treated with a mouse monoclonal antibody against the capsid protein (CP) of APV1 and subsequently labeled with FITC-labeled goat anti-mouse antibody. The entire digestive system was examined using a confocal laser scanning microscope, as described previously (Zhang et al. 2022).

Effects of different life stages of *P. cryptus* on APV1 transmission

To compare the transmission efficiency of *P. cryptus* at different stages, twenty first-instar and twenty third-instar mealybugs were allowed a 72 h AAP on the source seedlings carrying APV1. After a 48 h IAP, the inoculated plants were treated with the insecticide nitenpyram to eliminate the mealybugs. APV1 presence was detected by RT-PCR at 75 days post-inoculation (dpi), and transmission efficiency was analyzed using a χ^2 test between the two life stages.

Effects of starvation on APV1 ingestion by *P. cryptus*

After being starved for 6, 12, and 24 h, 3rd instar nymphs of *P. cryptus* were transferred to YLD source plants for a 1 h AAP. Subsequently, 20 third-instar nymphs were collected from each group to assess the acquisition rate of APV1 using RT-PCR.

Retention time of APV1 in *P. cryptus*

Third instar mealybugs were transferred to germinate non-host organic potatoes in glass jars (11 cm × 11 cm × 15 cm) and cultured in a greenhouse after a 72 h AAP of APV1. Mealybugs were individually removed for APV1 detection by RT-PCR at different time points (0, 1, 2, 4, 8, 16, and 32 d) on the non-host plants. The experiment was repeated twice, with each replicate consisting of ten mealybugs.

Effect of acquisition access period (AAP) and inoculation access period (IAP) on APV1 transmission efficiency

To assess the effect of AAP on APV1 transmission, third instar mealybugs were transferred from pumpkin to source betel palm seedlings carrying APV1 for AAP

determination with durations of 1, 3, 6, 12, 24, 48, or 72 h. Subsequently, the mealybugs were transferred to betel palm test plants for a 72 h IAP. To evaluate the effect of IAP on APV1 transmission efficiency, third instar mealybugs were transferred to test plants for IAP determination with durations of 1, 3, 6, 12, 24, 48, or 72 h after a 72 h AAP, respectively. The test plants were treated with insecticide after inoculation. Leaf samples from uninoculated lower crown leaves were collected for APV1 detection by RT-PCR at 75 dpi. Each betel palm seedling was inoculated with 20 mealybugs, and 18 seedlings were tested for each treatment, with each treatment repeated three times.

Abbreviations

AAP	Acquisition access period
APV1	Areca palm velarivirus 1
dpi	Day(s) post-inoculation
GLRaV	Grapevine leafroll-associated virus
IAP	Inoculation access period
ORF	Open reading frame
YLD	Yellow leaf disease

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-024-00228-x.

Additional file 1: Figure S1. a Auto-fluorescence of gut-tissue of *Pseudococcus cryptus*. Gut-tissue of *P. cryptus* was directly observed under confocal microscopy. **b** Immunofluorescence of *P. cryptus* carrying no APV1. Gut-tissue of *P. cryptus* was treated with mouse monoclonal antibody against APV1 and then with FITC-labeled goat anti-mouse IgG; Locations of stylets, forgut, midgut, hingut, and Malpighian tubule were indicated. **Figure S2.** Transmission efficiency of APV1 by *P. cryptus* after retention on non-host plants. The 3rd instar nymphs of *P. cryptus* carrying APV1 were transferred to potatoes (non-host plant) for 1 d and 2 d retention, then retransferred to healthy betel palm seedlings. RT-PCR was used to detect APV1 at 60 d after inoculation. **Figure S3.** APV1 was detected in insects infesting leaves of betel palm. **a** and **b** Areca palm virus 1 (APV1) was detected by RT-PCR in *I. seychellarum*, **c** and **d** *A. spiniferus* infesting on leaf of betel palm collected in Hainan. APV-infected leaf samples and symptomless samples were served as positive control (+) and negative control (−), respectively. M: DNA marker. B1–B4 and H1–H4 represent different *I. seychellarum* samples, and A1–A8 indicate different *A. spiniferus* samples collected in Hainan.

Additional file 2: Table S1. Effects of age of *P. cryptus* on the transmission of APV1. **Table S2.** Primers used in the work.

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Author contributions
XH and HW conserved and designed the project. XZ, RZ, and XC carried out experiments. XH supervised the work. All authors analyzed and discussed the data. XZ wrote the manuscript. All the authors reviewed and approved the manuscript.

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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors agree with the publication at the present status.

Competing interests
There is no competing interest.

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