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FULL RESEARCH PAPER

Presence of *Grapevine leafroll-associated virus 3* in primary salivary glands of the mealybug vector *Planococcus citri* suggests a circulative transmission mechanism

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Abstract *Grapevine leafroll-associated virus 3* (GLRaV-3) is a mealybug-transmissible ampelovirus. Though the transmission mechanism has been described as semipersistent on the basis of temporal parameters, definitive proof of this mechanism has never been provided. In the present study, we carried out preliminary assays to establish the location of the virus in its vector, *Planococcus citri.* After dissecting the insects, GLRaV-3 was detected by means of IC-RT-PCR in the salivary glands, intestine and Malpighian tubes, but not in the sucking apparatus. Immunogold labelling of the capsid protein revealed the presence of the virus in some cells of the primary salivary glands, but not in the alimentary channel of the stylet, or in the accessory salivary glands. The strong labelling of the electron-dense secretion vesicles in some cells of the primary salivary glands, together with the non-detection of the virus in the sucking apparatus suggests that the transmission mechanism may be different from that previously described. We propose a circulative transmission mechanism based on a specific transportation route for the viral particles from the midgut or hindgut to the salivary glands. As the transmission mechanism is generally a common feature of a viral genus, the existence of a circulative transmission mechanism for other mealybug-transmitted ampeloviruses is expected. Organ by organ analysis of GLRaV-1, another ampelovirus not transmissible by *P. citri*, showed the absence of the virus in the salivary glands, thus providing further, though indirect, evidence in favour of circulative transmission for this virus genus.

Keywords Ampelovirus · Citrus mealybug · GLRaV-3 · *Grapevine leafroll* · Semipersistent transmission

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Introduction

The order Hemiptera includes most insect vectors of plant viruses. Whiteflies (Family Aleyrodidae), leafhoppers (F. Cicadellidae), planthoppers (Superfamily Fulgoroidea that includes eight families), aphids (F. Aphididae), mealybugs (F. Pseudococcidae) and scale insects

(F. Coccidae) are the most important in terms of number of vector species and number of viruses transmitted (Nault, 1997). At least 21 species of mealybugs transmit plant viruses belonging to three genera of viruses: *Badnaviruses*, *Ampeloviruses* and *Vitiviruses* (Lockhart & Olszewski, 1999; Martelli et al., 2002; Adams et al., 2004).

Among grapevine virus diseases, leafroll is the most widespread throughout the world, and the economic importance of one of its associated viruses, *Grapevine leafroll-associated virus 3* (GLRaV-3), has been reported for many cultivars in different countries. GLRaV-3 is a phloem-limited virus which occurs in companion cells and in sieve tube elements of diseased plants, in which it typically induces vesiculations in mitochondria (Faoro, Tornaghi, Cinquanta, & Belli, 1992). The known hosts are all woody plants that are members of the genus *Vitis.* As regards other leafroll associated viruses, the main method of transmission is via grafting of infected material. Several species of mealybugs—*Heliococcus bohemicus*, *Phenacoccus aceris*, *Planococcus citri*, *Planococcus ficus*, *Pseudococcus calceolariae*, *Pseudococcus comstocki*, *Pseudococcus longispinus*, *Pseudococcus maritimus*, *Pseudococcus viburni* (Rosciglione & Gugerli, 1986; Engelbrecht & Kasdorf, 1990; Cabaleiro & Segura, 1997b; Petersen & Charles, 1997; Golino, Sim, Gill, & Rowani, 2002; Nakano, Nakaune, & Komazaki, 2003; Sforza, Boudon-Padieu, & Greif, 2003)—and one scale insect–*Pulvinaria vitis* (Belli et al., 1994)—are vectors of this virus. In all cases, the transmission mechanism remains unclear. Nevertheless, studies carried out to date to characterize transmission of ampeloviruses indicate a semipersistent transmission mechanism, on the basis of loss of infectivity and the acquisition times involved (Cabaleiro & Segura, 1997a; Krüger, Saccaggi, & Douglas, 2006). In spite of the lack of conclusive experimental evidence it was then generally assumed that transmission of GLRaV-3 and other members of the *Ampelovirus* genus is semipersistent (Martelli et al., 2002). Although GLRaV-1 is phylogenetically close to GLRaV-3 (Martelli et al., 2002), it has different vectors. Only the mealybugs *Phenacoccus aceris* and *Heliococcus bohemicus* (Sforza et al., 2003) can transmit both ampeloviruses. Golino et al. (2002) demonstrated that this virus species is not transmitted by *P. citri*.

The most widely used method for determining the presence of virions in vectors is by analysis of whole individuals. However, the increased sensitivity provided by PCR-based techniques has made possible the detection of virus in non-vector insects (Olmos, Bertolini, Gil, & Cambra, 2005). Assays of loss and acquisition of potential infectivity with entire insects are no longer considered reliable. In the present study, we attempted to detect GLRaV-3 and GLRaV-1 in different organs of *P. citri* to elucidate the mechanisms of transmission of leafroll by mealybugs.

Materials and methods

Plants and mealybugs

The plant material used was adult leaves from potted plants of known virus status, clones of plants maintained in the *Escola Politécnica Superior de Lugo*. Depending on the assay, the clones used were: C1 (cv. Albariño infected with GLRaV-3); CF+C1 (cv. Cabernet franc graft-infected with GLRaV-3); and A1, (cv. Albariño infected with GLRaV-1). The plants were maintained in a growth chamber at 25°C with a photoperiod of 16 h light, 8 h darkness. Specimens of *P. citri*, obtained from a vineyard where leafroll transmission has been demonstrated (Cabaleiro & Segura, 1997b), were reared on potato sprouts or pumpkins for several generations at 25°C in darkness.

Virus acquisition

The mealybugs were fed on leaves from clones C1, CF+C1 or A1 maintained in humid chambers at room temperature with the petiole inserted in a tube containing liquid culture medium (MS 1/2, Murashige & Skoog, 1962). In the first assay the mealybugs were maintained on leaves of CF+C1 or C1 for 10 or more days. In the second assay, immature adult mealybugs were maintained on clone CF+C1 or C1 for 7 days and only mealybugs that were still feeding after seven days were selected for analysis. For GLRaV-1, immature adult mealybugs were allowed to feed on clone

A1 for 7 days. The negative control consisted of mealybugs feeding on potato sprouts. In the immunogold labelling assays to determine virus location, the immature adult mealybugs were allowed to feed for five days on the leaves of CF+C1 plants.

Mealybug dissection and organ analysis

Mealybugs with the stylet clearly inserted were carefully removed from the leaves where they were feeding. After waiting until the stylet was retracted, the insects were fixed and maintained in FAE (ethanol 50°, formaldehyde 35%, acetic acid, 18:1:1) until dissection. Immediately before dissection, the mealybugs were dried and fixed onto slides with nail varnish. The mealybugs were then dissected, under a stereo microscope, in a drop of distilled water, with two entomological needles. The following parts were separated: intestine (midgut and hindgut), Malpighian tubes, mouth apparatus (comprising labium, stylet, and the alimentary channel from the stylet to the end of the oesophagus), reproductive system, suboesophageal ganglion, salivary glands and bacteriome. The body remains of each insect dissected were kept for later analysis. Analysis for the presence of the virus was performed by IC-CT-PCR. Microtubes for PCR (0.2 ml) were coated with 50 µl of commercial antibodies specific for GLRaV-3 or GLRaV-1 (Bioreba AC, Switzerland) diluted at 1:500 in coating buffer for 4 h at 37°C or overnight at 4°C. The tubes were washed three times for 3 min, and incubated with 50 µl of organ extract for 4 h at 37°C or overnight at 4°C. The extracts were obtained by grinding each organ with 75 µl of commercial grapevine extraction buffer (Bioreba, AG). The tubes were washed in the same way and kept for 15 min at 65°C to release the nucleic acids (Moury, Cardin, Onesto, Candresse, & Poupet, 2000). The reagents for one-step RT-PCR (cMaster^{TM} RTplusPCR System, Eppendorf) were then added, using the primers proposed by Minafra & Hadidi (1994) for GLRaV-3 (C547 and H229), or those proposed by Good & Monis (2001) for GLRaV-1(GSP3 and GSP4). The established cycles, performed in a Biorad iCycler, were: for GLRaV-1, one cycle at 50°C for 30 min and 94°C for 2 min, 40 cycles at 94°C for 30 s, 60°C for 30 s and 68°C for 20 s, and one final extension cycle at 68°C for 10 min; for GLRaV-3, one cycle at 50°C for 30 min and 94°C for 2 min, 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, and one final extension cycle at 72°C for 10 min. Aliquots of the PCR products were stained with SYBR Green and analysed by electrophoresis in 1% agarose gels.

Transmission electron microscopy (TEM) and immunogold labelling

Immature adult mealybugs were carefully removed from leaves of CF+C1 while they had the stylet still inserted. After waiting for the stylet to be retracted, the insects were washed with commercial dishwashing liquid (Mistol, Henkel Iberica) at 50% in distilled water and then rinsed in distilled water and dried. While keeping the mealybugs in a drop of fixative (paraformaldehyde 4%, glutaraldehyde 0.3% in phosphate buffer 0.1M, pH 7.4) and, with the aid of a stereo microscope, some cuts were made on the lateral part of the body to favour penetration of the fixative. The mealybugs were then kept in the same fixative for 2 h at 4°C and later dehydrated by immersion in a series of ethanol solutions (25, 50, 75, 90 and 100%). Finally the insects were embedded in London Resin White (LRW), as previously described (Faoro, Tornaghi, & Belli, 1991). Ultra-thin sections were labelled with a primary polyclonal serum anti-GLRaV-3 (1:100 and 1:1000) or a pre-immune serum as the negative control, and post-labelled with a goat anti-rabbit colloidal gold conjugate of 20 nm (BioCell, UK), as detailed in Faoro et al. (1991). Sections were then stained with uranyl acetate and lead citrate and examined with a Jeol 100SX (Japan) transmission electron microscope.

Results

GLRaV-3 and GLRaV-1 detection in *Planococcus citri* organs

After a minimum of 10 days of AAT (acquisition access time), GLRaV-3 was detected, in the first assay, in all salivary glands analysed and also in all

the midguts, hindguts and Malpighian tubes tested all together. The virus was also detected in the bacteriome (3/3), in the reproductive apparatus (2/3), in the suboesophageal ganglion (2/5), in the mouth apparatus (1/5) and always in the body remains of the mealybugs (4/4). In the second assay, GLRaV-3 was always detected in the midgut and hindgut (5/5), in all but one case in the salivary glands and in the Malpighian tubes (4/5) and, in 2 out of 5 cases, in the body remains. The virus was not detected in the ganglion, sucking apparatus, reproductive system or bacteriome. All organs originating from the three mealybugs feeding on potato sprouts were negative for GLRaV-3. Analysis results of the organs from mealybugs allowed to feed on leaves infected with GLRaV-1, were very different from those that had fed on GLRaV-3 infected ones. In fact, GLRaV-1 was detected only in the midgut and hindgut and in the Malpighian tubes and never in the salivary glands of all the examined insects. In 2 out of 5 mealybugs the virus was detected in the reproductive system and in one mealybug in the body parts remaining after dissection. GLRaV-1 was never detected in any organ of the three mealybugs feeding on potato sprouts.

Localization of GLRaV-3 in mealybugs by immunogold labelling and TEM

After applying antibody against GLRaV-3, diluted 1:100, intense specific gold labelling was observed only in part of the primary salivary gland cells, but not in the accessory glands. No labelling was detected in the nearby muscle fibres or in the haemocoel. The negative control, incubated in pre-immune serum, showed only a light background of unspecific labelling, uniformly distributed over all of the different organs (Fig. 1a), including both primary and accessory salivary glands. No labelling was detected in the alimentary channel of the stylet, although the

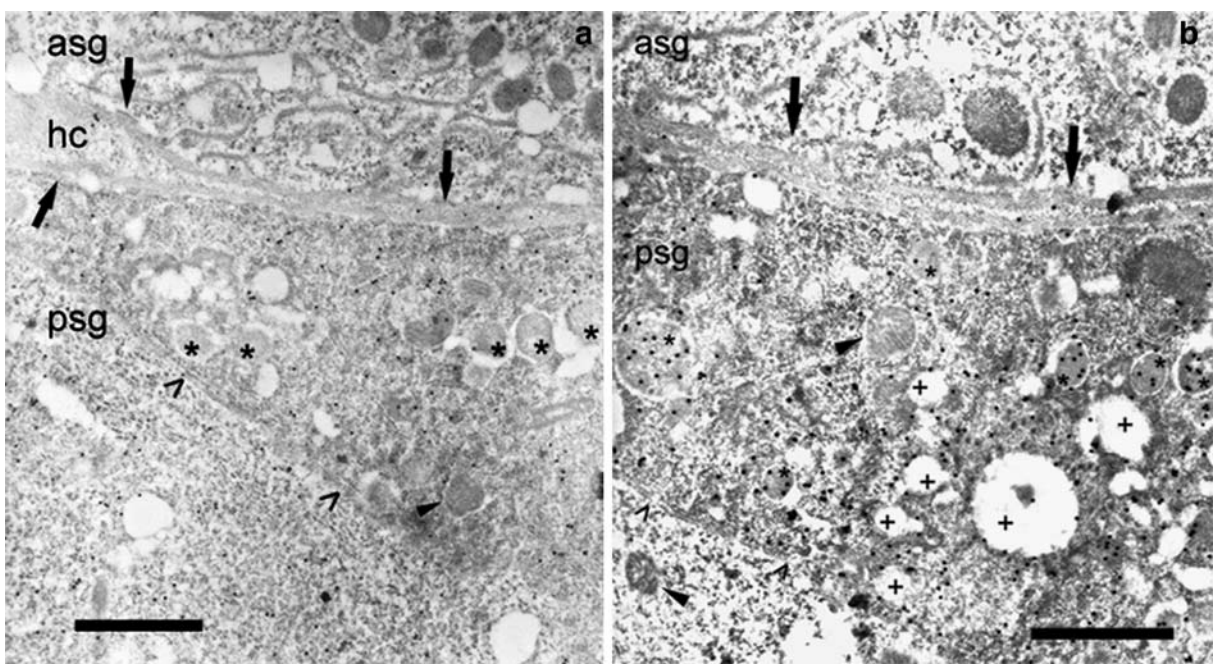

Fig. 1 Immunogold labelling of the coat protein of GLRaV-3 in *Planococcus citri*. (**a**) Negative control with preimmune serum of the accessory (asg) and primary (psg) salivary glands. The labelling is light and unspecific (bar = 1 μm); (**b**)Accessory and primary salivary glands. The accessory gland, unlabelled, is separated by the two basal lamina (arrow) from the primary gland. In the primary salivary gland a labelled cell (at the centrum) and a clearly unlabelled cell (at the bottom separated by the cytoplasmic membrane (>) are recognizable. The central cell has strongly labelled electrondense vesicles (*), electrolucent vesicles (+) few labelled and non-vesiculated mitochondria (arrowhead) (bar = 1 μm); hc = hemocoel

stylet walls and the mealybug cuticle showed labelling, possibly due to the affinity of chitin fibrils for antibodies, as observed in many other cases (F. Faoro, Istituto di Virologia Vegetale, CNR, Italy, pers. comm.). The accessory salivary glands (Fig. 1b) showed numerous large electron-dense secretion vesicles inside the cytoplasm. These corresponded to the description by Ponsen (1972) for *Myzus persicae* accessory glands. No labelling was observed in these glands when the antibody against GLRaV-3 was used.

In the primary salivary glands two cell types were observed: one with electron-dense cytoplasm and another with electron-transparent cytoplasm (Fig. 1b). The former showed large nuclei, and numerous electron-dense vesicles possibly containing proteinaceous material. These vesicles were heavily labelled by the anti-GLRaV-3 serum, although some labelling was also present in the cytoplasm but not in the nucleous or in the mitochondria. Another type of vesicle, usually larger than the above and almost completely electron transparent, was unlabelled (Fig. 1b). The cells with electron transparent cytoplasm contained both types of the above vesicles. However neither these vesicles or the surrounding cytoplasm were labelled (Fig. 1b).

We did not observe the typical viral bundles, or the mitochondrial vesiculations present in infected grapevine plants and where virus replication takes place (Faoro & Carzaniga, 1995).

Discussion

In the case of semipersistent transmission, the virus would specifically bind to the stylet alimentary channel or the foregut epicuticle (Ammar, Järlfors, & Pirone, 1994). However, we did not detect the virus, by IC-RT-PCR, in samples formed by the stylet and foregut and did not find any immunogold labelling in the stylet alimentary channel. Taken all together, these results provide evidence refuting the hypothesis of semipersistent transmission.

Detection of virus in the different organs may be explained in different ways. In the suboesophageal ganglion, separated by the perineural space, the presence of virus particles is explained only by the existence of an active entry mechanism or by contamination during the dissection process, which appears to be the most likely explanation. Detection of virus inside the bacteriome, the sucking apparatus or the body remains, can be attributed to the presence of virus from the haemolymph, as these organs are entirely suffused in the haemolymph, or to the contamination during the dissection process. Finally, the presence of virions in the reproductive system could be explained by the permanence of the haemolymph between the follicles, or by contamination with part of the hindgut during dissection, or because both organs share a common outlet.

Detection of virus by IC-RT-PCR in the salivary glands implies the presence the whole virion, as the technique allows the detection of protein-bound RNA. Transportation of the virion from the gut (where it is also found) to the salivary glands via the haemolymph should have then taken place. This type of transport in other hemiptera is possible because of the protection against the attack of the haemolymph by chaperonin GroEL homologues, synthesized by insect endosymbionts (Van Der Heuvel et al., 1997; Morin et al., 1999). Binding between virus and GroEL homologues occurs in many plant viruses (mainly luteoviruses and begomoviruses) and GroEL synthesized both by endosymbionts of vector and non-vector insects as well as by *Escherichia coli* (Van Der Heuvel et al., 1997; Akad, Dotan, & Czosnek, 2004). Like other filamentous plant viruses, GLRaV-3 does not bind the GroEL homologue of *E. coli* (Akad et al., 2004). The assumed lack of protection of GroEL synthesized by the ß-endosymbionts (Baumann, Thao, Hess, Johnson, & Baumann, 2002) means that the virus cannot escape destruction in the mealybug haemolymph. Thus, the amount of virus in the haemolymph will decline rapidly.

The route from the gut to the salivary glands includes three barriers that ensure the specificity of the transportation. The first is the gut epithelium and the second and third are the basal lamina and the plasmalemma of the salivary glands. (Peiffer, Gildow, & Gray, 1997). The likelihood of the IC-RT-PCR detected virions being absorbed in the basal lamina of the salivary

glands without penetrating can be discounted for two main reasons: (i) the coat protein is located specifically inside one part of the primary salivary gland cells; (ii) intense labelling of the basal layer of the salivary glands was never detected. Rather, the strong labelling in the salivary gland, in comparison with other insect organs, suggests specific transportation of virus to the gland. Furthermore, the specific presence of the virus in a certain part of the salivary glands indicates that these organs function differently, depending on the compartment considered. When present, labelling in a gland cell was quite uniformly distributed inside the cytoplasm and in the electrodense vesicles. However, the highest intensity of labelling was observed in the electrodense vesicles that form the saliva. Thus, the virions would be ready to be secreted and injected inside the plant during feeding.

In contrast, GLRaV-1 was not detected by IC-RT-PCR in the salivary glands or in other organs but only in the intestine and inside the Malpighian tubes, which are directly connected to the intestine. Detection of the virus in the reproductive system and body remains is probably due to contamination during dissection because of the rupture of the hindgut, which is extremely fragile. These results are consistent with those obtained in previous acquisition assays (Cabaleiro, unpublished data) and with unsuccessful transmission assays (Golino et al., 2002) of GLRaV-1 by *P. citri*. It therefore appears that a specific recognition and translocation mechanism, such as that described for GLRaV-3, does not exist for GLRaV-1, at least in *P. citri*. Considered all together, the present results indicate that transmission of GLRaV-3 by *P. citri* is circulative, although further studies are required to prove this.

The temporal parameters used to distinguish semipersistent and circulative transmissions are the existence or otherwise of a latency period and the duration of the infectivity retention period. However, the latency period is impossible to measure if the exact moment when feeding starts in the phloem is not established. Previous analysis of the feeding behaviour of *P. citri* on plants of cv. Cabernet franc, with the Electrical Penetration Graph (EPG) technique, indicated that feeding in the phloem started after more than 15 h (unpublished data), although it is not unusual for mealybugs to spend many hours with the stylet inserted into the xylem. Thus, if it is not known exactly when suction from the phloem starts, it is impossible to determine the time between acquisition and inoculation of the virus. The study of infectivity retention is also impaired by the alimentary behaviour of the mealybugs. It is difficult to determine how long mealybugs have fed without acquiring the virus at the time they are removed from the infected leaves. In previous studies, one involving *P. citri* feeding for 3–4 days on healthy leaves (Cid et al., 2006) and another involving *Planococcus ficus* feeding for 8 days on a non-host (Krüger et al., 2006), the virus was not detected by RT-PCR in whole mealybugs. These retention periods are shorter than those described for luteovirus circulative transmission by aphids (Gray & Gildow, 2003). This discrepancy may be due to the inability of GLRaV-3 to bind to GroEL, as the retention of non-binding mutants of luteovirus is shorter than wild-type and binding mutant virus, and 120 h post-injection the total amount of virus present was still 10% and 67% respectively (Van Der Heuvel et al., 1997). Thus the short retention period, evidence of non-circulative transmission, would correspond with a circulative non-GroEL-binding transmission.

Finally, virus bundles or replication sites were not detected in the mealybugs, and therefore it is likely that virus does not replicate in the insects (non-propagative transmission). This is consistent with the retention time because after 96 h feeding on healthy leaves the virus is not detected by IC-RT-PCR inside mealybugs (Cid et al., 2006). Since the transmission mechanism is usually a genera-distinctive characteristic (Nault, 1997), the transmission of other ampeloviruses may be similar to that proposed here for GLRaV-3.

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