Tropism, compartmentalization and retention of banana bunchy top virus (Nanoviridae) in the aphid vector Pentalonia nigronervosa

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Plant viruses of the families Luteoviridae and Geminiviridae rely on hemipteran vectors for the infection of their hosts. Several lines of evidence have revealed that these viruses are transmitted by competent vectors in a circulative manner, involving entry into the vector’s body and the crossing of epithelial tissues forming the alimentary tract and the salivary glands. Similar to luteovirids and geminiviruses, a third family of plant viruses, the family Nanoviridae, have also been reported to be transmitted by aphids in a circulative manner. However, there is limited direct evidence of a possible path of translocation through the aphid vectors. Here, we used time-course experiments and transmission assays coupled with real-time PCR and immunofluorescence assays on dissected tissues to examine the translocation, compartmentalization and retention of banana bunchy top virus (BBTV) into the aphid vector Pentalonia nigronervosa. Our results indicate that BBTV translocates rapidly through the aphid vector; it is internalized into the anterior midgut in which it accumulates and is retained at concentrations higher than either the haemolymph or the principal salivary glands. Despite the large increase in viral concentration, we have failed to detect BBTV transcripts with RT-PCR. When tissues were not permeabilized, BBTV localized as distinct puncta in the proximity of the basal surface of the cells forming the anterior midgut and principal salivary glands, suggesting an on-going process of virion escape and internalization, respectively. Interestingly, we document that those organs can have direct contact within the aphid body, suggesting a possible haemolymph-independent translocation path.

INTRODUCTION

Plant viruses of the families Luteoviridae, Geminiviridae and Nanoviridae comprise viruses transmitted by hemipteran insects in a persistent circulative manner (Hogenhout et al., 2008). During the process of transmission, circulative viruses are ingested with the sap of the infected plants, are internalized and cross the insect’s gut cells, from which they are translocated into the vector’s haemocoele. After internalization within the salivary glands, the virions can then be discharged into plant tissues along with the saliva produced during the feeding process.

The process of the internalization and retention of circulative viruses varies among different vector–virus combinations (Hogenhout et al., 2008). For instance, aphid-transmitted luteovirids are internalized through the hindgut and, in some cases, the posterior midgut (Gildow, 1993; Gray & Gildow, 2003; Reinbold et al., 2003) and specifically penetrate the accessory salivary glands (Gildow & Rochow, 1980). The process of virion penetration of the epithelial cells of the gut and salivary glands is known as transcytosis and is initiated through the recognition of specific receptors, i.e. cell surface proteins, through which virions can initiate the process of membrane binding and internalization (Tamborinde-guy et al., 2010). Several lines of evidence indicate that luteovirids are internalized through clathrin-mediated endocytosis (Brault et al., 2007; Gildow, 1982, 1993). The virions are transported into cell compartments through a specific endocytic pathway (Brault et al., 2007; Gildow, 1982, 1993; Gray & Gildow, 2003). The process of viral transport through different epithelial tissues appears to be highly specific; as a consequence, many luteovirids display a high level of vector specificity (Gray & Gildow, 2003).

The internalization of begomoviruses (family Geminiviridae), such as the tomato yellow leaf curl virus (TYLCV), occurs through the anterior midgut (AMG) and filter chamber of the whitefly vector Bemisia tabaci (Ghanim & Medina, 2007; Medina et al., 2006). Viral particles appear to be transmitted to plants after penetration of the principal salivary glands (PSGs) (Ghanim & Medina, 2007; Ghanim et al., 2001; Medina et al., 2006). A similar pattern of translocation has been described for another geminivirus, maize streak virus.
(MSV, genus Mastrevirus) in the leafhopper vector Cicadulina mbila (Ammar et al., 2009; Lett et al., 2002). However, there is no evidence of the possible cellular mechanism involved in the transcytosis of geminiviruses through the vector’s epithelial membranes.

Circulative viruses do not replicate in their vectors (see the exception of TYLCV; Czosnek et al., 2001; Sinisterra et al., 2005); however, they show a persistent pattern of transmission that generally lasts for several days to weeks (Czosnek et al., 2002; Reynaud & Peterschmitt, 1992), indicating that the virions are retained in the vector’s body while seemingly encountering little or no degradation. For example, MSV and other whitely transmitted begomoviruses appear to reach high concentration levels in the vector’s AMGs (Ammar et al., 2009; Czosnek et al., 2002). Although luteovirids, such as potato leafroll virus (PLRV, genus Polerovirus), are rapidly translocated through the epithelial cells of the posterior midgut, they also appear to accumulate within it (Garret et al., 1996). Haemolymph appears to be an important reservoir of virions because both luteovirids and geminiviruses can be detected at significant amounts (Garret et al., 1993; Hunter et al., 1998; Liu et al., 2006; Rosell et al., 1999). Many studies have reported an in vitro interaction of endosymbiotic bacteria-derived GroEL with circulative viruses. GroEL–virus interactions may prevent the proteolytic degradation of both luteovirids and geminiviruses in the haemolymph of their respective vectors (Filichkin et al., 1997; Gottlieb et al., 2010; Hogenhout et al., 2000; Morin et al., 1999; van den Heuvel et al., 1994). However, recent findings based on the localization of GroEL in the pea aphid Acyrthosiphon pisum Harris and the three-dimensional reconstruction of the GroEL polyprotein questions the possible protective function in vivo (Bouvaine et al., 2011).

Viruses of the family Nanoviridae differ substantially from both ssDNA viruses of the family Geminiviridae and ssRNA viruses of the family Luteoviridae with respect to particle morphology, genomic organization and mode of transcription. Nanovirids are non-enveloped small icosahedral particles that are 17–20 nm in diameter and contain circular, generally monocistronic, ssDNA molecules that are approximately 1 kb (Vetten et al., 2011). Nanovirids have a multi-component genome that, depending on the viral species and strain, consists of 6–11 DNA components separately encapsidated by a unique coat protein (Vetten et al., 2011). The family Nanoviridae currently includes at least eight formally recognized viral species clustered within two separate genera: Nanovirus and Babuvirus (Vetten et al., 2005). Faba bean necrotic yellows virus (FBNYV) and banana bunchy top virus (BBTV) are the type species of the genera Nanovirus and Babuvirus, respectively. Whereas FBNYV is transmitted by aphids that colonize legume crops, such as the cowpea aphid Aphis craccivora Koch, the bean aphid Aphis fabae Scopoli and the pea aphid (Katul et al., 1993), BBTV is specifically transmitted by the banana aphid Pentatonia nigronervosa (Hu et al., 1996; Magee, 1940; Wu & Su, 1990).

Information on the translocation path of nanovirids within the aphid vectors are limited and are mostly derived from indirect evidence from transmission assays rather than the direct visualization of virions (Anhalt & Almeida, 2008; Franz et al., 1998; Hu et al., 1996). Recently, we have developed an immunofluorescence assay to determine the localization of BBTV within its aphid vector. We found that BBTV antigens specifically localized to the AMG and specific cells forming the PSGs (Bressan & Watanabe, 2011). In this study, we used time-course experiments to examine the localization and concentration of BBTV in aphids that fed only on infected plants (uptake assay) or aphids that were allowed access to healthy plant tissues after having acquired virions (retention assay). Aphids were individually dissected, and the guts, haemolymph and salivary glands were separately processed for immunofluorescence and real-time PCR assays. Transmission assays were performed to examine the extent to which BBTV was aphid-transmissible. We conducted additional immunofluorescence assays to specifically localize BBTV antigens on the outer surface of guts and salivary glands. Based on the results obtained and on the internal distribution of tissues carrying BBTV antigens, we propose possible translocation, compartmentalization and retention of BBTV through the aphid vector.

**RESULTS AND DISCUSSION**

**Uptake and internalization**

The titre of BBTV-S, which contains an ORF coding for the coat protein (Vetten et al., 2011), was first quantified in aphid tissues by using real-time PCR assays. Fig. 1(a) represents the mean concentration of BBTV-S relative to the aphid’s actin gene, in the gut, salivary glands and haemolymph of aphids that fed from infected banana plants for 1, 2, 3, 4, 7 and 16 days (virus uptake assay). The mean titre of BBTV was increased 600-, 2750- and 3500-fold in the gut tissues relative to the banana aphid actin gene after 3, 7 and 16 days, respectively (Fig. 1a). An increase in BBTV concentrations was also observed in the haemolymph and salivary glands, but to a much lower extent than the gut (Fig. 1a). For instance, the relative concentration of BBTV in the haemolymph increased 0.5-, 17- and 140-fold after 3, 7 and 16 days, respectively, while in the salivary glands it increased 1-, 17- and 65-fold in the same time intervals (Fig. 1a).

Because of the rapid increase in the concentration of BBTV in the gut tissues, we examined the presence of RNA transcripts in viruliferous aphids. Although it was possible to detect BBTV-R viral transcripts in infected plants through RT-PCR, we did not find detectable levels of BBTV-R transcripts in the RNA isolated from aphids (Fig. S1, available in JGV Online). This result confirms previous assays conducted by Burns et al. (1995), who did not observe detectable levels of BBTV transcripts in aphids using Northern blotting assays. Therefore, the increase in
viral DNA observed over time in the aphid (Fig. 1a) is probably the result of the progressive uptake and internalization of virions from the infected plants. We selected a sampling timescale for viral uptake assay that roughly reflected the time required for the aphids to develop from newly born first instar nymphs into adults; therefore, the relative viral load detected may represent an estimation of the level aphids may ingest and internalize during their development in nature.

In parallel with real-time PCR, we performed immunofluorescence assays to determine the localization of BBTV antigens. Confirming the results from a previous study (Bressan & Watanabe, 2011), we obtained evidence of labelling in the AMG, but we failed to identify BBTV antigens in the foregut, posterior midgut or hindgut tissues (data not shown). Fig. 2(a) shows the change in labelling intensity over time in acetone-fixed anterior midguts incubated with polyclonal antibodies, which were detected with a biotin–streptavidin enhancement procedure. Virus labelling was observed in the AMG as early as 1 day after aphid feeding on infected plants; the labelling intensity increased along with the time that aphids fed on infected plants (Fig. 2a). There was no evidence of labelling in guts dissected from healthy aphids, which were used as controls (Fig. 2a). The kinetics of virus labelling in the AMG are in agreement with real-time PCR assays, revealing a large increase in BBTV-S DNA.

Confirming previous research (Bressan & Watanabe, 2011), we did not observe the labelling of BBTV in the accessory salivary glands (data not shown). However, a detectable level of labelling was observed in the PSGs (Fig. 3a), in which distinct puncta were observed. Labelling in the PSGs became apparent after 2 days of aphid feeding on infected plants, and we observed stronger labelling as time progressed (Fig. 3a). However, the degree of labelling varied between individual aphids, and a large proportion of salivary glands did not show obvious labelling patterns (Table 1). The differences in the labelling intensity of guts and salivary glands are in agreement with real-time PCR results (Fig. 1a).

We performed transmission experiments to examine whether the virions internalized into the aphid haemocoel were transmissible. The transmission experiments used the same sampling intervals as immunofluorescence and real-time PCR assays. Thus, aphids were collected after 1, 2, 3, 4, 7 and 16 days of feeding on BBTV-infected plants and were transferred to healthy banana leaf disks for an inoculation access period (IAP) of 2 days. The mean transmission rate was 0.55 after 1 day of feeding on infected plants (Fig. 4a), demonstrating that BBTV rapidly translocates through the epithelial membranes, and the relatively small amount of BBTV detected with real-time PCR after 1 day (Fig. 1a) is, in many instances, large enough to sustain inoculation. The probability of BBTV transmission increased with the time the aphids fed on the infected plants, reaching very high transmission rates (Fig. 4a).

Retention

Retention experiments were conducted by transferring viruliferous aphids to healthy banana plants, which were replaced every 4 days. To test if aphids may have ingested additional viral particles as a consequence of the plant infection, in a separate experiment we allowed healthy aphids to co-feed with BBTV-viruliferous aphids on a healthy banana plant. After a 4 day co-feeding on the same petioles, we determined the relative concentration of BBTV on aphids by using real-time PCR. As shown by the comparison with healthy (unexposed) aphids, there was no evidence that the healthy aphids exposed to co-feeding may have ingested viral particles (Fig. S2). Fig. 1(b) represents...
the mean concentration of BBTV-S in the guts, salivary glands and haemolymph of dissected aphids that developed as first instar nymphs on infected plants for 12 days and were subsequently transferred to healthy plants, from which they were sampled as adults after 2, 4, 7, 12 and 16 days. Aphids were also tested before being transferred to healthy plants, designated day 0 in Figs 1(b) and 2(b). BBTV was retained at a high concentration in the guts for at least 7 days. After 12 and 16 days of aphid feeding on healthy plants, the titre in the gut tissues decreased significantly ($P < 0.001$; Mann–Whitney test) (Fig. 1b).

The decrease in the mean viral concentration in the gut tissues was not accompanied with a significant increase in levels of BBTV DNA in the haemocoel (haemolymph and salivary glands) (Fig. 1b), as observed for MSV in the leafhopper vector *C. mbila* (Lett *et al.*, 2002).

Fig. 2(b) shows the typical pattern of labelling in acetone-fixed AMGs using polyclonal antibodies, which were detected with a biotin–streptavidin enhancement procedure. The kinetics of viral labelling in the AMGs were roughly in agreement with real-time PCR assays, revealing a decrease in the concentration of virions (Fig. 1b). BBTV antigens were detected as distinct puncta in the PSGs for at least 12 days of aphid feeding on healthy plants (Fig. 3b).

We also examined BBTV transmission by aphids that developed from first instar nymphs on infected banana plants for 12 days and were subsequently transferred to healthy plants. As for the immunofluorescence and real-time PCR assays, aphids were sampled at 0, 2, 4, 7 and 12 days from the healthy plants and were subsequently transferred to banana leaf disks for an IAP of 2 days. Aphids retained the ability to transmit the virus for up to

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**Fig. 2.** Immunofluorescence localization of BBTV in acetone-fixed anterior midguts of the banana aphid, *P. nigrornervosa* during the phase of uptake (a) and retention (b) (details on the uptake and retention assays are reported in the legend to Fig. 1). BBTV antigens were labelled with rabbit polyclonal antibodies, followed by biotin–streptavidin amplification and detection with Alexa Fluor 488 (green). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Bars, 100 μm.
16 days; however, the probability of BBTV transmission decreased over time (Fig. 4b).

**BBTV localization on the basal surface of guts and salivary glands**

The results obtained through real-time PCR and immunofluorescence assays strongly suggested that BBTV virions were internalized into the haemocoel through the AMG. To obtain more evidence to confirm the translocation of BBTV from the anterior midgut to the haemocoel, we performed immunofluorescence assays on paraformaldehyde-fixed guts either treated or not with Triton X-100 and incubated with BBTV mAbs. Triton X-100 is a non-ionic surfactant that permeabilizes the cell membranes, thus allowing antibodies to access the cell cytosol. By omitting Triton X-100, we prevented antibodies from penetrating the cell membranes and therefore selectively visualized antigens at or near the basal surface of the tissues, probably corresponding to the basal lamina and basal plasmalemma (Gildow, 1993). Compared with acetone (Fig. 2), paraformaldehyde allowed a much better preservation of the dissected organs and cell structures (Fig. 5). When Triton X-100 was applied, BBTV was labelled in the cell cytoplasm surrounding the cell nuclei of the AMG (Fig. 5a). However, when Triton X-100 was omitted, labelling was observed as distinct puncta on the basal surface of the AMGs (Fig. 5b; Table 2), suggesting that some virions crossed the epithelial cells. We performed similar immunofluorescence assays on the salivary glands. When Triton X-100 was applied, we observed labelling in the portion of the salivary glands corresponding to the main cells (Fig. 5a) (Bressan & Watanabe, 2011). There was no evidence of labelling within the cover cells. When Triton X-100 treatment was omitted, labelling was observed proximal to the basal surface of the principal cells, suggesting the selective penetration of the virions through these cells. Labelling was visible as puncta, similar to that observed in the gut tissues (Fig. 5b).

**Translocation path**

With the exception of our previous study (Bressan & Watanabe, 2011), data on BBTV–aphid interactions have been derived from transmission experiments only. BBTV is
transmitted for at least 15–20 days post-acquisition, and there is a detectable latent period estimated at 20–28 h (Anhalt & Almeida, 2008; Hu et al., 1996; Magee, 1940). Similar to other plant viruses transmitted in a circulative persistent manner, the presence of a latent period in the vector may reflect the need for virions to cross the cellular membranes of their vectors (Gray & Gildow, 2003).

As suggested by previous transmission experiments (Anhalt & Almeida, 2008; Magee, 1940) and results from the time-course and transmission assays reported in this study, BBTV is rapidly translocated into the aphid haemocoel. In fact, we obtained high transmission rates after only 1 day of acquisition access period followed by a 2 day IAP (Fig. 4a). In addition to rapid translocation, BBTV undergoes a process of accumulation within the aphid body, which appears to occur mostly in the AMG (Figs 1, 2 and 5). These results are similar to what has been observed for PLRV virions, which rapidly translocate through the posterior midgut of the aphid vector Myzus persicae, but also accumulate within it (Garrett et al., 1996).

The consistent detection of BBTV DNA in the haemolymph is of interest because it suggests that virions can be transported to the salivary glands through the haemolymph. Evidence suggests that both geminiviruses and luteovirids, such as TYLCV and PLRV, interact with GroEL produced by inherited endosymbiotic bacteria harboured within the vectors (Filichkin et al., 1997; Gottlieb et al., 2010; Hogenhout et al., 2000; Morin et al., 1999; van den Heuvel et al., 1994). We have recently detected the presence of the primary aphid endosymbiont Buchnera aphidicola within the banana aphid, and we have obtained the sequence of a gene coding for a putative GroEL protein. In addition, through a Western blotting assay, we detected a Buchnera GroEL homologue in the banana aphid haemolymph (unpublished). These results suggest that BBTV and Buchnera-derived GroEL co-occur in the banana aphid’s haemolymph and, therefore, may potentially interact.

Because BBTV DNA was first detected in the AMG, in which it reached concentrations greater than the haemolymph and salivary glands (Figs 1a and 2a), it is reasonable to suggest that the guts are the organs through which viral particles are first internalized. Conversely, the kinetics of BBTV DNA in the haemolymph and salivary glands follow similar temporal trends over time (Fig. 1a). The lack of temporal gaps in the detection of BBTV DNA in the haemolymph and salivary glands may be explained by the rapid translocation of the virions through the aphid haemocoel. However, these data may also suggest that virions are translocated to the salivary glands directly from the AMG. It is interesting to highlight that (i) serial transverse sections obtained from a whole banana aphid showed that the main cells forming the PSGs (but not the accessory salivary glands) can be in direct contact with the AMG (Fig. S3) and that (ii) BBTV antigens were labelled on or near the surface of both the AMG and the main cells forming the PSGs, suggesting an on-going process of virion

### Table 1. Detection of BBTV within the banana aphid, *P. nigronervosa* in time-course experiments by using immunofluorescence assays

<table>
<thead>
<tr>
<th>Labelling assay</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 12</th>
<th>Day 16</th>
<th>Day 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior midgut</td>
<td>10/16 (0.62)</td>
<td>13/19 (0.68)</td>
<td>16/17 (0.94)</td>
<td>13/15 (0.87)</td>
<td>16/16 (1.00)</td>
<td>23/23 (1.00)</td>
<td>16/16 (1.00)</td>
<td>18/18 (1.00)</td>
</tr>
<tr>
<td>PSGs</td>
<td>0/21 (0.00)</td>
<td>6/24 (0.25)</td>
<td>9/26 (0.35)</td>
<td>4/17 (0.24)</td>
<td>7/14 (0.50)</td>
<td>12/26 (0.46)</td>
<td>9/25 (0.36)</td>
<td>2/26 (0.08)</td>
</tr>
</tbody>
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*Uptake assay was performed by allowing 4th instar nymphs to feed on BBTV-infected banana plants for 1, 2, 3, 4, 7 and 16 days.

Retention assay was performed by allowing newly born, 1st instar nymphs to feed and develop on BBTV-infected banana plants for 12 days. Adults were then moved to healthy banana plants and were tested in immunofluorescence assays after 2, 4, 7, 12 and 16 days. Day 0 were aphids tested at the end of the development on infected plants, before transferring to healthy plants.

No. of dissected organs labelled for the virus over the total number of organs tested. Proportions are reported in parenthesis.
escape and internalization, respectively (Fig. 4). Recently, Cicero & Brown (2011) suggested a possibility of the direct transfer of squash leaf curl virus (genus \textit{Begomovirus}) from the midgut to the salivary glands of the whitefly \textit{B. tabaci}. The authors suggested that virion translocation may occur when the gut of the whitefly extends to its most anterior position in the thorax, making direct contact with the salivary glands. A similar path of virus translocation has also been proposed for tospoviruses (family \textit{Bunyaviridae}), which are propagatively transmitted by thrips (Moritz \textit{et al.}, 2004).

Collectively, our results suggest that BBTV translocation through its aphid vector has more in common with plant viruses of the family \textit{Geminiviridae} (transmitted by leafhoppers, treehoppers and whiteflies) than aphid-transmitted luteovirids. However, unlike other circulative plant viruses, there is evidence that nanovirids, such as FBNYV, require a virus-encoded helper factor for vector transmission (Franz \textit{et al.}, 1999), similar to non-persistently transmitted plant viruses of the genus \textit{Potyvirus} (Ng & Falk, 2006). Franz \textit{et al.} (1999) showed that the putative helper factor mediated viral transport through the

![Fig. 4. Probability of BBTV transmission by individual banana aphid, \textit{P. nigronervosa} during the phase of uptake (a) and retention (b) (details on the uptake and retention assays are reported in the legend to Fig. 1). Open dots report the observed transmission rates. Curves are from logistic regression analysis expressing the probability of transmission over time.](image)

![Fig. 5. Immunofluorescence localization of BBTV in paraformaldehyde-fixed guts and PSGs of the banana aphid, \textit{P. nigronervosa}. BBTV antigens were localized by using mouse mAbs followed by secondary antibodies labelled with Alexa Fluor 555 (red). Cell nuclei were stained with DAPI (blue). (a) Gut or PSG from a BBTV-infected aphid treated with Triton X-100. (b) Gut or principal salivary gland from a BBTV-infected aphid not treated with Triton X-100. (c) Gut or principal salivary gland from a healthy aphid treated with Triton X-100. am, Anterior midgut; pm; posterior midgut; cc, cover cells; f, foregut; mc, main cells; PSG, principal salivary glands. Bars, 100 \mu m.](image)
Controls were aphids collected from healthy banana plants. Labelling images have been reported in Fig. 5. The table combines the results from two replicates.

<table>
<thead>
<tr>
<th></th>
<th>Devoid of Triton X-100</th>
<th>Triton X-100</th>
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<tbody>
<tr>
<td></td>
<td>BBTV</td>
<td>CTL</td>
</tr>
<tr>
<td>Anterior midgut</td>
<td>21/23 (0.91)*</td>
<td>0/23 (0)</td>
</tr>
<tr>
<td>PSGs</td>
<td>8/10 (0.8)</td>
<td>0/11 (0)</td>
</tr>
</tbody>
</table>

*No. of organs labelled for the virus over the total number of organs tested. Proportions are reported in parentheses.

haemocoel–salivary gland interface of the pea aphid. On the basis of evidence that purified virions are not aphid-transmissible, it has been suggested that BBTV uses the same transmission strategy used by FBNYV (Thomas & Dietzgen, 1991). Further studies on nanovirid–aphid interactions may therefore shed new and exclusive light on vector–virus interactions.

METHODS

Aphid and virus maintenance. A strain of BBTV collected in August 2007 from a field-infected banana plant on the Island of Oahu (Hawaii; USA) was aphid-transmitted to potted micropropagated bananas, cv. Williams. The virus was characterized with a TAS ELISA (Agdia Inc.) and PCR assays using primer pairs for the detection of BBTV DNA components (Burns et al., 1995) according to previously described methods (Almeida et al., 2009; Xie & Hu, 1995). Sequencing of the PCR products identified nearly identical sequences as previous works (Almeida et al., 2009; Xie & Hu, 1995) and confirmed that BBTV from Hawaiian clusters within the Middle Eastern-South Pacific clade (Hu et al., 2007).

A colony of banana aphids, which was established from an individual aphid, was maintained on healthy potted banana plants. The BBTV-infected plants used in this research were produced by transferring 20–30 viruliferous aphids to healthy potted banana plants at a growth stage of six leaves. Plants were pruned at their base 1 month post-inoculation, allowing the suckers to sprout and grow for another 4 weeks. At that time, the suckers expressed strong symptoms of infection and were used for the experiments.

BBTV uptake and retention assays. We performed time-course experiments to analyse the pattern of BBTV uptake and retention within the banana aphid.

For BBTV uptake assays, we transferred fourth instar nymphs from the aphid colony to BBTV-infected plants. The aphids were deposited at the base of the plant petioles near the pseudostem on which the banana aphid feeds (Magee, 1927; Robson et al., 2007) and readily acquires the virus (Anhalt & Almeida, 2008; Magee, 1927). The aphids were subsequently sampled after 1, 2, 3, 4, 7 and 16 days and processed for immunofluorescence localization, real-time PCR and transmission assays.

At the end of aphid development, which lasted for 12 days, the emerging adults were transferred to healthy potted banana plants. Once they were transferred to the healthy plants, the aphids were sampled after 2, 4, 7, 12 and 16 days. In addition, the aphids were sampled just before being transferred to the healthy plants (designated day 0). The sampled aphids were processed for immunofluorescence localization, real-time PCR and transmission assays. For the retention assays, healthy banana plants were replaced every 4 days. We conducted a separate test to examine if during these 4 days aphids may have reacquired viral particles from the plants. Newly emerged BBTV-infected and healthy adult aphids were placed together on the petioles of a healthy five-leaf stage banana plant. To discriminate the BBTV-infected from the healthy aphids, the formers were marked on their abdomens with a mini-correction pen (L. P. Sanford, Oak Brook, IL, USA). After 4 days, aphids were collected from the plant and the DNA was extracted from single insects by using a QIAamp DNA mini kit (Qiagen). BBTV concentration was quantified by relative real-time PCR as described below. Healthy aphids raised from the rearing colony were used as a control. All of the experiments were performed in a greenhouse with a photoperiod of approximately 12 h and a temperature range of 27 ± 5 °C.

Dissection and haemolymph sampling. Aphids were dissected under a stereomicroscope essentially as described previously (Bressan & Watanabe, 2011). Briefly, individual 1 h starved aphids were immobilized on the surface of a dissection chamber (Electron Microscopy Sciences) and immersed in approximately 200 μl PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 2 mM KH2PO4; pH 7.4). PBS-immersed aphids were then dissected with a polytetra-fluoroethylene-coated stainless steel blade (Ted Pella Inc.) by cutting the aphid bodies behind their compound eyes and allowing both the salivary duct and the foregut to separate from the styles.

The haemolymph released after the decapitation was sampled with a glass capillary. The haemolymph of the banana aphid is red, whereas the internal organs are white-translucent (Fig. S4), thus allowing the selective collection of the haemolymph. The haemolymph sample in the capillary was transferred into a 1.5 ml microcentrifuge tube. Using small pins, the entire digestive tract and salivary glands were then dissected and removed from the aphid body, were washed multiple times in PBS and processed for immunofluorescence localization and real-time PCR assays. The haemolymph samples were evaluated with real-time PCR assays only.

Real-time PCR. DNA was extracted using a QIAamp DNA mini kit (Qiagen). Briefly, the dissected guts, salivary glands and haemolymph from three aphids were pooled into individual 1.5 ml ultracentrifuge tubes containing 180 μl lysis buffer. The procedure followed the manufacturer’s instruction. The DNA was eluted with 100 μl ultrapure water into clean 1.5 ml ultracentrifuge tubes. We first performed diagnostic PCR using primer pair CPXLPII and BBTV3C.EXP (Wanitchakorn et al., 2000). The DNA extracted from infected and healthy plants was used as positive and negative controls, respectively. We used a relative real-time PCR to target the BBTV DNA genomic component S and, as an internal reference, the banana aphid actin gene using a similar procedure described by Mason et al. (2008). The universal actin gene primers, actin1 (5′-CATCT-GCTGAAAGGT-3′) and actin2 (5′-CTGTAAGCCCTCCGTTGAC-CA-CAC-3′) (Boonham et al., 2002) were used to obtain a fragment of approximately 900 bp from the aphid actin gene. The amplicon was sequenced from both the 5′ and 3′ ends, and the sequence was analysed with the software Primer3 (v. 0.4.0) to design a primer pair specific for the banana aphid actin gene, ActPer (5′-CGGTTAATT-TCCATTATGATTGCT-3′) and ActPer (5′-GTGTTACGTTACGTTCA-GAAAG-3′). Similarly, a previously sequenced BBTV genomic component S was used to design the primer pair BBTV Si (5′-TGG-GGCTATATTATGTTGAT-3′) and BBTV S (5′-CGCCTGTTTIT-
BBTV DNA was normalized to the amount of actin DNA using the equation: $E^{\text{actin}} / E^{\text{BBTV}}$, where $E$ = the PCR efficiency of a given primer pair, and $C_t$ = threshold cycle, which was automatically calculated with the IQ software (Rotor-Gene 6000 Series Software Version 1.7, Corbett Research). The amplification efficiency was determined through linear regression obtained through the amplification of serially diluted DNA extracts. Efficiency values were calculated with the following formula: $10^{(-1/Slope)}$. Because $E$ = 2.00 was achieved for both BBTV and actin primer pairs, we adopted a simplified equation, $2^{C_t (\text{actin–BBTV})}$, to calculate the relative abundance of BBTV DNA in each sample. For each time point, we tested the DNA extracted from the guts, salivary glands and haemolymph of 18 aphids. The differences in the BBTV DNA concentration among different sampling date were evaluated with a Kruskal–Wallis test followed by Mann–Whitney test to perform pairwise comparisons. Statistical analyses were performed using the software SigmaStat Version 3.5 (San Jose, CA).

**Immunofluorescence localization.** Immunofluorescence localization was performed in the dissected and acetone-fixed guts and salivary glands with a biotin–streptavidin labelling procedure as described previously (Bressan & Watanabe, 2011). The slides were visualized under an Olympus BX-51 epifluorescence microscope with an Optronics MacroFire digital camera. To allow for the comparison of samples between the different tested time points, we standardized the time of the camera exposure to 300 ms for AMGs and 500 ms for the PSGs. In addition, we developed an immunofluorescence protocol to selectively determine the localization of BBTV antigens on or near the basal surface of the AMGs and PSGs. The organs were dissected from aphids that fed on BBTV-infected plants for 12 days or, as a control, aphids collected from healthy plants. The dissected organs were allowed to fully adhere to the surface of the slides and were then fixed in PBS containing 4% paraformaldehyde for 1 h. After extensive washing with PBS to remove the fixative, the slides were incubated with either PBS containing 1% Triton X-100 or PBS alone for 1 h. The samples were then incubated for an additional hour in a blocking solution of PBS with 10% normal goat serum. Anti-BBTV mouse mAbs (IgA/A24876 and IgA/A24877) diluted 1:200 in PBS and 1% normal goat serum were added to the slides, which were incubated overnight at 4°C. The slides were subsequently washed three times in PBS and incubated for 45 min with goat anti-mouse IgG conjugated with Alexa Fluor 555 (Invitrogen) diluted 1:500 in PBS. The slides were washed three times in PBS, rinsed in water and mounted with ProLong gold anti-fade mounting medium containing DAPI (Invitrogen) to counterstain the cell nuclei.

**Transmission assays.** The transmission efficiency of BBTV was analysed by using a leaf-disk assay as described previously (Bressan & Watanabe, 2011). Aphids were sampled during the uptake and retention assays and were individually transferred to healthy banana leaf disks for an IAP of 2 days. After removal of aphids, leaf disks were incubated for another 2 days to allow virus replication. Leaf disks were thereafter removed from the chamber, thoroughly washed with dish soap on both sides of the leaf and rinsed in water. The DNA was extracted from individual leaf disks using a cetyltrimethylammonium bromide procedure (Xie & Hu, 1995).

The presence of BBTV was analysed by a nested-PCR assay. A first round of amplification was performed using 1 µl DNA in 25 µl master mix, containing 5 pmol of primers 73F (5’-GGCTTTTAYC-CAGAAGACCA-3’) and 73R (5’-CCGATCATGTATATTGTTTT-3’) to specifically detect BBTV-S. The PCR products obtained from the first round of amplification were diluted 30 times and 1 µl of the diluted product was used in a second round of amplification, with a master mix containing 5 pmol of each primer CPX.PRI and BBTV3C.EXP (Wanitchakorn et al., 2000), to amplify an internal proportion of BBTV-S produced by the direct PCR. Bands were visualized in 1.2% agarose gel. Transmission experiments were replicated three times.

**Detection of BBTV transcripts in aphids and plants.** We isolated RNA from BBTV-infected or healthy aphids and from BBTV-infected or healthy plants using an RNeasy Mini kit (Qiagen). Prior to RNA extraction, aphids that developed by feeding on a BBTV-infected plant for 12 days were transferred to a healthy banana plant for 4 days to clear the alimentary tract (Sinisterra et al., 2005). Groups of 10 aphids or 20 mg of banana midribs were ground in a 1.5 ml tube containing 350 µl buffer RLT. Samples were centrifuged at high speed in QiAshredder columns (Qiagen). Thereafter, RNA extraction was performed according to manufacturer’s instructions. Samples were then reverse transcribed using QuantiTect Reverse Transcription kit (Qiagen). PCR assays were performed using primers R2f (5’-CC-TTCGATTTGATATTGATT-3’) and R2r (5’-GGCCATGATATTGCTTCCACCT-3’) to amplify a proportion of 236 bp within the ORF of the BBTV-R, encoding the master Rep. PCR was performed under the following conditions: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, extension at 72°C for 30 s and a final extension for 5 min at 72°C. Amplicons were visualized in 1.2% agarose gel. DNA extracted from BBTV-infected and healthy aphids were used as additional positive and negative controls, respectively.

**Salivary glands and anterior midgut spatial distribution.** We analysed the internal spatial distribution of the salivary glands and AMGs of the banana aphid. Briefly, entire aphid adults, devoid of appendages and permeabilized by puncturing the exoskeleton with small glass needles, were fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, followed by a wash in 0.1 M sodium cacodylate, and then post-fixed in 1% OsO4 in 0.1 M sodium cacodylate. Samples were dehydrated in a graded series of ethanol, substituted with propylene oxide, and embedded in LX-112 epoxy resin ( Electron Microscopy Sciences). Transverse 1 µm sections starting from the head of the aphids were produced with an Ultracut E Reichert-Jung Ultramicrotome. These were mounted on slides, stained with Richardson’s stain, and observed with a compound microscope.

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**REFERENCES**


