Assembly of the viroplasm by viral non-structural protein Pns10 is essential for persistent infection of rice ragged stunt virus in its insect vector

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Rice ragged stunt virus (RRSV), an oryzavirus, is transmitted by brown planthopper in a persistent propagative manner. In this study, sequential infection of RRSV in the internal organs of its insect vector after ingestion of virus was investigated by immunofluorescence microscopy. RRSV was first detected in the epithelial cells of the midgut, from where it proceeded to the visceral muscles surrounding the midgut, then throughout the visceral muscles of the midgut and hindgut, and finally into the salivary glands. Viromas, the sites of virus replication and assembly of progeny virions, were formed in the midgut epithelium, visceral muscles and salivary glands of infected insects and contained the non-structural protein Pns10 of RRSV, which appeared to be the major constituent of the viromas. Viroplasm-like structures formed in non-host insect cells following expression of Pns10 in a baculovirus system, suggesting that the viromas observed in RRSV-infected cells were composed basically of Pns10. RNA interference induced by ingestion of dsRNA from the Pns10 gene of RRSV strongly inhibited such viroplasm formation, preventing efficient virus infection and spread in its insect vectors. These results show that Pns10 of RRSV is essential for viroplasm formation and virus replication in the vector insect.

INTRODUCTION

Plant reoviruses are found in the genera Phytoreovirus, Fijivirus and Oryzavirus in the family Reoviridae (Boccardo & Milne, 1984). Rice ragged stunt virus (RRSV), an oryzavirus (Hibino, 1996; Hibino et al., 1977), has spread rapidly throughout southern China and Vietnam and causes severe damage to rice (Hoang et al., 2011). For example, since 2006, RRSV has spread widely in Fujian, Hainan, Yunnan, Guangxi and Guangdong provinces in China. RRSV has an icosahedral capsid, ~70 nm in diameter, to which spikes are attached (Miyazaki et al., 2008). The RRSV genome consists of ten dsRNA segments that encode at least seven structural proteins, P1, P2, P3, P4A, P5, P8B and P9, and three non-structural proteins, Pns6, Pns7 and Pns10 (Boccardo & Milne, 1984; Hagiwara et al., 1986; Upadhyaya et al., 1996, 1997, 1998). Among the structural proteins encoded by RRSV, P2, P3, P4A and P5 are a putative guanylyltransferase, capsid shell protein, putative RNA-dependent RNA polymerase and capping enzyme, respectively (Boccardo & Milne, 1984; Hagiwara et al., 1986; Supyani et al., 2007; Upadhyaya et al., 1998), P8 is a major outer-capsid protein (Hagiwara et al., 1986) and P9 is a spike protein involved in transmission via the insect vector (Zhou et al., 1999). Among the non-structural proteins encoded by RRSV, Pns6 functions as a viral RNA-silencing suppressor and a viral movement protein (MP) (Wu et al., 2010a, b), whilst Pns7 has been identified as an NTP-binding protein (Spear et al., 2012; Upadhyaya et al., 1997). The functions of the remaining proteins are unknown.

RRSV is transmitted by the brown planthopper (BPH), Nilaparvata lugens (Stål), in a persistent propagative manner (Hibino et al., 1977, 1979). As a persistent propagative plant virus that is transmitted by an insect vector following ingestion during feeding on diseased plants, RRSV must enter the epithelial cells of the alimentary canal in its insect vector, replicate and assemble progeny virions to move into the salivary glands from which RRSV can be introduced into a plant host during feeding (Hogenhout et al., 2008). Thus, RRSV must enter insect vector cells to establish persistent infection, that is, virus must replicate and accumulate progeny virions in the body of BPHs. During RRSV infection in BPHs, cytoplasmic inclusion bodies, known as viromas, form in the salivary glands, gut and muscles in
infected BPHs, as observed by electron microscopy (Hibino et al., 1979). Virus replication and assembly of progeny virions have been proposed to occur in viroplasms for plant reoviruses (Boccardo & Milne, 1984). Thus, the formation of a viroplasm for virus replication and assembly of progeny virions may play a crucial role in the propagation of RRSV in BPHs.

Viral non-structural proteins are essential for formation of the viroplasm matrix of plant reoviruses, such as P9-1 of three fijiviruses [rice black-streaked dwarf virus (RBSDV), Southern rice black-streaked dwarf virus (SRBSDV) and Mal de Río Cuarto virus (MRCV)], Pns12 of the phyto-reovirus rice dwarf virus (RDV) and Pns9 of rice gall dwarf virus (RGDV), also a phytoreovirus (Akita et al., 2011, 2012; Jia et al., 2012; Maroniche et al., 2010; Wei et al., 2006b). However, viral non-structural proteins involved in formation of the viroplasms induced by oryzaviruses are unknown.

RNA interference (RNAi), a conserved sequence-specific gene-silencing mechanism induced by dsRNA (Fire et al., 1998), has been developed into an important tool to investigate the functional role of fijivirus replication proteins in insect vectors (Jia et al., 2012). Ingestion of dsRNA via membrane feeding is an effective method of inducing RNAi in BPHs to knock down specific insect genes (Chen et al., 2010; Li et al., 2011). Thus, dsRNA-mediated gene silencing offers an opportunity for us to investigate the functional roles of viral non-structural proteins in the infection cycle of RRSV in BPHs.

In this study, using immunofluorescence microscopy and an RNAi strategy, the functional roles were determined for RRSV Pns10 in formation of the viroplasm and virus infection in BPHs. Our results suggested that Pns10 of RRSV is responsible for formation of the viroplasm matrix in which the assembly of progeny virions occurs in BPHs. RNAi induced by ingestion of dsRNA of the Pns10 gene strongly inhibited such viroplasm formation, preventing efficient virus infection and spread in BPHs. Our results indicated that assembly of the viroplasm by Pns10 is essential for persistent infection of RRSV in BPHs.

### RESULTS

#### Infection route of RRSV in BPHs revealed by confocal microscopy

To trace the infection route of RRSV within infected BPHs, immunofluorescence microscopy was used to elucidate the distribution of viral antigens in the body of BPHs after ingestion of RRSV from diseased plants. In preliminary tests, ~40% of BPHs became infected after a latent period of 9 days (Table 1). At 1, 3, 4, 6 and 9 days post-first access to diseased plants (p.a.d.p.), internal organs from 50 BPHs were dissected and processed for immunofluorescence microscopy. The actin-specific dye phalloidin–rhodamine was first used to stain the alimentary canal of BPHs. As in other types of planthopper (Tsai & Perrier, 1996), the alimentary canal of BPHs consists of the oesophagus, anterior diverticulum, midgut and hindgut (Fig. 1a). The midgut of BPHs consists of a single layer of epithelial cells, with extensive microvilli on the luminal side and basal lamina on the outer side, surrounded by visceral muscle tissues (Fig. 1a). Viral antigens were observed at 1 day p.a.d.p. in the midgut lumen in ~50% of BPHs examined (Fig. 1b), suggesting that RRSV had travelled through the oesophagus into the midgut lumen. At 3 days p.a.d.p., RRSV was mainly restricted to a few epithelial cells of the midgut in ~30% of the insects examined (Fig. 1c, Table 1). At 4 days p.a.d.p., RRSV in the epithelial cells had traversed the basal lamina and infected the visceral muscle tissues encircling the midgut epithelium in ~28% of BPHs examined (Fig. 1d, Table 1). By 6 days p.a.d.p., RRSV had spread to the oesophagus, anterior diverticulum, midgut, hindgut and salivary glands in a higher proportion of BPHs (~26%; Fig. 1e, Table 1); RRSV was present in the visceral muscle tissues of the entire midgut and hindgut but absent in the epithelium (Fig. 1f–h). By 9 days p.a.d.p., the presence of RRSV was extensive in the oesophagus, anterior diverticulum, muscles of the midgut and hindgut, and salivary glands in a high proportion of BPHs tested (~40%) (Fig. 1i, j, Table 1). At this time, RRSV was still absent in the epithelium of the midgut and hindgut (Table 1). Taken together, these results indicated that RRSV first accumulates in the midgut epithelium, procedes to the

### Table 1. Occurrence of RRSV antigens and viroplasms of Pns10 in various organs/tissues of BPHs as detected by immunofluorescence microscopy

<table>
<thead>
<tr>
<th>Organ/tissue examined</th>
<th>No. positive insects with viral antigens and viroplasms of Pns10 in different tissues (n=50)</th>
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<tbody>
<tr>
<td>Midgut epithelium</td>
<td>15</td>
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<tr>
<td>Visceral muscle (midgut)</td>
<td>0</td>
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<tr>
<td>Visceral muscle (hindgut)</td>
<td>0</td>
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<tr>
<td>Oesophagus</td>
<td>0</td>
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<td>Anterior diverticulum</td>
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<td>Salivary gland</td>
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Fig. 1. Infection route of RRSV in the insect vector. The internal organs of BPHs were stained for viral antigens with virus–FITC (green) and for actin with phalloidin–rhodamine (red) and examined by confocal microscopy. (a) The alimentary canal of BPH. Insets: single optical section of the lumen side (upper panel) and muscle side (lower panel) of the midgut. (b) At 1 day p.a.d.p., viral antigens accumulated in the midgut lumen. (c) By 3 days p.a.d.p., viral antigens were detected in a few epithelial cells of the midgut. Inset: enlarged image of the boxed area. (d) At 4 days p.a.d.p., viral antigens accumulated in the epithelium and visceral muscle of the midgut. The image is a projection of 12 optical sections taken at 0.5 μm intervals. Insets: single optical sections of the lumen side (left panel) and muscle side (right panel) of the midgut. (e) By 6 days p.a.d.p., viral antigens had accumulated throughout the digestive system. (f) Single optical sections of the lumen side (panel i) and muscle side (panel ii) of the midgut at 6 days p.a.d.p. (g, h) Viral antigens were detected in the visceral muscle of the midgut (g) and hindgut (h) at 6 days p.a.d.p. The inset in (h) is an enlargement of the boxed area. (i, j) At 9 days p.a.d.p., viral antigens accumulated throughout the digestive system. ad, Anterior diverticulum; mg, midgut; hg, hindgut; mt, Malpighian tubules; os, oesophagus; sg, salivary gland; gl, gut lumen; mv, microvilli; me, midgut epithelium; vm, visceral muscle. Bars, 70 μm.
visceral muscles surrounding the midgut, spreads throughout the visceral muscles of the midgut and hindgut, and finally spreads into the salivary glands.

**RRSV Pns10 is sufficient to induce the formation of viroplasm-like structures in non-host insect cells**

During infection of BPHs by RRSV, viroplasms, the putative sites for virus replication and assembly of progeny virions, are formed in the alimentary canal and salivary glands of BPHs, as revealed by electron microscopy (Boccardo & Milne, 1984; Hibino *et al.*, 1977, 1979). Viral non-structural proteins are essential for formation of the viroplasm matrix of plant reoviruses (Akita *et al.*, 2011, 2012; Maroniche *et al.*, 2010; Wei *et al.*, 2006b). To identify which non-structural protein encoded by RRSV had an inherent ability to form the viroplasm matrix, a baculovirus system was used to express each of the three non-structural proteins, Pns6, Pns7 and Pns10, fused to a 6×His tag (Pns6–His and Pns7–His) or Strep Tag II (Pns10–Strep). As seen by immunofluorescence microscopy, Pns6–His was associated exclusively with the plasma membrane (Fig. 2a, panel i), corresponding to previous evidence that Pns6 is a viral MP (Wu *et al.*, 2010b). Pns7–His formed filament-like structures in the cytoplasm or protruding from the plasma membrane (Fig. 2a, panel ii), whilst Pns10–Strep aggregated to form punctate inclusions in the cytoplasm (Fig. 2a, panel iii), resembling the viroplasm matrix in virus-infected cells (Hibino *et al.*, 1979). Our results indicated clearly that, among the three non-structural proteins of RRSV, Pns10 alone was sufficient for the formation of viroplasm-like structures in *Spodoptera frugiperda* (Sf9) cells. Taken together, our results suggested that Pns10 might self-aggregate to form the viroplasm matrix in RRSV-infected host cells.

To determine whether Pns6 or Pns7 could be recruited into viroplasm-like structures formed by Pns10, recombinant baculoviruses that expressed Pns6–His or Pns7–His were co-infected with recombinant baculoviruses expressing Pns10–Strep in Sf9 cells. Co-infection led to redistribution of Pns6–His into the viroplasm-like structures formed by Pns10–Strep (Fig. 2b). By contrast, Pns7–His was not observed in association with the viroplasm-like structures formed by Pns10–Strep (data not shown). Rabbit anti-6×His tag polyclonal antibody and anti-Strep Tag II mAb in non-infected cells were not observed to react with cellular structures (data not shown). Thus, our findings

![Fig. 2. RRSV Pns10 aggregates to form viroplasm-like inclusions in the absence of virus infection. Sf9 cells infected with recombinant baculoviruses containing Pns6–His, Pns7–His or Pns10–Strep were fixed 3 days after infection and prepared for immunofluorescence microscopy as described in Methods. (a) Pns6–His was associated with the plasma membrane (i), Pns7–His formed filament-like structures (ii) and Pns10–Strep formed punctate structures (iii). (b) Pns6–His was associated with the punctate structures formed by Pns10–Strep when co-infected with recombinant baculoviruses containing Pns6–His and Pns10–Strep. Bars, 5 μm.](https://www.microbiologyresearch.org/doi/abs/10.1099/00221287-2302-1)
suggested that Pns6 might be recruited to the viroplasm through the association of Pns6 with Pns10.

**RRSV Pns10 is the constituent of the viroplasm matrix in the body of infected BPHs**

To localize Pns10 in virus-infected cells, antibodies against this protein were prepared. Western blot analysis using anti-Pns10 antibodies showed a 33 kDa protein (in accordance with the expected size of the RRSV segment 10-encoded protein) present in protein extracts from infected rice plants (Fig. S1, available in JGV Online). No reaction was observed with proteins from uninfected plants, confirming that Pns10-specific antibodies were able to specifically recognize the protein produced by RRSV infection.

To determine whether Pns10 plays a key role in formation of the viroplasm, subcellular localization of Pns10 in the body of infected BPHs was examined by immunofluorescence microscopy. Double labelling showed that viral inclusions that stained with Pns10-specific IgG directly conjugated to rhodamine (Pns10–rhodamine) co-localized with punctate inclusions that stained with viral antigen-specific IgG directly conjugated to FITC (virus–FITC) in the midgut epithelium at 3 days p.a.d.p., in the visceral muscle tissues surrounding the midgut at 6 days p.a.d.p. and in the salivary gland at 9 days p.a.d.p. (Fig. 3a, b, Table 1). These results indicated that RRSV Pns10 is a constituent of viral inclusions where viral antigens accumulated.

To confirm whether the viral inclusions of Pns10 observed by immunofluorescence microscopy were the viroplasm

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**Fig. 3.** Virolasms containing Pns10 antigens co-localize with viral antigens in infected BPHs. The internal organs of BPHs were stained for viral antigens with virus–FITC (green), for virolasms with Pns10–rhodamine (red) or for actin with phalloidin–Alexa Fluor 647 carboxylic acid (blue) and then examined by confocal microscopy. Virolasms containing Pns10 antigens (red) and viral antigens (green) were distributed in the midgut epithelium at 3 days p.a.d.p. (a), in the visceral muscle tissues surrounding the midgut at 6 days p.a.d.p. (b) and in the salivary glands at 9 days p.a.d.p. (c). DIC, Differential interference contrast; me, midgut epithelium; mg, midgut; sg, salivary gland; vm, visceral muscle. Bars, 70 μm.
where viral particles accumulate, immunoelectron microscopy was used to localize Pns10 in the salivary glands of infected BPHs. As shown in Fig. 4(a), Pns10 antibodies reacted specifically with the viroplasm matrix in salivary glands, and the labelling was consistent with the results of the immunofluorescence microscopy (Fig. 3c). Careful analysis of the electron micrographs revealed that core-like particles of ~50 nm in diameter were distributed within the viroplasm matrix, whereas intact, double-layered viral particles of ~70 nm in diameter accumulated at the periphery of the viroplasm matrix (Fig. 4b). Moreover, RRSV particles aggregated to form paracrystalline arrays at the periphery of the viroplasm matrix (Fig. 4c). These results confirmed that Pns10 is the constituent of the viroplasm matrix induced by RRSV infection. Furthermore, our results clearly indicated that the viroplasm is the putative site of virus assembly in the body of infected BPHs.

**Ingestion of dsRNA of the Pns10 gene strongly inhibits virus infection in BPHs**

An RNAi strategy was used next to investigate the functional role of RRSV Pns10 in the virus replication cycle in BPHs. Second-instar nymphs of BPHs were fed 0.5 μg dsRNA μl−1 in 10% sucrose by membrane feeding for 1 day, allowed a 2-day acquisition on RRSV-infected rice plants and then fed on fresh rice seedlings. RT-PCR was used to determine the effects of dsRNA treatment on the transcript levels of viral genes of the non-structural protein Pns10 and the major outer-capsid protein P8 in infected insects that received either dsRNA or sucrose diet alone at 9 days p.a.d.p. (Upadhyaya et al., 1996, 1997). Our results showed that ~40% of BPHs (n=100, three repetitions) that received dsRNA of the *gfp* gene (dsGFP) contained transcripts for the *Pns10* and *P8* genes (Table 2). By contrast, ~12% (n=100, three repetitions) of BPHs that received dsRNA of the *Pns10* gene (dsPns10) contained transcripts for the *Pns10* and *P8* genes (Table 2). The number of positive samples found in BPHs that received dsGFP did not differ significantly from the controls that received the sucrose diet only (Table 2). These results indicated that ingestion of dsPns10 using the membrane feeding method can efficiently induce RNAi and inhibit virus infection in BPHs.

To analyse in more detail the inhibition of virus infection caused by ingestion of dsPns10 in the body of BPHs, internal organs from 50 BPHs receiving dsRNA or sucrose diet alone were dissected at 3, 6 and 9 days p.a.d.p. and processed for immunofluorescence microscopy. Virus infection was revealed by double labelling of internal organs with virus–FITC and Pns10–rhodamine. At 3 days p.a.d.p., Pns10 and viral antigens were restricted to a limited number of epithelial cells of the midgut in 30% of BPHs receiving dsGFP but only in 10% of those receiving dsPns10 (Table 2). These results suggested that RNAi induced by dsPns10 could significantly inhibit early RRSV infection in the cells of the midgut of insect vectors. At

![Fig. 4. RRSV Pns10 is the component of the viroplasm matrix. (a) Immunogold labelling of RRSV Pns10 in the viroplasm matrix in infected salivary glands. Salivary glands were immunostained using Pns10-specific antibodies as the primary antibody, followed by treatment with goat anti-rabbit antibodies conjugated to 15 nm gold particles as the secondary antibody. The inset shows an enlargement of the boxed area. Black arrows indicate virus particles, whilst white arrows indicate gold particles. (b, c) Morphogenesis of RRSV particles associated with the viroplasm matrix in infected salivary glands. The inset in (b) shows an enlargement of the boxed area. Black arrows indicate core-like particles, whilst white arrows indicate intact viral particles. VP, Viroplasm. Bars, 200 nm.](image-url)
6 days p.a.d.p., Pns10 and viral antigens were detected in both visceral muscle tissues and salivary glands in 20% of BPHs receiving dsGFP but only in 6% of BPHs receiving dsPns10 (Table 2). Furthermore, RRSV was absent from the midgut epithelium in BPHs receiving dsGFP, but virus could be detected in 12% of those receiving dsPns10 (Table 2). At 9 days p.a.d.p., Pns10 and viral antigens were detected in the visceral muscles of 40% of BPHs receiving dsGFP and in the salivary glands of 36% of BPHs receiving dsGFP (Table 2). However, Pns10 and viral antigens were only seen in ~12% of the visceral muscle tissues and 6% of the salivary glands in BPHs receiving dsPns10 (Table 2). No significant difference in the number of positive samples was found between BPHs that received dsGFP and diet alone (Table 2). Therefore, RNAi induced by dsPns10 inhibited efficient virus infection and spread in the body of insect vectors.

**DISCUSSION**

**RRSV Pns10 is responsible for formation of the viroplasm matrix in virus-infected cells**

Immunoelectron and immunofluorescence microscopy of RRSV-infected cells showed that the non-structural protein Pns10 of RRSV was a component of the viroplasm matrix where core-like particles and viral particles accumulated (Figs 3 and 4), confirming that viroplasms are the site of virus assembly for plant reoviruses. Among the three non-structural proteins encoded by RRSV, only expression of Pns10 in Sf9 cells, a non-host of RRSV, resulted in formation of viroplasm-like structures, whereas neither of the other two non-structural proteins, Pns6 and Pns7, appeared to form viroplasm-like structures in Sf9 cells (Fig. 2a). These results suggested that Pns10 was the minimal viral factor required for viroplasm formation during RRSV proliferation and that formation of the viroplasm matrix was not specific to host plant or insect vector cells and did not require host-specific components. Our studies also showed that Pns10 could recruit Pns6 into the viroplasm-like structures formed by Pns10 during co-expression of these two proteins in Sf9 cells (Fig. 2b). Thus, Pns6 might be recruited to the viroplasm through the association of Pns6 with Pns10 during virus infection. Our results suggested that RRSV Pns6, which functions as a viral MP in plant hosts (Wu et al., 2010b), is probably involved in virus replication. Furthermore, the filament-like structures protruding from the plasma membrane formed by RRSV Pns7 strongly resembled those formed by P7-1 of SRBSDV and Pns10 of RDV (Liu et al., 2011; Wei et al., 2006a). The efficient spread of RDV among insect vector cells is dependent on the formation of tubular structures induced by Pns10 (Wei et al., 2006a). It will be interesting to examine whether RRSV Pns7 also forms the same kind of structures and plays a similar role in the spread of virus among insect vector cells.

Non-structural proteins essential for formation of the viroplasm matrix have common characteristics among
viruses in the family *Reoviridae*. RRSV Pns10 contains the amino acid sequence motifs typical of an ATPase protein (Z.-X. Gong, unpublished data; http://www.doc88.com/p-33746844520.html) and RNA-binding protein (Upadhyaya et al., 1997), suggesting that Pns10 might have ATPase and RNA-binding abilities. The viroplasm matrix protein P9-1 of RBSDV and MRCV can bind RNA (Akita et al., 2012; Maroniche et al., 2010). In addition, P9-1 of MRCV has ATPase activity (Maroniche et al., 2010). The RNA-binding and NTPase activities of the viroplasm matrix protein NSP2 of rotavirus in the family *Reoviridae* have been studied in detail (Kumar et al., 2007; Vasquez-Del Carpio et al., 2006). All these proteins are able to form viroplasm-like structures in non-host cells (Akita et al., 2012; Fabbretti et al., 1999; Maroniche et al., 2010). The parallels among Pns10 of RRSV, NSP2 of rotavirus and P9-1 of MRCV and RBSDV suggest that these proteins might play similar roles in formation of the viroplasm matrix during virus replication cycles. It is interesting to note that viroplasm matrix proteins such as rotavirus NSP2, RBSDV P9-1 and RGDV Pns9 can form octameric structures (Akita et al., 2011, 2012; Jiang et al., 2006); thus, RRSV Pns10 may have a similar structure.

**Assembly of the viroplasm by Pns10 accompanies the sequential infection by RRSV of the internal organs of BPHs**

In our study of the sequential infection of RRSV in the internal organs of BPHs, the accumulation of virus and the formation of viroplasms were analysed by double labelling the internal organs with virus–FITC and Pns10–rhodamine. As early as 1 day after the virus was ingested, masses of viral particles had accumulated in the lumen or were attached to the microvillar membrane of the midgut (Fig. 1b). However, only a limited number of viral particles in the lumen had successfully crossed the microvilli into the epithelial cells of the midgut (Fig. 1c, Table 1). The fact that not all insects feeding on RRSV-infected rice plants became infected could be due to the infection barrier posed by the midgut. At an early stage of virus infection in the epithelial cells of the midgut, even in a single virus-infected cell, RRSV could initiate the formation of nascent viroplasms (Fig. 3a), which serve as the sites for assembly of progeny virions. It is interesting that most of the progeny RRSV virions traversed the basal lamina of the midgut to infect the visceral muscle tissues bordering the infected region, rather than spreading extensively into the adjacent epithelial cells of the midgut (Fig. 1d, Table 1). Subsequently, progeny virions spread to the visceral muscle tissues surrounding the midgut and hindgut (Figs 1f–h and 3b, Table 1). RRSV might disseminate directly from the visceral muscle tissues into the haemolymph and then into the salivary glands. After a latency period of ~9 days, the salivary glands were heavily infected, as shown by almost complete distribution of virus particles and viroplasms throughout the glands, which were proposed to support the assembly of a large number of progeny virions (Figs 1i, j and 3c). Based on the preceding discussion, formation of the viroplasm for assembly of progeny virions is probably essential for the establishment of a persistent infection of RRSV in insect vectors.

Our recent findings revealed a significantly different infection route for RDV, a phytoreovirus, in its leafhopper vector (Chen et al., 2011). RDV initially infected the epithelial cells of the leafhopper filter chamber. Following accumulation of progeny virions in these cells, most RDV particles spread to the epithelial cells of other organs such as the midgut. Furthermore, RDV may exploit nerves to spread into the visceral muscle tissues surrounding the anterior midgut, although we did not observe particles associated with the neural tissues of BPHs (data not shown).

**RRSV Pns10 is essential for virus infection in BPHs**

Our results showed that RNAi induced by ingestion of dsPns10 strongly inhibited efficient virus infection and spread in the body of BPHs. Treatment with dsPns10 reduced the number of positive BPHs with early virus infection in the epithelial cells of the midgut by ~67% (Table 2). In RRSV-positive BPHs that received dsPns10, RRSV still could infect a limited number of the epithelial cells of the midgut, but the subsequent spread of RRSV from the initial infection site to additional tissues was strongly inhibited (Table 2). Thus, RNAi induced by dsPns10 treatment remained activated, even in RRSV-positive BPHs, which may have caused the slower virus spread in the body of BPHs. RNAi induced by dsRNA is a conserved sequence-specific gene-silencing mechanism (Fire et al., 1998). Because the sequences of Pns10 and other viral genes of RRSV showed no homology (data not shown), we deduced that the RNAi response induced by dsPns10 most probably inhibited expression of the Pns10 gene in BPHs. Due to the essential role of RRSV Pns10 in biogenesis of the viroplasm matrix, we thus determined that knockdown of Pns10 expression would block formation of the viroplasm necessary for virus replication and assembly of progeny virions in the epithelial cells of the midgut, preventing efficient virus infection and spread in the body of BPHs. This conclusion agrees with our recent finding for SRBSDV, a fijivirus, on the inhibition of viroplasm assembly and virus replication in the body of white-backed planthoppers by RNAi induced by dsRNA targeting the viral gene for the viroplasm matrix protein P9-1 (Jia et al., 2012). Similarly, short-interfering RNAs against a viral gene for NSP2, the viroplasm matrix protein of rotavirus, inhibited viroplasm formation, genome replication, virion assembly and synthesis of the other viral proteins (Silvestri et al., 2004). Recently, transgenic rice plants, in which the expression of viroplasm matrix proteins of plant reoviruses, including RBSDV, RDV and RGDV, was silenced by RNAi, were shown to be strongly resistant to virus infection (Shimizu et al., 2009, 2011,
2012). These results all support the hypothesis that the viroplasm plays a pivotal role in replication of viruses in the family Reoviridae. In this context, identification of RRSV Pns10 as the driving force for viroplasm formation is a step towards identifying suitable targets for pathogen-derived resistance strategies to control disease. In this study, RNAi induced by synthesized dsRNA could overcome the lack of a reverse genetics system in RRSV and opens up new opportunities to understand the biological activities of viral proteins in replicative cycles in vivo.

METHODS

Antibody preparation. The preparation of RRSV antigen-specific IgGs has been described previously (Takahashi et al., 1991). Rabbit polyclonal antiserum against RRSV Pns10 was prepared as described by Akita et al. (2012). IgG was isolated from specific polyclonal antiserum using a protein A-Sepharose affinity column (Pierce). RRSV antigen-specific IgGs were conjugated directly to FITC (virus–FITC) and Pns10-specific IgGs were conjugated directly to rhodamine (Pns10–rhodamine) according to the manufacturer’s instructions (Invitrogen).

To determine the specificity of anti-Pns10 antibodies, total proteins were extracted from 1 g RRSV-infected and healthy rice plants. The proteins were separated by SDS-PAGE, transferred to a PVDF membrane and detected on immunoblots using prepared antibodies, as described previously (Spinelli et al., 2006).

Baculovirus expression of the non-structural proteins of RRSV. A baculovirus system was used to express the three non-structural proteins of RRSV, Pns6, Pns7 and Pns10, as described previously (Wei et al., 2006b). Recombinant baculovirus vectors containing Pns6 and Pns7 fused to a 6×His tag (Pns6–His and Pns7–His) and Pns10 fused to a Strep Tag II (Pns10–Strep) were introduced into DH10Bac (Invitrogen) for transposition into the bacmid. Recombinant bacmids were transfected into Sf9 cells in the presence of Cellfectin (Invitrogen) for transposition into the bacmid. Recombinant bacmids containing Pns6–His or Pns7–His were stained with rabbit anti-6×His tag polyclonal antibody (Abcam) and rhodamine-conjugated anti-rabbit IgG antibody (Sigma) as the secondary antibody. Cells infected with recombinant baculoviruses containing Pns6–His or Pns7–His were stained with rabbit anti-6×His tag polyclonal antibody (Abcam) and rhodamine-conjugated anti-rabbit IgG antibody (Sigma) as the secondary antibody. Cells infected with recombinant baculoviruses containing Pns10–Strep were stained with anti-Strep Tag II mAb (IBA) and FITC-conjugated anti-mouse IgG (Sigma) as the secondary antibody. Samples were examined under a Leica TCS SP5 inverted confocal microscope, as described previously (Wei et al., 2006a, b).

Immunofluorescence staining of the internal organs of BPHs after acquisition of virus. Second-instar nymphs of BPHs were allowed a 2-day AAP on rice plants infected with RRSV. On different days after the AAP, internal organs from BPHs were removed, fixed in 4% paraformaldehyde and processed for immunofluorescence microscopy, as described previously (Wei et al., 2006a, b). RRSV particles were stained with virus–FITC. Viroplasms were stained with Pns10–rhodamine. Actin was stained with phalloidin–rhodamine or phalloidin–Alexa Fluor 647 carboxylic acid (Invitrogen). The particles were then imaged by a Leica TCS SP5 inverted confocal microscope, as described previously (Wei et al., 2006a, b).

Electron microscopy. The salivary glands of infected BPHs were fixed, dehydrated and embedded, as described previously (Wei et al., 2006a, b). Cell sections were then incubated with antibodies against Pns10 and immunogold labelled with goat antibodies against rabbit IgG that had been conjugated to 15 nm gold particles (Sigma) (Wei et al., 2006a, b).

dsRNA production. A DNA fragment spanning a 793 bp segment of the Pns10 gene of RRSV was amplified by PCR using forward primer 5′-ATTCTCTAGAAGCTTAAATCGGAGAAGCTTACCTGAGC-3′ and reverse primer 5′-ATTCTCTAGAAGCTTAAATCGGAGAAGCTTACCTGAGC-3′, both possessing a T7 promoter (shown in italic) at the 5′ end. A DNA fragment spanning a 717 bp segment of the gfp gene was amplified by PCR using forward primer 5′-ATTCTCTAGAAGCTTAAATCGGAGAAGCTTACCTGAGC-3′ and reverse primer 5′-ATTCTCTAGAAGCTTAAATCGGAGAAGCTTACCTGAGC-3′, both possessing a T7 promoter (shown in italic) at the 5′ end. PCR products were used for dsRNA synthesis according to the protocol for the T7 Ribonuclease RNAi System kit (Promega).

Examination of the effect of dsRNAs on virus infection in the insect vectors. dsRNAs targeting viral genes were delivered to BPHs using a membrane feeding approach (Chen et al., 2010). Briefly, second-instar nymphs of BPHs were fed a diet of 0.5 μg dsRNA μ⁻1 diluted in 10% sucrose on a membrane for 1 day, allowed a 2-day AAP on RRSV-infected rice plants and then fed on uninfected rice seedlings. The transcript levels of viral genes for the targeted non-structural protein Pns10 and major outer-capsid protein P8 were determined by RT-PCR (Upadhyaya et al., 1996, 1997). In addition, internal organs from BPHs receiving dsRNAs or diet alone were fixed and processed for immunofluorescence microscopy, as described elsewhere (Wei et al., 2006a, b).

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