Interaction between non-structural protein Pns10 of rice dwarf virus and cytoplasmic actin of leafhoppers is correlated with insect vector specificity

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Many insect-transmissible pathogens are transmitted by specific insect species and not by others, even if the insect species are closely related. The molecular mechanisms underlying such strict pathogen–insect specificity are poorly understood. Rice dwarf virus (RDV), a plant reovirus, is transmitted mainly by the leafhopper species Nephotettix cincticeps but is transmitted ineffectively by the leafhopper Recilia dorsalis. Here, we demonstrated that virus-containing tubules composed of viral non-structural protein Pns10 of RDV associated with the intestinal microvilli of N. cincticeps but not with those of R. dorsalis. Furthermore, Pns10 of RDV specifically interacted with cytoplasmic actin, the main component of microvilli of N. cincticeps, but not with that of R. dorsalis, suggesting that the interaction of Pns10 with insect cytoplasmic actin is consistent with the transmissibility of RDV by leafhoppers. All these results suggested that the interaction of Pns10 of RDV with insect cytoplasmic actin may determine pathogen–vector specificity.

The vector specificity of plant viruses is common in nature, and describes the specific relationship between virus and vector (Hogenhout et al., 2008; Ammar, et al., 2009). Rice dwarf virus (RDV), a phytoreovirus in the family Reoviridae, is transmitted mainly by the leafhopper species Nephotettix cincticeps in a persistent-propagative manner (Honda et al., 2007). Our preliminary test indicated that another leafhopper species, Recilia dorsalis, was inefficient at transmitting RDV. Furthermore, our preliminary test also indicated that a continuous cell line of R. dorsalis displayed a low susceptibility to RDV infection compared with a cell line of N. cincticeps. The specificity of a plant virus for its vector can be explained by transmission barriers posed by different tissues in the insects (Hogenhout et al., 2008; Jia et al., 2012a; Markham et al., 1984). RDV encounters multiple barriers in its path from the intestine to the salivary gland of N. cincticeps (Chen et al., 2011). When ingested by N. cincticeps feeding on infected plants, RDV initially infects the filter chamber epithelium. Following assembly of progeny virions, RDV spreads to adjacent organs such as the anterior midgut, crosses the basal lamina of the midgut epithelium into the visceral muscles and then moves into the haemolymph and finally into the salivary glands, from which it can be introduced into rice hosts (Chen et al., 2011). Our recent study showed that RDV can exploit virus-containing tubules composed of viral non-structural protein Pns10 to facilitate virus spread within N. cincticeps (Chen et al., 2012). Furthermore, a Pns10-deficient isolate of RDV failed to be transmitted by N. cincticeps (Pu et al., 2011). These results suggested that Pns10 of RDV may act as a viral determinant for transmission. Thus, it is reasonable to assume that the specific interaction between Pns10 of RDV and leafhopper proteins would contribute to vector transmission specificity. Here, we revealed that the specific interaction between Pns10 of RDV and insect cytoplasmic actins may determine insect vector specificity.

In the present study, stock cultures of N. cincticeps and R. dorsalis, collected originally from Fujian Province in eastern China, were used for laboratory experiments. RDV was maintained on rice plants via transmission by N. cincticeps. To compare the ability of N. cincticeps and R. dorsalis to transmit RDV, second-instar nymphs were allowed a 2-day acquisition access period (AAP) on diseased rice plants and were then placed on healthy rice seedlings. At 20 days after first access to diseased plants, an individual adult insect was fed on a healthy rice seedling in test tubes for 2 days. The insect intestines and salivary glands were then examined by an immunofluorescence assay for the presence of viral antigen with viral particle-specific IgG directly conjugated to Alexa Fluor 633 and with the actin-specific dye phalloidin conjugated to FITC (Invitrogen), as described previously (Wei et al., 2006; Jia et al., 2012b). Inoculated seedlings were grown to allow the development of symptoms of infection. Immunofluorescence microscopy showed that RDV extensively infected the intestine and salivary gland of N. cincticeps (Fig. 1a). The viruliferous N. cincticeps was able to transmit the virus to rice seedlings, as judged by the appearance of symptoms.
on rice plants (Table 1). In contrast, *R. dorsalis* was rarely detected to be viruliferous, and viral infection was restricted to the small infection regions of the filter chamber (Fig. 1b). As expected, the viruliferous *R. dorsalis* failed to transmit RDV (Table 1). Thus, the inability of *R. dorsalis* to transmit RDV may be caused by the restriction
Table 1. Comparison of the ability of *N. cincticeps* and *R. dorsalis* to transmit RDV after 2 day AAP on diseased rice plants or after microinjection with purified viruses

<table>
<thead>
<tr>
<th>Method</th>
<th>Insect</th>
<th>No. viral antigen-positive insects that transmitted RDV to rice seedlings in experiment no.*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Feeding</td>
<td><em>N. cincticeps</em></td>
<td>15</td>
</tr>
<tr>
<td></td>
<td><em>R. dorsalis</em></td>
<td>0</td>
</tr>
<tr>
<td>Microinjection</td>
<td><em>N. cincticeps</em></td>
<td>37</td>
</tr>
<tr>
<td></td>
<td><em>R. dorsalis</em></td>
<td>10</td>
</tr>
</tbody>
</table>

*Data are for 50 insects.

of viral spread from the initially infected intestinal epithelium of *R. dorsalis*, and thus the virus could not spread to the salivary glands for subsequent transmission.

To establish that RDV indeed cannot pass through the intestine of *R. dorsalis*, which would prevent its spread, 50 nl purified virus (10 mg ml⁻¹) was microinjected into the haemocoel of third-instar nymphs of leafhoppers, as described previously (Omura *et al.*, 1982). The insects were kept on healthy rice plants for 20 days and then confined individually for 2 days to inoculate the rice seedlings. Our preliminary test indicated that there was no significant difference in transmission efficiency for the second- or third-instar nymphs of leafhoppers after a 2 day AAP on diseased rice plants, but the third-instar nymphs were more suitable for microinjection experiments. The salivary glands of insects were then examined by an immunofluorescence assay for the presence of viral antigen, as described above. Immunofluorescence microscopy indicated that RDV infected the salivary glands of injected *N. cincticeps* and *R. dorsalis* (Fig. 1c). These insects were also able to transmit RDV (Table 1). The results confirmed that the movement of RDV across the intestine into the leafhopper haemolymph was a significant barrier to transmission.

We then determined whether there was protein–protein interaction between Pns10 of RDV and actin of the leafhoppers. Because actin within the microvilli of the insect intestine is cytoplasmic actin (Popova-Butler & Dean, 2009), we firstly amplified the genes of the cytoplasmic actins of *N. cincticeps* and *R. dorsalis* by a reverse transcription-PCR method. The identity of the deduced amino acid sequences of the two cytoplasmic actin genes from *N. cincticeps* and *R. dorsalis* was 99.2%, with amino acids differing only at positions 11, 262 and 348 (Fig. 2a, b). Because Pns10 of RDV is a membrane-associated protein (Wei *et al.*, 2008; Liu *et al.*, 2011), we used the DUAL membrane system (Dualsysystems Biotech), a split-ubiquitin membrane-based yeast two-hybrid system, to detect the interaction between Pns10 of RDV and actins of *N. cincticeps* or *R. dorsalis*. Briefly, the Pns10 gene of RDV and two actin genes of *N. cincticeps* and *R. dorsalis* were cloned into the bait vector pBT-STE and the prey vector pPR3 N, respectively. The recombinant plasmids pBT-STE-Pns10 and pPR3-N-Actin-NC, pBT3-STE-Pns10 and pPR3-N-Actin-RD were used to co-transform yeast strain NMY51. The transformants were confirmed on SD-Trp-Leu-His-Ade plates for 3–4 days at 30 °C. This yeast two-hybrid assay demonstrated that Pns10 specifically interacted with cytoplasmic actin of *N. cincticeps* but failed to interact with that of *R. dorsalis* (Fig. 2c). On the basis of these analyses, we deduced that the loss in association of Pns10 with the microvilli from *R. dorsalis* was directly caused by the lack of interaction between Pns10 and the cytoplasmic actin from *R. dorsalis*.

Because only three amino acids differed between the two cytoplasmic actin genes of *N. cincticeps* and *R. dorsalis*, we then determined which amino acid was responsible for the specific interaction of RDV Pns10 and cytoplasmic actin of leafhoppers. We substituted the amino acids at positions of 11, 262 or 348 of the cytoplasmic actin gene of *R. dorsalis* by fusion PCR according to the corresponding gene sequence of *N. cincticeps* (Fig. 2b). The primers carrying the mutations at nt 31–33 (ATC→GTG), 784–786 (ATG→CTC) or 1042–1044 bp (GCC→GTC) of the DNA
## Fragment for the Cytoplasmic Actin of *R. dorsalis*

Amino acid sequence alignments of the cytoplasmic actin genes of *N. cincticeps* and *R. dorsalis*. Sequences were aligned using DNAMAN 7.0 software with default parameters. Identical residues between sequences of *R. dorsalis* and *N. cincticeps* are indicated with dashes. Yeast two-hybrid analysis for interactions of Pns10 with cytoplasmic actins and mutants. Transformants were grown on SD-Trp-Leu-His-Ade plates. +, Positive control (pTSU2-APP + pNubG-Fe65); –, negative control (pTSU2-APP + pR3N); *N. cincticeps*, pBT-STE-Pns10 + pPR3-N-Actin-NC; *R. dorsalis*, pBT-STE-Pns10 + pPR3-N-Actin-RD; 262m, pBT-STE-Pns10 + pPR3-N-262m; 11m, pBT-STE-Pns10 + pPR3-N-11m; 348m, pBT-STE-Pns10 + pPR3-N-348m.

### Yeast Two-Hybrid Analysis

Yeast two-hybrid analysis for interactions of Pns10 with cytoplasmic actins and mutants. Lysates from *Escherichia coli* strain BL21 cells expressing GST–Pns10 were incubated with cell lysate expressing His–actin of *N. cincticeps* and *R. dorsalis* and the three mutants. GST pull-down products were analysed by Western blotting (WB); antibody to GST was used to detect Pns10 and antibody to His to detect bound proteins. *N. cincticeps*, His–actin–*N. cincticeps*; *R. dorsalis*, His–actin–*R. dorsalis*; 262m, His–262m; 11m, His–11m; 348m, His–348m.

### GST Pull-Down Assay

GST pull-down assay to detect interactions of Pns10 with cytoplasmic actins and mutants. Lysates from *Escherichia coli* strain BL21 cells expressing GST–Pns10 were incubated with cell lysate expressing His–actin of *N. cincticeps* and *R. dorsalis* and the three mutants. GST pull-down products were analysed by Western blotting (WB); antibody to GST was used to detect Pns10 and antibody to His to detect bound proteins. *N. cincticeps*, His–actin–*N. cincticeps*; *R. dorsalis*, His–actin–*R. dorsalis*; 262m, His–262m; 11m, His–11m; 348m, His–348m.

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Fig. 2. Pns10 specifically interacts with cytoplasmic actin of *N. cincticeps* but not with that of *R. dorsalis*. (a) Amino acid sequence alignment of the cytoplasmic actin genes of *N. cincticeps* and *R. dorsalis*. Sequences were aligned using DNAMAN 7.0 software with default parameters. Identical residues between sequences of *R. dorsalis* and of *N. cincticeps* are indicated with dashes. (b) Schematic representations of cytoplasmic actin mutants of *R. dorsalis* generated by site-directed mutagenesis. (c) Yeast two-hybrid analysis for interactions of Pns10 with cytoplasmic actins and mutants. Transformants were grown on SD-Trp-Leu-His-Ade plates. +, Positive control (pTSU2-APP + pNubG-Fe65); –, negative control (pTSU2-APP + pRR3N); *N. cincticeps*, pBT-STE-Pns10 + pPR3-N-Actin-NC; *R. dorsalis*, pBT-STE-Pns10 + pPR3-N-Actin-RD; 262m, pBT-STE-Pns10 + pPR3-N-262m; 11m, pBT-STE-Pns10 + pPR3-N-11m; 348m, pBT-STE-Pns10 + pPR3-N-348m. (d) GST pull-down assay to detect interactions of Pns10 with cytoplasmic actins and mutants. Lysates from *Escherichia coli* strain BL21 cells expressing GST–Pns10 were incubated with cell lysate expressing His–actin of *N. cincticeps* and *R. dorsalis* and the three mutants. GST pull-down products were analysed by Western blotting (WB); antibody to GST was used to detect Pns10 and antibody to His to detect bound proteins. *N. cincticeps*, His–actin–*N. cincticeps*; *R. dorsalis*, His–actin–*R. dorsalis*; 262m, His–262m; 11m, His–11m; 348m, His–348m.

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The results showed that only the mutation at position 262 (Met→Leu, 262m) led to the specific interaction, whilst the reaction remained negative with the mutations at positions 11 (Ile→Val, 11m) and 348 (Ala→Val, 348m) (Fig. 2c).
To further confirm this interaction, the DNA fragments for the WT and three mutants of leafhopper cytoplasmic actins were cloned into plasmid vector pDEST17 to express His fusion proteins as the preys, and the Pns10 gene was cloned into pGEX-3X to construct a plasmid expressing glutathione S-transferase (GST) fusion protein as the bait. As described previously (Jia et al., 2014), the interaction was assayed using GST pull-down methodology, and binding between GST and His fusion proteins was detected by Western blotting. As shown in Fig. 2(d), positive interactions of Pns10 with the WT of cytoplasmic actin of N. cincticeps and mutant 262m were confirmed, but not with the WT of cytoplasmic actin of R. dorsalis and the 11m or 348m mutant. These results revealed that leucine at position 262 of leafhopper cytoplasmic actin plays a key role in determining the interaction of RDV Pns10 with the cytoplasmic actin from N. cincticeps and lack of interaction with actin from R. dorsalis.

In conclusion, we demonstrated that viral non-structural protein Pns10 of RDV specifically interacts with the cytoplasmic actin of the virus-transmitting leafhopper N. cincticeps but not with that of the non-transmitting leafhopper R. dorsalis, suggesting that the interaction of RDV Pns10 with insect cytoplasmic actin is consistent with the transmissibility of RDV by the leafhoppers. Pns10 tubules, which interacted with cytoplasmic actin within the microvilli of N. cincticeps, are able to pass through the intestinal microvilli, facilitating virus spread in the body of its insect vector (Chen et al., 2012). In contrast, the lack of interaction of RDV Pns10 with cytoplasmic actin within the microvilli of R. dorsalis restricted the virus in the initially infected epithelium in the intestine. Therefore, the interaction between RDV Pns10 and insect cytoplasmic actin may determine the insect vector specificity. We further determined that a change in one amino acid in leafhopper cytoplasmic actin may be enough to alter the insect vector specificity for RDV. Similarly, the interaction of antigenic membrane protein of a phytoplasma and leafhopper actin is correlated with the phytoplasma-transmitting capability of leafhoppers (Suzuki et al., 2006). Due to the lack of reverse-genetics and transgenic expression systems for hemipteran insects, direct evidence to support the conclusion that the interaction of pathogens with insect actin determines insect vector specificity is still not available.

Plant reoviruses, plant rhabdoviruses, tospoviruses and tenuiviruses are transmitted by insect vectors in a persistent-propagative manner (Hogenhout et al., 2008). These viruses induce the formation of virus inclusions composed of viral non-structural proteins to facilitate viral propagation in insect vectors (Hogenhout et al., 2008). In this study, we revealed that the interaction between the non-structural protein of a plant virus and insect actin was correlated with the specificity of the insect vector for the transmitted virus. The ability of viruses to pass through the insect intestine is an important factor in vector determination. Actin filaments are the major component of intestinal microvilli and visceral muscle, which would constitute a substantial barrier to the persistent transmission of viruses. Thus, the specific association of viruses and insect actin suggests that viruses may directly utilize insect actin filaments to overcome the transmission barriers. All these analyses support the conclusion that the interaction of viruses and insect actin may determine insect vector specificity.

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References


