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.
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

**Fig. 1.** Restriction of virus infection in the intestine in *R. dorsalis* is caused by the failure of spread of virus-containing Pns10 tubules. (a–d) Internal organs of *N. cincticeps* or *R. dorsalis* were immunostained for tubules with Pns10-specific IgG–rhodamine (red), for virions with virus-specific IgG–Alexa Fluor 633 (blue) and for actin filaments with phalloidin–FITC (green) and then examined by confocal microscopy. (a) RDV extensively infected the intestine (panel I) and salivary gland (panel II) of *N. cincticeps*. (b) RDV infected the small infection areas of the intestine (panel I) but failed to infect the salivary gland (panel II) of *R. dorsalis*. (c) RDV infected the salivary glands of *N. cincticeps* (panel I) and *R. dorsalis* (panel II) microinjected with purified viruses. (d) Pns10 tubules pass through microvilli of filter chamber epithelium of *N. cincticeps* (panel I) and only accumulate in the cytoplasm of filter chamber epithelium of *R. dorsalis* (panel II). (e) Electron micrographs showing that virus-containing tubules (arrows) are inserted into midgut microvilli in viruliferous *N. cincticeps* (panel I) but are only distributed in the cytoplasm of filter chamber epithelium in viruliferous *R. dorsalis* (panel II). Images in (a–c) were merged with blue fluorescence (viral antigen) and green fluorescence (actin). Images in (d) were merged with red fluorescence (Pns10 tubules), blue fluorescence (viral antigen) and green fluorescence (actin). es, Oesophagus; fc, filter chamber; amg, anterior midgut; mmg, middle midgut; pmg, posterior midgut; hg, hindgut; sg, salivary gland; Mv, microvilli; GL, gut lumen; Ec, epithelial cell; NC, *N. cincticeps*; RD, *R. dorsalis*. Bars, 100 μm (a, b, c); 20 μm (d); 200 nm (e).
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 µg 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 the restriction of virus infection in the intestine was caused by the failure of spread of virus-containing Pns10 tubules in *R. dorsalis*. The intestines from viruliferous *N. cincticeps* and *R. dorsalis* were examined by an immunofluorescence assay with virus-specific IgG–Alexa Fluor 633, Pns10-specific IgG–rhodamine and phalloidin–FITC, as described previously (Chen et al., 2012). Immunofluorescence microscopy indicated that Pns10 tubules completely passed through the actin-based epithelial microvilli of the intestine of *N. cincticeps*, as shown previously (Chen et al., 2012), but did not cross those of *R. dorsalis* (Fig. 1d). To confirm these results, the intestines from viruliferous *N. cincticeps* and *R. dorsalis* were examined by electron microscopy, as described previously (Chen et al., 2012). We observed that virus-containing tubules were associated with the midgut microvilli in the viruliferous *N. cincticeps* (Fig. 1e). The tubules were also observed in a limited number of epithelial cells of the filter chamber in the viruliferous *R. dorsalis* but were never found in association with the microvilli (Fig. 1e). All these results suggested that the failure of Pns10 tubules to associate with the actin-based intestinal microvilli may inhibit the efficient spread of RDV in *R. dorsalis*.

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 (Dualsystems 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 11, 262 or 348 of the cytoplasmic actin gene of *R. dorsalis* with 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

<table>
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<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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<tr>
<td>Feeding</td>
<td><em>N. cincticeps</em></td>
<td>15                        18                        17</td>
</tr>
<tr>
<td></td>
<td><em>R. dorsalis</em></td>
<td>0                           0                           0</td>
</tr>
<tr>
<td>Microinjection</td>
<td><em>N. cincticeps</em></td>
<td>37                        31                        33</td>
</tr>
<tr>
<td></td>
<td><em>R. dorsalis</em></td>
<td>10                        9                          11</td>
</tr>
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*Data are for 50 insects.*

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.
fragment for the cytoplasmic actin of *R. dorsalis* were designed to substitute the corresponding amino acids at positions 11 (Ile→Val), 262 (Met→Leu) or 348 (Ala→Val) (Fig. 2b). The PCR products were cloned into the prey vector pPR3 N and then used in the yeast two-hybrid assay described above. 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).

<table>
<thead>
<tr>
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<th><em>N. cincticeps</em></th>
<th><em>R. dorsalis</em></th>
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<tr>
<td>11m</td>
<td>ATC→GTG</td>
<td>ATG→CTC</td>
</tr>
<tr>
<td>262m</td>
<td>GCC→GTC</td>
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<td>348m</td>
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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 *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.
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**


