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A mitochondrial membrane protein is a target for rice ragged stunt virus in its insect vector

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ABSTRACT

Rice ragged stunt virus (RRSV; *Reoviridae*) is exclusively transmitted by the brown planthopper *Nilaparvata lugens* in a persistent-propagative manner. It is understood that RNA viral proliferation is associated with the intracellular membranes of the insect host cells. However, the molecular mechanisms of the interaction between the RRSV proliferation and the intracellular membranes remain essentially unknown. It will be of great interest to determine whether RRSV protein(s) directly interact with intracellular membrane components of its host cells. In this study, we identified a RRSV nonstructural protein Pns10 interacting with a host oligomycin-sensitivity conferral protein (OSCP) using yeast two-hybrid system. The interaction between RRSV Pns10 and *N. lugens* OSCP was verified by a glutathione S-transferase pull-down assay. Confocal miscopy revealed colocalization of these two proteins in the cytoplasm of the salivary gland cells during the viral infection. The virions were further detected in the mitochondria under confocal miscopy and transmission electron microscopy combined with western blotting assay. This is the first observation that RRSV protein has a direct link with mitochondria. Suppressing *OSCP* gene expression by RNA interference notably decreased the viral loads in RRSV-infected insects. These findings revealed novel aspects of a viral protein in targeting the host mitochondrial membrane and provide insights concerning the mitochondrial membrane protein-based virus proliferation mode in the insect vector.

1. Introduction

Rice ragged stunt virus (RRSV) is a member of the genus *Oryzavirus* in the family *Reoviridae* and causes rice ragged stunt disease. The disease is very destructive in the major rice-producing regions, especially in South East Asia and southern China (Hiraguri et al., 2015; Hoang et al., 2011). RRSV is transmitted to rice plant by brown planthopper *Nilaparvata lugens*, one of the most devastating insect pests of rice (Hibino et al., 1977). *N. lugens* acquires the virus by sucking the sap of infected rice plants. RRSV first enters epithelial cells of the insect midgut, then disperses throughout visceral muscles of midgut and hindgut, and finally reaches salivary glands (Jia et al., 2012). We previously reported a dynamic process of RRSV proliferation in salivary glands of *N. lugens* (Huang et al., 2015). However, the molecular mechanisms underlying the proliferation of this virus in salivary glands of its insect vector is not clear.

N. lugens transmits RRSV in a persistent-propagative manner and the insect itself is a host for viral replication. Intracellular membranes are critical for replication of many RNA viruses. Positive-sense RNA viruses, such as flock house virus (FHV; *Nodaviridae*), nodamura virus (NoV; *Nodaviridae*) that primarily infect insects, have established specific associations with mitochondrial membranes of infected cells. The viral infection induces mitochondrial membrane rearrangements and generates mitochondrial membrane-based RNA replication in host cells (Gant et al., 2014; Miller et al., 2001; Short et al., 2016). Viral proteins such as FHV and NoV RNA-dependent RNA polymerases (RdRp) interact with the outer mitochondrial membranes as an integral membrane protein and are essential for the viral replication (Gant et al., 2014; Miller and Ahlquist, 2002; Miller et al., 2003). Concerning double-stranded RNA (dsRNA) viruses, it was reported that rice gall dwarf virus (RGDV; *Reoviridae*), transmitted by the leafhopper *Nephotettix cincticeps*, was localized on surface of mitochondria of infected host cells during virus replication and assembly (Wei et al., 2011), indicating the importance of mitochondria (of yet unexplained molecular basis) in viral localization and activity during proliferation in the insect host.

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The RRSV genome comprises ten linear dsRNA segments (S1 to S10) that encode seven structural proteins (P1, P2, P3, P4A, P5, P8B and P9) and three nonstructural proteins (Pns6, Pns7 and Pns10) (Hagiwara et al., 1986; Upadhyaya et al., 1996, 1997, 1998; Jia et al., 2012). Pns10 constitutes a major viral component of the viroplasms (also known as inclusion or viral factories) in the cytoplasm of the infected insect vector, *N. lugens* (Jia et al., 2012). Viroplasms are thought to be the RNA viral replication and assembly sites, making virus proliferation rapid and effective, and also protecting viral genome from the host immune defense and preventing degradation by the gene-silencing machinery (Fernández-de-Castro and Risco, 2014). Several nonstructural proteins of plant reoviruses share similar functional properties; for example, P9-1 of Mal de Río Cuarto virus (MRCV) transmitted by planthoppers in a persistent-propagative manner, is an essential viral factor for viroplasm formation in the salivary gland cells of its insect vectors (Maroniche et al., 2010). Accordingly, P9-1 proteins of rice black-streaked dwarf virus and southern rice black-streaked dwarf virus, two closely related viruses of the genus *Fijivirus*, are the major constituents of viroplasm matrix and are required for viral replication in their insect vectors, the small brown planthopper *Laodelphax striatellus* and the white-backed planthopper *Sogatella furcifera*, respectively (Akita et al., 2012; Jia et al., 2012; Sun et al., 2013). The utilization of the host cell machinery implies that viruses may interact with as-yet-unknown host components. RRSV Pns10 possesses ATPase activity (Shao and Gong, 2004). This characteristic provides a possibility that Pns10 may be involved in host energy machinery during viral replication. To clarify this situation, we employed the RRSV Pns10 as a bait to screen a salivary gland-specific yeast-two hybrid (Y2H) library because this tissue is vital for RRSV proliferation and is an essential link for better understanding of insect vector–virus–plant host interactions. An oligomycin-sensitivity conferral protein (OSCP), a membrane component of the mitochondrial F_oF_1 proton ATP synthase/ATPase (F-type H^+-ATPase) was identified to bind with Pns10. The interaction between OSCP and Pns10 was verified by GST-pull down assay and immunofluorescence staining. The suppression of *OSCP* gene expression significantly reduced the viral loads in *N. lugens*. Our results establish a link between RRSV nonstructural protein Pns10 and the mitochondrial membrane component OSCP, which may regulate the viral propagation in the salivary glands of its insect host, *N. lugens*.

2. Material and methods

2.1. Insects and viruses

The *N. lugens* populations were originally collected from a rice field in Zhejiang University, Hangzhou, China. The insects were maintained on fresh rice seedlings (*Oryza sativa* strain Xiushui 134) at 26 ± 0.5 °C with 50 ± 5% humidity under a 16/8 h (light/dark) photoperiod as previously described (Huang et al., 2016). RRSV was kindly provided by Prof. Tai-Yun Wei of the Institute of Plant Virology of Fujian Agricultural and Forestry University and Prof. Xu-Dong Zhu of the China National Rice Research Institute and maintained in the rice Xiushui 134 strain.

2.2. Construction of bait vector

Viral RNA was extracted from RRSV-infected *N. lugens* nymphs using Trizol reagents (TaKaRa, Dalian, China). Following reverse transcription, PCR was performed to amplify an ORF region of S10 (GenBank accession no. NP_620539) using a pair of primers with *Noc*I/*BamH*I restriction site (Supplementary file: Table S1). PCR products and pGBKT7 DNA-BD cloning vector (Clontech, Mountain View, CA, USA) were digested with *Noc*I/*BamH*I at 37 °C for 2 h and purified using an AxyPrep DNA Gel Extraction Kit (Axygen, Union City, CA, USA). The bait vector was generated by ligating the PCR fragment into the pGBKT7 DNA-BD cloning vector at 4 °C overnight and transforming into *Saccharomyces cerevisiae* Y2HGold yeast strain. Auto-activation and toxicity assays of bait protein were performed according to the manufacturer's protocol.

2.3. Construction and screening the Y2H library of N. lugens salivary glands

The *N. lugens* salivary glands were dissected and washed in a phosphate-buffered saline (PBS) solution (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 and 1.47 mM KH_2PO_4 at pH 7.4). Total RNA was extracted from the salivary glands using Trizol reagent (TaKaRa). A cDNA library was constructed using a Matchmaker Gold Yeast Two-Hybrid System (Clontech). Briefly, the total RNA sample from the salivary glands was used as the starting material for reverse transcription. Synthesis of cDNA was conducted by long-distance PCR using a SMART cDNA Library Construction Kit (Clontech). Size fractionation to move the cDNAs less than 200 base pair was performed with a Trimmer-Direct cDNA Normalization Kit (Evrogen, Moscow, Russia). The purified cDNAs and *Sma*I-linearized pGADT7–Rec were cotransformed into *S. cerevisiae* Y187 yeast strain (Clontech). The generated transformants were spread on SD/Leu plates and incubated at 30 °C. All transformants were harvested and the library titer was determined by spreading 100 μL of a $1:10^2$, $1:10^3$ and $1:10^4$ dilutions on SD/Leu plates. Library screening was conducted by mating a 1-mL aliquot of the Y187 library with 5 mL of Y2HGold cells transformed with bait vector. Positive colonies were selected on quadruple dropout medium (SD/−adenine/−histidine/−leucine/−tryptophan/X-α-galactosidase/Aureobasidin A). The pGBKT7–Lam, empty pGBKT7 and empty pGADT7 were used as negative controls; pGADT7–T-antigen and pGBKT7–p53 were used as positive controls.

2.4. GST pull-down assay

The ORF sequences of RRSV S9 and S10 were amplified and cloned into PET-28a for fusion expression with His-tag; the ORF regions of *N. lugens OSCP* and *Aequorea victoria GFP* gene were cloned into PGEX-6P-1 for fusion expression with GST. The specific primers are shown in Supplementary file: Table S1. The recombinant proteins were expressed in *Escherichia coli* strain Transetta (TransGen Biotech, Beijing, China) by inducing with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) at 37 °C for 2 h. For pull-down assays, the GST-fused OSCP or GFP proteins were bound to glutathione-sepharose beads (GE Healthcare, Mickleton, NJ, USA) for 2 h, followed by centrifuging at 100 × *g* for 5 min. The supernatants were discarded and the beads were blocked with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) for 2 h. Then, the His-fused P9 or Pns10 proteins were loaded to beads and incubated at 4 °C overnight. The proteins were washed five times with PBST (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4 and 1% Triton-100 at pH 7.4) and eluted with elution buffer (50 mM Tris-HCl and 10 mM reduced glutathione at pH 8.0). The proteins were separated by SDS-PAGE and probed with an antibody against the His-tag (HuaAn Biotech, Hangzhou, China) at a dilution of 1:10,000. Detection was achieved with a goat anti-rabbit IgG-conjugated horseradish peroxidase at 1:1000 dilution (Jackson ImmunoResearch, West Grove, PA, USA). Western blots were imaged using a chemiluminescence detection kit (Bio-Rad, Hercules, CA, USA) on a Molecular Imager® ChemiDoc™ XRS+ System (Bio-Rad).