Plant viruses in the genera Tospovirus, Tenuivirus, Rhabdovirus, Phytoreovirus and Fijivirus are transmitted by their respective insect vectors in a persistent propagative manner (Ammar et al., 2009; Hogenhout et al., 2008). Insights into replication of these viruses in their vectors will help in development of new strategies to control the transmission of the viruses by the vectors. Continuous cultures of cells derived from insect vectors are powerful tools for studying the replication cycle of persistent propagative plant viruses because they are more uniform than primary cultures and thus provide more consistent data (Creamer, 1993). Continuous cultures of leafhopper cells provide a good system to study the replication cycle of phytoreoviruses in their insect vectors (Kimura & Omura, 1988; Omura & Kimura, 1994; Wei et al., 2006, 2007).

Rice stripe virus (RSV), the type species of the genus Tenuivirus, has filamentous ribonucleoprotein particles (RNPs) that contain nucleoprotein, RNA-dependent RNA polymerase and four negative-sense single-stranded RNA segments that encode seven proteins (Toriyama, 1986; Falk & Tsai, 1998; Wei et al., 2009; Xiao et al., 2010). RSV is transmitted by the small brown planthopper (SBPH; Laodelphax striatellus Fallén) in a persistent propagative manner (Toriyama, 1986; Wei et al., 2009). Currently, the mechanism that enables efficient multiplication of RSV in its vector is unknown due to the lack of SBPH cell lines. In previous attempts to establish primary cell cultures, only a small number of cells migrated occasionally from the embryonic explants dissected from SBPH eggs, and eventually the subculture died off (Mitsuhashi, 1969, 2001; Yamada et al., 1970). The present report describes the first successful establishment of continuous cultures of SBPHs for the study of RSV replication.

The SBPH cell line was established by adapting the protocol described by Kimura & Omura (1988). Non-viruliferous SBPHs eggs that had been oviposited 8 days earlier in leaf sheaths of rice plants were surface-sterilized with 70% ethanol and then rinsed in sterile Tyrode’s solution. Embryonic fragments were dissected from the eggs in Tyrode’s solution, treated with 0.25% trypsin in Tyrode’s solution for 15 min and then incubated with Kimura’s insect medium at 25 °C. The medium was changed every 7–10 days. New cells started to migrate from the SBPH embryonic explants within 48 h after the cultures were set up. Fig. 1(a) shows the epithelial-like cells that migrated from the embryonic explants to form monolayers by 12 days after preparation. When the cells grew almost confluently in the dish about 1 month after initiation, they were transferred to new dishes and again cultured to confluence (about 2 weeks after subculture). Such cells were then transferred to culture flasks for further subculturing. After 30 passages of subculturing at 10 day intervals, the dominant cell type in the established SBPH cell line was epithelial-like, approximately 25–50 μm in diameter, with dense cytoplasm around the nuclei, which were approximately 7.5–20 μm in diameter (Fig. 1b). This is the first report of the establishment of a continuous planthopper cell line.
Such SBPH vector cell monolayers (VCMs) were used for RSV infection. RSV inoculum was prepared from infected plants, essentially as described previously (Kimura & Omura, 1988; Omura & Kimura, 1994). Briefly, infected leaves were surface-sterilized with 70% ethanol and then rinsed with sterile distilled water. The sterilized leaves were ground in a solution of 0.1 M histidine that contained 0.01 M MgCl₂, pH 6.2 (His-Mg) and centrifuged. The supernatant was then used as the inoculum. VCMs were washed twice with His-Mg and then inoculated with RSV. After 2 h, VCMs were washed twice with His-Mg and covered with Kimura’s insect medium. VCMs were cultured to confluence in the culture flasks and then subcultured at intervals of 10 days through 10 passages. We then analysed the accumulation of RNP and non-structural protein NS3 of RSV in virus-infected VCMs by SDS-PAGE and immunoblotting with RNP- and NS3-specific antibodies, respectively, as reported previously (Zhang et al., 2008; Takahashi et al., 1991). Our results showed that RNP and NS3 of RSV accumulated in virus-infected VCMs even after 10 passages (Fig. 2a, b).

To further observe the subcellular localization of RNP and NS3, virus-infected VCMs fixed on coverslips with 4% paraformaldehyde were probed with RNP-specific antibodies conjugated to FITC (Invitrogen; RNP-FITC Abs) and NS3-specific antibodies conjugated to rhodamine (Invitrogen; NS3-rhodamine Abs) and then examined by confocal microscopy, as described previously (Wei et al., 2006, 2007). No specific fluorescence was detected in uninfected cells after incubation with either RNP-FITC or NS3-rhodamine Abs (Fig. 2c, d). In RSV-infected VCMs, RNP and NS3 were detected as discrete punctate inclusions throughout the cytoplasm (Fig. 2c, d). When the images for RNP and for NS3 were merged, RNP and NS3 co-localized with the viral inclusions (Fig. 2d), indicating that NS3 was associated with RSV RNPs during viral infection in VCMs.

To confirm our observations, we examined infected VCMs on coverslips using immunoelectron microscopy. VCMs were fixed, dehydrated and embedded as described previously (Jia et al., 2012; Wei et al., 2006). Cell sections were then incubated with RNP- and NS3-specific antibodies, respectively, as primary antibodies, followed by treatment with goat anti-rabbit IgG as secondary antibodies that had been conjugated to 15 nm gold particles, as described previously (Jia et al., 2012; Wei et al., 2006). Our results showed that both RNP and NS3 antibodies specifically reacted with the amorphous inclusion bodies in the cytoplasm of virus-infected VCMs (Fig. 2e, f), indicating that RNP and NS3 had the same subcellular distribution, as indicated by confocal microscopy (Fig. 2d). Our results confirmed previous data that RNP antibodies of RSV are specific for the amorphous inclusion bodies in the bodies of viruliferous SBPHs (Suzuki et al., 1992). Because RNPs are the infectious unit of RSV (Toriyama, 1986), the association of NS3 with RNPs suggested that NS3 may play a crucial role in viral replication or assembly in cultured vector cells. Furthermore, our observations using transmission electron and confocal microscopy showed that RSV infection of the SBPH cell line did not affect cellular structures and morphology (Fig. 2), suggesting that the SBPH cell line supported a non-cytopathic, persistent infection of RSV. Evidently, mechanisms have evolved in the vector cells to allow efficient replication of RSV without any significant pathology.

To confirm whether NS3 plays a crucial role in viral replication or assembly, we used an RNA interference (RNAi) strategy to knock down the expression of NS3 in virus-infected VCMs and then analysed its effect on the formation of viral inclusions and viral infection. The PCR products of a 633 bp segment of RSV NS3 gene (Xiong et al., 2009) and a 717 bp segment of green fluorescence

Fig. 1. Light micrographs of primary and continuous cultures of SBPH cells in vitro. (a) Epithelial-like cells that migrated from the embryonic explants (Ex) of SBPH to form a monolayer by 12 days after preparation. (b) Monolayer culture of SBPH cell line after 30 passages showing the predominantly epithelial-like cell population. Bars, 20 μm.
protein (GFP)-encoding gene as controls were used for dsRNA synthesis according to the protocol for the T7 Ribomax Express RNAi System kit (Promega). VCMs were transfected with 0.5 μg μl⁻¹ dsRNAs in the presence of Cellfectin (Invitrogen) for 24 h. VCMs were then inoculated with RSV, fixed with 4% paraformaldehyde 5 days later and immunostained with RNP-FITC and NS3-rhodamine Abs. Immunofluorescence showed that the

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**Fig. 2.** The association of viral nonstructural protein NS3 with RNPs of RSV in virus-infected VCMs. (a, b) Western blot analyses for RNP and NS3 of RSV. Protein extracts were separated by SDS-PAGE and detected with RNP-specific (a) or NS3-specific (b) antibodies, respectively. Lanes M, protein marker; lanes 1 and 5, extracts from rice plants infected with RSV; lanes 2 and 6, extracts from viruliferous SBPHs; lanes 3 and 7, extracts from VCMs infected with RSV; lanes 4 and 8, extracts from uninfected VCMs. (c, d) Confocal micrographs of VCMs which were treated with RNP-FITC Abs (green) or with NS3-rhodamine Abs (red). (c) Virus-infected (i) and uninfected (ii) VCMs stained for RNP. The images were merged under the background of transmitted light. Bars, 20 μm. (d) Images with green fluorescence [RNP Abs, (i)], red fluorescence [NS3 Abs, (ii)] and the merged green and red fluorescence [merged, (iii)] from virus-infected VCMs. No specific fluorescence was detected in uninfected VCMs after incubation with NS3-rhodamine Abs (iv). Bars, 5 μm. (e, f) Immunogold labelling of RNP (e) and NS3 (f) in the amorphous inclusion bodies in the cytoplasm of virus-infected VCMs. Bars, 100 nm. Cells were immunolabelled for RNP and NS3 with RNP- and NS3-specific Abs in (e) and (f), respectively, as primary antibodies, followed by treatment with goat anti-rabbit IgG conjugated with 15 nm gold particles as secondary antibodies.
treatment of dsRNAs from the GFP gene (dsGFP) did not prevent viral infection or the formation of viral inclusions containing RNPs and NS3 (Fig. 3a). In contrast, the treatment with dsRNAs from the NS3 gene (dsNS3) significantly inhibited viral infection and the accumulation of viral inclusions containing RNPs and NS3 (Fig. 3b). The effects of dsNS3 on the accumulation of RNPs and NS3 of RSV were further analysed by SDS-PAGE and immunoblotting with RNP- and NS3-specific antibodies, respectively, as reported previously (Zhang et al., 2008; Takahashi et al., 1991). As shown in Fig. 3c, transfection with dsNS3 resulted in a marked reduction of the accumulation of RNPs and NS3 in viral-infected VCMs. Because RNAi induced by dsRNA is a sequence-specific gene silencing mechanism (Fire et al., 1998; Chen et al., 2012; Jia et al., 2012) and the NS3 gene has no sequence homology with other RSV genes (data not shown), we determined that the knockdown of NS3 expression in VCMs was specifically caused by RNAi induced by dsNS3. Thus, our results indicated that the knockdown of NS3 expression led to the inhibition of the formation of viral inclusions containing NS3, with a significant simultaneous inhibition of the accumulation of RNPs in the VCMs. Due to the association of NS3 with RNPs, these results indicate that NS3 of RSV is essential for RSV infection in its insect vector and may be directly involved in viral replication or assembly. Since RSV NS3 was demonstrated to act as a suppressor of gene silencing in plant host (Xiong et al., 2009), it may have a similar function in insect vector cells.

In conclusion, the establishment of a SBPH cell line that supports a non-cytopathic, persistent infection of RSV opens new opportunities to elucidate the complex relationship between RSV and its insect vector.

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