Transmission of *Rice stripe virus* acquired from frozen infected leaves by the small brown planthopper (*Laodelphax striatellus* Fallen)

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Abstract

Rice stripe disease, caused by *Rice stripe virus* (RSV), is one of the most serious rice diseases in temperate and subtropical regions of the world. Since RSV is not transmissible mechanically, an insect transmission test was the original basis for identification of the viral population and cultivar resistance. A simple, rapid and reliable method is described by which virus-free small brown planthoppers acquired RSV from frozen infected rice leaves and transmitted the virus to healthy rice plants. Of 30 planthoppers tested, 9 insects fed on the frozen infected leaves acquired the virus as shown by an indirect-ELISA. In the transmission tests with a single insect, fed previously on frozen leaves, 5 of 30 plants (16.67%) became infected, compared to 7 of 30 plants (23.33%) became infected when a single insect fed on fresh infected leaves. All rice plants expressing stripe symptoms were identified with the virus by RT-PCR.

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1. Introduction

Rice stripe disease is one of the most serious rice diseases and occurs mainly in temperate and subtropical regions (Toriyama, 1986; Lin et al., 1990). Losses of 20–30% have been observed commonly in *japonica* rice-grown regions in China. Greater losses were found in 2001, 2003 and 2004 in Jiangsu, Shandong and Henan provinces of China due to climatic changes, changes in cultivation practices and loss of variety resistance (Zhou et al., 2004; Y. Zhou, personal communications). This disease is caused by *Rice stripe virus* (RSV), the typical member of the genus Tenuivirus (Ramirez and Haenni, 1994). RSV is transmitted only in the circulative/persistent manner by the small brown planthopper (*Laodelphax striatellus* Fallen) and three other delphacid species, *Unkanodes sapporona*, *U. albifascia* and *Terthron albovittatum* (Toriyama, 1986). As a typical member of the genus Tenuivirus, RSV has filamentous particles which appear folded, branched and supercoiled by electron microscopy and it replicates in both plant and insect vectors (Toriyama, 1983; Francki et al., 1991).

Planting disease-resistant cultivars is the ideal way to control rice viral diseases (Hibino, 1996). Knowledge of the pathogen population structure can help in the selection of disease resistance sources for crop breeding programs. Information on the number of viruliferous planthoppers in a given local population is very important for forecasting and chemical spraying warning schemes for potential crop threats (Kisimoto, 1993; Cai et al., 2003). Since RSV is not transmissible mechanically, insect transmission was the original basis for identification of the viral population and cultivar resistance. Three main approaches have been used to conduct this assay, all based on rendering artificially the insect vector viruliferous and then using the vector to transmit the virus to test plants (Rochow, 1960; for a review, see Nault, 1994). One approach was the direct injection of virus preparations into insects, used most successfully with leafhopper-transmitted plant viruses (Maramorosch, 1955). The second approach involved artificial feeding of insects through membranes on liquid virus preparations, used mostly for insect-transmitted plant viruses (Rochow, 1960). The third approach was feeding the insect vector on fresh virus-infected leaves. The
viruliferous vectors were then placed on healthy plants (Zhou et al., 1987; for a review, see D’Arcy et al., 1999). These methods are simple and inexpensive, and require no specialized reagents or equipment. They have several disadvantages, in the case of the first and second approaches the operator must be skilled and experienced, and for the third approach samples must be tested shortly after collection. A simple, rapid and reliable method is described by which the virus-free planthoppers acquired RSV from frozen rice leaves and transmitted it to healthy plants.

2. Materials and methods

2.1. Virus, small brown planthoppers, host plants and virus antibody

Virus-free small brown planthoppers (L. striatellus), were reared in the laboratory on rice seedlings (2–3 cm tall) in glass vessels at 26 °C with 14 h of light per day. The isolate of RSV used here was obtained in 2004 from a rice plant showing typical stripe symptoms from Yuanyang County, Henan Province, China. The isolate was identified as RSV by indirect enzyme-linked immunosorbent assay (indirect-ELISA) and reverse transcription polymerase chain reaction (RT-PCR), and infected leaves were frozen at −20 °C before experiments. A susceptible rice cultivar (Oryza sativa L. cv. Wuyujing No. 3) was used as a host for virus infection and planthopper rearing. The monoclonal antibody against RSV was provided by Dr. Y. Zhou (Institute of Plant Protection, Jiangsu Academy of Agriculture Sciences). The goat anti-mouse IgG (H + L) alkaline phosphatase was purchased from Promega (Madison, WI, USA).

2.2. Virus acquisition and transmission experiments

Each aviruliferous planthopper population was generated from a pair of recently hatched nymphs and confirmed as being aviruliferous by negative virus transmission tests and by indirect-ELISA (Zhou et al., 2004). Frozen rice leaves infected with RSV were thawed and cut into 5–6 cm lengths, and placed in Petri dishes containing wet filter papers that retained moisture for at least 5–6 h. The leaves were allowed to absorb water until they had spread out completely. Ten leaf pieces were transferred into an Erlenmeyer flask containing wet-absorbent cotton bedding. About 10–15 aviruliferous small brown planthopper nymphs (2nd instar), which had been starved for 5–6 h, were placed onto each leaf piece, by means of a camel-hair brush. After the nymphs were deposited in each flask, the flask was sealed with three layers of gauze, and then placed at 26 °C in the dark (Fig. 1). Blank controls were unfed planthoppers (i.e. placed on wet filter paper only). After a 48 h acquisition-feeding period, the surviving planthoppers were transferred from the leaf pieces with a brush to healthy rice seedlings. The rice was grown in the greenhouse in steam-sterilized soil (4 plants per 20 cm pot) for 7 day test-feeding to pass the virus through a circulative period. After the end of this 7-day period, 30 insects were removed with a brush for virus detection.

Fig. 1. A feeding flask, containing small brown planthoppers and frozen rice leaves.

Other 30 insects fed on frozen infected leaves as above were transferred to healthy rice seedlings. Each seedling was infected with one insect and caged individually during a 2-day inoculation test-feeding period. After the end of this 2-day period, the insects were killed by fumigation with lindane in a closed chamber. Plants were then placed under supplemental illumination in a greenhouse that was fumigated regularly to control insects. The plants were observed for at least 7–8 weeks. The insects fed on healthy leaves or fresh infected leaves are referred to as negative and positive controls, respectively.

2.3. Detection of RSV in viruliferous planthopper vector and rice plants

Indirect-ELISA was carried out (Zhou et al., 2004) using monoclonal antibody for virus detection in planthoppers fed on either frozen, healthy, or fresh infected rice leaves, respectively. Optical densities at 405 nm (OD405) were measured with an indirect-ELISA reader (Bio-Rad, Hercules, CA, USA). A positive reaction was recorded when the OD405 of a sample was twice that of samples from aviruliferous insects (Zhou et al., 2004).

After the removal of the insects, the plants were treated routinely with insecticide and kept in a greenhouse at approximately 25 °C until the development of symptoms. Samples of leaves either expressing typical stripe symptoms or not expressing disease symptoms were then collected for RNA extraction. Total RNA was extracted from the leaves 7–8 weeks post-inoculation, using Trizol reagent according to the manufacturer’s
Table 1

Percentages of viruliferous insects and infected plants to which RSV was transmitted by small brown planthopper (*Laodelphax striatellus*), that had acquired virus by either feeding on frozen or fresh infected leaves, respectively

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total no. of insects</th>
<th>No. of surviving insects</th>
<th>Proportion of viruliferous insects (%) (<em>n</em> = 30)</th>
<th>Proportion of infected plants (%) (<em>n</em> = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen infected leaves</td>
<td>120</td>
<td>71</td>
<td>30.00</td>
<td>16.67</td>
</tr>
<tr>
<td>Fresh infected leaves</td>
<td>120</td>
<td>105</td>
<td>36.67</td>
<td>23.33</td>
</tr>
<tr>
<td>Fresh healthy leaves</td>
<td>120</td>
<td>114</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wet filter paper</td>
<td>100</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Control diet, total insect number and survivor results are included. RSV analyzed by indirect-ELISA.

instructions (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using the Promega (Madison, WI, USA) cDNA synthesis system as recommended by the manufacturer. Virus specific cDNA was amplified using Taq DNA Polymerase (TAKARA, Dalian, China), using a downstream primer, CP2 (5′ TAGA ATGGGTACCAACAAGC 3′) according to the manufacturer’s instructions, followed by PCR amplification using upstream primer CP1 (5′ GTTCAGTCTAGTCATCTGCAC 3′) and CP2. CP1 and CP2 were developed from the known sequence of RSV capsid protein gene (Zhu et al., 1991, GenBank Accession number NC003776). Successful amplification of fragments of the expected size (979 bp) was confirmed by electrophoresis in 1% (w/v) agarose gels.

### 3. Results and discussion

#### 3.1. Detection of RSV in a single small brown planthoppers fed on frozen leaves

After feeding on either frozen infected, fresh infected or fresh healthy rice leaves for 48 h in the dark; 49, 15 and 6 small brown planthoppers died, respectively, all insects in the blank control also died (Table 1). The number of dead insects fed previously on frozen infected leaves was greater than those fed on fresh infected or healthy leaves, however, 71 of 120 insects survived after a 2-day feeding period. The results showed that the planthopper could feed successfully on frozen rice leaves. Thirty planthoppers (fed on either frozen infected, fresh infected or fresh healthy rice leaves, respectively), following a 7-day test-feeding period to pass the virus through a circulative period, were tested by indirect-ELISA. Of insects fed on frozen infected leaf, 30% reacted strongly with monoclonal antibody against RSV, suggesting that they had acquired the virus from infected leaves (Table 1). There were 36.7% RSV-positive insects among those fed on fresh infected rice leaves, but none of the insects fed on fresh healthy rice were RSV-positive. In subsequent replicated experiments, the number of viruliferous insects varied slightly. Generally, 23–30% of insects fed on frozen infected leaves were viruliferous, determined by indirect-ELISA.

#### 3.2. Symptom and virus detection in rice plants

Rice plants infested with a single viruliferous planthopper, fed previously on either frozen or fresh infected rice leaves for 48 h, began to express typical stripe symptoms (Fig. 2) after 50 days of supplemental illumination in a greenhouse at approximately 25 °C. In the virus transmission test with a single planthopper, insects fed on frozen infected leaves transmitted the virus to 16.7% of plants, and those fed on fresh infected leaves, transmitted the virus to 23.33% of plants (Table 1). None of the plants infested with a single non-viruliferous insect fed on fresh healthy leaves expressed any virus-like symptoms.

Plants infected with RSV by viruliferous insects were virus-positive by RT-PCR using the primers CP1 and CP2. An amplified fragment of the expected size (979 bp) was obtained from each plant expressing stripe symptoms, but not from symptom-free plants (Fig. 3). These results suggest that the planthoppers acquired the virus from frozen leaves and transmitted the virus to healthy plants.

Perhaps the most significant observation of this technique, which has been used in this laboratory for 2 years, is that at least some transmissions occurred by the insects fed on frozen infected leaves. Therefore, many field samples could be collected at the same time and stored for 1–2 years or longer before testing. This is an important implication for large-scale surveys as well as long-term epidemiological or ecological studies. On the other hand, each seedling was infected generally with 10 planthoppers in a transmission test. According to the proportions of viruliferous insects (30%) and of infected plants (16.67%) obtained in this study, 10 insects/plant would be needed for...
Agarose gel analysis of RT-PCR products obtained from different rice plants. The RT primer was CP2. The PCR primer pair was CP1 and CP2. The size of the RT-PCR fragment was about 1 kb. Lanes 1 and 21: DL2000 (TaKaRa, Dalian, China). Lanes 2 and 20: negative CK. Lanes 3 and 19: positive CK. Lanes 4, 8, 11, 16, 17 and 18: rice leaves with no symptoms. Other lanes: rice leaves expressed symptoms.

>90% transmission rates. This amount of transmission is usually sufficient for most studies. Since it is easy to use large numbers of the planthoppers, the relatively low transmission efficiency is not a major disadvantage. Using this method, 12 field samples collected from different regions of China and stored at −20°C for 1–2 years, were regenerated successfully.

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References


