Transmission of Rice Black-Streaked Dwarf Virus from Frozen Infected Leaves to Healthy Rice Plants by Small Brown Planthopper (*Laodelphax striatellus*)

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Abstract: In order to preserve virus for identifying resistance of rice varieties against rice black-streaked dwarf disease, a simple and reliable method was developed through which virus-free small brown planthopper (SBPH) acquired rice black-streaked dwarf virus (RBSDV) from frozen infected leaves and the virus was transmitted to healthy rice plants. RBSDV acquired from infected leaves frozen at -70°C by SBPH was transmitted to a susceptible rice variety. The experimental result showed that the SBPH could obtain virus from frozen infected leaves and RBSDV could be transmitted to the susceptible rice variety. For the ability to acquire RBSDV and transmit the virus to healthy plants by SBPH, there was no significant difference between frozen infected leaves and *in vitro* infected leaves. The novel method can be applied to resistance identification of rice varieties to rice black-streaked dwarf disease, which will facilitate the breeding process for rice black-streaked dwarf disease resistance.

Key words: rice black-streaked dwarf virus; frozen infected leaves; small brown planthopper

Rice black-streaked dwarf disease, which was discovered in 1963 at Yuyao County, Zhejiang province of China, mainly occurred in local areas of Zhejiang Province during the last century (Zhu et al, 1964; Chen and Zhang, 2005). With increasing amount of the small brown planthopper (*Laodelphax striatellus* Fallen, SPBH), rice black-streaked dwarf disease spreads and becomes one of the most serious rice diseases in East China. The disease is caused by rice black-streaked dwarf virus (RBSDV), a member of the genus Fijivirus of the family Reoviridae that is mostly transmitted by SBPH in a persistent-propagative manner, but not via its eggs (Ruan Y L et al. 1984). RBSDV forms non-enveloped, icosahedral, double-shelled particles with short surface spikes and contains 10 dsRNA segments (Zhang et al, 2002; Wang et al, 2002; Liu et al, 2007). RBSDV can infect cereal crops, such as rice, maize and wheat, leading to rice black streaked dwarf disease, maize rough dwarf disease and wheat dark-green dwarf disease, respectively, which caused severe economic damage (Zhou et al,1998; Yang et al, 2007; Zhang et al, 2002).

The development of disease-resistant cultivars is an ideal way to control rice viral diseases (Zhou et al, 2009; Sun et al, 2006; Hibino, 1996). It is necessary to establish a scientific method to identify the resistant varieties at first. Since RBSDV can not be transmitted mechanically, insect transmission was the original basis for identification of the viral population and cultivar resistance. However, diseased plants can not be long-term preserved for virus infection and needs to feed SBPH as soon as possible, which limit its application in crop improvement and breeding and genetic research for disease resistance. A simple, rapid and reliable method was developed, through which virus-free SBPH acquired rice stripe virus (RSV) from frozen infected rice leaves and transmitted the virus to healthy rice plants (Zhang et al, 2007). It provides a new idea to solve this problem and to establish basic research method in breeding and genetics research for rice black-streaked dwarf disease resistance.

MATERIALS AND METHODS

Virus resource

RBSDV used in the study was obtained in May...
2009 from wheat plants showing typical dark-green dwarf symptoms from Pizhou County, Jiangsu Province, China (Gong et al, 1981). The isolate was identified as RBSDV by reverse transcription polymerase chain reaction (RT-PCR). Primers were designed according to S9 sequence of RBSDV (R: 5′-GGATTACAACAHACACAMCGAAA-3′; F1: 5′-GR TAGACAGGCAAYMTAAGCGT-3′) (Zhang et al, 2001).

Total RNA was extracted from the wheat plants, using TRIzol reagent according to the manufacturer’s instructions (Reagent) and reverse transcribed using the Promega cDNA synthesis system as recommended by the manufacturer. Polymorphisms chain reaction (PCR) was analyzed as the following procedure. Each 25 µL PCR reaction contained 3.0 µL of template DNA, 2.5 µL 10×PCR reaction buffer (Mg2+), 2.5 µL of dNTPs (10 mmol/L), 1.0 µL of each primers (10 pmol/L), and 0.5 U of Taq DNA polymerase.

Amplification profiles consisted of 5 min of denaturation at 95°C, 35 cycles of 60 s denaturation at 94°C, 60 s annealing at 51°C, and 60 s extension at 72°C, followed by a final 10 min extension at 72°C and storage at 4°C. Denatured amplified products were electrophoresed on 1% agarose gels. Then the wheat plants were assayed positive for RSV by ELISA (Zhou et al; 2004). Some infected plants were frozen at –70°C before experiments, whereas the others were planted in greenhouse.

Vector populations

In April and May 2008, SBPHs were collected from the experimental station of Jiangsu Academy of Agricultural Sciences, China and maintained on the rice variety Wuyujing 3. One female SBPH was separated to oviposit after mating and assayed negative for RSV by ELISA. The 2nd and 3rd generation offsprings of these aviruliferous females were classified as a population. Each RSV-free SBPH population was confirmed as being aviruliferous by ELISA before transmission.

Virus acquisition experiments

Two freezing time treatments were set for 45 days and 140 days. Frozen wheat leaves infected with RBSDV were thawed in Petri dishes containing wet filter papers for at least 3–5 h. The leaves were allowed to absorb water until they had spread out completely. The leaves were transferred into an Erlenmeyer flask containing filter papers. Sixty aviruliferous SBPH nymphs (1st-2nd instar) pre-starved for 3 h were placed onto each flask (Fig. 1). Other 60 aviruliferous SBPH nymphs (1st-2nd instar) were fed by wheat leaves infected with RBSDV as control. Blank controls were fed by wheat leaves without RBSDV. After a 48 h acquisition-feeding period, the surviving SBPHs were calculated and transferred from the leaves with a brush to healthy rice seedlings.

Virus transmission experiments

After acquisition-feeding period, the surviving SBPHs were maintained on Wuyujing 3 for 15 days to pass the virus through a circulative period. Twenty-five insects fed on infected leaves as above were inoculated to a susceptible rice variety, Huajing 6. Each seedling was infected with one insect and caged individually in test tube (15 mm×150 mm). After 4-day inoculation test-feeding period, the insects were transferred to healthy rice seedlings. All seedlings from each tube were transplanted individually to the field after SBPH nymphs were removed. The incidence of rice black-streaked dwarf disease symptoms was evaluated 30 days later as described above and classified according to symptom expression. The main symptom is severe dwarfinf, short and broad and dark-green in disease leaves, white tumours, which subsequently changed dark brown, along the vein on the back of the leaves and on leaf sheaths (Liu et al, 2007). Plants infected with

Fig. 1. Virus-free small brown planthoppers acquire RBSDV form frozen infected leaves.
RBSDV by viruliferous insects were virus-positive by RT-PCR after investigation.

**Detection of RBSDV in viruliferous planthopper vector**

RT-PCR was carried out for the detection of RBSDV in single planthoppers after inoculation. Total RNA was extracted from the planthopper samples using TRIzol reagent according to the manufacturer’s instructions (Reagent). RT-PCR procedure was the same as stated above.

**RESULTS**

**Selection of wheat plants infected with RBSDV**

Twelve wheat plants with typical symptoms were identified to carry RBSDV by RT-PCR. After assayed by ELISA, nine of them were RSV-free. Four infected plants were kept at –70°C before use, while the other five were planted in greenhouse as control.

**Virus acquisition experiments**

After feeding SBPHs the frozen infected leaves (the preserved period for 45 d and 140 d), fresh infected and healthy rice leaves for two days, the number of alive planthoppers were 35, 27.7, 52.5 and 54, respectively, with the respective survival rates of 58.3%, 46.2%, 87.5% and 90% (Table 1). These results indicate that frozen rice leaves could be used for feeding the planthoppers, but not quite fit as compared to the fresh leaves under the experimental conditions. Twenty-five insects fed on infected leaves were selected randomly in each treatment to inoculate a susceptible rice variety. After inoculation, the insects were tested by RT-PCR for the presence of virus. The rates of viruliferous SBPHs fed on 45 d- and 140 d-frozen infected leaves and fresh infected leaves were 26.7%, 20.0% and 28.0%, respectively. There is no significant difference among the above three treatments by DPS 2.0 software analysis. However, no planthoppers fed on fresh healthy rice leaves were positive (Table 1).

**Virus transmission experiments**

Seedlings of the susceptible rice variety Huajing 6 were separately inoculated by 25 insects that fed on 45 d- and 140 d-frozen infected leaves and fresh infected leaves as above. Until 60 days after inoculation, 6.7%, 8.0% and 10.7% of the infected plants showed typical disease symptoms, respectively (Fig. 2, Table 2). There was no significant difference among the above three treatments by DPS 2.0 software analysis.

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**Table 1. Survival rates and percentages of viruliferous insects which acquire virus by feeding on frozen or fresh infected leaves.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total number of insects</th>
<th>Number of survival insects</th>
<th>Proportion of viruliferous insects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen infected leaves for 45 d</td>
<td>60</td>
<td>27.7a</td>
<td>20.0a</td>
</tr>
<tr>
<td>Frozen infected leaf for 140 d</td>
<td>60</td>
<td>35.0 b</td>
<td>26.7a</td>
</tr>
<tr>
<td>Fresh infected</td>
<td>60</td>
<td>52.5c</td>
<td>28.0a</td>
</tr>
<tr>
<td>Fresh healthy leaves</td>
<td>60</td>
<td>54.0 c</td>
<td>–</td>
</tr>
</tbody>
</table>

Within a column, data followed by the same lowercase letters are not significantly difference at $P<0.05$ level.

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**Table 2. Percentages of RBSDV infected plants by inoculation of SBPH fed on frozen or fresh infected leaves.**

<table>
<thead>
<tr>
<th>Infected leaf</th>
<th>Number of infected plant</th>
<th>Proportion of infected plants (n=25) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen for 40 d</td>
<td>2</td>
<td>8.0 a</td>
</tr>
<tr>
<td>Frozen for 140 d</td>
<td>1.67</td>
<td>6.7 a</td>
</tr>
<tr>
<td>Fresh infected</td>
<td>2.67</td>
<td>10.7 a</td>
</tr>
</tbody>
</table>

Within a column, data followed by the same lowercase letters are not significantly difference at $P<0.05$ level.
software analysis. These results indicate that the planthoppers acquiring the virus from frozen leaves could transmit the virus to healthy plants.

Twelve inoculated rice plants with symptoms were randomly selected and tested by RT-PCR. The expected size of the product was amplified from each plant expressing symptoms, but not in the symptom-free plants (Fig. 3). These results verified that the planthoppers acquiring the virus from frozen leaves could transmit the virus to healthy plants.

**DISCUSSION**

Following the outbreak of rice stripe disease, rice black-streaked dwarf disease, another virus transmitted by SBPH, had occurred and spread in East China and brought tremendous risk for food production. In 2007, there was about 2.05×10^5 ha rice infected by RBSDV. In 2008, the area of RBSDV-infected rice increased to 2.67×10^6 ha, and the disease ruined the harvest of about 2000 ha rice in Jiangsu province, China (disease incidence over 80%) (Chen et al, 2010). Development of resistant rice varieties is one of the most economically effective disease management strategies according to successful experiences from RSV (Sun et al, 2007). The implementation of this strategy is simply to establish a scientific and objective method for resistance identification of rice cultivars, while the acquirement of viruliferous insect vectors was the basic guarantee for the establishment of this approach. There were two methods used to obtain viruliferous vectors under experimental conditions. The first, direct catch of viruliferous vectors from disease areas, was less used for the shortcomings such as insect vulnerability, RSV interference and time limit. The second method, artificial feeding of insects through fresh virus-infected plant, was used widely. The method by direct injection of virus preparations into abdomens of 3rd to 4th instars insects using fine glass capillaries, was developed by Shikata et al (1977). The maize seedlings that inoculated by the injected insects that had passed a latent period of 15 days showed typical disease symptom. However, this method which needs skilled operators and large quantity of insect vectors, can hardly be adopted by most researchers. Another way widely used was the feeding of 1st to 2nd instars insects on fresh virus-infected leaves and then feeding the viruliferous vectors on healthy plants to pass a latent period. Since virus-infected plants is difficult for long-term preservation of RBSDV, the preservation of RBSDV had been a technical bottleneck of the research on identification of cultivar resistance. A method is described by which the virus-free SBPH acquired RBSDV from frozen leaves and transmitted it to healthy plants in this paper. The results indicate that the planthoppers acquired the virus from frozen leaves could transmit the virus to healthy plants. Xiong et al (1999) and Hollings et al (1960 and 1970) reported similar results in other research (cucumber mosaic virus, tobacco mosaic virus and potato virus Y). Zhang et al (2007) also reported that the virus-free SBPH acquired RSV from frozen rice plants could transmit the virus to healthy plants. Shikata et al (1977) reported that the injected insects artificially fed on frozen virus-infected plants could transmit the virus to healthy plants. These results all corroborated the feasibility of our proposed method.

Since RSV and RBSDV had occurred simultaneously, the virus-infected plants from the field often carried two kinds of viruses. The infected plants were identified as RBSDV-infected by RT-PCR.
and RSV-free by ELISA assay. On the other hand, because RSV can be transmitted to the progeny of the vector via eggs, the insect vectors used in the experiment were assayed negative for RSV by ELISA, which can reduce RSV interference. For the insects fed on frozen infected plants, 20.0%–30.0% of them were viruliferous, whereas 6.7% of RBSDV infected plants were inoculated by SBPH fed on frozen infected plants. The reason might be that the occurrence of SBPH in the field was far more than one insect per plants. For further exploration by increasing the amount of vaccination vectors, this method might be used in general identification experiments of plant varieties resistance to RBSDV. The method in this paper has applied for the National Invention Patent with application number 200910034147.1. It successfully solved the problem how to retain the virus resource, which can be used in screening the resistance of cultivars and genetic analysis of the cultivars resistant to RBSDV, also can accelerate the breeding for RBSDV resistance. There were no significant difference for the virus-free SBPH in acquiring the RBSDV virions after feeding on infected 45 d-frozen, 140 d-frozen and fresh leaves and transmitted it to healthy plants. Shikata et al (1977) reported that the injected insects that had artificially fed on frozen virus infected plants, which were frozen for 232d, could transmit the virus to healthy plants. Perhaps, the storage of frozen plants can be prolonged and need to be identified in further study.

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REFERENCES


