

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 the 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. The experimental results showed that SBPH could obtain RBSDV from frozen infected rice leaves and the virus could be transmitted to a 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 could be applied to identification of rice variety resistance to rice black-streaked dwarf disease, facilitating the breeding process for rice black-streaked dwarf disease resistance.

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

Rice black-streaked dwarf disease, discovered in 1963 at Yuyao County, Zhejiang Province, China, mainly occurred in local areas of Zhejiang Province during the last century (Zhu et al, 1964; Chen and Zhang, 2005). With the increasing amount of 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 et al. 1984). RBSDV forms non-enveloped, icosahedral, double-shelled particles with short surface spikes and contains 10 dsRNA segments (Zhang et al, 2002a; Wang et al, 2002; Liu et al, 2007). RBSDV can infect cereal crops, such as rice, maize and wheat, resulting in 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; Zhang et al, 2002b; Yang et al, 2007).

The development of disease-resistant varieties is an ideal way to control rice viral diseases (Hibino, 1996; Sun et al, 2006; Zhou et al, 2009). It is necessary to establish a scientific method to identify resistant varieties at first. Since RBSDV can not be transmitted mechanically, insect transmission was the original basis for identification of viral population and variety resistance. However, diseased plants can not be long-term preserved for virus infection and need to feed SBPH as soon as possible, which limits 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 a basic research method in breeding and genetic research for rice black-streaked dwarf disease resistance.

MATERIALS AND METHODS

Virus resource

RBSDV was obtained in May 2009 from wheat plants showing typical dark-green dwarf symptoms in 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 the S9 sequence of RBSDV (R: 5'GGATTACAACAHACACAMCG AAA3'; F1: 5'GRTAGACAGGCAAAYMTAAGCGT3') (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. Polymorphism chain reaction (PCR) was analyzed as the following procedure: Each 25 μ L of PCR reaction contained 3.0 μ L of template DNA, 2.5 μ L of 10 \times PCR reaction buffer (Mg²⁺), 2.5 μ L of dNTPs (10 mmol/L), 1.0 μ L of each primer (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

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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 enzyme linked immunosorbent assay (ELISA) (Zhou et al, 2004). Some infected plants were frozen at -70°C before experiments and 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, Jiangsu Province, China and maintained on a 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 d and 140 d. Frozen wheat leaves infected with RBSDV were thawed in Petri dishes containing wet filter paper 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 paper. 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 control was fed on wheat leaves without RBSDV. After a 48 h acquisition-feeding period, the surviving SBPHs were calculated and transferred from the leaves to healthy rice seedlings with a brush.

Virus transmission experiments

After the acquisition-feeding period, the surviving SBPHs were maintained on rice variety Wuyujing 3 for 15 d to pass the virus through a circulative period. Twenty-five insects fed on



Fig. 1. Virus-free small brown planthoppers acquire RBSDV from frozen infected leaves.

the infected leaves as above mentioned were inoculated to a susceptible rice variety Huajing 6. Each seedling was infected with one insect and caged individually in a 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 d later as described above and classified according to symptom expression. The main symptoms were severe dwarfing, 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 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 detection of RBSDV in a single planthopper 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 with the frozen infected leaves (the preserved periods were 45 d and 140 d), fresh infected and healthy rice leaves for 2 d, the numbers of alive planthoppers were 27.7, 35.0, 52.5 and 54.0, respectively, with the respective survival rates of 58.3%, 46.2%, 87.5% and 90.0% (Table 1). These results indicate that the frozen rice leaves could be used for feeding 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 SBPH fed on 45 d- and 140 d-frozen infected leaves and fresh infected leaves were 20.0%; 26.7% and 28.0%, respectively. There was no significant difference among the above three treatments by DPS 2.0 software analysis. However, no planthoppers fed on the fresh healthy rice leaves were positive (Table 1).

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

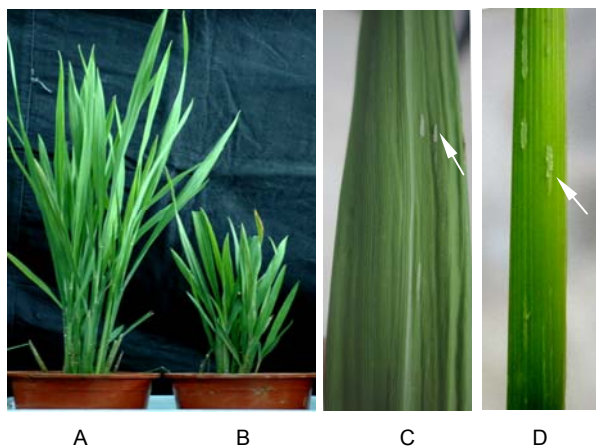
Treatment	Total number of insects	Number of survival insects	Proportion of viruliferous insects (%)
Frozen infected leaves for 45 d	60	27.7 a	20.0 a
Frozen infected leaf for 140 d	60	35.0 b	26.7 a
Fresh infected leaves	60	52.5 c	28.0 a
Fresh healthy leaves	60	54.0 c	–

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

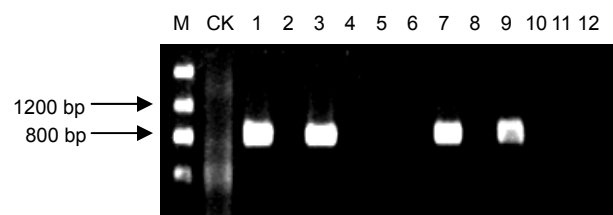
Table 2. Percentages of RBSDV infected plants by inoculation of SBPH fed on frozen or fresh infected leaves.

Infected leaf	Number of infected plants	Proportion of infected plants ($n=25$) (%)
Frozen for 45 d	2.00	8.0 a
Frozen for 140 d	1.67	6.7 a
Fresh infected leaves	2.67	10.7 a

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

**Fig. 2. Typical symptoms of rice plants inoculated with SBPH acquiring RBSDV from frozen infected leaves.**

A, Healthy plant; B, Infected plant; C, Typical symptoms on the leaves; D, Typical symptoms on the stem. Arrows indicate white or brown enations of short strip shape.

**Fig. 3. Detection of RBSDV in inoculated rice plants by RT-PCR.**

M, DNA marker; CK, Negative control; Lanes 1, 3, 7 and 9, Infected plants; Lanes 2, 4, 5, 6, 8, 10, 11 and 12, Healthy plants.

Virus transmission experiments

The 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, 8.0%, 6.7% 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. 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 hm² rice area infected by RBSDV. In 2008, the area of RBSDV-infected rice increased to 2.67×10^6 hm², and the disease ruined the harvest of about 2000 hm² 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 the successful experience from RSV (Sun et al, 2007). The implementation of this strategy is simply to establish a scientific and objective method for identification of resistant rice varieties, while the acquirement of viruliferous insect vectors is the basic guarantee for the establishment of this approach. There were two methods used to obtain viruliferous vectors under experimental conditions. The first method is to directly catch viruliferous vectors from disease areas, which is less used due to the shortcomings such as insect vulnerability, RSV interference and time limit. The second method is artificial feeding of insects through fresh virus-infected plants, which is used widely. The method for direct injection of virus preparations into abdomens of 3rd to 4th instar insects using fine glass capillaries, was developed by Shikata and Kitagawa (1977). The maize seedlings that inoculated by the injected insects that had passed a latent period of 15 d showed typical disease symptoms. However, this method needs skilled operators and large quantity of insect vectors, thus it could hardly be adopted by most researchers. Another way widely used was the feeding of 1st to 2nd instar insects on fresh virus-infected leaves and then feeding the viruliferous vectors on healthy plants to pass a latent period. Since virus-infected plants are difficult for long-term preservation of RBSDV, the

preservation of RBSDV had been a technical bottleneck of the research on identification of variety resistance. A method for the virus-free SBPH to acquire RBSDV from frozen leaves and transmit the virus to healthy plants is described. 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 occur simultaneously, the virus-infected plants from the field often carry two kinds of viruses. The infected plants were identified as RBSDV-infected by RT-PCR and RSV-free by ELISA assay. 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 the 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 plant. For further exploration by increasing the amount of vaccination vectors, this method might be used in general identification of plant variety resistance to RBSDV. The method has applied for the National Invention Patent with the application number 200910034147.1. It successfully solved the problem how to retain the virus resource. Therefore, it can be used in screening resistant varieties and genetic analysis of the variety resistance to RBSDV, also can accelerate the breeding for RBSDV resistance. There were no significant differences for the virus-free SBPH in acquiring RBSDV virions after fed on infected 45-day-frozen, 140-day-frozen and fresh leaves and transmitted it to healthy plants. Shikata et al (1977) reported that the injected insects which had artificially fed on 232-day-frozen virus infected plants, could transmit the virus to healthy plants. Perhaps, the storage of frozen plants can be prolonged, which needs to be identified in further study.

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