**Short Communication**

# A METHOD OF ACQUIRING INOCULUM OF *RICE BLACK STREAKED DWARF VIRUS* FROM WHEAT DARK-GREEN DWARF DISEASED PLANTS TO SCREEN MAIZE FOR RESISTANCE TO MAIZE ROUGH DWARF DISEASE

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**SUMMARY**

Maize rough dwarf disease (MRDD) is one of the main viral diseases of maize, and the cause of great yield losses; one of its causal agents is *Rice black-streaked dwarf virus* (RBSDV). Here, we developed a method for transmitting RBSDV to maize by *Laodelphax striatellus* Fallén, the small brown planthopper (SBPH) from wheat plants affected by wheat dark-green dwarf disease (WDGDD). The SBPH transmitted RBSDV more efficiently when it was acquired from wheat than from maize, with an efficiency of 36.7% and 12.7%, respectively. We also found that when the same number of vectors was used, transmission efficiency was similar after feeding on either WDGDD- or MRDD-infected plants. These results indicate that the method is efficient and viable, and is expected to facilitate the identification of maize cultivars that are resistant to RBSDV.

*Keywords*: Maize rough dwarf disease, wheat dark-green dwarf disease, RBSDV, resistance identification, planthopper vector.

Maize rough dwarf disease (MRDD) is a severe and widely spread disease caused by three closely related members of the genus *Fijivirus*, family *Reoviridae*: *Rice black streaked dwarf virus* (RBSDV), *Mal de Rio Cuarto virus* (MRCV) and *Maize rough dwarf virus* (MRDV) (Boccardo and Milne, 1984; Lenardon *et al*., 1998; Milne and Lovisolo, 1977). RBSDV is transmitted by *Laodelphax striatellus* Fallén, the small brown planthopper (SBPH) in a persistent and propagative manner, but not by transovarial transmission (Hibino, 1996; Ishii and Yoshimura, 1973). Typical symptoms induced by RBSDV in maize include stunting and development of white wax or black-streaked swellings along the veins on the back of the leaf blades and sheaths (Fang *et al*., 2001; Isogai *et al*., 2001; Lee *et al*., 2005; Luan *et al*., 2012; Wang *et al.,* 2011). An outbreak of MRDD was first reported in the 1950s in China, then in the northern, north-western and middle parts of this country in the 1990s. These MRDD outbreaks, severely affected the grain quality and total yield of maize (Zhang *et al*., 2001a; Chen *et al*., 1986). Recent studies have confirmed that the agent of MRDD in China is RBSDV rather than MRDV or MRCV (Zhang *et al*., 2001b; Wang *et al*., 2003).

Breeding resistant maize cultivars is considered an optimal strategy for controlling MRDD (Hibino, 1996). However, to identify resistant maize cultivars, it is necessary to establish a reliable method for the SBPH to acquire the virus from infected plants. Because maize is not a suitable host for SBPH, it is difficult to recover viruliferous SBPHs from RBSDV-infected maize (Maramorosch, 1955; Qiao *et al*., 2009). Several methods have been developed previously, such as feeding SBPHs on frozen rice leaves infected with RBSDV or injecting directly RBSDV into the SBPH abdomen by fine glass capillaries (Ruan *et al*., 1981; Zhou *et al*., 2011b). However, these methods are inconvenient for screening and evaluating resistant cultivars for a large number of viruliferous vectors is needed.

Wheat dark-green dwarf disease (WDGDD), recently reported from China, is also caused by RBSDV (Wu *et al*., 2013); thus, WDGDD-affected plants could be a potential RBSDV source for acquiring viruliferous SBPHs. Here, we present a method for RBSDV acquisition by SBPH from WDGDD-affected plants, which could accelerate the process for breeding RBSDV-resistant maize cultivars.

WDGDD symptomatic plants were collected from fields in Jianhu County, Jiangsu Province (China), in May 2014, tested for the presence of RBSDV by RT-PCR (Ji *et al*., 2011), and the RBSDV-positive ones were transplanted in a greenhouse. Maize plants with typical symptoms of MRDD were collected from Nanjing City, Jiangsu Province, in July 2014, tested by RT-PCR (Ji *et al*., 2011), and the RBSDV-positive ones were transplanted in a greenhouse.

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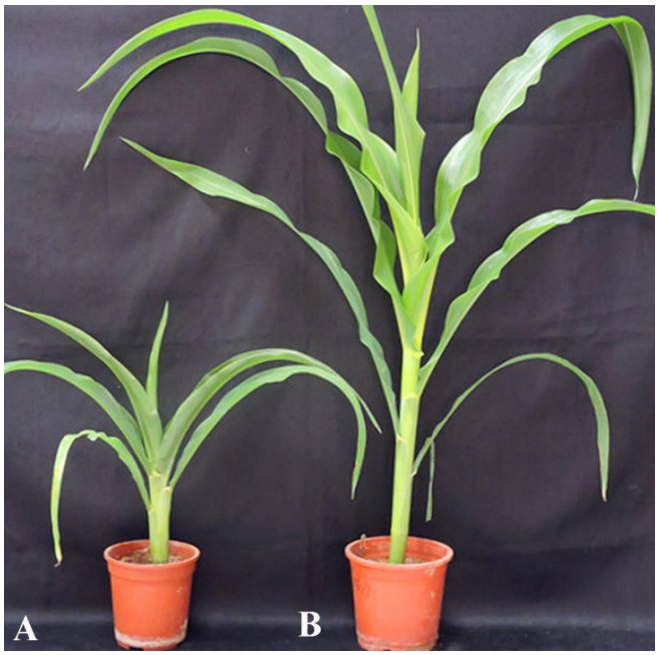


**Fig. 1.** A. Maize rough dwarf-diseased plant. B. Healthy plant.

For RT-PCR testing, total RNA was extracted with Trizol (Invitrogen, USA). Custom-synthesized primers (Shanghai Biological Engineering Technology Service, China) were: RB1-S9-F: 5'-GRTAGACAGGCAAAYM-TAAGCGT-3' (R: A/G; Y: T/C; M: A/C) and RB-S9-R: 5'-GGATTACAACAHACACAMCGAAA-3' (H: T/G; M: G/A/T) (Ji *et al.,* 2011). RT reactions were performed following the Promega cDNA synthesis system (Promega, USA). For PCR, 2 µl cDNA from the above RT step and 0.2 µM of the corresponding primers (RB1-S9-F and RB1-S9-F) were used in a 25 µl conventional reaction system. PCR was carried out using an S1000TM Thermal Cycler (BIO RAD, USA).

SBPHs were reared on rice seedlings of cv. Wuyujing No. 3 in a greenhouse at 25°C, with a 16 h day/8 h night photoperiod (Zhou *et al.,* 2011a). To obtain a SBPH population free of *Rice stripe virus* (RSV) and RBSDV, 100 SBPH individuals were randomly selected and assayed for the presence of these viruses by dot-ELISA (Wang *et al*., 2003; Zhou *et al*., 2004; Wu *et al*., 2013). A population established from the progeny of individuals free of RSV and RBSDV was used for transmission studies. Virus-free SBPH third instars were fed on WDGDD-affected plants or MRDD-affected plants for 48 h to acquire the virus, then transferred to healthy rice seedlings cv. Wuyujing No. 3 (1.5-2.0 leaf stage) and reared in a greenhouse at 25°C with a 16 h light/8 h dark photoperiod for 12 days, i.e the duration of the latent period of infection. A group of 100 SBPH individuals was selected, and the rate of infection by RBSDV was estimated by dot-ELISA (Wu *et al*., 2013).

The maize cv. Su No. 951 was selected for inoculation experiments. After being soaked in water for 48 h and germinated for 24 h, approximately 39 maize seeds were distributed in three 1000 ml beakers in a greenhouse at 25°C with a 16 h light/8 h dark photoperiod. Thirty healthy maize seedlings at the two-leaf stage, were inoculated by feeding SBPHs exposed to RBSDV for 48 h, and the insects were gently disturbed with a brush every 12 h to force them to distribute evenly among the maize seedlings, after which the insects were manually removed from the seedlings.

To ensure the consistency of inoculation intensity, the inoculation number of SBPHs, which had acquired RBSDV from either maize or wheat, used for maize inoculation was calculated according to the same effective inoculation number (inoculation number = effective inoculation number/viruliferous percent), and the effective inoculation number was 3 per seedling in this test (Zhou *et al*., 2011a). Therefore, 3/36.7% = *ca.* 8.17 SBPH per seedling (245 SBPH per 30 seedlings) were used from the RBSDV-infected wheat source, and 3/12.7% = *ca.* 23.62 SBPH per seedling (708 SBPH per 30 seedlings) were used from the RBSDV-infected maize source, to inoculate maize seedlings.

After manual removal of SBPH, maize seedlings were transplanted in the field, which were managed under standard practices without pesticide or antiviral during the growing period. Maize plants were examined for the presence of symptoms 30 and 37 days post inoculation. Plants displaying the "green dwarf" symptoms typical of maize rough dwarf disease were considered to be susceptible (Ruan *et al*., 1981), whereas symptomless plants were taken as resistant. Resistance against RBSDV was evaluated based on disease incidence, i.e. number of RBSDV-infected maize plants/the total number of maize plants × 100. All experiments were done in triplicate.

Nine wheat samples with stunting and darkened leaves were found to be RBSDV-positive by RT-PCR, and seven maize plants with typical symptoms were RBSDV-positive by RT-PCR. Positive plants were transplanted in a greenhouse.

Acquisition efficiency of RBSDV from wheat and maize was 36.7% and 12.7%, respectively. This was taken as eivdence that SBPH acquires RBSDV more easily from WDGDD-affected than from MRDD-affected plants. All of the inoculated plants of cv. Su No. 951 showed typical symptoms of MRDD at 30 days post inoculation (Fig. 1), which suggested that WDGDD-affected plants were a reliable virus source for RBSDV acquisition.

Using resistant maize cultivars is the most economical and effective strategy for controlling MRDD (Hibino, 1996; Chen and Zhang, 2005); therefore, an accurate and reliable inoculation method for RBSDV is needed for breeding and selecting resistant maize. Vector transmission is the only way to induce successful infection; thus, successful virus acquisition by vectors is the basic requirement (Li *et al*., 2011). Several methods have been used for obtaining viruliferous vectors. Shikata and Kitagawa (1977) injected the virus directly into the abdomens of 3$^{rd}$

to 4th instars with fine glass capillaries; Zhou *et al.* (2011b) used frozen RBSDV-infected rice leaves as a virus source; Ren *et al.* (2014) developed a novel, *in vivo*, indoor method to preserve RBSDV in small brown planthopper using wheat seedling as a bridge host. However, those methods are complex and, most importantly, unable to meet the requirements of large-scale breeding for resistant plants.

In wheat-rice and wheat-maize planting rotation areas, the first viruliferous SBPH generation that emerges from wheat is the main initial infection source of RBSDV (Ruan *et al*., 1981; Cai *et al*., 1964). This inspired us to use wheat as a RBSDV source. Moreover, "green dwarf" symptoms are currently found more easily in damaged fields after the wheat jointing stage compared with early stages of infection. Thus, it is more convenient to collect considerable numbers of infected wheat plants as virus source. In our study, SBPHs more easily acquired the virus from WDGDD-affected than that from MRDD-affected plants.

In our study, all the inoculated plants cv. Su No. 951 showed typical symptoms of MRDD, in concordance with field assessment results (data not shown), confirming that the WDGDD-affected plants are a steady and dependable RBSDV source.

The viruliferous SBPHs obtained by this method are useful asset for the identification of resistant maize cultivars and for research in the stability of resistance inheritance.

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