Brief Communication

Development of marker-free rice with stable and high resistance to rice black-streaked dwarf virus disease through RNA interference

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Dear Editor,

Rice black-streaked dwarf virus (RBSDV) disease transmitted by small brown planthoppers (Laodelphax striatellus Fallén, SBPH), causes severe rice yield losses in Asia (Zhou et al., 2015). Breeding resistant cultivars are one of the most economical and effective strategies to control the disease. In the past two decades, there were several studies on the identification of cultivars and the detection of QTLs for RBSDV resistance (Feng et al., 2019). However, few highly resistant germplasms or genes have been found (Sun et al., 2017), severely hindering the development of elite varieties with high RBSDV resistance through either conventional breeding or marker-assisted selection (MAS) breeding.

RNA interference (RNAi), an evolutionarily conserved defence mechanism against RNA viruses, has been successfully applied to develop antiviral varieties in plants (Cristina et al., 2018). The genome of RBSDV contains 10 double-stranded RNA (dsRNA) segments designated S1–S10 (Wang et al., 2003). Previously, Shimizu et al. (2011) generated RBSDV-resistant transgenic rice by silencing S9–T. Wang et al. (2016) constructed an RNAi vector simultaneously targeting 4 viral genes (S1, S2, S6 and S10) and obtained high RBSDV-resistant rice lines. However, it is not clear that targeting which specific one or the combination of four genes leading to the high resistance. Among the RNAi studies that targeting which specific one or the combination of four genes were equally effective (Shimizu et al., 2011b). Therefore, it is crucial to identify the targets and design effective RNAi fragments for a specific viral gene.

For more than 10 years, our group has been committed to developing varieties with high RBSDV resistance. We generated three RNAi constructs targeting the specific fragments of RBSDV on S1, S2 and S6, respectively, and transferred them into the highly susceptible japonica variety Wulingjing 1 (WLJ1). Three independent transgenic lines for each target with the accumulation of expected small interference RNA (siRNA) were selected for phenotyping test (Figure 1a). We first conducted an artificial inoculation using SBPH carrying an RBSDV viruliferous rate (VR) of 32% in 2010, then a large-scale field trial in 2011 in Yangzhou with a VR of 9.4%. We found that RNAi of S1 and S2 individually had partial effects, whereas RNAi of S6 conferred nearly full immunity to RBSDV (Figure 1b-c; China patent: ZL201310202564.9). To further evaluate the stability of the S6RNAi lines, we conducted field trials from 2012 to 2013 at 4 different locations randomly selected in Jiangsu and Shandong provinces where the RBSDV disease had severe outbreaks since 2009. We observed that the S6RNAi lines showed stable resistance to RBSDV in all 4 locations (Figure 1d and e), indicating its potential for practical application. In a further field trial in 2014 at Kaifeng, Henan province, which had a VR of 12%, the S6RNAi lines with a diseased/total plant ratio (DPR) of less than 0.3%, displayed drastically higher resistance than a commercialized japonica rice variety Liangjing 6 (LG6) with a DPR of 36.7%, which is considered as a relatively resistant variety to RBSDV (Figure 1f). We also noted that the S6RNAi lines had no inferior effects on agronomical or developmental traits. Collectively, these results demonstrate that S6 is an ideal target for genetic engineering of RBSDV resistance via RNAi strategy.

Introducing the selectable marker gene in the plant transformation procedure is a wide biosafety concern in genetic engineering. To obtain marker-free transgenic S6RNAi lines, we generated a double T-DNA expression construct for S6RNAi driven by maize ubiquitin-1 promoter (U6RNAi) and transferred it into rice variety WLJ1. We obtained 3 independent homozygous

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lines (US6R11, US6R7 and US6R2) from the T2 plants, which were confirmed carrying single-copy insertion and no selectable marker gene as well as the accumulation of expected siRNA by Southern and northern blot analyses (Figure 1g and h). These 3 marker-free US6RNAi lines and one resistant control S6R40 were confirmed to carry high levels of RBSDV resistance in artificial inoculation test in 2016 and in multiple field trials from 2015 to 2017 (Figure 1i-k). Notably, the 3 US6RNAi lines maintain the agronomic traits that

![Figure 1](https://wileyonlinelibrary.com)
are indistinguishable from those of the wild type (Figure 1l). Furthermore, through the inverse-PCR method, we identified the insertion of the US6RNAi construct is located in an intergenic site at 7.69 Mb on chromosome 6 in US6R11. We further validated this insertion site by PCR using three specific PCR primers (Figure 1m; China patent: CN201911243983.0). Through back-crossing and MAS, we then transferred the US6RNAi construct from US6R11 into a susceptible japonica rice cultivar Haidao 5 (HDS, as the recurrent parent), a widely cultivated variety in Jiangsu province nowadays. We found that three introgressed lines, containing homozygous US6RNAi selected from BC2F2 population, all displayed markedly higher resistance to RBSDV than HDS (Figure 1n). In addition, after the artificial inoculation of southern rice black-streaked dwarf virus (SRBSDV), a novel species closely related to RBSDV in the genus Fijivirus (Zhou et al., 2013), we found the marker-free US6RNAi lines also showed strong resistance to SRBSDV that outbreak recently in Southern China (Figure 1o and p). These results indicate that the US6R11 line has great potential for developing new resistant varieties to both RBSDV and SRBSDV.

Many experiments demonstrated that the promoter of the rice rbcS (small subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase) gene is useful to express target gene limited in green tissues but not in milled rice (Wang et al., 2015). To obtain green tissue-expressed S6RNAi rice for avoiding potential effects on grains to a great extent, we transferred S6RNAi driven by the rice rbcS promoter (RS6RNAi) into HDS using the double T-DNA construct. As a result, we obtained marker-free transgenic RS6RNAi lines with an ideal accumulation of the target siRNA (Figure 1q-r). All the RS6RNAi rice lines displayed stable and high resistance to RBSDV (Figure 1s-t).

In summary, our results demonstrate that RNAi targeting RBSDV S6 conferred rice with almost full immunity to this devastating plant virus, while RNAi of S1 or S2 only leads to partially increased resistance. The S6RNAi-mediated resistance is very stable at multiple locations throughout 8 years (2010–2017) in both artificial inoculation and field trials. Importantly, introducing the S6RNAi with distinct transformation vectors did not have adverse effects on agronomical or developmental traits in different rice varieties. In particular, we generated marker-free transgenic S6RNAi lines in elite rice background, which should have great potential in breeding of resistant varieties to both RBSDV and SRBSDV and provide a basis for further safety evaluation and commercial application.

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Conflict of interest

The authors have declared no conflict of interest.

Author contributions

S. Z., X. P. and G. L. W. conceived the project. Z. F., M. Y., J. Z., L. B. W., L. W., T. C., N. Z., W. X., Y. Z., Z. C. K.H. and W. L. performed the research and analysed the data. Z. F. and S. Z wrote the manuscript.

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