



Brief Communication

Transgenic rice overexpressing insect endogenous microRNA csu-novel-260 is resistant to striped stem borer under field conditions

Xixi Zheng¹, Zijin Weng¹, Han Li¹, Zichun Kong¹, Zaihui Zhou¹, Fei Li² , Weihua Ma¹, Yongjun Lin¹ and Hao Chen^{1,*} 

¹National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China

²Institute of Insect Sciences/Ministry of Agriculture Key Laboratory of Molecular Biology of Crop Pathogens and Insect Pests, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, China

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*Correspondence (Tel +86 27 87280916; fax +86 27 87287092; email hchen@mail.hzau.edu.cn)

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Dear editor,

RNA interference (RNAi) is a promising approach for developing insect-resistant crops. In the first two proof-of-concept studies, DNA fragments derived from essential insect genes were constructed into plant expression cassettes as inverted repeats, enabling long double-stranded RNAs (dsRNAs) to be transcribed in host plants (Baum *et al.*, 2007; Mao *et al.*, 2007). After ingestion, dsRNAs overexpressed in plants suppressed target gene expression via small interference RNA (siRNA)-mediated RNAi in western corn rootworm (*Diabrotica virgifera virgifera*; Baum *et al.*, 2007) and cotton bollworm (*Helicoverpa armigera*) (Mao *et al.*, 2007), and thereby reduced their viability. Subsequent studies have mostly employed a similar approach and attempted to develop RNAi crops against various insect species (Liu *et al.*, 2020). However, except for coleopterans that are generally susceptible to RNAi, most insects (e.g. lepidopterans, dipterans, hymenopterans and hemipterans) exhibit unpredictable responses to dsRNA-induced RNAi, and this has become a hurdle for ubiquitous adaption of this strategy (Cooper *et al.*, 2019). Potential factors influencing RNAi efficacy in insects involve dsRNA stability, cellular dsRNA absorption, core RNAi machinery integrity, systemic RNAi spread and target gene amenability (Cooper *et al.*, 2019).

Although most studies on RNAi-mediated insect-resistant plants are based on dsRNA (Liu *et al.*, 2020), limited studies have suggested that microRNA (miRNA)-mediated RNAi is also practically applicable for insect-resistant plant development. miRNA-mediated RNAi plants can be classified into four types based on the origin of miRNA effectors: artificially designed miRNAs (Guo *et al.*, 2014), natural insect miRNAs (Jiang *et al.*, 2017), natural plant miRNAs (Wamiq and Khan, 2018) and modified insect pre-miRNAs (Bally *et al.*, 2020).

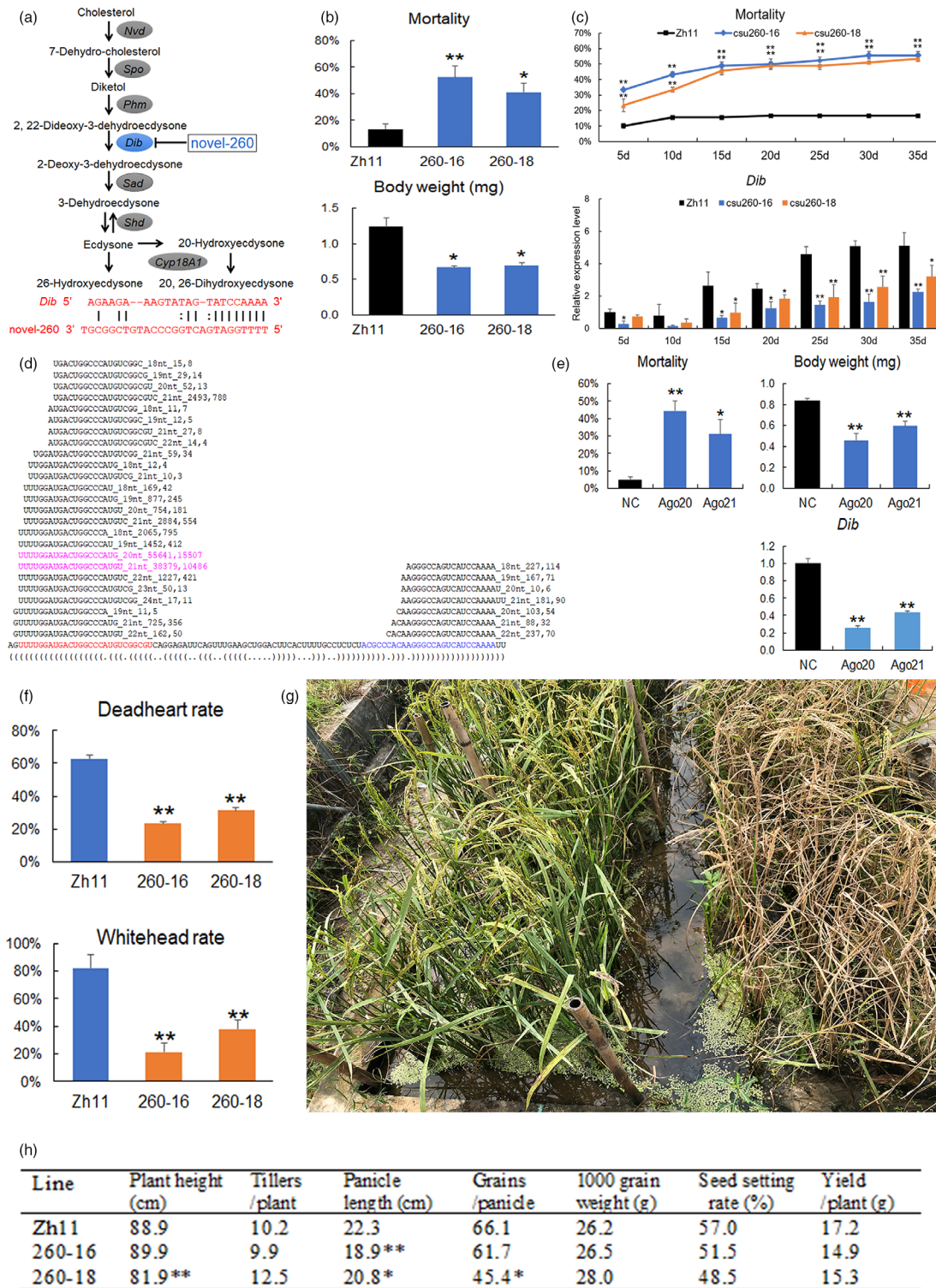
Our previous study demonstrated that striped stem borer (SSB; *Chilo suppressalis*), a destructive insect pest of rice (*Oryza sativa*),

is refractory to dsRNA-induced RNAi but relatively susceptible to artificial miRNA (amiRNA)-induced RNAi. Transgenic rice expressing SSB endogenous miRNA candidates with unknown functions retarded the growth of SSB larvae, without showing significant lethality (Jiang *et al.*, 2017).

In this study, we successfully engineered highly SSB-resistant rice (named csu260) expressing amiRNA of SSB endogenous miRNA csu-novel-miR260 through amiRNA expression technology. Cs-novel-miR260 negatively regulates ecdysteroid biosynthesis in SSB by suppressing the expression of disembodied (*dib*) that encodes a cytochrome P450 enzyme catalysing the C₂₂-hydroxylation of 2, 22-dideoxy-3-dehydroecdysone (Figure 1a; He *et al.*, 2017). Two representative homozygous csu260 rice lines (csu260-16 and csu260-18) with a single-copy insertion were obtained from 28 independent primary transformants.

A five-day stemcutting feeding assay was performed in triplicate with 15 first-instar SSB larvae for each replicate. It showed that csu260-16 and csu260-18 exhibited significant lethality against and retarded the growth of feeding SSB larvae compared with those fed the wild-type (WT) recipient variety Zhonghua11 (Zh11) (Figure 1b). Then, a consecutive stemcutting feeding assay was performed in triplicate with 30 first-instar SSB larvae for each replicate. It confirmed that csu260-16 and csu260-18 rice caused 55.6% and 53.3% larval mortality, respectively, at 35 days after feeding, whereas the mortality of the control larvae was less than 20% (Figure 1c). Correspondingly, the stem-loop quantitative polymerase chain reaction analysis showed consistently lower *dib* expression in the csu260-fed larvae than in the control larvae throughout the feeding assay (Figure 1c).

We used small RNA sequencing to characterize the amiRNA expression in csu260-16 and csu260-18 (Figure 1d). The sequenced amiRNA reads were normalized as reads per million, and the normalized amiRNA abundance was 1.8-fold higher in csu260-16 than in csu260-18. Consistently, csu260-16 showed higher and more stable insect resistance than csu260-18, and *dib* expression in SSB larvae fed csu260-16 was lower than that in larvae fed csu260-18 (Figure 1b, c). These results suggest that amiRNA dose is crucial for insect resistance of amiRNA plants. We used the *osa*-MIR528 precursor as a backbone for constructing amiRNA expression vectors (Jiang *et al.*, 2017). Small RNA sequencing showed that amiRNAs in csu260 rice are preferentially processed into 20- and 21-nt long sequences, similar to mature *osa*-MIR528 (21 nt), although the amiRNA sequence of csu-novel-260 originally constructed into amiRNA expression



vector is 27-nt long. The predominant 20 and 21 nt amiRNAs in csu260 rice possess the intact 5' end of original csu-novel-260, but they lacked 7mer and 6mer at the 3' end, respectively (Figure 1d). MIRNAs generally function effectively as long as the 'seed sequence' (positions 2–8 at the 5' end of miRNA) matches between miRNA and the 3' untranslated region (UTR) of messenger RNA (Brennecke *et al.*, 2005). An *in vitro* feeding assay was performed using 100 nmol synthetic miRNA mimics (agomirs) with the same sequences as the predominant 20

(Ago20) and 21 nt (Ago21) sequences. The agomirs were delivered via five-day germinated Zh11 seeds, which soaked in agomir solutions for 30 min before the assay. The result confirmed the efficacy of these trimmed amiRNAs (Figure 1e), indicating that the length of natural miRNAs would not be a major consideration when constructing the amiRNA expression vectors.

Moreover, we evaluated SSB resistance under field conditions, resembling practical rice production. Because SSB infestation causes

Figure 1 Overexpressing amiRNA of csu-novel-260 confers high resistance against striped stem borer (SSB). (a) The biosynthetic pathway of ecdysteroids and putative target site of csu-novel-260 in 5' UTR of *dib* (modified from He *et al.*, 2017). (b) Five-day stemcutting feeding assay. (c) Thirty-five day consecutive stemcutting feeding assay. (d) Small RNA sequencing of csu260-16 and csu260-18 rice. The bottom sequence is the amiRNA precursor of csu-novel-260. The red and blue bases in the amiRNA precursor are the positions of amiRNA and amiRNA*, respectively. Sequences above the amiRNA precursor of csu-novel-260 are the sequenced amiRNAs (threshold of read number ≥ 10 in csu260-16) matching the amiRNA precursor. The two sequences in pink are the two most abundant amiRNA sequences. The three numbers at the end of each amiRNA sequence are their length, and read counts of csu260-16 and csu260-18. (e) Agomir feeding assay. (f) Field assessment of SSB resistance in the tillering stage (deadheart rate) and the mature stage (whitehead rate) under manual infestation conditions. (g) Field performance under manual infestation in the maturity stage. Left: csu260-18; right: Zh11 control. (h) Agronomic assessment of csu260-16 and csu260-18 under field conditions. Data in (b), (c), (e) and (f) are presented as mean \pm SE. * and ** indicate statistically significant difference at $P < 0.05$ and $P < 0.01$, respectively, compared with the WT control, according to Student's *t*-test

'deadheart' in the tillering stage and 'whitehead' in the heading stage, we conducted two independent field assessments of SSB resistance in these two stages. In both field assessments of SSB resistance, each of csu260-16, csu260-18, and Zh11 was grown in three plots as three replicates. The deadheart rate of csu260-16 and csu260-18 decreased by 62.9% and 50.4%, respectively, compared with that of the WT control, 35 days after the manual inoculation of 30 second-instar SSB larvae per plant in the early tillering stage (Figure 1f). While the whitehead rate of the two csu260 lines decreased by 74.6% and 54.0%, respectively, compared with that of the WT control, 40 days after manual infestation with 1.4 SSB adult moths per plant in the early heading stage (Figure 1f, g). These results indicate that csu260 rice substantially prevented yield loss even under severe SSB infestation.

To evaluate agronomic performance in the field, each of csu260-16, csu260-18 and Zh11 was grown in three plots as three replicates. The result demonstrated that the most important trait yield per plant of two csu260 lines was not significantly different from that of the control. However, panicle length of csu260-16 and plant height, panicle length and grains per panicle of csu260-18 showed significant differences from those of the control (Figure 1h). Agronomic variations in transgenic lines are usually caused by somatic mutations occurring in transformation procedure. Therefore, most agronomic variations may be recovered via backcrosses with the WT and subsequent self-pollinations.

Our results demonstrated that amiRNA-induced RNAi is more applicable for controlling SSB than dsRNA-induced RNAi. Because endogenous miRNAs naturally regulate their gene targets in insects, their use theoretically avoids certain factors that cause inefficient RNAi, such as refractory target genes and relevant RNAi machinery deficiency (Cooper *et al.*, 2019). Moreover, critical siRNA and miRNA components associated with their biogenesis and function are significantly different (Cooper *et al.*, 2019), potentially causing variations among insects in terms of small RNA stability, cellular uptake and intercellular transport between siRNA and miRNA. These factors could explain why SSB is resistant to dsRNA-induced RNAi but relatively susceptible to miRNA-induced RNAi. Taken together, amiRNA-induced RNAi can be an alternative approach for controlling these insect pests insensitive to dsRNA-induced RNAi, which would broaden the applicability of RNAi-mediated insect pest management.

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Author contributions

H.C., F.L. and Y.L. designed the study; X.Z., Z.W., H.L., Z.K. and W.M. performed the experiments; H.C., X.Z. and Z.Z. analysed the data and prepared the figures; H.C. and X.Z. wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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