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Brief Communication

Anther-specific expression of *OsRIP1* causes dominant male sterility in rice

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Rice (*Oryza sativa* L.) is a major staple food crop feeding more than half of the world human population. Hybrid rice, using the three-line or two-line strategy for hybrid seed production, produces 10%–20% higher grain yield than the conventional rice and thus has been widely commercialized (Cheng *et al*., 2007). A number of genes are involved in the control of cytoplasmic male sterility (CMS) or photoperiod/thermos-sensitive genic male sterility (P/TMS), have been reported and successfully used in hybrid rice breeding (Chen and Liu, 2014). The Sanming-*Dms* line (named *Dms1* hereafter) has been widely used for recurrent selection in breeding programmes in China (Zhang *et al*., 2022). Use of *Dms1* can eliminate the hand emasculation procedure, thus enabling large-scale crossing with various parents. At each selection, only the male-sterile plants with traits of interest are chosen as female to cross with other breeding materials. The male sterility is controlled by a single dominant locus, and has been mapped on the short arm of chromosome 8 (Yang *et al*., 2012), however, the underlying gene has not been cloned.

To clone *Dms1*, we constructed a near-isogenic line (NIL)-*NJ4*^{Dms1} contains the genetic background of the *japonica* rice variety *Ningjing4* (*NJ4*) but carries a chromosome segment insertion with *Dms1* introduced from the mutant donor (Figure S1). Due to the dominant nature of male sterility in *Dms1*, NIL-*NJ4*^{Dms1} must be maintained as heterozygote at the *Dms1* locus. Compared to *NJ4*, anthers of NIL-*NJ4*^{Dms1} are much smaller (Figure 1a). In contrast, NIL-*Nip*^{Dms1} set seeds at a rate close to the recipient *Nipponbare* (*Nip*) when pollinated with wild-type pollens, indicating the *Dms1* locus has little effect on female gamete fertility (Figure S2). Further, iodine-potassium iodide staining assay revealed completely aborted pollens in NIL-*NJ4*^{Dms1}, again verifying the male specificity of the *Dms1*-associated reproductive sterility (Figure S3). No obvious morphological difference was observed between *NJ4* and NIL-*NJ4*^{Dms1} (Figure S3). Acetocarmine staining showed defective meiosis of the pollen mother cell in NIL-*NJ4*^{Dms1} (Figure S4). Detailed cytological observations uncovered that the pollen mother cell (PMC) in NIL-*NJ4*^{Dms1} failed to complete meiosis, resulting in collapsed anthers at the S7 and S8 stages. Ruptured cell membrane and vacuoles were present in PMC, a phenomenon similar to necroptosis in mammal cells suffering from toxin or other harmful external environments (Figure 1b and Figure S5). Thus, the pollen-less anther is the cause of male sterility in NIL-*NJ4*^{Dms1}.

To fine-map *Dms1*, an F_2 population with 934 individuals derived from the cross of NIL-*NJ4*^{Dms1} with *NJ4* was developed. *Dms1* was primarily located to a 290-kb interval between the molecular markers xhy45 and xhy54 on chromosome 8, and subsequently fine-mapped to a 5.4-kb region between the markers xhy128 and xhy129 using 19 348 plants (Figure 1c). This region contains a putative gene, *LOC_Os08g03820*, encodes a 32.5-kDa ribosome-inactivating protein (RIP), thus named *OsRIP1* herein. Sequence comparison found a 1978-bp insertion 98 bp upstream of the start codon of *OsRIP1* in NIL-*NJ4*^{Dms1} (Table S1). qRT-PCR analysis showed over threefold higher *OsRIP1* expression in young inflorescences of NIL-*NJ4*^{Dms1} than in *NJ4* (Figure 1d). We cloned the promoter region from *Nip* and NIL-*Nip*^{Dms1}, respectively, and used them to drive expression of the *GUS* reporter gene. Intriguingly, *GUS* expression was seen in the *pOsRIP1*^{Dms1}*::GUS* plants, but not in the *pOsRIP1*^{Nip}*::GUS* plants (Figure 1e). In situ hybridization to anthers at stages 8a and 8b further confirmed *OsRIP1* expression in NIL-*Nip*^{Dms1} but not in *Nip* (Figure 1e and Figure S6). Next, we knocked out *OsRIP1* in *Nip* and NIL-*Nip*^{Dms1}, respectively, using the CRISPR/Cas9 technology. We observed fertility restoration in NIL-*Nip*^{Dms1} knockout plants, while no fertility change was seen in *Nip* knockouts (Figure 1f). When using the anther-specific *EAT1* promoter to drive *OsRIP1* in *Nip*, the male sterility phenotype mimicking NIL-*Nip*^{Dms1} appeared (Figure 1g). Moreover, manipulated root- or leaf-specific expression of *OsRIP1* resulted in lethal plants (Figure S7), which

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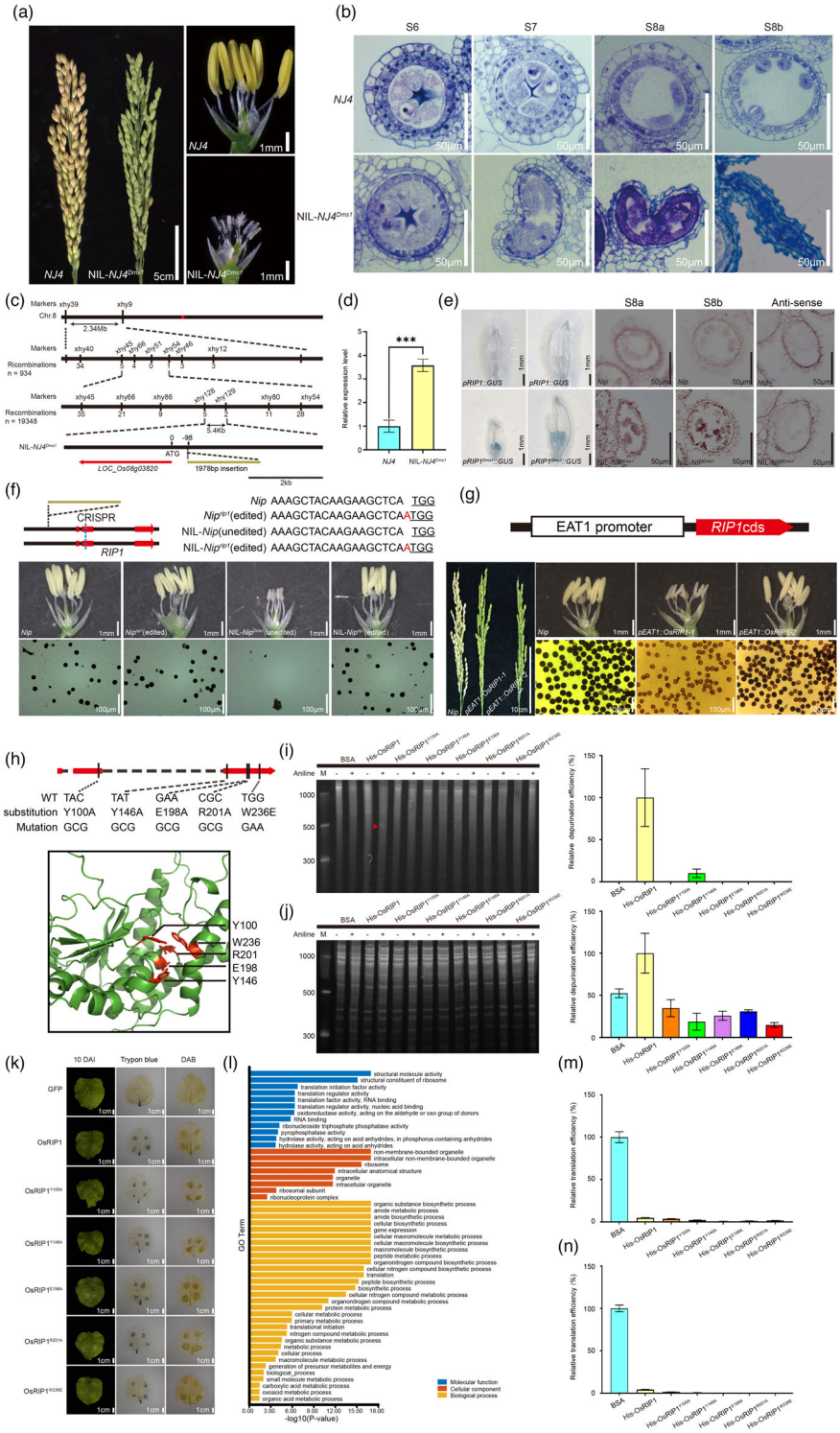

Figure 1 Cloning and genetic analysis of Dms1 in rice. (a) Sterile panicle and defective anther in NIL-NJ4Dms1. (b) Transverse sections showing anther and microspore development in NJ4 and NIL-NJ4Dms1. (c) Fine mapping of Dms1. (d) qRT-PCR analysis of OsRIP1 expression in anther. (e) GUS staining of transgenic spikelets and in situ hybridization detection of OsRIP1 transcripts in anthers. (f) Knock out of OsRIP1 in Nip and NIL-NipDms1. (g) Comparison of panicle, anther and pollen of Nip and transgenic plants carrying OsRIP1 under control of anther-specific promoter. (h) Homologous modelling of protein structure of OsRIP1. The substitution mutations are shown. (i,j) Recombinant His-tagged RIP1 and its mutant versions processed rRNA from rabbit (i) and wheat (j). depurinate quantified results are shown at right; red triangle indicates the depurinate product. (k) Transiently expressed OsRIP1 and its mutant versions in tobacco leaves. Images were taken after 10 days. Trypan blue staining indicates cell death in tobacco leaves and DAB staining indicates ROS accumulation in tobacco leaves. (l) GO analysis of the IP-MS results related to OsRIP1. (m) In vitro translation efficiency in Rabbit reticulocyte lysate treated with OsRIP1 and its mutant versions. (n) In vitro translation efficiency in wheat germ extract treated with OsRIP1 and its mutant versions.

demonstrate that OsRIP1 has the conserved toxicity to rice cells. Taken together, our results demonstrate that *LOC_Os08g03820* is responsible for *Dms1* and the enhanced expression by the insertion that causes male sterility.

The mechanism of RIP producing cytotoxicity in both mammal and plant are elusive (Grela *et al*., 2019). It is known that RIPs and homologous modelling of protein structure reveals five amino acids that aggregate to form active centres (Figure 1h). To check whether OsRIP1 has the similar enzymatic activity, the HIS-OsRIP1 and HIS-mutants (substitution at the five sites: HIS-OsRIP1^{Y100A}, HIS-OsRIP1^{Y146A}, HIS-OsRIP1^{E198A}, HIS-OsRIP1^{R201A} and HIS-OsRIP1^{W236E}) recombination proteins were expressed in *E. coli*. The HIS-OsRIP1, but not the other five mutant proteins, showed significant 28SrRNA depurinate activity in rabbit reticulocyte lysate (Figure 1i), suggesting that OsRIP1 is a typical ribosome-inactivating protein. Conversely, neither HIS-OsRIP1 nor its mutant versions had depurinate activity in wheat germ extract (Figure 1j). Surprisingly, these five non-enzyme-active mutant proteins still caused cell death after introduced into tobacco (Figure 1k). To investigate how OsRIP1 produces cytotoxicity in plants, semi-in vivo immunoprecipitation–mass spectrometry was performed. GO analysis (gene ontology) indicated that proteins involved in structure constituent of ribosome and translation process were enriched (Figure 1l), implying that OsRIP1 might produce cytotoxicity by inhibiting ribosome translation function. To test this possibility, we performed in vitro translation inhibition experiments using cell-free protein synthesis system. As anticipated, OsRIP1 and all the five mutant versions showed strongly inhibitory effects on translation in mammal and plant (Figure 1m, n). All those data suggest that OsRIP1 toxifies plant cells by inhibiting translation function rather than RNA depurating activity.

In China, the *Taigu*-dominant male-sterile gene *Ms2* has been widely used for population improvement by recurrent selection in wheat conventional breeding (Ni *et al*., 2017; *Xia et al*., 2017). However, such an approach has not been possible in rice. In this study, we have cloned the *Dms1* locus and demonstrated why the male sterility is dominant in the *Dms1*. The use of dominant male sterility (*Dms*) lines for recurrent selection can at least save one generation of selfing, compared to recessive male sterility lines. Thus, isolation of *Dms* causal genes will facilitate creation of *Dms* in other varieties. Male sterility in *Dms1* is a much-preferred line in rice breeding. The *Dms1* line is an ideal line widely used in the recurrent selection breeding strategy. Identification of *Dms1* provides opportunity to develop other dominant male sterility lines in various genetic backgrounds by transformation approach, which will increase efficiency and reduce cost in breeding programmes. Recent studies have discovered a number of genes involved in male gametogenesis, and their malfunctionization leads to male sterility. It is possible that the activated gametogenesis-specific expression of *OsRIP1* directly or indirectly affects translation of some, if not all, of those genes, resulting in defective gametogenesis. Mammals suffer from toxicity when accidently foraging plants containing RIPs, and the uptaken RIPs in mammals specifically interact with stalk subunit of ribosome, then depurinate 28SrRNA (Grela *et al*., 2019). The damaged ribosome is no longer a functional organelle for normal protein translation. However, RIPs exist in plants, but cannot depurinate plant ribosomes and its toxicity to plants is unclear (Nielsen and Boston, 2001; De Zaeytijd and Van Damme, 2017). Our results also indicate that OsRIP1 inhibits translation function of ribosome not by 28SrRNA depurination. As the cytotoxicity of OsRIP1 is likely conserved in monocot and dicot plants, we anticipate OsRIP1 can be employed to create new *Dms* lines, for example, by anther-specific expression in other crops.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Jianmin Wan, Zhigang Zhao and Shanshan Zhu supervised the project. Dekun Lei and Anqi Jian performed the experiments. Xianbo Huang provided the *Dms* material. Other authors provided technical supports. Dekun Lei, Anqi Jian prepared the article.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1-S7 Supplementary Figures.
Table S1-S2 Supplementary Tables.