Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2023), pp. 1–3 doi: 10.1111/pbi.14225

Brief Communication

Generating herbicide resistant and dwarf rice germplasms through precise sequence insertion or replacement

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Received 9 March 2023;
revised 24 October 2023;
accepted 26 October 2023.
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Keywords: prime editing, targeted insertion, targeted replacement, herbicide resistant.

Precise sequence insertion or replacement in plants is technically challenging but is of great importance in crop breeding because many agronomic traits are affected by DNA fragment variations. Although prime editing (PE) has been continuously optimized to improve its activity in plants (Jiang *et al.*, 2022; Li *et al.*, 2022a,b; Zong *et al.*, 2022), it is still inefficient for targeted insertion or replacement of longer sequences. Similar strategies, twinPE (Anzalone *et al.*, 2022) and GRAND editing (Wang *et al.*, 2022), in which a pair of PE guide RNAs (pegRNAs) are partially complementary to each other in their reverse transcriptase template (RTT) but are not homologous to the genomic sequences, were recently developed to facilitate longer sequence insertion (Figure 1a).

HPPD-inhibitor herbicides such as β-triketones are effective in controlling resistant weeds that have emerged. The *HIS1* gene in rice confers broad-spectrum resistance to triketone herbicides, whereas a dysfunctional *his1* allele with a 28-bp fragment deletion was found in triketone-sensitive *Indica* varieties (Maeda *et al.*, 2019). A genetic survey for 631 *Indica* varieties commonly used in rice breeding revealed that the 28-bp deletion is widely distributed, including 50.7% 3-line restorers, 40.7% 2-line restorers and 18.1% conventional varieties (Lv *et al.*, 2021), which causes a huge risk for applying HPPD-inhibitor herbicides in *Indica* rice cultivating area.

S1035 is an elite conventional *Indica* cultivar that sensitive to triketone due to the 28-bp deletion at *HIS1*. PE and GRAND editing strategies were tested to targeted insert the 28-bp fragment. Different from the design of PE (Figure S1), GRAND editing uses a pair of pegRNAs to delete the 18-bp genomic sequence between the two nicks and to insert a 46-bp designed sequence, including the to-be-inserted 28-bp and the to-be-replaced 18-bp sequences in which synonymous mutations were introduced to reduce the homology between RTT and genomic sequences (Figure 1b). It was reported that the sequence complementarity within the RTTs significantly affects the insertion efficiency (Wang *et al.*, 2022), 10-, 18- or 26-bp sequence complementarity was designed in our test (Figure 1b).

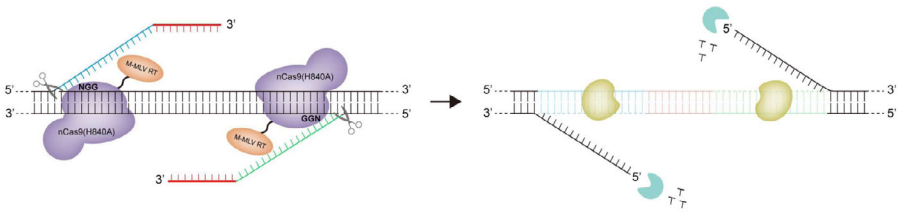
PE yielded 1.46% precise insertion events after protoplast transfection, indicating its low efficiency for DNA fragment insertion. GRAND editing with 10-bp complementary RTT sequences (RTT-10) achieved higher efficiency (9.88%) than that of RTT-18 (3.76%) or RTT-26 (0.59%) (Figure 1c). We then further evaluated GRAND editing during stable transformation. The transgenic lines were directly treated by 60 μM mesotrione, a widely used β-triketone herbicide. Nine (11.5%) resistant lines were generated from RTT-10 transformation, while only one was obtained each from RTT-18 and RTT-26 transformations (Figure 1d; Figures S2 and S3). Then RTT-10 construct was used to edit MingHui86, an elite 3-line restorer with the 28-bp deletion at *HIS1*. 13 (15.5%) of the 84 transgenic lines were recovered mesotrione-resistance (Figure 1d and Figure S4).

T-DNA free, homozygous offsprings were identified from S1035 edited lines in the T1 generation (Figure 1e and Figure S5, Table S1). The expression level of the repaired *HIS1* gene was comparable with that of wild type (WT; Figure 1f), but the mesotrione-resistance of the edited plants was similar with that of XiuShui134, a functional *HIS1* gene-containing *Japonica* rice variety (Figure 1g). These results indicated that GRAND editing can be used to rescue other defective varieties for quickly solving the risk of applying HPPD-inhibitor herbicides in *Indica* rice cultivating area.

PE and GRAND editing strategies were also tested for introducing the glyphosate-resistant $T^{173}IP^{177}S$ mutations ($C^{518}T + C^{529}T$) into the *OsEPSPS* gene (Figure 1h; Figure S6). Protoplast test showed the efficiency of GRAND editing was 1.86 fold of that of PE (9.98% vs. 5.37%; Figure S7). Only 3 (2.0%)

Please cite this article as: Liu, X., Wang, Y., Wang, H., He, Y., Song, Y., Li, Z., Li, M., Wei, C., Dong, Y., Xue, L., Zhang, J., Zhu, J-K. and Wang, M. (2023) Generating herbicide resistant and dwarf rice germplasms through precise sequence insertion or replacement. *Plant Biotechnol. J.*, https://doi.org/10.1111/pbi.14225.

(a)



(b)

GRAND editing design at *Oshis1*

Target: TGAACAAGTATGCATCATTCAGGCTATGGCCAAGCTTCTTGAGCTTGATGAG

Deletion: TTCAGGCTATGGCCAAGC

+28 bp

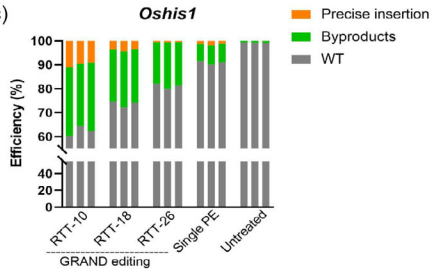
Insertion: GGAACCAAAAGAATTAGAGACGATATTATACAAGCAATGGCGAAAC

RTT-10: GGAACCAAAAGAATTAGAGACGATATTATACAAGCAATGGCGAAAC

RTT-18: GGAACCAAAAGAATTAGAGACGATATTATACAAGCAATGGCGAAAC

RTT-26: GGAACCAAAAGAATTAGAGACGATATTATACAAGCAATGGCGAAAC

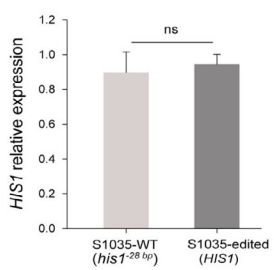
(c)



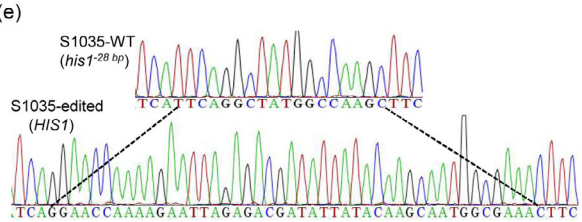
(d)

Variety	Complemental RTT length	Transgenic plants (T0)	Resistant plants	Plants with precise insertion			
				Ho	Bi	He	Chi
S1035	10 bp	78	9 (11.5%)	0	2 (2.6%)	5 (6.4%)	2 (2.6%)
	18 bp	71	1 (1.4%)	0	0	1 (1.4%)	0
	26 bp	78	1 (1.3%)	0	0	1 (1.3%)	0
MingHui 86	10 bp	84	13 (15.5%)	2 (2.3%)	2 (2.3%)	6 (7.0%)	3 (3.5%)

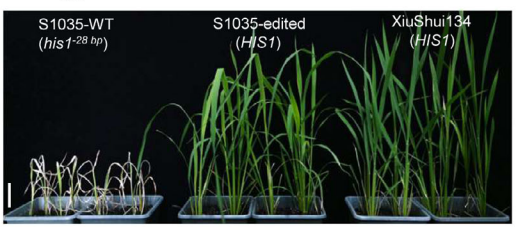
(f)



(e)



(g)



(h)

GRAND editing design at *OsEPSPS*

Target: CAACTCTTCTTGGGGAACGCTGGAAC^{518}TGCAATGCGAC^{529}CATTGACAGCAGCCGTGACTGC

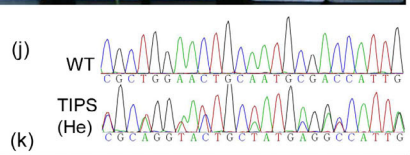
Deletion: CGCTGGAAC^{518}TGCAATGCGAC^{529}CATTG (--T^{173}AMRP^{177}--)

Replacement: TGCAGGTAT^{518}TGCTATGAGGT^{529}CATTA (--I^{173}AMRS^{177}--)

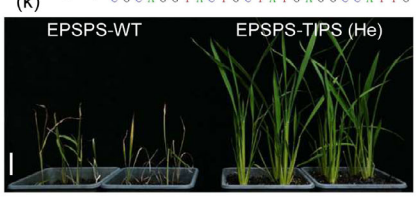
(i)

Total plants (T0)	Plants with precise replacement			Plants with byproducts only	WT
	Ho	He	Chi		
147	5 (3.4%)	18 (12.2%)	6 (4.1%)	5 (3.4%)	113 (76.9%)

(j)



(k)



(l)

GRAND editing design at *OsSLR1*

TVHYNP domain

Target: GTTCGTGTCGCACCTGGCCACGGACACCGTGCACTACAACCCCTCGGACCTCTCCTCCTGGG

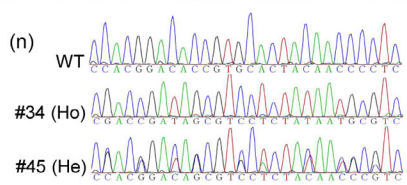
Deletion: CCACGGACACCGTGCACTACAACCCCTC (--TVHYNP--)

Replacement: CGACCGATAGCGTCCTCTATAATGCGTC (--SVLYNA--)

(m)

Total plants (T0)	Plants with precise replacement			Plants with byproducts only	WT
	Ho	He	Chi		
58	4 (6.9%)	19 (32.8%)	6 (10.3%)	5 (8.6%)	24 (41.4%)

(n)



(o)

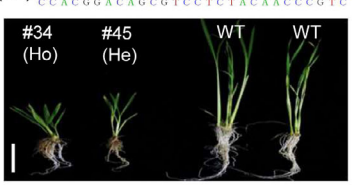


Figure 1 Efficient DNA fragment insertion/replacement in the rice genome. (a) Simplified schematic view of GRAND editing (Wang *et al*., 2022). (b, h, l) Designs of GRAND editing at *Oshis1*, *OsEPSPS* and *OsSLR1*. Boxes indicate guide-PAM sequences, PAMs are marked in bold and primer binding sites are underlined. The complementary RTTs are marked in yellow background and the designed mutations in RTT are marked in red. (c) PE and GRAND editing efficiencies of *Oshis1* in protoplast transfection. (d, i, m) Summary of the GRAND editing efficiencies in stable transformation. Ho: homozygous precise insertion/replacement. Bi: biallelic mutant, both alleles containing the desired insertion/replacement with different non-sense mutations. He: heterozygous insertion/replacement, with desired insertion/replacement occurring only at one allele, regardless the genotype of another allele. Chi: chimeric mutants that contain precise insertion/replacement events. (f) Relative *OsHIS1* expression level in S1035 WT and homozygous edited mutants. ns means no significant difference. (e, j, n) Sequencing chromatograms of the WT and mutants at target sites of *OsHIS1*, *OsEPSPS* and *OsSLR1*. Dashed lines in 1d indicate the deletion and insertion sequences designed in 1b. (g, k, o) Phenotypic difference between WT and the edited plants of *OsHIS1* (g, mesotrione treatment), *OsEPSPS* (k, glyphosate treatment) and *OsSLR1* (o). Bars equal to 5 cm.

heterozygous plants from PE contained desired TIPS mutations, other mutants were either chimeras or that the $C^{518}T$ and $C^{529}T$ substitutions occurred separately (Figures S6 and S8). In contrast, GRAND editing generated 5 (3.4%) homozygous and 18 (12.2%) heterozygous edited lines (Figure 1i; Figure S8). Since the plants carrying homozygous TIPS mutations were seriously affected in their growth or even died after transplanting to soil (Figure S9), the heterozygous TIPS mutants were tested by glyphosate and showed no symptoms of damage but the WT plants withered (Figure S10). Their offsprings in the T1 generation inherited the glyphosate-resistant trait (Figure 1j,k; Table S1). To further evaluate the activity of GRAND editing, we designed to replace a 28-bp fragment (containing three amino acid substitutions) in the TVHYNP domain of the *OsSLR1* gene (Figure 1l). We obtained 4 (6.9%) homozygous, 19 (32.8%) heterozygous plants from 58 transgenic lines (Figure 1m). As expected, both homozygous and heterozygous mutants displayed a dwarf phenotype (Figure 1n,o).

During preparation and review of our manuscript, Li *et al*. (2023) reported the use of GRAND editing in knock-in of protein tags. In combination with site-specific recombinases, large DNA fragments of up to 11.1 kb were targeted inserted in the rice genome (Sun *et al*., 2023). Collectively, ours and these results demonstrated the technical advancement for insertion or replacement of DNA fragments in the plant genome, which is of great importance in genetic research and crop breeding.

Acknowledgements

This work was supported by Bellagen Biotechnology Co. Ltd., Nanfan special project, CAAS (ZDXM04, ZDXM23014), National Key R&D Program of China (2021YFA1300404 to J.-K.Z.), National Natural Science Foundation of China (32188102 to J.-K.Z., 32271524 and 31901046 to M.W.), the Youth Innovation Promotion Association, CAS (2020272) to M.W., the Key R&D Program of Ningxia (2021BEB04075) and Ningxia Natural Science Foundation (2022AAC03007) to X.L.

Conflict of interest

The authors declare no competing interests.

Author contributions

J.-K.Z. and M.W. conceived of and designed the research. X.L., Y.W., H.W., Y.H., Y.S., Z.L., M.L., C.W., Y.D. L.X. and J.Z. conducted the experiments and analysed the data. J.-K.Z. and M.W. wrote the manuscript.

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Supporting information

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

Figures S1-S10 Supplementary Figures
Tables S1-S2 Supplementary Tables
Data S1 Materials and methods.

