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Exploiting the efficient Exo:Cas12i3-5M fusions for robust single and multiplex gene editing in rice[oo]

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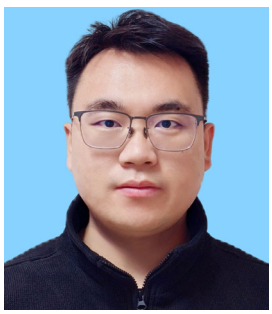

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ABSTRACT

The development of a single and multiplex gene editing system is highly desirable for either functional genomics or pyramiding beneficial alleles in crop improvement. CRISPR/Cas12i3, which belongs to the Class II Type V-I Cas system, has attracted extensive attention recently due to its smaller protein size and less restricted canonical "TTN" protospacer adjacent motif (PAM). However, due to its relatively lower editing efficiency, Cas12i3-mediated multiplex gene editing has not yet been documented in plants. Here, we fused four 5′ exonucleases (Exo) including T5E, UL12, PapE, ME15 to the N terminal of an optimized Cas12i3 variant (Cas12i3-5M), respectively, and systematically evaluated the editing activities of these Exo:Cas12i3-5M fusions across six endogenous targets in rice stable lines. We demonstrated that the Exo:Cas12i3-5M fusions increased the gene editing efficiencies by up to 12.46-fold and 1.25-fold compared with Cas12i3 and Cas12i3-5M, respectively. Notably, the UL12:Cas12i3-5M fusion enabled robust single gene editing with editing efficiencies of up to 90.42%–98.61% across the six tested endogenous genes. We further demonstrated that, although all the Exo:Cas12i5-5M fusions were capable of multiplex gene editing, UL12:Cas12i3-5M exhibited a superior performance in the simultaneous editing of three, four, five or six genes with efficiencies of 82.76%, 61.36%, 52.94%, and 51.06% in rice stable lines, respectively. Together, we evaluated different Exo:Cas12i3-5M fusions systemically and established UL12:Cas12i3-5M as the more robust system for single and multiplex gene editing in rice. The development of an alternative robust single and multiplex gene editing system will enrich plant genome editing toolkits and facilitate pyramiding of agronomically important traits for crop improvement.

Keywords: Cas12i3-5M, exonucleases, multiplex gene editing, rice (*Oryza sativa* L.)

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INTRODUCTION

The development of a single and multiplex gene editing system is highly desirable for either functional genomics or pyramiding beneficial alleles in crop improvement. Recently, a new Class II type V-I CRISPR/Cas system, CRISPR/Cas12i3, was exploited and used as a new alternative tool for genome editing (Bai et al., 2024; Duan et al., 2024; Lv et al., 2024). Like Cas12a, Cas12i3 is only guided by a single crRNA (~43 nt), which makes it an ideal tool for high-throughput multiplex

gene editing for crop improvement (Bai et al., 2024; Duan et al., 2024; Lv et al., 2024). Compared with Cas12a, Cas12i3 recognizes the less restricted thymidine-rich 5′-TTN-3′ protospacer adjacent motif (PAM) and produces similar double-stranded breaks (DSBs) with 5′-staggered ends (Duan et al., 2024; Lv et al., 2024). So far, only a few successful reports have been documented in plants due to the relatively lower editing efficacy of Cas12i3 (Bai et al., 2024; Duan et al., 2024; Lv et al., 2024). To improve the editing efficiency of Cas12i3, a Cas12i3-5M variant (S7R/D233R/D267R/N369R/S433R) was exploited by rational design and protein engineering that exhibited significantly improved gene editing activity in mammalian cells and rice (Duan et al., 2024). However, whether Cas12i3-5M could be used for efficient multiplex editing in plants remained to be investigated.

The DSBs containing 5′-staggered ends induced by Cas12i3/Cas12i-5M may compromise editing efficiency due to both cellular error-free non-homologous end joining (NHEJ) and microhomology-mediated end joining (MMEJ) repair pathways (Moore and Haber, 1996; Budman et al., 2007; Gu et al., 2007). Fusion of either 5′ or 3′ exonucleases to Cas endonucleases could significantly increase the insertion and deletion (InDel) frequencies by preventing implementation of the error-free NHEJ repair pathway, especially for DSBs containing 5′-staggered ends induced by Cas12a (Garforth and Sayers, 1997; Clements et al., 2017; Wu et al., 2020; Zhang et al., 2020; He et al., 2024). However, different exonucleases possess different exonuclease activities (Schreiber et al., 2024), and thus may affect the editing activity of the exonuclease and Cas fusion protein, as well as its editing profiles in plant stable lines. We reasoned that fusion of a more active 5′ endonuclease could resect the 5′ staggered ends induced by Cas12i3-5M more efficiently, and thus further improve its editing efficacy and enable multiplex gene editing. Here, we fused four 5′ endonucleases from different resources, including T5 endonuclease (T5E) from bacteriophages (Ceska et al., 1996), UL12 exonuclease from Herpes simplex virus 1 (HSV1) (Bronstein and Weber, 1996; Chung and Hsu, 1996), PapE (a UL12 ortholog from Papiine alpha Herpes Virus 2) (Black et al., 2014) and ME15 (the T7E homolog of bacteriophage IME15) (Huang et al., 2012), to the N-terminus of Cas12i3-5M to generate different exonuclease and Cas12i3-5M (Exo:Cas12i-5M) fusions. We systemically evaluated the editing activities of these Exo:Cas12i-5M fusions on single and multiplex gene editing in rice stable lines. We demonstrated that the UL12:Cas12i3-5M fusion exhibited superior performance and enabled robust gene editing of the six tested endogenous genes in rice stable lines. Different Exo:Cas12i-5M fusions produced different editing profiles. Furthermore, the UL12:Cas12i3-5M fusion outperformed other Exo:Cas12i-5M fusions in multiplex editing for simultaneous editing of three, four, five, or six endogenous genes in rice stable lines. Engineering of a robust Exo:Cas12i3-5M fusion-mediated single and multiplex gene editing system will not only enrich the plant genome editing toolkits, but also facilitate both fundamental biological research into dissecting a complicated pathway and pyramiding agronomically important traits in rice, and may be extended to other agriculturally important crop species.

RESULTS

To further improve the editing efficiency of Cas12i3-5M, we systemically evaluate the effect on editing efficacies of different 5′ exonucleases upon fusion to Cas12i3-5M. We fused the rice codon-optimized 5′ endonucleases from different resources, including T5E, UL12, PapE, and ME15 (Table S1), to the N-terminus of Cas12i3-5M to generate different Exo:Cas12i-5M fusions (Figure 1A). We used the 35S-CmYLCV-U6 composite promoter to drive the expression of the crRNA array (Figure 1A), as it was more efficient than the *Pol*III promoter (*U3* or *U6*) in CRISPR/Cas9-mediated prime editing in rice (Jiang et al., 2020). We chose seven rice endogenous genes/targets, including *OsHRC*, *OsSBEIIb*, *OsCKX2*, *OsARE1*, *OsBADH2*, *OsDEP1*, and *OsARF4*, as targeted genes for either single or multiplex gene editing (Figure 1B; Tables S2, S3). Once these targets were edited, traits of interest could be improved, including enhanced blast resistance (*OsHRC*) (Ding et al., 2023), improved resistant starch content with health benefits (*OsSBEIIb*) (Sun et al. 2016), enlarged grain size (*OsCKX2* or *OsARF4*) (Yeh et al., 2015; Xu et al., 2016; Hu et al., 2018; Rong et al., 2022), improved nitrogen use efficiency (*OsARE1*) (Wang et al., 2018), aromatic rice (*OsBADH2*) (Hui et al., 2022), and ideal plant architecture (*OsDEP1*) (Liu et al., 2021). For single gene editing, we also included Cas12i3 and Cas12i3-5M as controls (Figure 1A). For these endogenous genes, in order to be time-saving and cost-effective, we first evaluated the editing activities of Cas12i3-5M at these tested loci through RNP *in vitro* (Supplemental Material and Method). We then evaluated the editing performances of Cas12i3, Cas12i3-5M and different Exo:Cas12i3-5M fusions across six endogenous target loci in rice stable lines (Figure 1A, B). As expected, whereas Cas12i3 and Cas12i5-5M induced targeted gene knockout at average efficiencies of 0.00%–17.20% and 75.49%–91.50% across the six tested endogenous genes/targets (Figure 1C; Table S3), the average editing efficiencies of T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M were 83.84%–93.39%, 90.42%–98.61%, 82.79%–92.30%, and 83.33%–93.95%, respectively, at the six tested endogenous genes/targets (Figure 1C; Table S3). While Cas12i3 indeed exhibited very low efficiency as expected (Figure 1C), compared with Cas12i3-5M, the T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M fusions improved the editing efficiency, for example up to 1.11-fold (83.84%/75.49%), 1.25-fold (94.17%/75.49%), 1.19-fold (89.96%/75.49%), and 1.15-fold (86.86%/75.49%), respectively, at *OsARE1* (Figure 1C; Table S3). UL12:Cas12i3-5M, in particular, outperformed other Exo:Cas12i3-5M fusions, with average editing efficiencies of 90.49% at *OsHRC*, 90.42% at *OsSBEIIb*, 98.61% at *OsCKX2*, 94.16% at *OsARE1*, 96.02% at *OsBADH2*, and 94.01% at *OsDEP1* (Figure 1C; Table S3). Furthermore, except for the UL12:Cas12i3-5M fusion, other Exo:Cas12i3-5M fusions

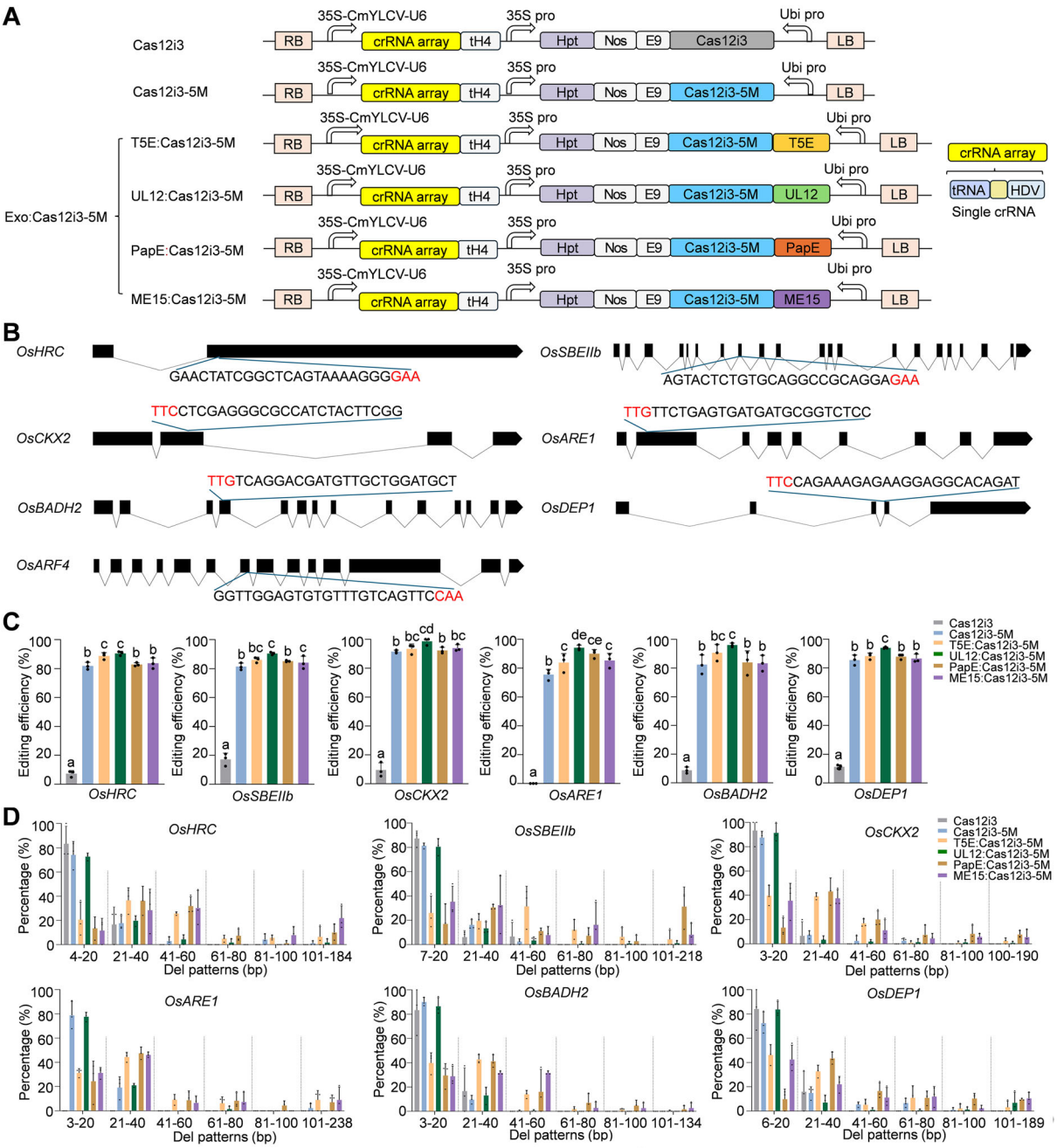

Figure 1. Evaluation of the editing efficiencies and outcomes at the six tested endogenous targets/genes derived from the Cas12i3, Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M in stable transgenic rice lines, respectively

(A) Schematic diagrams of Cas12i3, Cas12i3-5M, and Exo:Cas12i3-5M. The Cas12i3, Cas12i3-5M, and Exo:Cas12i3-5M are driven by a maize Ubiquitin promoter (Ubi). The 35S composite promoter (35S-CmYLCV-U6) is used to drive the expression of a tRNA–crRNA–HDV array and *hpt* gene served as a selection marker. Exo indicates T5E, UL12, PapE, or ME15. **(B)** The gene structures of target genes including *OsHRC*, *OsSBEIIb*, *OsCKX2*, *OsARE1*, *OsBADH2*, *OsDEP1*, and *OsARF4* in this study. Exon regions are shown as black boxes, and the PAM sites (5′-TTN-3′) are highlighted in red. **(C)** The editing efficiencies of Cas12i3, Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M across six endogenous genes/targets in independent transgenic rice lines. Bar plots show the on-target gene knockout frequencies. The frequencies Cas12i3, Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M are shown highlighted in gray, blue, orange, green, brown or purple, respectively. The editing efficiencies are calculated based on three biological repeats. The columns sharing the same letters indicate no significant difference from each other. **(D)** The deletion patterns induced by Cas12i3, Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M at six targeted gene loci. The deletion patterns are presented as the percentage of different lengths of deletions induced by Cas12i3, Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M at *OsHRC*, *OsSBEIIb, OsCKX2*, *OsARE1*, *OsBADH2*, and *OsDEP1* loci.

induced a higher proportion of larger deletions in the edited rice stable lines. For example, Cas12i3 and Cas12i3-5M predominantly generated 4–20 bp deletions (mainly composed of 8–18 bp deletions); T5E:Cas12i3-5M, PapE:Cas12i3-5M and ME15:Cas12i3-5M induced proportionally much larger deletions of 21-60 bp or longer than 100 bp (Figure 1D). Interestingly, despite its superior editing performance in single gene editing, we observed that UL12:Cas12i3-5M generated proportionally smaller deletions (Figure 1D) suggesting that, although UL12 had higher exonuclease activity, it may have a lower affinity for 5′-staggered end DNA substrates of DSBs *in vivo*. This was further reinforced by the depth of the 5′-end resection (Figure S1). In general, the depth of the 5′-end resection was similar for T5E:Cas12i3-5M, PapE:Cas12i3-5M and ME15:Cas12i3-5M fusions with 17–84, 16–116, and 20–115 bp deletions, respectively, in both PAM distal and PAM proximal directions (Figure S1). For UL12:Cas12i3-5M, there was a clear difference in the deletion pattern, with proportionally smaller deletions of 10–23 bp.

Based on the performances of Cas12i3, Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M in editing of single endogenous genes/targets in rice stable lines, we next chose Cas12i3-5M and four Exo:Cas12i3-5M fusions to further investigate their multiplex gene editing capacities by simultaneously editing of three, four, five, or six genes in rice stable lines (Figure 2A, B). For the 3X (HSC) (*OsHRC–OsSBEIIb–OsCKX2*) multiplexing combination, there were simultaneous editing efficiencies of Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M of 68.42% (26/38), 70.97% (22/31), 82.76% (24/29), 70.37% (38/54), and 72.41% (21/29), respectively, in T_0 independent lines (Figure 2B; Table S4). In comparison with Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M increased the simultaneous editing efficiency by up to 1.04-fold (70.97%/68.42%), 1.21-fold (82.76%/68.42%), 1.03-fold (70.37%/68.42%), and 1.06-fold (72.41%/68.42%), respectively. For the 4X (HSCA) (*OsHRC–OsSBEIIb–OsCKX2–OsARF4*) multiplexing combination, the simultaneous editing efficiencies of Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M were 52.63% (10/19), 57.14% (8/14), 61.36% (27/44), 57.14% (16/28), and 57.58% (19/33), respectively, in T_0 independent lines (Figure 2B; Table S4). In comparison with Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M increased the simultaneous editing efficiency by 1.09-fold (57.14%/52.63%), 1.17-fold (61.36%/52.63%), 1.09-fold (57.14%/52.63%), and 1.09-fold (57.58%/52.63%), respectively. For the 5X (HSCAA) (*OsHRC–OsSBEIIb–OsCKX2–OsARF4–OsARE1*) multiplexing combination, the simultaneous editing efficiencies of Cas12i3-5M, T5E:Cas12i3-5M, UL12:as12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M were 43.33% (13/30), 50.00% (10/20), 52.94% (18/34), 46.67% (14/30), and 45.45% (5/11), respectively, in T_0 independent lines (Figure 2B; Table S4). In comparison with Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M increased the simultaneous editing efficiency by 1.15-fold (50.00%/43.33%), 1.22-fold (52.94%/43.33%), 1.08-fold (46.67%/43.33%), and 1.05-fold (45.45%/43.33%), respectively. For the 6X (HSCAAB) (*OsHRC–OsSBEIIb–OsCKX2–OsARF4–OsARE1–OsBADH2*) multiplexing combination, the simultaneous editing efficiencies of Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M were 41.67% (20/48), 47.06% (16/34), 51.06% (24/47), 46.51% (20/43), and 42.86% (6/14), respectively, in T_0 independent lines (Figure 2B; Table S4). In comparison with Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M increased the simultaneous editing efficiency by 1.13-fold (47.06%/41.67%), 1.23-fold (51.06%/41.67%), 1.12-fold (46.51%/41.67%), and 1.03-fold (42.86%/41.67%), respectively (Table S4). Notably, this was consistent with the major editing outcomes of single gene editing, in which the depths of the 5′-end resection were similar for Cas12i3-5M and UL12:Cas12i3-5M with the 6–19 and 11–29 bp smaller deletions, respectively. In multiplex gene editing T5E:Cas12i3-5M, PapE:Cas12i3-5M and ME15:Cas12i3-5M fusions induced larger lesions of 21–60 bp or longer than 100 bp in rice stable lines (Figure S2).

To evaluate the specificity of Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M in rice stable lines, we examined the off-target possibility for each on-target site. Based on the predictions of the off-target website (http://skl.scau.edu.cn/offtarget/), we identified potential off-target sites of *OsHRC*, *OsSBEIIb*, *OsCKX2*, *OsARF4*, *OsARE1*, *OsBADH2*, and *OsDEP1* targets. Among the seven target genes, we only detected off-target effects at OsARF4-OFF1 and OsARF4-OFF2 sites with efficiencies of 6.25% (Table S5), which demonstrated the relatively higher specificity of the Exo:Cas12i3-5M fusions.

Furthermore, we analyzed the homozygous, biallelic, heterozygous or chimeric mutant lines in the T_1 generation for segregation analysis to investigate whether the targeted mutagenesis generated by Exo:Cas12i3-5M fusions could be transmitted to the next generation. Except for the chimeric lines, the mutations in the tested homozygous, biallelic, or heterozygous independent lines were transmitted to the next generation without the occurrence of new mutations (Table S6). Also, we performed segregation analysis of *Cas12i3*, *crRNA* cassette and *hpt* transgenes and successfully obtained transgene-free mutant lines in the T_1 generation (Table S6).

The development of an alternative and efficient Exo:Cas12i3-5M-mediated multiplex gene editing system will be of high desirability for breeders, as it will facilitate simultaneous improvement in several agronomic traits in one generation. For example, cereals high in amylose content (AC) and resistant starch (RS) offer potential health benefits (Regina et al., 2006; Chen et al., 2012; Li et al., 2021). Targeted mutagenesis in *OsSBEIIb* could generate high-amylose rice with significantly increased RS content (Sun et al., 2017). However, cereal grains higher in RS content usually have decreased width and length, resulting in a yield decrease (Sun et al., 2017; Li et al., 2021). However, balancing the improved RS content and yield performance remains a challenge. In this study, the targeted mutagenesis in *OsSBEIIb* resulted in smaller grains

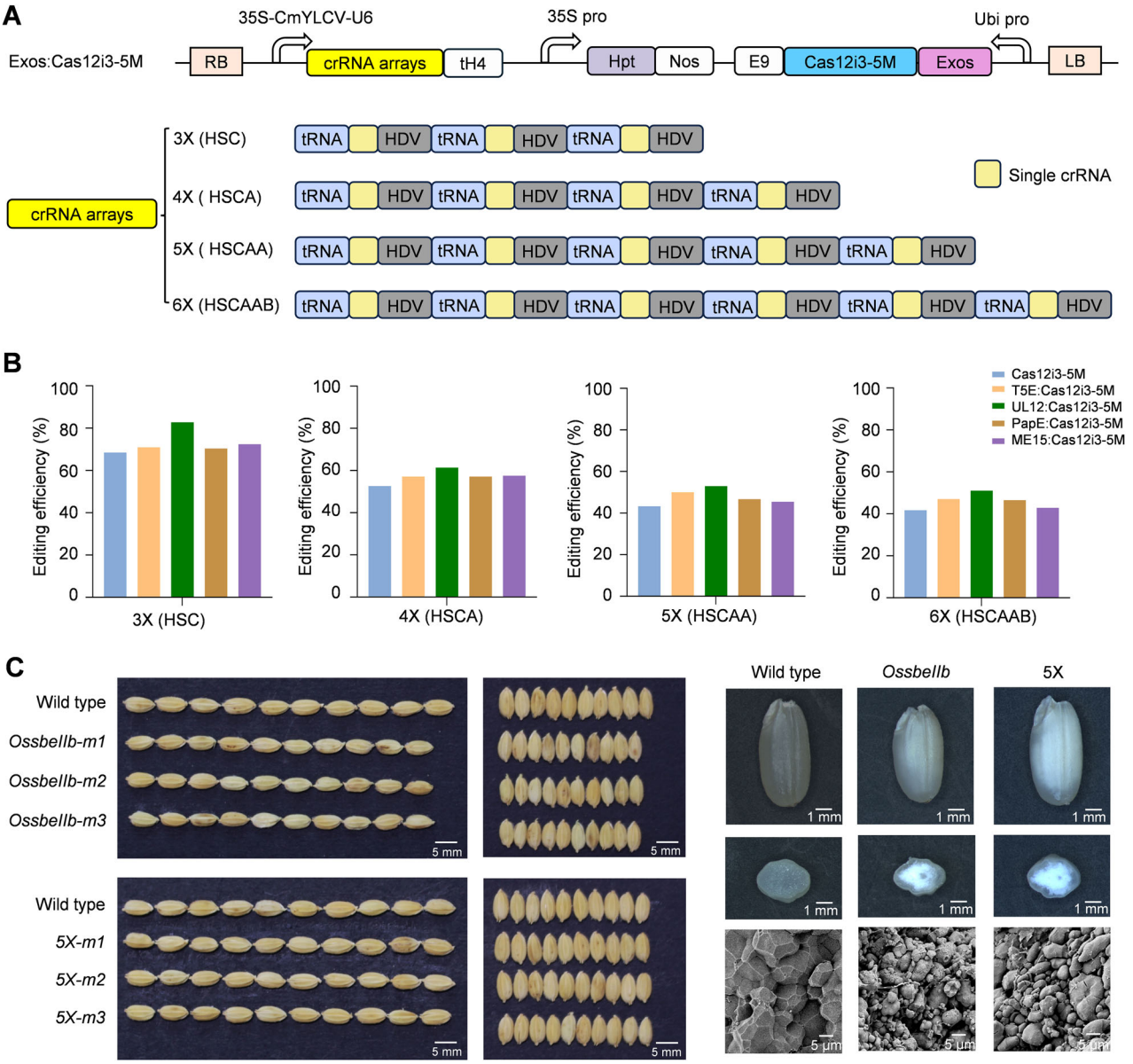

Figure 2. Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M-induced targeted mutagenesis at multiple gene loci in T_0 generation

(A) The tRNA–crRNA–HDV arrays of 3X, 4X, 5X, and 6X. (3X) HSC: *OsHRC* + *OsSBEIIb* + *OsCKX2*; 4X (HSCA):*OsHRC* + *OsSBEIIb* + *OsCKX2* + *OsARF4*; 5X (HSCAA): *OsHRC* + *OsSBEIIb* + *OsCKX2* + *OsARF4* + *OsARE1*; 6X (HSCAAB): *OsHRC* + *OsSBEIIb* + *OsCKX2* + *OsARF4* + *OsARE1* + *OsBADH2*. **(B)** The multiplex editing efficiencies of Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M in independent transgenic rice lines. The frequencies of Cas12i3-5M, T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M are shown highlighted in blue, orange, green, brown or purple, respectively. **(C)** The grain and starch morphologies of the *Ossbeilb* and *5X* mutant lines. (Left) The grain size of the wild type, *Ossbeilb* mutants and *5X* (Bars = 5 mm). (Right, upper) Morphologies and transverse sections of the brown grains from wild type, *Ossbeilb* mutants and *5X* mutants (Bars = 1 mm). (Right, lower) Scanning electron microscopy (SEM) images of the starch in wild type, *Ossbeilb* mutants and *5X* mutant grains (Bars = 5 μm).

with decreased length and width, as well as opaque endosperms, along with heterogeneous starch granules irregular in size and shape (Figure 2C). In contrast, the grains of the wild type had a transparent endosperm with compact, compound, and angular starch granules (Figure 2C). This finding is consistent with a previous report (Sun et al., 2017). Impressively, whereas the grains of the 5X (HSCAA) mutant lines retained the typical morphology characteristics of RS granules, such as heterogeneous starch granules in size and shape, without compound granular organization, due to simultaneous editing of *OsCKX2* and *OsARF4* genes (Yeh et al., 2015; Xu et al., 2016; Hu et al., 2018; Rong et al., 2022), the lengths and widths of the grains were almost the same as that of wild type (Figure 2C). The obtained rice germplasm through multiplex gene editing here in this study will facilitate breeding of rice varieties high in RS for population health benefits without compromising yield.

DISCUSSION

In this study, we systematically evaluated the editing activities of Exo:Cas12i3-5M fusions in rice stable lines, and established UL12:Cas12i3-5M as the more robust system for single and multiplex gene editing in rice. There has been a series of studies reporting that the fusion of exonucleases to CRISPR endonucleases could significantly increase insertion and deletion (InDel) frequencies (Clements et al., 2017; Wu et al., 2020; Zhang et al., 2020; He et al., 2024). However, different exonucleases may affect the editing activity and profiles of the exonuclease and Cas fusion protein due to different exonuclease activities (Schreiber et al., 2024). We first systematically evaluated the editing activities of T5E:Cas12i3-5M, UL12:Cas12i3-5M, PapE:Cas12i3-5M, and ME15:Cas12i3-5M fusions across six endogenous targets in rice stable lines (Figure 1A, B). Our results indicated that Exo:Cas12i3-5M fusions could improve the editing efficacy of Cas12i3-5M. Furthermore the UL12:Cas12i3-5M fusion exhibited superior performance and enabled robust gene editing around six targets in rice stable lines (Figure 1C). Compared with Cas12i3-5M, the UL12:Cas12i3-5M fusion could significantly increase editing efficiencies by up to 1.25-fold, reaching average efficiencies ranging from 90.42% to 98.61% across six endogenous target genes in rice stable lines, which was consistent with its higher exonuclease activities *in vitro* (Schreiber et al., 2024) (Table S3). We then further investigated the multiplex gene editing capacities of Cas12i3-5M and Exo:Cas12i3-5M fusions by simultaneously editing three, four, five or six genes in rice stable lines (Figure 2A). Our results indicated that, for 3X (HSC), 4X (HSCA), 5X (HSCAA), and 6X (HSCAAB) multiplexing combinations, the UL12: Cas12i3-5M fusion outperformed other Exo:Cas12i-5M fusions in multiplex editing in rice stable lines (Figure 2A). UL12: Cas12i3-5M could significantly increase the editing efficiency by up to 1.23-fold compared with Cas12i3-5M, with simultaneous editing efficiencies of 82.76%, 61.36%, 52.94%, and 51.06% for the three, four, five, and six genes multiplexing combinations, respectively (Figure 2B, Table S4). Given the advantages of the UL12:Cas12i3-5M fusion with less restricted canonical "TTN" PAM, expanded genome editing scope in AT-rich regions and the capability for robust single and multiplex gene editing, it will enrich plant genome editing toolkits and facilitate pyramiding of agronomically important traits for crop improvement.

In addition, fusion of exonucleases to the CRISPR endonuclease mostly leads to an increased rate of deletions, with a higher proportion of larger deletions (Clements et al., 2017; Wu et al., 2020; Zhang et al., 2020; He et al., 2024; Wang et al., 2024). Here, we demonstrated that, whereas Cas12i3 and Cas12i3-5M predominantly generated 4–20 bp deletions (mainly composed of 8–18 bp deletions), except for the UL12:Cas12i3-5M fusion, other Exo:Cas12i3-5M fusions induced a higher proportion of larger deletions in the edited rice stable lines. This is consistent with our previous report that fusion of T5E to Cas12i3-5M not only significantly enhanced genome editing efficacy, but also induced a higher proportion of larger deletions in edited wheat stable lines (Figure 1D) (Schreiber et al., 2024; Wang et al., 2024). In addition, different 5′ exonucleases may have different activities in processing resection of the 5′-end of the DNA strand at cleavage sites (Schreiber et al., 2024). We also observed that UL12:Cas12i3-5M generated proportionally smaller deletions of 10–23 bp, consistent with a previous report (Schreiber et al., 2024). This suggested that, although UL12 has a higher exonuclease activity, it may have a lower affinity for 5′-staggered end DNA substrates of DSBs *in vivo*; this aspect therefore needs further research. Next, it would be valuable to systemically evaluate the performances of different Exo:Cas12i3-5M fusions in genome editing of other agriculturally important polyploidy crop species.

In summary, we systemically evaluated different Exo: Cas12i3-5M fusions and established UL12:Cas12i3-5M as a robust system for single and multiplex gene editing in rice stable lines. We truly believe that the developed robust UL12:Cas12i3-5M gene editing system will serve as an alternative and effective tool for either functional genomics or pyramiding agronomically important traits in rice improvement, and may be extended to other crop species.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

W.W. and S.L. performed most of the research and drafted the manuscript. J.Y. carried out activity detection of crRNAs *in vitro*. L.Y. constructed the vectors. C.Z. carried out molecular characterization of the edited plants. Y.H. performed the off-target analysis. L.X. designed the experiments, supervised the study, and revised the manuscript. All the authors read and approved its content.

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SUPPORTING INFORMATION

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Figure S1. Sequencing results of the representative mutant lines around targets at *OsHRC*, *OsSBEIIb*, *OsCKX2*, *OsARE1*, *OsBADH2*, and *OsDEP* loci

Figure S2. Sequencing results of the representative mutant lines around targets at *OsHRC*, *OsSBEIIb*, *OsCKX2*, *OsARF4*, *OsARE1*, *OsBADH2* loci derived from multiplex gene editing

Table S1. The rice codon-optimized sequences of T5E, UL12, PapE, and ME15, respectively

Table S2. The primer sets used in this study

Table S3. The editing performances of Cas12i3, Cas12i3-5M, and Exo:Cas12i3-5M fusions in rice stable lines

Table S4. Summary of the Cas12i3-5M and Exo:Cas12i3-5M fusions-mediated multiplexing in T_0 plants

Table S5. Analysis of potential off-target effects

Table S6. Transmission and segregation of target mutations and transgenes from T_0 to T_1 generation

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