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Original research

Genome editing in plants with MAD7 nuclease

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ABSTRACT

MAD7 is an engineered nuclease of the Class 2 type V-A CRISPR-Cas (Cas12a/Cpf1) family with a low level of homology to canonical Cas12a nucleases. It has been publicly released as a royalty-free nuclease for both academic and commercial use. Here, we demonstrate that the CRISPR-MAD7 system can be used for genome editing and recognizes T-rich PAM sequences (YTTN) in plants. Its editing efficiency in rice and wheat is comparable to that of the widely used CRISPR-LbCas12a system. We develop two variants, MAD7-RR and MAD7-RVR that increase the target range of MAD7, as well as an M-AFID (a MAD7-APOBEC fusion-induced deletion) system that creates predictable deletions from 5′-deaminated Cs to the MAD7-cleavage site. Moreover, we show that MAD7 can be used for multiplex gene editing and that it is effective in generating indels when combined with other CRISPR RNA orthologs. Using the CRISPR-MAD7 system, we have obtained regenerated mutant rice and wheat plants with up to 65.6% efficiency.

Introduction

CRISPR systems are widespread prokaryotic adaptive immune systems that are best known as components of a new generation of genome-editing tools (Chen et al., 2019; Zhu et al., 2020; Gao, 2021). Cas9 recognizes 3′ G-rich PAMs; it is the most widely used CRISPR-Cas system and has been adapted for genome editing in various kingdoms, including Plantae (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013; Nekrasov et al., 2013; Li et al., 2013; Shan et al., 2013). Cas12a (Cpf1), a type V-A CRISPR system that belongs to Class 2 whose effector modules consist of a single, multidomain protein (Makarova et al., 2020), has been developed to achieve targeted DNA modification (Zetsche et al., 2015) and has been used to manipulate plant genomes (Endo et al., 2016; Hu et al., 2017; Kim et al., 2017; Tang et al., 2017; Xu et al., 2017; Zhang et al., 2019a, 2019b, 2020). Unlike Cas9, Cas12a recognizes 5′ T-rich PAMs and self-processes its CRISPR RNA (crRNA) (Zetsche et al., 2015; Fonfara et al., 2016), which expands the range of target genomes and makes it suitable for multiplex gene editing (Zetsche et al., 2017; Wang et al., 2018a). Furthermore, the CRISPR-Cas12a system generates a sticky end distal to the PAM sequence after cleavage of double-strand DNA (Zetsche et al., 2015), which facilitates homologous recombination-mediated gene targeting in plants (Wolter and Puchta, 2019; Li et al., 2020; Vu et al., 2020; Huang et al., 2021).

MAD7 is a nuclease that belongs to the Class 2 type V-A CRISPR family (Cas12a-like). It was identified in *Eubacterium rectale*, engineered by Inscripta (https://www.inscripta.com/madzymes/faq/) and publicly released as a royalty-free nuclease (freely available) for both academic and commercial use. It has only 31% amino acid homology with the canonical AsCpf1 from *Acidaminococcus* sp. Although it has been used for genome editing in microbial and mammalian cells (Wierson et al., 2019; Liu et al., 2020; Price et al., 2020), its application in plants has not yet been reported. We have adapted the CRISPR-MAD7 system for plant genome editing and find that it recognizes YTTN PAM sites. We demonstrate that it is a high-fidelity system and generates indel mutations with similar efficiency to the widely used CRISPR-LbCas12a. We have engineered two variants of MAD7 that

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have extended target ranges and recognize altered PAMs. In addition, by fusing MAD7 with APOBEC3A deaminase and UDG (Uracil-DNA Glycosylase), we have created an M-AFID system that generates predictable deletions from 5′-deaminated cytosines to the MAD7-cleavage site. We also show that MAD7 can be harnessed for multiplex gene editing in protoplasts. We have obtained MAD7-edited rice plants with up to 65.6% efficiency and have also managed to regenerate MAD7-free edited wheat in the T_0 generation.

Results

CRISPR-MAD7 efficiently generates indel mutations in plant protoplasts

To test whether MAD7 nuclease could be used for genome editing in plants, we codon optimized it for rice and expressed it under the maize ubiquitin-1 (*Ubi-1*) promoter (Fig. 1A; Sequence S1). We also constructed a CRISPR RNA (crRNA) driven by the Ubi-1 promoter and processed by ribozymes (Tang et al., 2017) (Fig. 1A). Two types of crRNA are recommended by Inscripta: (1) a 56 nt-crRNA (beginning with 35 nt of a direct repeat [DR] and followed by a 21 nt spacer sequence); (2) a 42 nt-crRNA (beginning with 21 nt of a DR followed by a 21 nt spacer sequence). These crRNAs were transformed separately along with MAD7 nuclease into wheat protoplasts (Sequence S2). We first evaluated the frequencies of indels induced by MAD7 at targets with TTTN (N = A, T, C, or G) PAMs and found frequencies of 38.7% at *TaDEP1* and 17.8% at *TaDME-T1*, using the 42 nt-crRNA, and frequencies of 44.5% at *TaDEP1* and 20.2% at *TaDME-T1* using the 56 nt-crRNA (Fig. 1B). These results demonstrate that CRISPR-MAD7 can be used for genome editing in wheat. Because the two crRNAs gave similar results, we used the smaller one in subsequent experiments.

We next expanded our evaluation to another cereal plant, rice, measuring the frequencies of indel mutations generated by MAD7 at 14 endogenous sites with TTTN (N = A, T, C, or G) PAMs in rice protoplasts. The indel frequencies obtained ranged from 1.2% to 34.8% (Fig. 1C). MAD7 was previously reported to recognize YTTV (Y = T or C, V = A, C, or G) PAM sites in microbes and mammalian cells (https://www.inscripta.com/madzymes/faq/). So we tested whether the CRISPR-MAD7 system could induce indels at plant sites with CTTN PAMs. CRISPR-MAD7 had cleavage frequencies of 1.6% and 4.5% at two target sites with CTTN PAMs in wheat (*TaDME-T2* and *TaDME-T3*) and frequencies ranging from 1.1% to 3.3% at five tested rice sites (Fig. 1C). However, the editing frequency of MAD7 at target sites with CTTN PAMs seemed to be lower than at target sites with TTTN PAMs (average frequency 28.2% at two sites in wheat and 14.0% at 14 sites in rice) (Fig. 1C), which indicated that MAD7 nuclease has higher affinity for TTTN PAMs than CTTN PAMs. Together these observations show that CRISPR-MAD7 can be used to modify plant genomes at sites with YTTN PAMs.

We then analyzed the characteristics of the indels created by CRISPR-MAD7. Deletions accounted for 81.2% of the indels induced by MAD7 at the 23 sites tested (Fig. S1). Most of the deletions were 6–10 bp in length, and the proportion of indels of that length ranged from 52.0% to 70.2% at the 4 sites (Fig. S2A). Most of the deletions extended from positions 13–15 to 22–24 distal to the PAM, taking the first 5′ base of the spacer as position 1 (Fig. S2B–S2D). These findings are in accord with studies of CRISPR-Cas12a in plants (Tang et al., 2017).

Optimizing MAD7 editing by varying spacer length and combining with different crRNAs

The spacer length for CRISPR-MAD7 recommended in microbes and mammalian cells by Inscripta (https://www.inscripta.com/madzymes/faq/) is 21 nt. When we tested whether spacer length (20–24 nt) affected MAD7 editing efficiency, we found that the indel frequencies generated by MAD7 using a 21 nt spacer as recommended by Inscripta (frequencies of 21.7% and 16.9% for *OsALS-T1* and *OsALS-T2*, respectively), were similar to those using 20 nt and

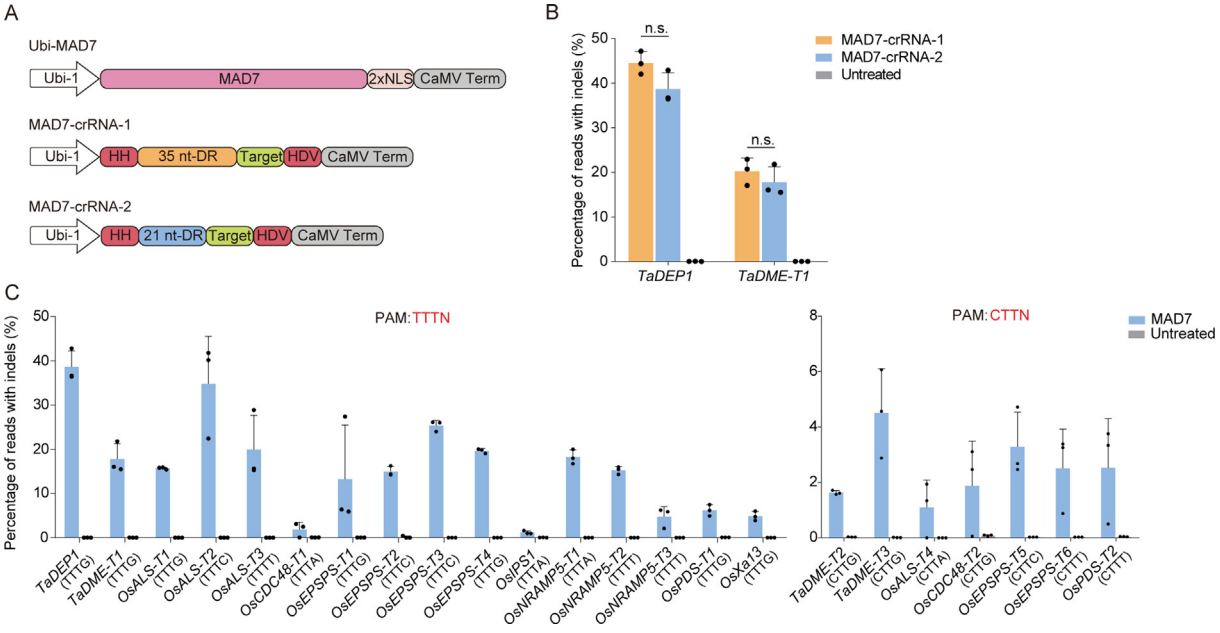

Fig. 1. CRISPR-MAD7-generated indel mutations in rice and wheat protoplasts. **A**: Schematic diagram of the CRISPR-MAD7 expression vector used in protoplasts. HH, hammerhead ribozyme; HDV, hepatitis delta virus ribozyme; DR, direct repeat. **B**: Indel frequencies induced by MAD7 using 56 nt-crRNA or 42 nt-crRNA in two wheat endogenous sites with TTTN PAMs. The percentages (mean ± SD) of mutation reads with indels among all reads are based on three independent biological experiments ($n = 3$). **C**: Indel frequencies induced by MAD7 at 23 endogenous target sites with YTTN PAMs in wheat and rice protoplasts. The percentages (mean ± SD) of mutation reads with indels among all reads are based on three independent biological experiments ($n = 3$). SD, standard deviation.

22–24 nt spacers (Fig. 2A). We therefore used 21 nt spacers in subsequent experiments.

The strong structural conservation of the DRs of crRNA in different bacterial species indicates that Cas12a would be able to perform genome editing with crRNA orthologs from other bacteria (Zetsche et al., 2015; Tu et al., 2017). We evaluated the editing efficiencies of MAD7 nuclease combined with the crRNAs of various Cas12a orthologs (Fig. S3). Editing efficiencies at *OsALS-T2* and *OsEPSPS-T3* were similar for the various crRNA orthologs tested (27.4%, 27.7%, 24.0%, and 23.0% on average for MAD7-crRNA, LbCas12a-crRNA, AsCas12a-crRNA, and FnCas12a-crRNA, respectively) (Fig. 2B) and the same was true when we compared the editing efficiencies of MAD7 combined with MAD7-crRNA or LbCas12a-crRNA at four additional sites (Fig. 2C).

Editing efficiencies of CRISPR-MAD7 and CRISPR-LbCas12a

As LbCas12a and AsCas12a are the most widely used CRISPR-Cas12a systems in plants, and LbCas12a seems to be more effective than AsCas12a (Tang et al., 2017; Bernabé-Orts et al., 2019), we compared the editing efficiencies of MAD7 and LbCas12a in plants. Two wheat sites were used, and we found the indel yields by using MAD7 were higher than those by using LbCas12a at *TaDEP1* (38.7% and 16.4% for MAD7 and LbCas12a, respectively) and similar to LbCas12a at *TaDME-T1* (17.8% and 16.6% for MAD7 and LbCas12a, respectively) (Fig. 2D). The editing efficiency of MAD7 was similar to that of LbCas12a at four rice sites (*OsEPSPS-T4*, *OsNRAMP5-T1*, *OsPDS-T1*, and *OsXa13*) (Fig. 2D). These results suggest that CRISPR-MAD7 is as effective as other CRISPR-Cas12a systems in plants.

MAD7 exhibits high specificity in plants

CRISPR-Cas12a has been reported to have higher specificity than the commonly used CRISPR-Cas9 system (Kim et al., 2016; Tang et al., 2018). To assess the specificity of CRISPR-MAD7 system, we synthesized six mismatched spacer sequences for each of four rice targets (Fig. 3), with the mismatches evenly distributed along the spacer sequences. The on-target editing efficiencies were high (6.2%–34.5% at these target sites), and there were essentially no indels in most of the spacers with two mismatches, apart from three spacers, two with indel yields of < 0.5% at the *OsEPSPS-T1* site and one with an indel frequency of < 2.0% at the *OsEPSPS-T3* site (Fig. 3A). Similar results were obtained with spacers with one mismatch (Fig. 3B). These results indicate that CRISPR-MAD7 is a high-fidelity system for genome editing in plants.

Engineered MAD7 variants with extended editing ranges

Previous studies have found that Cas12a can be modified to recognize TYCV and TATV PAMs by the mutations S542R/K607R and S542R/K548V/N552R, respectively (Gao et al., 2017; Yamano et al., 2017), and the target regions of LbCas12a and FnCas12a can also be expanded (Li et al., 2018; Zhong et al., 2018). We therefore analyzed the sequence homology between MAD7, AsCas12a, and LbCas12a (Fig. S4) and developed two MAD7 variants designated, respectively, MAD7-RR (harboring mutations

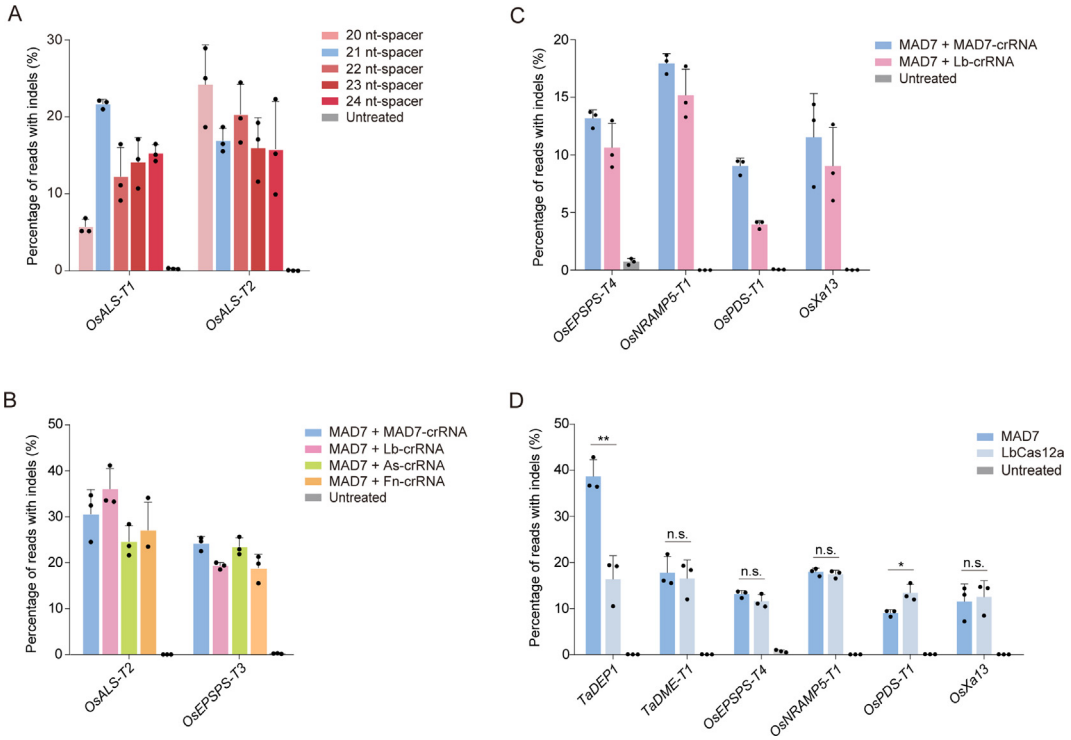

Fig. 2. Optimizing MAD7-mediated genome editing efficiency. **A**: Comparison of editing frequencies induced by MAD7 with different lengths of spacers in rice protoplasts at two endogenous rice sites. **B**: Genome editing frequencies induced by MAD7 using crRNAs from four Cas12a orthologs (MAD7, LbCas12a, AsCas12a, and FnCas12a) were compared at two rice sites. **C**: Four more rice endogenous sites were tested to compare the editing frequencies induced by MAD7 and by MAD7-crRNA and Lb-crRNA. **D**: Comparison of editing frequencies induced by optimized MAD7 and LbCas12a at six endogenous sites in wheat and rice protoplasts. The percentages (mean ± SD) of mutation reads with indels among all reads are based on three independent biological experiments ($n = 3$). *, $P < 0.05$; **, $P < 0.01$. SD, standard deviation.

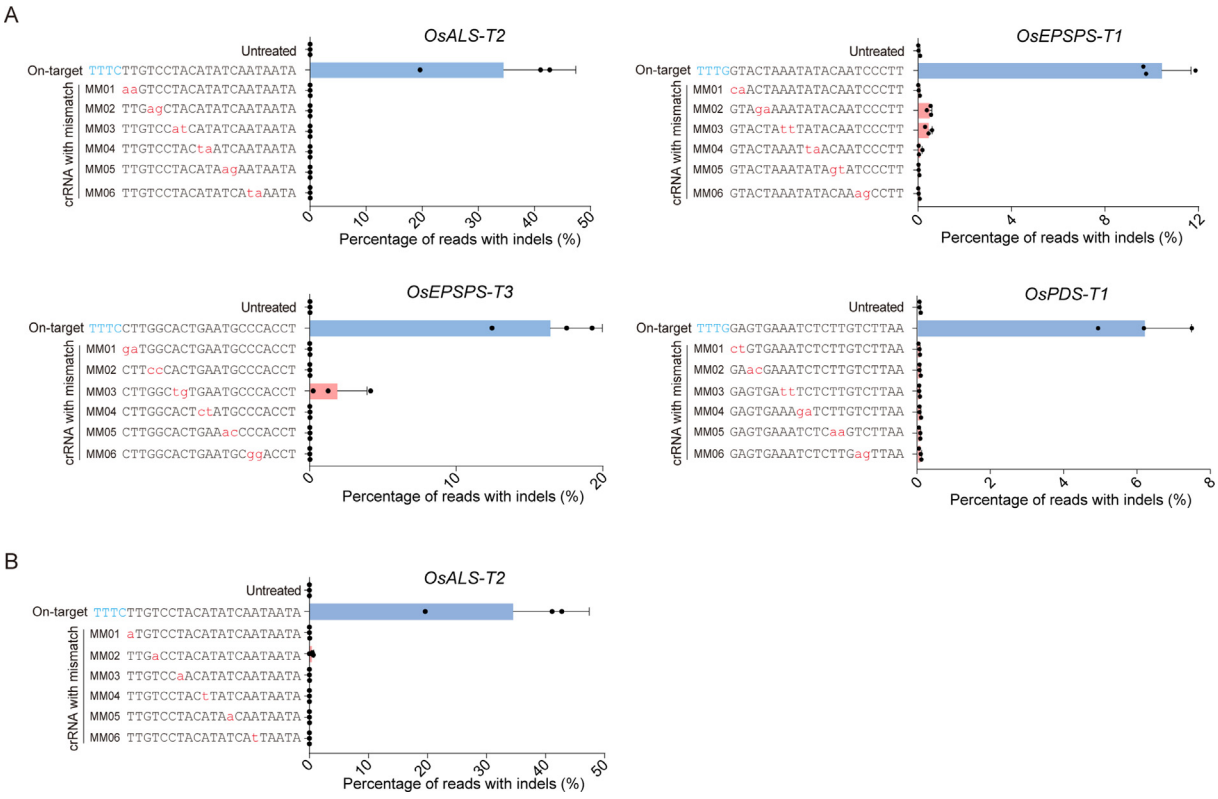

Fig. 3. Tolerance of MAD7 nuclease to mismatches in the crRNA spacer. **A**: Four rice sites were tested to evaluate the specificity of MAD7 when using sgRNA carrying two mismatches. Mismatched base pairs are in red and PAM sequence in blue. **B**: One rice site was tested to evaluate the specificity of MAD7 when using sgRNA carrying one mismatch. Mismatched base pairs are in red and PAM sequence in blue. The percentages of all reads that contained indels (mean ± SD) were measured in three biologically independent experiments ($n = 3$). SD, standard deviation.

D529R/K594R) and MAD7-RVR (harboring mutations D529R/K535V/N539R) (Fig. 4A; Sequence S1). MAD7-RR was found to induce indels at three of four targets with TYCV PAMs, with indel frequencies of 6.6%, 17.5%, and 1.9% at *OsEPSPS-RR-T2* (TTCA PAM), *OsPDS-RR* (TCCG PAM), and *OsSBEIIb-RR* (TTCC PAM), respectively, However indel frequencies were low at *OsEPSPS-RR-T1* (0.3% with TCCC PAM) (Fig. 4B). Similarly, MAD7-RVR generated indel yields of 12.7% and 1.1% at *OsEPSPS-RVR* (TATC PAM) and *OsROC5-RVR* (TATG PAM), respectively, but only 0.3% at *OsNRAMP5-RVR* (TATG PAM) (Fig. 4C). These results indicate that the PAM specificity of MAD7 can be modified to expand its target range.

Generation of predicable deletions by a MAD7-mediated M-AFID system

Base editors using human APOBEC3A (A3A) have been shown to generate C-to-T base conversion efficiently in animals and plants (Gehrke et al., 2018; Wang et al., 2018b; Zong et al., 2018; Lian et al., 2021). Recently, newly developed systems referred to as AFIDs (APOBEC-Cas9 fusion-induced deletion systems) have been used to generate predictable deletions, which are defined as extending from a 5′-deaminated C to the Cas9-cleavage site (Wang et al., 2020). We developed a similar system, designated M-AFID (MAD7-APOBEC

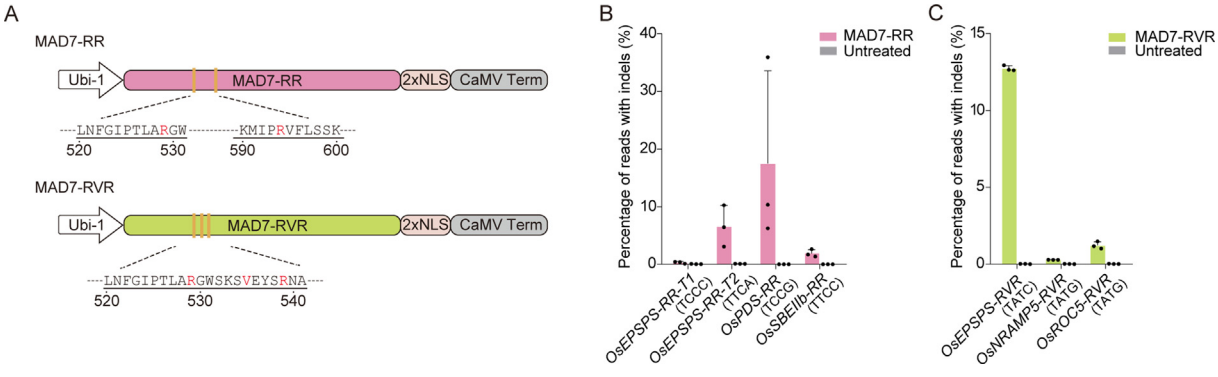

Fig. 4. The MAD7-RR and MAD7-RVR variants broaden the PAM range in plants. **A**: Schematic diagram of the CRISPR-MAD7 expression vector with an extended target range. **B**: Indel frequencies induced by MAD7-RR at four endogenous sites with TYCV PAMs. **C**: Indel frequencies induced by MAD7-RVR at three endogenous sites with TATV PAMs. The percentages of all reads that contained indels (mean ± SD) were measured in three biologically independent experiments ($n = 3$). SD, standard deviation.

fusion-induced deletion system), by fusing human APOBEC3A (A3A) and *Escherichia coli* UDG to the N-terminus and the C-terminus, respectively, of MAD7 (Figs. 5A and S5). The yield of indels induced by M-AFID was higher than that induced by MAD7 at *OsALS-T1*, similar to *OsALS-T1* at *OsEPSPS-T1* and *TaUbi10* and lower at *TaDEP1* (Fig. S6). We calculated the percentages of predictable deletions from C bases within the spacer sequence to the MAD7-cleavage site (position 22–24) among the total indels and found that M-AFID generated an average of 21.2% predictable deletions at the four tested targets, with an efficiency of up to 35.5% at *OsALS-T1* (Figs. 5B and S7). As a control, the yields of predictable deletions induced by MAD7 were much lower (ranging from 0 to 11.9%) (Figs. 5B and S7).

MAD7-mediated multiplex gene editing

Because MAD7 is a Cas12a ortholog and can process its own crRNA (Zetsche et al., 2015; Fonfara et al., 2016) and Cas12a has been used for multiplex gene editing (Zetsche et al., 2017; Wang et al., 2018a; Tang et al., 2021), we tested whether MAD7 could also be used to edit multiple targets simultaneously. We constructed three customized CRISPR arrays. For arrays 1, 2, and 3, the lengths of the DRs of the MAD7-crRNAs were set at 35 nt, 21 nt, and 21 nt, respectively. For array 3, each of the crRNAs was also processed by ribozymes (Fig. 5C; Sequence S2). Editing efficiencies of 3.7%, 2.8%, 2.8%, and 3.6% for array 1, 8.0%, 6.3%, 3.2%, and 2.9% for array 2, and 12.4%, 3.1%, 7.2%, and 3.8% for array 3, were observed at *OsALS-T2*, *OsEPSPS-T3*, *OsEPSPS-T1*, and *OsPDS-T1*, respectively (Fig. 5D). These results demonstrate that straightforward constructs using the CRISPR-MAD7 system can edit multiple targets simultaneously.

Inducing indel mutations in wheat and rice plants with MAD7

Finally, we attempted to use CRISPR-MAD7 to generate mutant wheat and rice plants. For wheat, we co-delivered Ubi-MAD7 vector and the 42 nt-crRNA vector by particle bombardment and regenerated plants without herbicide selection (Table 1). Analysis of individual T_0 wheat plants revealed 1.5% and 3.0% editing frequencies at *TaDEP1* and *TaVRN1*, respectively (Tables 1 and S1). Of 12 T_0 mutants targeting *TaDEP1*, three were homozygous, five were biallelic, and only one was heterozygous. Half of the 24 T_0 mutants targeting *TaVRN1* were homozygous, whereas seven were biallelic and three were heterozygous. To see whether these plants were MAD7-free, we used five primer sets specific for Ubi-MAD7 and Ubi-MAD7crRNA for polymerase chain reaction (PCR) screening (Fig. S8). All the *TaDEP1*-modified plants and all but seven of the 24 (29.2%) *TaVRN1*-modified plants harbored the transgene vector (Fig. S8; Table 1).

To generate mutant rice, we introduced pH-mono-MAD7 (Fig. S9) into rice calli by *Agrobacterium*-mediated transformation and targeted three sites, *OsALS-T2*, *OsEPSPS-T1*, and *OsNRAMP5-T1*, for CRISPR-MAD7 editing. Among 96 regenerated T_0 *OsALS-T2* plants, 47 (49.0%) were mutant, whereas 63 (65.6%) of 96 *OsEPSPS-T1* plants were mutant, and 82 (56.9%) of 144 *OsNRAMP5-T1* plants were mutant (Table 1). Most of the edited plants were biallelic (29/37 at *OsALS-T2*, 44/63 at *OsEPSPS-T1*, and 63/82 at *OsNRAMP5-T1*). Five and two *OsALS-T2*-modified plants, 13 and four *OsEPSPS-T1*-modified plants, ten and zero *OsNRAMP5-T1*-modified plants were homozygotes and heterozygotes, respectively (Tables 1 and S2). No off-target events were found at either the *OsEPSPS-T1* or *OsNRAMP5-T1* sites in the regenerated plants (Table S3).

Discussion

MAD7 is an engineered nuclease that shows low homology with canonical Cas12a nucleases. It was publicly released as a nuclease freely available for both academic and commercial use. However, the application of MAD7 has not yet been reported in plants. We have now demonstrated that the CRISPR-MAD7 system can be used for plant genome editing. The system recognizes sites with T-rich PAM sequences (YTTN), and it seems to prefer TTTN PAMs to CTTN PAMs in plants. Its editing efficiency is comparable to that of the CRISPR-LbCas12a systems widely used in wheat and rice, and the characteristics of the indels induced are similar to those of the indels induced by CRISPR-Cas12a. Similar to CRISPR-Cas12a (Tang et al., 2017), MAD7 is effective in creating indels (insertion/deletion), mainly of 6–10 bp and so may be suitable for inactivating both genes and regulatory elements. We also found that the yield of MAD7-induced indels at two targets was increased by incubating protoplasts at 37°C but decreased at two other targets (Fig. S10), indicating that

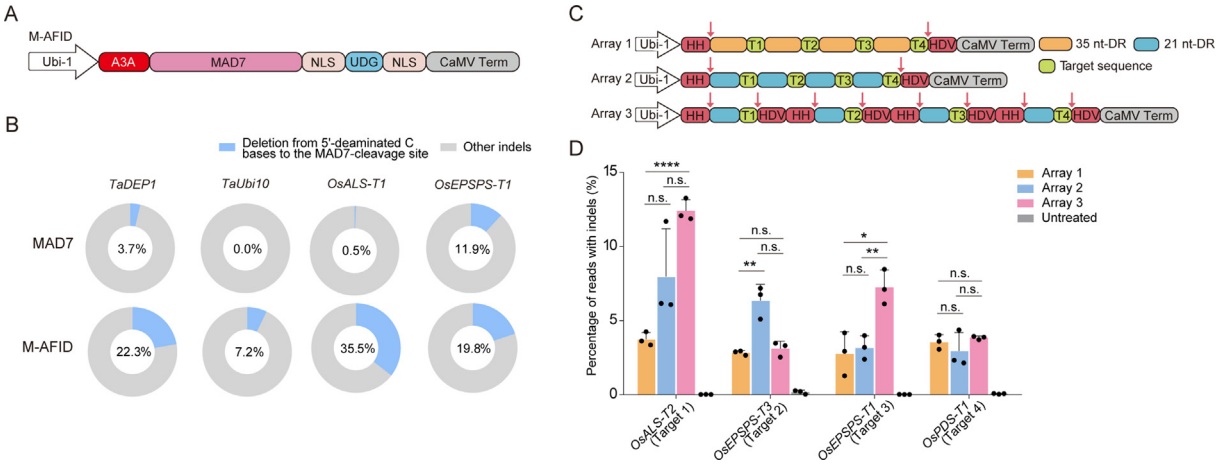

Fig. 5. CRISPR-MAD7-based M-AFID and CRISPR-MAD7-mediated multiplex gene editing systems in protoplasts. **A**: Schematic diagram of the CRISPR-MAD7-based M-AFID system. **B**: Comparison of the percentages of predictable deletions (from 5′-deaminated Cs to the MAD7-cleavage site) among all indels induced by MAD7 and M-AFID at four endogenous sites in protoplasts. **C**: Schematic diagram of three versions of the MAD7-based multiplex gene editing system. Red arrows represent the cleavage sites of the ribozymes. **D**: Comparison of the indel frequencies induced by the three versions of the MAD7-based multiplex gene editing system at the four endogenous rice sites. The percentages of all reads that contained indels (mean ± SD) were measured in three biologically independent experiments ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$. SD, standard deviation.

Table 1
CRISPR-MAD7-induced mutations in wheat and rice plants.

Target site	Species	Delivery form/selection (±)	Number of wheat embryos or transgenetic rice plants[a]	Number of mutants/ mutagenesis (%)	Number of homozygous/ biallelic/heterozygous/chimeric mutants	Number of transgene-free mutants/frequency (%)[b]
TaDEP1	Wheat	DNA/−	800	12 (1.5)	3/5/1/3	0 (0.0)
TaVRN1	Wheat	DNA/−	800	24 (3.0)	12/7/2/3	7 (29.2)
OsALS-T2	Rice	T-DNA/+	96	47 (49.0)	2/21/8/16	N/A
OsEPSPS-T1	Rice	T-DNA/+	96	63 (65.6)	4/41/13/5	N/A
OsNRAMP5-T1	Rice	T-DNA/+	144	82 (56.9)	0/64/9/9	N/A

N/A, not available.
[a] Gene gun bombardment was used for transformation of wheat embryos, and *Agrobacterium*-mediated transformation was used to transfect rice calluses.
[b] On the basis of the number of plants not harboring MAD7 vector among the total number of mutant plants detected.

MAD7, similar to other Cas12a nucleases (Malzahn et al., 2019), is temperature sensitive and has the potential to be engineered to a temperature-insensitive form (Schindele and Puchta, 2020). Because MAD7 also generates sticky ends distal to the PAM sequence, it may facilitate homologous recombination-mediated gene targeting in plants.

By introducing point mutations in the residues interacting with the PAM, we developed two variants, MAD7-RR and MAD7-RVR, that extend the target range of MAD7. These two variants are able to recognize TYCV and TATV PAMs, respectively, but their yields at some targets (such as *OsEPSPS-RR-T1* for MAD7-RR and *OsNRAMP5-RVR* for MAD7-RVR) are low, and their robustness needs to be further tested. Moreover, we also developed M-AFID, which generates predictable deletions. The A3A variants with narrow editing windows (Gehrke et al., 2018; Wang et al., 2018b) could theoretically greatly improve the precision of the editing products of M-AFID systems. Similar to ABE8e, which was developed for efficient adenine deamination (Richter et al., 2020), this system could also be modified to generate predictable deletions from deaminated As to the MAD7-cleavage site.

In conclusion, we have shown that MAD7, a royalty-free nuclease, is as efficient at gene editing as other Cas12a enzymes and could be a powerful tool for plant genome editing. The versatility of the MAD7 system should make it of use commercially, especially for agricultural and horticultural breeding.

Materials and methods

Plasmid construction

To construct vectors pUbi-MAD7, pUbi-MAD7-RR, and pUbi-MAD7-RVR, the MAD7, MAD7 variants, and NLS were codon optimized for rice, synthesized commercially (GENEWIZ, Suzhou, China), and the fusion protein sequences were cloned into the vector pJIT163 backbone (Shan et al., 2013; Wang et al., 2014), yielding the Ubi-1-driven nucleases plasmids. To construct the crRNA expression vectors, pUbi-MAD7crRNA, hammerhead ribozyme sequence, DRs, BsaI restriction enzyme sites, and hepatitis delta virus ribozyme sequence were synthesized commercially (GENEWIZ, Suzhou, China) and cloned into pJIT163 backbone, yielding the Ubi-1-driven crRNA plasmid, yielding Ubi-MAD7crRNA plasmid. The spacer sequence for each target are cloned into the resulting plasmid by DNA T4 ligase (NEB, Ipswich, MA, USA). For multiplexed gene editing, the customized CRISPR arrays were synthesized commercially (GENEWIZ, Suzhou, China) and cloned into Ubi-MAD7crRNA. PCR for vector constructions was performed using TransStart FastPfu DNA Polymerase (TransGen Biotech, Beijing, China).

To construct the binary vector pH-mono-MAD7 for *Agrobacterium*-mediated rice transformation, MAD7 nuclease and crRNA were driven by maize ubiquitin-1 (*Ubi-1*) promoter and switchgrass *Ubiquitin-1* (PvUbi-1) promoter (Čermák et al., 2017), respectively. ZmUbi-MAD7-2NLS and PvUbi-crRNA expression cassettes were cloned into the pHUE411 backbone (Xing et al., 2014) by using a ClonExpressII One Step Cloning Kit (Vazyme, Nanjing, China).

All the plasmid sequences are listed in Supplementary Data (Sequences S1 and S2). All the primer sets used in this work are listed and were synthesized by Beijing Genomics Institute (BGI) (Tables S4 and S5).

Protoplast transfection

The *Japonica* rice (*Oryza sativa*) variety Zhonghua11 and the winter wheat variety Kenong199 were used for protoplasts preparation. Protoplast isolation and transformation were performed as previously described (Zhang et al., 2011; Shan et al., 2013; Wang et al., 2014). The plasmids (10 μg per construct) were introduced into protoplasts by PEG-mediated transfection. The transfected protoplasts were incubated at 26°C for 48 h, followed by genomic DNA extraction.

DNA extraction

We used a DNA Quick Plant System (Tiangen Biotech, Beijing, China) for protoplasts and plant genomic DNA extraction. The targeted sequences were amplified with specific primers, and the amplicons were purified with an EasyPure PCR Purification Kit (TransGen Biotech, Beijing, China) and quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Deep amplicon sequencing

The target region was amplified from protoplasts genomic DNA with site-specific primers in the first-round PCR. In the second-round PCR, we added both forward and reverse barcodes to the ends of the PCR products for library construction. Equal amounts of PCR products were pooled and sequenced commercially (Novogene, Beijing, China) by the NovaSeq platform, and the sequenced reads of each crRNA target site were examined. Amplicon sequencing was repeated three times for each target site using genomic DNA extracted from three independent protoplast samples. Analyses of MAD7-editing processivity and indels were performed as previously described (Zong et al., 2017; Wang et al., 2020). The deep sequencing data have been deposited in an NCBI BioProject database (accession codes PRJNA702611).

Biolistic delivery of DNA constructs into wheat immature embryo cells

Plasmids harboring Ubi-MAD7 and Ubi-MAD7crRNA were delivered simultaneously into immature embryos of Kenong199 by particle bombardment (Shan et al., 2014). The embryos were cultured for plantlet regeneration after bombardment on medium without selective agent (Shan et al., 2014). Plants were cultivated at 26°C for regeneration.

Agrobacterium transformation of rice callus cells

The pH-mono-MAD7 binary vectors were transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation. Callus cells of Zhonghua11 were transformed as reported previously (Shan et al., 2014; Jin et al., 2021). Hygromycin (50 μg/mL) was used to select transgenic plants. Plants were cultivated at 28°C for regeneration.

Mutant identification by PCR-RE assays and Sanger sequencing

The rice and wheat mutants were identified by PCR-RE assay (Thermo Fisher Scientific, Waltham, MA, USA) or T7EI assay (Vazyme, Nanjing, China), and Sanger sequencing as described (Shan et al., 2014; Jin et al., 2021). T_0 transgenic rice plants were individually examined.

Statistical analysis

All numerical values are presented as means ± standard deviation (SD). Statistical differences between control and the treatments were tested using two-tailed Student's *t*-tests.

CRediT authorship contribution statement

Qiupeng Lin: Data curation, Methodology, Visualization, Software, Formal analysis, Investigation, Writing - Original draft preparation. **Zixu Zhu**: Data curation, Methodology, Visualization, Software, Formal analysis, Investigation, Writing - Original draft preparation. **Guanwen Liu**: Data curation, Methodology, Visualization, Software, Formal analysis, Investigation, Writing - Original draft preparation. **Chao Sun**: Formal analysis, Investigation. **Dexing Lin**: Formal analysis, Investigation. **Chenxiao Xue**: Visualization, Formal analysis. **Shengnan Li**: Visualization. **Dandan Zhang**: Data curation, Software. **Caixia Gao**: Conceptualization, Writing - Reviewing & Editing. **Yanpeng Wang**: Conceptualization, Supervision, Writing - Reviewing & Editing. **Jin-Long Qiu**: Conceptualization, Supervision, Writing - Reviewing & Editing.

Conflict of interest

The authors declare no conflict of interests.

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Supplementary data

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