Expanding plant genome-editing scope by an engineered iSpyMacCas9 system that targets A-rich PAM sequences

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ABSTRACT

The most popular CRISPR-SpCas9 system recognizes canonical NGG protospacer adjacent motifs (PAMs). Previously engineered SpCas9 variants, such as Cas9-NG, favor G-rich PAMs in genome editing. In this manuscript, we describe a new plant genome-editing system based on a hybrid iSpyMacCas9 platform that allows for targeted mutagenesis, C to T base editing, and A to G base editing at A-rich PAMs. This study fills a major technology gap in the CRISPR-Cas9 system for editing NAAR PAMs in plants, which greatly expands the targeting scope of CRISPR-Cas9. Finally, our vector systems are fully compatible with Gateway cloning and will work with all existing single-guide RNA expression systems, facilitating easy adoption of the systems by others. We anticipate that more tools, such as prime editing, homology-directed repair, CRISPR interference, and CRISPR activation, will be further developed based on our promising iSpyMac-Cas9 platform.

Key words: plant genome editing, iSpyMacCas9, PAM, cytosine base editing, adenine base editing

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INTRODUCTION

The rapid development of CRISPR-Cas9, -Cas12a, and -Cas12b systems has greatly accelerated genome-editing applications in plants (Zhang et al., 2019). The most popular *Streptococcus pyogenes* Cas9 (SpCas9 or SpyCas9) system recognizes simple NGG protospacer adjacent motifs (PAMs) (N = A, T, C, G) (Jinek et al., 2012). Despite its widespread use, the SpCas9 system has a limited targeting scope due to its NGG PAM requirement. Over the past few years, efforts to expand the scope of genome editing in plants have mainly focused on the assessment of different Cas orthologs and variants that possess altered PAM requirements other than NGG. For example, the Cas9 orthologs *Streptococcus thermophilus* Cas9 (StCas9) (Garneau et al., 2010; Muller et al., 2016) and *Staphylococcus aureus* Cas9 (SaCas9) (Ran et al., 2015) were shown to recognize PAMs of NNAGAAW (W = A, T) and NNGRRN (R = A or G), respectively, in *Arabidopsis* (Steinert et al., 2015), although the study did not fully validate the PAM scope of StCas9 and SaCas9. SaCas9 was demonstrated in additional plant species such as *Nicotiana benthamiana* (Kaya et al., 2017), rice (Kaya et al., 2016), and citrus (Jia et al., 2017). The engineered SpCas9 variant, SpCas9-VQR (Kleinstiver et al., 2015), was confirmed to edit PAMs of NGAN or NGNG in rice (Hu et al., 2016, 2018b). SpCas9-NG (Nishimasu et al., 2018) was shown to edit NG PAMs in rice and *Arabidopsis* (Endo et al., 2019; Ge et al., 2019; Hua et al., 2019; Ren et al., 2019; Zhong et al., 2019). In addition, type IV CRISPR systems, such as Cas12a and Cas12b, offer recognition of T-rich PAMs (Zhang et al., 2019), and the LbCas12a-RR variant enables the recognition of C-rich PAMs (Zhong et al., 2018) in plants.

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CRISPR-Cas systems are most effective in introducing indel (insertion and deletion) mutations through non-homologous end joining (Puchta, 2005). However, precise crop breeding often relies on the ability to swap alleles at single-base accuracy, which can be achieved by base editors. Currently, cytosine base editor (CBE) and adenine base editor (ABE) systems, first developed in human cells, have been applied in plants (Eid et al., 2018; Molla and Yang, 2019; Gurel et al., 2020). Among the multiple CBE configurations, the most efficient and popular one is base editor 3 (BE3) (Komor et al., 2016). In recent years, researchers have reported successful application of this system in rice (Li et al., 2017a; Lu and Zhu, 2017; Ren et al., 2017), wheat, and maize (Zong et al., 2017). Besides the APOBEC1 cytidine deaminase used in BE3, other deaminases have also been reported in plants, including Petromyzon marinus cytidine deaminase (PmCDA1) (Shimatani et al., 2017), a variant of human activation-induced cytidine deaminase (hAID*) (Ren et al., 2018) and human APOBEC3A (Zong et al., 2018). We recently showed that the PmCDA1 base editor is much more efficient than the APOBEC1 base editor, regardless of whether it is expressed as a single transcript unit (Tang et al., 2018) or coupled with Cas9-NG for targeting relaxed NG PAM sites (Zhong et al., 2019). Besides introducing missense and nonsense mutations, CBE systems were also demonstrated for altering splicing sites in plants (Xue et al., 2018; Li et al., 2019b).

Development of the first ABE system was based on an impressive study that applied seven rounds of extensive protein evolution to the bacterial tRNA adenosine deaminase TadA to generate a highly efficient DNA base editor, ABE-7.10, for A to G base changes (Gaudelli et al., 2017). More recently, an engineered ABE system, ABEmax, was reported to have enhanced base editing activity in human cells (Koblan et al., 2018). The application of ABE systems in plants has been reported in Arabidopsis, Brassica napus (Kang et al., 2018), rice (Yan et al., 2018; Negishi et al., 2019; Molla et al., 2020), and wheat (Li et al., 2018). The ABE systems are also potentially useful for introducing mutations at A-T rich cis-regulatory elements for regulating gene expression. Both CBE and ABE systems fill a major technology gap in precise plant genome editing. However, the base editing scope is restricted by the PAM requirements of Cas9 proteins in use.

To expand the scope of targeted mutagenesis and base editing, Chatterjee et al. (2020) swapped the PAM interacting (PI) domain of SpyCas9 with the PI domain from Streptococcus macacae Cas9 (SmacCas9), which recognizes NAA PAMs. Interestingly, the hybrid SpyMacCas9 gained NAA PAM-targeting capability while retaining the nuclease activity of SpyCas9 in vitro and in human cells. In addition, an improved SpyMacCas9 platform displaying high nuclease activity, named iSpyMacCas9, was further engineered (Chatterjee et al., 2020). Hence, we were very intrigued by the great potential of the iSpyMacCas9 system in broadening the genome-editing scope of CRISPR-Cas9 in plants. Prior to the publication of the human cell study (Chatterjee et al., 2020), we decided to develop iSpyMacCas9 systems for targeted mutagenesis, C to T base editing, and A to G base editing in plants.

RESULTS

Genome-wide PAM analysis in rice, maize, and wheat

To assess the potential targeting scope of SpyMacCas9 and iSpyMacCas9 systems in major crops, we conducted an in silico analysis of NAA, NGG, and VTTV (V = A, C, G) PAMs in the genomes of rice (O. sativa), maize (Z. mays), and wheat (T. aestivum). However, a CRISPR-Cas9 system for plant genome engineering capable of recognizing A-rich PAMs remains elusive.

Figure 1. Genome-wide analysis of PAM frequencies in rice, maize, and wheat.

In silico analysis of NAA, NGG, and VTTV (V = A, C, G) PAMs in the genomes of rice (O. sativa), maize (Z. mays), and wheat (T. aestivum).

Assessment of iSpyMacCas9 systems in rice cells

We synthesized a rice codon-optimized PI domain from SmacCas9 and replaced the corresponding PI domain in two versions of SpyCas9, pcoCas9 (Li et al., 2013), and zCas9 (Xing et al., 2014), which generated pco-SpyMacCas9 and z-SpyMacCas9. R221K and N394K mutations, previously identified through deep mutational scans, can increase SpyCas9 nuclease activity (Spencer and Zhang, 2017). We further introduced these two mutations to create pco-iSpyMacCas9 and z-iSpyMacCas9. Hybrid SpyMacCas9 and iSpyMacCas9 protein domains are presented in Figure 2A. Four independent target sites with NAA PAMs or VTTV PAMs (Figure 1) were used for rice protoplast transformation. The protoplast results showed that two out of four NAA PAM sites were edited, while retaining the nuclease activity of SpyCas9 in vitro and in human cells.
and z-iSpyMacCas9 appeared to be most potent, resulting in an editing frequency of >30% at one target site (Figure 2B and Supplemental Figure 1). Thus, we chose z-iSpyMacCas9 as our default iSpyMacCas9 system for all remaining experiments.

Our data suggested that iSpyMacCas9 may recognize PAMs that are more complex than NAA in rice. Therefore, we sought to investigate iSpyMacCas9 PAM requirements in a more comprehensive way. To accomplish this, we targeted a total of 32 sites in the rice genome associated with all 16 possible combinations of NAAN PAMs in duplicates. The protospacers were between 19 and 21 bp long, had a median GC% of 50%, and 90% of all the protospacers had a GC content between 30% and 70%. We assessed these constructs in rice protoplasts. The data indicated that iSpyMacCas9 could edit most NAAA and NAAG PAM sites but displayed low editing efficiencies at NAAC and NAAT PAM sites (Figure 2C and Supplemental Figure 2). Our data, along with recent reports in human cells (Chatterjee et al., 2020) and rabbit cells (Liu et al., 2019), suggest that while iSpyMacCas9 may generally target NAAN PAMs, its activity is greatly affected by the fourth nucleotide within the PAMs. Based on the editing data, we concluded that iSpyMacCas9 prefers NAAR (R = A, G) PAMs in rice.

Generation of stably edited T0 rice lines with iSpyMacCas9
We next evaluated (i)SpyMacCas9’s ability to generate OsPDS- and OsROC5-targeted mutants in stable transgenic rice lines using Agrobacterium-mediated T-DNA transformation. OsPDS encodes phytoene desaturase, an important enzyme in the carotenoid biosynthesis pathway (Miki and Shimamoto, 2004). OsROC5, rice outermost cell-specific gene 5, is responsible for leaf rolling control (Zou et al., 2011). We first targeted a site with OsPDS-sgRNA11 for an NAAG PAM. We compared SpyMacCas9 and iSpyMacCas9, which resulted in one out of five (20%) and four out of seven (57.1%) edited T0 lines, respectively (Table 1, Figure 3A and 3B). These data confirmed our earlier observation in rice protoplasts that iSpyMacCas9 showed higher editing efficiency than SpyMacCas9 (Figure 2B). We then focused on iSpyMacCas9 and evaluated two additional T-DNA constructs with sgRNAs targeting NAAA and NAAG PAMs in OsPDS and OsROC5, respectively. At the OsPDS-sgRNA09 site, 6 out of 16 (37.5%) T0 lines were edited (Table 1 and Figure 3C). At the OsROC5-sgRNA03 site, eight out of nine (88.9%) T0 lines were edited (Table 1 and Figure 3D), suggesting very high editing efficiency. In both cases, T0 plants with biallelic edits were readily identified (Figure 3C and 3D). Taken collectively, these data demonstrated that z-iSpyMacCas9 is capable of generating rice mutants at NAAR PAM sites with high efficiency.

Development of two C to T base editing systems with iSpyMacCas9
We reasoned that iSpyMacCas9, with its A-rich PAM requirements, would have great potential in expanding the base editing scope in plants. To develop efficient CBEs based on iSpyMacCas9 in plants, we compared a hyperactive hAID mutant and PmCDA1, two potent cytidine deaminases (Nishida et al., 2016; Nishida et al., 2016; Nishida et al., 2016).
Table 1. Summary of targeted mutagenesis by (i)SpyMacCas9 in rice T0 lines.

| Constructs    | PAM Index | Targeted Rice Sites       | Cas9 Variants | Tested TO Lines | Mutated TO Lines (Number; Ratio) | Biallelic Mutation Lines (Number; Ratio) |
|---------------|-----------|---------------------------|---------------|-----------------|----------------------------------|----------------------------------------|
| pLR1820       | GAAG      | OsPDS-sgRNA11-SM21        | z- SpyMacCas9 | 5               | 1; 20%                           | 0; 0.0%                                |
| pLR1829       | GAAA      | OsPDS-sgRNA09-SM17        | z- iSpyMacCas9| 16              | 6; 37.5%                         | 1; 6.3%                                |
| pLR1830       | GAAG      | OsPDS-sgRNA11-SM21        | z- iSpyMacCas9| 7               | 4; 57.1%                         | 1; 14.2%                               |
| pLR2223       | AAAG      | OsROC5-sgRNA3-SM6         | z- iSpyMacCas9| 9               | 8; 88.9%                         | 8; 88.9%                               |

DISCUSSION

Expanding the targeting scope of CRISPR-Cas9 has been pursued heavily in the field of genome editing so that many more sequences in a genome will become editable. There are two major routes for achieving Cas9 with altered PAM requirements, one relying on the investigation of Cas9 orthologs that have naturally evolved different PAM preferences than SpCas9 and the other relying on protein engineering. Within the protein engineering route, there are three approaches. The first approach is based on protein evolution. For example, xCas9 was generated with phage-assisted continuous evolution to target relaxed NG PAMs (Hu et al., 2018a). The second approach is based on structure-directed mutagenesis. For example, Cas9-NG, also claimed to target NG PAMs, was engineered in this manner (Nishimasu et al., 2018). The third approach is based on domain swap between Cas9 orthologs. This is how the SpyMacCas9 system was established (Chatterjee et al., 2020). For these engineered SpCas9 variants, all the initial assessments were made in human cells. Interestingly, plant researchers have recently tested xCas9 and found that it has extremely low editing efficiency at NG PAMs in plants (Wang et al., 2018; Li et al., 2019a; Hua et al., 2019b; Zhong et al., 2019; Zeng et al., 2020). While Cas9-NG can edit many NG PAM sites in plants (Endo et al., 2019; Hua et al., 2019; Ren et al., 2019; Zhong et al., 2019; Zeng et al., 2020), it prefers NGT PAMs rather than NGV PAMs, and its activity is very low at NGV PAMs (Zhong et al., 2019). Furthermore, its activity at canonical NGG PAM sites is much lower than that of the wild-type (WT) SpCas9 (Zhong et al., 2019). Thus, it is necessary to systematically evaluate the PAM requirements for a novel Cas9 protein in plants before its wide adoption by the plant research community.

In this study, we assessed the PAM requirements of the iSpyMacCas9 system in rice and found that it recognized NAAR PAMs. Our observation is in general agreement with the original report of iSpyMacCas9 with NAA PAMs in vitro and in human cells.
While we were conducting this research, another group assessed SpyMacCas9 in rabbits and found that it preferred NAAA PAMs (Liu et al., 2019). Thus, all these studies concluded that iSpyMacCas9 favors A-rich PAMs. It is likely that the PAM preference identified for iSpyMacCas9 in rice would largely hold true for other plant species, although this requires future exploration. In our study, we found that iSpyMacCas9 was indeed more potent than SpyMacCas9, revealing the importance of the R221K and N394K mutations for improved activity (Spencer and Zhang, 2017). It is conceivable that protein engineering may be further used to relax iSpyMacCas9 PAM requirements as well as enhance its nuclease activity.

Base editors are powerful tools for introducing targeted single-nucleotide polymorphisms for crop improvement. However, for a given base editor, the base changes only occur within a defined editing window, which typically only spans a few nucleotides. Thus, it is very important to develop base editors that have altered PAM requirements as they would open up additional sites for editing. Here, we developed two iSpyMacCas9 CBE systems based on PmCDA1 and hAID cytidine deaminases. We found that they both resulted in efficient C to T base conversions at NAAR PAM sites, albeit with different base editing windows. Our data are consistent with previous reports on the use of PmCDA1 and hAID in plants for base editing (Shimatani et al., 2017; Ren et al., 2018; Tang et al., 2018). Interestingly, we found frequent C to G transversions in stable transgenic rice plants, but not in mesophyll protoplasts, at certain target sites with our PmCDA1-based iSpyMacCas9 CBE. This may reflect differences in DNA repair in different cell types. While the mechanism warrants further investigation, similar observations were previously reported by others (Nishida et al., 2016; Shimatani et al., 2017). We also developed one iSpyMacCas9 ABE system that resulted in efficient A to G base changes at NAAR PAM sites. ABE systems typically generate quite pure A to G edits without...
Figure 4. Development and assessment of two iSpyMacCas9 C to T base systems in rice.

(A) Configurations of two cytidine base editors (CBEs) with PrmCDA1 or hAID and iSpyMacCas9-D10A nickase.

(B) Assessment of editing efficiency of two iSpyMacCas9 CBEs at four target sites in rice protoplasts.

(C) Deamination window of PrmCDA1 and hAID CBEs at the OsROC5-gRNA03 (SM6) target site.

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Expanding the plant genome-editing scope

| Constructs | PAM Index | Targeted Rice Sites | Base Editor | Tested TO Lines | Mutated TO Lines (Number; Ratio) | Base-Edited TO Lines (Number; Ratio) | Indels in TO Lines (Number; Ratio) |
|------------|-----------|---------------------|-------------|----------------|----------------------------------|--------------------------------------|----------------------------------|
| pLR2769   | TAAA      | OsROCS5-sgRNA05-SM10 | ISpymac zCas9-PmCDA1-UGI | 16              | 5; 31.3%                        | 3; 18.8%                              | 2; 12.5%                         |
| pLR2773   | CAAA      | OsROCS5-sgRNA13-SM26 | ISpymac zCas9-PmCDA1-UGI | 18              | 9; 50%                         | 7; 38.9%                              | 5; 27.8%                         |
| pLR2771   | TAAA      | OsROCS5-sgRNA05-SM10 | wtTadA1-TadA1’-ISpymac zCas9 | 24              | 3; 12.5%                        | 3; 12.5%                             | 0; 0.0%                          |

Table 2. Summary of base editing efficiency of two iSpyMacCas9 CBE vectors and one iSpyMacCas9 ABE vector in rice T0 lines. UGI, uracil DNA glycosylase inhibitor.

A unique advantage of our plant iSpyMacCas9 system over other existing CRISPR-Cas9 tools is its ability to target cis-regulatory elements that are typically A-T rich. For example, the quantitative trait variation in fruit size was generated by multiplexed promoter editing in tomatoes with the CRISPR-SpCas9 system (Rodriguez-Leal et al., 2017). Multiplexed editing of transcription activator-like effector binding sites in the promoters of SWEET genes in rice generated broad-spectrum disease resistance to bacterial blight (Oliva et al., 2019). The iSpyMacCas9 system uses the same sgRNA scaffold as the most popular SpCas9, making the system fully compatible with our previously developed multiplexed sgRNA expression systems (Lowder et al., 2015, 2017). Hence, the iSpyMacCas9 system can be easily applied for multiplexed promoter editing for crop trait engineering. In addition, iSpyMacCas9 base editors can be useful for introducing single-nucleotide polymorphisms in promotors to impact gene expression. At this point, this capability cannot be matched by A-T-rich PAM-targeting CRISPR-Cas12a and Cas12b systems as no base editors have been successfully developed in plants with these systems. Recently, SpCas9-based prime editors were demonstrated for precise genome editing in plants, although the editing efficiency was low (Hua et al., 2020a; Xu et al., 2020a, 2020b; Li et al., 2020; Lin et al., 2020; Tang et al., 2020). It will be useful to develop iSpyMacCas9 prime editors for directing specific changes at plant promoters to achieve desired gene expression. Finally, the A-rich PAM-targeting feature of iSpyMacCas9 also makes the system very appealing for transcriptional repression with CRISPR interference (CRISPRi) or transcriptional activation with CRISPR activation (CRISPRa) in plants. Previously, NGG PAMs targeting SpCas9 were predominantly used for CRISPRi (Piatek et al., 2014; Lowder et al., 2015) and CRISPRa (Lowder et al., 2015, 2018; Li et al., 2017b) in plants. By replacing SpCas9 with iSpyMacCas9 in such systems, more flexible promoter-targeting CRISPRi and CRISPRa systems could be engineered for plant applications.

In summary, we have developed an iSpyMacCas9 toolbox for plant genome editing at NAAR PAM sites. The four z-iSpyMacCas9 Gateway-compatible entry vectors that allow for targeted mutagenesis, C to T base editing, and A to G base editing have been deposited to Addgene for public dissemination. We envision that iSpyMacCas9 will enable the editing of A-T-rich cis elements for generating quantitative traits in addition to gene knockouts. Additionally, iSpyMacCas9 may facilitate other precise genome-editing applications, such as homology-directed repair and prime editing in plants. Finally, iSpyMacCas9 has the potential in developing CRISPRi and CRISPRa systems for transcriptional regulation in plants. These represent exciting fronts for future development and application of the iSpyMacCas9 systems.

MATERIALS AND METHODS

Genome-wide PAM analysis in rice, maize, and wheat

The genome of O. sativa Nipponbare (Oryza_sativa_nipponbare_v7.0_all-con) was downloaded from the Rice Genome Annotation Project (rice.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/). The genomes of T. aestivum (Triticum_aestivum.IWGSC.dna.toplevel.fa) and Z. mays (Zea_mays.B73_Ref-Gen_V4.dna.toplevel.fa) were downloaded from Ensembl (ftp://ftp.ensemblgenomes.org/pub/plants/release-45/fasta/tritium_aestivum/dna/ and ftp://ftp.ensemblgenomes.org/pub/plants/release-45/fasta/zea_mays/ dna, respectively). The FASTA files were scanned line by line for the appropriate PAM patterns and their reverse complements using a regular expression search in Perl (version 5.26). For instance, to search for the positions of VTTV sites on a line, the WHILE loop (/?=[ACGTTTACG][[ACGT][ACGT]...}
Independent T0 lines with clean A to G base editing at the biological replicates.

Assessment of base editing efficiency of the iSpyMacCas9 ABE at four target sites in rice protoplasts. Error bars represent standard deviations of three biological replicates.

(A) Configurations of the adenine base editor based on iSpyMacCas9-D10A nickase.

(B) Assessment of base editing efficiency of the iSpyMacCas9 ABE at four target sites in rice protoplasts. Error bars represent standard deviations of three biological replicates.

(C) Independent T0 lines with clean A to G base editing at the OsROCs-sgRNA05 (SM10) site. Base changes are indicated by asterisks.

For the design of the sgRNAs, the Nipponbare reference sequence of OsPDS and OsROCs was used. Using Phytozome V12.1 genomic data (https://phytozome.jgi.doe.gov/pz/portal.html), we validated that the Nipponbare reference sequences for both aforementioned genes were 100% identical in Kitaake, a rice variety used in this study. T-DNA vectors (Supplemental Table 1) for CRISPR-Cas9 were constructed using Gateway LR assembly reactions based on the protocols described previously (Lowder et al., 2015). In brief, forward and reverse primers (Supplemental Table 1) for sgRNAs were phosphorylated with T4 polynucleotide kinase (NEB, catalog no. M0201), annealed, and ligated with T4 DNA ligase (NEB, catalog no. M0202) into BsmBI (Thermo Fisher Scientific, catalog no. ER045) restriction-digested pYPQ141C (Addgene no. 69292) or pYPQ141D (Addgene no. 69293) sgRNA entry clones. Individual Gateway LR reactions consisted of the attL5-attL2 sgRNA entry clone, attL1-attR5 (iSpyMacCas9 entry clone), and attR1-attR2 destination vector pYPQ203 (Addgene no. 86 207) containing a ZmUb1 promoter for Cas9 expression. Both sgRNA and Cas9 entry clone recombination regions were confirmed by Sanger sequencing. The final T-DNA vectors were confirmed by restriction digestion with EcoRI (NEB, catalog no. R0101).

Rice protoplast isolation and transformation

The Japonica cultivar Kitaake was used in this study. The rice seedlings were grown on 1/2 MS solid medium for 12–14 days in the dark at 28°C. Rice protoplast extraction and transformation were performed according to our previously published protocols (Lowder et al., 2015; Tang et al., 2017). In brief, healthy leaves were cut into 0.5- to 1.0-mm strips and transferred into the enzyme solution, followed by vacuum-infiltration for 30 min and incubation at 70–80 rpm for 8 h at 25°C in the dark. Each digestion mixture was filtered through a 40-μm cell strainer. After washing twice with a W5 washing buffer, protoplasts were examined and counted under a microscope. The final protoplast concentration was adjusted to 2 x 10⁶ per ml. For protoplast transformation, 30 μg of plasmid DNA in 30 μl (1 μg/μl; prepared using a QIAGEN Midiprep kit) was used to transform 200 μl of protoplasts by gently mixing with 230 μl of 40% PEG transformation buffer. After incubation for 30 min in darkness, the reactions were stopped by adding 900 μl of W5 washing buffer. The protoplasts were centrifuged and transferred into a 12-well culture plate and incubated at 32°C in darkness for 48 h.

Validation of targeted mutagenesis in rice protoplasts

Two targets associated with each of 16 possible NAAN PAMs were chosen based on the presence of a type II restriction enzyme site...
Expanding the plant genome-editing scope

superimposed at the cleavage sites, allowing for the validation of target site mutations with restriction fragment length polymorphism assays. Genomic DNA was extracted from the protoplasts after 48 h of incubation in the dark at 32°C. Target sites were PCR amplified (primers listed in Supplemental Table 1), digested, and run on 2% gels. ImageJ 1.52p software was used to obtain the intensities of bands representing uncut and cut DNA fragments. Genome-editing efficiency was calculated as the ratio of uncut bands against all (cut and uncut) bands. Positive control (cut WT target site) and negative control (uncut WT target site) were carried out for system calibration.

Validation of base editing in rice protoplasts

The NGS of PCR amplicons was used for the detection and quantification of base editing mutations at the target sites. Genomic DNA was extracted from the protoplasts after 48 h of incubation in the dark at 32°C. Using protoplast DNA as the template, genome regions of targeted sites were amplified with barcoded primers (Supplemental Table 1) according to our previously published protocols (Zhong et al., 2019). The specificity of PCR reactions was verified by gel electrophoresis. Transcripts were column-purified, pooled, and sequenced by GENEWIZ (NJ, USA) with the Illumina MiSeq sequencing platform. The raw data were analyzed using BE-Analyzer online software (Hwang et al., 2018).

Stable transformation of rice

The Japonica cultivar Kitaake was used for rice stable transformation. Agrobacterium-mediated transformation was carried out by following a transformation protocol previously established in the lab (Lowder et al., 2015).

Validation of genetic modifications in T0 plants

Sanger sequencing was used to determine genetic modifications at the target sites in T0 plants. Genomic DNA was extracted from the protoplasts after 48 h of incubation in the dark at 32°C. Using T0 genomic DNA as the template, the genome regions of targeted sites were amplified with primers (Supplemental Table 1). The specificity of PCR reactions was verified by gel electrophoresis. PCR reactions were enzymatically cleaned with the ExoSAP kit (NEB) and Sanger sequenced by GENEWIZ. The raw data were analyzed using SnapGene software.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at Plant Communications Online.

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AUTHOR CONTRIBUTIONS

Y.Q. conceived and designed the experiments. S.S. designed the constructs. S.S., D.Y., and A.L. generated the cloning and expression vectors. S.S. and D.Y. conducted rice protoplast and stable transformation and analysis. S.S performed NGS sample preparation and data analysis. J.D.S. and S.M.M. performed the genome-wide PAM analyses in rice, maize, and wheat. S.S. and Y.Q. analyzed all the data and wrote the manuscript. All authors participated in the discussion and revision of the manuscript.

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