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LETTER TO THE EDITOR

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Efficient genome editing in plants using a CRISPR/Cas system

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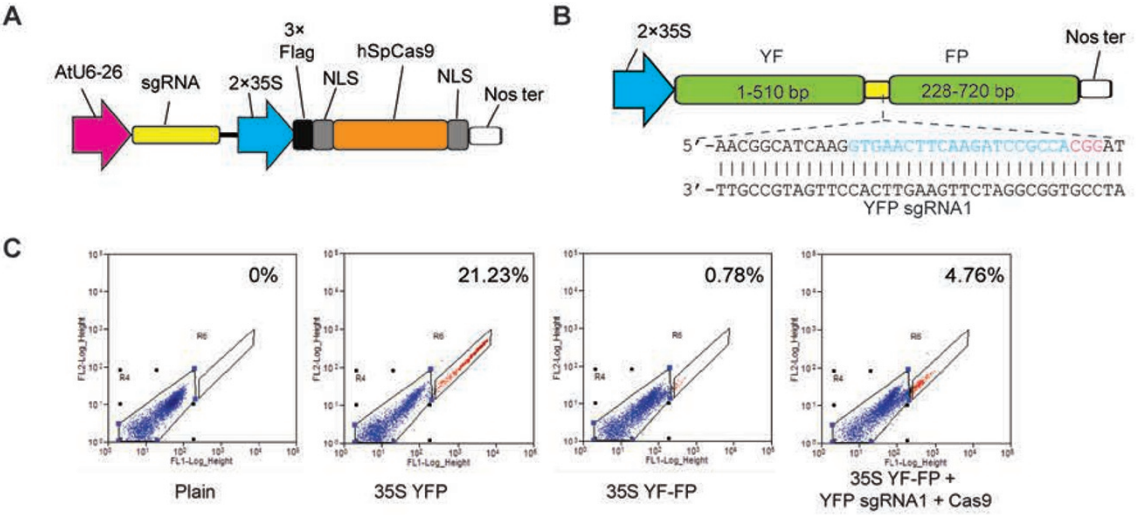
Dear Editor,

In the past few years, the development of sequence-specific DNA nucleases has progressed rapidly and such nucleases have shown their power in generating efficient targeted mutagenesis and other genome editing applications. For zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), an engineered array of sequence-specific DNA binding domains are fused with the DNA nuclease Fok1 [1, 2]. These nucleases have been successful in genome modifications by generating double strand breaks (DSBs), which are then repaired through non-homologous end joining (NHEJ) or homologous recombination (HR) in different species, including mouse, tobacco and rice [3-5]. Recently, another breakthrough technology for genome editing, the CRISPR/Cas system, was developed. CRISPR (clustered regulatory interspaced short palindromic repeats) loci are variable short spacers separated by short repeats, which are transcribed into non-coding RNAs. The non-coding RNAs form a functional complex with CRISPR-associated (Cas) proteins and guide the complex to cleave complementary invading DNA [6]. After the initial development of a programmable CRISPR/Cas system, it has been rapidly applied to achieve efficient genome editing in human cell lines, zebrafish and mouse [7-10]. However, there is still no successful application in plants reported.

We report here that the CRISPR/Cas system can be used to efficiently generate targeted gene mutations and corrections in plants. The *Cas9* gene was driven by the CaMV 35S promoter and the chimeric single guide RNA (sgRNA) was driven by the AtU6-26 promoter in *Arabidopsis* or the OsU6-2 promoter in rice. We show that the engineered CRISPR/Cas was active in creating DSBs when transiently expressed in *Arabidopsis* protoplasts and stably expressed in transgenic *Arabidopsis* and rice plants. Our results demonstrate the feasibility of using engineered CRISPR/Cas as molecular scissors to create DSBs at specific sites of the plant genome to achieve targeted genome modifications in both dicot and monocot plants.

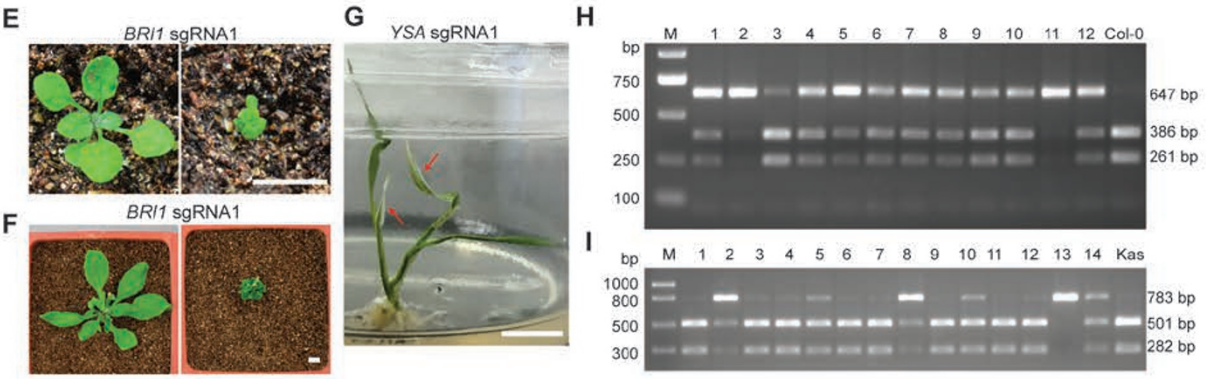
We used the optimized coding sequence of hSpCas9 [9] driven by the CaMV 35S promoter. For the non-coding RNA components of CRISPR, we expressed the sgRNA using native promoters for U6 RNAs in *Arabidopsis* (Figure 1A and Supplementary information, Figure S1A) or rice (Supplementary information, Figure S1A). The target site precedes an NGG, the requisite protospacer adjacent motif (PAM). To improve co-delivery, both the sgRNA and hSpCas9 were subcloned into one expression vector (Figure 1A). A split yellow fluorescent protein (YFP) reporter system, YF-FP, was used to test the functionality of the engineered CRISPR/Cas system in *Arabidopsis* protoplasts (Figure 1B). Co-transformation of the YF-FP reporter and the CRISPR/Cas construct led to the production of strong YFP signal with gene correction rate by HR at 18.8% ((4.76%–0.78%)/21.23%) (Figure 1C). The results suggest that the engineered CRISPR/Cas system is highly functional in generating DSBs on target DNA sequences in plant cells and that the DSBs can be repaired by HR to achieve gene correction.

Having successfully targeted a reporter gene in protoplasts, we started to target endogenous loci in plants. The *Arabidopsis* genes *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*), *JASMONATE-ZIM-DOMAIN PROTEIN 1* (*JAZ1*) and *GIBBERELLIC ACID INSENSITIVE* (*GAI*) and the rice genes *Rice Outermost Cell-specific gene5* (*ROC5*), *Stromal Processing Peptidase* (*SPP*) and *Young Seedling Albino* (*YSA*) were selected for CRISPR/Cas-based disruption (Supplementary information, Figure S1B). These genes were selected owing to obvious growth phenotypes when they are dysfunctional. We designed sgRNAs to target these genes (Supplementary information, Figure S1C). The targets contained restriction enzyme sites close to the PAM sequences, so that the restriction sites may be disrupted when successfully targeted by the CRISPR/Cas (Supplementary information, Figure S2), and RFLP (Restriction Fragment Length Polymorphism) analysis can be used to detect mutations in the target region. The vector containing the Cas9 and sgRNA expression cassette was introduced into plants by



D

	Target	No. of T1 trangenic plants	No. of T1 transgenic plants showing expected phenotype	No. of plants with mutation/ No. of genotyped T1 transgenic plants
Arabidopsis	*BRI1* sgRNA1	68	48	16/19
	BRI1 sgRNA2	55	40	5/15
	BRI1 sgRNA3	68	33	6/20
	GAI sgRNA1	89	24	8/17
	JAZ1 sgRNA1	72	N/A	49/60
Rice	*ROC5* sgRNA1	31	N/A	8/31
	SPP sgRNA1	21	N/A	1/21
	YSA sgRNA1	32	2	24/32
	YSA sgRNA2	21	2	10/21



J

Indels in *BRI1* locus of one *Arabidopsis* T1 transgenic plant targeted by *BRI1* sgRNA1

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                                                  PAM
5′-GGAGATTGGTTCGATGCCTTATCTGTTTATTCTCAATTTGGGTCATAACGATAT-----CTCTGGTTCGATTCC-3′   WT
   GGAGATTGGTTCGATGCCTTATCTGTTTATTCTCAATTTGGGTCATAACGATA------CTCTGGTTCGATTCC      D1
   GGAGATTGGTTCGATGCCTTATCTGTTTATTCTCAATTTGGGTCATAACGAT-------CTCTGGTTCGATTCC      D2
   GGAGATTGGTTCGATGCCTTATCTGTTTATTCTCAATTTGGGTCATAA----------------CGATTCC       D14
   GGAGAT-------------------------------------------------------TGGTTCGATTCC     D51
   GGAGATTGGTTCGATGCCTTATCTGTTTATTCTCAATTTGGGTCATA----------TCTCTGGTTCGATTCC      D7,+1
   GGAGATTGGTTCGATGCCTTATCTGTTTATTCTCAATTTGGGTCA----------ATCTGGTTCGATTCC        D10,+1
   GGAGATTGGTTCGATGCCTTATCTGTTTATTCTCAATTTGGGTCATAACGATATGTCAGCTCTGGTTCGATTCC    +5
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K

Indels in *YSA* locus of one rice T0 transgenic plant targeted by *YSA* sgRNA1

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                                        PAM
5′-AGGGCCGTGCCGGGAGGCGCGCCACCTCGGCCG-AAGCGGCCCTACTC-3′   WT
   AGGGCCGTGCCGGGAGGCGCGCCACCTCGGCC--AAGCGGCCCTACTC      D1
   AGGGCCGTGCCGGGAGGCGCGCCACCTCGGCCGAAAGCGGCCCTACTC      +1
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Agrobacterium-mediated transformation using floral dipping in *Arabidopsis* and tissue culture in rice.

More than 50 T1 and 20 T0 transgenic plants were generated for each target in *Arabidopsis* and rice, respectively (Figure 1D). We observed that a high percentage of the *Arabidopsis* T1 transgenic plants showed growth phenotypes at a very young stage (one week after transplanting in soil) (Figure 1D). For *BRI1*, more than 50% plants displayed retarded growth and rolling leaves (Figure 1D and 1E), which are expected for *bri1* mutant plants. More than a quarter of the T1 plants for *GAI* also showed a dwarf phenotype (Figure 1D). At later stages, some continued to exhibit a dwarf phenotype that was similar to *bri1* or *gai* mutant plants (Figure 1F and Supplementary information, Figure S1D). The designed target for *GAI* is located in the DELLA domain (Supplementary information, Figure S1C), which is important for GA-induced degradation of the GAI protein. It is known that amino acid substitutions or deletions in the DELLA domain of GAI would result in insensitivity to GA-induced degradation, leading to a dwarf phenotype. About 10% of T0 transgenic rice plants targeting *YSA* showed the expected albino leaf phenotype at the seedling stage (Figure 1D and 1G).

We genotyped transgenic plants first by RFLP analysis. Clear undigested bands were observed (Figure 1H and 1I). The failure of restriction enzyme digestion suggested the occurrence of DNA sequence mutations in the target regions. We then sequenced the PCR products to see whether there are additional sequence peaks in the target. Results from the two tests showed that the mutation frequency was very high in both *Arabidopsis* and rice, ranging from 26% (8 out of 31) to 84% (16 out of 19), except for the *SPP* sgRNA1 target (5%, 1 out of 21) (Figure 1D).

Furthermore, the undigested bands from RFLP analysis were cloned and sequenced. We found that in 24 out of the 27 *Arabidopsis* T1 transgenic plants and 14 out of the 24 rice T0 transgenic plants subjected to sequencing, there were 2 or more different mutated alleles in one single transgenic plant (Figure 1J-1K, Supplementary information, Tables S1 and S2). These plants all contained mutant alleles with small insertions or deletions (indels) at the target sites (Supplementary information, Figures S3-S11). The presence of multiple mutated alleles in the *Arabidopsis* transgenic plants indicated that in these plants the CRISPR/Cas did not function or certainly did not complete the genome editing during the fertilization stage, and the editing activity continued after the division of fertilized eggs. Regardless, the high frequency of *Arabidopsis* T1 transgenic plants showing the expected mutant phenotypes suggests that some of the mutations must have been generated very early in development and possibly in early meristematic cells. Therefore, germ line transmission of some of the mutations into T2 plants is expected for many, if not all, of the T1 plants. The identification of 3 bp deletions (which would result in an amino acid deletion) in 2 out of the 3 *GAI* sgRNA1 T1 transgenic plants (Supplementary information, Figure S6) could well explain the high-frequency dwarf phenotype observed (Supplementary information, Figure S1D). It is also worth noting that one rice T0 transgenic line for *ROC5* sgRNA1 (data not shown) and two each for *YSA* sgRNA1 (Figure 1I, lane 13 and data not shown) and sgRNA2 (data not shown) showed only mutated alleles and no wild-type allele in the RFLP analysis. Sequencing of individual clones revealed that the plants contained only or mostly mutated alleles (Supplementary informa-

Figure 1 High-efficiency targeted gene editing in *Arabidopsis* and rice plants. **(A)** The vector for transient transformation of *Arabidopsis* protoplasts. The expression cassette of hSpCas9 is driven by the 35S promoter, whereas the sgRNA is driven by the AtU6-26 promoter. **(B)** The HR-based YF-FP reporter. The PAM sequence of the sgRNA target is colored in magenta and the sgRNA target in cyan. **(C)** The activity of CRISPR/Cas was measured using the YF-FP reporter in *Arabidopsis* protoplasts. YFP-positive cells were quantified by flow cytometry. **(D)** Summary of observed phenotypes and detected mutations in T1 and T0 transgenic plants of *Arabidopsis* and rice, respectively. **(E** and **F)** Representative T1 transgenic plants of the *BRI1* sgRNA1 target. The left one is a T1 plant with normal vegetative growth, while the right one is a plant with a similar growth phenotype as *bri1* mutants. The plants were screened on MS plates for 5 days and transplanted in soil for 1 week **(E)** or 3 weeks **(F)** before photographing. **(G)** One representative rice T0 transgenic plant of the *YSA* sgRNA1 target showing an expected albino leaf phenotype at the regeneration stage. Arrows indicate the albino leaves. **(H)** Genotyping of 12 T1 transgenic *Arabidopsis* plants of the *BRI1* sgRNA1 target by RFLP analysis. The PCR products were digested with *Eco*RV. M, DNA marker. **(I)** Genotyping of 14 T0 transgenic rice plants of the *YSA* sgRNA1 target by RFLP analysis. The #13 sample corresponds to the plant in **G**. The PCR products were digested with *Sfi*I. M, DNA marker. Kas, wild-type rice Kasalath. **(J** and **K)** Representative sequences of mutant alleles identified from a single transgenic plant of the *BRI1* sgRNA1 target **(J)** and T0 plant of the *YSA* sgRNA1 target **(K)**. For the *YSA* locus, the plant corresponds to the plant in **G**. The wild-type sequence is shown at the top with the PAM sequence highlighted in magenta and the target sequence in cyan. Red dashes, deleted bases; red bases, insertions or mutations. The net change is to the right of each sequence (+, insertion; D, deletion). The scale bars equal to 1 cm **(E**, **F**, **G)**.

tion, Table S2, Figures S8, S10, S11). Especially for the *ROC5* sgRNA1 and *YSA* sgRNA1 lines, they contained one or two types of mutated alleles only. Importantly, the *YSA* sgRNA1 rice plants showed the expected albino leaf phenotype (Figure 1G). The result suggests that these rice plants are likely homozygous or bi-allelic mutants, which implies that in this case the CRISPR/Cas may have completed the generation of DSBs in the first meristematic cell during regeneration of the rice plants from transgenic calli.

To our knowledge, this is the first study demonstrating highly efficient targeted mutagenesis in multiple genes in *Arabidopsis* and rice using engineered CRISPR/Cas. Although future studies are needed to examine the germ line transmission and heritability of the CRISPR/Cas-induced mutations and to evaluate any potential off-target effects of the CRISPR/Cas, our results here suggest that the CRISPR/Cas technology will make targeted gene editing a routine practice not only in model plants but also in crops.

Detailed methods are described in the Supplementary information, Data S1 and Table S3.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)