**TALEN-Mediated Homologous Recombination Produces Site-Directed DNA Base
Change And Herbicide-Resistant Rice**

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ABSTRACT

Over the last decades, much endeavor has been made to advance genome editing technology due to its promising role in both basic and synthetic biology. The breakthrough has been made in recent years with the advent of sequence specific endonucleases, especially zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR) guided nucleases (e.g., Cas9). In higher eukaryotic organisms, site-directed mutagenesis usually can be achieved through non-homologous end joining (NHEJ) repair to the DNA double-strand breaks (DSB) caused by the exogenously applied nucleases. However, site-specific gene replacement or genuine genome editing through homologous recombination (HR) repair to DSBs remains a challenge. As a proof of concept gene replacement through TALEN-based HR in rice (*Oryza sativa*), we successfully produced double point mutations in rice acetolactate synthase gene (*OsALS*) and generated herbicide resistant rice lines by using TALENs and donor DNA carrying the desired mutations. After ballistic delivery into rice calli of TALEN construct and donor DNA, nine HR events with different genotypes of *OsALS* were obtained in T₀ generation at the efficiency of 1.4% to 6.3% from three experiments. The HR-mediated gene edits were heritable to the progeny of T₁ generation. The edited T₁ plants were as morphologically normal as the control plants while displayed strong herbicide resistance. The results demonstrate the feasibility of TALEN-mediated genome editing in rice and provide useful information for further genome editing by other nuclease-based genome editing platforms.

KEYWORDS: TALEN, genome editing, homologous recombination, gene replacement, site-directed mutagenesis, acetolactate synthase, herbicide resistance, rice.

INTRODUCTION

Genome editing, a process of precisely targeting and modifying predetermined genomic DNA sequences, is always challenging in the last decades though much effort has been made to artificially engineer sequence-specific endonucleases. Artificial endonucleases manipulate the cellular repair mechanisms through controlling the location and quantity of DNA double-strand breaks (DSB). Not until the advent of zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR) guided nucleases (e.g., Cas9 and Cpf1) did site directed mutagenesis or gene replacement become much easier (Christian et al., 2010; Jinek et al., 2012; Li et al., 2011; Smith et al., 2000). The rapid development of those powerful tools has been providing promise to rapid advance of basic and applied scientific research. However, genomic editing or gene replacement still remains a challenge in most of eukaryotic organisms.

Transcription activator-like effectors (TALE), a large group of highly conserved type III effector proteins, exist in *Xanthomonas* spp. and can be translocated into host cells by the bacterial type III secretion system (White et al., 2009). Different bacterial strains contain a varying number of TALEs, but majority of those effectors contain an N-terminal bacterial secretion and translocation signal, central DNA binding domain, and C-terminal eukaryotic nuclear localization signals (NLS) and a highly conserved acidic activation domain (AD) (Boch and Bonas, 2010). The DNA binding domain composes of an array of tandem repeats of 34 amino acids; and the repeats consist of nearly identical amino acids except those at the position 12th and 13th, which are termed Repeat Variable Di-residues (RVD). Collectively, the number of TALE repeats (ranging from 13-33) and composition of RVDs determine the specificity for DNA binding (Boch et al., 2009; Moscou and Bogdanove, 2009). The simple TALE/DNA recognition code is best described as one repeat corresponding one nucleotide and one type of RVD preferentially recognizing one type of nucleotide of target DNA. In a practical means, four types of repeats that predominantly occur in nature are the repeats with RVDs HD, NI, NG and

NN that recognize nucleotides cytosine (C), adenine (A), thiamine (T), and guanine (G) or A, respectively.

With high specificity of DNA recognition and relatively low dependence on genomic context of target DNA, TALE repeats, especially the four predominant ones, have been adopted to engineer DNA binding domains and assemble into sequence-specific endonucleases by fusing to the *Fok* I nuclease domain (Christian et al., 2010; Li et al., 2011). Accordingly, various modular assembly methods have been developed to engineer TALENs, including the most widely used golden-gate cloning method, automated solid-phase high-throughput method, PCR dependent or ligation independent subcloning techniques. TALEN genes can be made in laboratories or are available commercially (Cermak et al., 2011; Reyon et al., 2012; Schmid-Burgk et al., 2013). TALENs have been successfully used mostly for targeted mutagenesis in more than 25 organisms including yeast, animal, plant and human cells (Weeks, et al., 2016).

There are two DSB repair pathways in eukaryotic organisms, namely non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ is a process that directly joins the broken ends without homologous sequences or utilizes exposed micro-homologous ends to guide joining. The breakage can be precisely resealed or imprecisely repaired leading to small insertions or deletions (Mladenov and Iliakis, 2011). HR uses homologous region from donor DNA as a template to repair the breakage (Fig. 1) (San Filippo et al., 2008). In this case, the repair process is exploitable to generate site- or sequence-specific DNA mutation through sequence replacement, or to precisely integrate genes of interest such as florescent markers into target sites for various research and application purposes. However, relatively few studies have been reported for HR-mediated genome modifications largely due to the low intrinsic recombination efficiency in eukaryotes. For example, only a few cases have been reported in plants for ZFN or CRISPR (Wright et al., 2005; de Pater et al., 2009, 2013; Townsend et al., 2009; Li et al., 2013). In those studies, DNA was delivered through *Agrobacterium*-mediated floral dip in *Arabidopsis* or transfected into tobacco protoplasts.

Acetolactate synthase gene (*ALS*) encodes a protein that as an enzyme is involved in the biosynthesis of branched-chain amino acids valine, leucine and isoleucine (Chipman, et al., 1998). The gene product, acetolactate synthase (also known as

acetohydroxy acid synthase or AHAS) represents a major target for development of distinct classes of herbicides that inhibit its enzymatic activity and hence block the synthesis of these amino acids. Important herbicides such as chlorsulfuron and Bispyrobac-sodium (BS) have been developed to control a wide spectrum of grass and dicot weeds. To generate rice plant tolerant to the pyrimidinyl carboxy herbicide bispyribac-sodium, prior studies had identified an *OsALS* mutant gene (*OsALS^R*) that could tolerate BS through rice tissue culture and regeneration (Osakabe, et al., 2005; Kawai, et al., 2007). Two point mutations in *OsALS^R* are crucial for BS resistance: a G to T missense change leading to the amino acid tryptophan (TGG) to leucine (TTG) at the position 548 (W548L) and a second G to T missense change resulting in the substitution of serine (AGT) with isoleucine (ATT) at the position 627 (S627I). The double point mutations increase BS tolerance compared to single mutation. BS tolerant rice plants were reproduced by introducing the double point mutations to *OsALS* endogenous loci at a very low rate through conventional gene targeting without the aid of DSB-causing endonucleases (Endo et al., 2007). Additionally, this herbicide tolerant *ALS* has been successfully used as a selection marker for plant transformation (Endo et al., 2012).

In present study, as a proof of concept to demonstrate the feasibility of using TALENs for gene replacement in plant, we constructed a single construct that contained both the *OsALS*-targeting TALEN expression cassettes and the donor DNA fragment for the double point mutations. Through ballistic delivery, this construct yielded high efficiency of rice transformation and up to 6.3% of gene edits in 3 independent experiments. The HR mediated mutations were able to pass on to the T₁ generation progeny as tested. Furthermore, the T₁ plants showed strong BS resistance and were morphologically normal compared to the parent line. No HR event was detected in two sets of negative control experiments.

RESULTS

Rice ALS variant with two point mutations confers herbicide resistance in transgenic rice

The rice acetolactate synthase gene (*OsALS*) is transcribed from a single exon in chromosome 2 (*Os02g30630*) and its cDNA clone is 2279 bp (NCBI accession No.,

NM_001053466). To confirm the prior report that showed double point mutations (W548L and S627I) in *OsALS*, namely W548L/S627I or referred to here as *OsALS^R* allele, conferred strong resistance to bispyribac sodium (BS), a pyrimidinyl carboxylate type herbicide (Kawai, et al., 2007), we reconstructed the mutations based on the *ALS* gene amplified from the BS sensitive rice cultivar Kitaake and introduced the mutated gene (*OsALS^R*) into Kitaake through *Agrobacterium*-mediated gene transfer (Fig. 2A). Three 35S:*OsALS^R* transgenic rice lines were chosen for detailed characterization of *OsALS^R*. When grown in rice solution supplemented with 0.25 $\mu\text{mol/L}$ BS, one line grew as normally as the plants that were cultivated in rice solution without BS, while the negative control 35S:GFP transgenic Kitaake was sensitive to BS at the concentration of 0.25 $\mu\text{mol/L}$ (Fig. 2B). Transmission analysis of the transgene revealed the association of the presence of T-DNA and BS-resistance phenotype in the progeny of T₂ generation (data not shown). The other two T₀ lines were BS resistant, but their progeny plants lost the ability to grow in 0.25 $\mu\text{mol/L}$ BS solution probably due to silencing of transgene driven by the viral 35S promoter, which is common in our other experiments in rice and is also relatively common in *Arabidopsis* (Daxinger, et al., 2008). Nevertheless, the results confirm the *OsALS* variant *OsALS^R* is functional for BS herbicide resistance in rice including Kitaake.

Experimental design for TALEN-mediated gene editing through HR

In light of above finding, we sought to recapitulate the BS resistance by precisely editing the endogenous *OsALS* in Kitaake by employing TALEN technology. Three factors were considered for experimental design. First, TALENs capable of targeting *OsALS* for DSB near the BS resistance mutation site need to be designed and constructed. Second, a donor DNA fragment need to be constructed that contains the desired mutations for BS resistance and additional silent mutations for avoidance of cleavage by TALENs and for genotyping the edited plants. The donor would not be functional by itself when inserted randomly into the genome due to lack of expression and truncation of coding sequence. Finally, DNA delivery of TALEN construct and donor template to plant cells includes ballistic bombardment and *Agrobacterium*-mediated DNA transfer, ways that involve double-stranded DNA and single stranded DNA, respectively. Specifically, one pair of TALENs that each with 22 repeats binds to 22 at *OsALS* locus were assembled by using

four core TALE repeats of NI, NG, NN and HD (corresponding to nucleotide A, T, G and C, respectively) through golden gate cloning strategy (Fig. 3A). To shorten the relative distance between DSB and the desired DNA change, the TALEN target site was chosen approximate to the second mutation site (S627I) (Fig. 3B). We used an overlap PCR approach and subsequent DNA fragment swapping to incorporate the missense mutations for W548L and S627I and the silent mutations at the TALEN target site into the donor DNA (Fig. 3, A–D). The nucleotide changes in the donor fragment resulted in additional restriction sites that could be used for molecular screening of transformed rice plants and also for distinguishing spontaneous mutations from HR.

Resistant callus lines were obtained under hygromycin and BS selection

Single construct containing both the paired TALEN expression cassettes and donor DNA was introduced into the rice calli through ballistic delivery system (Fig. 4A). Almost every single callus line proliferated in selection medium supplemented with hygromycin, suggesting a high efficiency of transformation through ballistic DNA delivery, a rate that was much higher than the *Agrobacterium*-mediated DNA transfer. After two rounds of callus selection (first on hygromycin medium, then on BS-containing medium under a lower stringent selection, e.g., 0.25 $\mu\text{mol/L}$, a total of 11 BS resistant callus lines were obtained from 203 initial callus lines among three independent experiments (five from the experiment 1, four from experiment 2, and two from experiment 3) (Table 1). A construct that contained only one of paired TALENs (e.g., right TALEN) and the donor DNA fragment was also transferred into rice callus cells as a control. Similarly a construct containing only one of the paired TALENs and lacking donor DNA fragment was used as the second control. Ten and one BS resistant callus lines were picked up from 58 and 49 hygromycin resistant calli transformed with single TALEN with donor DNA and one TALEN only, respectively. All BS-resistant callus lines were transferred to plant regeneration medium, and plants from all lines were successfully generated and used for further molecular analysis (Table 1).

HR events occurred in plants derived from TALEN/donor experiments not from controls

We next extracted genomic DNA from leaf tissue of the primary transgenic plants and used the DNA for PCR-amplification of relevant region with specific primers at the

OsALS locus. To distinguish the donor DNA sequence from that of the endogenous *OsALS* and also to avoid the interference of T-DNA sequence, a pair of primers were designed that the forward primer was located 140 bp upstream of the W548L site in both endogenous *ALS* and donor template, and the reverse primer matched a region 75 bp beyond the 3' end of donor DNA. The primers would not yield PCR amplicon from the donor DNA were it randomly inserted in other genomic loci. The donor DNA was constructed to contain a new *Pf*MI site at the S627I position (located 380 bp from the 5' end of PCR product). It would be expected that digestion of PCR amplicons with *Pf*MI would yield three fragments (120, 260 and 420 bp) for the HR allele while two fragments (120 and 680 bp) for the endogenous *ALS* allele due to the original *Pf*MI site (20 bp upstream of W548L site or 120 bp from the 5' end of the PCR product (Fig. 4B). After PCR amplification and *Pf*MI restriction, different patterns of DNA bands were detected from the eleven independent T₀ lines. One line showed a HR pattern with only three fragments (120 bp, 260 bp and 420 bp); two lines had a wild type pattern of two bands (120 and 680 bp); while eight yielded a mixture of HR and wild type patterns, i.e. four fragments (120, 260, 420 and 680 bp) (Data not shown). We also sequenced the eleven PCR amplicons (Fig. S1). The line with the HR pattern only was a real edit in both *ALS* alleles (Fig. 4C). Among the eight lines with a mixture pattern, two lines contained a HR allele and another allele of wild type *ALS*, and the other six contained an allele of HR-mediated edit and another allele of wild type but a small deletion (4, 6 or 9 bp) (Fig. 4, C and D). The two lines with a DNA digestion pattern of wild type were not the edited *ALS* but diallelic *ALS* mutants instead that probably survived from a lower BS selection (Fig. 4, C and D). Taken together, the results indicate that the TALENs function as sequence-specific endonucleases to induce DSBs that facilitate either NHEJ-mediated mutagenesis or HR-mediated gene replacement or editing.

In summary, among the 11 BS-resistant rice lines, nine of them (four out of five, four out of four and one out of two from three experiments, respectively) turned out to be the real HR events (Table 1). Furthermore, eight of them showed heterozygous mutations with one HR allele and the other allele of different genotypes. Remarkably, in our study, one line with homozygous HR edit was obtained. The overall HR efficiency was up to 6%, which meant six HR mediated gene replacement lines could be obtained among one

hundred of transformants. When checking the BS-resistant rice lines from the two control groups with *Pf*/MI digestion, we found none of eleven lines producing the HR pattern (Table 1). Those plants probably resulted from the relaxed selection at low BS concentration or spontaneous mutation in *ALS* gene.

***ALS* edit and BS resistance were passed on to the T₁ generation**

We chose two rice lines for detailed characterization of *ALS* edit in the progeny of T₁ generation. T₁ seeds from two lines ALS^{HR} #1 and ALS^{HR} #2 with genotypes HR/-9 bp and HR/-6 bp, respectively, along with a control of 35S:GFP line were germinated on ½ MS medium, then transferred to ½ MS medium with or without 0.5 µmol/L BS. As shown in Figure 5, plants of line ALS^{HR} #1 and ALS^{HR} #2 showed BS resistance compared to the plants from the 35S:GFP line, while all plants grew normally and were healthy in ½ MS medium (Fig. 5, A and B). The genotyping results showed that T₁ plants from two ALS^{HR} lines (24 from ALS^{HR} #1, 4 from ALS^{HR} #2) were all homozygous HR edits without an allele of 6 bp or 9 bp deletion (Fig. 5C). There was no progeny plant heterozygous for HR edit and deletion mutations or homozygous for deletion mutations although 24 plants of ALS^{HR} #1 were investigated. The result suggests a negative effect of deletion mutations on the gametogenesis in the two rice lines. It is possible that deletions of 6 bp and 9 bp (i.e., 2 and 3 amino acids deletion, respectively) render the alleles nonfunctional and the haploid pollen or ovule with only the mutated *ALS* allele is defective. However, it is not for certainty that the two or three amino acids or the region are critical for function of acetolactate synthase activities.

Transfer DNA construct was segregated out in some T₁ progeny plants

It is important to breed plant or crop lines that contain the desired edits but lack the transferred DNA, in this case, the gene editing agent. Transgene-free plant or crop lines would reduce the regulatory burden for the producer and more likely be accepted by public if food or livestock is involved. We also investigated the presence of transfer DNA in the progeny plants of ALS^{HR} #1 and ALS^{HR} #2 lines. Two sets of primers that were specific for TALEN genes and hygromycin resistance gene were used for PCR analysis on the 28 plants. We were able to detect the presence of TALEN and hygromycin resistance genes in the twenty-four plants of line ALS^{HR} #1. However, we were not able to detect the two target regions in three of the four plants from line ALS^{HR} #2, suggesting

the elimination of T-DNA from those plants. It is worthy to point out that whole genome re-sequencing will conclusively demonstrate the entire removal of transgenes in those plants.

DISCUSSION

In this study, we established a simple but successful method to perform gene replacement in rice by using TALEN technology. *OsALS* gene is an ideal target to demonstrate the feasibility and efficiency of TALEN-mediated HR in plant because of the dominant phenotype of mutation for positive selection in T₀ generation. *ALS* orthologous genes have been used in this way in other species with different site-specific nucleases (Townsend, et al., 2009; Svtashev, et al., 2015; Sun, et al., 2016). In present study, 1.4-6.3% transformants were detected, either in one allele or both alleles, to acquire the donor DNA sequence at the endogenous locus, however, the control with either nonfunctional TALEN or no homologous donor DNA could not make HR occur. Several factors might influence the HR efficiency. First, the potency of nucleases to cause site-specific DSBs is the most important factor. The TALENs used in present study was highly active based on the 45% of NHEJ mutations caused in those 11 T₀ lines (Fig. 4C). Second, it is crucial to determine the distance between the DSB and the site for intended change. It has been shown that in tobacco, the HR frequency was increased from 0.2% at a distance of 1500 bp between DSB and the position of desired change to 4% with a window of 160 bp (Townsend et al., 2009). Unlike ZFN that is restricted by availability of target site, TALEN target is relatively flexible, enabling engineering nucleases to induce the DSBs at or near the site for desired DNA change to maximize the HR reaction. Third, length of donor DNA is another factor that may contribute to the efficiency. Long donor DNA fragments may be a good option. Endo et al. reported that they regenerated 66 independent recombined BS-resistant rice plants from 1500 calli through gene targeting without help from DSB-inducing nuclease (Endo et al., 2007). An 8 kb PCR fragment used for gene targeting might be the reason for high recombination rate in that case. Highly efficient transformation system they adopted might be another reason as a considerable number of transformation events occurred in each callus line as reported.

Third, copy number of donor DNA available in individual cells, in form of either genomic version or non-integrated and floating form, may increase the chance for DSB homologous recombination. The strategy that includes high quantity of donor DNA together with the DSB-inducing endonuclease construct through ballistic bombardment may improve the HR efficiency. It is worthy to mention that we also transformed the similar amount of callus cells through *Agrobacterium*-mediated gene transfer with the same construct for ballistic bombardment. In that experiment, only one BS-resistant callus line was obtained. Further molecular analysis of that line demonstrated a non-HR event (data not shown). Our finding is consistent with that in a study by Endo et al. that indicates that *Agrobacterium*-mediated template delivery is not efficient for homology-directed repair (HDR)-based gene editing (Endo et al., 2016).

Sequence specific nuclease mediated genome editing, especially genome targeting that can specifically change the gene sequence with predetermined donor DNA, is a powerful tool to investigate gene function. For genome targeting, it is achievable to investigate gene or/and protein spatiotemporal information at tissue or subcellular level when translational fusion of protein with fluoresce marker at an endogenous locus. The so called gene-tagging will more accurately reflect the natural expression pattern than the transgenic plants that contain a T-DNA of reporter cassette inserted randomly in the genome as different chromosome structure and epigenetic modification largely impact gene expression status. However, HR-mediated gene modification is rare in most eukaryotic organism wherein NHEJ pathway is dominant. Therefore, mutation screening process would be challenging if selection scheme would not be available. One solution would be to use a linker of self-cleaving 2A peptide between the gene of interest and a selection marker gene, making the targeted protein and selection marker relatively independent. In this case, it is possible to perform a positive selection instead of genotype screening for tagged insertion event from a large number of transformants.

MATERIALS AND METHODS

Construction of DNA plasmids

First, we made an expression construct of *OsALS^R* gene with double point mutations in *p35S:OsALS^R* to confirm the BS-resistant phenotype. To amplify *OsALS^R* genomic DNA

from the wild type rice cultivar Kitaake, three pairs of primers were designed and used respectively: ALS-F1 (5'-AATAGATCTCCACCCACCATGGCTACGAC-3') and ALS-R1 (5'-ACCTATCCTCCAATTGCACCAC-3'), ALS-F2 (5'-GTGGTGCAATTGGAGGATAGGT-3') and ALS-R2 (5'-TGAATGCACCACCAATTGGGATCATAGGCAGCACGTGTTCTTG-3'), and ALS-F3 (5'-CAGGAACACGTGCTGCCTATGATCCCAATTGGTGGTGCATTCA-3') and ALS-R3 (5'-CCGAGCTCATAGAAGTACTTTATTCTCTT-3'). Three individual PCR amplicons were joined by overlap-PCR amplification. The resulting 2.3 kb fragment was cloned into the binary vector *p35S* that contains CaMV 35S promoter, polylinker, nos terminator and hygromycin selection marker between the left and right border sequences through *Bam*H I/*Bgl* II and *Sac* I.

TALEN expression construct was generated using an established method that was described in detail previously (Li et al., 2014). Briefly, three arrays of 8-repeats of central fragments were assembled by using the TALEN assembly plasmid kit that was developed in our lab. The repeat fragments were cloned into TALEN encoding scaffold with restriction and ligation. One of the paired TALEN genes (e.g., TALEN-R) was cloned into the plant expression cassette by *Bgl* II/*Bam*H I and *Spe* I under the CaMV 35S promoter (*35S:TALEN-R*). Similarly, another TALEN gene was cloned into an intermediate vector under the maize ubiquitin 1 promoter in which the expression cassette (*Ubi:TALEN-L*) was mobilized into the binary vector by *Hind* III restriction and ligation.

To construct donor DNA template, *p35S:OsALS^R* was linearized with *Sac* I and partially digested with *Xho* I. Fragment with the 5' end 100 bp missing was collected through gel electrophoresis and cloned into pENTR4 vector. DNA fragment that contained silent mutations at the TALEN target site was synthesized from GenScript and swapped with the corresponding region in pENTR4 donor construct. Then the modified donor DNA was mobilized into the binary TALEN vector through Gateway recombination reaction. The final single construct with TALEN expression cassettes and donor fragment was used for ballistic bombardment.

For the negative control constructs, the donor fragment was mobilized from pENTR4 into *p35S:TALEN-R* through Gateway recombination reaction. Another negative control *p35S:TALEN-R* contained no donor DNA.

Ballistic delivery plasmid DNA plasmid into rice callus cells

The immature embryos of the *japonica* rice (*Oryza sativa* L.) cultivar Kitaake were used to initiate rice callus cells on MS 2,4-D callus initiation medium (MSD) for 14 days at 30°C under constant light. Before DNA bombardment, actively growing calli were transferred to an osmotic MSD medium and cultured for 4 hours. The gold particles were coated with DNA and used for DNA delivery using the Biolistic® PDS-1000/He Particle Delivery System from Bio-Rad. Two shots for each plate were performed with 650 psi of rupture disk and 6 cm distance for the first shot and 1100 psi of rupture disk and 10 cm distance for the second shot. The calli were cultured for 8 hours in the dark before transferred to selection medium.

Two-steps screening of HR positive calli

The transformed calli were first selected for hygromycin resistance at an interval of two weeks. After two rounds of selection (about 1 month), hygromycin resistant callus cells were visible and transferred to BS-supplemented medium (0.25 µmol/L). Callus cells were transferred at an interval of two weeks until BS-resistant cells were visible. The BS-resistant callus lines were transferred to rice regeneration medium supplemented with BS for generation of plantlets. Plantlets were finally put in in ½ MS+BS medium for root induction. Plantlets were grown in soil and in growth chamber at 28°C, 75% relative humidity and a photoperiod of 12 hours.

Molecular detection of HR events

Genomic DNA was extracted from leaf tissue of the primary transgenic (T₀) and progeny plants using CTAP method (Murray and Thompson, 1980). PCR amplification of the *OsALS* or derived loci was carried out with primers ALS-F5 (5'-ACCCAGGTGTCACAGTTGTTG-3') and ALS-R4 (5'-TCGTGTGATGACAGTTGAGCA-3'). The PCR amplicons in a size of 0.8 kb were digested with *Pf*MI and separated through gel electrophoresis for determination of genotypes. Each amplicon was further confirmed through Sanger sequencing at the Iowa State University DNA facility in USA.

BS-resistant phenotyping analysis

To assess the BS herbicide resistance of all plants, seeds were germinated and grown in ½ MS medium supplemented with or without BS at 0.25 or 0.5 µmol/L as specified in the

text. Transgenic plants with *35S::GFP* were used as control. For phenotype analysis on the HR rice lines, 1-week seedlings of T₁ generation were transferred to ½ MS medium with and without 0.5 µmol/L BS, respectively, for growth in two weeks.

Detection the presence of T-DNA in progeny plants

Genomic DNA was used for PCR-amplification of hygromycin phosphotransferase (*hpt*) gene and TALEN genes. Primers for the *hpt* gene were Hyg-F (5'-CCGCTCGTCTGGCTAAGATC-3') and 35S-R (5'-CGCTGAAATCACCAGTCTCTC-3'); and primers for the TALEN genes were FokI-F (5'-CAGCTAGTGAAATCTGAATTGG-3') and Nos-R (5'-CATCGCAAGACCGGCAACAGG-3').

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516

517 Table 1. TALEN-facilitated HR in *OsALS* in primary transgenic rice

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Construct	Experiment	Hyg ^R callus line	BS ^R callus line	HR event plant	HR rate
TALENs+Donor	Exp. 1	70	5	4	5.7%
	Exp. 2	63	4	4	6.3%
	Exp. 3	70	2	1	1.4%
Single TALEN+Donor	Exp. 1	58	10	0	0
TALENs	Exp. 1	49	1	0	0

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FIGURE LEGENDS

Fig. 1. Schematic diagram of TALEN-based homologous recombination.

After Paired TALENs recognize and bind to target sites, dimerized *Fok* I cleavage domains generate double-stranded DNA break within the spacer region (step 1). The free 3' end DNA, which is generated by repair enzyme excision, paired with donor DNA within homologous region (step 2). DNA repair enzymes extend DNA and fill the gap based on donor DNA as the template, resulting in genomic editing or gene replacement (step 3).

Fig. 2. Rice acetolactate synthase variant confers herbicide resistance.

A: Gene structure of the rice *ALS* gene. The 5' and 3' untranslated regions and coding region of the gene are presented as grey and solid black box, respectively. The size of each region is denoted above the boxes. Two amino acid substitutions in an *ALS* variant (*OsALS^R*) are indicated below the box. **B:** Phenotypes of transgenic plants with or without *OsALS^R* grown in medium supplemented with 0.25 $\mu\text{mol/L}$ bispyribac-sodium for three weeks.

Fig. 3. Donor template and TALENs for *OsALS* gene editing.

A: RVD sequence of TALENs and corresponding target DNA sequence in *OsALS*. Twenty-two repeats of each TALEN and the matching 22 nucleotides are shown. **B:** Donor fragment for *OsALS*. Sequences in endogenous *ALS* targeted by two TALENs are underlined. Spacer sequence between the target sites is depicted by dots. Donor template (mALS) is 2123 bp and contains 5' end truncation relative to *OsALS* and encodes two point-mutations (dots in red). **C.** Partial sequence of donor DNA around the two missense mutation sites with altered nucleotides (letter in red) resulting in three new restriction sites for *Mfe* I and *Pfi*MI. **D.** Alignment of the altered nucleotide sequences (letter in red) in donor compared to endogenous *ALS* sequence that contains two TALEN target sites (two sequences underlined).

Fig. 4. Genotype analysis of *OsALS* edited plants.

A: Construct used for rice transformation *via* ballistic bombardment and *Agrobacterium*-mediated gene transfer. **B:** Schematic diagram of PCR products amplified from *OsALS* locus. The blue line shows the HR-mediated integration of donor fragment with green bars denoting the two *Pfl*MI sites and red dots indicating the desired editing for BS resistance. Three fragments (120, 260 and 420 bp) would be produced after *Pfl*MI digestion. The black line indicates the endogenous *ALS* allele with only one *Pfl*MI site, resulting two fragments (120 and 680 bp) after *Pfl*MI digestion. The grey dots denote the corresponding sites to BS resistance site mutations in the non-HR alleles. **C:** Among the eleven mutant plant lines, one line is di-allelic HR, six mono-allelic edits and mono-allelic mutations derived from HR and NHEJ repair mechanism, respectively, two with mono-allelic edits and wild type allele, and two with di-allelic mutations. **D:** Sequences of different genotypes detected in the BS-resistant lines.

Fig. 5. Characterization of the *OsALS* edited plants.

A: TALEN-mediated *ALS* mutant rice plants are resistant to ALS-inhibiting herbicide (BS, bispyribac-sodium, 0.5 μ mol/L). **B:** Plants of the same category in (A) were grown in solution without BS. Photos were taken from 3 weeks old plants. **C:** HR-mediated *OsALS* edits are transmitted to the T₁ generation plants.

Fig. 1

Fig. 2

A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
TALEN-L	C	A	T	C	G	T	C	C	C	G	C	A	C	C	A	G	G	A	G	C	A	T
	HD	NI	NG	HD	NN	NG	HD	HD	HD	NN	HD	NI	HD	HD	NI	NN	NN	NI	NN	HD	NI	NG
TALEN-R	C	A	T	G	T	C	C	T	T	G	A	A	T	G	C	G	C	C	C	C	C	A
	HD	NI	NG	NN	NG	HD	HD	NG	NG	NN	NI	NI	NG	NN	HD	NN	HD	HD	HD	HD	HD	NI

B

TALEN-L
TCATCGTCCCGCACCAGGAGCAT●●●TGGGGGCGCATTCAAGGACATGA
AGTAGCAGGGCGTGGTCCTCGTA●●●ACCCCCGCGTAAGTTCCTGTACT

TALEN-R

OsALS

Chr 2

+1

mALS
(Donor DNA, 2131 bp)

+129

W548L

S627I

+2259 bp

C

W548L

S627I

V Q L E D ●●● E H V L P M I P I G G A

●●GTGCAATTGGAGGAT ●●●GAACACGTGCTGCCTATGATCCCAATTGGTGGTGC●●

Mfe I

Mfe I PflMI

D

WT

ATCATCGTCCCGCACCAGGAGCATGTGCTG ●● CCAAGTGGGGGCGCATTCAAGGACATGA

ATTTATGTTCACATCAAGAACACGTGCTG ●● CCAATTGGTGGTGC GTTTAAAGATATGA

Donor

I I V P H Q E H V L P I G G A F K D M

D

WT

Donor

Fig. 4

Fig. 5