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# Base-Editing-Mediated Artificial Evolution of *OsALS1 In Planta* to Develop Novel Herbicide-Tolerant Rice Germplasms

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## ABSTRACT

**Recently developed CRISPR-mediated base editors, which enable the generation of numerous nucleotide changes in target genomic regions, have been widely adopted for gene correction and generation of crop germplasms containing important gain-of-function genetic variations. However, to engineer target genes with unknown functional SNPs remains challenging. To address this issue, we present here a base-editing-mediated gene evolution (BEMGE) method, employing both Cas9n-based cytosine and adenine base editors as well as a single-guide RNA (sgRNA) library tiling the full-length coding region, for developing novel rice germplasms with mutations in any endogenous gene. To this end, *OsALS1* was artificially evolved in rice cells using BEMGE through both *Agrobacterium*-mediated and particle-bombardment-mediated transformation. Four different types of amino acid substitutions in the evolved OsALS1, derived from two sites that have never been targeted by natural or human selection during rice domestication, were identified, conferring varying levels of tolerance to the herbicide bispyribac-sodium. Furthermore, the P171F substitution identified in a strong *OsALS1* allele was quickly introduced into the commercial rice cultivar Nangeng 46 through precise base editing with the corresponding base editor and sgRNA. Collectively, these data indicate great potential of BEMGE in creating important genetic variants of target genes for crop improvement.**

**Key words:** CRISPR, base editor, gene evolution, *OsALS1*, herbicide resistance, *Oryza sativa* L

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## INTRODUCTION

Genetic diversity, which contributes to phenotypic variation of crop germplasm, is the basis of crop domestication and breeding. To date, a large number of elite genes associated with important agronomic traits have been identified and widely adopted in crop improvement to ensure food security. Among these, *sd1* and *Rf* in rice, *Rht* in wheat, *d8* in maize, and *sp* in tomato have even revolutionized agriculture across the globe (Fujioka et al., 1988; Peng et al., 1999; Sasaki et al., 2002; Eshed and Lippman, 2019). However, crop improvement using conventional crop breeding and modern transgenesis methods, which utilize natural mutations, artificially induced genetic variation, and transgenes, is presumably not keeping pace with the demands of climate change and the increasing human population. This is due to the fact that these methods are time-consuming, labor-intensive procedures and are hindered by public concerns. Fortunately, novel CRISPR-based genome-editing technologies, which have been reported to efficiently induce genetic modification in various crops, exhibit great potential in accelerating germplasm development and crop breeding (Zhou et al., 2014; Zong et al., 2017; Butt et al., 2019; Eshed and Lippman, 2019).

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Many efforts in recent years have been directed toward developing gain-of-function crop plants using targeted base editing methods that generate precise nucleotide changes instead of Indels. As a result, many cytosine base editors, which were engineered using Cas9n-guided cytosine deaminases and the uracil glycosylase inhibitor UGI, have been reported to efficiently induce cytosine (C) to thymine (T), adenine (A), or guanine (G) nucleotide changes at the targeted genomic loci in rice (Li et al., 2017; Lu and Zhu, 2017; Ren et al., 2017, 2018). Meanwhile, a number of adenine base editors, which were constructed using Cas9 variants and the tRNA adenosine deaminase TadA*, have also been developed to enable A-to-G conversion in rice target regions (Hua et al., 2018; Yan et al., 2018; Ren et al., 2019). These techniques, capable of directly manipulating DNA sequences with known effects on gene function, pave the way for next-generation molecular design breeding and crop improvement. However, the functional variations affecting a large number of trait-associated genes in crops have not yet been dissected.

Interestingly, the hyperactive hAID*Δ domain in rBE9 induces point mutations in a broader editing window, even beyond the single-guide RNA (sgRNA)-binding region, compared with APOBEC1-mediated editing tools (Ren et al., 2018). In addition, increasing evidence indicates that using multiple sgRNAs simultaneously with a base editor leads to robust mutagenesis at target sites due to a synergistic effect, presumably because a longer stretch of single-strand DNA allows more progressive action of base editors (Ma et al., 2016). All these findings imply the feasibility of artificially evolving functional proteins *in planta* through base editing with a limited number of sgRNAs against the target region. As a proof of concept, we present here the base-editing-mediated gene evolution (BEMGE) method, a novel crop-breeding procedure that efficiently induces sequence diversification of a target genomic locus through the use of cytidine and adenosine base editors as well as a tiled sgRNA library. As an example, the rice OsALS1 protein was successfully mutated to develop tolerance to the herbicide bispyribac-sodium (BS), and the novel genetic variation was introduced into an elite rice cultivar quickly and simply through precise base editing, allowing for potential agricultural applications in the future.

## RESULTS AND DISCUSSION

### BEMGE Principle and Procedure for Artificial Evolution of Endogenous Genes in Rice Cells

Acetolactate synthase (ALS) is the target of more than 50 commercial herbicides widely used for weed control in fields (Garcia et al., 2017). Discovery of novel *ALS* alleles associated with herbicide resistance is of great value in agricultural applications. In rice, there are three ALS homologs: *OsALS1* (*LOC_Os02g30630*), *OsALS2* (*LOC_Os04g32010*), and *OsALS3* (*LOC_Os04g31960*). Investigation of the transcript levels of the three *OsALS* genes using 284 publicly available transcriptomes from nine tissues (Xia et al., 2017) indicated that *OsALS1* was consistently expressed in all samples, whereas the transcripts of *OsALS2* and *OsALS3* were barely detected (Supplemental Figure 1A). Consistent with these data, only the *OsALS1* transcript was detected in leaves of Kitaake seedlings by RT–PCR analysis (Supplemental Figure 1B). Therefore, we chose the endogenous *OsALS1* gene as the target of artificial evolution to develop novel rice germplasms for herbicide resistance breeding.

A total of 63 sgRNAs, tiled on both the forward and reverse DNA strands, were designed against the 1935-bp open reading frame of intronless *OsALS1* in Kitaake (Supplemental Figure 2). To reduce the potential for gene silencing caused by high copy numbers of sgRNAs in transgenic plants, we divided the sgRNA library into six pools (10–11 sgRNAs each) according to the target region (Figure 1A). Each pool of sgRNAs was individually constructed and then incorporated into the well-studied cytosine base editor rBE9 and adenine base editor rBE14 vectors. rBE9 and rBE14 constructs carrying sgRNAs from the same pool were mixed together with gold particles and delivered into rice callus cells through particle bombardment (Figure 1B). Meanwhile, a mixture of rBE9 and rBE14 constructs carrying the same sgRNAs was introduced into *Agrobacterium* cells as well, and used for *Agrobacterium*-mediated rice transformation (Figure 1B). Regenerated plants from BS selection were genotyped by sequencing the endogenous *OsALS1* gene and *sgRNA* transgenes to identify the evolved OsALS1 variants of interest (Figure 1B).

### BEMGE Enables Rapid Artificial Evolution of *OsALS1* in Rice Cells

To explore the sequence diversification of *OsALS1* caused by BEMGE, which employs both cytosine and adenine base editors, we first cultured transgenic calli generated by *Agrobacterium*-mediated and particle-bombardment-mediated transformation with rBE/sgRNA pool no. 2 on callus induction medium (MSD plates) without BS selection. Genomic DNA was extracted from 600 independent calli obtained by both transformation techniques. Subsequently, the *sgRNA* transgenes and the targeted region of *OsALS1* were PCR amplified, pooled, and subjected to amplicon deep sequencing. As shown in Figure 2A, all 11 sgRNAs (sgRNA12 to sgRNA22) in pool no. 2 were present in rice cells after both transformation techniques (Figure 2A). Deep sequencing of the endogenous *OsALS1* gene revealed that approximately 15.2% and 23.8% of the sequencing reads from *Agrobacterium*- and particle bombardment-transformed calli, respectively, carried at least one base substitution. Single-nucleotide variations (SNVs) were enriched around the region targeted by sgRNA pool no. 2, 303 to 607 bp downstream of the *OsALS1* start codon, and numerous mutations were detected in the flanking regions (266–302 bp and 607–666 bp) and the spaces between sgRNA-binding sites (Figure 2B). This phenomenon likely resulted from the hyperactivity of hAID*Δ and the synergistic effect of multiple sgRNAs. Notably, sgRNAs achieved different editing efficiencies, resulting in mutational hotspots interspersed throughout the target region (Figure 2B). Because the target region of *OsALS1* has a GC content of around 70%, most of the identified nucleotide changes involved C and G nucleotides; there was a preference for C>T and G>A transitions and a minority of the changes were C to A or G substitutions (Figure 2C and Supplemental Figure 3A). Furthermore, we observed a substantial number of other nucleotide changes related to A and T nucleotides (Figure 2C). Combining all the data, we conclude that base editors guided by an sgRNA pool function more robustly compared with editors using a single sgRNA. We also compared SNVs generated by the two

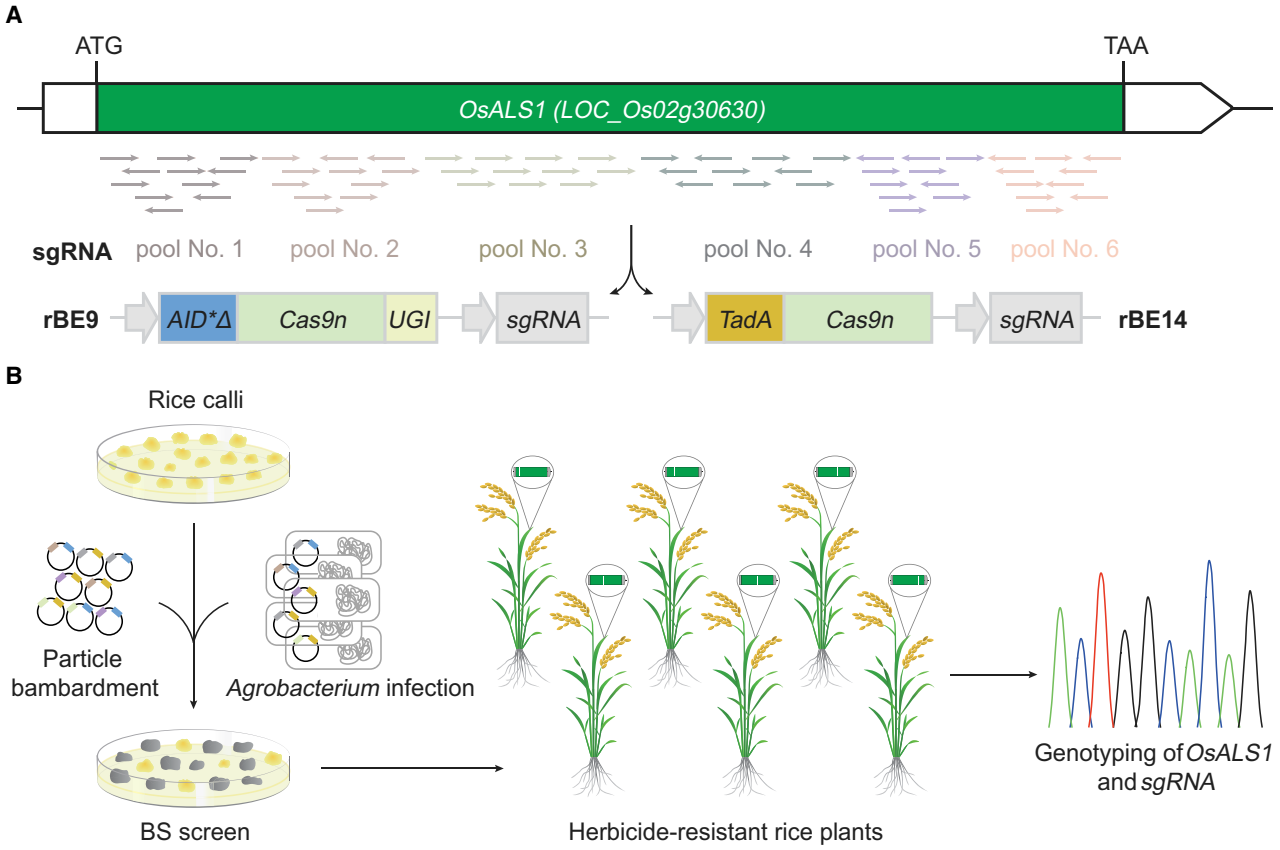



**Figure 1. BEMGE Principle and Procedure for Gene Evolution *In Planta*.**

**(A)** A tiled sgRNA library designed to target the coding sequence of *OsALS1* was divided into six pools of 10–11 sgRNAs according to the target region. Each sgRNA pool was cloned into the rBE9 and rBE14 vectors, then mixed in equal amounts for transformation. The sgRNA pools are shown in different colors.

**(B)** Schematic diagram of genetic screen for developing novel herbicide-tolerant rice germplasms. Six pools of rBE/sgRNA were delivered into rice cells through *Agrobacterium*-mediated and particle-bombardment-mediated transformation. Rice calli were selected on plates with both hygromycin and bispyribac-sodium (BS), and the regenerated plants from BS-tolerant calli were genotyped by Sanger sequencing of the endogenous *OsALS1* gene and sgRNA transgenes.

transformation techniques and found that the distribution of SNVs in rice calli generated by *Agrobacterium*-mediated transformation was highly correlated with the distribution in calli generated by particle bombardment (Figure 2D). In addition, we calculated the number of SNVs in each pair of sequencing reads, which revealed that particle bombardment led to a higher incidence of multiple SNVs in a single pair of sequencing reads as compared with *Agrobacterium*-mediated transformation (Figure 2E).

Subsequently, a total of 100 independent transgenic calli generated by both transformation methods were randomly chosen and genotyped for both the *sgRNA* transgenes and the endogenous *OsALS1* gene using Sanger sequencing. The results showed that *Agrobacterium* generally introduced only one sgRNA into the rice genome, whereas particle bombardment transferred more variable numbers of *sgRNAs* each time (Supplemental Figure 3B). *Agrobacterium*-mediated and particle-bombardment-mediated transformation achieved editing efficiencies of 24% and 28%, respectively. Regarding *Agrobacterium*-mediated transformation, nucleotide changes in *OsALS1* and the corresponding *sgRNA* were commonly detected simultaneously (Supplemental Figure 3C and 3D). Interestingly, mutations were also detected in the absence of the corresponding *sgRNA* (Supplemental Figure 3E). We believe that this is likely caused by the transient expression of *sgRNA* in rice cells in our experiments. On the other hand, particle-bombardment-mediated transformation generated more callus lines in which *OsALS1* alleles carried nucleotide changes at multiple sites (Supplemental Figure 3F and 3G). Combining all the data, we conclude that BEMGE using an sgRNA library is capable of inducing rapid artificial evolution of the endogenous *OsALS1* gene in rice cells and that its efficacy is dependent on the editing efficiency of each sgRNA.

## Screen for Herbicide-Tolerant Rice lines with *OsALS1* Variants Generated by BEMGE

Next, we carried out a screen for evolved *OsALS1* variants conferring BS tolerance. Approximately 3600 independent hygromycin-resistant calli, generated through both *Agrobacterium*-mediated and particle-bombardment-mediated transformation with the six rBE/sgRNA pools mentioned above, were subcultured on MSD plates containing 0.4 μM BS, which was sufficient to inhibit the growth of wild-type rice calli (Figure 3A and

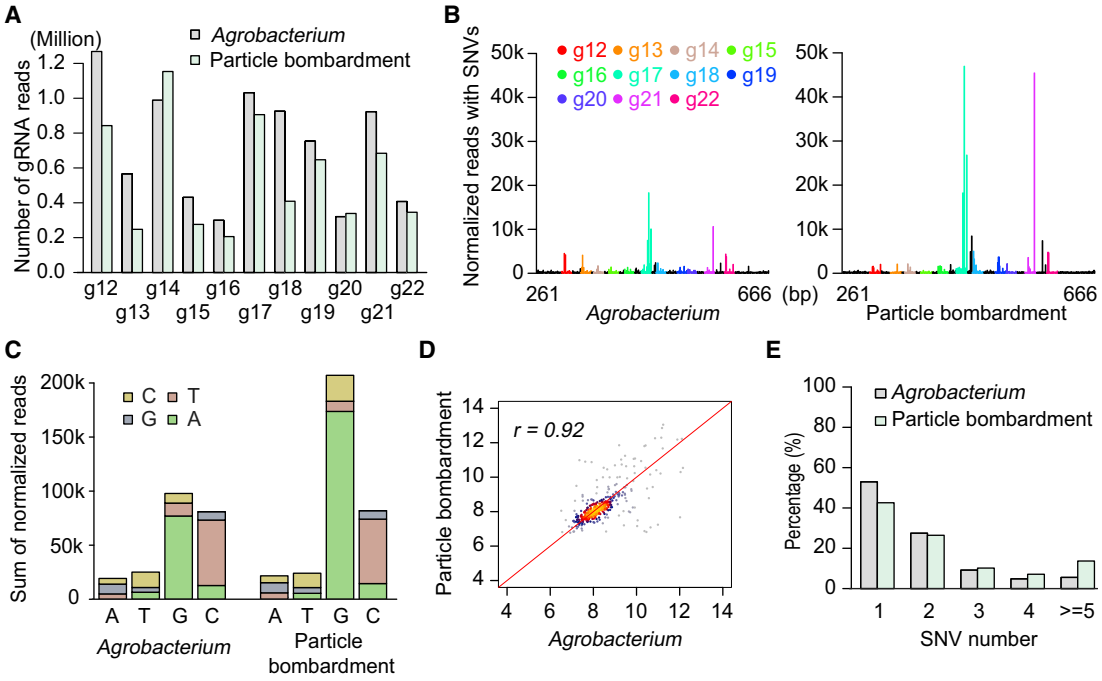



**Figure 2. sgRNA Distribution and Nucleotide Changes in *OsALS1* Gene Introduced by BEMGE in Rice Cells.**

**(A)** Number of sequencing reads for each sgRNA in pool no. 2 (sgRNA12-22) calculated from amplicon deep-sequencing data.

**(B)** Distribution of SNVs at each base position in the target region of *OsALS1* (261–666 bp) corresponding to sgRNA pool no. 2. sgRNA-binding regions are shown in different colors, and unbound regions are in black. Normalized reads were calculated as reads per million.

**(C)** Bar plots of nucleotide transitions and transversions identified in the target region of *OsALS1* (261–666 bp) corresponding to sgRNA pool no. 2. Normalized reads were calculated as reads per million.

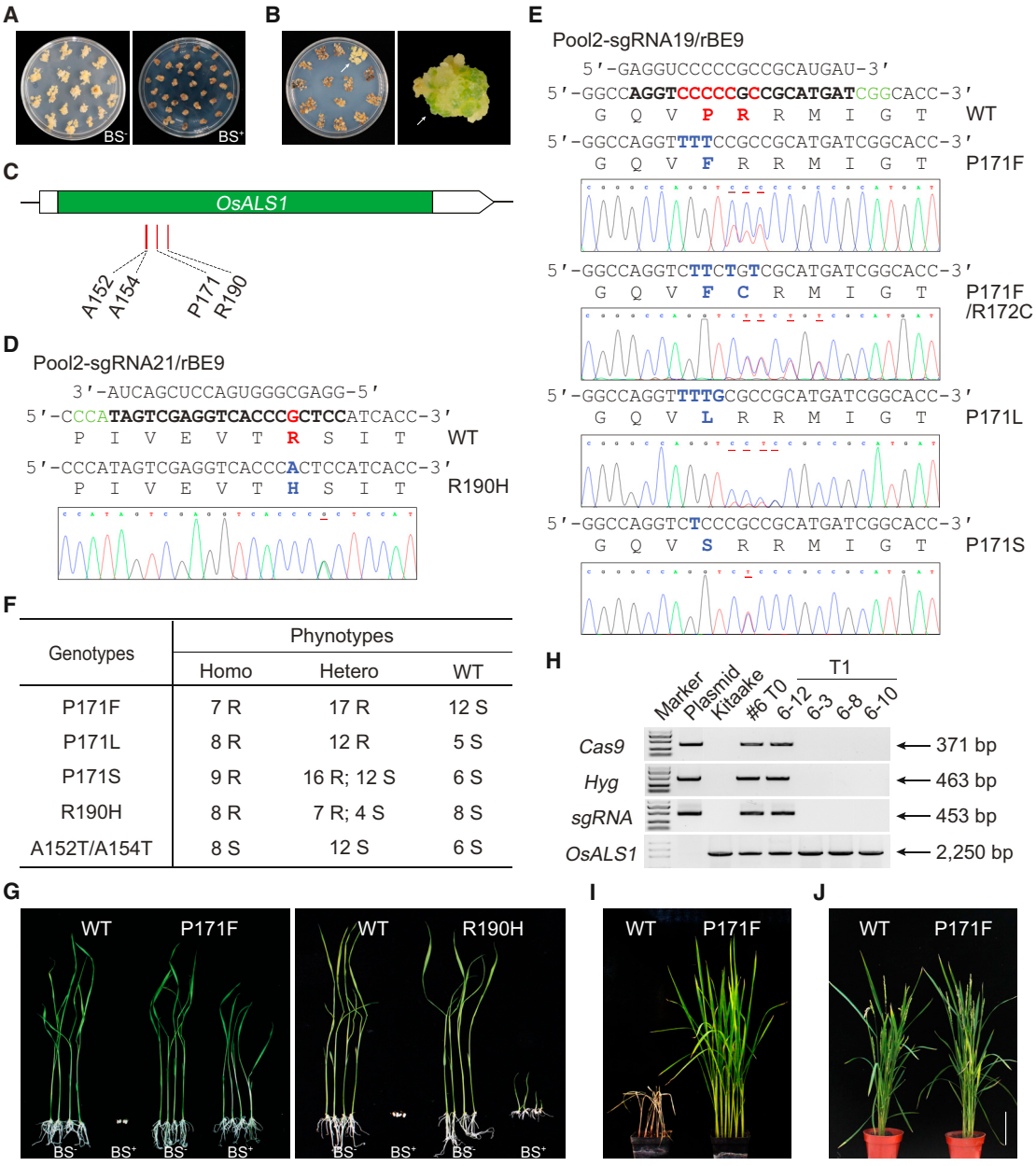
**(D)** A heat scatterplot showing pairwise comparison of $\log_2$ normalized reads with SNVs at each base position in *OsALS1* from calli obtained using the *Agrobacterium* and particle-bombardment techniques. The heat colors indicate density of data points calculated by kernel density estimation. Pearson correlation coefficient (*r*) is shown at the top left corner.

**(E)** Percentage of alleles with different numbers of SNVs calculated from each pair of sequencing reads.

3B). After 4 weeks of continuous selection, 62 and 51 calli from *Agrobacterium*-mediated and particle-bombardment-mediated transformation, respectively, were recovered. Regenerated plants were then genotyped to determine the identity of sgRNAs and potential *OsALS1* variants by PCR amplicon sequencing. We successfully identified six OsALS1 variants (mainly consisting of nucleotide changes at three genomic loci) and the corresponding *sgRNA*s in calli derived from both transformation techniques (Figure 3C–3E and Supplemental Figure 4). Among them, a single G569A base substitution, in heterozygous plants (edited by rBE9/sgRNA21) leads to a predicted R190H substitution in OsALS1 (Figure 3D). Various nucleotide changes including C>T and C>G around the P171 site were induced by rBE9/sgRNA19, resulting in the P171F, P171F/R172C, P171L, and P171S substitutions (Figure 3E). Moreover, A152T/A154T, which was caused by rBE9/sgRNA17, was identified together with the P171F substitution in an evolved OsALS1 variant generated by particle bombardment, indicating that simultaneous editing events occur when this method is utilized (Supplemental Figure 4). Therefore, we further constructed pUbi:rBE9/sgRNA17, pUbi:rBE9/sgRNA19, and pUbi:rBE9/sgRNA21 for the three identified genetic variants and used these vectors to generate *OsALS1*-edited rice plants using *Agrobacterium*-mediated transformation. Mutant plants were further identified by Sanger sequencing.

## BEMGE-Generated *OsALS1* Variants Confer Rice Plants with Stably Herited Tolerance to Herbicide

To examine whether the BS tolerance of rice plants is truly caused by the amino acid substitutions identified in these evolved OsALS1 variants, we carried out phenotypic and genotypic analysis using the progenies of heterozygous T0 plants. Rice seeds of each T1 population were germinated in cylinders containing half-strength Murashige and Skoog medium (1/2 MS) with 0.6 μM BS, which was sufficient to inhibit the hypocotyl and root growth of wild-type rice (Supplemental Figure 5). We observed that both homozygous and heterozygous seeds carrying the P171F substitution exhibited tolerance to BS treatment, characterized by the quite normal growth of seedlings in comparison with the complete growth arrest of wild-type seedlings (Figure 3F and 3G). Interestingly, seeds homozygous for P171L and P171S showed a lower degree of BS tolerance considering the obvious growth arrest of hypocotyls and roots (Supplemental Figure 6A). Notably, all (100%) heterozygous P171L seeds exhibited tolerance to BS, whereas 43% of heterozygous P171S seeds were susceptible to BS (Figure 3F). Therefore, *P171F*, *P171L*, and *P171S* were identified as strong, medium, and weak alleles in terms of BS tolerance, respectively. It is worthy of mention that P171 in rice OsALS1 corresponds to P197 of *Arabidopsis* ALS and P174 of wheat TaALS, which




| Genotypes | Phynotypes | | |
|---|---|---|---|
| | Homo | Hetero | WT |
| P171F | 7 R | 17 R | 12 S |
| P171L | 8 R | 12 R | 5 S |
| P171S | 9 R | 16 R; 12 S | 6 S |
| R190H | 8 R | 7 R; 4 S | 8 S |
| A152T/A154T | 8 S | 12 S | 6 S |

**Figure 3. Base-Editing-Mediated Artificial Evolution of *OsALS1 In Planta* to Breed Novel Herbicide-Tolerant Rice Varieties.**

**(A)** Assay of wild-type Kitaake rice calli for BS tolerance. The MSD plate contained 0.4 μM BS.

**(B)** Herbicide selection of transgenic rice calli on an MSD plate with 0.4 μM BS. BS-tolerant callus line is marked by an arrow.

**(C)** Summary of point mutations in *OsALS1* identified in BS-tolerant calli.

**(D and E)** Evolved OsALS1 variants identified in BS-tolerant plants carrying point mutations at R190 **(D)** and P171 **(E)**. The PAM sequences, the target sequences, the candidate bases in the putative editing window, and the detected nucleotide changes/corresponding amino acids are highlighted in green, bold, red, and blue, respectively. The heterozygous mutations are indicated by the double peaks, which are underlined in the sequencing chromatograms.

**(F)** Genotype–phenotype association analysis of BS-tolerant rice plants in the T1 generation. T1 seeds of independent BS-tolerant lines were germinated in 1/2 MS with 0.6 μM BS. R, resistance; S, susceptible; Homo, homozygous plants; Hetero, heterozygous plants; WT, wild type.

**(G)** Identification of transgene-free OsALS1(P171F) plants in the T1 generation. The presence and absence of individual genes were detected by PCR amplification with gene-specific primers.

**(H)** Germination assay of OsALS1(P171F) and OsALS1(R190H) seeds in cylinders containing 1/2 MS with 0.6 μM BS. Homozygous T2 seeds were used, and the emergence of hypocotyl and roots was considered evidence of tolerance.

**(I)** BS-tolerance assay of OsALS1(P171F) seedlings. Three-week-old seedlings were sprayed with bispyribac-sodium suspension concentrate 48*x* (*x* = 26.25 g a.i./ha) using a spray tower (0.3 MPa pressure) and photographed after 21 days.

**(J)** Morphology of wild-type and *OsALS1*-edited Kitaake plants at the heading stage. Scale bar, 10 cm.

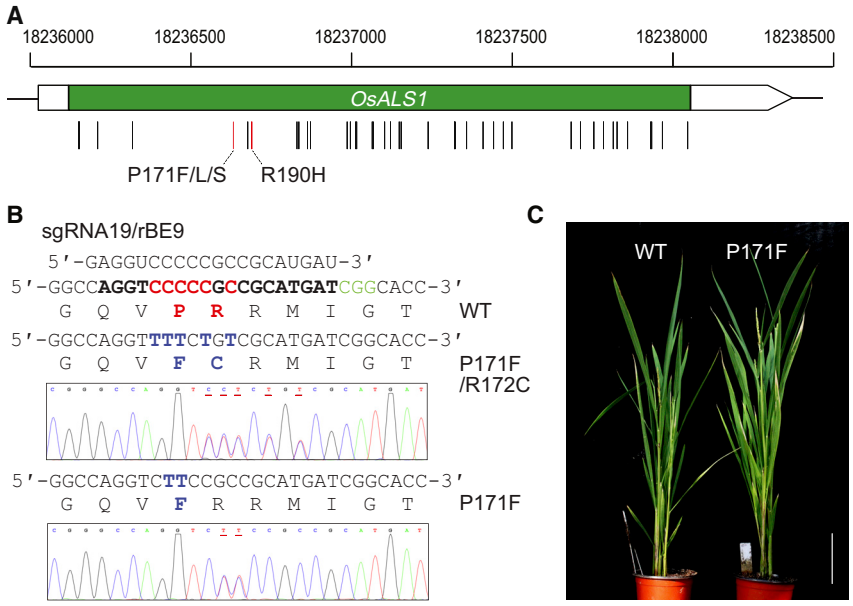



**Figure 4. Rapid Improvement of Commercial Rice Cultivar Nangeng 46 with Herbicide Resistance by Precise Base Editing of *OsALS1*.**

**(A)** Genetic variations of *OsALS1* in 4726 rice accessions (http://ricevarmap.ncpgr.cn/v2/). The naturally occurring SNVs and novel SNVs identified in this study are indicated by bars in black and red, respectively. The location of *OsALS1* on rice chromosome 2 is indicated on the top.

**(B)** Nucleotide changes in the endogenous *OsALS1* gene in Nangeng 46 caused by rBE9/sgRNA19. The PAM sequence, target sequence, candidate bases in the editing window, and detected nucleotide changes/corresponding amino acids are highlighted in green, bold, red, and blue, respectively. The heterozygous mutations are indicated by the double peaks, which are underlined in the sequencing chromatograms.

**(C)** Morphology of wild-type and OsALS1(P171F) plants of Nangeng 46 at the heading stage. Scale bar, 10 cm.

have been reported previously to confer herbicide resistance (Tranel and Wright, 2017; Zhang et al., 2019), verifying that BEMGE is a powerful and valid method to identify functional SNVs of target genes *in vivo*. The R190H substitution converted *OsALS1* into a weak allele for BS tolerance, similar to the P171S substitution. However, A152T/A154T did not contribute to BS tolerance in rice. Combining all the data, we conclude that OsALS1 was successfully evolved to contribute to BS tolerance, even to varying levels, and that all *OsALS1* variants are stably inherited in the next generation.

We also genotyped the T1 progenies of each herbicide-tolerant plant to assess for the presence of transgenes. PCR amplification with primers corresponding to the *Cas9*, *sgRNA*, and *Hyg* genes was carried out. The results showed that genetic segregation resulted in the loss of transgenes in some T1 plants (Figure 3H; Supplemental Figure 6B and 6C). Subsequently, transgene-free rice seedlings homozygous for the strong allele *P171F* at the three-leaf stage were sprayed with BS at a dose of 1260 g a.i./ha (active ingredient per hectare) using wild-type plants as a control. After 3 weeks, all wild-type rice seedlings died whereas P171F plants were healthy and exhibited strong tolerance to BS (Figure 3I). In addition, the P171F plants were morphologically indistinguishable from the wild type when grown under natural light conditions in the greenhouse (Figure 3J), suggesting that the P171F substitution in OsALS1 does not cause a growth penalty.

## Rapid Improvement of Elite Rice Cultivars via Precise Editing of the SNP Identified by Artificial Evolution of *OsALS1*

Analysis of the genetic diversity of *OsALS1* gene in 4726 resequenced rice accessions using RiceVarMap v2.0 (Zhao et al., 2015) revealed that P171 and R190 in OsALS1 have never been targeted by natural or human selection during rice domestication (Figure 4A). Given that the strong allele P171F rendered the rice plant tolerant at a high level to BS without fitness costs, this line represents a novel anti-herbicide rice germplasm with potential value in rice improvement. Rather than introducing the *OsALS1*(*P171*) allele from Kitaake into other elite rice cultivars through traditional time-consuming cross-breeding methods, we are more interested in quickly updating acceptor materials through precise base editing of the endogenous OsALS1 homologs. Nangeng 46 is a high-yield and good-quality rice cultivar widely planted in the area of southern Jiangsu Province of China. Given that no DNA variations were found at the P171 site in *OsALS1* (data not shown), we delivered the pUbi:rBE9/sgRNA19 construct into rice calli through *Agrobacterium*-mediated transformation. Three lines were identified with the nucleotide changes of interest after screening of 30 transgenic lines, achieving a frequency of approximately 10% (Figure 4B). Furthermore, two potential off-target loci with high scores in computational off-target site prediction were examined in the three positive lines, revealing that no other mutations occurred in these sites (Supplemental Figure 7). Similar to the wild type, OsALS1-edited Nangeng 46 plants grew normally in the greenhouse (Figure 4C). Hence, the evolved P171F substitution was successfully introduced into a commercial rice cultivar.

In conclusion, our study presents the artificial evolution of the endogenous *OsALS1* gene in rice cells for developing herbicide-tolerant rice germplasms through BEMGE, which employs both cytosine and adenine base editors as well as an sgRNA library. Technically, Cas9-NG- and ScCas9-mediated base editors, which recognize different photospacer adjacent motif (PAM) sequences (Ren et al., 2019; Wang et al., 2020), can be also included in BEMGE. By employing such a strategy, obtaining deeper coverage of the target gene by sgRNAs, and screening of a larger population will theoretically result in more novel alleles. In general, any endogenous gene in any crop related to a trait of interest (e.g., plant height, leaf angle, seed size, seed protein content) can be targeted for saturated mutagenesis *in planta* to identify novel alleles of different strength. Thus, BEMGE is a powerful tool that can be used in breeding programs to accurately identify functional

genetic variants and develop crops with specific traits in the near future.

## METHODS

### Plasmid Construction

For construction of the entry vector for high-throughput cloning of sgRNAs, the ccdB expression cassette in pENTR4 (Invitrogen) was PCR amplified with high-fidelity DNA polymerase Phusion (NEB) and the primer pair ccdB-F1/ccdB-R1, and integrated into *Bsa*I-digested pENTR4:sgRNA8 (Yan et al., 2018) using In-Fusion HD Enzyme Premix (Clontech), resulting in pENTR4:sgRNA10.

The constructs for base editing were generated as reported previously with modifications (Ren et al., 2018; Yan et al., 2018). In brief, the oligos (Supplemental Table 1) corresponding to the identified mutation region of *OsALS1* were chosen and incorporated into pENTR4:sgRNA10, and the sgRNA expression cassettes were then shuttled into pUbi:rBE9 or pUbi:rBE14 through Gateway recombination .

### sgRNA Design and sgRNA Library Assembly for Evolving *OsALS1*

The genomic DNA sequence of *OsALS1* (*LOC_Os02g30630*) was PCR amplified from Kitaake and Nangeng 46 with high-fidelity DNA polymerase Phusion (NEB) and the primer pair OsALS1-F1/OsALS1-R1, and determined by Sanger sequencing. Since *OsALS1* has no introns, the 1935-bp open reading frame sequence was scanned for the presence of an NGG PAM on both the sense and anti-sense strands by the naked eye. Spacers with cytidine or adenine candidates available in the editing window were identified . To cover most of the sections of *OsALS1*, we used the NAG PAM if no appropriate NGG was available. As a result, 63 spacers (Supplemental Table 1) were designed without considering their potential off-target effects.

Approximately 76-mer oligonucleotides 5′-CCC GCG CGC TGT CGC TTG TGT $GN_{18–19}$G TTT TAG AGC TAG AAA TAG CAA GTT AAA ATA AG-3′, in which $GN_{18–19}$ depicts each of the 63 spacer sequences, were individually synthesized (Sangon Biotech) and divided into six groups based on the sgRNA-targeti region of *OsALS1*. Each group of oligos was PCR amplified with the primer pair Array-F1/Array-R1 and the ~152-bp fragment was gel purified. pENTR4:sgRNA10 was digested with *Bsa*I and dephosphorylated by alkaline phosphatase (Fermentas). Both fragments were assembled together using In-Fusion HD Enzyme Premix (Clontech) following the manufacturer's instructions, resulting in six sgRNA pools of *OsALS1*, namely gOsALS1 pool no. 1 to pool no. 6. Furthermore, each sgRNA pool was introduced into both pUbi:rBE9 and pUbi:rBE14 through Gateway cloning by homologous recombination as reported previously (Ren et al., 2018; Yan et al., 2018). The resulting pUbi:rBE9 and pUbi:rBE14 constructs with the same sgRNAs were further mixed together, resulting in six rBE/sgRNA pools for rice transformation.

### High-Throughput Sequencing Analysis of *OsALS1* Mutations in Transgenic Rice Calli

Genomic DNA was extracted from 600 independent rice callus lines, which were generated through *Agrobacterium*- or particle-bombardment-mediated transformation with rBE/sgRNA pool no.2, with a DNeasy Plant Mini Kit (Tiangen). The sgRNA transgene and the target region of *OsALS1* were PCR amplified using I5 high-fidelity DNA polymerase (MCLAB) and specific primers with different barcodes (Supplemental Table 1). Equal amounts of each PCR amplicon were further pooled together and subjected to library construction and deep sequencing with the Illumina NextSeq 500 platform from Mega Genomics (Beijing). Sequencing reads were trimmed with trim_galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with a minimal length of 100 bp and were analyzed using Bowtie2, samtools, and in-house Python programs. Sequenced reads from the target region of *OsALS1* were mapped back using Bowtie2 with mismatches stored in the MD tag. The SNVs at each nucleotide with a minimal Phred score of 20 were counted, and normalized reads were calculated as reads per million sequenced reads.

## SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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## AUTHOR CONTRIBUTIONS

H.Z., X.Z., X.L., C.S., and S.L. designed the research; Y.K., B.R., and F.Y. conducted the experiments; S.L. performed the bioinformatics analysis; H.Z., X.Z., and S.L. supervised the research; H.Z., C.S., and S.L. wrote the original draft; all authors participated in discussion and revision of the manuscript.

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