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Gene replacements and insertions in rice by intron targeting using CRISPR–Cas9

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Sequence-specific nucleases have been exploited to create targeted gene knockouts in various plants[1], but replacing a fragment and even obtaining gene insertions at specific loci in plant genomes remain a serious challenge. Here, we report efficient intron-mediated site-specific gene replacement and insertion approaches that generate mutations using the non-homologous end joining (NHEJ) pathway using the clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9) system. Using a pair of single guide RNAs (sgRNAs) targeting adjacent introns and a donor DNA template including the same pair of sgRNA sites, we achieved gene replacements in the rice endogenous gene 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) at a frequency of 2.0%. We also obtained targeted gene insertions at a frequency of 2.2% using a sgRNA targeting one intron and a donor DNA template including the same sgRNA site. Rice plants harbouring the *OsEPSPS* gene with the intended substitutions were glyphosate-resistant. Furthermore, the site-specific gene replacements and insertions were faithfully transmitted to the next generation. These newly developed approaches can be generally used to replace targeted gene fragments and to insert exogenous DNA sequences into specific genomic sites in rice and other plants.

The type II prokaryotic CRISPR–Cas9 system has been used to generate targeted DNA double-strand breaks (DSBs)[2–4], which stimulate DNA repair by two main mechanisms: non-homologous end joining (NHEJ) and homologous recombination[5]. NHEJ is the dominant process; in it the broken DNA ends are simply rejoined[6]. However, this process is error prone, creating small insertions and/or deletions (indels) at the break, and has been exploited to create targeted gene knockouts in multiple cell types and organisms including plants[7,8]. Homologous recombination is a less frequent but high-fidelity process, in which targeted gene replacements and insertions can be created using a homologous donor DNA[9,10]. These changes have been of great value for functional genomics studies in plants and may help create agronomically valuable traits. However, generating precise modifications, such as point mutations, gene replacements and gene knock-ins by homologous recombination remains a serious challenge[11]. Only a handful of cases have been reported in which endogenous plant genes were accurately modified by homologous recombination, and even the success in these cases generally relied on the use of specifically designed selection schemes[11–19]. There is a pressing need for simple, universal and efficient methods for replacing or knocking-in DNA fragments to any targeted genes. Since NHEJ is the dominant repair process and minor alterations of intronic sequence may not affect alternative splicing and expression level of the target gene, we chose to modify introns with Cas9 to generate the same results as achieved by homologous recombination. Here, we describe intron-targeting methods using the NHEJ pathway to generate gene replacements and insertions that create glyphosate-resistant rice plants.

The enzyme EPSPS has a motif conserved in all plants and most bacterial EPSPS enzymes[20]. This motif is crucial for binding phosphoenolpyruvate (PEP) or its competitive inhibitor glyphosate, the active ingredient in Roundup. A natural double amino acid substitution (T102I + P106S (TIPS)) in the conserved motif leads to resistance to glyphosate in goosegrass[21]. To date, no spontaneous or induced TIPS double mutations have been reported in cultivated crops, probably because of the low probability of two simultaneous nucleotide substitutions. Hence we set out to obtain TIPS double amino acid substitutions of the endogenous *OsEPSPS* gene, generating glyphosate-resistant rice plants.

To replace the endogenous exon 2 with a new exon containing the TIPS substitutions, we designed several sgRNAs, targeting introns 1 and 2 of *OsEPSPS* (Fig. 1a and Supplementary Fig. 1a). The recognition sites of sgRNAs were all distant from the existing splicing donor and acceptor sites. The activities of the resulting sgRNAs were first checked in rice protoplasts by polymerase chain reaction (PCR) restriction enzyme digestion (PCR/RE) assays (Supplementary Fig. 1b and Supplementary Tables 1 and 2). The sgRNAs that target the C3 site in intron 1 and the C5 site in intron 2 were chosen on account of their high activity in protoplasts. We next predicted potential off-target sites for the sgRNAs targeting C3 and C5 in the rice genome using Cas-OFFinder[22]. The potential off-target sites to C3 have at least four nucleotide mismatches and those to C5 have at least three nucleotide mismatches. By the PCR/RE assay, we examined if mutations might occur in six and five of the most likely off-target sites of C3 and C5 in rice protoplasts, respectively. No off-target mutations were detected at any of these 11 sites (Supplementary Table 3), suggesting that C3 and C5 sgRNAs are specific for their respective recognition sites.

For rice transformation, we integrated C3 sgRNA (driven by the OsU3 promoter) and C5 sgRNA (driven by the TaU3 promoter) into the pHUN411 vector[23] carrying *hygromycin B phosphotransferase* (*hpt*) and *Cas9* expression cassettes, resulting in the construct pHUN411–C3C5 (Supplementary Fig. 2a,b and Methods). Concomitantly, we prepared a donor plasmid TB–TIPS–E2 consisting of three parts: a C3 target site including the protospacer-adjacent motif (PAM), the region spanning the genomic sequence between the C3 and C5 target sites and a C5 target site including the PAM (Supplementary Fig. 3). The sequence of exon 2 in the donor plasmid contained three nucleotide substitutions (C518T,

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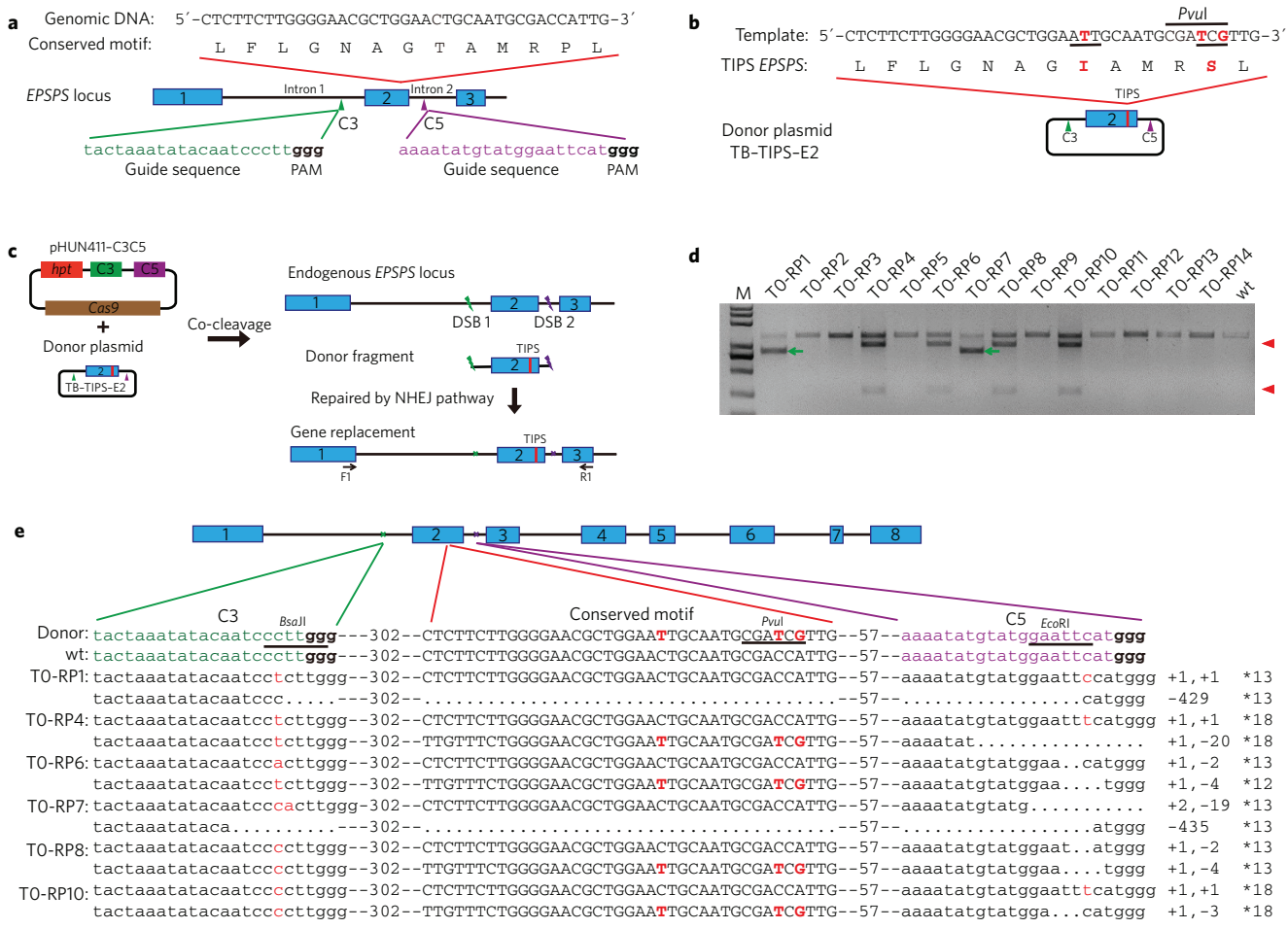

Figure 1 | Intron-targeted site-specific gene replacement. a, The target sequences in *OsEPSPS*. **b**, Scheme of the donor plasmid. The template spans the genomic sequence between the C3 and C5 target sites. **c**, Scheme of the strategy for site-specific gene replacement. The primers F1 and R1 are for detecting gene replacements. **d**, PCR/RE assays for 14 T_0 transgenic plants. The bands marked by red arrowheads indicate TIPS substitutions. The bands marked by green horizontal arrows indicate exon 2 deletion mutations. RP means mutants including mutations at C3 and C5 sites, deletions and TIPS substitutions obtained through gene replacement strategy. M, DNA marker; wt, wild type. **e**, Sequencing results of gene replacement and deletion plants. Primer F2 was used for sequencing. On the right, minus (-) and plus (+) signs indicate the number of nucleotides deleted and inserted at the C3 and C5 sites, respectively. The asterisk indicates the numbers of independent clones sequenced.

C529T and A531G) (Fig. 1b). The first two nucleotide substitutions provide the TIPS amino acid substitutions, and should confer resistance to glyphosate, whereas the substitution A531G, a synonymous mutation, is designed to create a *Pvu*I restriction enzyme site that can be used to detect gene replacement events (Fig. 1b).

The targeting vector pHUN411–C3C5 and the donor vector TB–TIPS–E2 were co-transformed into rice callus cells by particle bombardment. We reasoned that simultaneous cleavages of the sgRNA sites would result in the TIPS E2 fragment between the two sgRNA sites in the donor plasmid replacing the genomic region between the two sgRNA sites via the NHEJ pathway (Fig. 1c). Rice plantlets were regenerated from hygromycin-resistant calli after 10–12-week selection on 50 μg ml^{-1} hygromycin (the most efficient selectable marker for rice transformation[24]). Sequences containing the C3 target site, exon 2 and C5 target site were amplified from the genomic DNA of these transgenic seedlings and analysed individually by PCR/RE assays (Fig. 1d, Supplementary Fig. 4a,b and Supplementary Table 2). After *Pvu*I digestion, we identified eight gene replacement events in 390 T_0 transgenic lines (2.0%) (Table 1). The two predicted bands (1,002 and 276 bp) were recovered from plants T0-RP4, T0-RP6, T0-RP8 and T0-RP10 (Fig. 1d). Sequencing confirmed the presence of the C518T, C529T and A531G substitutions and indels in the C3 and C5 sites in these plants (Fig. 1e).

The targeted mutagenesis efficiency of sgRNA C3 was 82.8% (323/390) and that of sgRNA C5 was 80.2% (313/390) (Table 1). Most T_0 mutants were homozygous at C3, C5 or both sites (Supplementary Fig. 4c). Moreover, the frequencies of genomic fragment deletions, genomic fragment inversions, donor inversions and mutated exon 2 insertion at DSB1 induced by the two sgRNAs were 11.3, 1.8, 1.3 and 0.2%, respectively (Table 1 and Supplementary Fig. 4d,e). These results indicate that exon replacement can be achieved in an endogenous gene via the NHEJ pathway using pairs of sgRNAs targeting sites in the introns adjacent to the targeted exon.

Targeted gene insertion allows one to create clusters of genes that segregate as single loci. This procedure is commonly used to eliminate position effects and silencing of transgenes, so simplifying and speeding up the breeding process. We previously reported generating knock-ins at DSBs created by TALEN via the NHEJ pathway using ssDNA[25]. However, the inserted fragments were relatively small (<100 bp). Here we designed an intron-mediated targeted fragment (e.g. 1.6 kb) insertion approach via the NHEJ pathway using CRISPR–Cas9. To achieve targeted gene insertions, we used a single sgRNA, namely C3 (pHUN411-C3), targeting intron 1 of *OsEPSPS*. We designed a donor plasmid, TB–E2–TIPS–E8. It started from the C3 sgRNA site and extended up to the last base of intron 1, the complete cDNA of *OsEPSPS* except for the exon 1,

Table 1 | Summary of mutations generated by gene replacement and insertion through intron targeting using the CRISPR–Cas9 system

Approach	DSB	Efficiency*							
		C3 activity	C5 activity	Deletion of exon 2	Inversion		Insertion		TIPS substitutions†
					Endogenous exon 2	Donor	At DSB1	At DSB2	
Gene replacement	2	323/390 (82.8%)	313/390 (80.2%)	44/390 (11.3%)	7/390 (1.8%)	5/390 (1.3%)	1/390 (0.2%)	0	8/390 (2.0%)
Gene insertion	1	285/324 (88.0%)	-	-	-		7/324 (2.2%)	-	7/324 (2.2%)

DSB, double-stranded break; –, not applicable. *Based on the number of T_0 plants carrying the observed mutations over the total number of T_0 plants analysed. †Obtained through gene replacement or gene insertion.

with the 3′-UTR fused to another C3 sgRNA targeting site including the PAM (Fig. 2a and Supplementary Fig. 5). The exon 2 designed in the donor plasmid contained the three nucleotide substitutions (C518T, C529T and A531G) as in the above experiments.

We co-transformed the targeting vector pHUN411–C3 and the donor vector TB–E2–TIPS–E8 into rice callus cells. Concurrent cleavage of the sgRNA sites in both the donor plasmid and the *OsEPSPS* gene should result in insertion of the E2–TIPS–E8 fragment in the donor template into the chromosomal C3 site via the NHEJ pathway (Fig. 2a). Sequences containing the C3 target site and exon 2 were amplified from the genomic DNA of the resulting transgenic seedlings and analysed by PCR/RE assays (Fig. 2b, Supplementary Fig. 6 and Supplementary Table 2). After *Pvu*I digestion, we identified seven E2–TIPS–E8 inserted into the targeted chromosomal site events in 324 T_0 transgenic lines (2.2%) (Table 1). The two predicted cut bands (1,002 and 195 bp) were recovered in T0-IP8 and T0-IP11 (Fig. 2b). PCR analysis and sequencing of these two seedlings confirmed that the entire sequence (1.6 kb) between the two C3 sites in the donor had been inserted into the targeted chromosomal C3 site (Fig. 2c,d). PCR and junction sequencing showed that the insertion was accompanied by indels because of NHEJ at the 5′- and 3′-junctions (Fig. 2e,f and Supplementary Table 2). These results indicate that fragment-targeted insertion can be achieved via the NHEJ pathway using the CRISPR–Cas9 system. Furthermore, we investigated whether off-target mutations might happen in 15 mutants, including eight TIPS mutants from the exon replacement experiment and seven TIPS mutants from the insertion experiment. The 11 most likely off-target sites of C3 or C5 sgRNAs (see above) were examined by PCR/RE. No mutations were detected in any of these 11 sites for any of the 15 TIPS mutants (Supplementary Table 3). This further suggests that the sgRNAs used in this experiment are specific in inducing mutations.

There were minor modifications in the introns, which might generate alternative 5′ or 3′ splicing site and affect mRNA splicing. So it is necessary and important to examine the mRNA splicing of *EPSPS* in the mutants. We extracted total RNA samples from the TIPS substitution mutants (T0-RP4, T0-RP6, T0-RP8, T0-RP10, T0-IP8 and T0-IP11) and wild type, followed by amplifying the opening reading frames of *EPSPS* (1,548 bp) using RT–PCR. A single amplicon (~1.5 kb) was obtained for both wild type and each of the six mutants (Fig. 3a). Sequencing these amplicons confirmed that no splicing variant appeared and the intended substitutions C518T, C529T and A531G were present (Supplementary Fig. 7), suggesting that sequence alterations at C3, C5 and the TIPS substitution mutations in the six mutants do not affect pre-mRNA splicing of *EPSPS*. Quantitative RT–PCR (qRT–PCR) analysis showed that the expression level of *EPSPS* did not vary between the wild type and six mutants (Fig. 3b and Supplementary Table 2), demonstrating that our gene replacement and insertion approaches do not compromise the expression level of the targeted gene. However, it is well known that pre-mRNA splicing in eukaryotic organisms is regulated in a complex manner[26,27]. Therefore, it is worthwhile examining potential effects of the intronic sequence changes on *EPSPS* pre-mRNA splicing with RNA samples extracted from different rice organs at different developmental stages in further research.

To investigate whether the indels and TIPS mutations could be transmitted to the next generation, one T_0 plant (T0-RP3) with homozygous mutations at both C3 and C5, one T_0 plant (T0-IP1) with homozygous mutation at C3, four T_0 replacement plants (T0-RP4, T0-RP6, T0-RP8 and T0-RP10) with TIPS point mutations, two T_0 plants (T0-RP1 and T0-RP7) with exon 2 deletion and two T_0 insertion plants (T0-IP8 and T0-IP11) with TIPS point mutations were selfed and their T_1 progenies were analysed. Ninety-six T_1 individuals from each T_0 plant were analysed by PCR/RE and sequencing (Supplementary Fig. 8a,b). The examined T_1 plants of T0-RP3 and T0-IP1 were all homozygous mutations as their respective parents (Supplementary Table 4), and their T_1 seed-setting rates (83.5–84.2%) developed similarly to the wild-type plant (84.7%) (Supplementary Fig. 9). No T_1 plants homozygous for deleted exon 2 were detected in the progenies of T0-RP1 or T0-RP7, although plants heterozygous for deleted exon 2 were commonly found (Supplementary Table 5). The lack of T_1 plants homozygous for exon 2 deletion was not surprising, because EPSPS is critical for aromatic amino acid biosynthesis, and homozygous *OsEPSPS* mutants could not be obtained[28]. Furthermore, among the T_1 progenies of T0-RP4, T0-RP6, T0-RP8, T0-RP10, T0-IP8 or T0-IP11, no plants were found to be homozygous for TIPS point mutations (Supplementary Table 5). For example, among the 96 tested T_1 seedlings of T0-RP4, 55 T_1 progenies were heterozygous for TIPS point mutations and the remaining 41 seedlings were wild type. All these 96 tested plants carried the homozygous mutations in the targeting sites C3 (+1 bp, +1 bp) and C5 (+1 bp, −20 bp), which were identical to T0-RP4 (Supplementary Table 5). These and the sequencing results indicated that TIPS mutations in the T_0 population were faithfully transmitted to the next generation, although only in the heterozygous TIPS state (Supplementary Table 5). We observed that there were many underdeveloped T_1 seeds with no or abnormal embryos in the panicles of six T_0 TIPS mutants (T0-RP4, T0-RP6, T0-RP8, T0-RP10, T0-IP8, T0-IP11), as well as in the two deletion mutants (T0-RP1 or T0-RP7) (data not shown). In addition, we observed that the seed-setting rates of the six T_0 TIPS mutants (41.6–47.1%), similarly to the seed-setting rates of the two deletion mutants (41.8–42.8%), were substantially lower than that of the wild-type control (84.7%) (Supplementary Fig. 9). These results suggested that homozygous TIPS substitutions in the EPSPS protein may be lethal in rice.

To examine the possibility of achieving targeted gene replacements and insertions without foreign DNA being present in the T_1 generation rice genome, we designed PCR primers specific for *hpt* and *Cas9* gene in the plasmid pHUN411. PCR assays failed to detect the *hpt* and *Cas9* genes in 3 of 47 (6.4%) T_1 plants derived from T0-RP4 (Supplementary Table 6). This indicates that Cas9-free

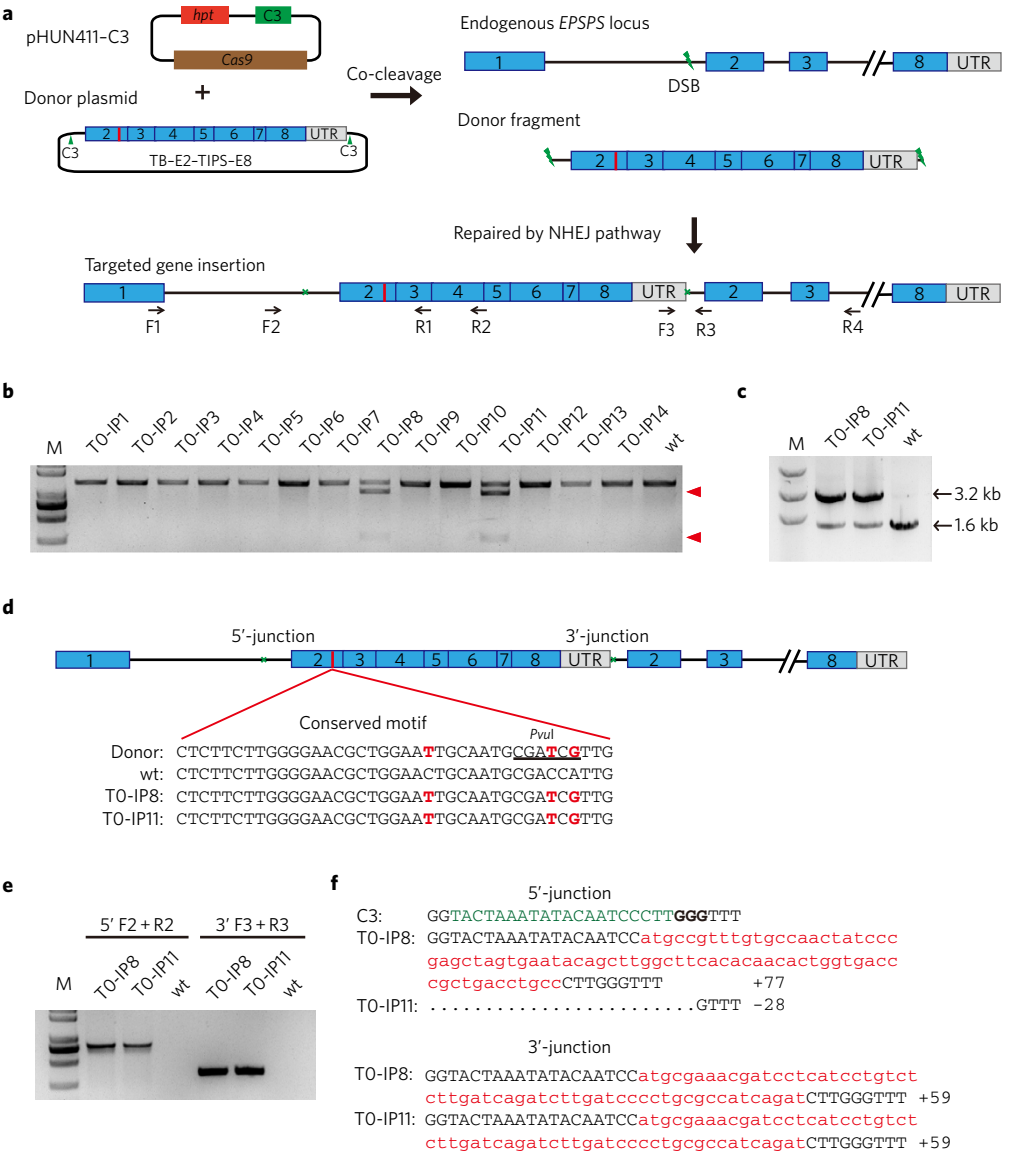

Figure 2 | Intron-mediated targeted gene insertions. a, Scheme of the strategy for gene insertions. **b**, Results of PCR/RE assays for detecting targeted gene insertions. The PCR fragments amplified using primers F1 and R1 were digested with *Pvu*I. The bands marked by red arrowheads indicate insertions. IP means mutants including mutations at C3 site and TIPS substitutions obtained by gene insertion strategy. **c**, PCR analysis of the entire sequence inserted using primers F1 and R4. **d**, Targeted gene insertion and TIPS substitutions identified by sequencing. **e**, PCR analysis of the 5′- and 3′-junctions. **f**, Sequencing results of 5′- and 3′-junctions.

plants carrying only the desired gene replacements and gene insertions can be obtained by segregation.

We assessed the glyphosate resistance of the T_0 and T_1 plants carrying the TIPS substitutions. After two weeks of growth on plates with 1 mg l^{-1} glyphosate, mutant plants T0-RP4 and T0-IP8 grew well and had normal phenotypes, but the wild-type plants were dead (Fig. 3c). To test the herbicide resistance of the T_1 generation of the TIPS mutants in the greenhouse, a 400× dilution of the glyphosate solution Roundup (with 41.0% of isopropylamine glyphosate salt as active ingredient, Monsanto) was sprayed onto 12-day-old seedlings. Eight days later all the heterozygous TIPS mutant plants segregated from lines T0-RP4 and T0-IP8 had normal phenotypes with no symptoms of damage but the wild type plants were dwarfs with withered leaves (Fig. 3d). These results show that the TIPS substitutions in the conserved EPSPS motif conferred glyphosate resistance. As EPSPS is critical for aromatic amino acid biosynthesis, it is highly likely that TIPS mutation in the conserved domain may influence the catalytic activity of EPSPS, and the plants carrying homozygous TIPS mutation could not survive, as has been found by the analysis of the homozygous knockout mutant of OsEPSPS[28]. However, the strategy of changing one of the genomes of the endogenous *EPSPS* to confer glyphosate resistance might be useful in polyploid plants, such as hexaploid wheat, tetraploid cotton and oilseed rape.

To test the applicability of our approach for other genomic sites, we constructed the vector pHUN411–D1D2 containing two active sgRNAs (D1 and D2) targeting the promoter region and intron 1 of *OsDEP1*, respectively, and prepared a donor plasmid TB–D1GFPD2 with the coding region of green fluorescent protein (GFP) flanking by the D1 and D2 sgRNA sites (Supplementary Fig. 10a). After co-transforming the two vectors into rice protoplasts, successful replacement of the fragment between the D1 and D2 sites in *OsDEP1* with *GFP* coding sequence via the NHEJ pathway was verified by PCR and junction sequencing analysis at the protoplast level (Supplementary Fig. 10b). This demonstrates that our approach is generally applicable.

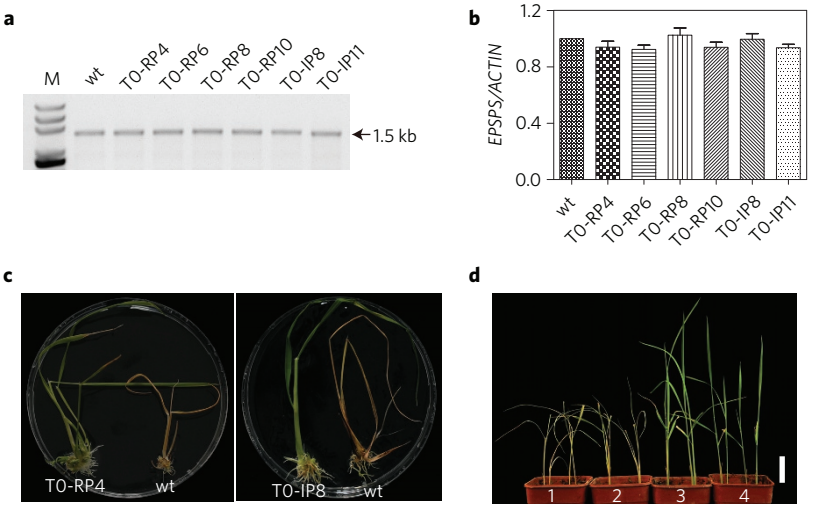

Figure 3 | EPSPS with TIPS substitutions confers resistance to glyphosate. a, Examination of potential alternative splicing of *EPSPS* in the plants with TIPS substitution by RT-PCR. **b**, Relative expression levels of *EPSPS* in leaves detected by qRT-PCR, with the data normalized to actin levels ($n = 3$). **c**, Phenotypes of the T_0 plants with TIPS substitutions in the petri dish with 1 mg l^{-1} glyphosate. **d**, Results of glyphosate resistance test of T_1 progenies. (1) Progenies of T0-RP1 with exon 2 deletion. (2) Wild type. (3) Progenies of T0-RP4 with TIPS substitutions. (4) Progenies of T0-IP8 with TIPS substitutions. Scale bar, 4 cm.

In conclusion, we have developed a general intron targeting gene replacement and insertion strategy via the NHEJ pathway using CRISPR–Cas9. This strategy was successfully used to introduce TIPS amino acid substitutions to rice EPSPS, which conferred resistance to glyphosate. This approach could serve as an alternative to homologous recombination mediated gene replacements and insertions in plants. Our findings extend the range of applications of CRISPR–Cas9, and may speed up the study of gene function and the improvement of crop traits.

Methods

Construction of Cas9 and sgRNA expression vectors. We used the CRISPR–Cas9 system as previously described[23]. Pairs of oligonucleotides (oligos) including targeting sequences (Supplementary Table 1) were synthesized as primers, annealed and cloned into *Bsa*I-digested pHUN411 vector. To integrate these two sgRNAs (targeting C3 and C5) driven by different Pol III promoters in a single vector, the wheat U3 promoter was tested to compare with rice U3 promoter targeting the same site (C6 in exon 2 of *EPSPS*). TaU3 promoter combined with Cas9 induced targeted gene mutations in rice protoplasts as efficiently as OsU3 promoter (Supplementary Fig. 2a). PCR fragment containing two sgRNAs was purified and inserted into *Bsa*I-digested pHUN411 by the Golden Gate method.

Rice protoplast transformation. Rice protoplasts were transformed as previously described[29]. The average transformation efficiency was 70–80%. Transformation was carried out with aliquots of 20 μg of sgRNA plasmid.

The PCR/RE assay in rice protoplasts. Genomic DNA was extracted from pooled protoplasts transformed with the sgRNA plasmid, and the genotyping primers were used to amplify fragments containing the sgRNA target. The PCR/RE assay was as previously described[30]. Mutation frequencies (indels (%)) in protoplasts were calculated by measuring band intensities with UVP VisionWorks LS Image Acquisition Analysis Software 7.0[29].

Construction of donor vectors. Primers ED-1F and ED-1R (Supplementary Table 2) were used to amplify E2–EPSPS gDNA from rice variety Nipponbare. The resulting PCR product was cloned into the pEASY-Blunt cloning vector (TransGen Biotech). With this plasmid as the template, site-directed mutagenesis was performed to obtain the donor vector TB–TIPS–E2 containing the three point mutations (C518T, C529T and A531G) (Fig. 1b).

To generate the donor plasmid for targeted gene insertion, the DNA fragment E2–TIPS was amplified from the donor plasmid TB–TIPS–E2. The E3-UTR DNA fragment fused with a C3 sgRNA was amplified from the cDNA of rice. Then E2–TIPS–E8 was amplified by overlap PCR and the fragment cloned into pEASY-Blunt cloning vector to generate TB–E2–TIPS–E8 (used in Fig. 2a). Primers used are listed in Supplementary Table 2.

Biolistic transformation of rice. The targeting vector and donor vector were extracted using a wizard plus midipreps kit (Promega). The final concentration of vectors should be more than 1 μg μl^{-1}. In the gene replacement and insertion strategy, the targeting vector and donor vector were mixed in 1:1 molar ratio before bombardment. One-month-old embryogenic calli (60–80 pieces) of rice cultivar Nipponbare were bombarded using the previously reported protocol[31]. Biolistic transformation was performed using a PDS1000/He particle bombardment system with a target distance of 6.0 cm from the stopping plate at helium pressure 1,100 psi. Hygromycin (50 μg ml^{-1}) was present in all subsequent tissue culture procedures. After 10–12 weeks, T_0 transgenic plants were obtained.

Screening of intron-targeted TIPS substitutions. Genomic DNA from individual rice plants was extracted using a high-throughput Automation Workstation Biomek FX (Beckman Coulter) with a magnetic bead-based DNA extraction kit (GeneOn Biotech). *Pvu*I was used to detect the TIPS substitutions, and *Bsa*JI and *Eco*RI were used to detect the activities of sgRNAs C3 and C5, respectively.

Genotyping. The PCR products of mutants were purified, and cloned into pEASY-Blunt cloning vector. Bacterial colony PCR was conducted and positive clones were picked for sequencing to identify mutations. For the T0-RP1 and T0-RP7 mutants, the positive clones were divided into two groups based on their amplification size. An equal number of positive clones carrying the large or small band were sequenced.

RNA extraction and quantitative RT–PCR. Total RNA was extracted from plant tissues using TRIzol reagent (Life Technologies) and treated with RNase-free DNase I (Invitrogen). Two micrograms of RNA were reverse transcribed using oligo (dT) primer and AMV reverse transcriptase (Promega). Quantitative PCR experiments were performed. Each qRT–PCR assay was replicated at least three times with three independent RNA preparations. The rice *actin1* gene was used as internal control. Primers used are listed in Supplementary Table 2.

Glyphosate test in T_0 and T_1 generation plants with TIPS mutations. T_0 plantlets with TIPS mutations and the wild type were transferred to a plate containing rooting medium with 1 mg l^{-1} glyphosate and cultured in a growth chamber (28 °C, 16-h light/8-h dark). T_1 rice seedlings were grown on 1/2 MS (Murashige and Skoog) medium in a growth chamber (28 °C, 16-h light/8-h dark). After 8 days, the plantlets with TIPS mutants segregated from lines T0-RP4 and T0-IP8 and the wild type were transferred to greenhouse. Glyphosate solution Roundup (41.0% of isopropylamine glyphosate salt as active ingredient) was sprayed at 400× dilution on 12-day-old seedlings T_1 TIPS mutations.

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Author contributions

C.X.G., J.Y.L. and J.L. designed the experiments; J.L., X.B.M., Y.Z., K.L.C., H.W.Z. and J.X.L. performed the experiments; and C.X.G., J.Y.L. and J.L. wrote the manuscript.

Additional information

Supplementary information is available for this paper. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to J.L. and C.G.

Competing interests

The authors have filed a patent application based on the results reported in this paper.