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Generating broad-spectrum tolerance to ALS-inhibiting herbicides in rice by base editing

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Herbicide-tolerant rice varieties generated by genome editing are highly desirable for weed control. We have used a cytosine base editor to create a series of missense mutations in the P171 and/or G628 codons of the *acetolactate synthase* (*ALS*) gene to confer herbicide tolerance in rice. The four different missense mutations in the P171 codon, P171S, P171A, P171Y and P171F, exhibited different patterns of tolerance towards five representative herbicides from five chemical families of ALS inhibitors. For example, P171S and P171A had lower levels of tolerance than P171Y and P171F to bispyribac but not to the other herbicides. Interestingly, a novel triple mutant (P171F/G628E/G629S) had the highest tolerance to all five tested herbicides. Field trials showed that both P171F and P171F/G628E/G629S could potentially be used with nicosulfuron. Our work illustrates an effective way of using base editing to generate herbicide tolerance in elite rice varieties.

base editing, herbicide tolerance, rice, acetolactate synthase (ALS)

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INTRODUCTION

Weeds are a major threat to global food production. To tackle this problem, herbicide-tolerant crops have been developed by transgenic and traditional mutation breeding. The commercialization of transgenic cultivars is limited by long and costly regulatory evaluation processes, as well as public concerns. For staple crops such as rice, this has been a major barrier to broader adoption of transgenic technology. Although non-transgenic rice varieties tolerant to herbicides have been developed by traditional breeding (Sudianto et al., 2013), the high cost and complexity of creating herbicide-tolerant point mutations via traditional mutagenesis and backcrossing make it difficult to introduce non-transgenic herbicide-tolerant traits into the hundreds of local elite crop varieties. In recent years, CIRSPR-Cas9-based homologous recombination technology has been used to generate herbicide-tolerant crops, including rice, maize and soybean (Li et al., 2015; Sun et al., 2016; Svitashev et al., 2015). However, the low efficiency of homologous recombination in plants limits the widespread use of this technology.

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Base editing has emerged as an alternative to mutagenesis or homologous recombination, facilitating precise editing of endogenous target genes in a programmable manner without involving double-strand breaks and donor repair templates (Gaudelli et al., 2017; Komor et al., 2016). Cytosine base editing uses cytidine deaminase and Cas9 fusion proteins to alter nucleotides in target DNA *in situ*, generating C:G>T:A base transitions (Komor et al., 2016; Xue et al., 2018). So far point mutations have been introduced into several genes, including *acetolactate synthase* (*ALS*), *acetyl-coenzyme A carboxylase* and *tubulin* genes, to generate herbicide-tolerant plants (Fan et al., 2020; Li et al., 2018; Li et al., 2020; Liu et al., 2020a; Liu et al., 2020b; Zhang et al., 2019). *ALS* encodes a key enzyme in the biosynthesis of branched-chain amino acids, which is the target of a large number of herbicides with dissimilar chemistries, including sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine (TP), pyrimidinyl-thiobenzoates (PTB) and sulfonyl-aminocarbonyl-triazolinone (SCT). Tolerance to ALS-inhibiting herbicides has been generated using cytosine base editing in a number of species, including rice, wheat, maize, *Arabidopsis*, watermelon, tomato, potato, and canola (Chen et al., 2017; Kuang et al., 2020; Li et al., 2020; Shimatani et al., 2017; Tian et al., 2018; Veillet et al., 2019; Wu et al., 2020; Zhang et al., 2019). However, few studies have examined the field application of edited crop lines with herbicide-tolerant point mutations.

Rice is the most important food crop in the world, especially in Asian counties. No-till, direct-sown cropping systems are valued by farmers for their economic and environmental benefits in rice cultivation, but hindered by difficulties of efficient weed control. Moreover, weedy rice, which is a close relative of cultivated rice and a weed, is worsening in the paddy field because of limited chemical management. To address these problems, we used a cytosine base editor to generate a number of rice mutants tolerant to multiple herbicides by targeting different positions of *OsALS*. Paddy trials showed that two mutants, P171F and P171F/G628E/G629S, could potentially be used with nicosulfuron without yield penalty, allowing more effective crop rotations and better weed management for rice farmers.

RESULTS

Development of herbicide-tolerant mutants using a base editor to target OsALS-P171 and/or OsALS-G628

As different families of ALS-inhibiting herbicides bind to different parts of the herbicide binding domain, point mutations in a particular ALS codon confer tolerance to a specific spectrum of ALS-inhibiting herbicides (Garcia et al., 2017). For example, mutations of P197 in *Arabidopsis AtALS* confer tolerance to SU herbicides, while those at G654 confer tolerance to IMI herbicides. Based on the amino acid sequence alignment of OsALS and AtALS, we identified two amino acids suitable for base editing, OsALS-P171 and OsALS-G628, in positions homologous to AtALS-P197 and AtALS-G654 (Figure S1 in Supporting Information), expecting to obtain rice mutants with tolerance to different herbicides. The binary vector pH-BE3 (Jin et al., 2019) was used as backbone to construct base editor plasmids targeting OsALS-P171 and OsALS-G628 separately (Figure 1A and B). We then delivered plasmids pH-BE3-OsALS-P171 and pH-BE3-OsALS-G628 into rice calli via *Agrobacterium*-mediated transformation. Twenty and eight transgenic T_0 plants from OsALS-P171 and OsALS-G628 transformation were selected for genotyping, respectively. Sanger sequencing showed that OsALS-P171 and OsALS-G628 transformation respectively produced seven (35.0%) and four (50.0%) T_0 plants that only harbored base substitutions, and 11 (55.0%) and three (37.5%) T_0 plants that contained indel alleles, while the remaining two (10.0%) and one (12.5%) T_0 plants were wild type (Figure 1C). OsALS-P171-sgRNA transformation generated five different missense mutations P171S, P171F, P171A, P171Y, and P171F/R172C (Figure 1D). Of these, P171S, P171F and P171F/R172C resulted from C to T conversions of two, three and five consecutive C's; while the P171A and P171Y were caused by non-canonical C to G and C to A conversion, respectively. OsALS-G628-sgRNA generated two missense mutations, G629S and G628E/G629S, containing two and three G to A transitions, respectively (Figure 1E).

Since multiple point mutations in the herbicide-tolerant target gene are likely to confer higher-level and broader-spectrum tolerance (Lee et al., 1988; Yu et al., 2015), we used the base editor to target both the OsALS-P171 and OsALS-G628 sites (Figure 1B). The double-site targeting construct pH-BE3-OsALS-P171+G628 was delivered into rice calli via *Agrobacterium*-mediated transformation. Twenty-six transgenic T_0 plants were selected for genotyping. Sanger sequencing showed that five (19.2%) T_0 plants harbored only base substitutions and 16 (61.5%) T_0 plants harbored indel alleles, and the remaining five (19.2%) T_0 plants were wild type (Figure 1C). Four combined missense mutations, P171S/G629S, P171S/G628E/G629S, P171F/G629S, and P171F/G628E/G629S, were obtained from OsALS-P171+G628-sgRNA transformation (Figure 1F).

To see whether the edited alleles were heritable, two indel-harboring lines (P171-#3 and #4) and two base substitution lines (P171-#7 and #10) from the OsALS-P171 construct were examined (Table S1 in Supporting Information). The indel alleles in lines P171-#3 and #4 failed to pass to the T_1 generation, which is not surprising as loss of ALS function is lethal, so that the indel alleles would be lost during segregation; however, all three missense mutations P171F, P171A and P171F/R172C, in homozygous or heterozygous form,

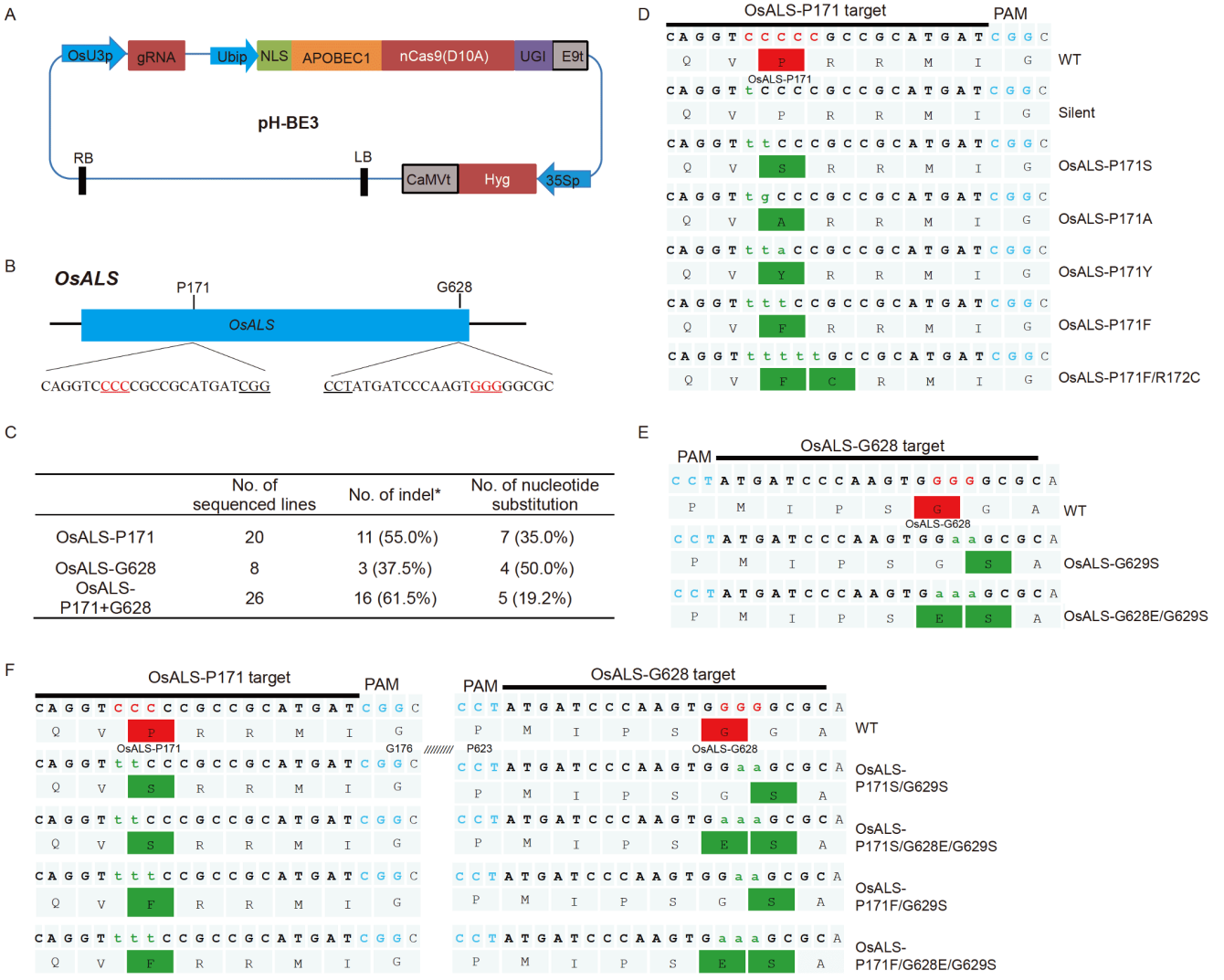

Figure 1 Generation of missense mutations in OsALS-P171 and/or OsALS-G628 in rice by base editing. A, Structure of vector pH-BE3 used for base editing in rice. UGI, uracil DNA glycosylase inhibitor; NLS, nuclear localization signal. B, Sequences of the sgRNAs targeting OsALS-P171 and OsALS-G628. The OsALS-P171 and OsALS-G628 codons are highlighted in red and underlined. The protospacer-adjacent motif (PAM) sequence is underlined. C, Base editing frequencies of OsALS-P171, OsALS-G628 and OsALS-P171+G628 sgRNAs. *indel indicates plants harboring indel alleles. D, Base editing events at the OsALS-P171 site. E, Base editing events at the OsALS-G628 site. F, Combinations of edits generated by simultaneous editing of OsALS-P171 and OsALS-G628. In figures D–F, target sequences, PAM sequences, DNA modifications and editable cytosines or guanosine are indicated in bold, blue, green and red text, respectively; changed amino acid residues, and amino acids that when changed confer herbicide tolerance, are indicated by green and red filled boxes, respectively.

were identified in the next generation. The three missense mutations P171S, P171F, and P171Y in two lines, P171-#7 and #10, were also transmitted to the T_1 generation. Interestingly, the P171-#10 line was heterozygous for the WT and P171F alleles in the T_0 generation, but it produced T_1 offspring carrying P171S alleles. This suggested that the WT allele could be re-edited when passed to the next generation. One indel-harboring line (G628-#6) and one base substitution line (G628-#7) from OsALS-G628 were also examined (Table S1 in Supporting Information). The indel allele was detected in the T_1 generation of line G628-#6, along with the WT allele. We also sprayed T_1 seedlings generated from the OsALS-P171 and OsALS-P171+G628 constructs (P171-#1, #2, #3, #4 and P171+G628-#9, #17) with nicosulfuron or imazapic, and the alleles of the tolerant seedlings were found to contain substitution alleles. These results indicate that the substitutions generating herbicide tolerance are heritable.

Analysis of off-target events

To examine the potential for Cas9-dependent off-target events, we searched likely off-target sites with CRISPR-P (Liu et al., 2017). The top five off-target sites containing a maximum of 4-nt mismatches and their related genomic positions are listed in Table S2 in Supporting Information. For the OsALS-P171 site, the first two potential off-target

sites contained only 1-nt and 2-nt mismatches in the region distal to the PAM, and editing of these two off-targets had been detected in our previous work (Jin et al., 2019). In this study, we also detected off-target edits at off-target sites with both 1- and 2-nt mismatches in the T_1 mutants harboring homozygous on-target mutations (Table S2 in Supporting Information). Both of two off-target genes were ALS homologs that are barely expressed in rice (Kuang et al., 2020; Xia et al., 2017), suggesting that the effects of homologous off-target substitutions in OsALS-OFF1-P188 and OsALS-OFF2-P184 are probably negligible (Figure S2 in Supporting Information). For the OsALS-G628 site, the potential off-target sites had at least 4-nt mismatches, with a low probability of off-target editing (Kim et al., 2017). We sequenced the top five predicted off-target sites and did not detect any mutations (Table S2 in Supporting Information).

Herbicide tolerance of mutants in the greenhouse

To evaluate the herbicide tolerance level of the various mutants, we obtained transgene-free homozygotes for OsALS-P171, OsALS-G628 and their combination by segregation in the T_1 generation (Figure S3 in Supporting Information) and conducted herbicidal tolerance assays using nicosulfuron and imazapic. All the OsALS-P171 mutants, P171S, P171Y, P171A, P171F, and P171F/R172C survived the nicosulfuron treatment at 40 g ai ha^{-1}, whereas the wild type died (Figure S4A in Supporting Information). However, only G628E/G629S conferred some tolerance to imazapic at 108 g ai ha^{-1} (Figure S4B in Supporting Information). In addition, we observed that the G628E/G629S mutant was growth-retarded in the field in the absence of herbicides, suggesting that these mutations entail significant fitness costs and are of limited value for field application (Figure S4C in Supporting Information). However, the P171F/G628E/G629S mutant generated by the combination sgRNA was tolerant to both nicosulfuron and imazapic (Figure S4D in Supporting Information) without obvious retardation.

As ALS is the target for a large number of herbicides across dissimilar herbicide chemistries, including SU, IMI, TP, PTB, and SCT, we further tested five mutants (P171S, P171Y, P171A, P171F, and P171F/G628E/G629S) with representative herbicides from each of the five chemical groups at various multiples of the field-recommended rates at the two-leaf stage (Figure 2). The triple amino acid mutant P171F/G628E/G629S had greater tolerance to both nicosulfuron and imazapic than the other mutants, which died or showed severely injured even at 1X the field-recommended rate (Figure 2). All five mutants were only slightly growth-retarded at 32X the field-recommended rates of pyroxsulam and flucarbazone, whereas the wild type showed obvious growth stunning at 1/4X the field-recommended rates (Figure 2). Interestingly, the five mutants displayed dissimilar tolerance levels to bispyribac, a PTB herbicide registered for weed control for rice (Figure 2). The P171S and P171A mutants showed increasing growth stunning as the bispyribac rate was increased from 1X to 32X the field-recommended rates, but not the mutants P171Y, P171F and P171F/G628E/G629S (Figure 2). We also tested mutant G628E/G629S with the five herbicides at 1X the field-recommended rate at the three-to-four-leaf stage (Figure S5 in Supporting Information). G628E/G629S conferred slight tolerance to nicosulfuron, imazapic, pyroxsulam, and bispyribac, and moderate tolerance to flucarbazone (Figure S5 in Supporting Information). Overall, the triple amino acid mutant P171F/G628E/G629S was the most tolerant to the various ALS inhibitors; P171Y, P171F, and P171F/G628E/G629S exhibited the best tolerance to bispyribac; and all five mutants were tolerant to up to 32X pyroxsulam and flucarbazone.

The herbicide-tolerant mutants suffer no yield penalty in the field

To assess the potential value of these herbicide-tolerant mutations, P171F and P171F/G628E/G629S were first evaluated under field conditions along with the wild type in the absence of herbicide (Figure S6A in Supporting Information). Although both mutants were slightly shorter than the wild type, neither their tiller numbers, grain numbers per panicle, fertility nor 1,000-grain weights were significantly different from those of the wild type (Figure S6A in Supporting Information). As a result, the grain yields per plant of P171F and P171F/G628E/G629S were not significantly different from that of the wild type (Figure S6A in Supporting Information). The fact that both mutants were shorter than the wild type points to possible resistance to lodging. These results indicate that P171F and P171F/G628E/G629S do not suffer a yield penalty and may display lodging resistance under natural paddy field conditions.

Next, we compared the field agronomic characteristics of P171F, P171F/G628E/G629S and the wild type at various multiples of the field-recommended levels of nicosulfuron. Both mutants survived 1X the field-recommended rate of nicosulfuron (40 g ai ha^{-1}), whereas the wild type died (Figure 3). P171F displayed more growth stunting than P171F/G628E/G629S over 21 days in the field (Figure 3), consistent with the results of the green house herbicidal assays (Figure 2). Notably, the grain yields per plant of P171F and P171F/G628E/G629S treated with 1X nicosulfuron were not significantly different from that of untreated wild type (Figure 4B). Detailed analysis revealed no differences in tiller number, grain number per panicle, fertility or 1,000-grain weight (Figure 4C–F). Similar results were obtained in the 1/3X nicosulfuron set (Figure S6B in Supporting Information), whereas in the 3X set, P171F suffered a sig-

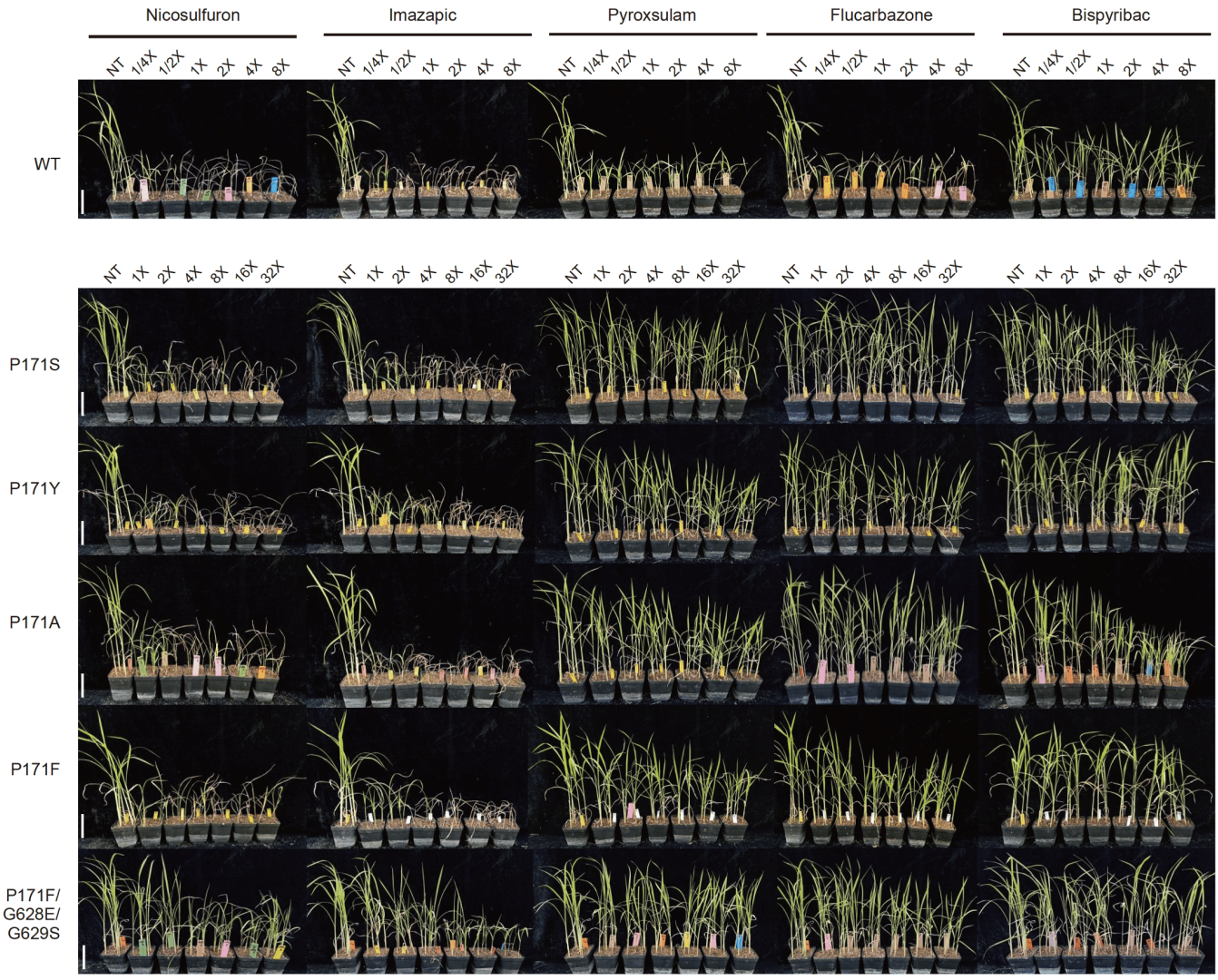

Figure 2 Phenotypes of T2 rice edited at the OsALS-P171 and OsALS-P171+G628 sites after exposure to five representative herbicides at various multiples of field-recommended levels. Five mutant lines (P171S, P171Y, P171A, P171F, P171F/G628E/G629S) and the wild type were treated with herbicides at the two-to-three-leaf stage, and photos were taken at 21 days after treatment. The five herbicides, nicosulfuron, imazapic, pyroxsulam, flucarbazone and bispyribac, were applied to the wild type at 1/4, 1/2, 1, 2, 4 and 8X field-recommended levels and to the five mutant lines at 1, 2, 4, 8, 16 and 32X field-recommended levels. NT, no treatment. Scale bar, 8 cm.

nificant yield penalty in terms of reduced main panicle grain number, fertility and 1,000-grain weight (Figure S6C in Supporting Information). However, P171F/G628E/G629S showed negligible yield penalty at higher herbicide concentrations (Figure S6C in Supporting Information). These results demonstrate that both mutants, especially the triple mutants, could potentially be used with nicosulfuron for weed control.

We also challenged P171S, P171F, P171F/G628E/G629S and the wild type with 9X bispyribac (Figure 5). As bispyribac is a post-emergence herbicide for rice, herbicide treatment resulted in no obvious differences in grain yield per plant (Figure 5B). However, mutants P171F and P171F/G628E/G629S exhibited higher fertility than the wild type and P171S in the presence of bispyribac treatment, but no difference in the absence of bispyribac (Figure 5E). These results indicate that P171F and P171F/G628E/G629S have a significantly increased ability to protect rice varieties from damage by bispyribac under natural paddy field conditions.

Structural basis of tolerance to multiple herbicides

To understand the molecular basis of the tolerance mutations, we developed homology models of OsALS in complex with monosulfuron or imazaquin, representing herbicides of the SU and IMI herbicide families, respectively, based on the crystal structure of AtALS (Figure 6). OsALS-P171, the site homologous to AtALS-P197, is located at the entrance to the access channel to the active-site and interacts with the aromatic ring of SUs (Figure 6A). For this reason, mutation P171F is expected to confer tolerance to SUs (Figure 6B). However, as IMIs bind at a greater distance from P171

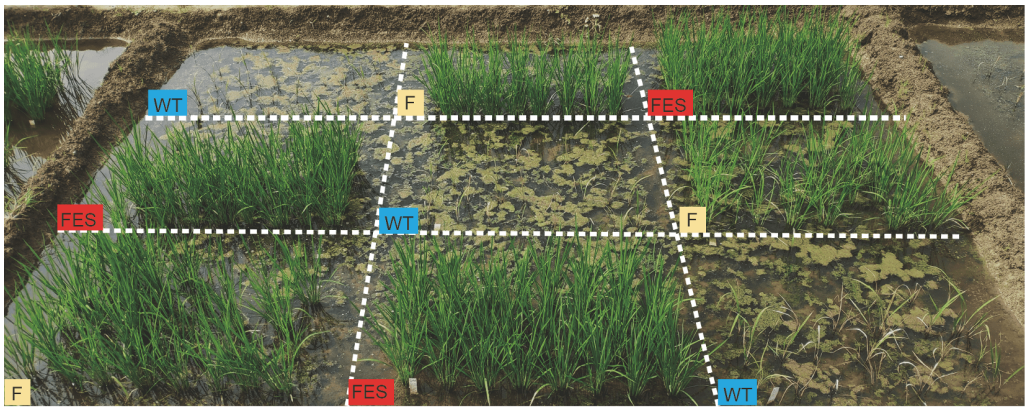

Figure 3 Field phenotypes of the wild type, P171F and P171F/G628E/G629S plants at 21 days after nicosulfuron treatment at 1X the field-recommended rate (40 g ai ha^{-1}) in Beijing (2019). F refers to mutant P171F and FES to mutant P171F/G628E/G629S.

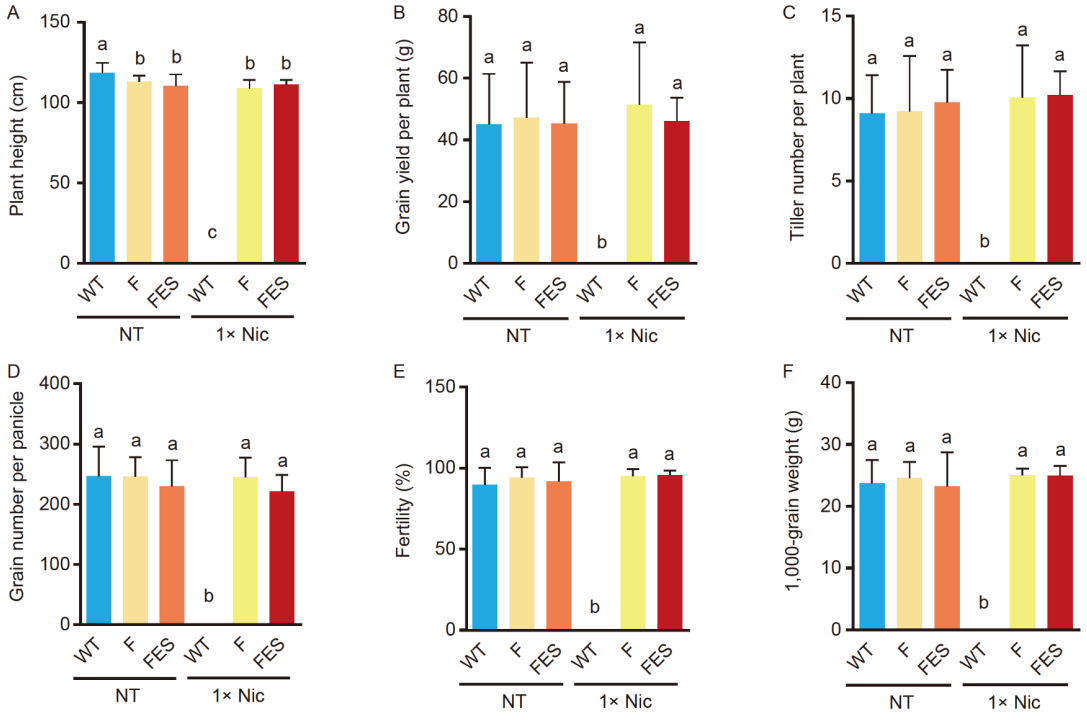

Figure 4 Agronomic characteristics of the wild type, P171F and P171F/G628E/G629S plants with or without one-time nicosulfuron treatment in a field trial in Beijing (2019). Plant heights (A), grain yields per plant (B), tiller numbers per plant (C), grain numbers per panicle (D), fertility (E) and 1,000-grain weights (F) of the wild type, P171F and P171F/G628E/G629S with or without nicosulfuron treatment. F refers to mutant P171F and FES to mutant P171F/G628E/G629S. NT, no treatment; Nic, nicosulfuron. Values in A–F are means+SD (18 replicates for each characteristic). Means marked with the same letters above the error bars represent no significant differences among each other by one-way ANOVA using Tukey's multiple comparisons test at 0.05 level.

(Figure 6D), mutations at P171 would not be significant contributors to IMI tolerance (Figure 6E). Since double mutations P171F and G628E shrink the situation space of both monosulfuron and imazaquin from different directions (Figure 6C and F), this combination confers tolerance to both agents. Another mutation, G629S, does not block herbicide binding (Figure 6C and F), which is consistent with the lethal effect of imazapic on this mutant (Figure S4B in Supporting Information). As the binding sites of the five herbicide families overlap (Garcia et al., 2017), the P171F/G628E mutation is expected to confer tolerance to all five herbicide families.

DISCUSSION

In this study, we performed base editing in rice and showed that multiple missense alleles created by editing OsALS-P171 and OsALS-P171+G628 codons endowed rice plants with significant tolerance to various ALS-in-

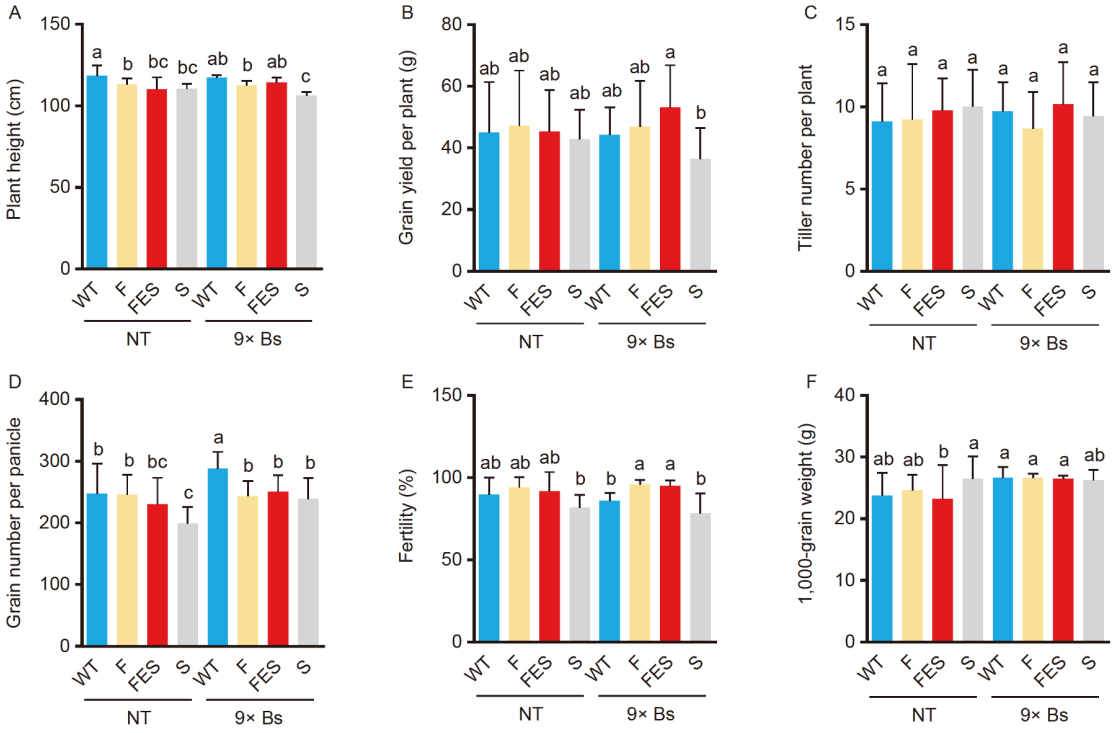

Figure 5 Agronomic characteristics of the wild type, and P171F, P171F/G628E/G629S and P171S mutants with and without bispyribac treatment at 9X recommended rates in a field trial in Beijing (2019). Plant heights (A), grain yields per plant (B), tiller numbers per plant (C), grain numbers per panicle (D), fertility (E) and 1,000-grain weights (F) of the wild type, P171F, P171F/G628E/G629S and P171S with or without bispyribac treatment. F refers to mutant P171F, FES to mutant P171F/G628E/G629S and S refers to mutant P171S. NT, no treatment; Bs, bispyribac. Values are means+SD (18 replicates for all characteristics). Means marked with the same letters above the error bars represent no significant differences among each other by one-way ANOVA using Tukey's multiple comparisons test at 0.05 level.

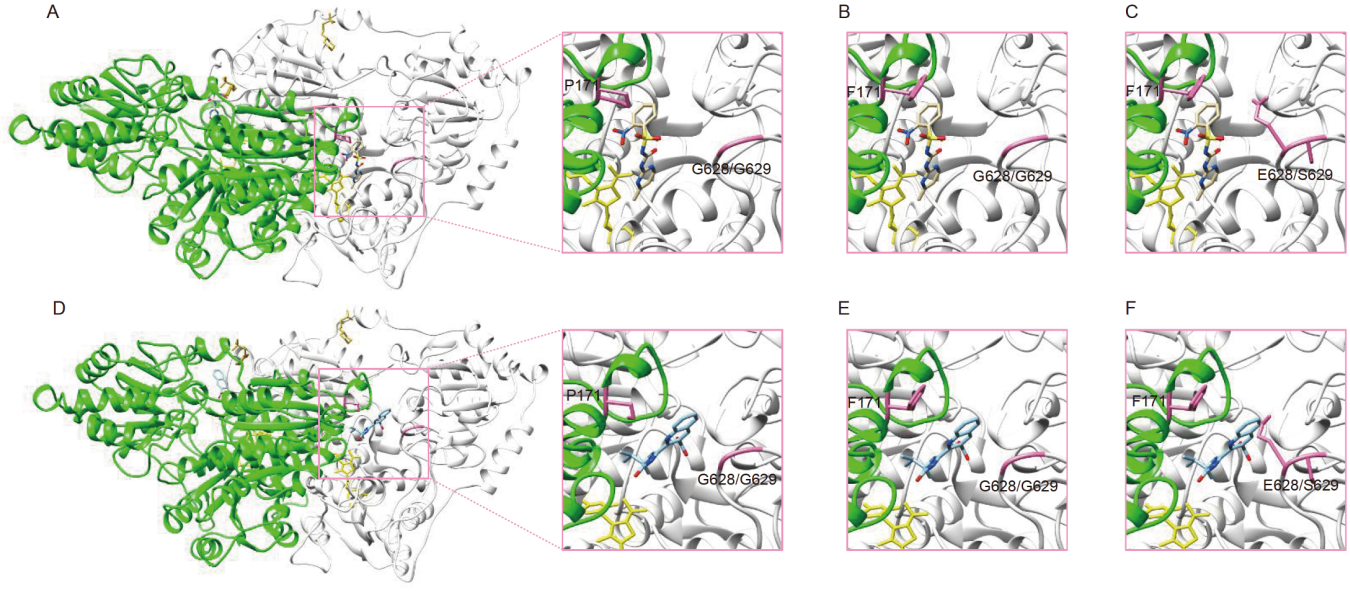

Figure 6 Structural models of OsALS in complexes with herbicides, based on the structure of AtALS. A, 3D model of the OsALS-monosulfuron complex. B and C, Molecular basis of herbicide tolerance to SU in the OsALS mutants P171F (B) and P171F/G628E/G629S (C). D, 3D model of the OsALS-imazaquin complex. E and F, Molecular basis of herbicide tolerance to IMI in the OsALS mutants P171F (E) and P171F/G628E/G629S (F). Key residues in the wild type (A, D), P171F (B, E), and P171F/G628E/G629S (C, F) are shown as sticks and in purple. The two monomers of OsALS are shown in grey and green, respectively.

hibiting herbicides applied at field-recommended rates. This is the first time that a number of tolerance substitution alleles are installed in native context of plant genome and evaluated systemically for cross-tolerance and fitness.

Prior to our study, most IMI-herbicide-tolerant strains were created by mutation breeding (Tan et al., 2005). Since SU herbicides such as nicosulfuron do not persist in the soil, the use of SU herbicide-tolerant rice varieties coupled with short-lived SU herbicides would offer farmers more opportunities to plant rotational crops. TP and SCT herbicides such as pyroxsulam and flucarbazone are also potential choices.

Multiple point mutations in herbicide target genes tend to confer higher levels of tolerance than single mutations. For instance, P197A&W574L in the *ALS* gene of tobacco (Lee et al., 1988) and T102I/P106S in the *EPSPS* gene of *Eleusine indica* (Yu et al., 2015) confer higher levels of tolerance to ALS herbicides and glyphosate, respectively. In this study, the novel triple amino acid mutations that we introduced into ALS conferred greater herbicide tolerance than single point mutations. Moreover, the triple mutation expanded the tolerance spectrum and conferred high-level tolerance to all five chemical groups of ALS herbicides. The G628E/G629S mutant showed obvious growth retardation but the triple mutant did not. Thus, strategies of multiple point mutations could be used to increase tolerance levels and generate cross-tolerant strains of other herbicide-tolerant crops with acceptable fitness.

In this study, we used a cytosine base editor to generate a number of multiple ALS herbicide-tolerant rice mutants. Due to the characteristics of the cytidine deaminase in the cytosine base editor BE3, genome-wide single nucleotide variants may occur (Jin et al., 2019). Nevertheless, mutants P171F and P171F/G628E/G629S did not suffer a yield penalty, suggesting unpredictable DNA off-target events may be negligible in plant breeding. Moreover, sgRNA-independent DNA off-target can be dramatically reduced by using more precise base editors (Jin et al., 2020). The slightly reduced height of the herbicide-tolerant plants may be caused by the amino acid substitutions in ALS and confer resistance to lodging, a potential side benefit that needs to be investigated in future.

MATERIALS AND METHODS

Plant growth

Rice seeds were surface-sterilized with 1% (v/v) H_2O_2 for 12 h, rinsed with distilled water three times, then pre-imbibed in distilled water at 37°C for 2 days. After pre-germination, they were transferred to pots or 96-well plates containing Kimura B nutrient solution and cultured in a growth chamber (30°C, 16-h light/8-h dark) to the two-to-four-leaf stage for herbicide assays. Two-to-four-leaf stage seedlings were transferred to the field in Beijing during the rice growing season or to a greenhouse (16-h light at 30°C/8-h dark at 22°C).

Construction of vectors

To make construction easier, a binary expression vector without a *Bsa* I restriction enzyme site, pH-BE3, was made as follows. APOBEC partial nCas9 and UGI sequences without *Bsa* I were synthesized commercially (GenScript, Nanjing, China) and cloned into pUC57 as an intermediate vector. The other part of nCas9 was excised from pHUE411 with *Sda* I and *Mlu* I, and ligated to an intermediate vector digested with the same two enzymes, yielding the plasmid pUC57-APOBEC1-nCas9-UGI. Full length APOBEC1-nCas9-UGI was excised using *Xba* I and *Sac* I, and sub-cloned into pHUE411 pre-digested with the same two enzymes. The resulting pH-BE3 vector was used to construct sgRNA expression plasmids as previously described using the *Bsa* I restriction enzyme site (Shan et al., 2013). pCBC-MT1T2 was used as PCR template for constructing the two sgRNAs (Xing et al., 2014). All the primer sets used for sgRNA construction are listed in Table S3 in Supporting Information.

Agrobacterium-mediated transformation

Oryza sativa L. (cv. Zhonghua11) was used in this study. *Agrobacterium*-mediated transformation was performed as reported (Shan et al., 2015). Hygromycin (50 μg mL^{-1}) was used to select transgenic plants carrying *HPT*. Each transgenic line contained one to four seedlings originating from one single callus.

PCR, PCR-RE assays and Sanger sequencing

PCR and Sanger sequencing were performed as described previously to identify base changes in target regions (Zong et al., 2017). Primers used in this study are listed in Table S4 in Supporting Information. PCR products from T_0 transgenic plants were analyzed by Sanger sequencing.

Herbicide tolerance tests in the greenhouse and field

Rice seedlings were cultured to the two-to-four-leaf stage in a greenhouse (16-h light at 30°C/8-h dark at 22°C) and sprayed once with nicosulfuron (40 g ai ha^{-1}), imazapic (108 g ai ha^{-1}), pyroxsulam (3.375 g ai ha^{-1}), flucarbazone-sodium (94.5 g ai ha^{-1}) and bispyribac-sodium (12.6 g ai ha^{-1}) at the field-recommended rate. The concentrations of active ingredient of commercial nicosulfuron (ISK Biosciences Corporation Ltd), imazapic (BASF), pyroxsulam (Dow AgroScience), flucarbazone-sodium (Arysta LifeScience Corporation Ltd) and bispyribac-sodium (Shanghai Hulian Biopharmaceutical Corporation Ltd) were 4%, 24%, 7.5%, 70% and 20%, respectively. Field tests of herbicide tolerance were performed during the regular rice

cultivation season. Four-leaf-stage seedlings were transplanted to the paddy field at a density of 15 cm×15 cm. After two weeks' recovery, various multiples of the field-recommended levels of herbicides were applied using pressurized equipment at 0.2 MPa with a spraying volume of 450 L ha $^{-1}$.

Detection of off-target events

Potential off-target sites were searched with CRISPR-P (Liu et al., 2017) (http://crispr.hzau.edu.cn/CRISPR2/). Off-target sites containing one- to four-nucleotide mismatches were examined by Sanger sequencing. The primer sets used are listed in Table S4 in Supporting Information.

Modeling the 3D structure of OsALS complexes with herbicides

Homology models of the structures of the complexes formed by OsALS with monosulfuron and imazaquin were generated based on the crystal structures of the complexes of *Arabidopsis* orthologs (PDB id: 3e9y and 1z8n) (McCourt et al., 2006; Wang et al., 2009), using SwissModel. The structures of mutant proteins were generated with UCSF Chimera (http://www.cgl.ucsf.edu/chimera/).

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

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