LETTERS
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Generation of herbicide tolerance traits and a new selectable marker in wheat using base editing

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Developing herbicide-tolerant varieties by genome editing holds great promise for addressing the worsening weed problems in wheat cultivation[1]. Here, we generated transgene-free wheat germplasms harbouring herbicide tolerance mutations that confer tolerance to sulfonylurea-, imidazolinone- and aryloxyphenoxy propionate-type herbicides by base editing the acetolactate synthase (ALS) and acetyl-coenzyme A carboxylase genes. These stackable herbicide tolerance traits provide a potentially powerful tool for weed management. In addition, we found that base editing at the wheat ALS Pro-174 codon (TaALS-P174) endowed wheat with sufficient resistance to nicosulfuron herbicide in MS growth medium to allow selection. When the TaALS-P174 editor was coupled with editors for other targets of interest, co-editing occurred in the nicosulfuron-resistant plants, and selection for resistance in growth medium enriched the frequency of coupled targets by several-fold. This selectable co-editing system has the potential to greatly bolster adoption of base editing for crop improvement applications.

Weeds are a major threat to world food production, and herbicide-tolerant varieties have been cost-effective tools for helping farmers manage weeds[2]. Jointed goatgrass (*Aegilops tauschii*), a close relative to wheat, is becoming an especially damaging weed species due to the limited availability of tools for chemical control[1]. To address this issue, non-transgenic wheat varieties made tolerant to imidazolinone (IMI) herbicides by point mutations have been developed by traditional breeding and adopted by farmers especially in developed countries[3,4]. However, IMI herbicides persist in the soil and severely damage sensitive crops planted months and even years later[4,5]. As a result, in many countries such as China, where food supplies and farmers' incomes heavily rely on multiple harvests of different crops from the same field per year, IMI-herbicide-tolerant wheat varieties are not a practical choice and not commercially available. Currently, the sulfonylurea (SU) herbicide, mesosulfuron, is the only wheat-registered foliar-applied herbicide that provides control of jointed goatgrass in China[1]; it is thus being increasingly adopted by farmers despite it often causing damage to their wheat[6]. Therefore, non-transgenic crops with herbicide tolerance traits, coupled with low-risk herbicides, are badly needed by millions of multi-cropping farmers in their battle against weeds.

An obvious solution is to generate non-transgenic herbicide tolerance traits, by introducing point mutations that confer tolerance to herbicides in addition to IMIs. This task would be very challenging if carried out by traditional breeding since each individual mutation would confer a relatively low level of resistance due to the polyploid nature of wheat[7]. Recently developed cytidine base-editing technology offers an effective alternative[8]. Cytidine base editing uses cytidine deaminase–Cas9 fusion proteins to mutate nucleotides in target genes without generating double-strand breaks[8]. Acetolactate synthase (ALS), a key enzyme in the biosynthesis of branched-chain amino acids, is an ideal herbicide tolerance target for base editing in wheat, as ALS genes can harbour point mutations that confer sufficient tolerance to herbicides with little penalty to plant productivity[9]. For this reason, mutations in ALS genes have been generated using cytidine base editing in some diploid plant species (for example, IMI tolerance in rice[10], and tribenuron tolerance in *Arabidopsis*[11] and watermelon[12]).

As wheat mutations at ALS-P174 probably confer tolerance to SU herbicides[9,13], many of which are short-lived in the soil[14], we set out to generate mutations at this position. pnCas9-plant base editor (PBE)[15] and single-guide RNA (sgRNA) TaALS-P174 constructs targeting the TaALS-P174 site were delivered into ~640 immature embryos of the bread wheat variety Kenong199 by particle bombardment using the previously described DNA-based transient nuclease expression system[16]. PCR restriction enzyme digestion (PCR-RE) assays and Sanger sequencing revealed that 16 (2.5%) of the T0 plants had base-editing mutations and, among them, 10 were transgene-free (Table 1 and Supplementary Fig. 5). Most of the base changes were C to T conversions at positions 6, 7 and 8 of the protospacer, while some were C to G changes (Fig. 1a). Of the 16 mutant plants, 8 harboured heterozygous, biallelic or homozygous missense mutations across all 3 subgenomes, 2 had heterozygous or homozygous missense mutations in 2 of the 3 subgenomes, 3 had heterozygous or homozygous missense mutations in 1 of the 3 subgenomes, and the remaining 3 had silent mutations (Table 1). In addition to the expected P174S and P174F mutations, we obtained P174A substitutions caused by C to G transversions rather than C to T transitions, and P174F&R175C double missense mutations caused by additional dual C to T transitions at the ninth and tenth position of the spacer sequence (Fig. 1a and Table 1).

To determine whether the acquired missense mutations conferred herbicide tolerance, the 16 base-edited T0 plants with emerging rootlets were transferred to medium supplemented with 0.25 mg l^{-1} nicosulfuron, an SU herbicide with a relatively low risk

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Table 1 | T0 wheat plants (Kenong199) harbouring multiallelic edits at the TaALS-P174 position survived nicosulfuron

	A genome substitution	B genome substitution	D genome substitution	Resistance to nicosulfuron	Transgene-free
T0-1	F/S	F/S	F/S	R	No
T0-2	F/–	F	S	R	No
T0-3	S/–	F/S	S/–	R	No
T0-4	-	-	SM	NR	Yes
T0-5	S	S/–	F/S	R	No
T0-6	S	FC/–	-	R	Yes
T0-7	S	FC/–	-	R	Yes
T0-8	-	-	F/–	NR	Yes
T0-9	F	F/S	S/–	R	Yes
T0-10	F	F/S	S/–	R	Yes
T0-11	A	-	-	NR	Yes
T0-12	A	-	-	NR	Yes
T0-13	S/–	F/–	F/S	R	No
T0-14	S	F/–	F/–	R	No
T0-15	-	SM	-	NR	Yes
T0-16	-	SM	-	NR	Yes

FC, P174F&R175C; SM, silent mutation; -, wild type; NR, not resistant; R, resistant. T0 wheat plants were generated on non-selective MS medium and plantlets with emerging rootlets were challenged with nicosulfuron herbicide at 0.25 mg l^{-1} in the medium. Stunted T0-8 and T0-11 plants were transferred to non-selective MS medium and later produced sufficient seeds. Nicosulfuron-tolerant T0 plants produced a similar amount of seeds compared to the wild-type control. The T2 generation of the T0-11 mutant was used for the herbicidal assay.

to subsequently planted crops[14]. After three weeks' exposure to nicosulfuron, ten plants were healthy while the remaining six were dead or severely damaged (Table 1). Genotyping revealed that the resistant plants had three or more edited alleles, while the edited but sensitive ones had one or two missense or silent edits. The mutant $aa^{F/F}bb^{F/S}Dd^{S}$ harbouring five edited alleles is shown as an example in Fig. 1b. These results indicated that greater numbers of resistance alleles conferred higher levels of herbicide tolerance.

To examine whether these mutants potentially could be used for weed control in the field, transgene-free T2 plants bearing different numbers of edited alleles were exposed to nicosulfuron at the field-recommended rate of 40 g active ingredient (ai) ha^{-1} (Table 1, Supplementary Fig. 5 and Supplementary Table 7). Homozygous mutants with four or six edited alleles at the P174 codon grew normally whereas wild-type (WT) plants died within 30 days (Fig. 1c). The mutants grew as well as untreated mutant and WT controls, pointing to the potential for immediate field application (Fig. 1c). Homozygous mutants with two edited alleles at the P174 codon showed different levels of growth retardation in the presence of nicosulfuron, and their resistance was ranked as $aa^{S/S}$BBDD > AABBdd$^{F/F}$ > $aa^{A/A}$BBDD (Fig. 1c), suggesting that P174S conferred higher resistance than P174A in subgenome A. In addition, biased expression of homologous alleles may also contribute to the different tolerance level among the mutants[17]. Moreover, as mesosulfuron often causes damage to wheat[18], increased tolerance of wheat varieties to mesosulfuron could greatly facilitate weed control by this herbicide. We therefore tested transgene-free T2 mutants with six edited alleles ($aa^{S/S}bb^{S/S}dd^{F/F}$) at various multiples of the field-recommended levels of mesosulfuron at the two-leaf stage (Fig. 1d and Supplementary Table 8). The mutants grew better at 1, 3 and 9 times the field-recommended rates (13, 39 and 117 g ai ha^{-1}) over 21 days, whereas the WT exhibited visible injury, especially stunting, at 3 and 9 times the field-recommended rates (Fig. 1d). This result suggested that the mutants potentially could be immediately used to address the safety issue of mesosulfuron, and also lead to the registration of new SU herbicides for better weed control.

To check whether this base-editing strategy could allow us to confer herbicide tolerance on other elite wheat varieties, we delivered the same construct by particle bombardment into Kenong9204. T0 plants regenerated on non-selective Murashige and Skoog (MS) medium were immediately put on medium supplemented with 0.25 mg l^{-1} nicosulfuron and we obtained 10 resistant mutants of Kenong9204 with genotypes similar to Kenong199 mutants (Supplementary Table 1). This result also suggests that nicosulfuron can be used to directly select base-edited plants at the codon of TaALS-P174.

As process-based regulatory frameworks deal with plant products made using DNA intermediates, we tried to use RNA to generate herbicide-tolerant traits. In vitro-transcribed messenger RNAs of nCas9-PBE and TaALS-P174 sgRNA were delivered into ~800 immature embryos of Kenong199 by particle bombardment. PCR-RE assays and Sanger sequencing revealed that four plants harboured resistance-conferring substitutions (Supplementary Table 2). Three of them had heterozygous P174S missense mutations in the A subgenome, and one had heterozygous P174F missense mutations in the B and D subgenomes. It is likely that plants harbouring $aa^{S/S}bb^{F/F}dd^{F/F}$ could be generated by crossing and should possess adequate levels of herbicide resistance. These results indicate that DNA-free base editing can potentially be used to create herbicide tolerance traits in wheat.

As co-editing of two independent target sites is likely to occur when two different sgRNAs are cloned into the same base-editing vector, we supposed that the nicosulfuron tolerance resulting from base editing at ALS-P174 could be a potential co-editing marker for other target sites. Since different mutations in ALS confer resistance to different herbicides[19,20], we used this co-editing idea to introduce a second mutation at Gly 631 (G631) for resistance to IMI herbicides. A vector expressing sgRNAs targeting the P174 and G631 positions in *TaALS* coupled with pnCas9-PBE was introduced by particle bombardment into ~1,200 immature Kenong199 embryos. Since missense edits by P174-sgRNA confer adequate resistance to nicosulfuron, we collected thousands of plants emerging from non-selective MS medium and transferred them to nicosulfuron-supplemented medium. A total of 50 plants survived and grew, and genotyping revealed that all of them indeed had multiallelic edits in P174, and 27 (54%) had additional missense edits in the

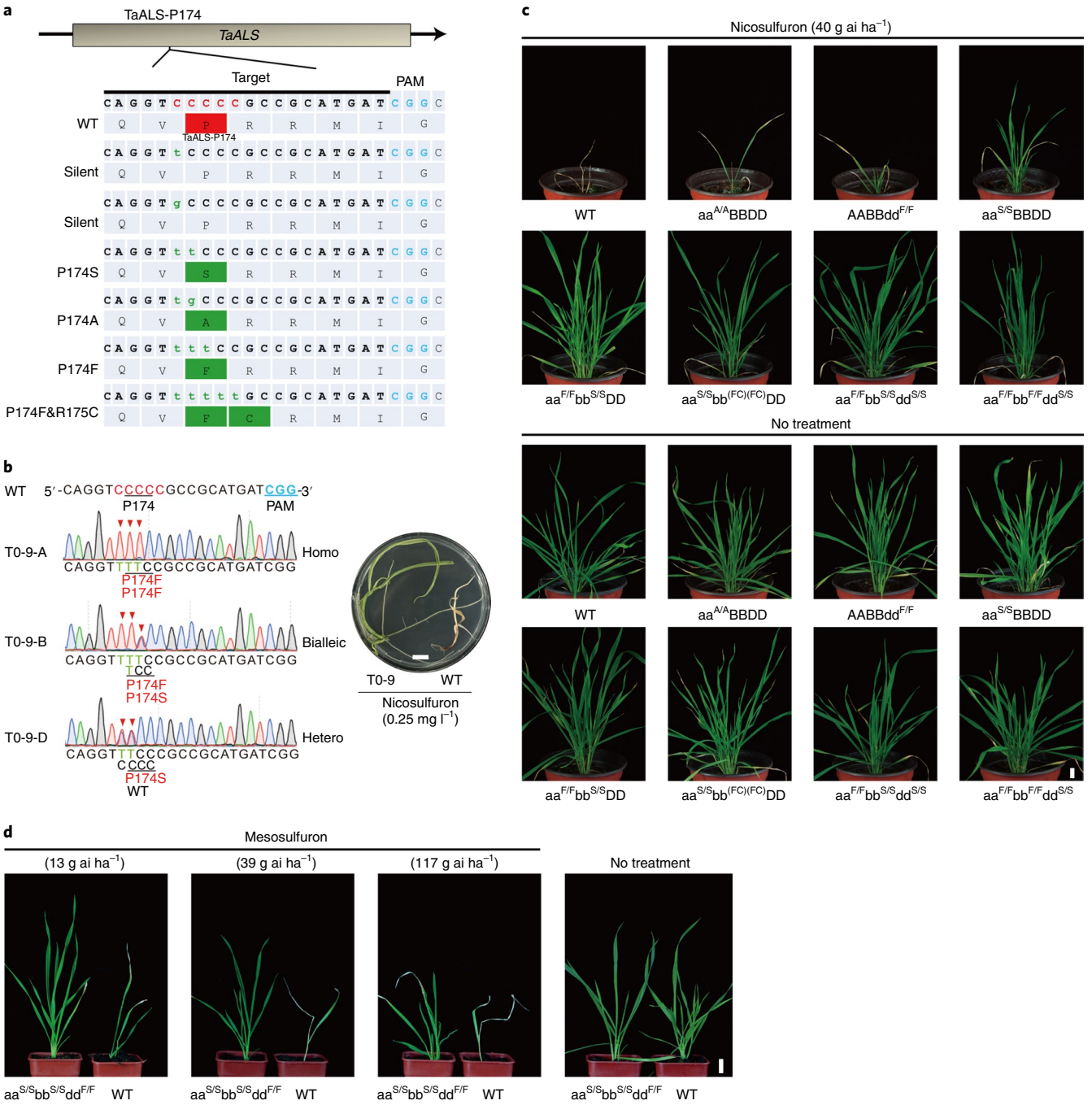
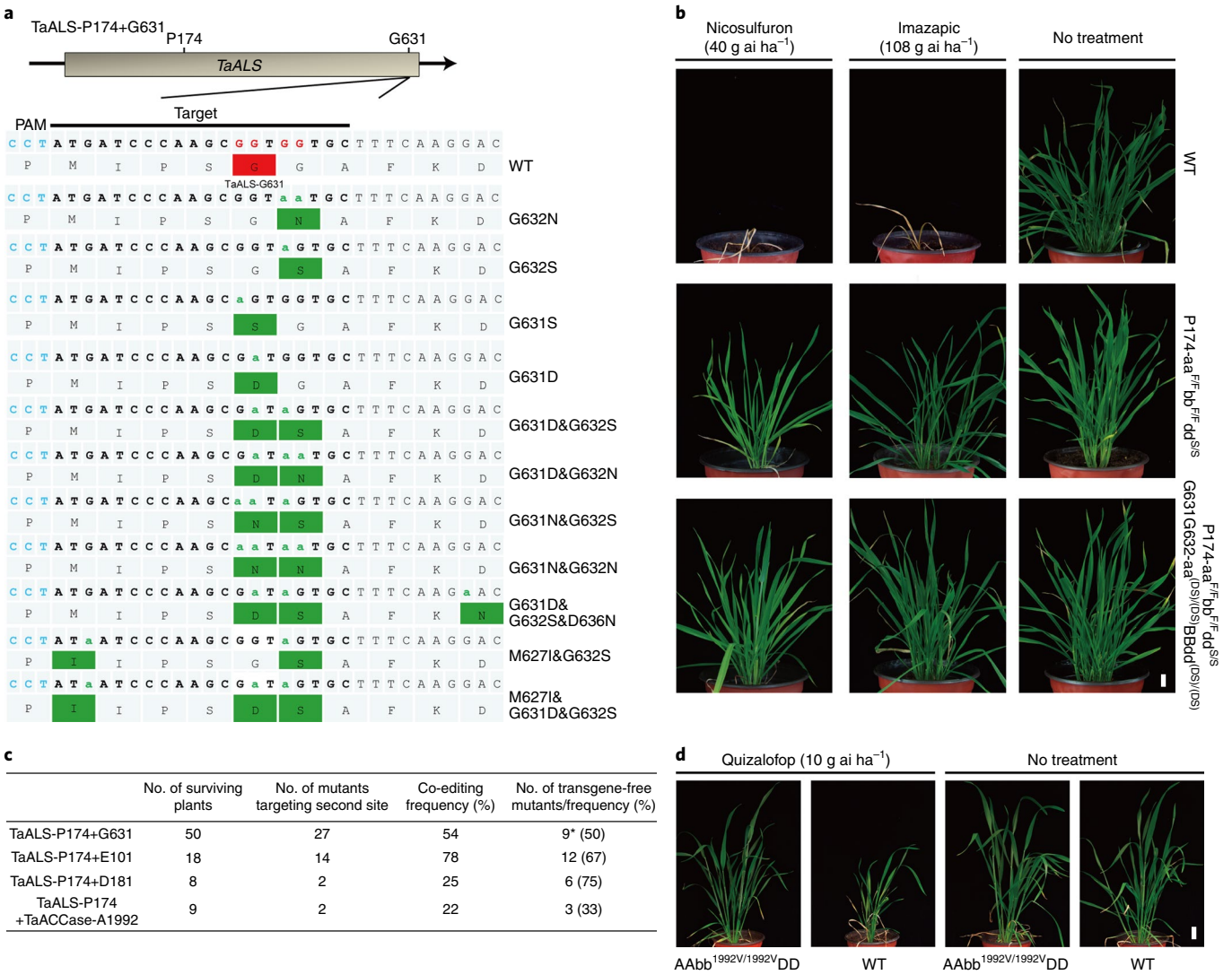


Fig 1 | Multiallelic editing of TaALS-P174 codons endowed wheat tolerance to multiple herbicides. a, Sequence alignment of TaALS-P174-targeted T0 plants. The TaALS-P174 site is conserved in the three subgenomes and the amino acid numbering refers to the TaALS-A subgenome. Target sequences, PAM sequences, DNA modifications and editable cytosines are indicated by bold, blue, green and red text, respectively. Changed amino acid residues and the amino acid at which mutations confer herbicide tolerance are indicated by green and red filled boxes, respectively. **b**, Simultaneous edits across wheat subgenomes at the TaALS-P174 position (left); mutants with missense edits survived nicosulfuron at 0.25 mg l^{-1} in MS medium, whereas wild-type (WT) plants were killed (right). Scale bar, 1 cm. As in **a**, PAM sequences, DNA modifications and editable cytosines are indicated by blue, green and red text, respectively. **c**, Phenotypes of T2 wheat edited at the TaALS-P174 site after nicosulfuron treatment. Mutants bearing different edited alleles in the TaALS-P174 site and WT were treated with nicosulfuron (40 g ai ha^{-1}, the field recommended rate in maize field) at the four-leaf stage, and photos were taken 30 days after treatment. The photos shown are typical of three separate tests, with each test using three to four replicate pots for each genotype. FC refers to P174F&R175C. Scale bar, 2 cm. **d**, Phenotypes of T2 wheat edited in the TaALS-P174 site after mesosulfuron treatment. Mutants and WT were treated with mesosulfuron at the two-leaf stage and photos were taken 21 days after treatment. The photos shown are typical of three separate tests, with each test using three replicate pots for each genotype. Mesosulfuron is a wheat-registered herbicide with a field recommended rate at 13 g ai ha^{-1}. Scale bar, 2 cm.

G631-sgRNA region (Fig. 2c). Detailed genotyping of the co-edited alleles revealed that the 2 sgRNAs had generated different numbers of missense mutations (Fig. 2a and Supplementary Table 3): TaALS-P174 had created 4 types of amino acid changes, while TaALS-G631 had generated 11, probably because there were more editable bases in the case of TaALS-G631. In addition, point



	No. of surviving plants	No. of mutants targeting second site	Co-editing frequency (%)	No. of transgene-free mutants/frequency (%)
TaALS-P174+G631	50	27	54	9* (50)
TaALS-P174+E101	18	14	78	12 (67)
TaALS-P174+D181	8	2	25	6 (75)
TaALS-P174 +TaACCase-A1992	9	2	22	3 (33)

Fig. 2 | The TaALS-P174-based co-editing strategy efficiently generates mutations at desired positions via nicosulfuron selection. a, Diverse co-editing events at the TaALS-G631 site. The TaALS-G631 site is conserved in the three subgenomes and the amino acid numbering refers to the TaALS-A subgenome. Target sequences, PAM sequences, DNA modifications and editable cytosines are indicated by bold, blue, green and red text, respectively. Changed amino acid residues and the amino acid at which mutations confer herbicide tolerance are indicated by green and red filled boxes, respectively. **b**, Co-editing generated mutants (P174-aa$^{F/F}$bb$^{F/F}$dd$^{S/S}$&G631G632-aa$^{(DS)/(DS)}$BBdd$^{(DS)/(DS)}$) resistant to both nicosulfuron and imazapic. Plants were treated with herbicide at the four-leaf stage and photos were taken 30 days after treatment. The photos shown are typical of three separate tests, with each test using three replicate pots for each genotype. DS refers to G631D&G632S. Scale bar, 2 cm. **c**, Co-editing frequency of TaALS-P174 sgRNA and four other different sgRNAs. *Eighteen of fifty surviving plants were randomly selected for transgene detection. **d**, Co-editing generated double mutations in TaACCase-A1992V conferring resistance to quizalofop at 10 g ai ha^{-1}. The AAbb$^{1992V/1992V}$DD plant harbours both TaALS-P174-aa$^{S/S}$bb$^{S/S}$dd$^{F/F}$ and TaACCase-A1992-AAbb$^{V/V}$DD edits. Plants were treated with herbicide at the three-leaf stage and photos were taken 40 days after treatment. The photos shown are typical of three separate tests, with each test using three replicate pots for each genotype. Scale bar, 4 cm.

mutations created by G631-sgRNA were occasionally encountered at the −8 and 18 positions, outside the typical base-editing window between positions 3 and 9 of the protospacer region[15] (Fig. 2a). Along with C to G or A conversions, these factors contributed to the unexpected abundant diversity of the resulting genotypes at the TaALS-G631 position. It should be noted that a vast number of unique genotypes in wheat could be generated in this way because TaALS-P174 and TaALS-G631 are intragenic alleles that are base-editable across all three subgenomes. This result indicates that a single vector expressing two sgRNAs targeting two intragenic targets in wheat could theoretically generate extensive genetic diversity.

To determine whether the missense mutations in the TaALS-G631 region conferred IMI tolerance, transgene-free T2 plants with six allelic edits in TaALS-P174 alone (P174-aa$^{F/F}$bb$^{F/F}$dd$^{S/S}$), and plants with all six allelic edits in TaALS-P174 and four allelic edits in the TaALS-G631 region (P174-aa$^{F/F}$bb$^{F/F}$dd$^{S/S}$&G631G632-aa$^{(DS)/(DS)}$BBdd$^{(DS)/(DS)}$), were treated with imazapic (Fig. 2b, Supplementary Figs. 1 and 5 and Supplementary Table 9). The mutants with edits in TaALS-P174 and those with edits in TaALS-P174+G631 displayed tolerance to imazapic at the field-recommended rate of 108 g ai ha^{-1} over 30 days (Fig. 2b). Although markedly stunted, the latter displayed a low degree of herbicide tolerance at 3 times (324 g ai ha^{-1}) and 5 times (540 g ai ha^{-1}) the field-recommended rate (Supplementary Fig. 1). Similarly, double mutants had a slightly higher level of nicosulfuron resistance than single mutants (Fig. 2b). This higher tolerance to SU and IMI could be due to

synergistic effects of mutations at TaALS-P174 and TaALS-G631G632, since co-occurrence of multiple mutations in the same gene in rice confers a higher level of herbicide tolerance[19]. It is notable that double mutants were more tolerant to imazapic than PI653509, an IMI-herbicide-tolerant wheat germplasm generated by chemical mutagenesis carrying the S630N mutation in subgenome D alone (Supplementary Fig. 1).

To test whether this TaALS-P174-based co-editing selection system works for other target sites, we selected two intragenic positions, TaALS-E101 and TaALS-D181, as well as the A1992 position of the acetyl-coenzyme A carboxylase (TaACCase-A1992) gene, as targets to be coupled with TaALS-P174. ACCase is a key enzyme in plant lipid biosynthesis that carboxylates acetyl-CoA to form malonyl-CoA, and mutations at A1992 are reported to confer resistance to quizalofop[20]. Single vectors expressing TaALS-P174 sgRNA and one of the second sgRNAs described above coupled with pnCas9-PBE were introduced into ~1,000 immature Kenong199 embryos by particle bombardment, and plants regenerated on non-selective medium were challenged with nicosulfuron. Genotyping revealed that 22–78% of the nicosulfuron-resistant plants contained appropriate edits at the second target site, thus showing that herbicide tolerance at TaALS-P174 can be an efficient selective marker for base editing in wheat (Fig. 2c, Supplementary Figs. 2–4 and Supplementary Tables 4–6). Compared with PCR-RE or Sanger sequencing methods for identifying base-edited plants at TaALS-P174 (2.5%), this base-editing selection method achieves a roughly 9- to 31-fold increase in efficiency. Moreover, 33–75% of the nicosulfuron-resistant plants without detectable transgenes were obtained in the T0 generation according to the PCR analysis (Fig. 2c and Supplementary Fig. 5).

As mutations at TaACCase-A1992 may confer resistance to quizalofop[21], transgene-free and homozygous T2 mutants carrying A1992V in subgenome B were exposed to quizalofop (Supplementary Fig. 5 and Supplementary Tables 6 and 10), and the herbicidal assay showed that the mutations endowed wheat plants with tolerance to quizalofop (Fig. 2d). ACCase-inhibiting herbicides are used to control grass weed species, but some are registered on wheat due to their rapid detoxification in wheat. Under some field conditions, crop injury can happen due to impaired detoxification processes. Thus, TaACCase-A1992V germplasm should not only address potential damage by wheat-registered ACCase herbicides but also introduce other highly effective ACCase herbicides to better manage grasses in wheat fields. Moreover, since herbicide tolerance mutations in TaALS and TaACCase are stackable, wheat varieties that tolerate herbicides with different modes of action should also help to manage current herbicide-resistant weeds and suppress the appearance of additional herbicide-resistant weeds in agricultural fields.

As the APOBEC1 base editor tolerates 1-nucleotide (nt) mismatches at almost every position and 2-nt mismatches in the region distal to the protospacer adjacent motif (PAM) but does not tolerate PAM-proximal or PAM-distal 3-nt or 4-nt mismatches[22], we sequenced potential off-target sites containing up to 3-nt mismatches generated by the tool CasOT[23]. No off-target events were detected in the sites highly homologous to those conferring herbicide resistance (Supplementary Figs. 6–8 and Supplementary Table 11).

Collectively, we have generated mutations in wheat conferring tolerance to multiple herbicides. These new herbicide tolerance traits could be introduced into elite wheat varieties via base editing or backcrossing to greatly improve weed control. Moreover, we have developed a novel selectable co-editing strategy for detecting edited plants by coupling an sgRNA of interest with TaALS-P174 sgRNA; in this strategy, the high incidence of co-editing makes herbicide-tolerant plants obtained by selection likely to carry edits in the desired targets. As *ALS* genes contain several base-editable codons conferring different herbicide resistances and are conserved across plant species, similar selectable co-editing systems could be readily established to facilitate transgene-free editing with deaminase–Cas9 fusion proteins in other plant species.

Methods

Plant materials and growth conditions. Seeds of wheat germplasm PI653509 were provided by D. Doohan at the Ohio State University. Wheat seeds were grown in a greenhouse (23 °C, 16-h light/8-h dark), and seedlings at the two-leaf stage were kept at 4 °C for 4 weeks for vernalization. Seedlings were then transferred back to the greenhouse (23 °C, 16-h light/16 °C, 8-h dark) and herbicide assays were performed at the two-to-four-leaf stage.

Construction of plasmids. The construction of sgRNA expression vectors was performed as previously described[24]. To construct pairs of sgRNAs, pCBC-MT1T2 was used as a PCR template[25]. All primers used for sgRNA construction are listed in Supplementary Table 13.

Biolistic delivery of DNA constructs into immature embryo cells. Plasmids for pnCas9-PBE and sgRNA expression vectors driven by the promoter TaU6 were simultaneously delivered into immature embryos of Kenong199 or Kenong9204 via particle bombardment as previously described[16]. The embryos were cultured for plantlet regeneration on medium without selective agent.

In vitro transcription of RNA and biolistic delivery of RNA. The cassette NLS-rAPOBEC1-nCas9-UGI-NLS in pnCas9-PBE was cloned into pLZT7[16], and named pLZT7-PBE. pLZT7-PBE was linearized with XbaI and used as a template for in vitro transcription with an Ambion mMESSAGE mMACHINE kit (AM1344). The template for the synthesis sgRNA was amplified from the TaALS-P174 sgRNA plasmid using the primer set TaALS-P174-RNA-F/RNA-R (Supplementary Table 12). TaALS-P174 sgRNA was synthesized using a HiScribe T7 High Yield RNA Synthesis Kit (NEB, E2040). PBE mRNA and TaALS-P174 sgRNA were prepared and delivered into immature embryos of Kenong199 via particle bombardment as previously described[16].

PCR, PCR-RE assays and sequencing analysis. PCR, PCR-RE and Sanger sequencing to identify nucleotide conversions in the target regions were conducted as described previously[15]. The primers used in this study are listed in Supplementary Table 12. Plantlets (usually 3–4) derived from bombarded immature embryos were pooled for the PCR-RE assays, and positive plantlets were examined by Sanger sequencing to identify base changes. Transgene-free mutants were selected by PCR using four pairs of primers (Supplementary Table 12).

Herbicide resistance test. T0 mutants and the wild type with emerging rootlets were transferred to a plate containing rooting medium with 0.254 mg l^{-1} nicosulfuron and cultured in a growth chamber (23 °C, 16-h light/8-h dark). Transgene-free seedlings with homozygous edits as a result of segregation were identified by PCR and used for herbicide tests (Supplementary Fig. 5 and Supplementary Tables 7–10). Seedlings at the two-to-four-leaf stage in the greenhouse (23 °C, 16-h light/16 °C, 8-h dark) were treated with various rates of commercial nicosulfuron (ISK Biosciences Corporation Ltd), mesosulfuron (Bayer Cropscience Ltd), imazapic (BASF) and quizalofop (Nissan Chemical Industries Ltd). Herbicides were applied using pressurized equipment at 0.2 MPa, with a spraying volume of 450 l ha^{-1}. Each treatment contained at least three to four replicate pots that were randomly placed to avoid possible location effects. Photos of plants with and without herbicide treatment were taken at the same period of time following treatment with the herbicide. Representative pictures out of at least 3 biological replicates were taken 21–40 days after treatment.

Off-target detection. Potential off-target sites were searched by CasOT[23], and off-target sites containing up to three-nucleotide mismatches were analysed by Sanger sequencing. Four independent transgene-free mutant plants were examined for each off-target site. The T1-targeted TaALS-P174 mutants T1-7-17, T1-10-1, T1-10-39 and T1-10-46 were subjected to TaALS-P174 off-target assessment. The T1-targeted TaALS-P174+G631 mutants T1-17-1~4 were subjected to TaALS-G631 off-target assessment. The T1-targeted TaALS-P174+TaACCase-A1992 mutants T1-7-3, T1-7-5, T1-7-7 and T1-7-9 were subjected to TaACCase-A1992 off-target assessment. Representative Sanger sequencing chromatograms are shown in Supplementary Figs. 6–8.

Statistics and reproducibility. Representative Sanger sequencing chromatograms of at least three biological replicates with similar results are shown in Fig. 1b and Supplementary Figs. 6–8. Representative gel images of at least three biological replicates with similar results are shown in Supplementary Fig. 5b.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data supporting the findings of this study are available in the article or its Supplementary Information, or are available from the corresponding author upon

reasonable request. Sequence data in this article can be found in the Ensembl Genomes database (http://plants.ensembl.org/Triticum_aestivum/Info/Index) under the following accession codes: TaALS-A (TraesCS6A02G288000), TaALS-B (TraesCS6B02G317400), TaALS-D (TraesCS6D02G268700), TaACCase-A (TraesCS2A02G069400), TaACCase-B (TraesCS2B02G082500) and TaACCase-D (TraesCS2D02G068100)

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Author contributions

C.G., L.J. and J. Li designed the experiments; R.Z., Z.C., S.C. and Y.B. performed most of the experiments; J. Liu generated mutant plants. Y.Z. and K.C. analysed the results; C.G., L.J. and J. Li supervised the project; C.G., L.J., J. Li and R.Z. wrote the manuscript.

Competing interests

C.G. and L.J. are inventors on patent applications covering generation of herbicide torlerance traits in wheat described in this work. C.G., R.Z. and J. Liu are inventors on patent applications describing generation of a selectable marker in wheat using base editing.

Additional information

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Software and code

Policy information about availability of computer code

Data collection	Sanger sequencing was used to analyze the mutations of the target genes; DNAMAN 6.0 was used to to align and analyze the sequences.
Data analysis	All the genes sequence data were obtained from Ensembl Genomes database (http://ensemblgenomes.org/); CasOT-1.0 was used to predict the potential off-target sites; SnapGene Viewer 4.0.5 was used to view DNA sequence traces.

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The authors declare that all data supporting the findings of this study are available in the article and its Supplementary Information files or are available from the corresponding author on request. Sequence data in this article can be found in Ensembl Genomes database (http://plants.ensembl.org/Triticum_aestivum/Info/

Index) under the following accession numbers:
TaALS-A (TraesCS6A02G288000), TaALS-B (TraesCS6B02G317400), TaALS-D (TraesCS6D02G268700), TaACCase-A (TraesCS2A02G069400), TaACCase-B (TraesCS2B02G082500) and TaACCase-D (TraesCS2D02G068100).

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Life sciences study design

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Sample size	All the herbicidal assay experiments were performed with three biological repeats and each biological repeat contained at least three to four replicate pots. According to our experimental trials as described in methods section, this sample size is sufficient to ensure reproducibility.
Data exclusions	No data exclusion.
Replication	All attempts for replication were successful. A minimum of three biological replicates were included.
Randomization	Plants used for herbicidal assay were randomly placed to avoid possible location effects.
Blinding	Not applicable, as samples were processed identically through standard and in some cases automated procedures (DNA sequencing, DNA isolation) that should not bias outcomes.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☒	☐ Unique biological materials
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
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Methods

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☒	☐ ChIP-seq
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