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A nonsynonymous mutation in an acetolactate synthase gene (*Gh_D10G1253*) is required for tolerance to imidazolinone herbicides in cotton

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Abstract

Background Herbicide tolerance in crops enables them to survive when lethal doses of herbicides are applied to surrounding weeds. Herbicide-tolerant crops can be developed through transgenic approaches or traditional mutagenesis approaches. At present, no transgenic herbicide tolerant cotton have been commercialized in China due to the genetically-modified organism (GMO) regulation law. We aim to develop a non-transgenic herbicide-tolerant cotton through ethyl methanesulfonate (EMS) mutagenesis, offering an alternative choice for weed management.

Results Seeds of an elite cotton cultivar Lumianyan 37 (Lu37) were treated with EMS, and a mutant Lu37-1 showed strong tolerance to imidazolinone (IMI) herbicides was identified. A novel nonsynonymous substitution mutation Ser642Asn at acetolactate synthase (ALS) (*Gh_D10G1253*) in Lu37-1 mutant line was found to be the potential cause to the IMI herbicides tolerance in cotton. The Ser642Asn mutation in ALS did not present among the genomes of natural *Gossypium* species. Cleaved amplified polymorphic sequence (CAPS) markers were developed to identify the ALS mutant allele. The *Arabidopsis* overexpressing the mutanted ALS also showed high tolerance to IMI herbicides.

Conclusion The nonsynonymous substitution mutation Ser642Asn of the ALS gene *Gh_D10G1253* is a novel identified mutation in cotton. This substitution mutation has also been identified in the orthologous ALS genes in other crops. This mutant ALS allele can be used to develop IMI herbicide-tolerant crops via a non-transgenic or transgenic approach.

Keywords Acetolactate synthase, Cotton, EMS mutagenesis, Herbicide tolerance, Imidazolinone

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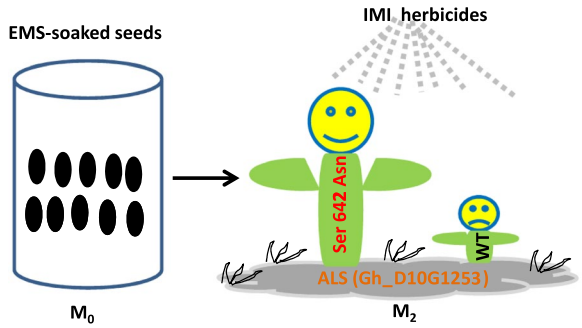
Graphical abstract



Background

Cotton (*Gossypium hirsutum* L.) is the most important source of widely used natural fiber. According to FAO statistics (https://www.fao.org/faostat/en/#data/QCL), China, India, the United States, Brazil, Pakistan, Uzbekistan, Turkey, and Australia are the top 8 cotton producing countries in the world. Weeds negatively affect cotton growth and yield by competing for nutrients, sunlight, space, and water. Poor weed management may lead to up to 90% yield reduction in cotton (Manalil et al. 2017). Herbicide-resistant cotton cultivars, mainly available with the genetically-modified glyphosate-resistant cotton, make weed management more convenient and efficient. However, overreliance on glyphosate has led to superweed problems (Heap and Duke 2018). The application of herbicides with different sites of action in weed control, in addition to glyphosate, will delay the evolution of resistance in weeds.

Acetolactate synthase (ALS), also known as acetohydroxyacid synthase (AHAS), is the first enzyme in the branched-chain amino acid (i.e., valine, leucine, and isoleucine) biosynthesis pathway that has been found in plants and microorganisms but not in animals. ALS is the target of 58 commercial herbicides from five herbicide chemical groups: imidazolinone (IMI), sulfonylurea (SU), triazolopyrimidine (TP), pyrimidinyl-thiobenzoates (PTB), and sulfonyl-aminocarbonyl-triazolinone (SCT). Those herbicides are used globally to protect stable crops, including corn, rice, wheat, cotton and more others, owing to their wide crop selectivity, low application rates, and low mammalian toxicity (Garcia et al. 2017). Point mutations in the target ALS gene improved plant tolerance to these ALS-inhibitor herbicides. As of 25 December 2022, 29 tolerance endowing-amino acid substitutions at 8 conserved positions of *Arabidopsis thaliana* ALS protein (AT3G48560: Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653, and Gly654) have been identified in weeds (http://www.weedscience.org/Mutations/MutationDisplayAll.aspx). Some of these mutations, generated through cell culture, seed mutagenesis, microspore (pollen) mutagenesis, or natural selection, were used to develop ALS herbicide-tolerant crops commercialized as non-transgenic Clearfield® crops, including rice, wheat, maize, oilseed rape, and sunflower (Tan et al. 2005).

Cotton (*Gossypium hirsutum* L.) consists of 6 *ALS* genes. Two of them, on the A5 and A19 on the genomic clones, respectively are the most conserved genes (Grula et al. 1995). Cotton can also develop tolerance to ALS-inhibitor herbicides. Two cotton mutants continuously selected with TP insuspension cultures showed variable resistance to TP and cross-resistance to other ALS herbicides from IMI, SU, and PTB families (Subramanian et al. 1990). The embryogenic cell line with Trp563Ser mutation in the A19 gene and the Trp563Cys mutation in the A5 gene were screened with an SU herbicide, which conferred tolerance to both SU and IMI herbicides (Rajasekaran et al. 1996a). However, no fertile plants have been regenerated from these mutant cell lines. Subsequently, dual mutantion on both sites of Trp563Ser and Ser642Asn at A19 gene were introduced to cotton genome. Transgenic cotton Trp563Ser mutation plants exhibite tolerance to IMI and SU herbicides, while the Ser642Asn mutants are tolerant to IMI herbicides only (Rajasekaran et al. 1996b). Four cotton mutants tolerant to the IMI herbicide imazamox have been obtained through ethyl methanesulfonate (EMS) mutagenesis of upland cotton seeds (Bechere et al. 2009, 2010). Genetic analysis revealed that the herbicide tolerant traits in the four mutant alleles are either from the same gene locus or the tightly linked gene loci. However, no resistance gene had been cloned. According to the current understanding of the mechanism of herbicide tolerance in cotton, we speculated that this resistance was attributed to

a mutanted ALS gene that encoded an enzyme insensitive to the IMI herbicide imazamox. In addition to ALS genes, the P450 gene *CYP749A16* (*Gh_D10G1401*) is also involved in acquiring the tolerance to the SU herbicide trifloxysulfuron sodium in cotton (Thyssen et al. 2014, 2018). And the mutation or silencing of *CYP749A16* generated a sensitive phenotype to the SU herbicide in cotton.

In this study, we aim to develop non-transgenic herbicide-tolerant cotton and identify the corresponding gene underlying herbicide tolerance. We obtained a mutant cotton (Lu37-1) tolerant to IMI herbicides by EMS mutagenesis. A novel nonsynonymous substitution mutation (Ser642Asn) in an ALS gene (*Gh_D10G1253*) contributed to the tolerance to IMI herbicides in the mutant cotton Lu37-1. The ectopic expression of the mutanted cotton ALS gene in *Arabidopsis* also conferred the increased tolerance level to IMI herbicides.

Results

Identification of an IMI-tolerant Lu37-1 cotton mutant

Approximately 50 kg of M_2 cotton seeds were sown in field and then subjected to the imazapic at 6 × the recommended rate. At 40 days after planting, most of the plantlets showed symptoms of chlorosis, stunting, and leaf distortion, while the imazapic tolerant plants were taller and had fully expanded new leaves (Fig. 1a). A total of 9 putative tolerant M_2 plantlets were obtained in the field, and 8 of them produced seeds after transplantation under greenhouse growth conditions. M_3 seeds obtained from those 8 M_2 plants were further screened by spraying with imazapic. At 15 days after treatment (DAT), the progeny of 7 M_2 mutant lines were all susceptible to imazapic, showing stunted shoots and yellow-spotting leaves (Fig. 1b). Only one M_2 mutant line produced 2 tolerant and 2 sensitive M_3 plantlets. These two tolerant M_3 plantlets grew normally (Fig. 1c) and were designated as Lu37-1 (i.e., Lu37-1–1 and Lu37-1–2) for further study.

Mutant Lu37-1 was tolerant to a high rate of imazapic

Higher dose of imazapic was applied to test the tolerance level of the Lu37-1 mutant in the field. At 40 DAT, the wild type plant was stunted, and the newly developed leaves were chlorotic and very small when treated with 3 × the recommended rate of imazapic (Fig. 1d). In contrast, the M_4 generation of Lu37-1 developed normally with fully expanded and green leaves andexhibited resistance to imazapic of 3× and 6 × the recommended rate (Fig. 1e, f).

The M_5 generation of Lu37-1 was further tested in the greenhouse growth condition. At 26 DAT, the shoot apical meristem of the wild type under the 3× and 6 × the recommended rate of imazapic was damaged and the growth was terminated (Fig. 2a). In contrast, only a minor inhibition of the development of Lu37-1 mutant was observed in the imazapic treatment of 3× and 6 × the recommended concentration comparing to the treatment with water (Fig. 2b, c).The degree of stunting and chlorosis phenotype was increased gradually with the increasing application rates of imazapic. However, newly formed leaves were still found in the two mutant lines under 21× and 24 × the recommended rate. At 64 DAT, the growth of the wild type treated with 3 × the recommended rate of imazapic was inhibited severely, showing chlorotic and distorted shoot tips, while the mutant plantlets under 21× and 24 × the recommended rate showed slightly crinkled and chlorotic leaves. And the mutants treated with imazapic at 12 × or less than 12× the recommended rate showed normal growth with regular plant height (Fig. 2d). This result suggested that the Lu37-1 mutant possessed at least eight fold elevated tolerance to imazapic than the wild type.

Mutant Lu37-1 was tolerant to other IMI herbicides but not to PTB or SU

Other ALS-inhibitor herbicides were used to test the cross-resistance in the Lu37-1 mutant. For imazethapyr and imazamox, chlorotic and stunted leaves were observed, and no new leaf was developed in the wild-type Lu37 at 26 DAT, while at least three new expanded leaves were developed in the mutant Lu37-1 (Fig. 3), indicating the increased tolerance level of Lu37-1 to these two IMI herbicides. There was no cross-resistance to the PTB herbicide bispyribac-sodium since both the wild type and the mutant had a similar sensitive phenotype. For the SU herbicides, both the wild-type Lu37 and mutant Lu37-1 showed similar tolerance responses to nicosulfuron and mesosulfuron-methyl, although slight chlorosis occurred in the leaves of the wild-type Lu37 treated with nicosulfuron. Thus, the mutant Lu37-1 gained tolerance to IMI herbicides but not to PTB and SU herbicides.

ALS enzyme of mutant Lu37-1 was insensitive to imazapic

We further tested whether the ALS enzyme was involved in the tolerance to the herbicide imazapic. Various concentrations of imazapic (0.01–100,000 $\mu mol \cdot L^{-1}$) were used to analyze the inhibition effect on ALS activity *in vitro*. The ALS enzyme from the wild-type Lu37 was sensitive to imazapic, and the enzyme activity decreased at 0.1 $\mu mol \cdot L^{-1}$ imazapic, and the enzyme activity only remained 35% at 10 $\mu mol \cdot L^{-1}$ imazapic (Fig. 4a). In contrast, the ALS from the mutant Lu37-1 remained normal activity at 100 $\mu mol \cdot L^{-1}$ imazapic and still had

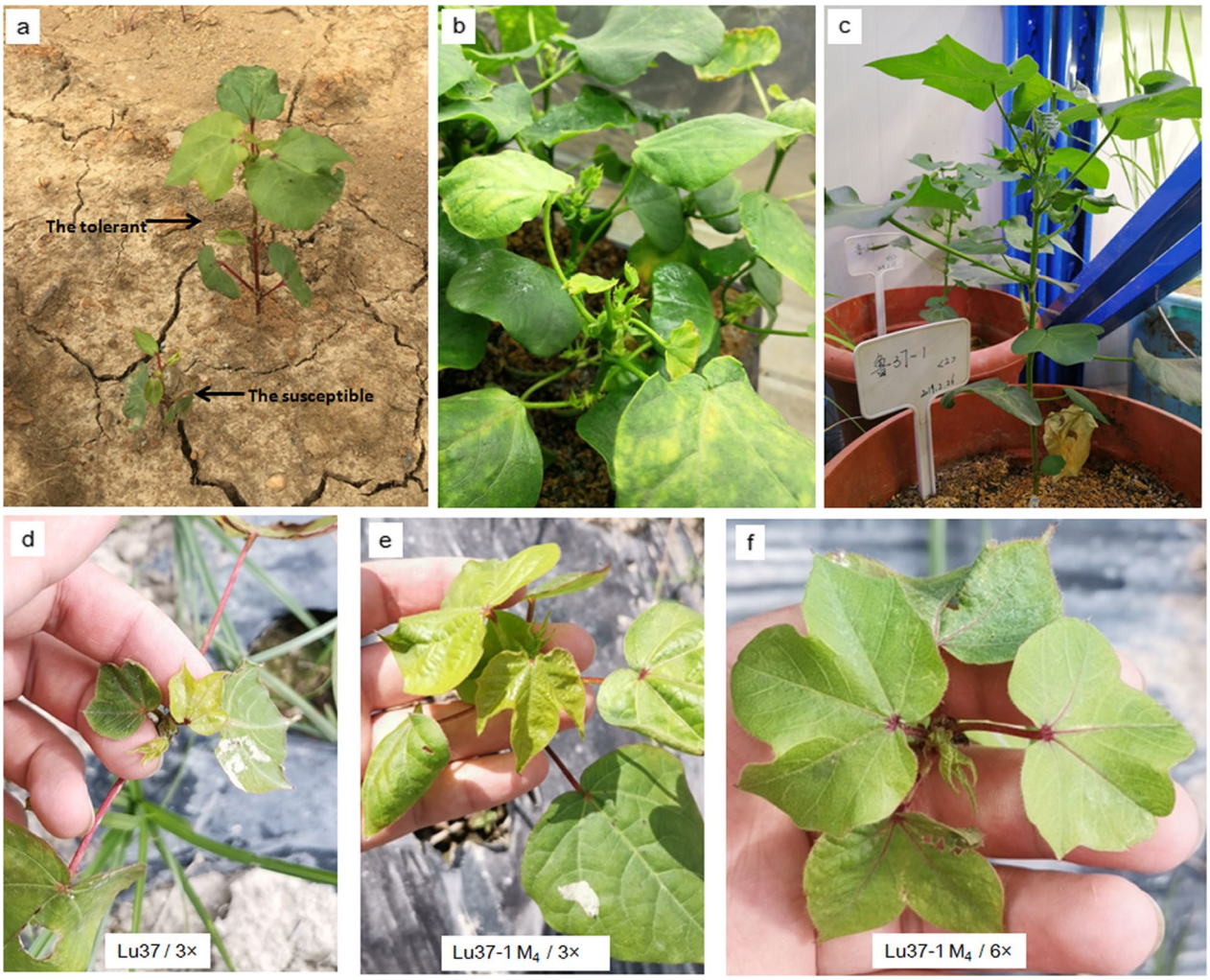


Fig. 1 Herbicide screening of the Lu37-1 cotton mutant with imazapic. **a** Field screening of the cotton M_2 generation by pre-emergent application of the 6 × the recommended rate. **b** At 44 DAT, susceptible M_3 plantlets under imazapic treatment in the growth chamber. **c** At 70 DAT, two imazapic-tolerant M_3 plantlets (i.e., Lu37-1-1 and Lu37-1-2) grown normally under treatment of imazapic in the growth chamber. **d** At 45 DAT, herbicide response of the wild-type Lu37 seedlings under treatment with 3 × the recommended rate of imazapic in the field. **e** At 45 DAT, herbicide response of the mutant Lu37-1 seedlings (M_4 generation) under treatment with 3 × the recommended rate of imazapic in the field. **f** At 45 DAT, herbicide response of the mutant Lu37-1 seedlings (M_4 generation) under treatment with 6 × the recommended rate of imazapic in the field

43% activity at 10 000 μmol·L^{-1} imazapic. The IC_{50} of imazapic to the ALS enzyme in the mutant Lu37-1 was 7 633.026 μmol·L^{-1}, increased by approximately 850 fold in comparison to the wild-type version of enzyme which had an IC_{50} of 8.983 μmol·L^{-1}. These results indicated that the tolerance of Lu37-1 to imazapic was closely related to the insensitivity of the mutanted ALS to this herbicide.

Identification of the ALS mutation site in the Lu37-1 mutant

Because plant resistance to the ALS-inhibitor herbicides was mostly attributed to mutations in ALS genes (Yu and Powles 2014), we firstly tested whether the Lu37-1 mutant had mutations in the cotton ALS genes. Therefore, the sequences of two ALS genes (*Gh_D10G1253* and *Gh_A10G1238*) amplified using the same primer pair could be easily clarified by their SNPs. Among the 20 sequencing clones, there are 4 clones from Lu37-1-1 and 4 clones from Lu37-1-2 containing the *Gh_D10G1253* sequence, which all had a G-to-A transition at position 1925 in the CDS of *Gh_D10G1253*, resulting in the conversion of Ser642 to Asn642 in the amino acid sequence (Fig. 4b), while the other 12 clones contained *Gh_A10G1238* sequence, which were 100% identical to the wild-type allele. Moreover, no wild-type sequence

Fig. 2 Greenhouse tests of the herbicide response of the Lu37-1 cotton mutant (M_5 generation) to different doses of imazapic. **a** At 26 DAT, wild-type Lu37 seedlings under treatment with 3× and 6 × the recommended rate of imazapic. **b** At 26 DAT, herbicide-tolerant Lu37-1-2 seedlings under treatment with 3×, 6×, 18×, 21×, and 24 × the recommended rate of imazapic. **c** At 26 DAT, herbicide-tolerant Lu37-1-1 seedlings under treatment with 3×, 6×, 9×, 12×, 18×, 21×, and 24 × the recommended rate of imazapic. **d** At 64 DAT, herbicide-tolerant Lu37-1-2 seedlings under treatment with 12×, 18×, 21×, and 24 × the recommended rate of imazapic

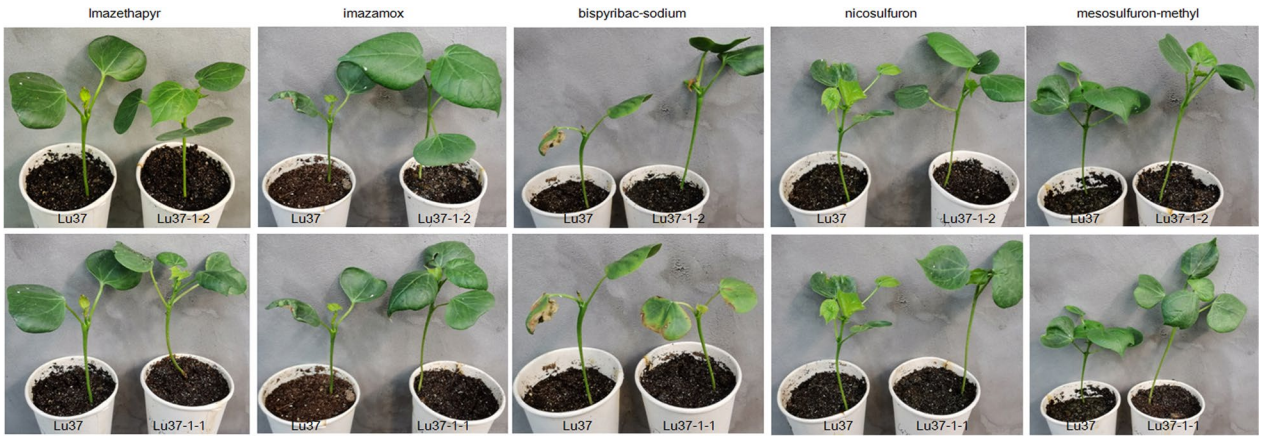


Fig. 3 Tests of the herbicide response of the Lu37-1 cotton mutant (M_5 generation) to other ALS-inhibitor herbicides under greenhouse growth condition. The herbicide-tolerant Lu37-1 and the wild-type Lu37 seedlings were sprayed with 3 × the recommended rate of ALS-inhibitor herbicides. Photos were taken at 26 DAT

of *Gh_D10G1253* was identified from these sequencing clones, indicating that the G-to-A mutation was homozygous in the *Gh_D10G1253* gene of the Lu37-1 mutant (i.e., Lu37-1-1 and Lu37-1-2).

The genetic diversity among 1 913 cotton accessions, including 1 623 *G. hirsutum*, 261 *G. barbadense*, and 29 other *Gossypium* species, was recently investigated against the reference genome of *G. hirsutum* TM-1 HAU v1.0 (Li et al. 2021). We retrieved the SNPs at the genomic region of *Gh_D10G1253* from this public database. Thirteen SNPs were found in the CDS of *Gh_D10G1253*, but no nucleotide diversity was found at Ser642 of *Gh_D10G1253* (Fig. 4c), suggesting that the Ser642 was highly conserved during domestication and the Ser642Asn mutation of *Gh_D10G1253* was a novel one.

CAPS marker for the ALS alleles

Sanger sequencing confirmed that the 416 bp polymerase chain reaction (PCR) products amplified by the primers GhD10ALS642-F1/Gh642M-R were the specific fragments amplified from the *Gh_D10G1253* gene based on the characteristic SNPs(data not shown). Next, the SNP (G/A) between the wild-type and the mutant allele of *Gh_D10G1253* was used to develop the CAPS marker for genotype detection. The 416 bp PCR product from the mutant contained the recognition sequence CAATTG for *Mfe*I, which cut the sequence product into 2 fragment of 383 bp and 33 bp, while the PCR product from the wild type plant remain undigested since there was no digestion site in its sequences (Fig. 4d).

Transgenic *Arabidopsis* was tolerant to a high rate of imazapic

In order to further confirm the mutation at the *Gh_D10G1253* was responsible for the increased tolerance to imazapic in cotton, the mutant allele of *Gh_D10G1253* was transformed to *Arabidopsis*. The T_1 and T_2 generations of transgenic *Arabidopsis* were screened on 1/2 MS medium supplemented with 50 mg·L^{-1}

(See figure on next page.)

Fig. 4 Molecular characteristics of the Lu37-1 cotton mutant. **a** The relative ALS activity of wild-type Lu37 and mutant Lu37-1 in response to different concentrations of imazapic. The absorbance of the sample without imazapic (0 μmol·L^{-1}) was set as 100%, while the others were presented as a percentage value relative to that of 0 μmol·L^{-1}. The values are the means ± SDs of two independent determinations. **b** Demonstration of the G-to-A mutation at 1 925 bp of the CDS (G1925A, upper case in red) which led to the Ser642Asn mutation in the amino acid sequence of *Gh_D10G1253*. The primers for the CAPS marker are marked with arrows, and a mismatched base pair (lower case letters in red) was designed in the reverse primer to create a recognition site (CAATTG, marked with the solid box) of endonuclease *Mfe*I. **c** The illustration of the Ser642Asn (marked by blue line) in the CDS sequnece of *Gh_D10G1253*. The 1 980-bp *Gh_D10G1253* CDS (green arrow) were blasted against the *G. hirsutum* HAU v1.0 genome on the CottonGen website. The SNPs among 1913 cotton accessions within the region of Ghir_D10: 22854388..22852409 from the results of Li et al. (2021) were marked in red line with corresponding chromosomal site. **d** Gel image of mutant allele and wild type allele of ALS digested by *Mfe*I. The ALS wild type allele was the 416bp band, while the mutant allele was cleaved into a smaller 383bp band by *Mfe*I

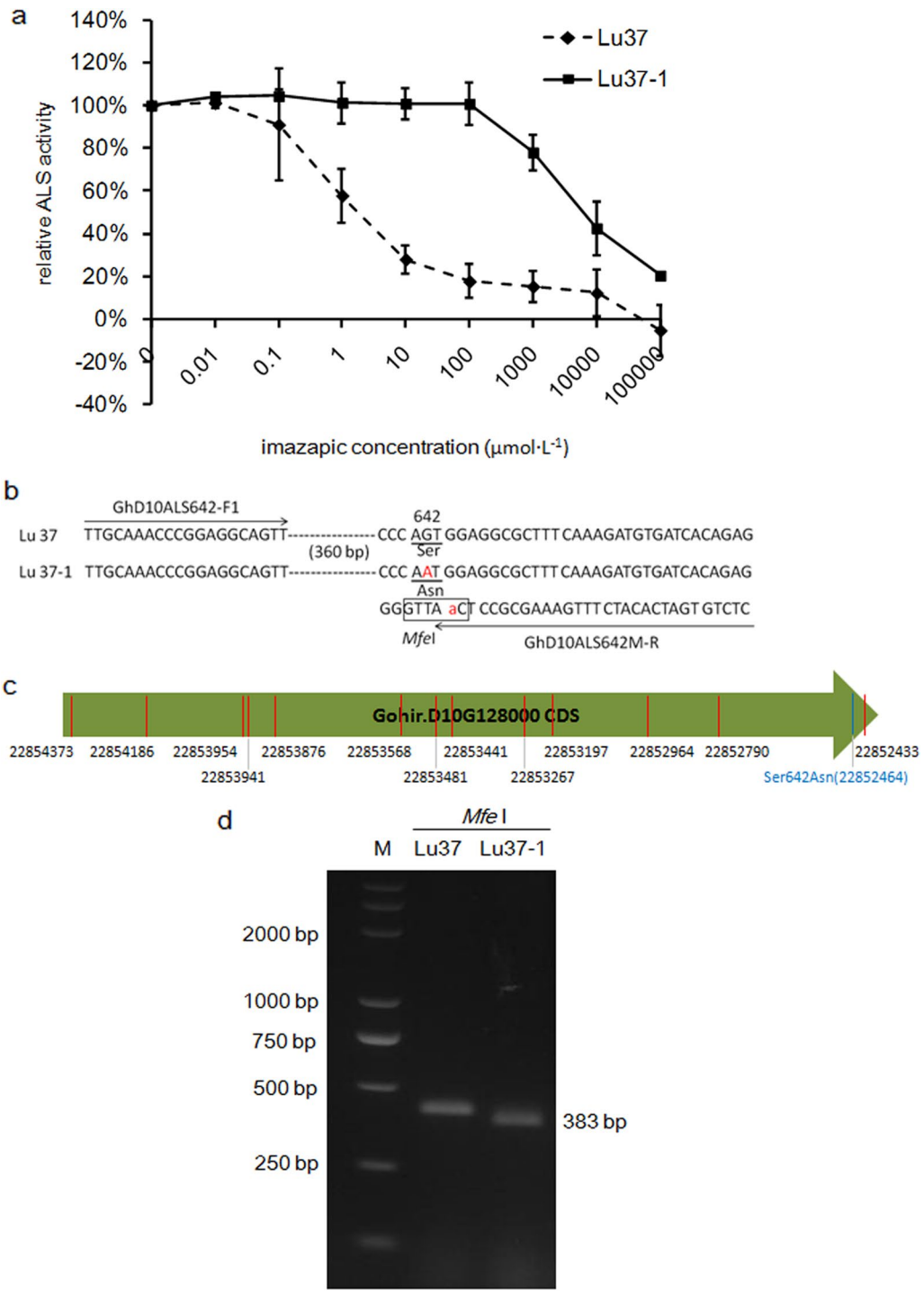


Fig. 4 (See legend on previous page.)

kanamycin to generate T_3 seeds. The T_3 plantlets as well as the wild-type Col were sown on the 1/2 MS supplemented with 0∼2 μmol·L^{-1} imazapic. At 21 DAT, the wild-type Col and some transgenic lines showed a chlorosis phenotype on 0.1 μmol·L^{-1} imazapic plate, while the resistant transgenic lines showed green leaves even on 2 μmol·L^{-1} imazapic plate (Fig. 5a), indicating at least 20-fold increased tolerance to continuous selection on imazapic plate. Based on the herbicide selection of T_3, the resistant T_4 lines were further confirmed on 1/2 MS medium supplemented with 0.5 μmol·L^{-1} imazapic

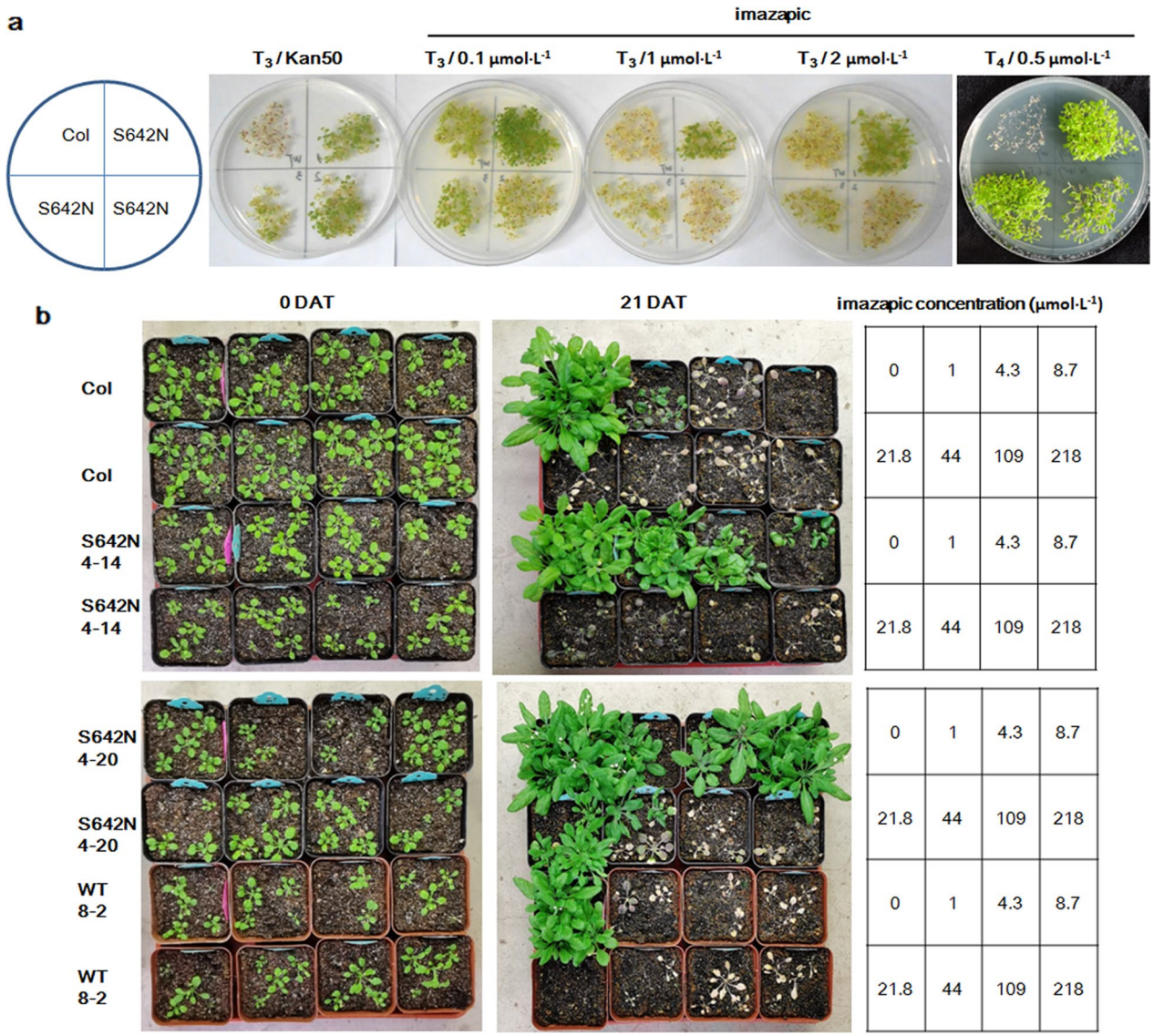


Fig. 5 Overexpression of mutant *Gh_D10G1253* conferred herbicide tolerance in *Arabidopsis*. **a** At 21 DAT, transgenic *Arabidopsis* S642N (T_3 and T_4 generations) cultured on 1/2 MS supplemented with different concentrations of imazapic. Transgenic lines of the T_3 generation and the wild-type Columbia (Col) were sown on 1/2 MS supplemented with 50 mg·L^{-1} kanamycin to confirm the transgenics. **b** At 21 DAT, transgenic *Arabidopsis* S642N (T_4 generations) sprayed with 2 mL of 0, 1, 4.3, 8.7, 21.8, 44, 109, and 218 μmol·L^{-1} of imazapic. Col is the wild-type line Columbia, S642N 4-14 and S642N 4-20 are transgenic *Arabidopsis* S642N lines, and WT 8-2 is a transgenic line with a wild-type allele of ALS gene serving as a transgenic control

(Fig. 5a). The resistant T_4 lines grown in pots were also sprayed with 2 mL imazapic at different concentrations to test their resistance levels. At 21 DAT, the wild-type Col and the transgenic *Arabidopsis* expressing the wild-type ALS (WT 8-2) developed a phenotype with purplish color and stunted shoot tips and eventually died at 1 μmol·L^{-1} imazapic (Fig. 5b). In contrast, the resistant transgenic *S642N* lines (S642N 4-14, 4-20) were tolerant to 8.7 μmol·L^{-1} imazapic, and some lines (i.e., S642N 4-20) were even tolerant to 44 μmol·L^{-1} imazapic, with at least 40-fold higher tolerance than the susceptible *Arabidopsis* lines.

Discussions

The Lu37-1 cotton mutant tolerant to IMI herbicides offers an alternative choice for weed management

Herbicide resistance is an important and valuable trait in agricultural production since it offers an effective and economic approach to control weeds when used together withherbicides. As of 25 December 2022, there

are 45 transgenic herbicide tolerance events commercially available in cotton globally, which integrate one, two, or three traits of herbicide tolerance to glufosinate, glyphosate, oxynil, 2,4-D, dicamba, sulfonylurea, or isoxaflutole, according to the GM Approval Database (https://www.isaaa.org/gmapprovaldatabase/commercialtrait/default.asp?TraitTypeID=1&TraitType=Herbicide%20Tolerance). However, none of these transgenic cotton events have been commercialized in China. Some transgenic cotton lines overexpressing the *G2-aroA* or *pGR79 EPSPS-pGAT* genes, which possess resistance to glyphosate, have been developed in China (Liang et al. 2017; Zhang et al. 2017). However, the commercialization of these transgenic events still have a long way to go in China since genetically-modified crops are under strict regulation and must undergo long approval processes with expensive registration cost. In contrast, herbicide-tolerant crops obtained by traditional mutation breeding methods are easier to be accepted by the public and governments. Here, we report the cotton mutant Lu37-1 from EMS mutagenesis that was highly tolerant to IMI-herbicides, offering a non-transgenic choice for weed management.

Cotton is sensitive to IMI herbicides. When planted in rotation with peanut, cotton plants have shown visibly injury (19%∼58% injury) by the imazapic residual from the previous application of 70 g a.i. ha^{-1} (manufacturer's recommended rate) or 140 g a.i. ha^{-1} in the peanut field, which resulting in delayed cotton maturity and reduced yield (York et al. 2000). Here, the cotton mutant Lu37-1 possesses tolerance to 24 × the recommended rate of imazapic, or by eight fold elevated tolerance to imazapic when compared with the wild type whose growth has been completely inhibited at 3 × the recommended rate (Fig. 2). The high IMI-tolerant cotton Lu37-1 may protect the cotton plant from damage by IMI herbicides in crop rotation. Notably, 24 × the recommended rate of imazapic inhibited the growth of the Lu37-1 mutant plants, resulting in a dwarf phenotype when compared with plants treated with 12 × the recommended rate (Fig. 2). Therefore, the rate above 24 × the recommended rate of imazapic has not been tested in this study.

Molecular mechanism of Lu37-1 tolerance to IMI herbicides

The mechanisms of plant resistance to herbicides can be broadly classified into target-site resistance and nontarget-site resistance. Target-site resistance can occur when gene mutation confers an amino acid change in the target site of action of an herbicide, resulting in an insensitive or less sensitive target protein to the herbicide, or it can be conferred by the overexpression or amplification of the target gene of the herbicide (Powles and Yu 2010). Plant resistance to ALS-inhibitor herbicides is ofen associated with point mutations in the target *ALS* gene (Yu and Powles 2014), meanwhile, cytochrome P450 mediated metabolism may also occur in certain wheat cultivars (Rojano-Delgado et al. 2014). The *ALS* gene family of cotton (*G. hirsutum*) contains 6 *ALS* genes, and the A5 (herein Gh_A10G1238/Gohir.A10G138800 in CottonGen) and A19 (herein *Gh_D10G1253*/*Gohir.D10G128000* in CottonGen) genes are the most conserved genes of *ALS* (Grula et al. 1995). Both the analysis of gene sequences and enzyme activity of ALS have confirmed that the herbicide tolerance of cotton mutant Lu37-1 is a target-site resistance attributed by a less sensitive mutanted ALS enzyme, which has a G-to-A mutation at the position which encode Ser642 of *Gh_D10G1253*. The G-to-A mutation was consistent with the finding that C/G to T/A transitions were the predominant mutations in EMS mutagenesis (Shirasawa et al. 2016). Moreover, the mutation in Ser642 of *Gh_D10G1253* was not found in the natural 1 913 cotton accessions (Li et al. 2021). Transgenic *Arabidopsis* overexpressed the mutant *Gh_D10G1253* phenotype further confirm its tolerance to the IMI herbicide. Together, the Ser642Asn mutation in the cotton ALS gene *Gh_D10G1253* confers tolerance to IMI herbicides.

The comparison of ALS proteins revealed that Ser642Asn in the cotton mutant Lu37-1 corresponded to the mutant Ser653Asn in *Arabidopsis* and Ser627Asn in rice, which has been widely used to develop commercialized IMI-tolerant maize, oilseed rape, wheat, and rice (Edi et al. 2013; Tan et al. 2005). In addition to Ser642Asn, Ser642Thr (in cotton ALS numbering) mutant of *Arabidopsis* and tobacco are also IMI-resistant, and Ser642Phe *Arabidopsis* mutant is resistant to both IMI and SU (Chong and Choi 2000; Lee et al. 1999). These results together suggest that Ser642 is a highly conserved and herbicide tolerance-endowing mutant site in ALS among plant species. Therefore, it is technically possible to develop IMI-tolerant traits in cotton and other crops, either using a native mutant form such as the EMS cotton mutant Lu37-1 in this study or in a transgenic approach (Rajasekaran et al. 1996b).

Conclusions

The cotton mutant Lu37-1 generated from EMS mutagenesis was strongly tolerant to IMI herbicides. The herbicide tolerance was attributed to the G-to-A transition at the position which encode Ser642 of the ALS gene *Gh_D10G1253*. The G-to-A (Ser642Asn) allele in *Gh_D10G1253* has not been found in natural cotton species. A CAPS marker has been developed to genotype the herbicide-tolerant ALS allele. Transgenic *Arabidopsis* overexpressing the mutant *Gh_D10G1253* confer high tolerance to IMI herbicides. The *Gh_D10G1253* mutant

ALS gene can be used to develop IMI herbicide-tolerant crops via a non-transgenic or transgenic approach.

Methods

Seed mutagenesis and selection of herbicide-tolerant cotton

According to the pre-experiment for the determination of the median lethal dose (LD_{50}), delinted cotton seeds of cultivar Lumianyan 37 (Lu37) were imbibed in tap water for 8 h and then treated with 0.8% (w/v) of EMS (Sigma, USA; product number: M0880) for 14 h, followed by a treatment with 3.5% (w/v) of sodium thiosulfate (Sinopharm, Shanghai, China) for 30 min. The mutagenized seeds were rinsed in tap water 5 times for 5 min each time, and then planted in field. The plants arising from these seeds were M_1 (mutant generation 1) plants, whose M_2 seeds were harvested in bulk. M_2 seeds were individually sown in field, immediately followed by a single pre-emergent application of the IMI herbicide PLATEAU® (BASF, Shanghai, China) at a rate of 518.4 g a.i. ha^{-1} (6 × the recommended rate in peanut field). The PLATEAU® contains the active ingredient imazapic at a concentration of 240 $mg{\cdot}mL^{-1}$, and the recommended application rate in peanut field is 86.4 g a.i. ha^{-1}. Four to eight M_3 seeds obtained from each tolerant M_2 plant were sown in 32-well trays (54 × 28 × 6.3 cm) and then placed in the plant growth chamber. The M_3 plantlets at the 2–3 leaf stage were sprayed with PLATEAU® 4 times within 15 days, i.e., 1 × the recommended rate on the 1st and 5th days and 3 × the recommended rate on the 10th and 15th days. One M_2-tolerant mutant line and its progenies were designated as Lu37-1 for further study.

Herbicide tolerance level assay

To determine of the resistance levels of Lu37-1 to ALS-inhibitor herbicides, the M_4 seeds of Lu37-1 as well as the wild-type Lu37 were sown in the field and sprayed with PLATEAU® at 3 and 6 × of the recommended rate at the 3–4 leaf stage. The M_5 generation was screened in the greenhouse growth condition with imazapic and other ALS-inhibitor herbicides. Seeds of both Lu37-1 and Lu37 were sown in pots in greenhouse, and seedlings at the 1-leaf stage were treated with imazapic (IMI class), imazethapyr (IMI class), imazamox (IMI class), bispyribac-sodium (PTB class), nicosulfuron (SU class) and mesosulfuron-methyl (SU class), respectively. The greenhouse was maintained at 30 ± 3/25 ± 3 °C day/night temperature with natural sunlight. For imazapic treatment, the seedlings of mutant Lu37-1 were sprayed with different doses of PLATEAU®, including 3×, 6×, 9×, 12×, 18×, 21×, and 24 × the recommended rate, while the wild-type Lu37 seedlings were treated with 3× and 6 × the recommended rate. For treatments with imazethapyr, imazamox, bispyribac-sodium, nicosulfuron and mesosulfuron-methyl, the application rate at 3 × the recommended rate was tested in both mutant and wild type. The treatment with spraying water only served as a control at 0 × the recommended rate. The recommended rate was 97.5 g a.i. ha^{-1} for imazethapyr (Shandong Vicome Greenland Chemical Co., Ltd, Shandong, China), 45 g a.i. ha^{-1} for imazamox (Jiangsu Agrochem Laboratory Co., Ltd, Nanjing, China), 30 g a.i. ha^{-1} for bispyribac-sodium (Jiangsu Agrochem Laboratory Co., Ltd, Nanjing, China), 51 g a.i. ha^{-1} for nicosulfuron (Beijing Green Agricultural Science and Technology Group Co., Ltd, Beijing, China), and 13.5 g a.i. ha^{-1} for mesosulfuron-methyl (Jiangsu Agrochem Laboratory Co., Ltd, Nanjing, China) respectively.

ALS enzyme activity assay

A modified protocol according to Chen et al. (1996) and Singh et al. (1988) was used to test ALS enzyme activity in cotton. Cotton calli were induced from cotyledon explants in MS medium + 0.1 $mg{\cdot}L^{-1}$ 2,4-D + 0.1 $mg{\cdot}L^{-1}$ KT + 30 $g{\cdot}L^{-1}$ glucose + 2.5 $g{\cdot}L^{-1}$ Phytagel for 40 d. For crude enzyme extraction, 1 g calli was harvested and ground in 1 mL extraction buffer (100 $mmol{\cdot}L^{-1}$ potassium phosphate buffer (pH 7.5) containing 5 $mmol{\cdot}L^{-1}$ $MgCl_2$, 0.5 $mmol{\cdot}L^{-1}$ thiamine pyrophosphate (TPP), and 1 $mmol{\cdot}L^{-1}$ dithiothreitol) in a mortar. The homogenate was centrifuged at 25 000 × *g* for 20 min, and the clear supernatant was used directly for the assay. All samples were kept at 4 °C or on ice during this process. The assay mixture was prepared by adding the following solution to a final volume of 0.9 mL: 650 µL of reaction buffer (50 $mmol{\cdot}L^{-1}$ potassium phosphate buffer (pH 7.0), 1 $mmol{\cdot}L^{-1}$ $MgCl_2$, and 1 $mmol{\cdot}L^{-1}$ TPP), 100 µL of imazapic dissolved in the reaction buffer with final concentrations of 0∼100 000 $\mu mol{\cdot}L^{-1}$, 100 µL of crude enzyme extraction, and 50 µL of 3.6 $mol{\cdot}L^{-1}$ pyruvate. For the blank control, pyruvate and imazapic were omitted from the assay mixture. All mixtures were incubated at 37 °C for 30 min, followed by mixing with 50 µL of 3 $mol{\cdot}L^{-1}$ H_2SO_4, and then incubated at 60 °C for 15 min. Then, 0.5 mL of 0.83 $g{\cdot}L^{-1}$ creatine and 0.5 mL of 8.3 $g{\cdot}L^{-1}$ 1-naphthol were added to the mixture and incubated at 60 °C for 15 min and then incubated at 37 °C for 30 min. The absorption of the mixture was measured at 530 nm with a ScanDrop® 250 spectrophotometer (Analytik Jena, Germany). The relative ALS activities were presented as the percentage value of the absorption values in the presence of the herbicide imazapic to that of the treatment without herbicide. The IC_{50} (half maximal inhibitory concentration) of imazapic to the ALS

enzyme was estimated in SPSS Statistics version 22 (IBM, Armonk, NY, USA) using the PROBIT procedure.

Identification of mutation sites in the ALS gene

The A19 gene (NCBI accession: Z46959.1) and A5 gene (NCBI accession: Z46960.1) were considered to be the most conserved ALS genes in cotton (Grula et al. 1995). The A19 and A5 genes were blasted against the reference genome of *G. hirsutum* L. acc. TM-1 in the CottonGen website (https://www.cottongen.org/blast), and gene ID were corresponded to the *Gh_D10G1253*/*Gohir.D10G128000* and Gh_A10G1238/Gohir.A10G138800 in the TM-1, respectively. Both ALS genes only have one extron without intron. According to the conserved sequences between the two cotton ALS genes, the primers GhALS-F1 (5′-CAC CACAAGCCTCTCATCGAC-3′) and GhALS-R1 (5′-CCATAACCCACCCTCATTC-3′) were used to amplify the CDS of the two ALS genes. Cotton genome DNA was extracted from the leaves of the wild type Lu37 and the herbicide tolerant cotton plants (Lu37-1–1 and Lu37-1–2). The PCR solution contained 10 μL of 2 × Phanta Max buffer, 0.4 μL of dNTP mix (10 mmol·L^{-1} each), 0.2 μL of Phanta Max SuperFidelity DNA polymerase (Vazyme, Nanjing, China), 0.2 μL of dimethyl sulfoxide (DMSO), 1 μL of primers (10 μmol·L^{-1} of each), 1 μL of genomic DNA, and 6.2 μL of ddH_2O. The PCR program was 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C C for 2 min; and then followed by 72 °C for 5 min and 16 °C for 1 min. The PCR products were TOPO-cloned into the pESI-Blunt vector (Yeasen, Shanghai, China) and then 10 independent PCR positive clones from each line were subjected to Sanger sequencing (Tsingke, Nangjing, China). The SnapGene software was used for DNA sequence alignment to identify the mutation site.

CAPS marker in ALS alleles

The forward primer GhD10ALS642-F1 (5'-TTGCAA ACCCGGAGGCAGTT-3') specifically matched to *Gh_D10G1253* was designed with SnapGene software. According to the web-based dCAPS Finder 2.0 (Neff et al. 2002), an additional C-to-A mismatch was designed in the primer GhD10ALS642M-R (5'-CTCTGTGATCACATCTTTGAA AGCGCCTCaA-3') to create a restriction site CaATTG for endonuclease *Mfe*I to identify the mutant ALS sequence. The PCR solution contained 10 μL of 2 × Rapid Taq Master Mix (Vazyme, Nanjing, China), 0.5 μL of primers (10 μmol·L^{-1} each), 1 μL of genomic DNA, and 8 μL of ddH_2O. The PCR program was 95 °C for 3 min; 35 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C C for 15 s; and then followed by 72 °C for 5 min and 16 °C for 1 min. The PCR product (416 bp) was first subjected to Sanger sequencing and then to endonuclease digestion with *Mfe*I. For *Mfe*I digestion, 1 μL of 10 × Cutsmart buffer, 4 μL of 416 bp PCR product, 1 μL of *Mfe*I (NEB, Beijing, China), and 4 μL of ddH_2O were well mixed and incubated at 37 °C for 3 h. The digestion products were run on 2% agarose gels at 100 V for 1 h.

Herbicide resistance assay in transgenic *Arabidopsis*

The coding sequences (CDS) of the wild type and the mutant *Gh_D10G1253* ALS genes were amplified with the primers GhALS-F3 5'- CGGGGGACTCTAGAGGAT CACACCACAAGCCTCTCATCGAC-3' and GhALS-R3 5'- GGGGAAATTCGAGCTCGGTACCCCATAACC CACCCTCATTC-3' using the corresponding TOPO clones, respectively. The PCR products were then cloned into the cassette under the control of the CaMV35S promoter in the binary vector pCAMBIA2301 by the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). Transgenic T_1 and T_2 *Arabidopsis* plants were screened by 50 mg·L^{-1} kanamycin supplied in 1/2 MS medium after transformation by the floral dip method (Clough and Bent 1998). The T_3 and T_4 generations of transgenic *Arabidopsis* as well as the wild-type Columbia (Col) were sown and screened on 1/2 MS medium supplemented with 0∼2 μmol·L^{-1} imazapic. Herbicide-resistant T_4 plantlets were further tested on 1/2 MS medium supplemented with 0.5 μmol·L^{-1} imazapic and in pots by spraying with 2 mL of imazapic at different concentrations (0, 1, 4.3, 8.7, 21.8, 44, 109, and 218 μmol·L^{-1}).

Abbreviations

ALS	Acetolactate synthase
AHAS	Acetohydroxyacid synthase
CAPS	Cleaved amplified polymorphic sequences
EMS	Ethyl methanesulfonate
IMI	Imidazolinone
SU	Sulfonylurea
SCT	Sulfonyl-aminocarbonyl-triazolinone
TP	Triazolopyrimidine
PTB	Pyrimidinyl-thiobenzoates

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Author contributions

Zhang BL raised the funding and conceived the experiments. Chen TZ designed, performed the experiments, analyzed the data, and prepared the manuscript. Ling XT and Yu Y performed the experiments. All authors have read and approved the final manuscript.

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The authors declare that they have no competing interests.

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