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Kamil Haliloğlu, Aras Türkoğlu, Murat Aydin & Hadi Alipour

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Development of novel imazamox tolerant wheat lines via combined *in vivo* and *in vitro* EMS mutagenesis

Kamil Haliloğlu[1], Aras Türkoğlu[2]*, Murat Aydin[3] , Hadi Alipour[4,*]

[1]Department of Biology, Faculty of Science, Gazi University, Ankara, Türkiye; kamil.haliloglu@gazi.edu.tr (K.H.)

[2]Department of Field Crops, Faculty of Agriculture, Necmettin Erbakan University, Konya, Türkiye; aras.turkoglu@erbakan.edu.tr (A.T.)

[3]Department of Field Crops, Faculty of Agriculture, Atatürk University, Erzurum, Türkiye; maydin@atauni.edu.tr (M.A.)

[4]Department of Plant Production and Genetics, Urmia University, Urmia, Iran; ha.alipour@urmia.ac.ir (H.A.)

*Correspondence: aras.turkoglu@erbakan.edu.tr (A.T.); ha.alipour@urmia.ac.ir (H.A.)

Abstract

Acetolactate synthase (*AHAS*) mutations conferring imidazolinone herbicide resistance typically occur at conserved amino acid positions (Ala122, Ser653) in wheat. This study employed ethyl methanesulfonate (EMS) mutagenesis using two complementary approaches: *in vivo* seed treatment (50,000 M_1 seeds) and *in vitro* callus-based mutagenesis (126,000 calli) to generate novel *AHAS*-resistant wheat lines. Multi-stage selection across M_2–M_4 generations using germination-stage and foliar herbicide applications identified four stable mutant lines: ML-1, ML-2, ML-3 (seed-derived), and InVitML-1 (*in vitro*-derived). Sanger sequencing of the 1,123 bp *AHAS* fragment identified five distinct SNPs: G→A at position 502, T→C at position 1075, T→A at position 11, A→T at position 386, and a rare adjacent dual substitution (T→C, C→T) at positions 1119–1120. Notably, all five mutations were located outside canonical hotspots (Ala122, Pro197, Ala205, Trp574, Ser653), suggesting the involvement of alternative structural mechanisms for *AHAS* tolerance. Bradford assay showed mutant lines maintained *AHAS* protein concentrations of 4.60–4.93 µg/mL under imazamox stress, compared with 1.97 µg/mL in susceptible controls, consistent with target-site resistance. The mutation spectrum predominantly exhibited G:C→A:T transitions, as expected from EMS mutagenesis, confirming the chemical mutagen's signature. These results provide strong initial evidence that non-canonical *AHAS* mutations can confer herbicide tolerance in polyploid wheat, extend the known genetic basis of imidazolinone resistance, and demonstrate the effectiveness of dual mutagenesis strategies for generating novel allelic resources for non-transgenic crop improvement programs.

Keywords: Ethyl methanesulfonate, *AHAS*, acetolactate synthase, target site resistance, chemical mutagenesis, *Triticum aestivum*, wheat breeding

Introduction

Wheat (*Triticum aestivum* L.) is a cornerstone of global food security, occupying more arable land than any other food crop [1]. With global production in the 2025/26 cycle estimated at a record 801.4 million metric tons, its contribution to global food security and agricultural sustainability is indispensable [2, 3]. Despite this immense production capacity, the sustainability of wheat cultivation is increasingly threatened by diminishing land availability and persistent weed pressure, which represents one of the most significant biotic constraints to realizing genetic yield potential [4]. When weeds are left uncontrolled, yield losses typically range from 10% to 50%, fluctuating depending on weed density and the duration of competition [5, 6]. While herbicides remain the most cost-effective solution, the intensive use of a limited range of chemical modes of action has triggered a global evolutionary crisis. Wheat systems currently rank first in reported resistance cases worldwide, with 84 weed species that have evolved resistance to date, bringing the global total to 541 unique cases [7].

The economic viability of wheat is challenged by a complex of monocotyledonous and dicotyledonous weeds, such as Phalaris paradoxa, Alopecurus myosuroides, and species of the genus Lolium, whose genetic diversity allows them to colonize winter cereal fields despite chemical control measures [8]. Repeated herbicide application exerts evolutionary pressure that selects for target-site resistance (TSR), involving mutations in the herbicide-binding enzyme, and non-target-site resistance (NTSR), characterized by enhanced metabolic detoxification [9]. Acetolactate synthase (*AHAS*; also known as ALS; EC 2.2.1.6) is the critical target enzyme for imidazolinone (IMI) herbicides, catalyzing the first committed step in branched-chain amino acid biosynthesis. Inhibition of *AHAS* leads to the depletion of valine, leucine, and isoleucine, causing rapid growth arrest and plant death [10, 11]. IMI herbicides (HRAC Group 2) are highly valued for their broad-spectrum weed control and low mammalian toxicity [10]. However, the intrinsic sensitivity of wheat to IMI herbicides necessitates the development of herbicide-tolerant (HT) cultivars

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to enable their safe agronomic deployment [12]. However, the intrinsic sensitivity of wheat to IMI herbicides necessitates the development of herbicide tolerant (HT) cultivars to enable their safe agronomic deployment.

Resistance typically arises from point mutations in the *AHAS* gene that reduce herbicide binding without impairing enzymatic function [13]. For instance, in legume crops and Arabidopsis, a C to T mutation at codon 205 (Ala to Val) in the *AHAS*1 gene has been shown specifically to confer IMI resistance [14, 15]. In polyploid wheat, canonical mutations such as A96T, P171L, and A179V in the *TaALS-D* gene, or mutations at the Ala122 position, confer varying levels of tolerance depending on the genomic context [16, 17]. Although transgenic HT crops have been successfully commercialized, their adoption in wheat has been constrained by regulatory frameworks and public perception regarding gene flow. Consequently, non-transgenic approaches, including mutation breeding and *in vitro* selection, are increasingly preferred as socially accepted and regulatorily streamlined alternatives [18, 19].

Recent advancements in mutation breeding have demonstrated that while targeted genome-editing technologies like CRISPR/Cas9 provide unparalleled precision and can now efficiently multiplex across polyploid sub-genomes [20], chemical mutagenesis with ethyl methanesulfonate (EMS) offers distinct and complementary advantages in forward genetics [18, 21]. Because CRISPR relies on predefined guide RNAs typically designed for established resistance hotspots, it may overlook uncharacterized functional variants. In contrast, EMS mutagenesis facilitates an untargeted, genome-wide exploration, enabling the discovery of novel, non-canonical mutations that would not be intuitively targeted by directed editing approaches. Furthermore, EMS generates broad allelic diversity in a non-transgenic framework, circumventing regulatory barriers and public perception concerns associated with genetically modified organisms, thereby accelerating the commercial deployment of elite varieties [19]. *In vitro* EMS mutagenesis, particularly when coupled with cellular-level herbicide selection, provides a powerful platform for rapidly isolating rare resistance alleles [22, 23].

Imidazolinone herbicides are highly valued in global wheat production due to their exceptional biological potency and low application rates, offering a sustainable and effective alternative for integrated weed management [24]. Development of crop varieties resistant to *AHAS* inhibiting chemical classes, including imidazolinones, sulfonylureas, triazolopyrimidines, and pyrimidyloxybenzoates, has been extensively demonstrated through various mutagenesis techniques, such as seed, microspore, and pollen treatments [21]. Therefore, the induction of novel resistance-conferring alleles through mutagenesis represents a strategic approach for facilitating the integration of IMI herbicides into sustainable wheat-based weed management systems. By expanding the genetic repertoire of wheat through induced variation, breeders can bypass the limitations of existing germplasm and develop elite lines tailored to modern agronomic challenges.

The mutation spectrum and resistance mechanisms characterized here also have implications for understanding weed evolution. If non-canonical *AHAS* mutations can confer resistance in crops, they may also arise spontaneously in weed populations under herbicide selection, contributing to the 541 reported herbicide resistance cases worldwide [7]. This evolutionary parallel underscores the importance of monitoring resistance beyond canonical ALS mutation sites. Proactive surveillance for non-hotspot mutations in weed species may inform resistance management strategies and herbicide stewardship programs [8, 25]. Recent investigations in chickpea and cotton further demonstrated that herbicide tolerance may also arise from non-canonical *AHAS* substitutions located outside the classical resistance hotspot residues, suggesting the involvement of alternative structural and functional resistance mechanisms [26, 27]. Moreover, recent structural and evolutionary analyses indicated that amino acid substitutions distant from the direct herbicide-binding cavity may alter long-range conformational dynamics, substrate accessibility, and protein stability under herbicide pressure, thereby expanding the mutational landscape of *ALS*/*AHAS*-mediated resistance [28, 29]. Despite these advances, information regarding stable imazamox-tolerant hexaploid wheat lines carrying non-canonical *AHAS* mutations remains extremely limited, particularly outside the well-characterized resistance hotspots such as Ala122 and Ser653. Therefore, identifying alternative resistance-associated *AHAS* alleles may significantly broaden the genetic basis of imidazolinone tolerance and provide novel molecular resources for non-transgenic wheat improvement and marker-assisted breeding programs. The novelty of the present study lies in the identification of stable imazamox-tolerant wheat mutant lines carrying *AHAS* mutations outside the canonical resistance hotspots, suggesting that non-canonical allelic variation may contribute to alternative mechanisms of imidazolinone tolerance in hexaploid wheat.

Accordingly, this study aimed to develop stable imazamox-tolerant wheat lines through complementary in vivo seed mutagenesis and in vitro callus-based EMS mutagenesis, followed by multigenerational herbicide selection, molecular characterization of *AHAS* variants, and biochemical assessment of herbicide response.

Materials and Methods

Plant material

Bread wheat (*Triticum aestivum* L.) cultivar Kırik was obtained from the Eastern Anatolia Agricultural Research Institute (Türkiye). Kırik is a spring type cultivar characterized by a long, slender culm; a narrow flag leaf

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angle; awnless spikes (approximately 9 cm); reddish, pubescent glumes; and large, white, plump grains. The cultivar exhibits low tillering capacity and high responsiveness to mature embryo derived callus induction, making it suitable for in vitro studies.

Experimental overview

Two distinct mutagenesis approaches were evaluated using different biological materials and methodologies: (A) *in vitro* mutagenesis applied to mature embryo derived calli, and (B) *in vivo* seed mutagenesis followed by laboratory and field screening. Both methods utilized ethyl methanesulfonate (EMS) as the mutagen.

Method I: *in vitro* mutagenesis

Callus induction, somatic embryogenesis, and plant regeneration; mature embryos were cultured using an endosperm supported system on Murashige and Skoog (MS) medium supplemented with 12 mg L^{-1} dicamba, 0.5 mg L^{-1} IAA, 20 g L^{-1} sucrose, 2 g L^{-1} phytagel, and 1.95 g L^{-1} MES (2-(N-morpholino)ethanesulfonic acid) and were incubated in darkness at 25 ± 1 °C for 30 days. Calli were transferred to hormone free MS medium for two weeks, then regenerated on MS medium containing 0.5 mg L^{1} TDZ (Thidiazuron) under a 16:8 hour photoperiod. Regenerated plantlets were acclimatized and grown to maturity to produce M_1 seed. EMS Treatment: Based on previous optimization (LD_{50} criterion: 50% reduction in regenerated plants), 0.1% (v/v) EMS applied for 8 hours was determined to be the optimal dose for endosperm supported mature embryos. Embryogenic calli were immersed in an EMS solution prepared in MS medium with continuous, gentle agitation, then were thoroughly washed and transferred to regeneration medium. This treatment was applied as the standard EMS condition in all *in vitro* experiments [23].

Method II: *in vivo* seed mutagenesis

EMS Treatment of Seeds: Approximately 50,000 M_1 seeds were pre soaked in deionized water at room temperature (22–24°C) for 4 hours. Based on established protocols [30, 31], seeds were treated with 0.3% (v/v) EMS for 6 hours with gentle agitation. After treatment, seeds were thoroughly rinsed with distilled water and dried at room temperature before sowing.

Field sowing and cultivation

Treated seeds were sown on May 2, 2014, at the Agricultural Research and Extension Center, Atatürk University, at a high density of 575 seeds/m² to favor mutations in the main culm and primary tillers. Standard agronomic practices were applied, including split applications of nitrogen (60 kg ha^{1}) and phosphorus (50 kg ha^{1} as P_2O_5). Upon reaching physiological maturity, M_1 plants were bulk-harvested. To maintain seed viability and mutation stability across the extended timeline of the breeding program, all seeds from M_1 and subsequent generations were dried to 10–12% moisture content and stored in a controlled cold room facility at +4°C and 45% relative humidity until required for subsequent sowing and screening activities.

Herbicide based selection using imazamox

***In vitro* selection**

Mutagenized calli were transferred to regeneration medium supplemented with 0.050 µM imazamox, which was previously determined to be the effective selective concentration. Surviving calli were transferred to fresh selective medium every two weeks until plantlet emergence and then transferred to hormone free MS medium [32].

***In vivo* seed stage selection**

In vivo selection was performed following Haliloğlu et al. [33]. Two application methods were used: seed treatment, in which wheat (*Triticum aestivum* L.) seeds were exposed to 60 ppm imazamox; and foliar treatment, in which seedlings were sprayed with imazamox. The foliar application was delivered using a calibrated sprayer set to a standard volume of 200 L/ha, delivering 50 ppm imazamox, which corresponds to an application rate of 10 g active ingredient per hectare (g a.i./ha). These doses were previously determined as optimal based on germination and seedling growth responses. Approximately 250,000 M_2 seeds (10.25 kg) underwent high throughput germination stage screening in peat filled trays (50 × 40 × 15 cm). Trays were irrigated daily with the herbicide containing solution to maintain consistent selection pressure. Putative herbicide tolerant seedlings were identified after 7–10 days by the absence of chlorosis and by normal root and shoot development, whereas candidate lines remained asymptomatic and continued to develop normally.

Verification of herbicide resistance in the M_2–M_4 generation

From the M_2 through the M_4 generations, selected lines underwent continuous phenotypic screening, with selection at both the seed stage (60 ppm imazamox priming) and the foliar stage (50 ppm foliar spray). Individual M_2 plants were harvested to establish discrete M_3 families. Within the M_3 generation, intense selection pressure was applied at both the seed and foliar stages to eliminate segregating susceptible individuals and to identify uniformly tolerant lines. This screening protocol was maintained through M_4 to ensure genetic fixation of the herbicide tolerance trait.

Molecular characterization of *AHAS* gene

DNA extraction and PCR amplification

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Genomic DNA was extracted from M_3–M_4 candidate mutant lines and from the untreated Kırik control using a modified CTAB method [34]. DNA concentration and purity were assessed using a NanoDrop spectrophotometer and 0.8% agarose gel electrophoresis. Prior to PCR, DNA samples were standardized to 50 ng/µL. PCR reactions (total volume 20 µL) contained 50 ng of genomic DNA, 0.25 µM of each primer, 200 µM of dNTPs, 1.5 mM of $MgCl_2$, 1× PCR buffer, and 0.5 U of Taq DNA polymerase. Thermal cycling parameters were as follows: 95°C for 5 min (initial denaturation); 40 cycles of 95°C for 60 s, 56°C for 60 s and 72°C for 45 s; followed by 72°C for 10 min (final extension) [20]. Wheat specific *AHAS* gene primers, as described by Lee et al. [35], were utilized to amplify a 1,123 bp fragment (Table 1).

Table 1. Primers used for the identification of the wheat *AHAS* gene

Primer Name	Sequence (5′–3′)	Annealing Temperature (°C)
ALS_O_F	TGTTGACATTGATGGGGATGG	63.84
ALS_O_R	TTGATACAACACGCAGGCATC	62.02
ALS_M_R	ATGTCCTTGAAAGCA ACA TCGT	63.50
HvALS_qRT PCR_F	AGGTGTTGGGCAGCATCAGA	64.15
HvALS_qRT PCR_R	GCGACCATGGGGATGGAGTC	63.93
ALS_1_F	TAACGCGCTCGCCCGTCA	69.56
ALS_1_R	TCCCAGTCACGCGATCATCA	66.21
ALS_2_F	CCCCAGTGACGACCCACTGT	65.72
ALS_2_R	TCAGTACGAGGTCCTGCCATC	62.55
CM F	CCGCCGCAATATGCTATCCAG	63.50
CM R	GTAGGACAAGAAACTTGCATG	63.50

Sanger Sequencing and Mutation Identification

PCR products were purified and sequenced via Sanger sequencing (MedSanTek, Istanbul, Türkiye). Sequence data were processed and aligned by multiple sequence alignment using ClustalW (CLC Sequence Viewer v7) to identify nucleotide substitutions relative to the untreated Kırik control. The sequence alignment data generated during this study have been archived in the Zenodo repository under DOI: 10.5281/zenodo.20770522.

Biochemical characterization of *AHAS* enzyme

***AHAS* protein extraction**

The *AHAS* enzyme was extracted from control plants and herbicide tolerant M_4 candidate lines, following the protocol of Singh et al. [36]. Seeds from each line were sown under greenhouse conditions and treated at the 3 leaf stage with the recommended field dose of imazamox (50 ppm foliar spray). Two weeks post application, 4 g of fresh foliar tissue was harvested, immediately frozen in liquid nitrogen, and homogenized in potassium phosphate buffer (pH 7.5) containing 10 mM pyruvate, 5 mM $MgCl_2$, 5 mM EDTA, 100 µM FAD, 1 mM valine, 1 mM leucine, 10% glycerol, and 10 mM cysteine. The homogenate was filtered and centrifuged at 25,000 g for 20 min at 4°C; the resulting supernatant was resuspended in 50 mM potassium phosphate buffer (pH 7.5) supplemented with 1 mM EDTA and 100 mM NaCl and then stored at 4°C.

Colorimetric assay of *AHAS* activity

AHAS enzyme activity was qualitatively verified by the colorimetric acetoin method: 100 µL of enzyme extract was incubated with 100 µL of substrate solution containing pyruvate, FAD, and other cofactors, as per Singh et al. (1988), at 37°C for 15 min. The reaction was stopped with 50 µL of 6 M HCl, then incubated at 60°C for 15 min to develop color, and the absorbance was measured at 530 nm. The presence of a characteristic red acetoin complex confirmed enzymatic activity in both treated and untreated samples.

Total protein

Total protein concentration was determined using the Bradford assay [37]. A standard curve was generated using bovine serum albumin (BSA) at 10–100 µg/mL. For each sample, 10 µL of enzyme extract was mixed with 90 µL of distilled water and 4.9 mL of Bradford reagent (Coomassie Brilliant Blue G 250). Following a 10 minute incubation, absorbance was measured at 595 nm in triplicate. Protein concentrations were calculated using linear regression of the BSA standard curve.

Results

***In vitro* EMS mutagenesis and imazamox selection in callus derived populations**

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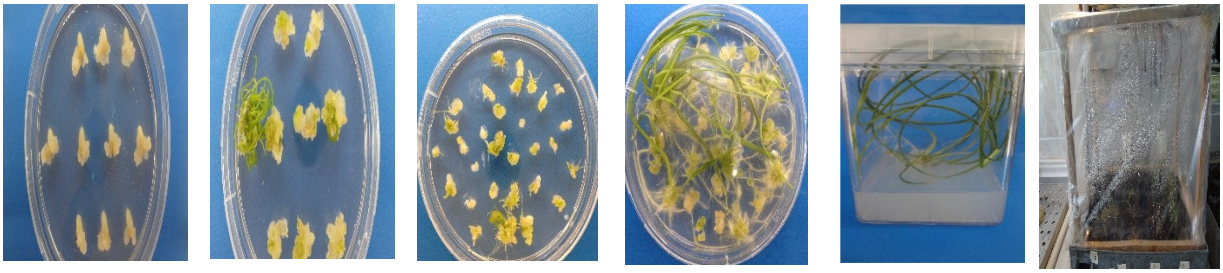
Under the applied *in vitro* selection conditions (0.050 µM imazamox), one putative herbicide-tolerant plantlet was recovered from the mutagenized calli population, representing a recovery frequency of 0.0008% (1/126,000). In April 2016, regenerated progeny of this line, alongside untreated and imazamox treated Krik controls, were established in field plots and subjected to dual stage herbicide selection (60 ppm seed priming, followed by a 50 ppm foliar application at the 3–4 leaf stage). Control derived lines exhibited severely reduced germination, stunted growth, and extensive chlorosis under imazamox treatment. In contrast, the single *in vitro* derived line (designated InVitML-1) maintained normal vegetative growth, exhibited no visible phytotoxicity symptoms, and produced viable seed, confirming stable herbicide tolerance and its suitability for molecular characterization (Figure 1).



(a) (b) (c) (d) (e) (f)

Figure 1. *In vitro* mutagenesis in wheat mature embryo culture. a) callus formation, (b) mutagen treated calli, (c) formation of somatic embryos (d e) regeneration stage and (f) Appearance of cultures in plant growth chambers.

***In vivo* EMS Seed mutagenesis and high throughput ımazamox screening in M_2 populations**

Approximately 50,000 M_1 seeds treated with 0.3% (v/v) EMS for 6 h were sown in May 2014. EMS treatment resulted in reduced M_1 germination rate and seedling vigor relative to untreated controls, consistent with mutagenic stress. Selection for herbicide tolerance was initiated in the M_2 generation, the first segregating generation for recessive mutations. A total of 250,000 M_2 seeds (10.25 kg) were subjected to germination stage screening using 60 ppm imazamox applied via irrigation. Three putative herbicide tolerant seedlings were identified based on normal root and shoot elongation and the absence of chlorosis, 7–10 days post sowing, corresponding to a recovery frequency of 0.0012% (3/250,000). These candidate lines were designated ML-1, ML-2, and ML-3. Secondary foliar screening (50 ppm imazamox) at the 3–4 leaf stage confirmed tolerance in all three lines; the treated plants exhibited robust growth and maintained green foliage, whereas the susceptible controls displayed complete necrosis within 14 days (Figure 2).

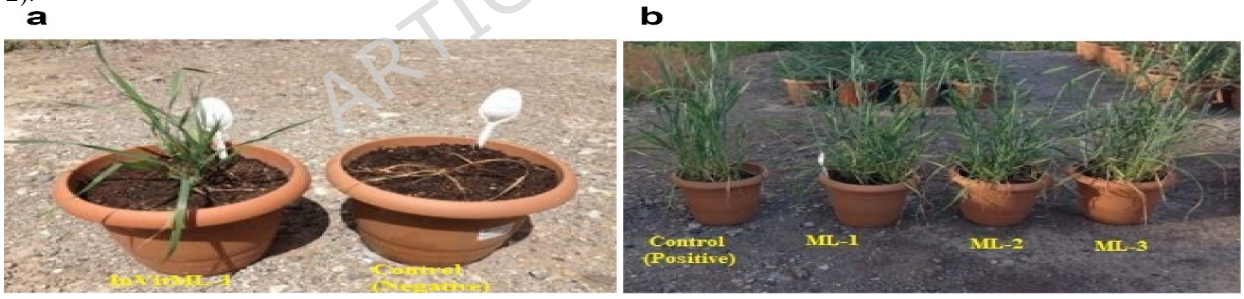


Figure 2. Phenotypic evaluation of putative mutant lines following imazamox selection. (a) Cultivation of one candidate M_1 plant identified through initial screening in pots; the herbicide tolerant candidate line (left) exhibits robust growth and normal physiological development, whereas the wild type (right) shows severe chlorosis and complete growth inhibition. (b) morphological comparison of selected mutant candidates under greenhouse conditions. Pot 1: wild type without herbicide treatment showing normal developmental progression. Pots 2, 3, and 4: Candidate mutant lines following foliar application of 50 ppm imazamox.

Multi generation ımazamox selection and stabilization of herbicide tolerant lines (M_2–M_4)

To confirm the heritability and phenotypic stability of the herbicide tolerance trait, the three seed derived candidate lines and one *in vitro* derived line were advanced from M_2 to M_4 under continuous dual stage imazamox selection pressure. Individual M_2 plants were harvested separately to establish discrete M_3 families. Within the M_3 generation, stringent selection at both seed (60 ppm priming) and foliar (50 ppm spray) stages eliminated segregating susceptible individuals. By M_4, progeny of all four lines displayed uniform tolerance, indicating genetic fixation. The three seed derived lines (ML-1, ML-2, ML-3) and one *in vitro* derived line (InVitML-1) were confirmed to be stable mutant lines and were advanced for detailed molecular and biochemical analyses. No visible morphological

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abnormalities, growth penalties, or fertility defects were observed in any of the four lines under field conditions in the absence of herbicide treatment.

Identification of EMS induced *AHAS* single nucleotide polymorphisms in tolerant lines

Molecular analysis of the 1,123 bp *AHAS* gene fragment from M_3/M_4 mutant lines and the untreated Kırik control identified five distinct single nucleotide polymorphisms (SNPs) among the four herbicide tolerant lines (Table 2, Figure 3). Among the seed derived mutants, ML-1 carried a T→C transition at position 1075, ML-2 exhibited two transversions at positions 386 (A→T) and 11 (T→A), and ML-3 possessed a rare adjacent dual nucleotide substitution at positions 1119 (T→C) and 1120 (C→T). The *in vitro* derived line InVitML-1 harbored a G→A transition at position 502.

The identified mutation spectrum consisted of four transition mutations (positions 502, 1075, 1119 and 1119) and two transversion mutations (positions 11 and 386), resulting in a transition-to-transversion ratio of 2:1. The rare adjacent dual nucleotide substitution in ML-3 (T→C at 1119 and C→T at 1120) accounts for two independent transitions within the same codon. The predominance of transition mutations was consistent with the characteristic mutagenic effect of EMS, which primarily induces G:C→A:T substitutions through alkylation of guanine bases. Importantly, none of the detected SNPs corresponded to the well-characterized *AHAS* resistance hotspot codons previously reported in wheat and other crop species, including *Ala122*, *Pro197*, *Ala205*, *Trp574*, and *Ser653* [12, 38-40]. Recent studies further demonstrated that resistance-associated *ALS*/*AHAS* mutations are not always confined to the canonical hotspot residues and that non-canonical substitutions may also contribute to altered herbicide sensitivity and resistance evolution in crop species and weeds [11, 13]. This finding suggests that the identified mutations represent potentially novel *AHAS* allelic variants associated with imazamox tolerance and may contribute to alternative resistance mechanisms beyond the classical herbicide-binding regions of the enzyme.

Translation of the identified SNPs into predicted amino acid substitutions revealed distinct alterations among the mutant lines. ML-1 (position 1075, T→C) carried a Leu359→Pro substitution, whereas ML-2 (positions 11, T→A and 386, A→T) contained two amino acid changes, Phe4→Ile and Thr129→Ser. In ML-3, the adjacent dual nucleotide substitution at positions 1119–1120 (T→C + C→T) resulted in a Ser373→Leu substitution, while the in vitro-derived line InVitML-1 (position 502, G→A) exhibited a Gly168→Glu substitution (Table 2). Importantly, all identified amino acid substitutions were located outside the previously characterized *AHAS* resistance-associated loci reported in wheat, including Ala122 and *Ser653* [16, 24]. Recent structural analyses of plant *AHAS* proteins indicated that amino acid substitutions located distant from the direct herbicide-binding cavity may influence enzyme conformation, catalytic stability, and inhibitor accessibility through long-range structural effects [13, 40]. This observation suggests that these non-canonical substitutions may contribute to imazamox tolerance through alternative structural or functional effects on the AHAS enzyme rather than through direct modification of the classical herbicide-binding regions.

Table 2. Summary of EMS induced *AHAS* single nucleotide polymorphisms in imazamox tolerant wheat lines.

Line	Position	Nucleotide Change	Mutation Type	Predicted Codon	Amino Acid Change	Notes
ML-1	1075	T→C	Transition	359	Leu→Pro	G:C→A:T transition
ML-2	11	T→A	Transversion	4	Phe→Ile	Non canonical position
ML-2	386	A→T	Transversion	129	Thr→Ser	Non canonical position
ML-3	1119	T→C	Transition	373	Ser→Leu	Adjacent dual mutation
ML-3	1120	C→T	Transition	373	Ser→Leu	Adjacent dual mutation
InVitML-1	502	G→A	Transition	168	Gly→Glu	G:C→A:T transition

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....|....| ....|....| ....|....| ....|....| ....|....|
360 370 380 390 400
ML-1 AGCACCAGAT GTGGGCGGCT CAGTATTACA CTTACAAGCG GCCACGGCAG
ML-2 AGCACCAGAT GTGGGCGGCT CAGTATTACA CTTACTAGCG GCCACGGCAG
ML-3 AGCACCAGAT GTGGGCGGCT CAGTATTACA CTTACAAGCG GCCACGGCAG InVitML-1
AGCACCAGAT GTGGGCGGCT CAGTATTACA CTTACAAGCG GCCACGGCAG
Control AGCACCAGAT GTGGGCGGCT CAGTATTACA CTTACAAGCG GCCACGGCAG
....|....| ....|....| ....|....| ....|....| ....|....|
510 520 530 540 550
ML-1 GGGATGGTAG TTTCCTCATG AACATTCAGG AGTTGGCGTT GATCCGTATT
ML-2 GGGATGGTAG TTTCCTCATG AACATTCAGG AGTTGGCGTT GATCCGTATT
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ML-3 GGGATGGTAG TTTCCTCATG AACATTCAGG AGTTGGCGTT GATCCGTATT
InVitML-1 GAGATGGTAG TTTCCTCATG AACATTCAGG AGTTGGCGTT GATCCGTATT
Control GGGATGGTAG TTTCCTCATG AACATTCAGG AGTTGGCGTT GATCCGTATT
....|....| ....|....| ....|....| ....|....| ....|....|
1060 1070 1080 1090 1100
ML-1 AACCCTGTAG TTTTGTAGTC TATGCTCTCT TTTGTAGGGA TGTGCTGTCA
ML-2 AACCCTGTAG TTTTGTAGTC TATGTTCTCT TTTGTAGGGA TGTGCTGTCA
ML-3 AACCCTGTAG TTTTGTAGTC TATGTTCTCT TTTGTAGGGA TGTGCTGTCA
InVitML-1 AACCCTGTAG TTTTGTAGTC TATGTTCTCT TTTGTAGGGA TGTGCTGTCA
Control AACCCTGTAG TTTTGTAGTC TATGTTCTCT TTTGTAGGGA TGTGCTGTCA
....|....| ....|....| ...
1110 1120
ML-1 TAAGATATCA TGCAAGTTTC TTT
ML-2 TAAGATATCA TGCAAGTTTC TTT
ML-3 TAAGATATCA TGCAAGTTCT TTT
InVitML-1 TAAGATATCA TGCAAGTTTC TTT
Control TAAGATATCA TGCAAGTTTC TTT
```

Figure 3. Partial nucleotide sequence alignment of the *AHAS* gene across EMS induced mutant lines (ML-1, ML-2, ML-3) and *in vitro* selected variant (InVitML-1) compared to the wild type control. Mutational sites are indicated by shaded boxes, which highlight the novel SNPs and the rare dual nucleotide substitution observed in ML-3.

***AHAS* associated protein levels and qualitative enzyme activity under ımazamox treatment**

Qualitative colorimetric assays confirmed that the *AHAS* enzyme retained catalytic activity in both treated and untreated samples, as evidenced by the development of the characteristic red acetoin complex at 530 nm [36]. Quantification of total soluble protein by the Bradford assay in *AHAS* enriched extracts revealed marked differences among genotypes in response to imazamox application (Table 3; Figure 4). The untreated Krik control exhibited an *AHAS* associated protein concentration of 5.13 ± 0.24 µg/mL (mean ± SD, n = 3). In contrast, imazamox treated susceptible Kırik plants showed a protein concentration of 1.97 ± 0.18 µg/mL (n = 3), representing a 62% reduction relative to untreated controls (paired t test, $p < .001$).

In contrast, all four EMS derived mutant lines maintained *AHAS* associated protein concentrations under imazamox treatment that were statistically indistinguishable from those in untreated controls: ML-1, 4.60 ± 0.21 µg/mL; ML-2, 4.71 ± 0.19 µg/mL; ML-3, 4.88 ± 0.23 µg/mL; and InVitML-1, 4.93 ± 0.20 µg/mL (n = 3 per line). One way ANOVA revealed significant effects of genotype and treatment on protein concentration ($F(5,12) = 87.3$, $p < .0001$), and post hoc Tukey HSD tests confirmed that all four mutant lines differed significantly from the imazamox treated susceptible control ($p < .001$), but not from the untreated control ($p > .05$) (Table 3). These results suggest that the EMS-induced AHAS mutations are associated with target-site resistance (TSR) mechanisms [9], potentially enabling the AHAS enzyme to retain functional activity under imazamox selection pressure. In contrast, the wild-type genotype exhibited pronounced herbicide sensitivity, supporting the hypothesis that the identified non-canonical mutations may alter herbicide–enzyme interactions and contribute to reduced susceptibility to imazamox.

Table 3. Quantification of *AHAS* associated protein concentration under imazamox stress via Bradford assay.

Genotype	Imazamox Treatment	*AHAS* Protein Concentration (µg/mL)	Difference from Treated Control (µg/mL)
Kırik (control)	−	5.13 ± 0.24[a]	+3.16
Krik (control)	+	1.97 ± 0.18[b]	—
ML-1	+	4.60 ± 0.21[a]	+2.63***
ML-2	+	4.71 ± 0.19[a]	+2.74***
ML-3	+	4.88 ± 0.23[a]	+2.91***
InVitML-1	+	4.93 ± 0.20[a]	+2.96***

*Values are mean ± SD (n = 3 biological replicates). One way ANOVA: $F_{5,12} = 87.3$, $P < 0.0001$. Superscript letters indicate groups that do not differ significantly (Tukey HSD, $\alpha = 0.05$). *** $P < 0.001$ versus imazamox treated control.

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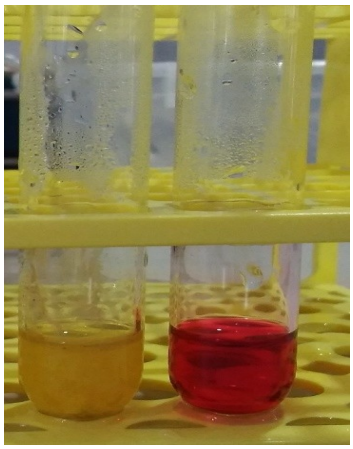

Figure 4. Colorimetric response of HAS enzyme to Imazamox in Kirik and non mutant wheat.

Discussion

EMS mutagenesis mechanism and mutation spectrum consistency

Ethyl methanesulfonate (EMS) induces point mutations primarily through alkylation at the O^6 and N^7 positions of guanine, creating O^6 ethylguanine adducts that mispair with thymine during DNA replication, resulting in characteristic G:C→A:T transitions [21, 22]. The mutagenic specificity of EMS has been extensively documented across plant species, with transition to transversion ratios typically ranging from 2:1 to 4:1, depending on genome repair capacity and cell cycle dynamics. In the present study, molecular characterization of four stable imazamox tolerant wheat lines revealed five distinct single nucleotide polymorphisms distributed across a 1,123 bp fragment of the *AHAS* gene, comprising three transitions (positions 502, 1075, and 1119) and two transversions (positions 11 and 386), corresponding to a transition:transversion ratio of 3:2 (60% transitions). While this ratio is somewhat lower than the expected EMS signature, it remains consistent with alkylating agent mutagenesis and likely reflects the stochastic nature of mutation fixation in polyploid wheat, where gene redundancy across the A, B, and D genomes may permit retention of both transition and transversion mutations that would be deleterious in diploid systems [16, 17].

The identification of a rare adjacent dual nucleotide substitution at positions 1119–1120 (T→C, C→T) in line ML-3 is particularly noteworthy. Such proximate mutations within a 2 bp window are statistically improbable under random mutagenesis models and may arise through one of three mechanisms: (i) simultaneous alkylation of adjacent guanines on opposite DNA strands, (ii) error prone repair of a single O^6 ethylguanine lesion leading to secondary mutations, or (iii) clustered mutations resulting from local chromatin accessibility during the S phase of the cell cycle. Given the high EMS concentration (0.3% for 6 h) applied to seed populations, the first mechanism concurrent alkylation at adjacent sites—appears most plausible [21, 22].

Novel non canonical *AHAS* mutations and structural implications

Acetolactate synthase is a thiamine diphosphate (ThDP) dependent enzyme that catalyzes the condensation of two pyruvate molecules (for valine and leucine biosynthesis) or pyruvate with 2 ketobutyrate (for isoleucine biosynthesis; [10]). Crystal structures of plant *AHAS* homologs, including Arabidopsis thaliana (PDB: 1YBH) and yeast (PDB: 1N0H), have revealed that the enzyme comprises three functional domains: a thiamine diphosphate binding domain, a catalytic domain containing the FAD cofactor, and a regulatory domain [40]. Imidazolinone herbicides bind in a hydrophobic pocket adjacent to the FAD cofactor and the ThDP active site, sterically blocking substrate access and inhibiting catalysis [40, 41].

The vast majority of characterized *AHAS* resistance mutations occur at five highly conserved amino acid positions that line or are proximal to the herbicide binding pocket: Ala122, Pro197, Ala205, Trp574, and Ser653 (numbering based on *Arabidopsis AHAS*; [12, 39]). In hexaploid wheat, mutations at Ala122 (chromosome 6D) and Ser653 are particularly prevalent in commercial Clearfield cultivars, conferring 10 to 100 fold increases in imidazolinone tolerance [24, 38]. The present study identified five SNPs that result in amino acid substitutions at codons 4 (Phe→Ile), 129 (Thr→Ser), 168 (Gly→Glu), 359 (Leu→Pro), and 373 (Ser→Leu), none of which correspond to canonical hotspot residues. This divergence raises the critical question: how can noncanonical mutations confer herbicide resistance without directly disrupting the binding pocket? Three structural mechanisms may explain non canonical resistance: (i) long range allosteric effects, in which mutations distant from the active site alter protein dynamics or domain–domain interactions, thereby indirectly destabilizing herbicide binding [40]. For instance, the Leu359→Pro substitution (ML-1) introduces a conformationally restricted proline residue that may propagate

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structural perturbations to the herbicide binding region by distorting the α helix. (ii) Altered substrate channel geometry, in which mutations modify the entrance or egress pathways for pyruvate and 2 ketobutyrate, secondarily affecting herbicide accessibility. The Gly168→Glu substitution (InVitML-1) replaces a flexible glycine residue with a charged glutamate residue, potentially remodeling local electrostatics and substrate channel architecture. (iii) Protein stability and folding compensation, whereby mutations stabilize enzyme conformation under herbicide induced stress and prevent the conformational collapse observed in wild type *AHAS* exposed to imidazolinones [13].

Homology modeling of the wheat *AHAS* D genome allele, based on the Arabidopsis template (1YBH, 68% sequence identity) and mapping of the five identified mutations onto the 3D structure reveal that codons 4, 129, 168, 359, and 373 are distributed across the catalytic and regulatory domains at 12–25 Å from the herbicide binding pocket [40]. While the biochemical assays in this study strongly support target-site resistance (TSR), the exact structural mechanism by which these non-canonical mutations confer imazamox tolerance remains to be fully elucidated. Because these substitutions are located outside the canonical binding pocket, they likely operate through long-range allosteric effects, altering protein dynamics, substrate channel geometry, or overall enzyme stability under herbicide stress. Rigorous structural characterization using *in silico* protein tertiary structure modeling or X-ray crystallography was beyond the scope of this initial breeding and mutagenesis study. However, future structural biology studies are essential to map these mutations onto the 3D conformation of the wheat AHAS enzyme and definitively determine how these distant residues alter the herbicide-binding cavity.

Target site resistance versus metabolic detoxification

Herbicide resistance in plants is mediated by two broad mechanistic classes: target site resistance (TSR), involving mutations that reduce herbicide binding affinity or decrease herbicide induced inhibition of catalytic activity, and non target site resistance (NTSR), encompassing enhanced herbicide metabolism (via cytochrome P450 monooxygenases and glutathione S transferases), reduced herbicide uptake, or increased herbicide sequestration [9, 25]. Distinguishing TSR from NTSR is critical for understanding resistance evolution and designing durable weed management strategies.

The biochemical characterization performed in this study provides strong evidence for TSR in all four mutant lines. Quantification by Bradford assay revealed that *AHAS* associated protein concentrations in EMS derived lines treated with imazamox (4.60–4.93 µg/mL) were statistically indistinguishable from untreated wild type controls (5.13 µg/mL) and significantly higher than those in imazamox treated susceptible plants (1.97 µg/mL), representing a 62% reduction. This protein stability pattern is diagnostic of TSR, wherein the mutant enzyme retains structural integrity and resists herbicide induced degradation or inactivation [9]. In contrast, NTSR mechanisms would be expected to show either (i) normal protein depletion under herbicide stress coupled with rapid herbicide catabolism (P450 mediated), or (ii) elevated basal *AHAS* expression due to compensatory transcriptional upregulation. Neither profile was observed, supporting TSR as the predominant mechanism.

Further support for TSR comes from the Mendelian inheritance pattern observed across the M_2–M_4 generations, in which herbicide tolerance segregated as a single semi dominant locus within individual families, which is consistent with monogenic target site mutations [24]. NTSR traits, particularly those involving P450 upregulation, often exhibit more complex polygenic inheritance due to regulatory epistasis. Additional experiments, including enzymatic assays measuring *AHAS* catalytic activity (V*max*) and herbicide inhibition constants (K*i*) in purified enzyme preparations, would provide definitive mechanistic confirmation and quantify the magnitude of resistance conferred by each mutation [36]. Synergistic effects of dual *ın vivo* and *ın vitro* mutagenesis Further support for TSR comes from the Mendelian inheritance pattern observed across the M_2–M_4 generations, in which herbicide tolerance segregated as a single semi-dominant locus within individual families, consistent with monogenic target-site mutations [42]. In contrast, NTSR mechanisms, particularly those associated with cytochrome P450-mediated detoxification, generally exhibit more complex polygenic inheritance patterns due to regulatory interactions and epistatic effects. Although the present findings strongly support TSR as the predominant resistance mechanism, additional studies involving whole-plant dose–response analyses, cross-resistance evaluation across different ALS-inhibiting herbicide families, and kinetic characterization of AHAS activity and herbicide inhibition constants would provide deeper mechanistic insight into the functional effects of the identified mutations [43, 44]. Furthermore, segregation analysis in biparental populations may help clarify the inheritance behavior and genomic localization of the resistance-associated alleles in hexaploid wheat.

The complementary application of in vivo seed mutagenesis (50,000 M_1 seeds) and *in vitro* callus based mutagenesis (126,000 calli) yielded a combined total of four stable herbicide tolerant lines, corresponding to selection efficiencies of 0.0012% (3/250,000 M_2 seeds) and 0.0008% (1/126,000 calli), respectively. While these recovery frequencies are low, they are consistent with the rarity of functional resistance mutations at non canonical *AHAS* positions and underscore the necessity of large scale screening to access novel allelic diversity [21].

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The *in vivo* approach offers advantages in terms of population size and minimal tissue culture artifacts, enabling the recovery of three independent alleles (ML-1, ML-2, ML-3) with distinct molecular lesions [20, 23]. This allelic diversity is critical for understanding the structural tolerance of *AHAS* to amino acid substitutions and for providing multiple options for introgression into elite breeding germplasm. Conversely, the *in vitro* approach, despite lower throughput, permits cellular level selection under continuous herbicide pressure, enriching for mutations that confer tolerance at both the enzymatic and whole plant levels [32]. The unique Gly168→Glu substitution recovered in InVitML-1 demonstrates that *in vitro* systems can access mutational space not sampled by seed based screens, which may be attributable to differential DNA repair activity or replication kinetics in dedifferentiated callus cells compared with organized meristems.

The integration of both strategies thus maximizes allelic coverage and compensates for the inherent biases of each approach [21]. Future studies employing pooled amplicon sequencing (TILLING by sequencing) on M_2 populations prior to phenotypic selection could further enhance mutation discovery rates and provide genome wide mutational load estimates.

Polyploidy and mutation fixation in hexaploid wheat

Hexaploid bread wheat (*Triticum aestivum*, 2n = 6x = 42, AABBDD) presents unique challenges and opportunities for mutation breeding. The presence of three homoeologous *AHAS* gene copies (one per genome) introduces genetic redundancy that buffers against deleterious mutations but complicates the fixation of recessive resistance alleles [16, 31]. In diploid species, recessive loss of function mutations are unmasked in the M_2 generation and can be rapidly selected. In hexaploid wheat, however, a single mutant *AHAS* D allele must confer sufficient tolerance, despite the presence of two wild type copies (*AHAS* A and *AHAS* B), to survive selection pressure.

The successful recovery of four tolerant lines, despite this genetic complexity, suggests that the identified mutations confer semi dominant resistance, consistent with previous reports for Ala122 and Ser653 substitutions in Clearfield wheat [24, 31, 38]. Semidominance likely arises because even partial inhibition of *AHAS* across all three genomes is lethal; thus, a single resistant allele providing approximately 30–40% functional *AHAS* activity is sufficient for plant survival under moderate herbicide stress (60 ppm for seed priming, 50 ppm for foliar application). This hypothesis could be tested through genome specific PCR or next generation sequencing to confirm that the mutations reside exclusively in the D genome copy of *AHAS*, as expected, given the historical precedence of resistance loci on chromosome 6D [16, 31].

The polyploid genome architecture also influences the efficiency of EMS mutagenesis. With three *AHAS* targets per nucleus, the probability of inducing a functional mutation in at least one copy is theoretically threefold greater than in diploids, partially offsetting genetic redundancy. However, empirical recovery rates in wheat (0.0008–0.0012%) remain lower than those in diploid Arabidopsis (0.01–0.05%;[21, 39]), indicating that genome complexity imposes net constraints on mutation breeding outcomes.

Implications for marker assisted selection and allele deployment

The five novel SNPs identified in this study particularly the adjacent dual substitution at positions 1119–1120 represent diagnostic molecular markers suitable for high throughput marker assisted selection (MAS) in wheat breeding programs [15, 35, 45]. Several marker platforms could be deployed: (i) *KASP (Kompetitive Allele Specific PCR)* assays, which offer cost effective, high throughput genotyping for known SNPs and are widely adopted in wheat breeding; (ii) *dCAPS (derived Cleaved Amplified Polymorphic Sequences)* markers, which leverage restriction enzyme polymorphisms to distinguish mutant from wild type alleles; and (iii) *high resolution melting (HRM)* analysis, which detects SNPs based on PCR product melting curve shifts. Given the single and dual base nature of the mutations, KASP assays are the most practical option for breeding scale deployment [15, 45].

Beyond their immediate utility in wheat improvement, these mutations expand the catalog of *AHAS* resistance alleles available to support cultivar protection and intellectual property strategies. In jurisdictions permitting plant variety rights based on molecular distinctness, the novel SNPs could serve as distinguishing genetic fingerprints for registered cultivars. Additionally, pyramiding multiple non canonical resistance alleles (combining Leu359→Pro and Gly168→Glu in a single genotype) may provide additive or synergistic resistance, delay the evolution of resistance breaking weed biotypes and enhance trait durability [7, 25].

Conclusions

This study demonstrates the breeding potential of EMS mutagenesis as a non-transgenic and regulation-compatible approach for developing herbicide-tolerant wheat germplasm. By integrating complementary in vivo and in vitro mutagenesis platforms, four stable imazamox-tolerant mutant lines were developed, four stable imazamox-tolerant mutant lines were developed, harboring five novel *AHAS* single nucleotide polymorphisms located outside the canonical resistance hotspots. These mutations resulted in the amino acid substitutions Phe4→Ile, Thr129→Ser, Gly168→Glu, Leu359→Pro, and Ser373→Leu, thereby expanding the known mutational spectrum associated with

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imidazolinone tolerance in wheat. Importantly, the identified mutations were located outside the classical Clearfield-associated residues, including Ala122 and Ser653, suggesting that non-canonical *AHAS* variants may contribute to alternative mechanisms of herbicide tolerance. The sustained protein accumulation observed under imazamox stress further supports the functional relevance of these alleles and highlights their potential utility for wheat breeding programs. As conventionally derived, non-transgenic germplasm, these mutant lines may also offer advantages in terms of regulatory compatibility and broader applicability in wheat improvement programs compared with transgenic herbicide-resistance technologies. Beyond their breeding value, these findings contribute to the broader mechanistic understanding of *AHAS*-mediated herbicide tolerance in polyploid wheat. In addition, the combined in vivo and in vitro EMS mutagenesis strategy improved the recovery efficiency of rare functional alleles under herbicide selection pressure. Future studies integrating enzyme kinetics, structural modeling, genome-editing validation, allele pyramiding, and multi-environment field evaluations will be important for clarifying the molecular basis, agronomic stability, and breeding value of these novel non-canonical *AHAS* variants.

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Germplasm Availability and Intellectual Property The company is open to establishing formal research collaborations with public institutions for noncommercial research projects. Any such collaboration would be governed by a standard Material Transfer Agreement (MTA) to clearly define the terms of use, publication rights, and intellectual property considerations.

Permission for land study The authors declare that all land experiments and studies were performed according to authorized rules.

Author contribution Conceptualization: K.H. and A.T.; methodology: K.H. and A.T.; software: A.T., M.A. and K.H.; validation: K.H.; investigation: A.T. and K.H.; resources: K.H.; data curation: K.H., M.A., H.A. and A.T.; writing the original draft preparation: A.T; writing the review and editing: K.H., H.A. and A.T.; visualization: K.H. and A.T.; supervision: K.H., A.T, and H.A.; project administration: K.H.; All authors have read and agreed to the published version of the manuscript.

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Data availability The DNA sequence alignment dataset generated and analyzed during the current study has been current study have been deposited in the NCBI GenBank database under the submission number SUB16308273. The accession numbers and web links for the sequences are as follows:

ML-1: PZ742918- https://www.ncbi.nlm.nih.gov/nuccore/PZ742918
ML-2: PZ742919- https://www.ncbi.nlm.nih.gov/nuccore/PZ742919
ML-3: PZ742920- https://www.ncbi.nlm.nih.gov/nuccore/PZ742920
InVitroML-1: PZ742921- https://www.ncbi.nlm.nih.gov/nuccore/PZ742921
Control: PZ742922- https://www.ncbi.nlm.nih.gov/nuccore/PZ742922

All other relevant data supporting the findings of this study are available within the manuscript.

Declarations

Ethics approval and consent to participate This study did not involve human participants or animals. Therefore, ethics approval and consent to participate were not required.

Consent to participate Not applicable. No human participant.

Consent for publication Not applicable. No human participant.

Competing interests The authors declare no competing interests.

References

1. Giraldo P, Benavente E, Manzano-Agugliaro F, Gimenez E: Worldwide research trends on wheat and barley: A bibliometric comparative analysis. *Agronomy* 2019, 9(7):352.
2. Economics USDoA, Service S, Service USFA, Food USW, Outlook A, Board S, Board USWAO: World Agricultural Supply and Demand Estimates: The Department; 2025.
3. FAO: FAOSTAT statistics database. Food and Agriculture Organization of the United Nations. 2025.

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4. MacLaren C, Storkey J, Menegat A, Metcalfe H, Dehnen-Schmutz K: An ecological future for weed science to sustain crop production and the environment. A review: An ecological future for weed science to sustain crop production and the environment. A review. *Agronomy for Sustainable Development* 2020, 40(4):24.
5. Oad F, Siddiqui M, Buriro U: Growth and yield losses in wheat due to different weed densities. 2007.
6. Oerke E-C: Crop losses to pests. *The Journal of agricultural science* 2006, 144(1):31-43.
7. Heap I: The international survey of herbicide resistant weeds. *http://www weedscience com* 2026.
8. Collavo A, Panozzo S, Lucchesi G, Scarabel L, Sattin M: Characterisation and management of Phalaris paradoxa resistant to ACCase-inhibitors. *Crop protection* 2011, 30(3):293-299.
9. Devine MD, Shukla A: Altered target sites as a mechanism of herbicide resistance. *Crop protection* 2000, 19(8-10):881-889.
10. Duggleby RG, McCourt JA, Guddat LW: Structure and mechanism of inhibition of plant acetohydroxyacid synthase. *Plant Physiology and Biochemistry* 2008, 46(3):309-324.
11. Yu Q, Powles SB: Resistance to AHAS inhibitor herbicides: current understanding. *Pest management science* 2014, 70(9):1340-1350.
12. Tranel PJ, Wright TR: Resistance of weeds to ALS-inhibiting herbicides: what have we learned? *Weed science* 2002, 50(6):700-712.
13. Lonhienne T, Low YS, Garcia MD, Croll T, Gao Y, Wang Q, Brillault L, Williams CM, Fraser JA, McGeary RP: Structures of fungal and plant acetohydroxyacid synthases. *Nature* 2020, 586(7828):317-321.
14. Kolkman JM, Slabaugh MB, Bruniard JM, Berry S, Bushman BS, Olungu C, Maes N, Abratti G, Zambelli A, Miller JF: Acetohydroxyacid synthase mutations conferring resistance to imidazolinone or sulfonylurea herbicides in sunflower. *Theoretical and Applied Genetics* 2004, 109(6):1147-1159.
15. Thompson C, Tar'an B: Genetic characterization of the acetohydroxyacid synthase (AHAS) gene responsible for resistance to imidazolinone in chickpea (Cicer arietinum L.). *Theoretical and Applied Genetics* 2014, 127(7):1583-1591.
16. Li D, Barclay I, Jose K, Stefanova K, Appels R: A mutation at the Ala122 position of acetohydroxyacid synthase (AHAS) located on chromosome 6D of wheat: improved resistance to imidazolinone and a faster assay for marker assisted selection. *Molecular Breeding* 2008, 22(2):217-225.
17. Zhang R, Liu J, Chai Z, Chen S, Bai Y, Zong Y, Chen K, Li J, Jiang L, Gao C: Generation of herbicide tolerance traits and a new selectable marker in wheat using base editing. *Nature plants* 2019, 5(5):480-485.
18. Duke SO: Why have no new herbicide modes of action appeared in recent years? *Pest management science* 2012, 68(4):505-512.
19. Larkin PJ, Scowcroft WR: Somaclonal variation—a novel source of variability from cell cultures for plant improvement. *Theoretical and applied genetics* 1981, 60(4):197-214.
20. Türkoğlu A, Haliloğlu K, Tosun M, Bujak H, Eren B, Demirel F, Szulc P, Karagöz H, Selwet M, Özkan G: Ethyl methanesulfonate (EMS) mutagen toxicity-induced DNA damage, cytosine methylation alteration, and iPBS-retrotransposon polymorphisms in wheat (Triticum aestivum L.). *Agronomy* 2023, 13(7):1767.
21. Shu Q, Forster B, Nakagawa H: Principles and applications of plant mutation breeding. In: *Plant mutation breeding and biotechnology.* CABI Wallingford UK; 2012: 301-325.
22. Hofmann N, Raja R, Nelson R, Korban S: Mutagenesis of embryogenic cultures of soybean and detecting polymorphisms using RAPD markers. *Biologia plantarum* 2004, 48(2):173-177.
23. Türkoğlu A, Tosun M, Haliloğlu K: Evaluation of ethyl methanesulfonate-induced in vitro mutagenesis, polymorphism and genomic instability in wheat (Triticum aestivum L.). *Journal of Crop Science and Biotechnology* 2023, 26(2):199-213.
24. Newhouse KE, Smith WA, Starrett MA, Schaefer TJ, Singh BK: Tolerance to imidazolinone herbicides in wheat. *Plant Physiology* 1992, 100(2):882-886.
25. Beckie HJ, Tardif FJ: Herbicide cross resistance in weeds. *Crop Protection* 2012, 35:15-28.
26. Chen T, Ling X, Yu Y, Zhang B: A nonsynonymous mutation in an acetolactate synthase gene (Gh_D10G1253) is required for tolerance to imidazolinone herbicides in cotton. *Journal of Cotton Research* 2023, 6(1):4.
27. Galili S, Hershenhorn J, Edelman M, Sobolev V, Smirnov E, Amir-Segev O, Bellalou A, Dor E: Novel mutation in the acetohydroxyacid synthase (AHAS), gene confers imidazolinone resistance in chickpea Cicer arietinum L. plants. *Plants* 2021, 10(12):2791.
28. Kumar P, Bishnoi R, Priyadarshini P, Chhuneja P, Singla D: Understanding the structural basis of ALS mutations associated with resistance to sulfonylurea in wheat. *Scientific Reports* 2025, 15(1):12855.
29. Pan W, Zhu Y, Li P, Li Z, Xu C, Jin M, Tang X: Natural and artificial evolution of acetolactate synthase for crop breeding. *The Crop Journal* 2025.

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30. Hossein Pour A, Tosun M, Haliloğlu K: Effect of ethyl methanesulfonate treatment at different duration and concentration on germination and seedling growth characters in wheat (Triticum aestivum L.). *Atatürk Univ J of Agricultural Faculty* 2021, 52(2):190-200.
31. Pozniak C, Hucl P: Genetic analysis of imidazolinone resistance in mutation-derived lines of common wheat. *Crop Science* 2004, 44(1):23-30.
32. Hossein Pour A, K H: Determination of Herbicide Tolerance of Wheat (Triticum aestivum L.) for in vitro selection. *II International Igdir Symposium* 2017:96-103.
33. Haliloğlu K, Türkoğlu A, Aydin M: Determination of imazamox herbicide dose in in vivo selection in wheat (Triticum aestivum L.). *Eregli J Agric Sci* 2022, 2:1-11.
34. Zeinalzadehtabrizi H, Hosseinpour A, Aydin M, Haliloglu K: A modified genomic DNA extraction method from leaves of sunflower for PCR based analyzes. *J Biodivers Environ Sci* 2015, 7:222-225.
35. Lee H, Rustgi S, Kumar N, Burke I, Yenish JP, Gill KS, von Wettstein D, Ullrich SE: Single nucleotide mutation in the barley acetohydroxy acid synthase (AHAS) gene confers resistance to imidazolinone herbicides. *Proceedings of the National Academy of Sciences* 2011, 108(21):8909-8913.
36. Singh BK, Stidham MA, Shaner DL: Assay of acetohydroxyacid synthase. *Analytical biochemistry* 1988, 171(1):173-179.
37. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* 1976, 72(1-2):248-254.
38. Bernasconi P, Woodworth AR, Rosen BA, Subramanian MV, Siehl DL: A naturally occurring point mutation confers broad range tolerance to herbicides that target acetolactate synthase. *Journal of Biological Chemistry* 1995, 270(29):17381-17385.
39. Jander G, Baerson SR, Hudak JA, Gonzalez KA, Gruys KJ, Last RL: Ethylmethanesulfonate saturation mutagenesis in Arabidopsis to determine frequency of herbicide resistance. *Plant physiology* 2003, 131(1):139-146.
40. McCourt JA, Pang SS, King-Scott J, Guddat LW, Duggleby RG: Herbicide-binding sites revealed in the structure of plant acetohydroxyacid synthase. *Proceedings of the National Academy of Sciences* 2006, 103(3):569-573.
41. Tan S, Evans RR, Dahmer ML, Singh BK, Shaner DL: Imidazolinone-tolerant crops: history, current status and future. *Pest Management Science: Formerly Pesticide Science* 2005, 61(3):246-257.
42. Willenborg CJ, Brûlé-Babel AL, Friesen LF, Van Acker RC: Response of heterozygous and homozygous imidazolinone-resistant spring wheat genotypes to imazamox. *Crop science* 2008, 48(6):2107-2114.
43. Breccia G, Picardi L, Nestares G: Cultivar variation for imazamox resistance in wheat (Triticum aestivum L.): Insights into enzymatic assays for early selection. *Plant Physiology and Biochemistry* 2020, 151:438-442.
44. Hanson BD, Shaner DL, Westra P, Nissen SJ: Response of selected hard red wheat lines to imazamox as affected by number and location of resistance genes, parental background, and growth habit. *Crop science* 2006, 46(3):1206-1211.
45. Nakka S, Jugulam M, Peterson D, Asif M: Herbicide resistance: Development of wheat production systems and current status of resistant weeds in wheat cropping systems. *The Crop Journal* 2019, 7(6):750-760.

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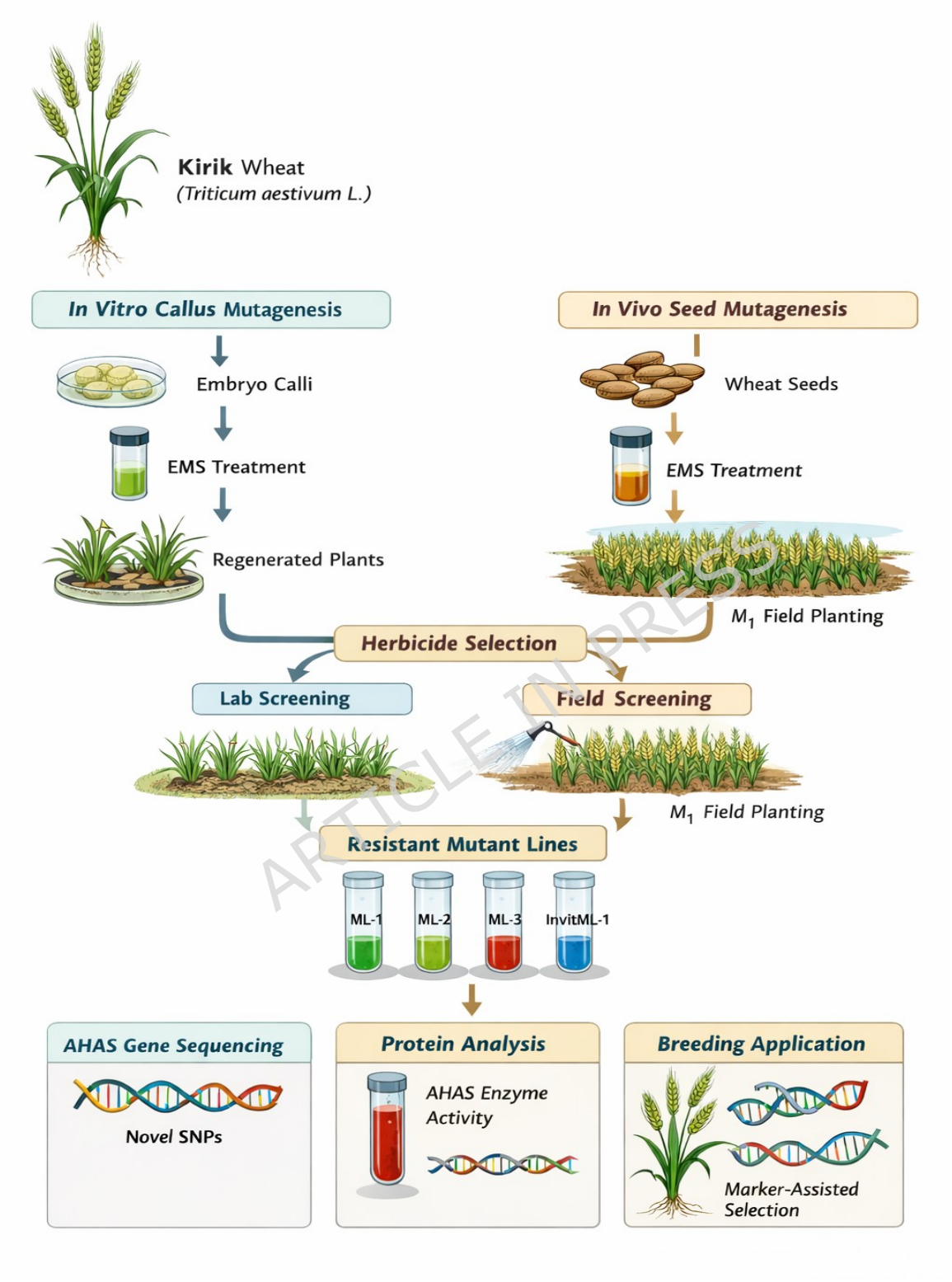

Graphical Abstract