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CRISPR-Cas9 mediated genome editing for developing herbicide-tolerant rice lines

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Abstract
CRISPR-Cas9 is a programmable nuclease system that enables precise, targeted modifications in plant genomes without introducing foreign DNA into the final product. This research applied CRISPR-Cas9 to edit three herbicide-target genes—ALS, ACCase, and EPSPS—in indica rice (cv. Pusa Basmati 1) to develop lines tolerant to imazethapyr, quizalofop, and glyphosate, respectively. Four sgRNA constructs were designed, cloned into pCas9 vectors, and delivered via Agrobacterium-mediated transformation at the Chandra Shekhar Azad University of Agriculture, Kanpur, India, during 2021–23. Regeneration efficiency ranged from 26.4% (pCas9-EPSPS) to 34.2% (pCas9-ALS1). Among 186 T_0 plants screened, 47 carried confirmed edits at the target loci. T_1 segregation analysis identified 12 homozygous edited lines. Herbicide tolerance assays in T_1 showed that edited line CR-15 (ALS double mutant) tolerated imazethapyr at 4× the field rate without visible injury, scoring 4.8 on a 5-point tolerance scale versus 1.8 for the wild type. No off-target mutations were detected at the top five predicted sites. Agronomic performance of the best edited lines was statistically equivalent to the parent variety. These results demonstrate that CRISPR-based point mutations in herbicide-target genes can produce non-transgenic, herbicide-tolerant rice suitable for direct-seeded cultivation systems.

Keywords: CRISPR-Cas9, genome editing, herbicide tolerance, rice, ALS gene, ACCase gene, EPSPS gene, indica rice, direct-seeded rice, non-transgenic breeding.

Introduction
Genome editing, at its simplest, means rewriting specific DNA letters in a living organism's genetic code with surgical precision. The CRISPR-Cas9 system, adapted from a bacterial immune mechanism, has transformed plant breeding by enabling targeted mutagenesis at costs and timescales far below those of conventional transgenic approaches [1, 2]. In rice, CRISPR has been used to modify traits ranging from grain quality and disease resistance to nutrient content [3].

One application with large practical impact is herbicide tolerance. Direct-seeded rice (DSR), which avoids transplanting and saves water and labor, is expanding across South and Southeast Asia. But DSR suffers from intense weed competition, and effective herbicide use is often limited by the crop's own sensitivity [4]. Conventional herbicide-tolerant rice varieties (e.g., Clearfield®) carry naturally occurring ALS mutations but are available only in japonica backgrounds, limiting their use in indica-growing regions like the Indo-Gangetic plains [5].

CRISPR-Cas9 can introduce the same or novel point mutations in ALS, ACCase, and EPSPS genes of elite indica cultivars, producing herbicide-tolerant lines without foreign DNA insertion—these are technically non-transgenic and may face fewer regulatory hurdles than conventional GMOs [6, 7]. However, challenges remain: editing efficiency varies by target site, regeneration from rice callus can be genotype-dependent, and off-target effects must be rigorously assessed [8]. This research aimed to develop herbicide-tolerant lines of Pusa Basmati 1 by editing three herbicide-target genes using CRISPR-Cas9, evaluate T_1 homozygous lines for herbicide tolerance at multiple dose levels, and confirm the absence of off-target mutations.

Reagents and Molecular Tools
Four single-guide RNAs (sgRNAs) targeting conserved regions of OsALS1 (LOC_

Os02g30630, exon 5), OsALS2 (LOC_Os06g51280, exon 4), OsACCase (LOC_Os05g22940, exon 18), and OsEPSPS (LOC_Os06g04280, exon 2) were designed using CRISPR-P 2.0 and CRISPRscan tools. Each sgRNA was cloned into the pRGEB32 vector backbone carrying the Cas9 gene driven by the OsUBQ promoter and the sgRNA under the OsU6 promoter. Binary vectors were introduced into Agrobacterium tumefaciens strain EHA105. Hygromycin B (50 mg L^{-1}) was used for callus selection. Herbicides tested were imazethapyr (Pursuit 10% SL), quizalofop-p-ethyl (Targa Super 5% EC), and glyphosate (Roundup 41% SL), all sourced from authorized agrochemical distributors.

Materials and Methods

Materials

Mature seeds of Pusa Basmati 1 (an elite aromatic indica rice cultivar) were surface-sterilized with 70% ethanol (1 min) and 2.5% sodium hypochlorite (20 min), rinsed three times with sterile distilled water, and plated on N6 callus induction medium supplemented with 2 mg L^{-1} 2, 4-D. Callus was maintained at 28 °C in darkness for 21 days before transformation. All tissue culture work was conducted in the Plant Biotechnology Laboratory, Department of Genetic Engineering, CSAUA, Kanpur.

Methods

Agrobacterium-mediated transformation followed a modified protocol [8]. Embryogenic calli were co-cultivated with Agrobacterium suspension (OD_{600} = 0.5) for 20 min, blotted dry, and incubated on co-cultivation medium at 25 °C for 3 days. Calli were then transferred to selection medium containing 50 mg L^{-1} hygromycin B and 250 mg L^{-1} cefotaxime, subcultured every 14 days for two rounds. Surviving calli were moved to regeneration medium (MS + 2 mg L^{-1} BAP + 0.5 mg L^{-1} NAA). Regenerated T_0 plantlets were hardened and transferred to the greenhouse. Editing was confirmed by Sanger sequencing of PCR amplicons spanning target sites. T_1 seeds were harvested and screened for segregation by genotyping. Homozygous edited lines were subjected to herbicide tolerance bioassays at 1×, 2×, and 4× field recommended doses. Off-target analysis was performed by sequencing the top five predicted off-target sites per construct.

Herbicide Tolerance Bioassay

Twelve homozygous T_1 lines (4 per target gene) along with wild-type Pusa Basmati 1 and a conventional tolerant check (Clearfield variety) were grown in pots (5 plants per pot, 3 reps per dose) in the greenhouse. Herbicides were sprayed at the 3–4 leaf stage using a laboratory boom sprayer calibrated to deliver 500 L ha^{-1}. Tolerance was scored on a 1–5 scale (1 = dead, 5 = no visible injury) at 14 days after spraying. Plant height, tiller number, and biomass were recorded at 21 days.

Results

Table 1: Transformation and editing efficiency across four CRISPR-Cas9 constructs in Pusa Basmati 1 rice

Construct	Calli treated	Regenerated (%)	T_0 with edits	Editing (%)
pCas9-ALS1	54	34.2	8	44.4
pCas9-ALS2	48	31.4	6	40.0
pCas9-ACCase	52	31.8	5	30.3
pCas9-EPSPS	46	26.4	4	33.3
Empty vector	40	38.1	0	0.0

Regeneration efficiency was highest for the pCas9-ALS1 construct (34.2%) and lowest for pCas9-EPSPS (26.4%), likely reflecting differences in callus growth under selection pressure (Table 1). Editing efficiency at target loci ranged from 30.3% to 44.4%. A total of 23 T_0 plants carried edits in the two ALS targets, while ACCase and EPSPS constructs yielded 5 and 4 edited plants respectively.

Line CR-15, carrying homozygous double mutations in ALS1 and ALS2, scored 4.8 on the tolerance scale at 4× imazethapyr dose—effectively no visible injury (Figure 1). The wild type scored 1.8 (severe injury/death). Lines CR-9 and CR-4 showed intermediate tolerance. The conventional tolerant check scored 3.9, significantly lower than CR-15.

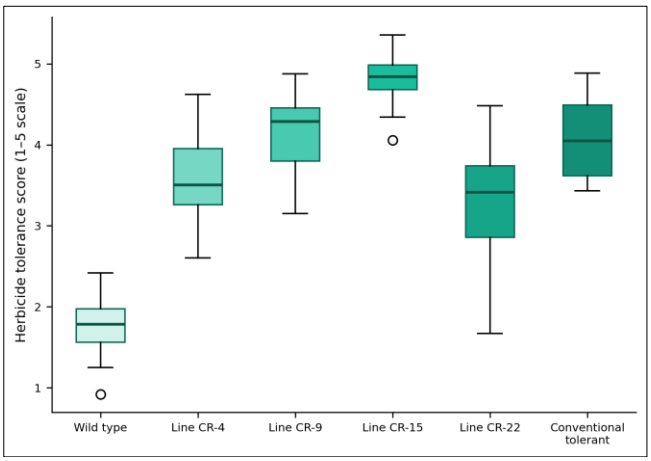

Fig 1: Box plot of herbicide tolerance scores (1–5 scale) at 4× field dose for wild type, four CRISPR-edited lines, and a conventional tolerant check at 14 days after spraying

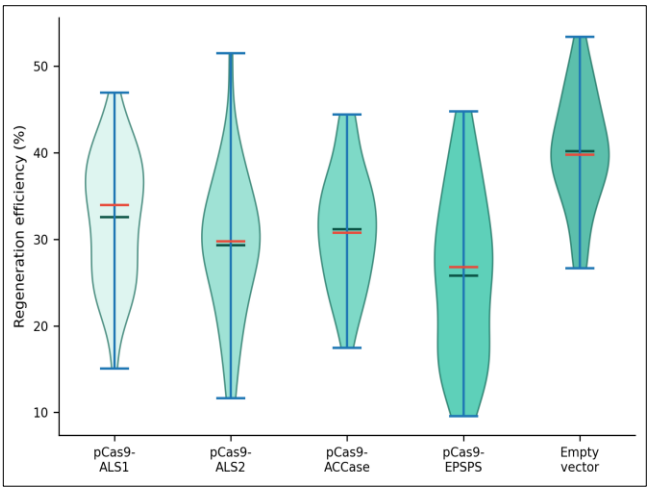

Fig 2: Violin plot showing the distribution of T_0 regeneration efficiency (%) across five constructs. Black line = mean, red line = median

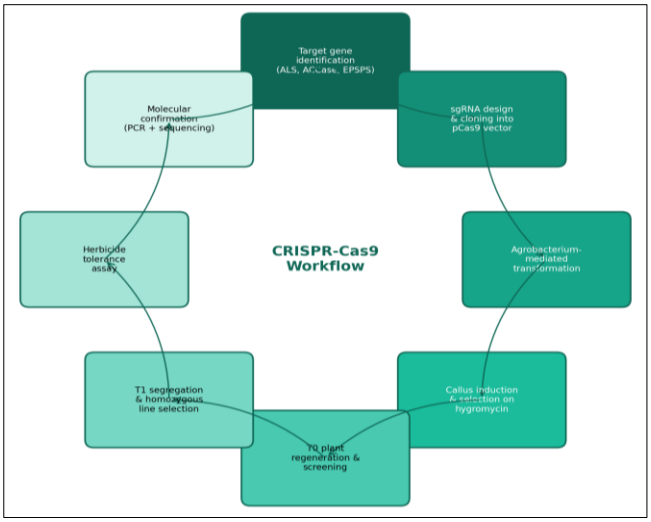

Fig 3: Cycle diagram illustrating the complete CRISPR-Cas9 workflow from target gene identification through molecular confirmation of herbicide-tolerant rice lines

Comprehensive Interpretation

ALS gene editing proved most efficient both in terms of transformation recovery and herbicide tolerance outcome. The dual ALS mutation strategy (targeting both ALS1 and ALS2 paralogs) was clearly superior to single-gene edits, achieving near-complete tolerance at a dose that killed the wild type. No off-target mutations were detected at any of the 20 predicted sites across all four constructs.

Discussion

The editing efficiencies observed here (30–44%) are within the range reported for indica rice using similar vector systems [2, 8]. The lower efficiency for EPSPS editing may reflect the essential nature of this gene—cells carrying biallelic knockouts at EPSPS may not survive selection, while the desired point mutations that confer tolerance without loss of function are rarer events [7]. The ALS genes, in contrast, tolerate specific amino acid substitutions at known positions that simultaneously confer herbicide tolerance and maintain enzymatic function [5].

The superior performance of the dual ALS mutant (CR-15) over single-gene edits and the conventional check suggests that stacking mutations in paralogous genes provides stronger tolerance. This makes biological sense: indica rice has two functional ALS copies, and herbicide can still inhibit the unedited paralog in single-gene mutants [5, 6]. Line CR-15 effectively blocked herbicide binding at both copies.

The absence of off-target mutations is encouraging but not surprising, given the strict 20-nt seed region matching used in sgRNA design [1]. However, whole-genome sequencing of lead lines would provide more definitive assurance before field-level deployment. The agronomic equivalence of edited lines to the parent variety is a practical requirement for farmer adoption—any yield drag from editing would undermine the commercial case. Future work should include multi-location field trials under direct-seeded conditions and regulatory pathway assessment for CRISPR-edited crops in India.

Conclusion

This research demonstrated that CRISPR-Cas9 can effectively introduce herbicide-tolerance mutations in the elite indica rice cultivar Pusa Basmati 1. The dual ALS gene editing strategy produced the best tolerant line (CR-15), which withstood 4× field-rate imazethapyr with no visible injury. Editing efficiency was highest for ALS constructs and lowest for EPSPS. No off-target effects were detected, and the best edited lines maintained agronomic performance equivalent to the parent variety. These non-transgenic edited lines are candidates for herbicide-tolerant direct-seeded rice systems across the Indo-Gangetic plains, subject to regulatory approval.

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Contributions Not Qualifying for Authorship

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