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REVIEW ARTICLE

Exploring *ALS* (Acetolactate synthase) gene as a target for herbicide resistance through CRISPR/Cas mediated genome editing

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Abstract

The increasing global population, combined with escalating climate change, has necessitated the adoption of innovative agricultural practices to ensure sustainable food production. Traditional crop cultivation system relies on conventional weed management practices that are labour and resource intensive. To address this challenge, the development of herbicide-resistant (HR) crops has emerged as a critical solution. CRISPR/Cas-based genome editing has revolutionized HR crop development, offering precision and efficiency over conventional breeding and transgenic approaches. The acetolactate synthase (*ALS*) gene, which is essential for biosynthesis of branched-chain amino acids (BCAAs) such as leucine, isoleucine and valine, serves as a key target for ALS-inhibiting herbicides to control broad spectrum of weed species. Notably, several weed species were identified with natural mutations in *ALS* gene that confers strong level of resistance to different ALS-inhibiting herbicides. Advanced genome editing technologies like CRISPR/Cas9, CRISPR/Cas mediated base editing (BE) and prime editing (PE) have been successfully utilized to mimic these natural mutations or introduce novel mutations in *ALS* gene, thereby creating heritable herbicide resistance in various crops. In this review, we summarize the remarkable achievements reported so far in the development of herbicide-resistant crops specifically by targeting the *ALS* gene using CRISPR/Cas genome editing approach. Furthermore, we propose forward-looking perspectives on integrating multiplex gene editing and gene stacking. The synergy between CRISPR (Clustered Regularly Interspaced Palindromic Repeat) technology and advanced phenotyping platforms holds significant promise for next generation of climate-resilient herbicide resistance crop varieties. These insights offer a valuable roadmap for researchers to design tailored, sustainable weed management strategies and accelerate the global transition towards precision agriculture.

Keywords: *ALS*-gene; CRISPR/Cas; crop improvement; genome editing; herbicide resistance

Introduction

Modern agriculture is facing significant pressure to boost food production in response to the growing global demand caused by rapid population growth (1). Nevertheless, crop production is significantly impacted by drastic climate change, biotic and abiotic stresses, labour and water scarcity, weed management and other environmental constraints. Weeds compete with crop for nutrient resources and sunlight, drastically reducing the potential yield. Weeds pose a major threat to agricultural productivity, causing up to 31.5 % of global crop losses, equivalent to nearly $32 billion in annual economic loss. They are particularly harmful during the early stages of crop growth accounting for approximately 46.2 %–61.5 % yield loss in grains such as rice, wheat and maize. Weeds are responsible for almost one-third of all pest-related crop losses, emphasizing the importance of implementing effective weed management strategies (2). Among the chemical and physical methods of controlling weeds, spraying of non-selective herbicides in field grown with herbicide-resistant crops is a viable method for controlling weeds. One promising approach to address this issue is the development of herbicide-tolerant crop varieties that reduce reliance on labour-intensive methods and make large-scale farming more practical and cost-effective.

Herbicides function by inhibiting essential enzymes or disrupting key metabolic pathways thereby interfering with the normal growth and development of target plants. Various genes have been identified and used as targets for different class of herbicides. Among these, the ALS enzyme is one of the prime targets, as it is involved in the biosynthesis of essential branched-chain amino acids like valine, leucine and isoleucine (3). Apart from ALS, acetyl-CoA carboxylase (*ACCase*) is targeted by *ACCase*-inhibiting herbicides, disrupting lipid biosynthesis (4). 4-hydroxyphenylpyruvate dioxygenase (*HPPD*) is the target of HPPD-inhibiting herbicides like mesotrione (5). Another important target is tubulin gene family, which is affected by

dinitroaniline herbicides that inhibit plant cell division. Crops can develop resistance to these herbicides by altering the specific target genes, thereby preventing herbicide binding and ensuring continued plant growth (6).

Initially, traditional breeding and mutagenesis were employed to select or induce herbicide-tolerant trait in crops. These traits were introduced to cultivars by creating mutations at the herbicide target site by induced mutagenesis (7). Mutations were introduced through chemical mutations (Ethyl Methane sulfonate) or physical mutagens and identified by screening mutant populations for target trait. Herbicide tolerant crops such as maize, rice, wheat, canola were developed using non transgenic approach through induced mutagenesis (8). The first herbicide tolerant rice variety developed through induced mutagenesis in the *ALS* gene was developed by Louisiana State University Agricultural Centre and American Cyanamid-BASF and it was commercialized in 2002 (9).

In India, true breeding rice mutant, HTM-N22 (HTM), tolerant to herbicide Imazethapyr was developed through chemical mutagenesis technique and this mutant line can be used as a genetic stock for developing herbicide tolerant crops (10). Owing to the drawbacks of multiple mutations and off-target effects, precise modification of genome sequences through genome engineering technologies has been developed. With the advent of transgenic approaches, scientists began to introduce foreign genes into crops to confer resistance to specific herbicides. The commercially cultivated herbicide tolerant crops have been developed against the broad-spectrum herbicides: glyphosate and glufosinate by using transgenic approach (11). The herbicide tolerant rice varieties *viz*., Liberty Link, Provisia, Clearfield and Jietian were released and cultivated in USA (12). The two Liberty link rice varieties LLRICE 62 and LLRICE 06 were developed by targeting the *pat* gene and it shows tolerance to glufosinate. These herbicide tolerant rice varieties were approved for commercial cultivation in USA and Canada (13). Genetically modified (GM) or transgenic crops are currently regulated under strict guidelines in most countries, with cultivation banned in numerous regions. In contrast, non-GM herbicide-tolerant varieties have seen widespread regulatory approval and adoption. Recent advancement in genome editing, especially CRISPR/Cas system have revolutionized the HR crop development by enabling precise, efficient and targeted modification in plant genomes (14). This method allows modification of the target genes with highest precision offering farmers greater flexibility in selecting herbicides to manage herbicide resistant weed populations.

Among the various genes targeted for HR, the *ALS* gene stands out due to its critical role in amino acid biosynthesis and its proven effectiveness in conferring resistance to a broad spectrum of herbicides. Using CRISPR/Cas technology, targeted mutations can be introduced in the *ALS* gene to develop herbicide-resistant crops (15). These precise edits mimic naturally occurring or previously identified resistance alleles, thereby offering a non-transgenic approach that can be more readily accepted in both the regulatory framework and public perception. This comprehensive review focuses on the herbicide resistance in various crops developed through CRISPR/Cas mediated genome editing with particular emphasis on the acetolactate synthase gene as a key target.

2. ALS catalyzed biosynthesis pathway and its inhibitors

The *ALS* plays a major role in the biosynthesis of essential branched chain amino acids (BCAAs) like valine, leucine and isoleucine in plants, bacteria and fungi. The BCAAs are essential for normal plant growth and protein synthesis. The first step in BCAAs biosynthesis involves the condensation of two molecules of pyruvate and one molecule of acetyl-CoA to form acetolactate, a reaction catalyzed by ALS. This pathway involves several enzymatic reactions: Acetolactate and acetohydroxybutyrate (AHB) are synthesized by acetolactate synthase (*ALS*) from pyruvate and 2-ketobutyrate, respectively. Acetolactate serves as a precursor for valine and leucine biosynthesis, whereas AHB is a key intermediate in the isoleucine biosynthesis pathway (16) (Fig. 1). The *ALS* gene is conserved in many plant species and is the primary target for ALS-inhibiting herbicides due to its significance in the production of essential amino acids (3).

Over the past two decades, one of the most significant achievements in herbicide chemistry has been the discovery and widespread use of *ALS*-inhibiting herbicides (17). These herbicides bind to the active site of the ALS enzyme, disrupting the BCAAs biosynthesis pathway, leading to the deficiency of amino acids and ultimately result in the death of plants. ALS-inhibitors are categorized into five main classes, namely imidazolinone, pyrimidine thiobenzoates, sulfonyl-amino carbonyl-triazolinone, sulfonylureas and triazolopyrimidines. These herbicides are highly preferred by farmers, due to their low application rates, broad-spectrum efficacy against various

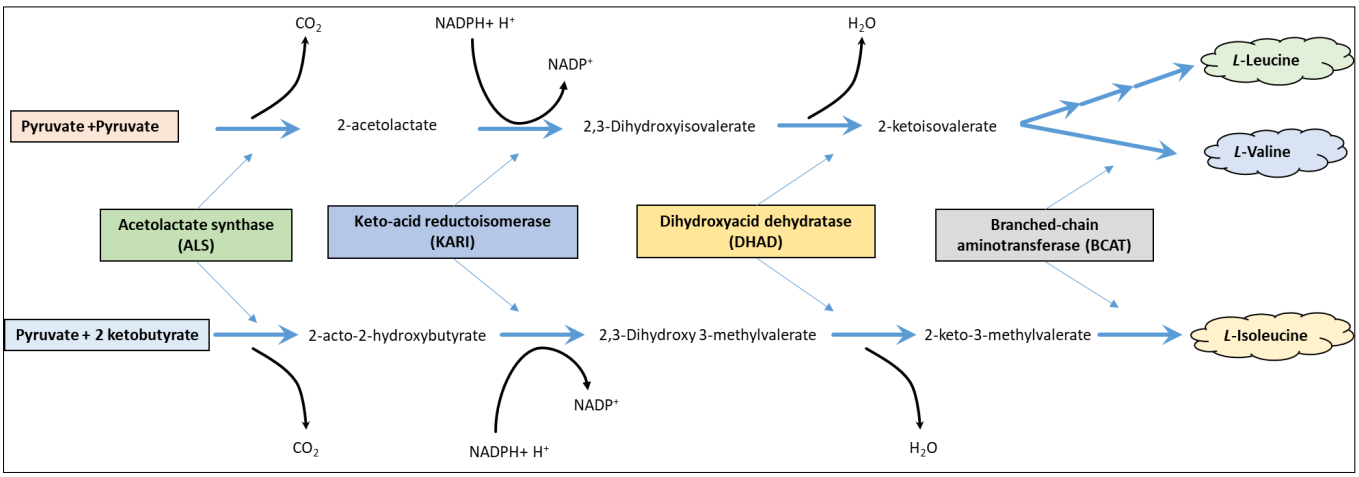

Fig. 1. Schematic representation of the branched chain amino acid biosynthesis pathway in plants.

weed species and low toxicity to mammals (18).

Resistance to *ALS*-inhibiting herbicide is primarily the result of genetic mutation in the *ALS* gene. These mutations alter the structure of the ALS enzyme, reducing the herbicide's ability to bind effectively to its active site, thereby diminishing its inhibitory effect (19). Point mutations in the *ALS* gene lead to change in amino acid that confer resistance. Studies have reported that 66 different weed species exhibit herbicide resistance. Usually, this resistance mechanism entails the substitution of amino acids at particular conserved positions within the enzyme. Genetic analysis of weeds has identified 29 distinct types of mutations occurring at eight key amino acid positions in the *ALS* gene, namely, A122, P197, A205, D376, R377, W574, S653 and G654. Notably, the P197 position can be substituted with 11 different amino acids, including arginine, asparagine, glutamine, histidine, isoleucine, leucine, serine, threonine, tyrosine and alanine. Each of these amino acid substitutions contributes to a different resistance phenotype (20). The major resistant weeds and their associated natural mutation position are summarized in Table 1.

Alterations in the tertiary structure or spatial conformation of acetolactate synthase due to point mutations significantly reduce the enzyme's affinity for *ALS*-inhibiting herbicides. The level of resistance is largely determined by the specific amino acid substitution and the precise location of the mutation. Because of its well-established function in the BCAA pathway and the naturally occurring resistance mutations identified in weed species, *ALS* has emerged as a prime target for CRISPR/Cas mediated genome editing to develop HR in crops. Targeting this gene may also provide broad-spectrum resistance to multiple classes of *ALS*-inhibiting herbicides (30).

3. Mechanism of CRISPR/Cas9 system

The potential and impacts of genome editing technologies for crop development have become increasingly evident over the past decade. The targeted double-stand breaks (DSBs) in DNA were induced by using zinc finger nuclease (ZFNs) and transcription activator-like nucleases (TALENs). However, these methods were time-consuming, expensive and technically demanding, as each target site required the custom engineering of a specific protein (31). In contrast, the CRISPR/Cas9 system, offers immense advantages over traditional gene editing techniques.

CRISPRs (Clustered Regularly Interspaced Short Palindromic repeats) are distinct DNA elements consisting of short, repetitive sequences, typically 23 to 47 bp in length, separated by unique spacer sequence that vary among the different organisms. This system act as part of the adaptive immune response in bacteria and archaea, where CRISPR RNAs (crRNAs) guide the recognition and silencing of foreign genetic material, such as viral DNA. In *E. coli* a series of non-repetitive and repeat sequence located downstream of the *iap* gene were first identified now popularly known as CRISPR (32).

The main key components of CRISPR-Cas9 system are a single guide RNA (SgRNA) and the cas9 endonuclease. The Cas9 is DNA-Cleaving enzyme that has been isolated from several bacterial species like *Brevibacillus laterosporus*, *Staphylococcus aureus*, *Streptococcus thermophilus* and *Streptococcus pyogenes* (33, 34). Among these, Cas9 from *S. pyogenes* is widely used in CRISPR based gene editing due to its effectiveness and extensive validation across the systems. The synthetic single-guide RNA (SgRNA), typically around 100 nucleotides in length, contains 20 nucleotide target sequence at its 5' end. The recognition and cleavage by Cas9 also require a Protospacer Adjacent Motif (PAM) typically characterized by 5'-NGG-3' sequence, present on DNA immediately downstream of target site (35). The Cas9 endonuclease contains two nuclease domains, RuvC domain which cuts the non-complementary DNA strand and HNH domain which cuts the strand complementary to guide RNA. The secondary structure formed by SgRNA, particularly its 3'-terminal stem-loop facilitates the formation of stable complex with Cas9, which is essential for precise DNA cleavage generally observed at three base pair upstream of PAM site (36).

The SgRNA-Cas9 complex generates a DBS upstream of protospacer adjacent motif (PAM) site specifically, NGG sequence in case of Cas9. The type of mutation introduced depends on one of the two DNA repair mechanism viz. Non-Homologous End Joining (NHEJ) and Homology-Directed Repair (HDR). NHEJ is an error–prone mechanism that

Table 1. Summary of CRISPR/Cas system in HR targeting ALS gene

Amino Acid	Substitution reported	Weed Species	Scientific Name	Herbicides Resistance	Reference
Ala122	Tyr / Val / Thr	Barnyard grass, Wild radish	*Echinochloa crus-galli, Raphanus raphanistrum*	Imidazolinone (IMI), Sulfonylureas (SU)	21, 22
Pro197	Thr	Flixweed	*Descurainiasophia*	Sulfonylureas (SU), Triazolopyrimidines (TP), Sulfonylamino-carbonyl-triazolinones (SCT)	23
Pro197	Glu	Water chickweed	*Myosoton aquaticum*	Sulfonylureas (SU), Pyrimidinylthiobenzoates (PTB), Triazolopyrimidines (TP), Imidazolinones (IMI)	24
Ala205	Phe	Annual bluegrass	*Poa annua*	Imidazolinones (IMI), Sulfonylureas (SU), Triazolopyrimidines (TP), Sulfonylamino-carbonyl-triazolinones (SCT)	25
Asp376	Glu	Rush	*Schoenoplectusjuncoides*	Sulfonylureas (SU)	26
Trp574	Leu	Flixweed, Crabgrass, Corn gromwell	*Descurainiasophia, Digitariasanguinalis, Lithospermum arvense*	Sulfonylureas (SU), Imidazolinones (IMI), Triazolopyrimidines (TP), Sulfonylamino-carbonyl-triazolinones (SCT), Pyrimidinylthiobenzoates (PTB)	23, 27, 28
Ser653	Thr/Asn/Ile	Green foxtail	*Setariaviridis*	Imidazolinones (IMI)	29

introduce insertions or deletion (indels) at the site of DSB, resulting in gene knockout. HDR repair mechanism requires a repair template (RT), allowing precise nucleotide changes such as point mutations or targeted insertions (37, 38).

3.1 Advancements in CRISPR/Cas9 system

The CRISPR/Cas system has been enhanced by utilizing various Cas nuclease protein variants. One such variant is CRISPR/Cas 12a (also known as CRISPR-Cpf1) produces staggered DNA ends that produce discrete double-strand breaks. This enables efficient editing in A-rich areas in the genome. Cas12a target sites with TTTN PAM sequences, modified Cas12a variants have been developed to expand their PAM compatibility and target sites. Additionally, advanced base editing and prime editing technologies offer greater precision and flexibility in introducing mutations. Base editors were developed to modify specific bases in the sequence which produces favourable phenotype Base editing combines Cas9 nickase with cytidine or adenosine deaminases, enabling specific base substitutions such as C→T or A→G without introducing double-strand breaks. (39). Cytosine Base Editor (CBE1) was developed by fusing a rat cytidine deaminase enzyme (rAPOBEC1) with a catalytically inactive Cas9 (dCas9), which carries the D10A and H840A mutations. In this system, a cytosine on the non-target DNA strand is deaminated to uracil, which is then recognized and converted to thymine by the cell's DNA repair mechanisms. To improve editing efficiency, a uracil DNA glycosylase inhibitor (UGI) was added to the C-terminal end of the construct, creating rAPOBEC1-dCas9-UGI, which prevents uracil excision by blocking UNG activity. Further enhancements were made by replacing dCas9 with a Cas9 nickase (nCas9, D10A mutant) and fusing two UGI molecules to its C-terminus, increasing base editing efficiency (39).

In the development of Adenine Base Editors (ABEs), researchers fused nCas9 (D10A) with an engineered adenosine deaminase to convert adenine (A) to guanine (G). The first generation, ABE7.10, combined wild-type TadA with an evolved variant TadA7.10 and showed efficient editing within a defined window of 4-8 nucleotides from the PAM site. Later, ABE8e was developed by incorporating a more active deaminase, TadA8e, improving editing efficiency. Building on this, the PhieABE system was created by fusing TadA8e with a single-stranded DNA-binding domain, resulting in a broader editing window and higher editing efficiency compared to earlier ABE versions (40).

Prime editing is a search and replace genome editing method that enables the precise insertion or deletion of small DNA sequences and allows for a wide range of base substitutions, including both transitions and transversions, without inducing double-strand breaks (DSBs). This system relies on a fused complex of a reverse transcriptase enzyme (derived from Moloney murine leukemia virus, M-MLV-RT) and a nickase version of Cas9 (nCas9, with the H840A mutation). The reverse transcriptase uses a specially designed RNA molecule called the prime editing guide RNA (pegRNA) to change the desired genetic sequence into the target DNA site. Notably, this process does not require donor DNA templates or DSBs. Prime editing facilitates the targeted alteration of multiple bases or the incorporation of defined insertions and deletions at endogenous loci through a pegRNA-directed reverse transcription mechanism (41).

Also multiplex editing creates mutation using multiple guide RNAs targeting different traits/genes to develop climate resilient crops. Multiplexed genome editing has emerged as a powerful strategy for the coordinated regulation of gene expression, the pyramiding of beneficial traits and the manipulation of complex regulatory networks. The most employed approach involves the incorporation of multiple single-guide RNA (sgRNA) expression cassettes within a single plasmid vector. Alternatively, multiplex editing can be achieved through the co-delivery of Cas9 protein and distinct sgRNA constructs, or by employing Csy4 endoribonuclease-mediated processing or tRNA-based cleavage systems to generate individual sgRNAs from a polycistronic transcript. These multiplex platforms enable the simultaneous and precise editing of multiple genomic loci, thereby accelerating functional genomics studies and the development of complex trait architectures in plants and other organisms (42).

3.2 Workflow and strategic applications of CRISPR/Cas9 in crop development

The CRISPR/Cas9-mediated gene editing has emerged as a powerful tool for crop improvement, enabling targeted modification of plant genomes to enhance agronomic traits such as yield, biotic and abiotic stress resistance. The process begins with the identification of target gene associated with desirable traits, followed by the design of single-guide RNA (sgRNA) that direct the Cas9 nuclease to specific DNA sequences. Once the CRISPR/Cas9 construct is delivered into plant cells *via* direct (Particle Bombardment, Electroporation and Microinjection) or indirect (*Agrobacterium*-mediated transformation and Virus-mediated transformation) gene delivery methods, site-specific cleavage is introduced by Cas9, which will be further repaired by the plant's endogenous repair pathways. The screening and validation of induced mutation is then confirmed by various molecular techniques like DNA based PCR analysis and sanger sequencing of target regions. The putative mutants are then selected for further field trials and trait evaluation. Transgene-free mutants are obtained through generation advancement of positive mutants (43). This process facilitates the segregation of the desired mutation and molecular analysis of the segregating population helps to identify transgene free lines (Fig. 2).

The efficient use of CRISPR/Cas9 technique to enhance a wide range of agronomic traits across diverse crop species. In soybean, the *GmSNAP02* gene was identified as a susceptibility factor for soybean cyst nematode (SCN) and precisely knocked out using dual-guide RNA constructs delivered via *Agrobacterium rhizogenes*, resulting in transgenic hairy root systems that exhibited a significant reduction in SCN cyst formation, thereby validating GmSNAP02 as a negative regulator of resistance and highlighting its potential in nematode-resilient soybean breeding (44). In *Camelina sativa,* a hexaploid oilseed crop, researchers used CRISPR/Cas9 to disrupt all three homeologs of the TT8 gene, which regulates proanthocyanidin biosynthesis. The resulting tt8 mutants displayed yellow seeds with thinner seed coats, reduced fiber content and enhanced oil accumulation up to 41.2 % compared to 36.8 % in the wild type without affecting growth or yield (45). In tomato (*Solanum lycopersicum*), disruption of

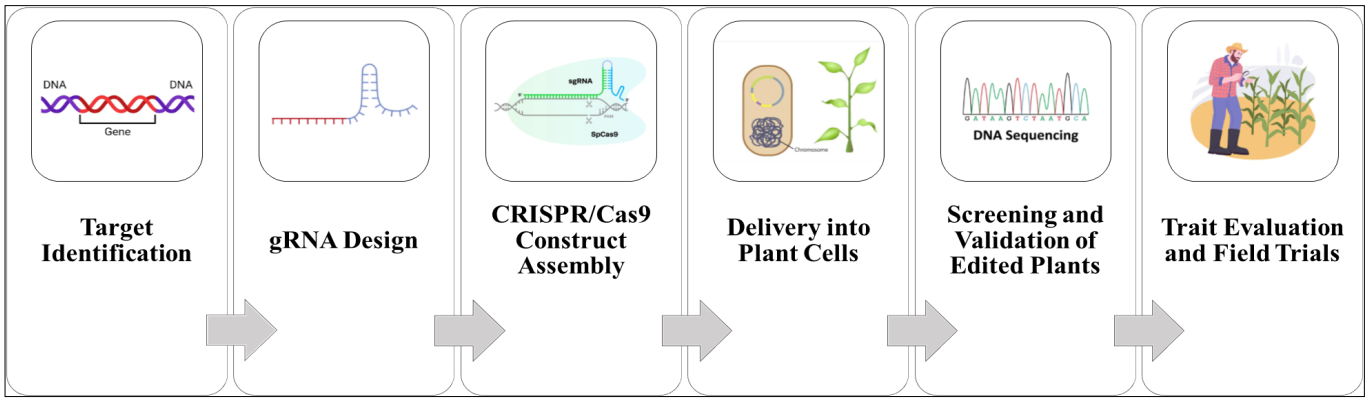

Fig. 2. Steps involved in CRISPR/Cas9 mediated gene editing for crop improvement.

the *SlPG* gene, encoding the polygalacturonase enzyme responsible for fruit softening, significantly delayed post-harvest ripening and improved fruit firmness without altering key quality traits, showcasing the gene's relevance in enhancing shelf life and marketability (46). In rice, two groundbreaking studies underscore the versatility of CRISPR technology. One study achieved clonal seed production by editing four pivotal genes *OSD1*, *PAIR1*, *REC8 and MTL* to induce mitosis-like division and parthenogenesis, resulting in diploid clonal seeds that preserved hybrid vigor across generations (47). Another rice study focused on Knockout or knockdown of *STRONG1* gene through CRISPR/Cas9, significantly increased culm diameter, wall thickness, cellulose content and lodging resistance. The mutant lines also showed improved panicle architecture, grain number and plant structure, leading to a 9.3 –15.4 % increase in grain yield. related genes through altered chromatin marks, without compromising other agronomic traits (48). Adding further to these advancements, a recent study of CRISPR/Cas9 mutagenesis successfully created novel alleles of yield gene *An-1* leading to increased grain number in rice. the homozygous transgene free mutants were found to be free from off-targets, affirming *An-1* as a novel domestication gene and a powerful breeding target (49). Collectively, these studies showcase the unprecedented power of CRISPR/Cas9 genome editing to dissect gene functions, enhance yield, improve nutritional value, prolong shelf life and build resilience against biotic and abiotic stresses.

The primary objective when targeting the *ALS* gene is to introduce specific point mutations that confer herbicide resistance without disrupting gene function, rather than to completely knock out the gene. In this context, the innovative CRISPR-based genome editing techniques, such as base editing and prime editing, have emerged as powerful tools for inducing specific mutations within the *ALS* gene. Using base editing, targeted nucleotide substitutions can be introduced directly into the *ALS* gene without creating double-strand breaks. The schematic representation of the application of various CRISPR/Cas based approach for targeting *ALS* in different crops with respect to developed resistant against the ALS-inhibitor is illustrated in Fig. 3.

4. Gene editing of the *ALS* Gene in commercial crops

4.1 Rice

Various genome editing techniques have been employed to develop herbicide-resistant rice (*Oryza sativa*), each with specific advantages and limitations. Early methods such as TALENs and ZFNs (50) used HDR to introduce point mutations like W548L and S627I into the *OsALS* gene. However, these systems produced predominantly heterozygous edits with modest efficiency (0.5 %-6.3 %). Subsequent studies utilized CRISPR/Cas9-mediated HDR to improve targeting efficiency. Approaches such as dual-gRNA delivery (51), Lig4 suppression to enhance HDR rate (52) and cgRNA-based HDR (53), enabled the generation of homozygous herbicide resistant lines. Cas9-VirD2 fusions (54) and Cas12a-mediated gene targeting (55) subsequently enhanced HDR precision and biallelic recovery, these methods were still limited by limited editing efficiencies.

Later advancements made use of prime and base editors to provide cleaner and more accurate edits without introducing double-strand breaks. These platforms enabled targeted modifications at key amino acids such as P171, G628 and A96, leading to broad-spectrum resistance to multiple ALS -inhibiting herbicides. Base editors such as Target-AID (56), BE3 (57) and dual-base editors (pDuBE1) (58, 59) have successfully introduced these mutations in *ALS* genes. Notably, base-editing-mediated directed mutagenesis was demonstrated in studies referenced 57 and 60, resulting in triple mutants P171F/G628E/G629S and novel resistance-conferring mutations e.g. P171F and G628E. In parallel, prime editing platforms such as PE-P2 (61), Sp-PE3 (62) introduced specific edits like W548L, S627N and P171S. These systems enabled clean, template-free editing, but exhibited limited editing efficiency (less than 10 %) and variable heritability. The editing scope was expanded with SpRY-based PAM-less base editors (63) and T7 RNAP-driven synthetic evolution (64), which facilitated the discovery of additional functional *ALS* variants and broadened the range of editable genomic sites.

Taken together, these studies highlight three major technological approaches: base editing, prime editing and HDR-based precise editing for developing herbicide-resistant rice. While HDR remains the most accurate method, its low efficiency, dependence on transformation techniques such as biolistic and frequent interference from the NHEJ pathway limit its practical application. Base editing offers higher efficiency and a broader mutation spectrum; however, it also presents challenges including chimerism, restricted editing windows and potential off -target effects. Similarly, prime editing and T7 RNA polymerase-driven methods show potential for multiplexed genome editing and *in vivo* directed evolution, delivering clean edits without DSBs. However, they are hindered by low editing efficiency, complex pegRNA design requirements and inconsistent performance across genomic loci. Commercial adoption of these technologies limited by the lack of scalability tests in elite

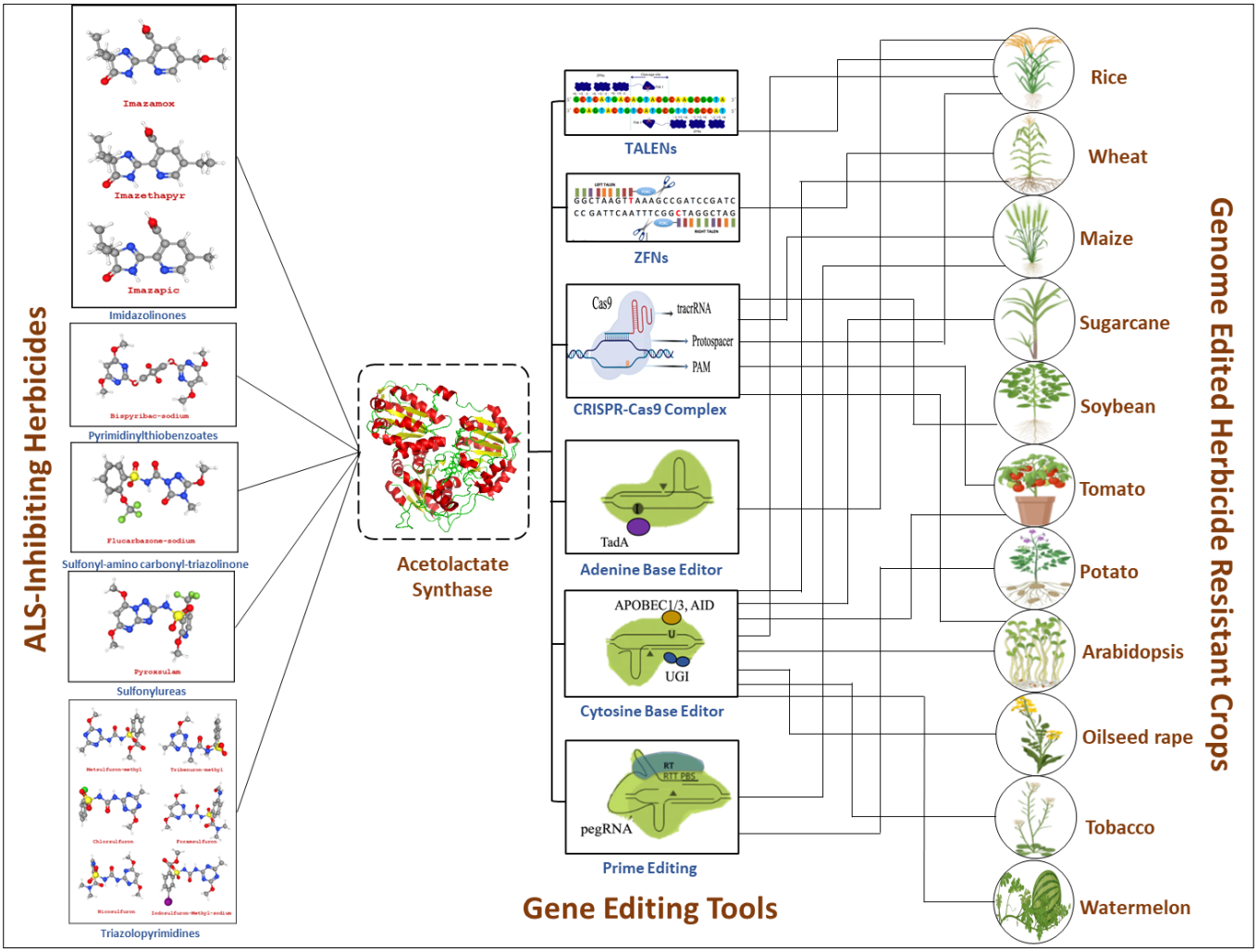

Fig. 3. Crops targeted to develop ALS- Inhibiting Herbicide resistant by using various genome editing tools.

cultivars and the requirement for transgene-free, regulatory-compliant editing pipelines. To fully realize the potential of genome editing in sustainable herbicide resistance breeding in rice, future research should focus on optimizing editing fidelity, delivery mechanisms and trait stacking strategies.

4.2 Wheat

Ran and colleagues provided one of the first examples of targeted gene editing for herbicide resistance in wheat by using ZFNs to introduce the S653N mutation in the *ALS* gene, conferring resistance to the herbicide, imazamox. The mutation was achieved through NHEJ-mediated allele insertion. Although insertions and deletions (indels) were occasionally observed at the repair sites, the efficiency of precise editing remained low, especially with the dual-ZFN approach. Despite these limitations, the study offered an important proof-of-concept for trait editing in complex polyploid wheat genomes, paving the way for future genome editing efforts in cereals (65).

Subsequent research improved the accuracy and efficiency of editing through the use of cytidine base editors (CBEs). To overcome the limited editing scope of earlier base editors, Zong and team developed the A3A-PBE system by combining APOBEC3A with Cas9 nickase (66). This system enabled C-to-T conversions that confer resistance to herbicides nicosulfuron, by targeting the conserved Pro174 location in the *TaALS* gene. Using this method, the researchers successfully generated 27 edited T_0 plants, including lines with edits in all three wheat sub-genomes (A, B and D) and were able to produce transgene-free plants. The A3A-PBE system offered several advantages, including a broadened editing window (C6-C13), support for multiplexed editing and DNA-free delivery, thereby enhancing its relevance for regulatory approval and plant breeding. However, the approach still faced limitations such as low homozygous recovery, reduced efficiency with RNP-based delivery and a moderate overall editing efficiency (~22.5 %).

Building on this work, Zhang and team used a plant base editor (PBE) derived from APOBEC1 to target both *ALS* genes in wheat, resulting in herbicide resistance to sulfonylureas class of herbicide (67). The study introduced precise mutations such as P174S, P174F and P174A in the *TaALS* gene, as well as combinatorial edits like P174F and R175C. By co-editing, the researchers were able to stack tolerance to multiple herbicides, including imazapic, nicosulfuron and mesosulfuron. Notably, 10 out of 16 edited lines were transgene-free and homozygous mutants across all three sub-genomes (A, B and D) exhibited strong herbicide resistance, tolerating dosages up to 9× the recommended field rate without exhibiting adverse agronomic traits. However, editing efficiencies varied across sub-genomes and the study did not extensively evaluate long-term field performance or potential off-target effects.

A definite technical progression is evident when comparing these three studies, from ZFN-induced allele insertion to more extensive and accurate base editing tools. While ZFNs, as demonstrated by Ran and team (65), provided an early proof-of-concept for allele-specific editing, the A3A-PBE and PBE systems, on the other hand, greatly increased their

potential for regulatory acceptability and breeding adoption by providing transgene-free lines with greater precision and compatibility with co-editing. Notably, while PBE enabled multi-gene targeting for stacked herbicide resistance, the A3A-PBE platform demonstrated superiority in multiplexing and editing window flexibility.

4.3 Maize

Three primary genome editing technologies, such as Cytidine Base Editing (CBE), Prime Editing (PE) and CRISPR/Cas9-mediated HDR have been employed in various studies to introduce precise mutations into acetolactate synthase (*ALS*) genes for conferring herbicide resistance in maize (*Zea mays*). The P165S mutation in *ZmALS2* was initially introduced using HDR, conferring herbicide resistance (68). The resulting edited lines exhibited high resistance to chlorsulfuron. Later, Jiang and team used PE to introduce W542L and S621I mutations in *ZmALS1* and *ZmALS2*, successfully generating homozygous double mutants that tolerated multiple herbicide classes and exhibiting higher editing efficiency, especially with the S621I target site (69). More recently, Li and team introduced a C→T substitution in *ALS* genes using a cytidine deaminase-based CBE system leads to P165A/S/L/W mutations, which likewise produced high-level Chlorsulfuron herbicide resistance (70). Interestingly, homozygous double mutants produced *via* PE and CBE exhibited tolerance to high herbicide doses while retaining normal growth and fertility.

Each of these technologies has its own advantages and disadvantages. A comparison reveals a progressive improvement in both traits stacking capacity and editing precision. Although HDR, laid the groundwork for precise ALS editing in maize, it was constrained by low editing efficiency (~0.45 %) and reliance on robust selection mechanisms (68). prime editing (PE), previous studies offered a significant advancement by enhancing editing efficiency and enabling multiplexed edits across multiple loci, all without introducing double-strand breaks (69).

4.4 Soybean

CRISPR/Cas9-mediated targeted mutagenesis was successfully applied by Li and colleagues in soybean (*Glycine max*) to introduce herbicide resistance against sulfonylurea herbicide (71). A primary focus was the P178S mutation in the *ALS1* gene, a well-characterized modification associated with resistance to chlorsulfuron. The researchers employed particle bombardment to deliver two Cas9-gRNA constructs into soybean tissues. Through homology-directed repair (HDR), five distinct nucleotide substitutions were introduced at the *ALS1* locus, resulting in the desired P178S amino acid change. Southern blotting and PCR confirmed the accurate insertion of transgene and the transgenic callus were regenerated into T_0 and T_1 plants. Notably, sequence analysis confirmed the precise introduction of five nucleotide substitutions that resulted in effective and inheritable herbicide resistance and clean homozygous T_1 plants lacking the transgenic backbone. The overall HDR efficiency was modest (~4 %) and most HDR-positive callus lines failed to generate stable edited plants, likely due to somatic chimerism. In contrast, NHEJ mediated mutation rates were substantial, reaching up to 76 %. The co-delivery of donor DNA and Cas9-gRNA constructs led to multiple random insertions in a few events, necessitating extensive segregation in subsequent generations to isolate transgene-free lines. Additionally, the strategy mostly depends on external selection markers such as resistance to hygromycin and chlorsulfuron, which poses regulatory challenges for commercial deployment. Despite the specificity of edits targeting ALS1, potential off-target effects on other ALS homologs were not thoroughly examined. Future research should aim to enhance HDR precision, mitigate somatic mosaicism, develop marker-free editing systems and expand multiplexing capabilities to facilitate broad-scale genome editing in elite soybean germplasm.

4.5 Potato

Recent genome editing efforts have targeted herbicide resistance in potato (*Solanum tuberosum*), specifically through targeted modifications of the acetolactate synthase (*ALS*) gene, which is essential for conferring resistance to ALS-inhibiting herbicides such as imazamox and chlorsulfuron. Former researchers investigated the use of Prime Editing, specifically, a CRISPR-Cas9 nickase fused to a reverse transcriptase, to introduce a precise P186S point mutation in the *ALS* gene of potato (72). However, editing efficiency in potato was extremely low, with only one successful PE2 event (pegRNA-only), in contrast to the higher success observed in the model moss *Physcomitrium patens*. The PE3 approach, which employs an additional sgRNA to nick the opposing strand, failed entirely and led to undesired deletions, highlighting significant limitations in applying PE to complex polyploid crops like potato. In a similar manner, Butler and team used TALENs in conjunction with BeYDV-based replicons and CRISPR/Cas9 to induce mutations such as W563L and S642T in *ALS*1 (73). To overcome somatic mosaicism and isolate stable edits, this approach required multiple regeneration cycles and strict dual selection (kanamycin and herbicide), which resulted in higher editing frequencies (up to 34.5 %). In the tetraploid potato, 10 % of herbicide-resistant mutants were transgene-free and base conversions at the 14th position (C-14) predominantly yielded Pro186 substitutions to Ser, Ala, or Thr, all of which conferred herbicide tolerance.

Despite employing distinct approaches, both studies collectively underscore the persistent challenges in achieving efficient and commercially viable herbicide resistance in polyploid crops such as potato. PE is hampered by low editing efficiency, while HR-mediated editing is constrained by a dependence on selectable markers and recurring regeneration cycles. Additionally, both strategies suffer from limitations such as a lack of coordination among editing components in the PE system. The primary causes of inefficiencies in the PE appear to be pegRNA instability, complex chromatin environments in polyploid genomes and insufficient expression or functionality of Cas9-RT fusion proteins.

4.6 Tomato

The CRISPR/Cas9-mediated cytidine base editor (CBE) was utilized to develop herbicide resistance in two economically significant solanaceous crops, potato and tomato, by targeting the acetolactate synthase (*ALS*) gene (74). Point mutations at the Pro186 codon (which is analogous to Pro197 in *Arabidopsis*) confer resistance to ALS-inhibitor herbicides such as chlorsulfuron. In tomato, the editing efficiency of 71.4 % was observed using the vector Target-AID CBE (nCas9 linked with cytidine deaminase) delivered *via Agrobacterium*. Furthermore, off-target activity in the homologous *ALS2* gene was closely observed in the study and transgene-free lines exhibited

noticeably no off-target editing. Despite the high editing efficiency, uracil excision was not sufficiently suppressed, as evidenced by a significant proportion of edited plants displaying indels (up to 75 %). This highlights the need to enhance base editor fidelity, possibly using alternative or improved uracil glycosylase inhibitor (UGI) elements. While the approach proved effective in vegetatively propagated crop like potato, it has yet to be widely validated in other herbicide-resistant or polyploid crops. These limitations present opportunities to improve sgRNA design, optimize base editor performance and extend this strategy to additional plant species and traits.

4.7 Arabidopsis

The CRISPR/Cas9-mediated cytidine base-editing (BE3) system was successfully established in *Arabidopsis thaliana* to generate herbicide-resistant plants through precise point mutations in the acetolactate synthase (*AtALS*) gene (75). The researchers targeted codon Pro197 of *ALS*, a highly conserved site across plant species known to confer resistance to *ALS*-inhibiting herbicides. Out of 240 examined plants, four carried C→T edits within the "deamination window," resulting in three distinct mutations, P197L, P197S and P197F. While P197L and P197S are well established resistance mutations, P197F was newly characterized in this study. The T_1 progeny confirmed both the heritability and phenotypic expression of herbicide resistance, with 75–91 % seedlings survival rate with tribenuron treatment. Notably, several transgene-free herbicide-resistant plants were identified. Additionally, an unexpected mutation G202D was observed in the PAM region, suggesting a potential new editable site. The editing efficiency in T_1 was relatively low (~1.7 %), significantly below the 2.7–40 % range reported in rice using same editing platforms. This reduced efficiency may be attributed to the low predicted targeting performance for the selected sgRNA sequence as well as the use of an egg-cell-specific promoter, which, although favorable for heritable editing, may limit expression levels. Unexpected re-editing events and novel PAM-site mutations, while insightful, raise concerns about off-target activity and unpredictability in genomic outcomes. Furthermore, although this study successfully demonstrated herbicide resistance in model plant *Arabidopsis*, its translational potential in crop species, particularly those with complex polyploid genomes, remains unassessed.

4.8 Watermelon

Tian and team successfully engineered herbicide-resistant watermelon using a CRISPR/Cas9-mediated cytidine base-editing system, marking the first report of success in cucurbits (76). The base-editing tool employed was the BE3 system (nCas9 fused to rat APOBEC1 and UGI), driven by a double 35S promoter and delivered using *Agrobacterium*-mediated transformation into the watermelon cultivar ZG94. Among 199 T_0transgenic lines, 45 (23 %) carried precise C-to-T mutations at codon Pro190 of *ClALS* gene, resulting in P190S or P190L amino acid substitutions. These point mutations are well-characterized for conferring resistance to sulfonylurea herbicides, including tribenuron-methyl. Genotyping of T_1 progeny confirmed stable inheritance, including the recovery of transgene-free, homozygous P190S mutants. Herbicide assays confirmed that these mutants exhibited strong resistance to tribenuron, even at four times the recommended field dose, with no adverse effects on yield or fruit quality. While base-editing efficiency reached 23 % in T_0 plants, most of transgenic lines remained unedited, highlighting the need for improved delivery or expression systems.

4.9 Oilseed rape

Wu and team reported the development of herbicide resistance in oilseed rape (*Brassica napus*) using CRISPR/Cas9-mediated cytidine base editing (CBE) (77). The paralogs *BnALS1* and *BnALS3* genes encoding acetolactate synthase (ALS) were targeted by base-editing construct included rat APOBEC1 (cytidine deaminase), nCas9 (D10A nickase) and uracil glycosylase inhibitor (UGI) under the control of a ubiquitin promoter. Transformation was performed *via Agrobacterium tumefaciens*, producing 217 transgenic T_0 plants. Of these, 7 plants exhibited edits in the *BnALS1*, 4 showed precise C→T substitutions leading to Pro197 to Ser (P197S) or Phe (P197F) mutations, while three contained indels. Notably, only *BnALS1*was successfully edited, despite targeting *BnALS3* and *BnALS2*. The P197S mutation conferred strong resistance to tribenuron-methyl, with homozygous plants surviving herbicide concentrations up to 30 mg ai/L. The T_1 progeny displayed Mendelian segregation and transgene-free homozygous lines were recovered. However, the editing efficiency was low, with only ~1.8 % of T_0 plants exhibiting the precise C→T substitutions in *BnALS1.* The lack of edits in *BnALS*3 and *BnALS2* highlights the difficulty of simultaneously editing multiple homoeologous loci in polyploid genomes such as that of *Brassica napus*, likely due to factors such as chromatin accessibility or sequence divergence.

4.10 Tobacco

CRISPR-Cas9-mediated cytidine base editing for the generation of herbicide-resistant plants in tobacco (*Nicotiana tabacum*) was reported (78). The study tested two base editors, specifically nCas9-PmCDA1 and APOBEC1-nCas9, both fused with an uracil DNA glycosylase inhibitor (UGI) to prevent uracil excision. By targeting protoplasts of *Nicotiana tabacum,* the researchers mapped specific nucleotide substitution windows and found nCas9-PmCDA1 to be more efficient than APOBEC1-nCas9. The most effective system, nCas9-PmCDA1-N19 successfully generated sulfonylurea (SU) herbicide-resistant plants by inducing a Proline to Leucine substitution at position 194 in the *ALS* gene. Moreover, base-editing efficiency was substantially improved when using a ubiquitin promoter over the commonly used 35S promoter, resulting in improved regeneration rates of herbicide-resistant shoots. Although APOBEC1-nCas9 exhibited lower editing activity, the study did not rigorously control for potential confounding variables such as codon optimization or construct-specific expression levels, leaving the comparison of the base editors incomplete. Moreover, the majority of base-editing tests were conducted in protoplasts, which may not fully represent the editing dynamics in whole plants or callus tissues.

4.11 Alfalfa

Cytidine base editing (CBE) was successfully applied in alfalfa (*Medicago sativa*) (79), marking the first report of gene editing in this important forage crop. Using a CRISPR/Cas9 system combined with a cytidine deaminase, the researchers targeted the *ALS1* and *ALS2* genes to introduce C-to-T point mutations that conferred herbicide tolerance. Out of 150 transformation events, 17 lines showed edits in *ALS1* and nine in *ALS2*, all exhibiting improved tolerance to both sulfonylurea herbicides

(metsulfuron-methyl, foramsulfuron, iodosulfuron-methyl-Na) and imidazoline herbicides (imazapic). The introduced mutations led to a Proline (P) to Leucine (L) amino acid substitution known to disrupt herbicide binding at the ALS enzyme. Notably, the Argentina government recognized the resulting trait as a conventional, non-transgenic modification, potentially facilitating commercial adoption without the regulatory burdens associated with transgenic crops. However, the tetraploid nature of alfalfa complicates editing outcomes and the study did not specify how many alleles per locus were edited or assess zygosity effects. Additionally, analyses were restricted to controlled growth chamber conditions, without field trial data to evaluate performance under realistic agricultural stressors. Potential off-target mutations were also not investigated, leaving questions about the precision and broader impact of the edits.

4.12 Sugarcane

A major breakthrough in sugarcane genome editing was demonstrated through the precise editing of the *ALS* gene using CRISPR/Cas9-mediated homology-directed repair (HDR) (80). The study engineered herbicide resistance by introducing two well-characterized point mutations, W574L and S653I, which are known to confer strong resistance to herbicides such as nicosulfuron and bispyribac sodium. Among 146 regenerated plants, 54 lines (11.6 %) showed successful HDR edits: 25 carried W574L, 18 carried S653I and 11 carried both mutations. This represents a relatively high HDR efficiency, especially given sugarcane's highly polyploid genome and exceeds most HDR-based editing efficiencies reported in other polyploid crops. Although the study successfully achieved multiplex editing in sugarcane's complex genome, it did not provide data on the long-term stability, agronomic performance, or potential pleiotropic effects of the introduced mutations. Future efforts should focus on implementing base editing or prime editing strategies to improve precision and reduce reliance on the relatively low-efficiency of the HDR pathway. A brief summary of use of CRISPR/Cas for development of herbicide resistant by targeting Acetolactate synthase gene in various crop is presented crop-wise (Table 2) (81-83).

5. Challenges and limitations of CRISPR/Cas based gene editing

Although significant progress has been made in recent years, the application of CRISPR/Cas technologies for targeting the *ALS* gene to enhance herbicide resistance remains in a developmental stage. Transitioning from proof-of-concept studies to real-world agricultural impact involves overcoming multiple challenges, including technical limitations, regulatory restrictions and socio-economic considerations. Simultaneously, new scientific advancements present exciting possibilities to expand the scope and efficiency of *ALS* gene editing and other

Table 2. Summary of CRISPR/Cas system in HR targeting ALS gene

Crop	Genome Editing System	Delivery Method	Mutation	Target Herbicide	Reference
Rice (*Oryza sativa*)	TALEN	Ballistic delivery	W548L/S627I	Bispyribac-sodium	50
	CRISPR/Cas9	Particle bombardment	W548L/S627I	Bispyribac-sodium	51
	CRISPR/Cas9	*Agrobacterium*-mediated transformation	W548 L/S627I	Bispyribac-sodium	52
	CRISPR/Cas9	*Agrobacterium*-mediated transformation	W548L	Bispyribac-sodium	53
	CBE (Target-AID)	*Agrobacterium*-mediated transformation	A96V	Imazamox	47
	CRISPR/Cas9	Particle bombardment	W548L	Bispyribac-sodium	54
	CRISPR/Cas12a	Particle bombardment	W548, S627	Bispyribac-sodium	55
	Prime editing	*Agrobacterium* mediated transformation	W548L	Bispyribac-sodium	61
	Prime editing	*Agrobacterium* mediated transformation	W548L, P171S	-	60
	BEMGE	*Agrobacterium*-mediated and Particle bombardment	P171F, P171L, P171S, R190H	Bispyribac-sodium	49
	CRISPR/Cas9	*Agrobacterium* mediated transformation	G628W	Imazethapyr, imazapic	81
	Prime editing	*Agrobacterium*-mediated transformation	S627N	Imidazoline	62
	ABE	*Agrobacterium*-mediated transformation	S627G	Imidazolinone,	82
	DuBE	*Agrobacterium*-mediated transformation	P171F	Bispyribac-sodium	58
	A/C/DBE	*Agrobacterium*-mediated transformation	P171S, P171Y, P171A, P171F, P171F/G628E/G629S	Nicosulfuron, Imazapic, Pyroxsulam flucarbazone, Bispyribac sodium	58
	A/C/DBE	*Agrobacterium*-mediated transformation	G628 E/R/K, G629S	Bispyribac sodium	63
	A/C/DBE	*Agrobacterium*-mediated transformation	A39T, P70R, P93S, G94R, W548C, F575C, K591E, G629R, G629S	Bispyribac sodium	64
	CRISPR/Cas9	*Agrobacterium*-mediated transformation	W548L	Bispyribac sodium	83
Wheat (*Triticum aestivum*)	ZFN	Particle bombardment	S653N	Imidazolinone	65
	CBE	Particle bombardment	P174F & R175C	Nicosulfuron	66
	CBE	Particle bombardment	P174 S/F, P174F&R175C, P174F&P171S/G631D & G632S	Nicosulfuron, Mesosulfuron, Imazapic	67

	CRISPR/Cas9	Particle bombardment	P165S	Chlorsulfuron	68
Maize (*Zea mays*)	Prime editing	*Agrobacterium*-mediated transformation	W542 L/S621I	/	69
	CBE	*Agrobacterium*-mediated transformation	P165A/S/L/W	Chlorsulfuron	70
Soybean (*Glycine max*)	CRISPR/Cas9	Particle bombardment	P178S	Chlorsulfuron	71
	TALEN, CRISPR/Cas9	*Agrobacterium*-mediated transformation	W563 L/S642T	Imidazoline	74
Potato (*Solanum tuberosum*)	CBE	*Agrobacterium*-mediated transformation	P184	Chlorsulfuron	73
	Prime editing	*Agrobacterium*-mediated transformation	P186S	Chlorsulfuron	72
Tomato (*Solanum lycopersicum*)	CBE	*Agrobacterium*-mediated transformation	P186	Chlorsulfuron	74
Arabidopsis (*Arabidopsis thaliana*)	CBE		P197S, P197L, P197F, G202D	Tribenuron	75
Watermelon (*Citrullus lanatus*)	CBE	*Agrobacterium*-mediated transformation	P190S	Tribenuron	76
Oilseed rape (*Brassica napus*)	CBE	*Agrobacterium*-mediated transformation	P197S	Tribenuron-methyl	77
Tobacco (*Nicotiana tabacum*)	CBE	*Agrobacterium*-mediated transformation	P194L	Chlorsulfuron	78
Alfalfa (*Medicago sativa*	CBE		P177L P182L	Metsulfuron, Foramsulfuron, Iodosulfuron-Methyl-sodium, Imazapic	79
Sugarcane (*Saccharum officinarum*)	CRISPR/Cas9	Biolistic delivery	W574L, S653I	Nicosulfuron, Bispyribac sodium	80

genome targets. One of the foremost areas of ongoing development is the enhancement of HDR efficiency in plants. Homology-directed repair is essential for achieving precise and programmable base substitutions; however, its application is limited by low efficiency, particularly in non-dividing somatic tissues. To overcome this bottleneck, researchers are exploring several innovative strategies, such as cell cycle synchronization, use of geminiviral replicons, inhibition of non-homologous end joining (NHEJ) pathways, such as through DNA Ligase IV suppression and the incorporation of single-stranded oligodeoxynucleotides (*ssODNs*) as donor templates (84). These approaches have demonstrated encouraging results in model species like rice and tobacco; however, further optimization is required for broader implementation in polyploid and transformation-recalcitrant crops, such as wheat and soybean (85).

In addition to HDR-based strategies, base editing and prime editing are emerging as leading platforms for precise, transgene-free gene modifications in crop species. Nevertheless, these technologies still face several limitations. Current base editors are constrained to nucleotide transitions (C→T and A→G), thereby limiting the range of editable mutations within the ALS gene and other important loci. This restriction can preclude the generation of herbicide resistance variants that require transversions (e.g., G→C or T→A). Prime editing, while theoretically capable of introducing all types of point mutations, remains less efficient in plant systems compared to base editing. Major challenges include the low stability and delivery efficiency of prime editing guide RNAs (pegRNAs) and reverse transcriptase fusion proteins in plant cells. Enhancing these components is essential to realize the full potential of prime editing for precision crop improvement. Another promising avenue involves the development and deployment of novel CRISPR-associated nucleases. Beyond the canonical SpCas9, alternative enzymes such as Cas12b, Cas13, CasΦ (Cas phi) and the engineered SpRY are being actively explored for plant genome editing. These variants offer significant advantages, including greater protospacer adjacent motif (PAM) flexibility, smaller protein sizes conducive to vector packaging and, in the case of Cas13, RNA-targeting capability. Expanding the CRISPR toolbox with these new nucleases can improve editing efficiency and broaden the applicability of genome engineering across diverse crop species.

The delivery of CRISPR reagents is also undergoing significant innovation. While traditional *Agrobacterium*-mediated transformation remains widely used and effective in many dicotyledonous species, its applicability is limited in monocots and several economically important fruit crops. Biolistic delivery methods often suffer from tissue damage and inconsistent transformation efficiencies. The future lies in DNA-free delivery methods, such as RNP (ribonucleoprotein) complexes, viral vectors (e.g. Gemini virus replicons), nanoparticle-based systems and even spray-induced gene editing (SIGE) (86). These strategies not only enhance delivery efficiency but also avoid stable genomic integration, thereby simplifying regulatory approval processes and improving public acceptance. Such transient delivery methods align well with Non-Genetically Modified Organism (non-GMO) labelling frameworks and could facilitate wider adoption of genome editing technologies in agriculture. The development of transgene-free mutants through precise genome editing techniques offers the potential for classification as non-GMO, subject to rigorous evaluation and approval by regulatory agencies in India and globally, though regulatory definitions and policies differ among countries.

6. Global comparative regulatory frameworks for gene-edited plants

CRISPR-based herbicide-resistant crops hold immense promise for enhancing agricultural productivity, but their success hinges on overcoming regulatory, ecological and

technological challenges. The regulatory landscape for genome editing of the acetolactate synthase (*ALS*) gene to develop herbicide-resistant crops varies globally, with policies emphasizing safety, environmental impact and public acceptance. International collaboration among researchers, regulators and policymakers will be crucial to ensure that scientific advancements translate into accessible and sustainable technologies for farmers worldwide.

In countries such as the United States, Japan and Argentina, gene-edited plants that do not contain foreign DNA are often exempted from stringent genetically modified organisms (GMO) regulations, thereby facilitating rapid field deployment (87) For example, in the United States, the USDA's SECURE rule (2020) exempts genome-edited crops without foreign DNA, such as ALS-edited plants, from regulation if they do not pose a plant pest risk (88). The concept of mechanism of action (MOA) is pivotal in this framework; once a particular ALS -related MOA is approved, similar edits may not require additional regulatory review (89). Furthermore, oversight in the U.S. is supplemented by the Food and Drug Administration (FDA), which evaluates food safety and the Environmental Protection Agency (EPA), which oversees herbicide usage. Canada follows a product-based regulatory approach, wherein *ALS*-edited crops are categorized as plants with novel traits (PNTs) under the Canadian Food Inspection Agency (CFIA). Regulatory assessments focus on the novelty of the trait rather than the editing technique and exemptions are possible if the genetic changes are similar to those achieved through conventional breeding (90). Japan follows a similarly permissive stance; gene-edited crops that do not contain foreign DNA are not classified as GMOs and only require government notification prior to commercialization (91). In India, the 2022 biosafety guidelines issued by the Ministry of Environment, Forest and Climate Change (MoEFCC) exempt genome-edited plants developed using site-directed nucleases (SDN-1 and SDN-2), such as knockouts, from biosafety assessments, provided they are free of exogenous DNA (92, 93).

In contrast, the European Union (EU) maintains a more stringent regulatory stance. Following a 2018 Court of Justice of the European Union ruling, all genome-edited organisms are classified as genetically modified organisms (GMOs) under Directive 2001/18/EC. As a result, gene-edited crops are subject to the same regulatory scrutiny as conventional GMOs, including comprehensive risk assessments conducted by the European Food Safety Authority (EFSA), which assess off-target effects and broader ecological risk (94). Similarly, countries like Switzerland and New Zealand have adopted precautionary regulatory frameworks, reflecting heightened public and environmental concerns (95). While CRISPR-based herbicide-resistant crops offer transformative potential, their widespread adoption depends on navigating the diverse regulatory landscapes across nations. Achieving policy harmonization and fostering international collaboration will be essential to ensure equitable access to these technologies and to fully realize their benefits for global food security.

7. Future Prospects

The CRISPR/Cas-based technologies offer a potential approach in the development of herbicide-resistant crops by targeted editing of acetolactate synthase gene. The practical implementation of these proof-of concept studies, several scientific and technological challenges must be overcome. Enhancing the efficiency and precision of CRISPR/Cas-based technologies remains major objective. In particular, low efficiency of homology directed repair (HDR) limits precise and programmable base substitutions in non-dividing plant tissues. To overcome this limitation, novel strategies such as cell cycle synchronization, the use of geminiviral replicons and the inhibition of non-homologous end joining (NHEJ) pathways are being employed to enhance HDR efficiency. Moreover, the CRISPR-based base editing and prime editing system must be further optimized for implementation in various crops as only limited studies are currently available in this area. Although currently available base editors are limited to the C-to-T and A-to-G transitions, they are unable to induce potential resistance-conferring mutations in the ALS gene that require other types of base changes. Advanced CRISPR-based prime editing allows the editing multiple bases simultaneously, offering greater versatility. However, its application in plant systems remains constrained due to instability of pegRNA and low efficiency of reverse transcriptase fusion proteins. Another major area of concern is the gene delivery system used for genome editing, although the conventional methods such as *Agrobacterium*-mediated transformation and biolistic method have been effective, they are often inefficient or damaging in in certain crops, particularly monocots and polyploids. The future direction focused on the development of DNA-free delivery systems such as ribonucleoprotein (RNP) complexes, nanoparticle-mediated delivery, viral vectors and spray-induced gene editing (SIGE). These methods bypass the stable genomic integration and are more compatible with non-GMO regulatory frameworks.

The improvement in the durability and efficacy of herbicide resistance depends on the efficient utilization of available techniques. Editing only the *ALS* gene may lead to increased selection pressure on weed populations, accelerating the evolution of cross-resistance. Therefore, future strategies must focus on stacking of multiple resistance genes for multiple herbicide resistance. The incorporation of additional herbicide resistance genes, including those involved in metabolic detoxification pathways or targeting other herbicide-binding enzymes, such as *ACCase* or *HPPD*, could provide broader and more sustainable weed management. A major research gap in available studies is the limited focus on improving agronomic traits despite the *ALS* is directly involved in essential plant development pathways. The future efforts should aim to integrate agronomic trait improvement with herbicide resistance. This can be achieved through multiplex gene editing by simultaneously targeting genes for herbicide resistance and agronomic traits, such as salinity, drought or disease tolerance.

Moreover, field-level validation is indispensable for the commercial readiness of CRISPR-edited crops. Most current studies are limited to laboratory or greenhouse conditions. Future research should prioritize multi-location field trials to assess the long-term agronomic performance, trait stability, herbicide efficacy and ecological interactions of edited plants. Integrating genome editing into conventional breeding

programs will also be important to introgress beneficial alleles into elite cultivars and landraces. Transgene-free editing pipelines should be refined to produce regulatory-compliant varieties without the introduction of foreign DNA.

Conclusion

The acetolactate synthase gene has emerged as a scientifically validated and agronomically relevant target for developing herbicide-resistant crops through CRISPR/Cas-mediated genome editing. Researchers have enabled the generation of herbicide-resistant varieties across a wide range of crops including rice, wheat, maize, soybean, potato and others. These innovations offer significant benefits such as reduced chemical inputs, enhanced weed management and improved sustainability. These advances must be paired with ecological stewardship and sustainable agricultural practices to ensure long-term success and mitigate the risk of resistance evolution in weed populations. The future of *ALS* gene editing lies not only in the laboratory but also in the field, where real-world implementation will define its impact on global food security and sustainable crop production.

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Authors' contributions

RM and MS aided in planning the outline of this study. SRS drafted the first manuscript. SAR and SPR reviewed and edited the manuscript. RM, MS, VRR, WM and BA overviewed and finalised the manuscript. All the authors have read and approved the final manuscript.

Compliance with ethical standards

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