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REVIEW ARTICLE

Advancements in CRISPR/Cas technology for generating herbicide tolerant crops: a comprehensive review

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Abstract

Weeds, which compete with crops for essential resources, have long posed a significant challenge to agriculture, impacting several crops. Although some weeds offer ecological benefits, their overall detrimental effects on crop yields necessitate effective management strategies. Herbicides have become the primary tool for weed control, and the development of herbicide-tolerant (HT) crops has revolutionized weed management by providing a viable substitute to traditional methods. However, the widespread use of herbicides has led to the rapid development of herbicide-resistant weeds, reducing the efficacy of existing herbicides and emphasizing the need for innovative solutions. The CRISPR/Cas system has emerged as an innovative approach in genome editing, offering precise and efficient methods for introducing or modifying traits in crops. This review aims to consolidate advancements in CRISPR/Cas technology for generating herbicide-tolerant crops, exploring the mechanisms, successes, and challenges associated with this approach. This review is an overview of the current state and future prospects of CRISPR/Cas-based herbicide tolerance, highlighting its potential to enhance agricultural productivity and sustainability in the face of evolving weed resistance.

Keywords CRISPR/Cas system · Herbicide tolerant crops · Genome editing · Herbicides · Target mutations

Abbreviations

CRISPR	Clustered regularly interspaced short palindromic repeats
Cas	CRISPR-associated protein
gRNA	Guide RNA
sgRNA	Single guide RNA
PAM	Protospacer adjacent motif
NHEJ	Non-homologous end joining

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HDR	Homology-directed repair
SDN	Site-directed nucleases
HT	Herbicide tolerant
MOA	Modes of action
EPSPS	5-Enolpyruvoylshikimate-3-phosphate synthase
PPT	Phosphinothricin
GS	Glutamine synthetase
ALS	Acetolactate synthase
ACC	Acetyl-coenzyme A carboxylase
HPPD	Hydroxyphenypyruvate dixygenase

Introduction

Weeds, which are plants that compete with crops for resources such as soil moisture, nutrients, CO_2, light, and space, have long posed a significant challenge to agriculture. Out of approximately 200,000 plant species on Earth, only about 1% are cultivated as crops, with around 0.1% classified as weeds (Sundstrom et al. 2014). These weeds can severely impact crop productivity and agricultural systems, affecting major crops like rice (Sreekanth et al. 2024; Pawar et al. 2022), wheat (Sondhia et al. 2023), soybean (Chander et al. 2023), and potato (Chethan et al. 2023) etc. Weeds not only compete with crops but also pose additional challenges under climate change situation (Sreekanth et al. 2023; Mahawar et al. 2023; Roy et al. 2023) and environmental concerns (Sreekanth et al. 2022). Some weeds even offer positive ecological benefits, such as the ability to absorb heavy metals from contaminated soils (Roy et al. 2021). Despite these benefits, their detrimental effects on crop yields necessitate effective management strategies (Heap 2014; Green 2014). In modern agriculture, herbicides have become the primary tool for weed control (Gage et al. 2019; Green and Owen 2011). The development and extensive adoption of herbicide-tolerant (HT) crops over the past two decades have provided farmers with a powerful tool to manage weeds more efficiently. This shift was driven by the increasing cost and complexity of traditional weed management methods, along with rising farm sizes and a declining agricultural workforce (Green 2012). The advent of HT crops, made possible by advances in biotechnology, revolutionized weed management by offering a viable alternative to non-chemical methods (Awan et al. 2015; Green and Owen 2011).

The Weed Science Society of America (WSSA) describes herbicide resistance as "the inherited capability of a plant to survive and reproduce after exposure to a dose of herbicide normally lethal to the wild type." This can arise naturally or be induced by genetic engineering or selection (WSSA 2021). Herbicide tolerance, on the other hand, refers to the "inherent ability of a plant to survive and reproduce after herbicide treatment without selection or genetic manipulation" (WSSA 2021). Herbicide-tolerant technology has become a cornerstone of modern weed management, significantly enhancing agricultural productivity (Gressel 2009; Busi et al. 2013). While herbicides are effective against weeds, they can also negatively impact crop plants (Zhang et al. 2018). Therefore, developing HT crops that can withstand herbicide applications without adverse effects is crucial for improving crop production and managing weed infestations. HT crops not only mitigate crop phytotoxicity from herbicide use but also expand the spectrum of effective herbicides and reduce weeding costs (Glick 2001; Shaner 2000; Green 2012). Consequently, creating herbicide-resistant crops is an efficient strategy for controlling weed proliferation and boosting crop productivity (Kumar et al. 2008).

However, the widespread use of herbicides will result in the development of herbicide-resistant weeds, which has significantly reduced the efficacy of existing herbicides. As of now, 267 herbicide-resistant weeds have been found worldwide, rising resistance to 21 of the 31 known herbicide sites of action and to 165 different herbicides (Heap 2022). The herbicide site of action (SOA) refers to the specific biochemical target within a plant that a herbicide disrupts, leading to plant injury or death. This target is usually an enzyme, protein, or cellular process essential for plant growth and development. The site of action determines how the herbicide affects the plant and plays a crucial role in classifying herbicides based on their mode of action. The slow and costly development of new herbicides exacerbates this issue, with the evolutionary rate of herbicide-resistant weeds potentially surpassing the rate of novel herbicide discovery (Green 2014; Gaines et al. 2021). Therefore, developing herbicide-tolerant crops remains a critical approach to managing weeds and enhancing agricultural output by enabling the selective use of herbicides, reducing crop-weed competition, and ensuring effective weed control (Dong et al. 2021; Kumar et al. 2008). However, the overuse of herbicides can lead to the evolution of herbicide-resistant weeds, necessitating the adoption of integrated weed management strategies to sustain the benefits of herbicide-tolerant crops. Traditional methods for developing HT crops include mutation breeding, transgenic technology, and genome editing. Transgenic crops, while effective, face regulatory hurdles and public skepticism, particularly for staple crops like rice. Non-transgenic herbicide-tolerant crops have been developed through traditional breeding, but the complexity and cost associated with creating herbicide-tolerant traits via mutagenesis and backcrossing present challenges (Sudianto et al. 2013).

Herbicides typically target essential metabolic or physiological processes in plants, such as those disrupted by glyphosate, glufosinate, synthetic auxins, and imidazolinones (Endo and Toki 2013). Both transgenic and non-transgenic approaches have been used to develop herbicide-tolerant crops, with mutations in herbicide target sites or detoxification genes being common strategies. While the

development of herbicide-tolerant crops has revolutionized weed management, concerns about the evolution of herbicide-resistant weeds necessitate proactive strategies. Integrated weed management (IWM) approaches, such as herbicide rotation, tank mixtures with different modes of action, stacking multiple resistance traits, cover cropping, and mechanical weed control, can help delay resistance development. Additionally, advancements in genome editing, such as targeted modifications for metabolic resistance and herbicide detoxification pathways, offer promising solutions to sustain herbicide efficacy while reducing reliance on single-mode-of-action herbicides. The CRISPR/Cas system has arisen as a groundbreaking tool in genome editing, offering precise and efficient methods for introducing or modifying traits in crops (Metje-Sprink et al. 2020). The integration of CRISPR/Cas tool for developing herbicide-tolerant crops represents a significant advancement in agricultural biotechnology.

In this comprehensive review, we aim to consolidate the advancements in CRISPR/Cas technology for generating herbicide-tolerant crops. We will explore the mechanisms, successes, and challenges associated with CRISPR/Cas-based herbicide tolerance, providing an overview of the current state and future prospects of this innovative approach in sustainable agriculture.

Mechanisms and strategies for developing herbicide-tolerant crops

Understanding the pathways and highlighting key genes associated with herbicide tolerance

Herbicide-tolerant (HT) crops have been generated through numerous mechanisms, including:

1. Target site modification: Altering the target sites of herbicides to prevent their binding and action.

2. Enzyme introduction or enhancement: Incorporating or enhancing herbicide-degrading or inactivating enzymes within the plants.

3. Mechanism alteration: Modifying the plant to induce mechanisms that prevent herbicides from reaching their molecular targets. This includes upsurge in sequestration or diminishing translocation and uptake of herbicides. Metabolic inactivation or degradation remains the principal natural technique of crop tolerance against selective herbicides (Duke and Cerdeira 2010). HT crops, such as glyphosate-resistant cotton, maize, canola, and soybean, have significantly impacted weed management. Their effectiveness and simplicity have led to widespread adoption, contributing to increased yields and economic savings (Duke 2015). Various approaches, including genome editing, have been employed to develop HT crops (Schütte et al. 2017). Given the current challenges with weeds, reliance on herbicides alone is insufficient for effective control. Combining gene-edited HT crops with new modes of action (MOAs) and integrated weed management (IWM) strategies offers a more comprehensive strategies to manage weeds and minimizing environmental impacts.

Herbicide target enzymes often have multiple homologs across crop species, impacting the effectiveness of genome editing for herbicide tolerance. The number of homologous copies and their functional redundancy dictate whether modifying one, some, or all copies is necessary for resistance. In crops with a single-copy target gene, a single nucleotide change can be sufficient, as observed in *Brassica napus*, where a base substitution in the *ALS* (*AHAS*) gene conferred imidazolinone resistance without compromising plant fitness (Tan et al. 2005). However, in species with multiple homologs, altering a single copy may not be adequate due to functional redundancy. For example, in wheat (*Triticum aestivum*), which contains three *AHAS* homeologs, targeted editing of specific copies can confer resistance without modifying all homologs (Zhang et al. 2021). In crops with multiple functionally redundant homologs, such as rice (*Oryza sativa),* which carries three *AHAS* homologs (*OsALS1*, *OsALS2*, *OsALS3*), editing all copies is often essential to achieve complete resistance. The successful modification of all homologs in rice has led to strong herbicide tolerance without major growth penalties (Kuang et al. 2020).

Herbicides act by binding to specific enzyme domains, inhibiting their function and causing plant mortality. CRISPR/Cas-based genome editing has been widely used to introduce precise mutations in these herbicide-binding or recognition domains, blocking herbicide interaction while maintaining enzymatic activity. This approach has successfully conferred herbicide tolerance in multiple crops. The recognition domain comprises key amino acid residues that interact with herbicides, and mutations in these sites can prevent herbicide binding without disrupting enzyme function. For example, modifications at T102I and P106S in the glyphosate-targeted 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme reduce glyphosate affinity while maintaining enzymatic function (Yu et al. 2015). CRISPR/Cas technology enables precise base substitutions at these recognition domains with minimal off-target effects. CRISPR/Cas9-mediated editing has introduced resistance-conferring C-to-T or G-to-A mutations at Pro197, Trp574, and Ser653 in wheat and rice (Zhang et al. 2021; Kuang et al. 2020), while CRISPR/Cas9 and prime editing have successfully altered T102I and P106S to confer glyphosate resistance in maize and soybean (Chen et al. 2021).

Key genes associated with herbicide tolerance in weeds

Glyphosate and EPSPS

Glyphosate, a phosphonic compound, acts by targeting the enzyme 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS) in plants (Duke and Powles 2008). EPSPS is crucial in the chloroplasts for the formation of 5-Enolpyruvylshikimate-3-phosphate (EPSP) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P). This enzymatic process is essential for the production of tryptophan, tyrosine, and phenylalanine, as well as various plant compounds including hormones, flavonoids, lignin, and phenolics. Glyphosate disrupts EPSPS activity by forming a stable complex with EPSPS-S3P, competing with PEP, thereby inhibiting the synthesis of essential amino acids needed for protein production. This disruption leads to an accumulation of shikimate, ultimately causing plant death (Dill 2005). Currently, 56 weed species worldwide have developed varying degrees of resistance to glyphosate, attributed to 344 unique cases of EPSPS mutations (Heap 2022). Key mutation sites include Thr102 to Ile/Ser, Ala103 to Val, and Pro106 to Ser/Ala/Leu/Thr. Among these mutations, Thr102 to Ile and Pro106 to Ser are particularly associated with the highest levels of glyphosate resistance (Gaines and Heap 2020). The following table presents weed species with target site mutations and amino acid substitutions that confer resistance to glyphosate (Table 1).

Glufosinate and glutamine synthetase

Glufosinate (phosphinothricin, PPT) is a non-selective inhibitor targeting glutamine synthetase (GS) in plants. GS converts glutamate and ammonia into glutamine. Glufosinate, structurally analogous to glutamate, competes with the usual substrate of GS, disrupting N_2 absorption. This inhibition leads to the accumulation of toxic levels of ammonia within the plant cells, which in turn hampers photosynthetic activity, damages chloroplast structure, disrupts glyoxylic acid metabolism, and ultimately causes plant death (James et al. 2018). Resistance to glufosinate has been found in only a limited number of weed species, including *Amaranthus palmeri, Eleusine indica, Poa annua, and Lolium perenne*, with documented cases of resistance involving specific mutations (Heap 2022). For instance, studies on glufosinate-resistant Italian ryegrass have identified the substitution of Asp171 with Asn, altering the amino acid polarity and reducing GS sensitivity to glufosinate (Avila-Garcia et al. 2012). Similarly, investigations on alfalfa GS have highlighted specific amino acid positions—such as Gly at position 207, any amino acid except Gly at position 245, and Arg or Lys at position 332—that confer varying levels of resistance to glufosinate (Goodman et al. 1990). In rice, mutants of glutamine synthetase (OsGS1;1) with Gly at position 59 and Arg at position 296 have shown greater tolerance to glufosinate (Deng et al. 2019). The following table presents weed species with target site mutations and amino acid substitutions that confer resistance to Glufosinate ammonium. To date, several weed species like Palmer Amaranth (*Amaranthus palmeri*), Goosegrass (*Eleusine indica*), Perennial Ryegrass (*Lolium perenne*), Italian Ryegrass (*Lolium perenne* ssp. Multiflorum), Rigid Ryegrass (*Lolium rigidum*), Annual Bluegrass (*Poa annua*) have showed resistance to glufosiate-ammonium (Heap 2024).

Acetolactate synthase (ALS) inhibitor herbicides

ALS is primarily involved in the biogenesis of valine, leucine, and isoleucine in plants (Tan et al. 2005). Herbicides targeting ALS hinder its activity, thereby blocking the synthesis of these essential amino acids. This disruption interferes with translation, cell division, and overall plant performance, finally leading to the death of the plant. The ALS

Table 1 Weed species exhibiting target site mutations and amino acid substitutions conferring resistance to Glyphosate

Sl. no.	Weed species	Target site mutation	Amino acid substitution	References
1	*Amaranthus palmeri* S.Watson	Pro106Ser	Proline to serine	Gaines et al. (2010a, b, c)
2	*Lolium rigidum* Gaudin	Pro106 Thr	Proline to threonine	Powles and Preston (2006)
3	*Eleusine indica* (L.) Gaertn	Thr102Iso, Pro106Ser	Threonine to isoleucine, proline to serine	Yu et al. (2007)
4	*Ambrosia trifida* L	Pro106Ser	Proline to serine	VanGessel et al. (2009)
5	*Amaranthus tuberculatus* (Moq.) J.D.Sauer	Pro106Ser	Proline to serine	Nandula et al. (2013)
6	*Conyza canadensis* (L.) Cronquist	Pro106 Ala	Proline to alanine	Koger et al. (2004)
7	*Digitaria insularis* (L.) Mez ex Ekman	Thr102Iso, Pro106Ser	Threonine to isoleucine, proline to serine	Takano et al. (2020)
8	*Echinochloa colona* Raole & R.J.Desai	Pro106Ser	Proline to serine	Alarcón-Reverte et al. (2015)
9	*Sorghum halepense* (L.) Pers	Pro106Ser	Proline to serine	Vila-Aiub et al. (2007)

inhibitor herbicide group includes widely used chemicals such as imidazolinones (IMI), sulfonylureas (SU), triazolopyrimidines (TP), pyrimidinylthiobenzoates (PTB), and sulfonylamino-carbonyl-triazolinones (SCT) (Tan et al. 2005). Experiments on herbicide-resistant weeds have revealed that while few got resistance by enhancing their ability to detoxify herbicides, mutations in the ALS target genes predominantly confer resistance. Currently, 66 weeds have evolved resistance to ALS inhibitor herbicides through mutations in ALS, with documented mutations occurring at eight key amino acid positions: Ala122 (5), Pro197 (11), Ala205 (2), Asp376 (1), Arg377 (1), Trp574 (4), Ser653 (3), and Gly654 (2). Notably, the Pro197 position alone shows substitution with 11 different amino acids, i.e., Thr, Ala, Arg, Asn, Gln, His, Ile, Leu, Ser, Thr, and Tyr (Tranel et al. 2020). These amino acid changes alter the enzyme's structure, lowering its affinity for herbicides and thereby conferring resistance. The specific mutation sites and the types of amino acid substitutions dictate the level and spectrum of resistance to ALS inhibitor herbicides. The following table presents weed species with target site mutations and amino acid substitutions that confer resistance to ALS inhibitor herbicides (Table 2).

Acetyl-CoA carboxylase (ACCase) inhibitor herbicides

ACCase is involved the carboxylation of acetyl-CoA to generate malonyl-CoA, a key precursor for fatty acid formation and the production of various secondary metabolites (Tang et al. 2014). ACCase inhibitor herbicides target this enzyme, disrupting fatty acid synthesis and subsequently impairing the production of membrane lipids. This disruption leads to damage to cellular structures and ultimately results in plant death.

ACCase inhibitor herbicides can be categorized into three main classes:

1. Aryloxyphenoxypropionates (APPs): Includes quizalofop-p-ethyl, diclofop-methyl, and metamifop.

2. Cyclohexanediones (CHDs): Includes sethoxydim, clethodim, and tralkoxydim.

3. Phenylpyrazolines (DEN): Includes pinoxaden.

These herbicides specifically target the carboxyl transferase (CT) domain of the plastid ACCase (Yu et al. 2010). Research on herbicide-resistant weeds has identified several mutations within this domain, including Ile1781Leu, Trp1999Ser/Cys, Trp2027-Cys, Ile2041 Asn, Asp2078Gly, Cys2088 Arg, and Gly2096 Ala. These mutations contribute to resistance against various ACCase inhibitors. In these mutations, the Ile1781Leu mutation is the utmost commonly observed in resistant plants (Jang et al. 2013). The following table presents weed species with target site mutations and amino acid substitutions that confer resistance to ACCase inhibitors (Table 3).

Hydroxyphenypyruvate dixygenase (HPPD) inhibtor herbicides and HPPD

The enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) is ubiquitously present in various organisms, facilitating the conversion of hydroxyphenylpyruvic

Table 2 Weed species exhibiting target site mutations and amino acid substitutions conferring resistance to ALS inhibitor herbicides

Sl. no.	Weed species	Target site mutation	Amino acid substitution	Herbicide	References
1	*Amaranthus palmeri* S.Watson	Trp574Leu	Tryptophan to leucine	Chlorsulfuron, imidazolinones	Tranel et al. (2020)
2	*Ambrosia artemisiifolia* L	Ser653 Asn	Serine to asparagine	Chlorsulfuron, imidazolinones	Zheng et al. (2011)
3	*Lolium rigidum* Gaudin	Pro197 Thr	Proline to threonine	Chlorsulfuron, imidazolinones	Yu et al. (2013)
4	*Kochia scoparia* (L.) Schrad	Thr197Ile	Threonine to isoleucine	Chlorsulfuron, imidazolinones	Beckie et al. (2015)
5	*Echinochloa crus-galli* (L.) P.Beauv	Ala122 Thr	Alanine to threonine	Chlorsulfuron, imidazolinones	Wang et al. (2016)
6	*Lolium multiflorum* Lam	Pro197Ser	Proline to serine	Chlorsulfuron, imidazolinones	Saari et al. (1994)
7	*Poa annua* Schltdl. & Cham	Trp574Leu	Tryptophan to leucine	Chlorsulfuron, imidazolinones	McCullough et al. (2013)
8	*Raphanus raphanistrum* L	Ala122 Thr	Alanine to threonine	Chlorsulfuron, imidazolinones	Yu et al. (2012)
9	*Amaranthus tuberculatus* (Moq.) J.D.Sauer	Ser653 Thr	Serine to threonine	Chlorsulfuron, imidazolinones	Whaley et al. (2007)
10	*Bromus tectorum* L.	Pro197Ser	Proline to serine	Chlorsulfuron, imidazolinones	Park et al. (2004)

Table 3 Weed species exhibiting target site mutations and amino acid substitutions conferring resistance to ACCase inhibitors

Sl. no.	Weed species	Target site mutation	Amino acid substitution	Herbicide	References
1	*Alopecurus myosuroides* Huds	Serine 627	Ile-1781-Leu, Trp-2027-Cys, Ile-2041-Asn, Asp-2078-Gly, Gly-2096-Ala	Clodinafop, Fenoxaprop, Quizalofop	Delye et al. (2005)
2	*Echinochloa crus-galli* (L.) P.Beauv	Proline 197	Pro197Leu, Pro197Ser	Fluazifop, Quizalofop	Gaines et al. (2010a, b, c)
3	*Lolium rigidum* Gaudin	Serine 627	Ser627 Asn, Ser627Ile, Ser627 Thr	Aryloxyphenoxypropionate (AOPP) herbicides	Boutsalis et al. (2012)
4	*Setaria viridis* (L.) P.Beauv	Proline 197	Pro197Leu	Fluazifop, Clethodim	Wang et al. (2020a, b)
5	*Amaranthus palmeri* S.Watson	Leucine 178	Leu178Ile	Acetyl-CoA carboxylase inhibitors (various)	Heap (2020)
6	*Chenopodium album* L	Serine 627	Ser627 Asn, Ser627Ile	Clethodim, Fluazifop	Feng et al. (2007)

acid (HPPA) into homogentisic acid (HGA). In plants, HPPD plays a pivotal role in the biosynthesis of essential compounds such as plastoquinone and tocopherol, which are substrate for carotenoid and other metabolites important for carbon reduction (Moran 2005). HPPD inhibitor herbicides function by binding to the Fe^{2+} ion in the enzyme's active site, thereby preventing HPPD from interacting with its substrate HPPA. This competitive inhibition disrupts the synthesis of plastoquinone and tocopherol, leading to the impairment of photosynthetic electron transfer. Consequently, affected plants experience bleaching and ultimately succumb to herbicide-induced death (Ndikuryayo et al. 2017). Recent research by Maeda et al. (2019) recognized a rice gene termed HIS1 (HPPD Inhibitor Sensitive 1), which confers sensitivity to b-triketone like benzobicyclon (BBC). HIS1 is responsible for the formation of an $Fe^{2+}/^{2-}$ oxoglutarate-dependent oxygenase that detoxifies b-triketone by catalyzing their hydroxylation. In contrast, rice plants lacking HIS1 due to a 28-bp deletion in its coding region exhibit resistance to HPPD inhibitor herbicides (Maeda et al. 2019). The following table presents weed species with target site mutations and amino acid substitutions that confer resistance to HPPD inhibitor herbicides (Table 4).

Dinitroaniline herbicides and tubulin genes

Dinitroaniline herbicides, such as trifluralin, pendimethalin, and ethalfluralin, have been extensively utilized for weed control in crops like soybeans and cotton (Chu et al. 2018). These herbicides exert their effects by binding to tubulin, a key protein involved in microtubule formation and function. By disrupting tubulin polymerization, dinitroaniline herbicides inhibit normal cell division and elongation processes in plants, ultimately leading to plant death. Studies employing molecular structural modeling and analysis of tubulin have provided insights into how dinitroaniline herbicides interact with α-tubulin, specifically disrupting the polymerization of microtubules (Chu et al. 2018). This disruption interferes with crucial cellular processes, highlighting the mechanism by which dinitroaniline herbicides exert their herbicidal effects. The following table presents weed species with target site mutations and amino acid substitutions that confer resistance to Dinitroaniline herbicides (Table 5).

Cytochrome P450 s

The cytochrome P450 superfamily is crucial in various metabolic processes, including hormone, lipid, and secondary metabolite metabolism. It also plays a significant role in herbicide detoxification by hydroxylating or dealkylating

Table 4 Weed species exhibiting target site mutations and amino acid substitutions conferring resistance to HPPD inhibitor herbicides

Sl. no.	Weed species	Target site mutation	Amino acid substitution	Herbicide	References
1	*Amaranthus tuberculatus* (Moq.) J.D.Sauer	Pro197Ser, Pro197Leu	Proline to serine/leucine	Mesotrione, tembotrione	Jugulam et al. (2019), Ma et al. (2013)
2	*Amaranthus palmeri* S.Watson	Pro197Ser, Pro197Leu	Proline to serine/leucine	Mesotrione, tembotriobe, isoxaflutole	Jugulam et al. (2019), Ma et al. (2013)
3	*Ambrosia artemisiifolia* L	Pro197Ser	Proline to serine	Tembotrione	Ge et al. (2019)
4	*Conyza canadensis* (L.) Cronquist	Pro197Ser	Proline to serine	Tembotrione	Ge et al. (2019)

Table 5 Weed species exhibiting target site mutations and amino acid substitutions conferring resistance to Dinitroaniline herbicides

Sl. no.	Weed species	Target site mutation	Amino acid substitution	Herbicide	References
1	*Echinochloa crus-galli* (L.) P.Beauv	Gly255 Asn	Glycine to aspartic acid	Dinitroanilines	Cochrane et al. (2016)
2	*Chenopodium album* L	Glu78Gly	Glutamic acid to glycine	Dinitroanilines	Gressel (2002)
3	*Eluecine indica* (L.) Gaertn	Thr261Pro	Threonine to proline	Dinitroanilines	Heap (2020)
4	*Amaranthus hybridus* L	Gly242 Ala	Glycine to alanine	Dinitroanilines	Riggins et al. (2009)
5	*Eriochloa villosa* (Thunb.) Kunth	Ala-264Ser	Alanine to serine	Dinitroanilines	Cruz-Hipolito et al. (2011)

inhibitors of ACCase, ALS, and photosystem II (PS II) (Yu and Powles 2014). So far, only one cytochrome P450 gene has been recognized in rice that confers increased tolerance to Bentazone, a selective herbicide that inhibits carbon reduction. Interruption of this gene results in heightened sensitivity to Bentazone (Yu and Powles 2014). Exploring the cytochrome P450 superfamily further could enhance our understanding of herbicide detoxification mechanisms and improve the molecular manipulation and breeding of herbicide-tolerant crops. The following table presents weed species with target site mutations and amino acid substitutions that confer resistance to Cytochrome P450 s inhibitor herbicides (Table 6).

Approaches for development of herbicide tolerant crops using CRISPR Technology

CRISPR/Cas9 has been efficiently applied in several crops due to its easiness, adaptability, and accuracy (Ma et al. 2015; Xu et al. 2016). This technology, recognized for its simplicity and precision, has revolutionized agricultural biotechnology by enabling targeted mutagenesis and gene editing (Najera et al. 2019). By integrating genome editing with modern breeding methods, significant improvements in crop yields can be achieved sustainably.

CRISPR/Cas system

The CRISPR/Cas system comprises a guide RNA (sgRNA) and the Cas9 endonuclease.

Guide RNA (gRNA): A short RNA molecule with CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The sgRNA is engineered from crRNA and tracrRNA, guiding Cas9 to specific DNA sequences to induce double-stranded breaks (Jinek et al. 2012, 2014).

Cas9 Protein: A DNA endonuclease that forms a complex with gRNA. Cas9 recognizes and binds to definite DNA sequences through a protospacer-adjacent motif (PAM) on the non-target strand of the double-stranded DNA. The crRNA guides Cas9 to the target DNA, where it forms a DNA heteroduplex, creating an R-loop structure that enables Cas9 to induce a double-stranded break (Jinek et al. 2012; Sternberg et al. 2014).

Cas9 Protein Domains:

Nuclease (NUC) Lobe: Contains two nuclease domains, RuvC and HNH.

Recognition (REC) Lobe: Contain three recognition domains, REC1, REC2, and REC3 (Jiang et al. 2016).

Cas9-sgRNA complexes make precise cuts in the DNA, allowing for targeted gene modification (Miglani 2017).

The CRISPR/Cas9 system, along with its modified versions, has been effectively applied to foremost crops and model plants due to its adaptability and accuracy (Ma et al. 2015; Xu et al. 2016).

Table 6 Weed species exhibiting target site mutations (*CYP71 A1* gene) and amino acid substitutions conferring resistance to Cytochrome P450 s inhibitor herbicides

Sl. no.	Weed species	Target site mutation	Amino acid substitution	Herbicide	References
1	*Alopecurus myosuroides* Huds	Phe251Leu	Phenylalanine to leucine	Cyhalofop-butyl	Molin et al. (2021)
2	*Amaranthus palmeri* S.Watson	Trp214 Arg	Tryptophan to arginine	Glyphosate	Gaines et al. (2010a, b, c)
3	*Echinochloa colona* (L.) Link	Arg124Leu	Arginine to leucine	Fenoxaprop-P-ethyl	Nandula et al. (2011)
4	*Lolium rigidum* Gaudin	Ser306 Asn	Serine to asparagine	Diclofop-methyl	Powles et al. (2005)
5	*Conyza canadensis* (L.) Cronquist	Gly162 Cys	Glycine to cystine	Imazethapyr	Yu et al. (2009)
6	*Setaria viridis* (L.) P.Beauv	Ser285 Asn	Serine to asparagine	Sulfometuron-methyl	Zhou et al. (2020)

Mechanisms of CRISPR/Cas9 genome editing

Homology-directed repair (HDR)

HDR and targeted base editing (CBEs and ABEs) are used in precision plant breeding for creating herbicide-tolerant crops (Zong et al. 2017; Hua et al. 2018; Kuang et al. 2020; Zhang et al. 2020a, b, c). Examples include the creation of herbicide-tolerant rice mutants through CRISPR/Cas9-mediated homologous recombination (Sun et al. 2016) and base editing to induce specific amino acid changes (Wang et al. 2019).

Site-directed nucleases (SDNs)

SDN-1: Introduces point mutations via the error-prone non-homologous end joining (NHEJ) pathway, resulting in small insertions or deletions (Grohmann et al. 2019).

SDN-2: Utilizes HDR with a template sequence to make precise changes (Grohmann et al. 2019).

SDN-3: Similar to SDN-2 but uses longer DNA templates for more extensive modifications (Grohmann et al. 2019).

Base editing

Enables precise edits at target sites without DSBs or donor templates by using a fusion of Cas9 nickase and DNA deaminase (Komor et al. 2016a, b; Gaudelli et al. 2017a, b).

Prime editing

Introduces an RNA-programmable nickase coupled with reverse transcriptase and a prime editing guide RNA, enabling all twelve probable single-base variations and slight insertions or deletions (Anzalone et al. 2019; Kantor et al. 2020).

Cpf1

A class 2 CRISPR effector that enhances genome editing capabilities (Zetsche et al. 2015).

These innovations continue to advance the field of genome editing, offering new opportunities for creating herbicide-resistant crops and other agricultural improvements.

MAD7

A CRISPR-associated protein from the Type V-A nuclease family, has emerged as an alternative to Cas9 and Cpf1 for genome editing. It offers advantages such as a distinct PAM recognition sequence and potential benefits for gene editing applications, including the development of herbicide-tolerant crops.

CRISPR/Cas-mediated herbicide tolerance

Engineered Cas variants and their impact on specificity and efficiency

In spite of the versatility and usefulness of CRISPR/Cas9 over previous genome engineering techniques, a significant disadvantage is its tendency to cleave off-target DNAs that do not entirely match the crRNA (Pattanayak et al. 2013). To address this, various engineered Cas9 variants have been established in two main approaches:

Rational design: Variants such as Cas9-HF1, eCas9, and HypaCas9 target the REC3 domain to disrupt over-stabilized interactions between mismatched crRNA and the target DNA (Casini et al. 2018; Kleinstiver et al. 2016; Chen et al. 2017a, b).

Directed evolution: Variants like evoCas9, Sniper-Cas9, and xCas9 have been developed, with Sniper-Cas9 improving target specificity and xCas9 expanding PAM compatibility (Lee et al. 2018; Hu et al. 2018). The aim of these engineered variants is to actively destabilize the Cas9:gRNA complex, enhancing its ability to distinguish between on-target and off-target bindings. For example, in eCas9, mutations in the RuvC domain, which regulates the stability of the non-target strand post-heteroduplex formation, reduce off-target cleavage efficiency (Slaymaker et al. 2016). Single-molecule studies have linked off-target discrimination to inactive states of the Cas9:gRNA complex. The HNH domain's transition between active and inactive states is crucial; it remains active for on-target DNA but shifts to inactive states with PAM-distal mismatches (Okafor et al. 2019). Engineered variants like Cas9-HF1, eCas9, and HypaCas9 show a declined population of the HNH active state for off-target sequences relative to wild-type Cas9 (Chen et al. 2017a, b).

Direct monitoring of R-loop formation dynamics via dual labeling on both sides of the NTS and target strand shows that engineered Cas9 variants shift towards cleavage-incompetent conformations for off-target DNAs, indicating a crucial role for inactive conformations in off-target discrimination (Singh et al. 2018; Okafor et al. 2019). Heteroduplex dynamics, the initial step for Cas9 nuclease function, is essential for a comprehensive understanding of engineered Cas9 variants (Lim et al. 2016).

Novel delivery methods for improved CRISPR/Cas editing in crops

CRISPR/Cas-based genome editing relies on the genetic transformation of cells, requiring the delivery of Cas enzymes and gRNAs to work in tandem to edit DNA.

Therefore, plant transformation is a crucial initial step in the genome editing process. Effective delivery of the CRISPR/Cas9 components into cells is vital for the achievement of gene editing. The primary methods for introducing the CRISPR/Cas9 complex into cells: physical, chemical, and viral vectors. Non-viral approaches are preferred for ex vivo CRISPR/Cas9-based gene editing therapies. Physical procedures include electroporation, microinjection, and hydrodynamic injection. Electroporation uses a high electric field to increase cell membrane permeability, though it can result in significant cell death (Zhang et al. 2020a, b, c). In microinjection directly injecting the CRISPR/Cas9 complex into cells, but it is technically challenging and limited to small cell numbers (Fajrial et al. 2020).

Chemical delivery methods utilize lipid- and polymer-based nanoparticles. Lipid nanoparticles, like those created with Lipofectamine, form spherical structures that enable the union of the CRISPR/Cas9 complex across cell membranes (Xu et al. 2019).

Viral vectors are highly effective for in vivo CRISPR/Cas9 delivery, with adenoviral vectors (AVs), adeno-associated viruses (AAVs), and lentivirus vectors (LVs) being commonly used. However, the large size of the Cas9 protein and the inadequate cloning capacity of viruses present challenges. One solution is to package Cas9 and sgRNA separately and co-transfect them into cells, or use a smaller Cas9 variant from Staphylococcus aureus (SaCas9) instead of the more common SpCas9 (Horodecka and Düchler 2021).

Extracellular vehicles (EVs) have shown promise for in vivo CRISPR/Cas9 delivery, potentially overcoming some limitations of viral and non-viral methods. Off-target effects, where the sgRNA mismatches with non-target DNA, pose significant risks, including mutations, deletions, rearrangements, immune responses, and oncogene activation. Strategies to minimize off-target effects include optimizing sgRNA design, modifying Cas9 nucleases, using alternative Cas variants, and employing anti-CRISPR proteins (Pausch et al. 2020; Yılmaz 2021).

Direct RNA delivery

Both Cas proteins and sgRNAs can be synthesized or transcribed in vitro and subsequently transferred into plant cells through direct procedures such as protoplast transformation and particle bombardment. For example, Zhang et al. (2016) utilized a gene gun to deliver in vitro created Cas9 RNAs and sgRNAs into immature wheat embryos, successfully producing genome-edited mutants.

Virus-mediated delivery

Plant virus vectors

Researchers have explored plant virus vectors for delivering Cas9 and sgRNAs. Mei et al. (2019) and Alagoz et al. (2016) demonstrated successful genome editing in several crops using plant virus vectors. Cody et al. (2017) utilized tobacco mosaic virus-derived vectors to deliver sgRNAs, achieving genome editing in all tested genes.

Engineered virus vectors

Recent advancements include using engineered viruses for direct delivery of the CRISPR/Cas9 system. Ma et al. (2020) reported delivering the entire CRISPR/Cas9 cassette into plant cells via an engineered plant negative-strand RNA virus, sonchus yellow net rhabdovirus (SYNV). This method achieved high frequencies of single, multiplex mutagenesis, and chromosome deletions in tobacco. These advancements in virus-mediated CRISPR/Cas9 delivery are promising, offering DNA-free methods for genome editing that could revolutionize plant genetic engineering (Liu and Zhang 2020). This approach was also successful in editing wheat (Liang et al. 2017), potato (Andersson et al. 2018), Brassica (Murovec et al. 2018), and rice (Banakar et al. 2019). In addition to gene knockout, RNP-mediated genome editing has been effectively combined with donor DNA for knockin mutants via HDR. These studies highlight RNP-mediated genome editing as a novel approach for generating targeted mutations in plants. One significant advantage of RNP-mediated editing is that all resulting genome edits are transgene-free, thereby streamlining regulatory approval processes typically required for transgenic products destined for field and market use. Another advantage is its potential to reduce off-target effects compared to Agrobacterium-mediated CRISPR/Cas genome editing (Liang et al. 2017). Due to the transient nature of RNPs, the CRISPR/Cas system is less likely to induce unintended cuts at locations other than the intended target sites. The table below showcases case studies of successful CRISPR/Cas applications in creating herbicide-tolerant crops (Table 7).

Regulatory hurdles and public acceptance of CRISPR-modified crops

General targeted mutagenesis in crops using the CRISPR/Cas9 system is poised to revolutionize plant biology research. Unlike traditional random mutagenesis, which struggles to access every gene due to its random nature, CRISPR/Cas9 offers precise gene editing, allowing for targeted inactivation studies, multiple loci mutations, and large

Table 7 Case studies of successful CRISPR/Cas applications in generating herbicide tolerant crops

Sl. no.	Gene	Mutation sites	Genome editing approach	References
Rice				
1	*OsALS*	Ala96	CRISPR/Cas9	Wang et al. (2017)
2	*OsALS*	Trp548	CRISPR/Cas9	Wang et al. (2017)
3	*OsALS*	Gly628	CRISPR/Cas9	Zhang et al. (2020a, b, c)
4	*OsALS*	Ser627	CRISPR/Cas9	Packer and Liu (2015)
5	*OsALS*	Pro171/Gly628	CRISPR/Cas9	Li et al. (2020)
6	*OsALS*	Trp548/Ser627	CRISPR/Cas9	Ali et al. (2020)
7	*ACCase*	Cys2186	CRISPR/Cas9	Packer and Liu (2015)
8	*EPSPS*	Thr102/Pro106	CRISPR/Cas9	Weeks et al. (2016)
9	*TubA2*	Met268	CRISPR/Cas9	Yan et al. (2021)
10	*TubA2*	*Met268*	CRISPR/Cas9	Packer and Liu (2015)
Maize				
11	*ALS*	*Pro165*	CRISPR/Cas9	Endo et al. (2016)
12	*ALS*	*Trp542/Ser621*	CRISPR/Cas9	Butt et al. (2020)
Wheat				
13	*ALS*	*Pro174*	CRISPR/Cas9	Tian et al. (2018)
14	*ACCase*	*Ala1992*	CRISPR/Cas9	Zong et al. (2018)

deletions. This precision accelerates plant breeding without introducing transgenes. Although genetically modified (GM) crops could address crop improvement challenges, they face controversies regarding potential environmental and health impacts (Hilbeck et al. 2011). Traditional genetic engineering involves random insertion of DNA constructs into plant chromosomes, which can lead to undesirable effects and complications in making large-scale changes, such as integrating entire metabolic pathways (Lau et al. 2014).

In contrast, CRISPR/Cas9 technology allows for precise, inheritable modifications without necessarily introducing foreign DNA. The Cas9/sgRNA system is part of the new plant breeding techniques (NPBTs). NPBTs offer faster, more targeted modifications compared to traditional methods, and can generate null segregant lines lacking transgenic inserts (Araki and Ishii 2015). Evaluations should focus on the final product rather than the process of creation (Woo et al. 2015). While CRISPR-edited plants might bypass current GMO regulations, they pose new challenges for regulatory and social acceptance (Araki and Ishii 2015; Kanchiswamy et al. 2015). The debate over NPBTs involves whether genome-edited crops should be classified as GMOs based on their process or product. The European Union and Russia focus on the process, while countries like the United States, Canada, Argentina, and Brazil evaluate the product, regardless of the procedure applied (CTNBIO 2018; Kuluev et al. 2019; GAO 2021). Several investigators claim that transgene-free edited crops should not undergo special safety assessments, as they resemble natural mutants (Kuluev et al. 2019; Van Eenennaam et al. 2019; Zhang et al. 2020b). However, stringent regulations and social rejection of genome-edited crops could hinder the benefits of NPBTs and negatively impact the field's evolution (Lassoued et al. 2019).

Regulatory frameworks for NBTs, including CRISPR-Cas, are still evolving. Policymakers are considering process-based versus product-based approaches, with countries differing in their regulations (Scheben and Edwards 2018; Lassoued et al. 2020). For example, Brazil categorizes SDN1 mutations as non-GMO, while SDN2 and SDN3 systems are evaluated on a case-by-case basis (Sprink et al. 2016; CTNBIO 2018). In the USA, over 100 genome-edited plants, including high-oil camelina and powdery mildew-resistant wheat, have been deemed non-regulated (Waltz 2018). Conversely, the European Union treats genome-edited organisms with the same regulations as GMOs. The lack of consensus on regulatory approaches could affect international trade, commercial viability, and policy enforcement. Traceability of mutations, whether natural or CRISPR-induced, and product labeling are significant concerns (Gao 2018; Van Eenennaam et al. 2019). Addressing these issues carefully will prevent delays and backlash similar to past experiences with GMOs, ensuring the successful integration of gene-edited foods into the market. A balanced regulatory approach that accommodates the complexity of CRISPR technology will be crucial for advancing agriculture and addressing future challenges (Macnaghten and Habets 2020). Enhanced public and regulatory awareness is essential for achieving reasonable interpretations and decisions, fostering innovation while ensuring safety (Duensing et al. 2018).

Environmental impact of herbicide tolerant crops

Transgenic crops have had significant positive impacts on the environment across various dimensions. Firstly, there has been a notable shift in herbicide use patterns, particularly with Glyphosate-resistant (GR) crops dominating the market. This shift led to a substantial increase in glyphosate use, from 2.9 million kg/year in soybean crops before the commercialization of GR soybeans in 1995 to 41.7 million kg/year by 2006 (USDA 2012). This indicates a 14-fold increase in glyphosate application. Concurrently, there was a reduction in the use of other herbicides, contributing to an overall decrease in pesticide applications across croplands. From 1996 to 2009, this reduction amounted to an estimated 393 million kg of active ingredients, resulting in significant environmental benefits corresponding to a 17.1% reduction in associated environmental impacts (James 2011). In 2009 alone, this drop translated to 39.1 million kg of pesticide active ingredient saved. Secondly, transgenic crops have facilitated reduced fossil fuel combustion and subsequent CO_2 emissions by promoting zero or reduced tillage practices. This approach led to a substantial decrease in CO_2 emissions, totaling 17.6 billion kg in 2009 alone. This decrease is comparable to removing 7.8 million cars from the road, exhibiting the environmental benefits of reduced tillage (Brookes and Barfoot 2011).

Thirdly, the adoption of transgenic crops has contributed to soil and moisture conservation through optimized agricultural practices. In the United States, the cultivation of GR soybean, maize, and cotton collectively reduced soil erosion by an estimated 1 billion tons annually by 2010 (Smith 2010). These findings underscore the multifaceted environmental benefits of transgenic crop technologies, emphasizing their role in sustainable agriculture practices and environmental stewardship.

Challenges and limitations

Off-target effects and potential unintended consequences

Genome editing techniques are generally regarded as safe for crop improvement due to their ability to preserve the native genomic structure. In spite of this, there are ongoing apprehensions about the biosafety of crops produced via these tools, particularly regarding potential nontarget effects of synthetic nucleases. The primary concerns focus on the efficiency and specificity of engineered nucleases, which are critical for minimizing unintended outcomes. The efficiency of DNA cleavage at both target and non-target sites be subject to on several factors: the nuclease activity (such as FokI domains in ZFNs and Ruv domains in Cas9 proteins), the accessibility of the target site, and the affinity of the DNA-binding domain (such as TAL effector domains or guide RNA in CRISPR systems). Specificity is influenced by the binding affinity between the nuclease and DNA, as well as interactions like zinc finger binding in ZFNs, TAL effector binding in TALENs, and gRNA hybridization in CRISPR. Additionally, dimerization of the FokI domain and Cas9 interaction with the protospacer adjacent motif (PAM) contribute to specificity. For instance, while canonical C2H2 ZFNs are well-studied, noncanonical ZFNs with C3H1 binding domains have shown high binding efficiency (Cai et al. 2015). To mitigate nontarget effects, it is essential to carefully select target sites by conducting thorough bioinformatics analyses. This involves avoiding sites with repeated sequences or high homology with other genomic regions. Several software tools have been created to aid in the design and validation of nuclease target sites, which help in reducing the risk of unintended effects and ensuring the accuracy of genome editing (Khatodia et al. 2016; Lee et al. 2016; Koo et al. 2015).

Conclusion

The advancements in CRISPR/Cas technology have marked a transformative era in the development of herbicide-tolerant crops, offering precise and efficient tools for addressing one of agriculture's most persistent challenges. This review highlights the significant progress made in applying CRISPR/Cas systems to engineer herbicide tolerance across various crop species, demonstrating their potential to enhance agricultural productivity and sustainability. CRISPR/Cas technology has enabled the development of crops with tailored herbicide resistance, overcoming limitations associated with traditional breeding and transgenic methods. By targeting specific genes related to herbicide action or detoxification, CRISPR/Cas offers the ability to develop crops that not only withstand herbicide applications but also reduce crop phytotoxicity and enhance the effectiveness of weed management strategies. Despite these advancements, challenges remain, including regulatory hurdles, public acceptance, and the need for robust safety assessments. The rapid evolution of herbicide-resistant weeds also underscores the necessity for ongoing innovation in herbicide-tolerant crop development to keep pace with emerging resistance issues. Future research should focus on optimizing CRISPR/Cas systems for broader application across diverse crops, exploring novel resistance mechanisms, and addressing ecological

and environmental considerations. Integrating these developments with sustainable agricultural practices will be crucial for maximizing the benefits of herbicide-tolerant crops and achieving long-term agricultural resilience. In summary, CRISPR/Cas technology signifies a remarkable leap forward in crop biotechnology, with the ability to transform weed management and contribute to food security. Continued advancements and interdisciplinary collaboration will be essential to harness its full potential and address the complex challenges facing modern agriculture.

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Declarations

Conflict of interest The authors declare that they do not have any conflict of interest.

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