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# CRISPR-Cas based precision genome editing: current advances and associated challenges in crop improvement and trait enhancement

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## Keywords

*CRISPR-Cas, plant genome editing, crop improvement, agricultural biotechnology, current status, food security, abiotic stressors*

## Abstract

The CRISPR-Cas system has revolutionized precision genome editing and opened up enormous prospects for crop improvement and trait enhancement. This review covers recent advances related to the application of CRISPR-Cas in agricultural biotechnology, with an emphasis on its potential to contribute to solving critical issues of food security and sustainability. Global population is expected to reach 10 billion by 2050, presenting a challenge that demands new approaches to raise agricultural productivity. The review investigates the mechanisms by which CRISPR-Cas systems, especially Cas9 and Cas12a, facilitate precise alterations in plant genomes, leading to augmented resilience against both biological and environmental challenges. Significant accomplishments comprise increased drought resistance, enhanced disease tolerance, and improved nutritional quality in agricultural species such as wheat, maize, and soybean. However, the application of CRISPR-Cas technology encounters challenges, including regulatory hurdles and public acceptance. This paper underlines modern achievements together with future possibilities, at the same time paying much attention to the connected challenges and emphasizing the crucial role of CRISPR-Cas technology for the advancement of sustainable agriculture. Using such a powerful tool, scientists aim at creating resistant crop species that will be able to meet the needs of the growing population while providing food safety and quality.

## Introduction

With the global population projected to reach 10 billion by 2050, the demand for food is expected to surge significantly, requiring a 60% to 100% increase in production to sustain the growing needs of humanity (Chen, 2019; Ribeiro, 2020). Achieving this goal aligns with the United Nations' Sustainable Development Goal (SDG) to eradicate hunger and ensure access to nutritious food by 2030 (Fanzo, 2019). The existing agricultural production still faces difficulty reaching the necessary level of productivity needed to feed 10 billion people by 2050, despite of significant attempts to enhance the global food system (Hickey, 2019). It is due to the challenges such as diminishing arable land, biotic stressors (e.g., fungi, bacteria, pests), and abiotic stressors (e.g., heat, drought, salinity) (Babalola, Berner and Amusa, 2007; Vermeulen, 2012; Razzaq, 2019; Akanmu, 2021). Thus it is essential to create a safe and secure food system to face the rising food demand globally (Chukwuka, 2020; Mamphogoro, 2020). The purpose of crop improvement is to increase yields, enhance quality, and improve nutritional values by improving resistance against different biotic and abiotic stressors. Today, genetic improvement in food crops has been a promising measure for attaining growing population's need for food, but at the same time safeguarding the preference and health of the consumers. Genetic engineering systems include mega nucleases, transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and CRISPR-Cas9 with its orthologs (Jinek, 2012; Zetsche, 2015; Pant, 2022). The review's main emphasis, the CRISPR-associated proteins (Cas9 and Cas12a), have been reported as precise, practical, and effective genome editing technologies that have created new avenues for use across a range of industries (Mohanraju, 2016; Macovei, 2018; Swartjes, 2019; Zhang, 2019). Due to its wide application, massive interest has been given to the CRISPR-Cas9 system for its usage within plant breeding in agricultural crop development and biological research. Notable examples include disease resistance (Katoch, 2020), drought tolerance (Shi, 2016) and salt tolerance (Zhang, 2019), in major crops such as wheat (Liang, 2019), maize (Barman, 2019), and soybean (Chilcoat, 2017), have been investigated.

Moreover, CRISPR technology has the potential to improve sustainable agriculture by mitigating ecological impacts associated along with traditional farming methods. By targeted genome editing, researchers can engineer crops that require fewer chemical inputs, such as pesticides and fertilizers, thus reducing environmental pollution, and soil health conservation (Schindele, Wolter and Puchta, 2018). Additionally, CRISPR edited crops with improved nutritional profiles offer a viable solution to malnutrition and food insecurity, especially in the regions where staple crops are deficient in vitamins and minerals (Wurtzel et al., 2019). Although CRISPR-Cas9 holds great potential to be the game-changer in agriculture, the complex regulatory system, ethical issues, and public perception of this technology remain daunting, despite efforts to address these concerns (Waltz, 2016). Different jurisdictions have adopted different regulatory regimes for growing GMOs and commercializing them, which creates barriers to the widespread adoption of CRISPR-edited crops globally (Chawla et al. 2017). Further, there is the concern that off-target mutations may occur unintendedly, or that genes from the modified plant would flow to the related wild species

highlighting the necessity for stringent risk assessment and environmental monitoring (Bortesi and Fischer, 2015).

Thus, this review aims to provide an in-depth analysis of the CRISPR-Cas revolution in the agricultural sector, covering all its aspects from applications, underlying mechanisms, regulatory frameworks, and ethics. We have synthesized recent research findings and emerging trends to illustrate the transformative potential of CRISPR technology in achieving sustainable crop improvement and trait enhancement to meet the multi-faceted challenges faced by modern agriculture.

## Material and methods

A comprehensive review of available articles was performed regarding the status and application of CRISPR-Cas technology on improving plants from a holistic point of view for sustainable crop production. Literatures were searched non-selectively through Web of Science, Scopus, ScienceDirect, PubMed, and Google Scholar. Their search results were then carefully compiled to organize the relevant articles within the scope of the study. Additionally, the titles, abstracts, and conclusions of the literature were evaluated to identify the most pertinent articles.

### *CRISPR-Cas: A breakthrough in precision plant breeding and crop improvement*

CRISPR-Cas technology is designed to introduce specific modifications into a genome of interest either by gene insertion or deletion, thus being incredibly effective for enhancing plant breeding and promoting crop development accordingly. The cell's natural repair mechanisms fix the cut, introducing specific changes to the targeted chromosomes in the process (Wu, 2018). The CRISPR-Cas technology leverages the crop's existing genetic traits and, in cases of gene disruption, does not involve the introduction of foreign genes (Soda, 2017). Recent breakthroughs in science have revealed that CRISPR-Cas systems, especially type II CRISPR-SpCas9 from *Streptococcus pyogenes* and type V CRISPR-LbCas12a from *Lachnospiraceae bacterium*, hold immense untapped potential in human biology (Li, 2023; Li et al., 2023), agriculture, and microbiology (Tomlinson, 2018; Brandt, 2019; Hickey, 2019) as versatile technologies. CRISPR-Cas systems and other genome-editing technologies have the potential to contribute to food security and safety by providing the means to enhance the productivity of existing cropland sustainably in the face of climate change, while preserving wild ecosystems. This technology will reduce fertilizer use by farmers through the engineering of microbes capable of producing fertilizing nitrogen to crops.

With CRISPR-Cas, the development of new attributes in crops can include improved efficiency with increased carbon sequestration, improved nutrient composition, and more food per acre using fewer inputs (Brandt, 2019). This technology involves developing one single-guide RNA to target a particular gene, with CRISPR/Cas9 used for delivering the genetic information into the plant cell. This is commonly applied through either a bacterial delivery system, like Agrobacterium-mediated transformation method or ribonucleoproteins (RNP). This process typically includes: (i) using tissue culture to induce callus formation, (ii) regenerating plants from CRISPR/Cas9-modified tissues, (iii) creating T0 CRISPR/Cas9-modified transgenic plants, (iv) screening these transgenic plants to assess the

accuracy of CRISPR-Cas9 in targeting genes, (v) self-pollinating T0 transgenic plants to produce homozygous T1 plants, and (vi) analysing the traits of T1 plants (Figure 1). The most common strategy for applications of CRISPR-Cas involves frameshift mutations that include the insertion of small indels, or premature stop codons, which generally produce gene knockouts/null alleles (Zhang, 2018). Due to this reason, the technology has been quickly adopted and rapidly exploited for plant improvements and provides effective solutions to many challenges in plant breeding (Li, 2016; Ricroch, 2017; Gao, 2018). Additionally, it has been utilized to produce genetically modified plants, which have the potential to greatly increase agricultural yields and other crucial aspects of commercially produced genetically modified crops (Stout, 2017; Zhang, Massel, et al., 2018; Verma, 2023). It has advantages over conventional breeding methods which will enable the researcher to develop desirable disease-resistant and herbicide-resistant germplasm with improved yield and quality. This is achieved either through the removal of undesirable genetic elements or by introducing gain-of-function mutations through the precise editing of genomes. To date, the technique has been employed in the improvement of a wide array of crops, including wheat, cassava, tomatoes, and corn (Castiglioni, 2008; Zaidi, 2016; Khatodia, 2017; Ribeiro, 2020). Current research on its applications demonstrates enhanced biotic and abiotic traits, including the development of resistance to powdery mildew in wheat and tomatoes, viral resistance in potatoes and

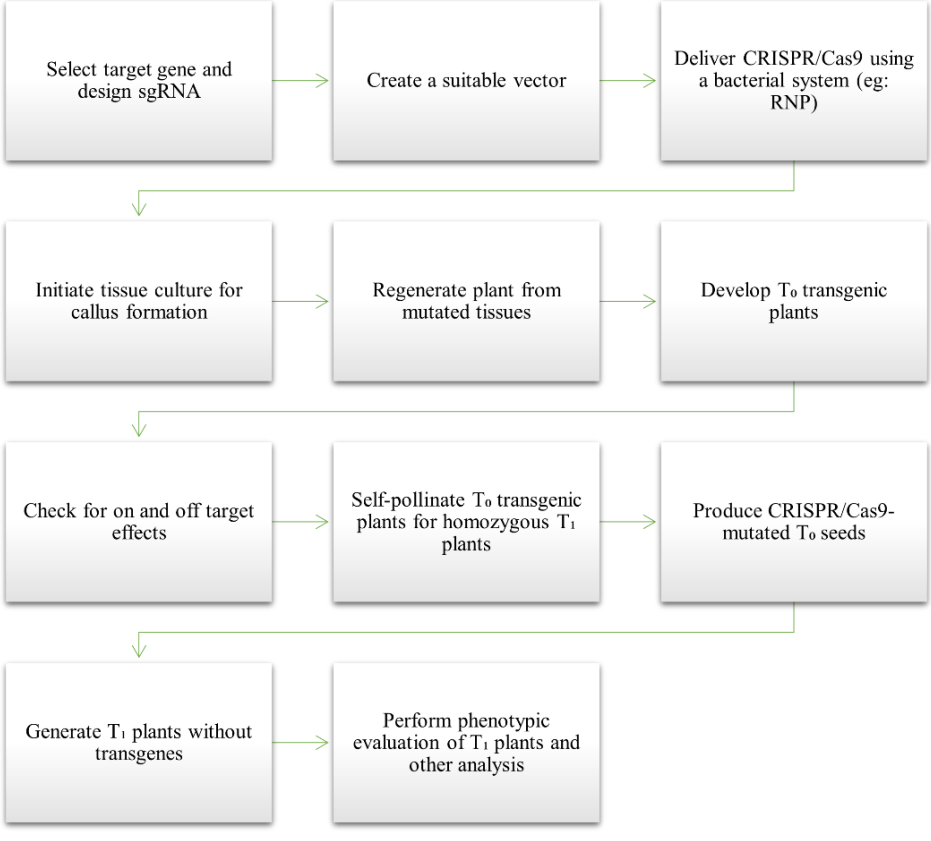


**Figure 1.** Procedure for implementing CRISPR-Cas9-mediated gene editing in plants

cucumbers, and drought tolerance in soybeans, among other improvements (Table 3). Furthermore, CRISPR-Cas technology has facilitated enhancements in yield and nutritional quality in certain crops, such as increased protein and amylose content in wheat, elevated lycopene levels, improved fruit colour, shape, and extended shelf life in tomatoes, as well as the breeding of lipoxygenase-free soybeans and the production of cyanide-free cassava (Table 2). Likewise, CRISPR-Cas technology is pivotal in augmenting fruit size, grain length, width, and weight across various crops, including wheat, tomatoes, groundcherries, and rice. It has also been effectively employed to enhance tillering in wheat (Table 1). However, many of these engineered crops are at different stages of research, development, and production, with a significant number awaiting regulatory approval (Chen, 2019).

*Advancing crop improvement through CRISPR-Cas as gene editing tool*

The wider availability of genomic information on various plant species today, as a result of the advancement in sequencing technologies, has paved the way for precision gene editing (Ahmar, 2020). Present crop breeding methodologies are oriented to a great extent on the research of various biological mechanisms and the unravelling of functions by genetic and epigenetic factors, which in turn will allow one to understand how these elements might interact and thus contribute toward crop trait improvements for plant breeding and agricultural advancement (Gallusci, 2017; Richardson, 2023). The principle of modern genetic engineering is thus based on the possibility of coupling specific DNA sequences with the ability to induce targeted breaks in the genome. When combined with a particular DNA binding domain (DBD), a nuclease may be precisely guided to target a site of choice (Ranjha, 2018). The DNA cleavage is followed by the host's DNA repair machinery, which introduces mutations that can, in turn, result in the functional knockout of the gene of interest (Jinek, 2012; Zetsche, 2015; Globus, 2017).

The rDNA technology provides the avenue, therefore, for transferring desirable genes from any plant or microorganism into crops, producing new genotypes and phenotypes that can increase yield and improve crop quality (Das, 2023). The main four approaches to engineered nucleases developed for genome editing include homing meganucleases, zinc finger nucleases, transcription activator-like effector nucleases, and CRISPR-Cas9 and -Cas12a orthologs. The first three approaches, however, have a closed architectural design; as such, manipulation of target specificity is difficult and these processes can be problematic, long-winded, and expensive to perform (Guha, 2017; Chandran, 2023). Unlike the typical protein-DNA complexes, CRISPR-Cas systems operate under a different ability because their identification of target sequences for purposes of cleavage, modification, deletion, insertion, or replication are mediated by RNA guides and ribonucleotide-DNA complexes. This system confers upon them an exceptional degree of versatility, as is evident from various studies (Jinek, 2012; Zetsche, 2015; Globus, 2017). Of the

various CRISPR systems, the tools most widely used in plant genetics applications are CRISPR-Cas9 and CRISPR-Cas12a. The first biochemical process detailing how the CRISPR-Cas9 system works was reported in 2012 (Jinek, 2012). This was a seminal moment that has, to this date, fully changed genetic research throughout the life sciences. Both CRISPR-Cas9 and CRISPR-Cas12a can be targeted to DNA with high specificity, inducing double-strand breaks (DSBS).

This break can be repaired through two main pathways: Non-Homologous End Joining (NHEJ), which may introduce small mutations by imperfect repair, or Homology-Directed Repair (HDR),

**Table 1.** Improved growth and development in CRISPR-Edited Crops

| Crop species | Target gene | Improved Trait | Reference |
|---|---|---|---|
| Wheat (Tritocum aestivum) | *LAZY1* | Spreading of plant tillers | (Miao, 2013) |
| | *TaGW2,α-gliadin genes* | Increase grain size | (Sánchez-León, 2017) |
| | *GW2* | Enhances grain weight | (Zhang, 2018) |
| Tomato (Solanum lycopersicum L.) | *SP5G* | Earlier harvest time | (Soyk, 2016) |
| | *fas, lc* | Fruit size | (Rodríguez-Leal, 2017) |
| | *ENO* | Fruit size | (Yuste-Lisbona, 2020) |
| | *CLV3* | Fruit size | (Zsögön, 2018) |
| Rice (*Oryza sativa*) | *GS3, Gn1a* | Grain length | (Zhao, 2018) |
| | *GW2, GW5, TGW6* | Grain length and width | (Rongfang, 2016) |
| | *GL2/OsGRF4, OsGRF3* | Grain size | (Hao, 2019) |
| | *GS9* | Slender grain shape | (Zhao, 2018) |
| | *GW5* | Grain width | (Liu, 2017) |
| | *OsGS3, OsGW2 and OsGn1a* | Grain length and width | (Zhou, 2018) |
| | *Gn1a, GS3, DEP1* | Larger grain size, enhanced grain number, and dense erect panicles | (Li, 2016) |
| Groundcherry (*Physalis* sp.) | *ClV1* | Fruit size | (Lemmon, 2018) |

which allows for precise DNA modifications using a template. These processes ensure targeted gene editing but also raise challenges like off-target effects, which could impact related genes or proteins.

These characteristics enable them to effectively facilitate delivery of DNAs, RNAs, or even protein-RNA complexes into site-directed nucleases (SDN) in plant cells. It is due to this quality that CRISPR technologies have become imperative in the advancement and improvement of crops through genetic engineering (Zhang, 2016; Zhang, Massel, et al., 2018). Because of its unparalleled capacity to precisely alter plant genomes, CRISPR-Cas has become an invaluable instrument in the field of agriculture (Khan, 2022).

**Table 2.** Improved yield traits in CRISPR-Edited Crops

| Crop species | Target gene | Improved Trait | Reference |
|---|---|---|---|
| Wheat (Tritocum aestivum) | *TaGW2,α-gliadin genes* | Low gluten content | (Wang, 2018) |
| | *SBEIIb* | Increased amylose content | (Sun, 2017) |
| | *GW2* | Increase in protein content | (Zhang, Li, *et al.*, 2018) |
| Tomato (Solanum lycopersicum L.) | *SP, SP5G, CLV3, WUS, GGP1* | Domestication of tomato | (Li, 2018) |
| | *SlAGL6* | Parthenocarpy | (Klap, 2016, p. 201) |
| | *ANT1* | Fruit color (purple) | (Cermak, 2015; Van Vu, 2020) |
| | *SlMYB12* | Fruit color (pink) | (Deng, 2017) |
| | *CRTISO* | Fruit color (tangerine) | (Shlush, 2020) |
| | *Psy1, CrtR-b2* | Fruit color (yellow) | (D'Ambrosio, 2018) |
| | *OVATE, Fas, Fw2.2* | Fruit size, oval fruit shape | (Zsögön, 2018) |
| | *TaGW7* | Grain shape | (Wang, 2019) |
| | *ALC* | Long shelf life | (Yu, 2017) |
| | *PL, PG2a, TBG4* | Long shelf life | (Uluisik, 2016) |
| | *slyPDS* | Increased lycopene content | (Li, 2018) |
| | *BnFAD2* | High oleic acid proportion | (Okuzaki, 2018) |
| Maize (Zea mays L.) | *TMS5* | Thermosensitive male-sterile | (Liu, 2017) |
| | *Psy1* | Seed color | (Shi, 2016) |
| | *Wx1* | Waxy corn | (Gao, 2020) |
| Potato (*Solanum tuberosum* L.) | *StGBSS* | Low amylose content | (Veillet, 2019) |
| | *StSBE1, StSBE2* | High amylose content | (Tuncel, 2019) |
| | *OsBEI and OsBEIIb* | High amylose content | (Sun, 2017) |
| | *CrtI, PSY* | High *β*-carotene content | (Dong, 2020) |
| | *Wx1* | High amylopectin content | (Andersson, 2016) |
| Sweet potato (*Ipomoea batatas*) | *IbGBSSI* | Low amylose content | (Wang, 2019) |
| | *IbGBSSI, IbSBEII* | High amylose content | (Wang, 2019) |

| | | | |
|---|---|---|---|
| Grapefruit (*Citrus paradisi*) | *ldnDH* | Low tartaric acid | (Ren, 2016) |
| Mushroom | *PPO* | Anti-browning phenotype | (Waltz, 2016) |
| Soybean (*Glycine max* [L.] Merr. | *GmFAD2–1A and GmFAD2–1B* | Improvement of seed oil composition | (T., 2019) |
| | *GmLox genes (GmLox1, GmLox2, and GmLox3)* | Breeding for lipoxygenase-free soybean varieties | (Wang, 2020) |
| Cassava (*Manihot esculenta* Crantz) | *CYP79D1 and CYP79D2* | Production of Cyanide-free cassava | ('2020 World Congress on In Vitro Biology, June 6-10, Virtual Pre-Recorded Meeting: Late Submission Abstracts.', 2020, pp. 6–1) |
| | *GBSS and PTST/PTST1* | Production of waxy starch | (Bull, 2018) |
| | *PTST1, GBSS* | Low amylose content | (Liu, 2021) |
| Flax (*Camelina sativa*) | *FAD2* | Reduced polyunsaturated fatty acids | (Jiang, 2017) |
| Rice (*Oryza sativa*) | *GS9* | Slender grain shape | (Zhao, 2018) |
| | *OsGAD3* | High GABA content | (Akama, 2020) |
| | *OsNramp5* | Low Cd accumulations | (Li, 2017) |
| | *OsFAD2-1* | High oleic acid proportion | (Abe, 2018) |
| | *OsPLD* | Low phytic acid content | (Khan, 2019, p. 201) |
| | *SlGAD2, SlGAD3* | High GABA content | (Nonaka, 2017) |
| | *OsGBSSI* | Low amylose content | (Huang *et al.*, 2020) |
| | *OsAAP6, OsAAP10* | Reduce GPC | (Wang, 2020) |
| | *OsBADH2* | Fragrant rice | (Ashokkumar, 2020) |
| | *SH2, GBSS* | Supersweet and waxy corn | (Dong, 2019) |
| Carrot (*Daucus carota*) | *DcMYB7* | Root color | (Xu, 2019) |
| Kale (*Brassica oleracea*) | *CRTISO* | Yellow leaves and stems | (Sun, 2020) |
| Banana (*Musa paradisiaca*) | *MaACO1* | Long shelf life | (Hu, 2021) |
| | *OsGBSSI* | Low amylose content | (Xu, 2020) |
| Barley (*Hordeum vulgare L.*) | *HvGBSSIa* | Low amylose content | (Zhong, 2018) |
| | *Aux/IAA gene family (StIAA2)* | Involved in Auxin/indole-3-acetid acid proteins synthesis | (Wang, 2015) |
| | *Acetolactate synthase1 gene (ALS1)* | Biosynthesis of branched-chain amino acids | (Butler, 2015) |
| | *Granule-bound starch synthase (GBBS)* | Involved in starch synthesis pathway | (Andersson, 2016) |
| | *Wx1* | High amylopectin content | (Bahagiawati, 2019) |
| Rapeseed (*Brassica* | *BnITPK* | Low phytic acid content | (Sashidhar, 2020) |

| | | | |
|---|---|---|---|
| *napus*) | *BnTT8* | High oil production and GPC | (Zhai, 2019) |
| Camelina (*Camelina sativa*) | *CsFAD2* | High oleic acid proportion | (Khan, 2019) |
| Banana (*Musa paradisiaca*) | *MaACO1* | Long shelf life | (Hu, 2021, p. 2) |

This technology revolutionized contemporary breeding processes and, therefore, was able to foster the creation of new varieties with desirable traits in crops. Thus, enabling many other species to be domesticated within a relatively short timeframe (Wolter, 2019). Recent research has demonstrated that manipulating cytokinin homeostasis, among various factors influencing yield, can effectively enhance plant productivity which has emerged as a practical strategy for increasing grain production (Zhu, 2020). An example of this includes the new high-yield wheat phenotype created through downregulation of the gene for cytokinin oxidase/dehydrogenase (CKX), an enzyme that degrades cytokinins (Nadolska-Orczyk, 2017). A rice cultivar was developed by knocking out the gene encoding amino acid permease 3, which is a key gene in nutrient partitioning. The resultant plants exhibited maintained grain quality and higher tiller numbers, resulting in better yields. In addition, rice carrying terminus C gene (LOGL5) responsible for the activation of cytokinin enzymes has also been edited to ensure increased grain production in different climatic conditions (Zhu, 2020). Similarly, CRISPR technology has been applied to alter complex characteristics in corn and tomatoes to achieve improved yields. Site-directed nucleases (InDel) were used to induce specific mutations in the regulatory genetic elements responsible for yield traits in tomatoes. This approach had the added advantage of enhancing genetic variability and achieving higher yields in a much shorter duration than achieved with traditional breeding programs (Kawall, 2021; Tiwari, 2023).

Various works explored CRISPR-Cas technology against plant biotic stress through introduction of dominant resistant genes that may encourage the development of resistance in pathogenic organisms (Ahmad, 2021). A commonly referenced instance is the application of InDel in wheat to develop a resistant variety against the fungus powdery mildew, an obligate host-specific fungus called *Blumeria graminis* f. sp. *Tritici*. This particular fungus is known to be of major global losses associated with the crop (Lyzenga, 2021). It has also been used to modify a promoter sequence of Argose, one type of maize lines known as Zars, to enable the expression of the endogenous gene constitutively. This improved yield potential dramatically even under conditions of drought stress (Manna, 2023). Another example is the development of rice varieties resistant against *Xanthomonas oryzae* pv. oryzae, achieved by using CRISPR-Cas genome editing in order to modify the promoter elements of the O. sativa SWEET11, SWEET13, and SWEET14 genes (Oliva, 2019). Meanwhile, a wheat variety resistant to powdery mildew across a broad spectrum of resistance was generated by co-mutating all three homologues of the MLO EDR gene controlling resistance to *B. graminis* f.sp. *tritici* (Zhang, 2018). Similarly, the TaMLO-A1

and TaMLO-B1 genes have also been mutated with CRISPR-Cas9 to yield bread wheat resistant to powdery mildew (Tyagi, 2020). In tomatoes, the use of CRISPR-Cas that targeted the MLO1 gene of *Solanum lycopersicum* has conferred resistance against the powdery mildew pathogen *Oidium neolycopersici*. Further enhancement in resistance against RNA viruses was also enabled by RNA-targeting methods mediated through Cas13a, Cas13b, Cas13d, and Cas12a from *Francisella tularensis* subsp. *novicida* (strain U112) (Wang, 2014). Another promising approach to developing broad-spectrum viral resistance involves knocking out plant susceptibility genes.

This approach has been explored in *Cucumis sativus* for Potyviruses. By knocking down the gene eIF4E (eukaryotic translation initiation factor) in cucumber, necessary for viral infection but not for the growth of the plant, scientists achieved broad resistance to potyviruses without compromising the overall fitness (Bastet, 2017).

**Table 3.** Improved Biotic and Abiotic Traits in CRISPR-Edited Crops

| Crop species | Target gene | Improved Trait | Reference |
|---|---|---|---|
| Wheat *(Triticum aestivum)* | *OsERF922* | Resistance to rice blast disease | (Wang, 2016) |
| | *ALS, EPSPS* | Resistance to herbicide | (Endo, 2015; Li, 2016; Sun, 2016; Butt, 2017) |
| | *EDR1* | Developing resistance to powdery mildew disease caused by *Blumeria graminis* f.sp. (Btg) Tricitici | (Zhang, 2017) |
| | *OsERF922* | Resistance to rice blast disease | (Wang, 2016) |
| Maize *(Zea mays L.)* | *ALS* | Herbicide resistance | (Svitashev, 2015) |
| | *ARGOS8* | Tolerance to drought | (Shi, 2016, p. 8) |
| Potato *(Solanum tuberosum L.)* | *ALS* | Herbicide resistance | (Butler, 2016) |
| | *elF4E and elF(iso)4E)* | Resistance | (Hameed, 2021) |

| | | | |
|---|---|---|---|
| | | against viruses and cold-induced sweetening | |
| Tomato (*Solanum lycopersicum* L.) | *SlMLO1* | Resistance to powdery mildew disease | (Nekrasov, 2017) |
| | *SlJAZ2* | Resistance to bacteria speck disease | (Ortigosa, 2018) |
| Cucumber (*Cucumis sativus*) | *eIF4E* | Resistance to viral disease | (Chandrasekaran, 2016) |
| Orange (*Citrus sinensis*) | *CsLOB1 promoter* | Resistance to citrus canker disease | (Peng, 2017) |
| Grapefruit (*Citrus paradisi*) | *CsLOB1 promoter* | Resistance to citrus canker disease | (Jia, 2015) |
| Soybean (*Glycine max* [L.] Merr. | *ALS* | Herbicide resistance | (Li, 2015) |
| | *HaHB4* | Drought tolerance | (Martignago, 2020) |
| Cassava (*Manihot esculenta Crantz*) | *EPSPS* | Herbicide resistance | (Hummel, 2018) |
| | *eIF4E isoforms nCBP-1 and nCBP-2* | Reduction in cassava brown streak disease symptom severity and incidence | (Gomez, 2018) |
| Kale (*Brassica oleracea*) | *CRTISO* | Yellow leaves and stems | (Sun, 2020) |
| Flax (*Camelina sativa*) | *EPSPS* | Herbicide resistance | (Sauer, 2016) |

*Reshaping crop stress responses with CRISP-Cas technology*

Abiotic stressors include extreme temperatures (both high and low); drought; salinity; UV radiation; and environmental pollution, considered major factors impeding plant growth, development, and yield, with generally negative consequences for quality (Wang, 2003; Li et al., 2023). These key players, which have been identified to mediate the crops' responses to these environmental challenges, include many structural and regulatory genes and non-coding RNAs, all of which constitute valuable targets for enhancing the stress tolerance of plants (Zhang, 2021). The CRISPR-Cas system has shown considerable promise in improving plants' resilience to abiotic stressors. For instance, genetic manipulation of the negative regulator of the ethylene response pathway, such as the AGROS8 gene, has been shown to enhance the drought tolerance in maize significantly (Hameed, 2021). The knockout of SlAGAMOUS-Like 6 mediated through CRISPR-Cas9 promoted the growth and fruiting of tomatoes under heat stress conditions (Wan, 2021). Recent studies demonstrated that, in Arabidopsis, CRISPR-Cas-mediated mutants of dpa4-sod7-aitr256 exhibited enhanced drought tolerance (Zhang, 2021). However, not all CRISPR-Cas9 interventions yield positive results; for example, the knockdown of the non-expressor of pathogenesis-related gene 1 (npr1) in tomato plants led to a reduction in drought tolerance, demonstrating the complexity of gene editing outcomes (Zhang, 2021).

Climate change has become one of the biggest threats to food security and food safety due to unforeseeable weather conditions, increased CO2 levels, drought stress, interference with plant defence mechanisms, and a rise in pest populations and pathogen virulence (Gangurde, 2019). Significantly, it has been estimated that for each 1°C rise in Earth temperature, pests will wipe out 10 to 25% of key staple food crops like corn, rice, and wheat. Expected yields have therefore taken an observable plunge, with returns in food staples like rice experiencing a 0.3% drop, wheat experiencing a 0.9% drop, and oil palm experiencing a 13.4% drop on an annual basis during recent years (Basnet, 2012; Ray, 2019). Simultaneously, crop production must double by 2050 to meet the demands of a growing global population, putting further pressure on the food system (Pastor, 2019).

Traditional approaches to overcoming the impact of climate change on food production have yielded limited success quantitatively and qualitatively. Conversely, CRISPR-Cas technology has emerged as a very precise and more cost- and time-effective approach for adapting plants to future challenges. This gene-editing system has been employed to enhance the salt tolerance of rice by knocking out the *OSRR22* gene, ensuring the continued safe consumption of a crop that sustains 3.5 billion people globally (Zhang, 2019). It has also been used to improve draught tolerance of maize grains by editing the gene ARGOS8, which enhances cell division and hence reduces the negative impact of shortage of water on plants. The technology also has been used in the development of disease-resistant tomatoes by knocking out the gene *DMR6* that makes the plant broadly resistant against pathogens without affecting the growth and

development of the plant. In these genome-edited tomato plants, high resistance was noted against *Pseudomonas syringae*, *Phytophthora capsici*, and *Xanthomonas species* (Kieu, 2021).

Herbicide-resistant weeds are a major cause of crop losses and pose a growing threat to global food security (Tyczewska, 2018; Manning, 2019). In response, the development of herbicide-resistant crops through CRISPR-Cas technology has proven to be an effective method for weed control, offering innovative approaches for transferring herbicide resistance to plants (Han, 2019). This is primarily accomplished by targeting endogenous genes such as acetolactate synthase (*ALS*), cellulose synthase A catalytic subunit 3 (*CESA3*), 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*), and splicing factor 3B subunit 1 (*SF3B1*) (Hussain, 2021; Khan, 2022). Research has shown that herbicide tolerance can be achieved by introducing amino acid changes in the *ALS* gene, as observed in studies of naturally occurring point mutations (McNaughton, 2005). Additionally, cytosine base editors (CBEs) have been employed to induce precise base transitions in the *ALS* gene of *Oryza sativa*, conferring herbicide resistance to rice while preserving *ALS* enzymatic function (Preston, 2006).

Unlike traditional breeding methods, gene editing by means of CRISPR technology is more time-efficient and stable. Because the genetic variants that emerge as a result of editing with the use of CRISPR are no different from those which emerge when natural mutations take place, this technology is extremely reliable (Anders, 2022; Marone, 2023). Increased precision and efficiency are the main factors contributing to the successful acceptance of CRISPR-edited crops in the commercial marketplace, increasing its potential for adoption for crop improvement (Brandt, 2019).

*Global regulatory frameworks and challenges in CRISPR-Cas crop development*

Over the past couple of years, approximately 190 million hectares of the transgenic crop have been grown in 26 different countries, comprising of 21 developing and five industrialized countries. Recently, Brazil, Argentina, and India was in the top five countries with the largest biotech crop production areas, taking up 54% of the total growth in developing regions (Turnbull, 2021). On the other side, the industrialized nations including the United States, Canada, Australia, Spain, and Portugal account for 46% of the total biotech crops produced worldwide. Irrespective of the extensive consumption of these improved crops, most governments and legislators take it up as their core responsibility to ensure that their people, the ecosystems, and the environment are protected by imposing various regulations on feeds, plants, and crops for consumption of the people (Hilbeck et al., 2020). The regulatory regimes differ in their approach depending upon whether they are process-oriented or product-oriented. Consequently, the governance of genetic engineering, particularly the development of transgenic organisms, is shaped by two primary legal approaches (Eckerstorfer, 2019).

The product-based approach, classically accepted by the United States, Canada, and a number of other countries in the Americas, considers GMOs no different from those obtained by conventional breeding (Medvedieva, 2018). For that reason, such products are subject to already long-standing general legislation designed to protect human health and the environment, and therefore the

creation of special regulations is not required (Turnbull, 2021). The European Union, on the other hand, has conventionally followed the process-based approach (Medvedieva, 2018). Gene-editing technologies, therefore, from this perspective, are unique and exceptionally new in nature; the development of particular laws with their regulations becomes necessary (Duensing, 2018). European Union legislation already governs the production and release of many genetically modified crops, such as maize, soybeans, rapeseed, and cotton, and incorporates various methods with appropriate risk assessments. However, techniques like mutation breeding, used before the 2001 directive, are exempt from such regulations (Wolt, 2016; VAN DER MEER, 2021). It is possible that the technology of CRISPR/Cas9 could fall under the process-based current EU regime if it will be viewed as a variation of the more traditional genetic engineering leading to GMOs (Zhang, 2020).

With the use of CRISPR-Cas9, more than 25 plant species have been genetically edited to date, with over 100 genes being modified for a wide range of desirable traits in key crops (Manghwar, 2020). Although CRISPR-Cas has, to date, been recognized as a highly potent tool in editing genetic makeup toward enhanced agricultural output, the debate on its advantages and disadvantages regarding food production resulted in a landmark case before the Court of Justice of the European Union back in July 2018 (Purnhagen, 2020). The court ruled that it was a landmark decision that CRISPR-Cas falls under the "mutagenesis exception" inducted in Appendix 1 B of rules on Genetically Modified Organisms, as created upon the case of Confédération Paysanne, C-528/16 (Siebert, 2021). However, new plant breeding techniques (NPBT), such as ‘oligonucleotide-directed mutagenesis’ were concluded not to qualify for the mutagenesis exemption after they were classified as being technologies of GMOs in origin (Urnov, 2018). This legal standing has posed a problem for those biotechnology companies using CRISPR-Cas, given the added regulatory requirements which they must address and which include marketing authorization and product labelling that involves considerable costs (Turnbull, 2021). As a result, the societal acceptance and application of CRISPR-Cas as a new gene-editing technology have become subjects of great debate, especially among researchers and interested parties directly involved with the technology.

Countries have taken distinct regulatory approaches concerning genome editing and genetically modified organisms (GMOs). For instance, Argentina established itself as the inaugural nation to enact targeted legislation regarding novel plant breeding technologies, encompassing genome editing, in 2015. This legislative framework provides an exemption for transgene-free products from the current GMO regulations (Menz, 2020). The United States is also regarded as one of the leaders in the development and commercialization of genetically modified crops, controlling about 30% of the global agricultural biotechnology market. However, unlike many other nations, the U.S. does not have a unified federal law governing GMOs. In contrast, genetically modified (GM) products are governed by the Coordinated Framework for Regulation of Biotechnology, which imposes identical health, safety, and environmental criteria as those applied to traditional products (Wolt, 2016; Turnbull, 2021). Likewise, Canada employs a risk assessment methodology grounded in scientific principles, emphasizing the potential

allergenic, toxic, and off-target consequences associated with the product (Arpaia, 2020). It is considered a PNT when a plant expresses a trait that is different from conventionally cultivated varieties by about 20–30%, rather than being called a genetically modified organism (GMO) (Ferreira, 2023). Applications must be approved by the e Canadian Food Inspection Agency (CFIA) before environmental deployment (Smyth, 2019). In Africa, countries such as South Africa, Ethiopia, Malawi, Kenya, Nigeria, Sudan, and Eswatini have embraced the cultivation of GM crops, despite the challenges posed by population growth and climate change to food security (Endale, 2022). South Africa, the region's leading GM crop producer, was the first African country to establish a regulatory framework for the cultivation, import, and export of GM crops. Other countries implemented the same regulations; for example, Burkina Faso, though it has never started commercial production of GM crops, it has kept a Biosafety Law enacted since 2012 (Turnbull, 2021).

The expansion in the use of CRISPR-Cas9 technology should be based on an estimation of the possible limitations and risks associated with its application. So far, two major issues have been emphasized: off-target unintended editing and unethical or illegal scientific activities. Regarding off-target editing, two main risks have been identified. First, there is the risk of unintended editing when genomic regions of high sequence similarity to the intended target occur. The second aspect pertains to off-target modifications occurring in areas that bear no relevance to the intended target sequence (Kaul, 2020). It can also have unintended effects on gene networks and protein interactions. Editing a specific gene may inadvertently alter the expression of neighbouring or interacting genes due to interconnected pathways. For instance, modifying a resistance gene might disrupt metabolic or signalling networks critical to plant growth. Similarly, changes in gene-protein interactions can lead to unanticipated effects on protein functionality, stability, or downstream processes, potentially impacting overall plant performance. Understanding these interactions is crucial to ensure that genome edits produce stable and predictable traits without compromising plant health. Off-target effects, characterized by the CRISPR-Cas9 system binding to and cleaving unintended loci, may lead to the inactivation of otherwise functional genes or erroneous repair of genes linked to various diseases, thereby posing substantial obstacles in therapeutic (Klein, 2018). Furthermore, unintended editing may result in chromosomal reorganizations and various modifications, including DNA mismatches that arise within the PAM-distal region of the complementary sequence of the sgRNA (Manghwar, 2020). In addition, the risk of off-target mutations caused by CRISPR-Cas9 has the potential to influence not just the modified organism but also its descendants (Zhang, Li, et al., 2018). With the advancement of technology, forthcoming investigations may reveal novel, unanticipated off-target effects that possess unpredictable implications (Bin Moon, 2019).

Despite significant advances that have been made in understanding CRISPR-Cas9 technology, challenges still remain regarding its targeting efficiency. The potential for unwanted off-target effects has raised concern, particularly with respect to applications in plants, and must be addressed if

this technology is to see widespread use in gene therapy and crop improvement. During the breeding process, off-target effects are managed effectively by the validation and selection of phenotypes that are of interest, which facilitates the exclusion of mutations that may lead to unwanted traits (Bishop, 2020). Additionally, contemporary studies concerning CRISPR-Cas9 applications in plant genome editing indicate that occurrences of off-target mutations are comparatively infrequent (Wolt, 2016). In contrast to gene therapy and clinical trials involving humans, which undergo considerable ethical examination (Hunt, 2023), research involving plants does not encounter the same level of ethical concerns, thereby rendering off-target effects more acceptable in agricultural settings.

## Conclusion

The CRISPR-Cas technology is a powerful tool due to its ability to make quick changes in plant genomes and to create desirable traits with efficiency in overcoming both biotic and abiotic stressors. However, from the high level of its promise, the technology has been a controversial topic, more so with respect to ethical considerations and the safety of its wide application. The major challenge for agricultural production using CRISPR-Cas does not lie in the scientific application of this technology but rather in societal views on genetically modified products and regulatory frameworks that govern their application. While traits developed through conventional plant breeding are often considered ‘nature-identical’ CRISPR techniques cover a broader range of uses, including the addition of transgenes, which has raised concerns about the implications of genetically modified organisms in agricultural uses.

The greatest expectation for CRISPR-Cas technology use in agriculture should rest with improving "nature-identical" traits. Its use in biomedical and human research requires in-depth ethical consideration, while evidence testifies that its use in agricultural contexts-at least in crop production-is relatively safe. However, at present, despite the demonstrated success of CRISPR-Cas in increasing crop yields, the technology is not utilized because of global regulatory challenges. More refined CRISPR tools have emerged in recent years that go beyond DSB-based editing. These tools are able to recognize and target highly specific DNA sequences. Examples include fusions of enzymatic domains with DNA-targeting proteins such as nuclease-dead Cas9 or Cas12a that can execute precise modifications. A typical example is represented by the complexation of dead Cas9 with a deaminase enzyme, allowing in such a way the conversion of one DNA nucleotide without inducing DSBs. These advances notwithstanding, the availability of base-editing platforms is still limited, especially for C-T or A-G conversions, thus restricting the range of sequence editing. It is envisaged that broader base-editing platforms will become available shortly, overcoming these limitations. As these technologies continue to evolve, CRISPR technologies are likely to offer a broader array of possibilities, including the visible editing of specific genomic locations, the direct modification of gene expression, and the triggering of targeted epigenetic changes.

In conclusion, the wide diffusion of CRISPR technology requires political will and public acceptance, besides ongoing research and proactive effort for global integration. Scientists should be open about their challenges in

communicating and presenting CRISPR-based breeding technology to the general public and regulatory bodies. Breeding approaches are fundamental to the long-term sustainability of agricultural systems, which will see formidable enhancement upon incorporation of the CRISPR technologies. Meeting these scientific and regulatory challenges will enable sustainable and productive crop production.

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## Authors' contributions

**Samanwaya Niraula:**

Conceptualization; Formal Analysis; Investigation; Review; Writing – original draft.

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## Conflict of interest

Authors declare no conflict of interest.

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