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REVIEW

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Exploring the potential of CRISPR/Cas genome editing for vegetable crop improvement: An overview of challenges and approaches

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Abstract

Vegetables provide many nutrients in the form of fiber, vitamins, and minerals, which make them an important part of our diet. Numerous biotic and abiotic stresses can affect crop growth, quality, and yield. Traditional and modern breeding strategies to improve plant traits are slow and resource intensive. Therefore, it is necessary to find new approaches for crop improvement. Clustered regularly interspaced short palindromic repeats/CRISPR associated 9 (CRISPR/Cas9) is a genome editing tool that can be used to modify targeted genes for desirable traits with greater efficiency and accuracy. By using CRISPR/Cas9 editing to precisely mutate key genes, it is possible to rapidly generate new germplasm resources for the promotion of important agronomic traits. This is made possible by the availability of whole genome sequencing data and information on the function of genes responsible for important traits. In addition, CRISPR/Cas9 systems have revolutionized agriculture,

Abbreviations: 16DOX, 16α-hydroxylase; ALS, acetolactate synthase; ALMT9, Al-activated malate transporter 9; ANT1, Anthocyanin mutant 1; BZR1, Brassinazole resistant 1; CCD8, cleavage dioxygenase 8; CHS, chitin synthase 1; CRISPRi, CRISPR interference; CRISPR-BEST, CRISPR-Base Editing SysTem; *CRTISO*, carotenoid isomerase; DMR6, downy mildew resistance 6; DSB, double-strand break; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; eIF4E, eukaryotic translation initiation factor 4E; GABA, γ-aminobutyric acid; FAD2, fatty acid desaturase 2 gene; FAE1, fatty acid elongase 1; G3P, glyceraldehyde 3-phosphate; GAD, glutamate decarboxylase; GBSSI, granule-bound starch synthase I; GGPPS, geranylgeranyl pyrophosphate synthase; IAA9, auxin-induced 9; IAA9 gene, indole-3-acetic acid inducible 9; MAGIC, multifunctional genome-wide CRISPR; Mlo1, mildew resistant locus O; MAPK, mitogen-activated protein kinase; MAX1, more axillary growth 1; MYB12, MYB transcription factor 12; PAM, protospacer adjacent motif; PDS, phytoene desaturase; PPO, polyphenol oxidase; PSY1, phytoene synthase; RBOHD, respiratory burst oxidase homolog D; SBEII, starch branching enzyme II; SFAR, seed fatty acid reducer; SFAR4, seed fatty acid reducer 4; SFAR5, seed fatty acid reducer 5; SlAGL6, SlAGAMOUS-LIKE6; SpCas9, *Streptococcus pyogenes* Cas9; ZISO, z-carotene isomerase.

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making genome editing more versatile. Currently, genome editing of vegetable crops is limited to a few vegetable varieties (tomato, sweet potato, potato, carrot, squash, eggplant, etc.) due to lack of regeneration protocols and sufficient genome sequencing data. In this article, we summarize recent studies on the application of CRISPR/Cas9 in improving vegetable trait development and the potential for future improvement.

KEYWORDS

CRISPR/Cas9, gene knockout, regulatory framework, stress tolerance, trait improvement, vegetable breeding

1 | INTRODUCTION

By 2050, the human population on earth will be about 9.9 billion, which means that food demand will also increase accordingly. Climate change and biodiversity loss are also accelerating rapidly. Plants also face various biotic and abiotic stresses due to sudden or ever-changing environmental factors. These stresses have led to the reduction in the limits of agricultural crop productivity. On the positive side, developments in agricultural technology have led to the development of crops with higher yield potential and greater climate resilience. With conventional plant breeding, we can select, combine and select plants with desirable traits to improve the quality and quantity of our crops, but this type of breeding can only be done if the plants can be sexually mated (Schaart et al., 2016). Genetic diversity is lost through conventional plant breeding (Louwaars, 2018; Rauf et al., 2010). Variation breeding is another technique that uses chemicals to increase the mutation rate. However, this method of breeding is uncontrolled and does not result in the desired dominant alleles. These obstacles make it difficult to address global food security challenges. New molecular genome editing techniques are emerging. CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9) is the latest technique for gene editing to modify desirable traits (Bhattacharyya et al., 2022; Biswas et al., 2022; Cong et al., 2013; Mitra et al., 2022; Nidhi et al., 2021; Pickar-Oliver & Gersbach, 2019; Sirohi et al., 2022). Only a few bacteria and archaea have CRISPR/Cas9 systems that function as part of their immune system. Foreign particles such as viruses and plasmids are eliminated by this system (Koonin & Makarova, 2009, 2013). SgRNA (single-stranded guide RNA) and Cas9 are both components of CRISPR/Cas9 (Negi et al., 2022). The complex (Cas9 and sgRNA) cleaves the target DNA by the nucleases RuvC and HNH, resulting in complementary and noncomplementary breaks, respectively. SgRNAs are designed to target specific DNA sequences at sites 3 bp upstream of the PAM (protospacer adjacent motif) (Barrangou et al., 2015; D. Liu et al., 2016). In addition, a DSB (double-strand break) is generated by cleavage of Cas9, which initiates the DNA repair mechanism (Figure 1). DSBs are repaired by either nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). The most likely outcome of a DSB is the activation of NHEJ mechanisms leading to various mutations, including indels and substitutions (Barakate & Stephens, 2016). CRISPR/Cas9 is a highly effective and simple tool for initiating mutagenesis (Cui et al., 2018). The target genes are completely eliminated using this tool, and the genetic changes are stable and are passed from generation to generation (Barrangou et al., 2015; D. Liu et al., 2016). The rapid development of next-generation sequencing technologies and the availability of vegetables in the public domain have made it possible to manipulate their genome with precision and accuracy to achieve desired traits.

Vegetables are of great benefit to humans as they contain important nutritional components for our daily diet that prevent diseases (Iqbal, 2014). Due to the rapidly growing population, vegetable breeding technology must be improved and breeding processes must be accelerated to meet the needs of our daily vegetable consumption. Although it is possible to increase the yield of vegetables through breeding techniques, this approach is time-consuming. On the other hand, trait improvement using genome editing techniques can overcome such obstacles and promises the timely delivery of improved crops for a rapidly growing population with increased food demands. In this review, we have introduced CRISPR/Cas9 and its applications in vegetable crops. We have also discussed the challenges and prospects of genome editing by CRISPR/Cas9 to improve vegetable crops.

2 | APPLICATIONS OF THE CRISPR/CAS9 SYSTEMS IN VEGETABLES

To successfully apply CRISPR/Cas9 technology to a crop, its genome sequence must be available. This helps in developing guide RNAs that are more specific and have minimal or no off-targets. The first plant was edited with the CRISPR/Cas9 system in 2013 (J. F. Li et al., 2013; Shan et al., 2013) and has subsequently been applied to numerous agronomically important crops. CRISPR/Cas9-mediated genome editing is a revolutionary tool for cost-effectively modifying plant genomes and improving plant traits (Nazir et al., 2022; Verma et al., 2021). Multigene targeting, precise base editing, and gene activation or suppression are possible with CRISPR-based

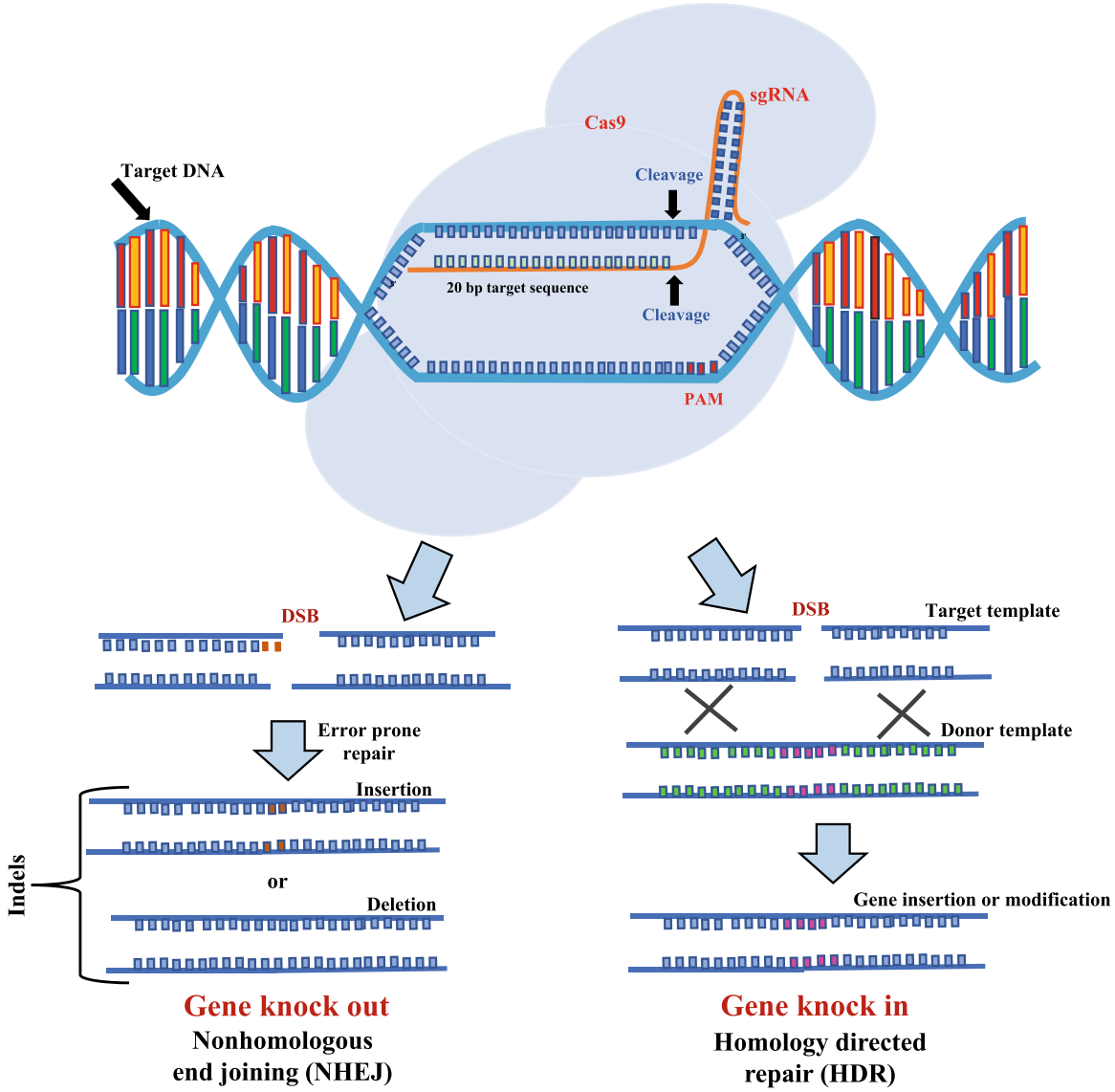

FIGURE 1 Schematic illustration of CRISPR/Cas9-mediated genomic modification. Cas9, directed by a single-strand guide (sgRNA), cuts double-stranded DNA and creates a double-strand break (DSB). After that, DNA damage is repaired by one of two different pathways: homology-directed repair (HDR) or nonhomologous end-joining (NHEJ). CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9; PAM, protospacer adjacent motif.

approaches, making it easy to create the desired changes. This method has been used extensively for editing genomes of fruits, vegetables, and cereals (Figure 2). The list of genes and traits that have been modified in vegetable plants using the CRISPR/Cas9 system is summarized in Table 1.

2.1 | Enhancing abiotic stress tolerance

Environmental factors such as heat, cold, UV radiation, salinity, and drought can negatively affect vegetable crops. Genomic editing with CRISPR/Cas9 can reveal the function of genes involved in stress-related proteins. This will help improve high-yielding vegetable crops by increasing their adaptability to environmental conditions. *BRASSINAZOLE RESISTANT 1 (BZR1)* role in stress is not yet fully understood, but this gene modulates brassinosteroid-promoted growth. BZR1 was mutated by gene editing with CRISPR/Cas9 and resulted in a reduction in the induction of respiratory burst oxidase homolog1 (RBOH1) and H_2O_2 production in tomato. Suppression of *BZR1* reduced heat stress (Heckman et al., 2018). Drought stress to which tomato plants are exposed is regulated by mitogen-activated protein kinases (*MAPKs*). Silencing of *SLMAPK3* with CRISPR/Cas9 genome editing resulted in increased H_2O_2 production and decreased antioxidant enzymes. *SIMAPK3* appears to be involved in the response of tomato plants to drought by protecting cell membranes from oxidative damage (L. Wang et al., 2017). Gene editing techniques can be used to create new cold-tolerant germplasms, such as CBF1 (C-repeat binding factor 1), which controls cold

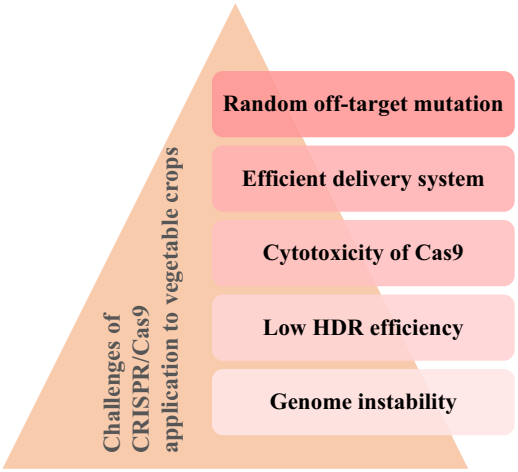

FIGURE 2 Challenges in the applications of CRISPR/Cas9 in vegetable crops. CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9.

tolerance in plants (R. Li, Zhang, et al., 2018). The physiological functions of tomato UV-B photoreceptors (SlUVR8) were also investigated using the CRISPR/Cas9 genome editing approach. Manipulation of UV-B photoreceptors in tomato plants using this editing method is an efficient way to increase the tolerance of tomato plants to high doses of UV-B (X. Liu et al., 2020). Salinity stress increased the expression of *RBOHD (respiratory burst oxidase homolog D)*, GRF12 (14-3-3 protein), and AHA1 (plasma membrane H^+-ATPase) in pumpkin. Knockout of *RBOHD* by CRISPR/Cas9 resulted in salt-sensitive traits via lower root apex H_2O_2 and K+ content, GRF12, AHA1, and HAK5 expression (Huang et al., 2019).

2.2 | Enhancing biotic stress tolerance

Plant resistance to biotic stress caused by viruses, bacteria, nematodes, and fungi can be improved by the CRISPR/Cas9 tool. Numerous diseases caused by fungi result in significant yield and crop quality losses. Downy mildew and powdery mildew, for example, pose a serious threat to tomato crops. The fungal disease (*Oidium neolycopersici*) resistant tomato cultivar "Tomelo" was developed using CRISPR/Cas9 by deleting homozygous SlMlo1 (Nekrasov et al., 2017). The tomato *DMR6* (downy mildew resistance 6) gene was edited to confer resistance to *Pseudomonas syringae*, *Phytophthora capsica*, and *Xanthomonas* spp. (Paula de Toledo Thomazella et al., 2016). The biosynthetic gene of strigolactones (SLs), that is, *MAX1 (MORE AXILLARY GROWTH 1)* was modified using CRISPR/Cas9 for prevention against *Phelipanche aegyptiaca*, a root parasitic weed. Genetic analysis showed that next-generation plants inherited mutations from their parents. The mutant plants also showed a reduction in height and adventitious root, and an increase in axillary buds growth (Bari et al., 2021). *P. syringae*, a bacterial plant pathogen causes bacterial speck disease on the economically and agronomically important tomato cultivar Moneymaker. Deletion of the Jas domain of *SlJAZ2* using CRISPR/Cas9 results in resistance to *P. syringae* pv. *Tomato* (Ortigosa et al., 2019). Another fungal pathogen, *Botrytis cinerea*, causes gray mold disease in crops, heavily infecting fruits and vegetables. As a result of the CRISPR/Cas9 mutation of SAMPK3, ROS was increased and the activities of defense enzymes were reduced, further remodeling the SA and JA defense signaling pathways and conferring resistance to *B. cinerea* (Zhang et al., 2018). The fungal pathogen *Sclerotinia sclerotiorum* can cause stem rot in *Brassica napus*. knockout of WRKY70 via CRISPR/Cas9 resulted in high resistance against *Sclerotinia* (Q. Sun et al., 2018). Also, *eIF4E* (eukaryotic translation initiation factor 4E) gene editing by CRISPR/Cas9 was exhibited in enhanced virus-resistance cucumber plants (Chandrasekaran et al., 2016).

2.3 | Enhancing herbicide resistance

Weeds pose a serious threat to vegetable crops. Weeds affect vegetable yields by causing stress as they compete with plants for space, light, water, and nutrient resources. CRISPR/Cas9 gene editing is used in the production of herbicide-tolerant fruits and vegetables. An important aspect of gene editing is selecting the right target genes. *P. aegyptiaca* is an obligate plant parasite that promotes seed germination with the help of SL from the roots of its host plants. CRISPR/Cas9 mutated *MAX1* genes involved in the biosynthesis of SL and produced resistance tomato against *P. aegyptiaca*. The next generation plants showed targeted mutations transmitted from the parent plants to their progeny. In addition, plant height and adventitious root formation were reduced (Bari et al., 2021). The *acetolactate synthase* gene (*ALS*) is targeted by CRISPR/Cas9 gene editing for base-editing in watermelon to generate herbicide-resistant germplasm (Tian et al., 2018). The potato *StALS1* template was successfully replaced by a herbicide-inhibiting point mutation using a geminivirus replicon or via *Agrobacterium tumefaciens* using a conventional 35S T-DNA expression vector in potato (Butler et al., 2015). In tomato and potato, C-to-T base conversion was used as a method to edit the cytidine of *ASL*, which induces herbicide resistance. In tomatoes and potatoes, 12.9% and 10% of first-generation plants were edited but transgene-free, respectively (Veillet et al., 2019).

2.4 | Fruit maturity and ripening time

The regulation of ripening is one of the most important concerns in the study of fleshy fruit/vegetable species. For the study growth and ripening, tomato plants are an ideal model because of their short life cycle, easy of transformation, and effective propagation. Fruit maturation is a complex and irreversible developmental process involving many biochemical and physiological processes. With the help of CRISPR/Cas9, the fruit ripening process can be slowed down by genetically modifying genes that directly or indirectly regulate ripening (Martín-Pizarro & Posé, 2018). Silencing *of SlORRM4* gene

TABLE 1 Application of the CRISPR/Cas9 system to enhance vegetable crop yield, nutritional quality, and other agronomic traits.

S. No	Vegetable crops	Targeted gene(s)	Mutation	Mode of delivery	Trait modified	Key observations	References
1.	Cabbage (*Brassica oleracea* var. *capitata* L.) Brassicaceae	*PDS*	Knockout	*Agrobacterium tumefaciens* strain EHA105 mediated hypocotyl transformation	Phenotype	–	Ma et al. (2019)
		FRI, *PDS*	–	PEG-mediated protoplast transfection	Phenotype	–	Murovec et al. (2018)
2.	*Camelina sativa* (L.) Crantz Brassicaceae	*FAD2*	Knockout	*A. tumefaciens* mediated transformation	Quality improvement	Linoleic acid ↓ (~16% to <4%), linolenic acid ↓ (~35% to <10%)	Jiang et al. (2017)
		FAD2	Knockout	*A. tumefaciens* mediated transformation	Quality improvement	Oleic acid ↑ (10%–62%)	Morineau et al. (2017)
		FAD2	Knockout	*A. tumefaciens* mediated transformation	Quality improvement	Oleic acid ↑	Okuzaki et al. (2018)
		FAE1	Knockout	*A. tumefaciens* mediated vacuum infiltration transformation	Quality improvement	Very long-chain fatty acids ↓ (60%)	Ozseyhan et al. (2018)
3.	Cucumber (*Cucumis sativus* L.) Cucurbitaceae	*eIF4E*	Knockout	*Agrobacterium*-mediated cotyledon transformation	Virus resistance	Resistance against *Cucumber vein yellowing virus*, *Zucchini yellow mosaic virus*, *Papaya ring spot mosaic virus-W*	Chandrasekaran et al. (2016)
4.	Eggplant (*Solanum melongena* L.) Solanaceae	*SmelPPO1-10*	Knockout	*A. tumefaciens* strain LBA4404 mediated transformation	Enzymatic browning	Browning ↓	Maioli et al. (2020)
5.	Kale (*Brassica oleracea* var. *alboglabra*) Brassicaceae	*BoaCRTISO*	Knocked down	*A. tumefaciens* strain GV3101 mediated transformation	Color	Carotenoid and chlorophyll biosynthesis ↓	B. Sun et al. (2020)
6.	Rapeseed *(Brassica napus* L.*)* Brassicaceae	*SFAR*	Knockout	*A. tumefaciens* mediated hypocotyl transformation	Oil degradation	Seed oil content ↑	Karunarathna et al. (2020)
7.	Tomato *(Solanum lycopersicum* L.) Solanaceae	*ANT1*	Knockout	*A. tumefaciens* mediated transformation	Color	Anthocyanin ↑	Čermák et al. (2015)
		RIN	Knockout	*A. tumefaciens* mediated method	Fruit ripening	Red color pigmentation in mutant ↓	Ito et al. (2015)
		DMR6	Knockout	*Agrobacterium tumefaciens* strain GV3101 mediated cotyledon transformation	Disease resistance	Resistance against *Pseudomonas syringae* pv. *tomato* and *Phytophthora capsica*, *Xanthomonas* spp.	Paula de Toledo Thomazella et al. (2016)
		PSY1	–	*A. tumefaciens* strain GV3101 using cotyledon transformation	Color	–	Hayut et al. (2017)

(Continues)

TABLE 1 (Continued)

S. No	Vegetable crops	Targeted gene(s)	Mutation	Mode of delivery	Trait modified	Key observations	References
		SlDML2	Knockout	*A. tumefaciens* mediated transformation	Fruit ripening	–	Lang et al. (2017)
		Mlo1	Knockout	*A. tumefaciens* mediated cotyledon transformation resistance	Disease resistance	Resistance against *Oidium neolycopersici*	Nekrasov et al. (2017)
		GAD	Knockout	*A. tumefaciens* mediated transformation	Quality improvement	GABA ↑	Nonaka et al. (2017)
		IAA9	Knockout	*A. tumefaciens* mediated leaf disk method	Parthenocarpy	Leaf shape changed	Ueta et al. (2017)
		Sl-ALMT9	Knockout	*A. tumefaciens* strain C58 mediated electroporation method	Quality improvement	Malate ↑	Ye et al. (2017)
		SlMYB12	Knockout	*A. tumefaciens* mediated transformation	Color	Naringenin ↓, chalcone ↓, *CHS1* ↓, *CHS2* ↓	Deng et al. (2018)
		G3P, *DXS*, *GGPPS*, *PDS*, *ZISO*	Knockout	*A. tumefaciens* mediated transformation method	Phenotype	Lycopene content ↑	Li, Wang, et al. (2018)
		MAPK3	Knockout	*A. tumefaciens* mediated cotyledon transformation	Disease resistance	Resistance to *Botrytis cinerea*	Zhang et al. (2018)
		lncRNA1459	Knockout	*A. tumefaciens* mediated transformation	Fruit ripening	Ethylene ↓, lycopene↓	R. Li, Fu, et al. (2018)
		CCD8	Knockout	*A. tumefaciens* strain EHA105 mediated transformation	Herbicide resistance	Resistance against *Phelipanche aegyptiaca*	Bari et al. (2019)
		JAZ2	Knockout	*A. tumefaciens* mediated transformation	Disease resistance	Resistance against *Pseudomonas syringae* pv. *tomato* DC3000	Ortigosa et al. (2019)
		MAX1	Knockout	*A. tumefaciens* strain EHA105 mediated transformation	Disease resistance	Resistance against *Phelipanche aegyptiaca*	Bari et al. (2021)
8.	Potato (*Solanum tubersoum* L.) Solanaceae	*StALS1*	Knockout	*A. tumefaciens* mediated transformation	Herbicide resistance	–	Butler et al. (2015)
		St16DOX	Knockout	*A. rhizogenes* strain ATCC15834 mediated transformation	–	22,26-dihydroxycholesterol ↑	Nakayasu et al. (2018)
		StPPO2	Knockout	–	Enzymatic browning	PPO ↓	González et al. (2020)

TABLE 1 (Continued)

S. No	Vegetable crops	Targeted gene(s)	Mutation	Mode of delivery	Trait modified	Key observations	References
9.	Pumpkin (*Cucurbita moschata* Duchesne) Cucurbitaceae	*RBOHD*	Knockout	*A. rhizogenes* mediated transformation	Salt tolerance	H_2O_2 ↓, K^+ ↓	Huang et al. (2019)
10.	Sweet potato (*Ipomoea batatas* (L.) Lam.) Convolvulaceae	*GBSSI*, *SBEII*	Knockout	*A. tumefaciens* strain LB4404 mediated transformation	Quality improvement	Amylose ↓, amylopectin ↓	H. Wang et al. (2019)

Abbreviations: CHS, chitin synthase 1; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9; GABA, γ-aminobutyric acid; PEG, polyethylene glycol; PPO, polyphenol oxidase; ↑, increase; ↓, decrease.

using CRISPR/Cas9 genome editing dramatically delayed tomato fruit ripening. Moreover, loss of function of *SlORRM4* significantly alters mitochondrial functions (Yang et al., 2017). CRISPR/Cas9-mediated loss-of-function of tomato DNA demethylase gene *SlDML2* resulted in increased DNA methylation. In addition to ripening-induced genes, ripening-supressed genes were also found, suggesting that DNA demethylation plays an important role in tomato ripening (Lang et al., 2017). Especially in tomato, one of the regulators of ethylene biosynthesis is the protein *RIN (RIPENING INHIBITOR)*. Ito et al. (2015) studied the insertion or deletion of a single base in *RIN* induced by the CRISPR/Cas9-system and showed the production of incomplete-ripening fruits with lower red color pigmentation than that of wild type (Ito et al., 2015). Another study reported that editing *lncRNA1459* by the CRISPR/Cas9-system significantly suppressed ethylene production and lycopene accumulation (R. Li, Fu, et al., 2018). These results suggest that CRISPR/Cas9 can effectively modify genes to delay maturation, highlighting a future research gap.

2.5 | Parthenocarpy

Parthenocarpy describes fruit development without pollination and fertilization. In general, parthenocarpy in fruits and vegetables is characterized by the absence of seeds or a small number of seeds, which is preferred by consumers. Parthenocarpic fruits and vegetables are more resistant to a number of environmental stresses and have better fruit set, quality, and yield (Acciarri et al., 2011; Pandolfini et al., 2002). To develop parthenocarpic plants, the latest genome editing techniques CRISPR/Cas9 can be used as a breeding tool (Rao et al., 2018). Using the CRISPR/Cas9 system, Ueta et al. (2017) knocked out the SlIAA9 (indole-3-acetic acid inducible 9) gene that controls parthenocarpy. The mutant tomato plants were able to produce seedless fruits (Ueta et al., 2017). In another study, the CRISPR/Cas9 editing system was involved in knockout *SlAGAMOUS-LIKE6 (SlAGL6), which* is capable of producing parthenocarpic tomato plants under high-temperature stress. The results of mutagenesis did not change the weight, fruit shape, or vitality of pollen, which makes *SlAGL6* an attractive gene (Klap et al., 2017).

2.6 | Quality improvement

In recent years, consumers have paid much more attention to the quality of vegetables because of their health benefits. Taste, fruit size, color, and the presence of nutrient-rich and health-promoting compounds play a large role in determining the quality of a vegetable. One method of improving the quality of vegetables is organic farming. However, it has been proven that organic vegetables yield less (Patel et al., 2015; Rembialkowska, 2003). The process of improving the quality of vegetables, followed by a prolonged shelf life of the vegetables, can also be achieved by treating different approaches together (Toivonen, 2009). The use of biotechnological approaches such as double haploids and marker-assisted selection in

the breeding program can successfully improve quality with shorter generation cycles (Ghuge & Mirza, 2021). Unfortunately, these techniques have become less useful because they produce high levels of homozygosity and false-negative errors. Vegetables provide essential nutrients for humans. Genes involved in the synthesis of amino acids, fatty acids, carbohydrates, vitamins, or carotenoids can be successfully edited by CRISPR/Cas9 genome editing. Total starch and seed oil content of sweet potato and rapeseed were successfully increased by CRISPR/Cas9 technology with *IbGBSSI* (granule-bound starch synthase I) or *IbSBEII* (*starch-branching enzyme II*) (H. Wang et al., 2019) and *BnSFAR4* (*seed fatty acid reducer 4*) and *BnSFAR5* (*seed fatty acid reducer 5*) (Karunarathna et al., 2020) as target sites, respectively. Malate and ɣ-aminobutyric acid (GABA) have several benefits for human health. CRISPR/Cas9 manipulation of *SlALMT9* (*Al-activated malate transporter 9)* resulted in malate accumulation in tomatoes (Ye et al., 2017). Nonproteinogenic amino acid GABA is elevated in tomato leaves or fruit (Nonaka et al., 2017). There have been many attempts to improve oil quality by targeting genes in *Brassica napus* L. and *Camelina sativa* (L.) Crantz is involved in fatty acid metabolism (Jiang et al., 2017; Morineau et al., 2017; Okuzaki et al., 2018; Ozseyhan et al., 2018). X. Li, Wang, et al. (2018) designed a bidirectional strategy to promote lycopene accumulation while inhibiting the conversion from lycopene to β- and α-carotene using targeted site-specific CRISPR/Cas9 genome editing techniques (Li et al., 2018). Loss of function of the carotenoid isomerase gene of Chinese kale (*BoaCRTISO*) via using CRISPR/Cas9 resulted in accumulation of lycopene and a change in color from green to yellow (B. Sun et al., 2020). Recently, CRISPR/Cas9 modification was established in carrot cells and proved to be a promising application. Klimek-Chodacka et al. (2018) showed that a mutation of the anthocyanin biosynthesis gene *F3H* resulted in reduced anthocyanin accumulation and callus discoloration in carrots (Klimek-Chodacka et al., 2018).

Commercial varieties differ from their wild counterparts in size, texture, and fruit color. Chinese and Japanese consumers prefer pink tomatoes, while white, and red tomatoes are more popular in Europe and the United States. The pink phenotype in tomatoes is caused by a deficiency of yellow-colored flavonoids, namely naringenin chalcone. The development of the phenotype is controlled by SlMYB12, a transcription factor associated with R2R3-MYB that encodes the monogenic recessive yellow (y) locus. Silencing of the *SlMYB12 gene (MYB transcription factor 12)* suppresses naringenin chalcone biosynthesis by CRISPR/Cas9 modification. It was also found that the editing efficiency in this experiment was high (90.9%). Furthermore, the study suggests that CRISPR/Cas9 manipulation requires less time (within 1 year) compared with traditional breeding (Ballester et al., 2010; Deng et al., 2018). Purple and yellow tomatoes were successfully cultivated using CRISPR/Cas9 technology targeting the anthocyanin mutant gene 1 (*ANT1*) (Čermák et al., 2015) and the phytoene synthase gene 1 (*PSY1*) (Hayut et al., 2017), respectively.

Another way to develop high-quality fruits and vegetables is to reduce unwanted compounds. Potato contains two steroidal glycoalkaloids (α-solanine and α-chaconine) that are bitter and toxic to various organisms. Silencing the *St16DOX* gene using CRISPR/Cas9 completely prevents the accumulation of these molecules in potato tubers (Nakayasu et al., 2018). Polyphenol oxidase (*PPO*) genes were deleted in potato and eggplant using the CRISPR/Cas9 method. The PPO enzyme causes browning by mediating the oxidation of polyphenols. After the induction of mutations in potato, a significant decrease in PPO activity (69% in tuber) and browning (73%) was observed (González et al., 2020; Maioli et al., 2020).

3 | CHALLENGES OF CRISPR/CAS9 GENOME EDITING IN VEGETABLE CROPS

The ability of CRISPR/Cas9 to edit the genome of plants still poses many challenges, despite its extensive applications in plant breeding (Figure 3).

3.1 | Off-target effects

Occasionally, genome editing gets out of control by triggering off-target effects through CRISPR that limit the beneficial effects of this technique. We have already pointed out many studies in which very small or minimal off-target effects were found, but it is still impossible to avoid all off-target mutations. However, editing with the CRISPR tool to improve desirable traits has off-target effects that we cannot ignore. Off-target effects include unwanted mutations of genes such as insertions, deletions, translocations, and inversions. The main strategy to reduce off-target risk is to improve the sgRNA target to reduce mismatches (Doench et al., 2016) and limit Cas9 levels in cells to minimize off-target binding (Shen et al., 2019).

3.2 | Cytotoxicity of Cas9

Genome editing is often performed with the endonuclease enzyme SpCas9 in bacterial genomes, and expression of the Cas9 enzyme sometimes triggers toxicity. Editing by the CRISPR/Cas9 system is toxic to a variety of organisms, leading to chromosome breaks and eventual gene editing failure (Zhao et al., 2020). The toxicity of Cas9 can be reduced by either replacing strong promoters with weak ones, or by using alternative nucleases (SpCas9-HF1, eSpCas9, and HypaCas9) to lower endogenous Cas9 levels (Standage-Beier et al., 2015). The toxicity of Cas9 can also be reduced by using base editors to reduce DNA DSBs and thus cytotoxicity (Chen et al., 2018; Kleinstiver et al., 2016). So far, no reports on the toxicity of Cas9 in plants have been found.

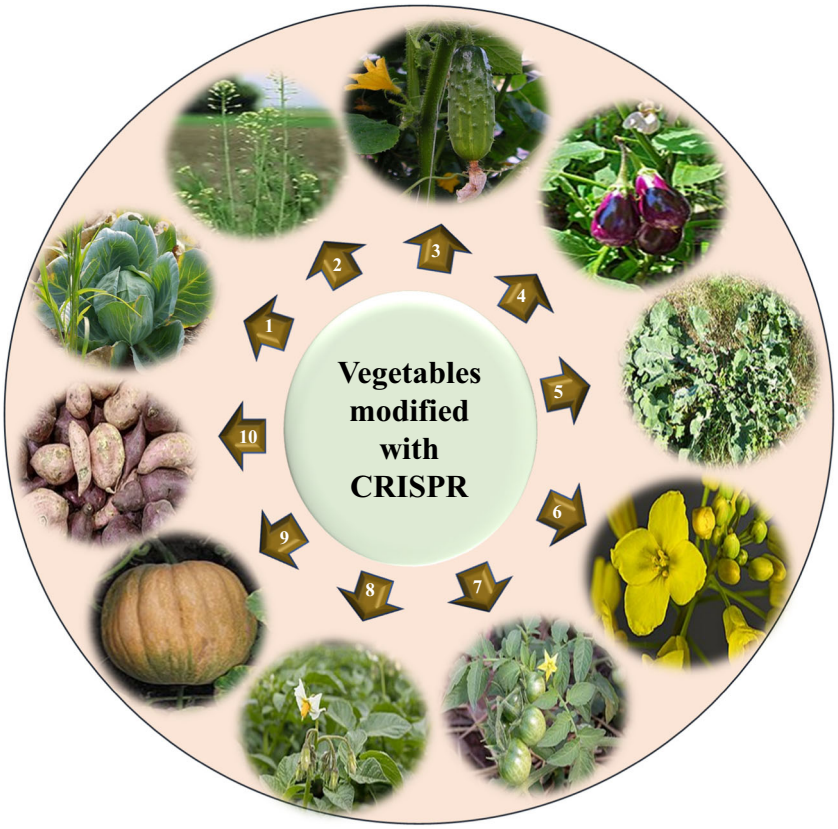

FIGURE 3 Diagram showing the list of vegetables genome editing with CRISPR/Cas; (1) Cabbage (source: Wikimedia commons; Creative Commons Attribution-Share Alike 2.05); (2) Camelina sativa (source: Wikimedia commons; Creative Commons Attribution-Share Alike 3.0); (3) Cucumber (source: Wikimedia commons; Creative Commons Attribution-Share Alike 3.0); (4) Eggplant (source: Wikimedia commons; Creative Commons Attribution-Share Alike 3.0); (5) Kale (source: Wikimedia commons; Creative Commons Attribution-Share Alike 3.0); (6) Rapeseed (source: Wikimedia commons; Creative Commons Attribution-Share Alike 4.0); (7) Tomato (source: Wikimedia commons; Creative Commons Attribution-Share Alike 2.0); (8) Potato (source: Wikimedia commons; Creative Commons Attribution-Share Alike 4.0); (9) Pumpkin (source: Wikimedia commons; Creative Commons Attribution-Share Alike 4.0); (10) Sweet potato (source: Wikimedia commons; Creative Commons Attribution-Share Alike 4.0). CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9; HDR, homology-directed repair.

3.3 | Introducing HDR induction instead of NHEJ

NHEJ causes indel mutations after repair of DSBs in most plant genome editing via CRISPR/Cas9. When this type of mutation occurs, gene knockout occurs. NHEJ has its drawbacks. HDR, on the other hand, repairs a single strand of DNA by recombination with a repair template. The HDR method allows more precise DNA repair than NHEJ and it allows integration of the entire DNA. Many attempts have been made to initiate HDR repair. One idea is to add NHEJ inhibitors (Scr7, resveratrol, and L755507) or inducers to HDR repair to speed up the process (Aird et al., 2018; Chu et al., 2015).

3.4 | Delivery method

Inserting CRISPR/Cas9 components into solid plant cells is difficult. To modify the genome of a plant, the following methods are usually used: Agrobacterium-mediated transformation, floral-dip-mediated transfer, PEG-mediated protoplast transformation, and particle bombardment. The main disadvantage of Agrobacterium-mediated transfer is that it requires binary vectors, while floral-dip transfer is effective only in plants that produce a sufficient number of flowers and seeds. Transformation by particle bombardment is more costly and less efficient (Baltes et al., 2017). These problems can be solved by using pollen magnetofection and nanoparticle-mediated delivery (Sandhya et al., 2020). The most commonly used method for delivering CRISPR/Cas9 components is *Agrobacterium*-mediated transformation. It requires cloning of an appropriate fragment to be edited in CRISPR vectors such as pRGEB31, pRGEB32, and so forth, and subsequent insertion into *Agrobacterium*. Callus/plants are later transformed with these bacteria containing the CRISPR components. Floral dip is quite simple and user-friendly compared to transformation of explants by tissue culture. It is commonly used in *Arabidopsis thaliana*. The inflorescences of the plants to be transformed are dipped in buffer containing transformed *Agrobacterium* cells. Seeds developing from these plants are later grown on selection media to select transformants. Further validation is done by polymerase chain reaction, and the transgenic lines are propagated over several generations until homozygous lines are achieved. Other transformation methods such as particle bombardment and PEG-mediated protoplast transformation are also commonly used. However, their use varies from plant to plant because different plants respond differently to the different transformation methods.

3.5 | Phenotype to genotype linking

In CRISPR/Cas9 editing, it is difficult to precisely identify the edits, so their effectiveness and efficiency are limited. The multifunctional CRISPR/Cas9 systems have yet to be implemented at the whole genome level. The CRISPR/Cas9 system is limited to high-throughput genomics and phenotypic editing. High-throughput biosensors are capable of assessing both genotypes and desired phenotypes simultaneously. A genome-wide CRISPR system (MAGIC) has been

developed to examine whole-genome expression levels and link genotypes to desired phenotypes (Lian et al., 2019; Tong et al., 2019).

3.6 | Genome instability

DSBs can lead to cell death if not properly repaired. They lead to genome instability (chromosomal rearrangements or aneuploidy), which limits the use of CRISPR/Cas9. Alternative strategies, CRISPR/Base Editing SysTem (CRISPR-BEST) and CRISPRi, are highly efficient DSB-free base editors that introduce mutations within the coding region (Eid et al., 2018).

4 | REGULATORY APPROACHES FOR GENE EDITING

Since the mid-1990s, when the first genetically modified (GM) crops appeared in the market, there has been a public and political debate about them. As a result of this debate, several regulations have been enacted to ensure the safety of genetically modified organisms (GMOs) and to provide a high level of protection for humans, the environment, and animals. Generally, GM crops are controlled at the national or regional level, resulting in a fragmented regulatory system worldwide. In addition, the United States has adopted the concept of significant equivalence, which states that GM commodities that are identical to those available in commerce should be considered conventional. In contrast, the European Union GMO law includes the precautionary approach, which was adopted as a guideline in Directive 2001/18/EC. According to the revision of the Federal Law No. 358- FZ -2016, Russia prohibits the cultivation of GM plants, but not the import of approved GM food and feed. In addition, Canadian regulations distinguish between products based on evaluated innovative traits rather than technical processes. In India, regulations are made on a case-by-case basis, while in Japan, only products that do not contain inserted DNA or RNA are considered GMOs. Finally, cultivation of GM crops is banned in Australia, New Zealand, Venezuela, Ecuador, and Peru, although Brazil, Argentina, and China are among the top five GM agricultural countries.

After more than 20 years of consuming GM food, no negative health or environmental effects have been found so far. Currently, scientists agree that a different regulatory system should be applied that focuses on the risk assessment of the trait/product rather than the technology used to produce it (Hartung & Schiemann, 2014; Heap, 2013; Morris & Spillane, 2008, Podevin et al., 2012). With the advancement of the New Breeding Technologies (NBT) platform in recent years, the controversy surrounding GMOs has been reignited. There are major concerns about the regulation of these new approaches (Hartung & Schiemann, 2014). In particular, genome editing strategies should overcome challenges such as public acceptance of the technology and government regulatory requirements (Hua et al., 2019; Khatodia et al., 2016), and some progress has been made in this regard. For example, GM crops without foreign DNA were not classified as GMOs by the USDA in 2016 and were allowed to be sold as DuPont Wx1 corn, which was created to shift the starch metabolism pathway toward amylopectin production (Globus & Qimron, 2018). According to the European Academies of Agriculture, Food and Natural Sciences (Union Européenne des Académies d'Agriculture [UEAA], Paris, France), the GMO Directive adopted by the European Union in 2001 is inadequate because it was drafted before the discovery of NBTs. Therefore, a new regulatory framework is needed to accommodate the use of these technologies for precision editing of plant genomes. Genome-edited plants may still fall under the 2001/18/EC law, even though the European Court of Justice (ECJ) has ruled that plants developed using genome-editing technology are covered by this law.

5 | CONCLUSIONS

CRISPR/Cas9 is now widely used and is a promising tool for precision plant breeding. However, there are also some limitations, but we are optimistic that these will be addressed in the near future. CRISPR/Cas9 has already demonstrated its potential in modifying important vegetable crops. The GABA-overproducing tomato developed using the CRISPR approach has already been approved for consumption (Waltz, 2022). It is speculated that the use of genome-edited crops will accelerate in the near future as demand for food increases exponentially. India and China, the world's most populous countries, have already exempted CRISPR-edited crops from biosafety regulations. Ultimately, combining gene editing with other breeding strategies will produce tastier and more nutritious vegetables that will ultimately improve our quality of life and extend our lives. To fully implement CRISPR/Cas9, we must continue to develop it. This includes increasing accuracy, ensuring that there are no off-target effects, flexibility, ensuring that the system is free of donor DNA and PAM, compatibility, ensuring that the transfer is not specific to one species or cell type, and liability, where modifications must be traceable. Ultimately, the biggest challenge and concern is: Will people buy and consume GM vegetables?

AUTHOR CONTRIBUTIONS

Tuyelee Das and Uttpal Anand: review structure, contributed to study ideas, wrote the first draft of the manuscript, and prepared the table and figures. **Tarun Pal and Sayanti Mandal**: participated in review structure, wrote the manuscript, and arranged the references. **Manoj Kumar, Radha, and Abilash Valsala Gopalakrishnan**: writing-review & editing, data validation, response, suggestions, overall revision. **José M. Pérez de la Lastra**: conceptualization, revised the manuscript, formal analysis, supervision, project administration and funding acquisition. **Abhijit Dey**: conceptualization, review structure, revised the manuscript, formal analysis, suggestions, supervision, and final draft. All authors have read and approved the final version of the manuscript for submission to this journal. All authors listed have contributed to the concept, literature mining, writing and methodology of the review, provided critical feedback and revised the

manuscript critically. All authors contributed to the writing or revision of the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

This is a review article. Therefore, all data are taken from the cited sources or indicated in the manuscript at the appropriate place. The data supporting this study are available in the cited sources. Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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