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Chapter

Enhancement of Agricultural Crops: A CRISPR/Cas9-Based Approach

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Abstract

Horticultural crops are indispensable agricultural food materials with all essential nutrients. Though, severe threats like pests, diseases, and adverse abiotic factors will affect their productivity and quality. This permits to promote sustainable agriculture by utilizing the recent biotechnological approach to tackle the mentioned issues. In recent year's genome editing technologies has become one of the most executed genetic tools which altered plant molecular biology. Recently, CRISPR-Cas utilizes for its high target specificity, easier design, and higher success rate. This chapter deals with recent advances in CRISPR/Cas9 technology in horticultural crops in response to the enrichment of essential metabolites, which was achieved by introducing the viral genome to the host via CRISPR-mediated targeted mutation. Furthermore, the strategies based on CRISPR/Cas9 targeted modifications of genes in crop species such as rice, wheat, and soy will be discussed. Finally, we discuss the challenges, improvements, and prospective applications of this cutting-edge technology.

Keywords: CRISPR/Cas9, horticultural crops, genome editing, cereals, pulses

1. Introduction

Vegetable growing is a fundamental component of the economic system, which efforts to produce horticultural crops such as vegetables, fruits and spices, tubers, and medicinal plants. A substantial part of food and nutritional security is provided by these crops [1]. As the world's population grows, we need to increase agricultural output to maintain a sustainable food supply. The development of next-generation crops plays a significant role since conventional breeding techniques have been extensively used and time-consuming [2, 3]. Transgenesis is the possible alternate plant breeding approach where public acceptance is a significant concern in terms of commercialization. Recently, recombinant DNA technology using nucleases such as ZFNs [4], TALENs [5, 6], and CRISPR/Cas9 [7] has proven to be a viable technique to modify the targeted location in the genome and is widely utilized in many agricultural crops. The technology is expected to be widely used shortly.

2. Genome editing

It is a collection of sophisticated molecular biology techniques that enable the accurate, efficient, and targeted modification of particular nucleotide sequences [8–10]. Researchers employ this technology to better understand the genome's function and develop crops resistant to insects, have higher nutritional value, and thrive in dry regions. The application of genome editing methods based on site-specific nucleases (SSNs) has proven extensive gene editing across flora and fauna species during the last generation. SSNs work by assembling endonucleases capable of cleaving DNA inside a specific region in the genome. The active domain of SSN is connected to it via a DNA-binding region or an RNA sequence [11–13]. These SSNs are responsible for causing double-stranded breaks (DSBs) in the target DNA. Nonhomologous end joining (NHEJ) or homology-directed recombination (HDR) methods are used to repair DSBs, which result in insertion/deletion (INDELS) and replacement mutations in the host locus [14, 15]. Following SSN breakage of the target sequence, cellular DNA repair processes result in gene expression changes at the target sites.

1. Engineered meganuclease (MegaN),

2. Zinc finger nucleases (ZFNs),

3. Transcription activator-like effector nucleases (TALENs),

4. Clustered regularly interspaced short palindromic repeat/CRISPR/Cas9nuclease systems are the four kinds of engineered nucleases being employed in genome editing [16, 17]. All of these approaches made it possible to alter the genome in a straightforward, fast, and cost-effective way.

2.1 Engineered meganuclease (MegaN)

Meganucleases (MegaN) are endonucleases found in nature and were discovered most often in the late 1980s. Endonucleases are capable of detecting and cleaving large nucleotide sequences (ranging from 12 to 40 base pairs), which are considerably different across many genomes [13, 18]. I-SceI, a yeast mitochondrial enzyme, and I-CreI, an algal photosynthetic enzyme, are both good meganucleases. Meganucleases have been modified to recognize previous target sequences even though meganuclease receptors are still infrequent in relevant genomes. Because of the slightly longer template strand, there is more discrimination and much more minor off-target trimming. On the other hand, engineered meganucleases had a much lower utilization than some other sequential nucleases, in addition to the issue of changing meganucleases to accept novel specificities [19, 20].

2.2 Zinc finger nuclease-based engineering

Artificial sequential nucleases known as zinc finger nucleases (ZFNs) have transformed the area of programmable nucleases. ZFNs were created by attaching numerous zinc finger DNA-binding domains to the restriction endonuclease FokI's nonspecific cutting pattern [21]. The protein molecules can enlighten the difference between two DNA sequences separated by only a few nucleotides. This allows the two endonucleases to form a dimer, breaking double-stranded DNA [22]. Furthermore, because each motif in the zinc finger array reads a distinct

3-nucleotide complementary strand, the domain composition variable sequence can always be chosen to fit the particular destination. ZFNs were originally used for sequence-specific mutagenesis in tobacco in the early 2000s, which was most likely the first time a designed endonuclease identified and fractured chromosomal DNA [23, 24]. With these remarkable achievements, ZFN usage in agriculture has indeed been limited due to factors such as the technical complexity of manufacturing and the scarcity of aiming places in genomes in comparison to more recently established functional genomics approaches.

2.3 Transcription activator-like effector nucleases (TALENs)

The TALEN (transcription activator-like effector nucleases) technology was developed in 2011 to optimize the effectiveness, reliability, and availability of genome editing. Transcription activator-like effectors (TALES) were discovered [25, 26]. The TALEN, like ZFNs, creates proteins artificially with a flexible array of DNA-binding regions joined to FokI's nonspecific fragmentation site. Each repeat consists of 33–35 amino acids and identifies just one nucleotide. The last repetition is considered as a "half-repeat" since it frequently contains 20 amino acids. The varied amino acids at positions 12 and 13 provide DNA identifying distinctiveness (for example, NI accepts adenine, HD detects cytosine, NG detects thymine, and NN recognizes both guanine and adenine) [27, 28]. TAL effectors have natural segmentation grace to facilitate genome editing in TALENs, where these repetitions are organized to find individual regions of expression. Additional TALENs and gene-specific stimulators and regulatory proteins were employed as gene targeting reagents in conjunction with TAL effector assemblies [29–31]. TALENs are more adaptable compared to meganucleases and ZFNs and are used extensively in plant genome editing. However, a large number of experiments renders TALEN production as well as transport throughout plant tissue problematic.

2.4 Clustered regularly interspaced short palindromic repeat/CRISPR/Cas9 nuclease system

The CRISPR-Cas system, like TALENS, draws its inspiration from biology. The CRISPR-Cas nucleases had first been found in the adaptive immune system function of archaea and bacteria. CRISPR codes for "spacer" RNA molecules that create associations between CRISPR-associated (Cas) nucleases and instruct them to break down external nucleic acids. The spacer segments within those biological systems were obtained from bacteriophage components caused by a bacterium the prokaryote progenitor. The system's aiming specialization is based upon basic genetic platform principles. A lateral or segmental short sequence-specific element is necessary for a target sequence site to be accepted and bisected; the above offers assurance that the prokaryote utilizing the CRISPR/Cas system will not focus its genome; these representations are just not available among exogenously linked patterns. The *Streptococcus pyogenes* CRISPR-Cas9 system (CRISPR-*Sp*Cas9) was the first to be discovered for genetic manipulation, and also the term "CRISPR-Cas9" is generally used to describe this technology [32–34]. To avoid misunderstanding, CRISPR-Cas9 can only be used to relate to properties that CRISPR-SpCas9 and its orthologs share. The designed CRISPR-Cas9 system is made up of two parts—(1) the Cas9 nuclease and (2) a single guide RNA (sgRNA), which is made up of two RNA molecules—the spacer-containing CRISPR RNA (crRNA), and they serve to enhance crRNA (tracrRNA), which itself is needed again for retired moiety's growth and development. The sgRNA

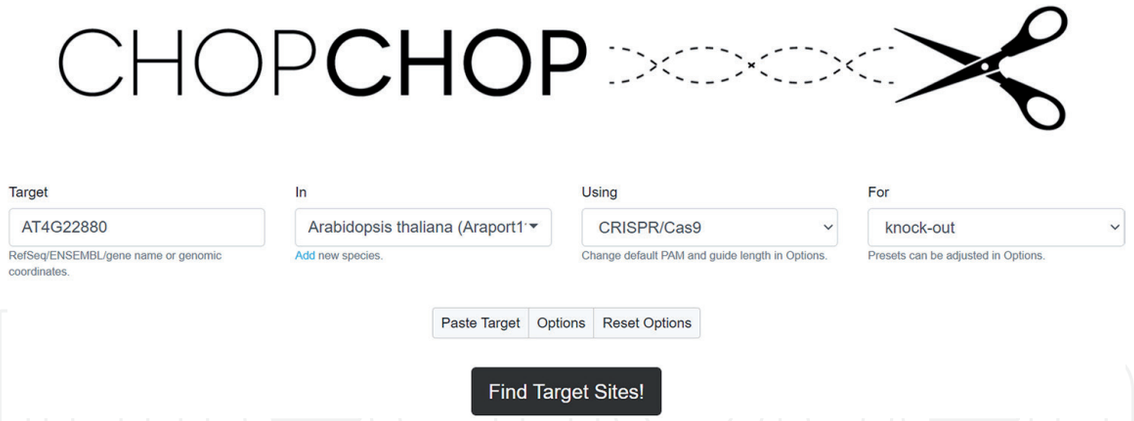
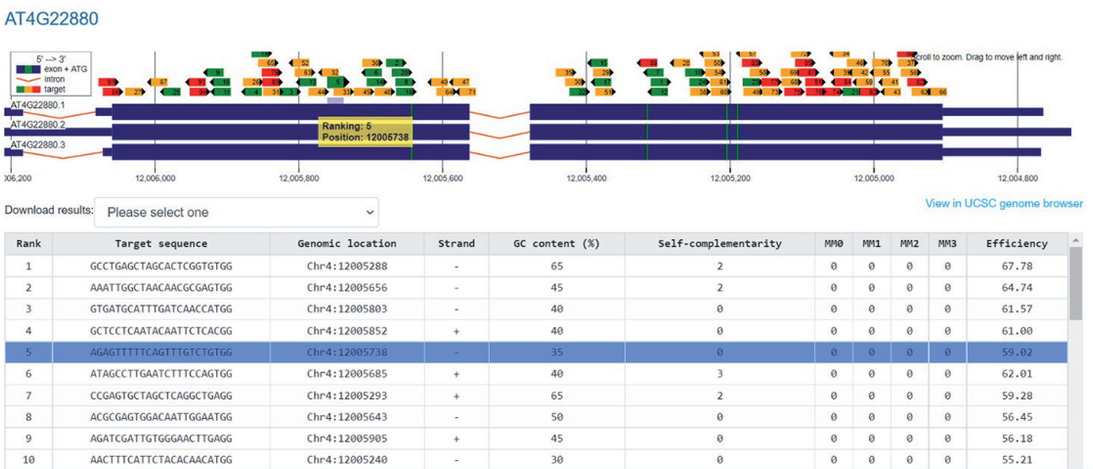
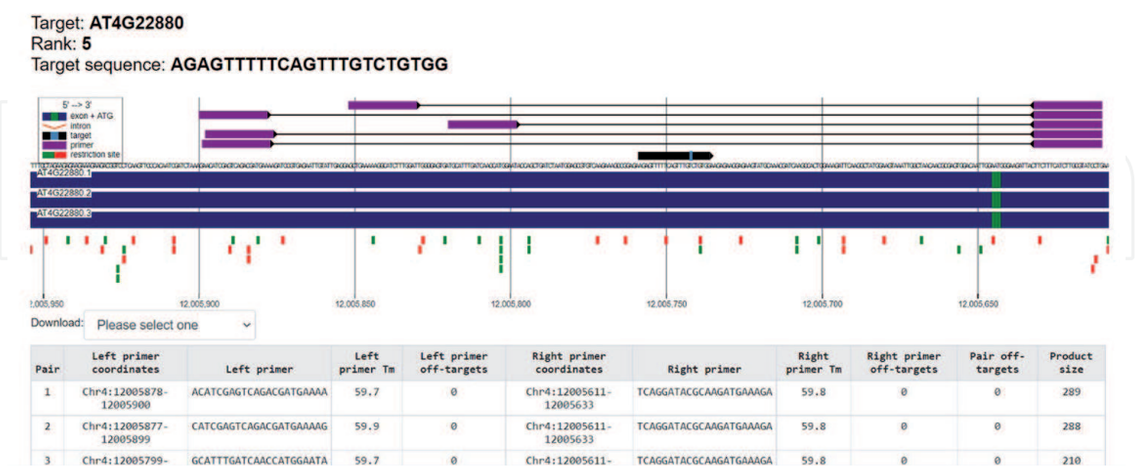


Figure 1.
Home page of CHOPCHOP bioinformatics tool for designing of sgRNA.



Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	GCCTGAGCTAGCACTCGGTGTGG	Chr4:12005288	-	65	2	0	0	0	0	67.78
2	AAATTGGCTAACAACGCGAGTGG	Chr4:12005656	-	45	2	0	0	0	0	64.74
3	GTGATGCATTTGATCAACCATGG	Chr4:12005803	-	40	0	0	0	0	0	61.57
4	GCTCCTCAATACAATTCTCACGG	Chr4:12005852	+	40	0	0	0	0	0	61.00
5	AGAGTTTTTCAGTTTGTCTGTGG	Chr4:12005738	-	35	0	0	0	0	0	59.02
6	ATAGCCTTGAATCTTTCCAGTGG	Chr4:12005685	+	40	3	0	0	0	0	62.01
7	CCGAGTGCTAGCTCAGGCTGAGG	Chr4:12005293	+	65	2	0	0	0	0	59.28
8	ACGCGAGTGGACAATTGGAATGG	Chr4:12005643	-	50	0	0	0	0	0	56.45
9	AGATCGATTGTGGGAACTTGAGG	Chr4:12005905	+	45	0	0	0	0	0	56.18
10	AACTTTCATTCTACACAACATGG	Chr4:12005240	-	30	0	0	0	0	0	55.21

Figure 2.
Preliminary stage for the identification of off target regions of our desired genomic sequence.



Pair	Left primer coordinates	Left primer	Left primer Tm	Left primer off-targets	Right primer coordinates	Right primer	Right primer Tm	Right primer off-targets	Pair off-targets	Product size
1	Chr4:12005878-12005900	ACATCGAGTCAGACGATGAAAA	59.7	0	Chr4:12005611-12005633	TCAGGATACGCAAGATGAAAGA	59.8	0	0	289
2	Chr4:12005877-12005899	CATCGAGTCAGACGATGAAAAG	59.9	0	Chr4:12005611-12005633	TCAGGATACGCAAGATGAAAGA	59.8	0	0	288
3	Chr4:12005799-	GCATTTGATCAACCATGGAATA	59.7	0	Chr4:12005611-	TCAGGATACGCAAGATGAAAGA	59.8	0	0	210

Figure 3.
Intermediate stage for identification of off target regions of our desired genomic sequence. In this view, we can identify the %GC, levels of off targets and primer sequences.

leads the nuclease combination to a specific DNA location, causing the identical nucleotide sequence to be cleaved [20, 21]. Cas9 has a composite morphology, having dual nuclease domains, RuvC and HNH [14, 20, 35]. The CRISPRCas9 system has gone through several modifications in the area of plant genome engineering methods [36], including simplicity of design, cloning, and transport into



Off-targets		
Location	Number of mismatches	Sequence (including mismatches)
There are no off-targets.		

Figure 4.
Final stage of off target analysis or our desired genomic sequence.

plant cells, which also has resulted in a significant success rate in genome editing technology. The design of guide RNAs using the CHOPCHOP software tool is summarized in **Figures 1–4**.

3. CRISPR/Cas9 exotic variants and challenges

CRISPR/Cas9 genome editing utilizing the SpCas9 enzyme from *Streptococcus pyogenes* continuously transforming the area of genome editing by offering very accurate, simple, and highly efficient gene alterations by creating nicks on the double-stranded genome of the targeted organism. Since 2013 scientists have extensively used and still exploring its vast possibilities in genome editing. Even though the efficiency of Cas9 is still high, there are some setbacks regarding their use in gene editing. One of the limitations of using them in plant biotechnology is the indistinct regulations using CRISPR/Cas9 edited plants. Another drawback is the unavailability of a standardized transformation protocol to deliver the CRISPR/Cas9 construct to some plants. These problems are now solved by the availability of novel exotic variants of genome editing enzymes that have been tested as equally efficient or perhaps more efficient than *Sp*Cas9 [37]. Scientists have identified and characterized many other kinds of microbial communities; CRISPR-RNA-guided adaptive immune systems are found. Two primary classes are distinguished, with five types and 16 subclasses [38]. These enzymes require multisubunit proteins to bind to crRNA and cleave the target genome, which is all found in class 1. "Class 2" consists mostly of two types of effectors—Type II and Type VI. Each of these types of effectors binds and cleaves crRNA and target nucleic acids. Cpf1 [38, 39], C2c1 or C2c3 [40], and C2c2 with two HEPN RNase (higher eukaryotes and prokaryotes nucleotide-binding) domains are used in Class 2 Type V and Class 2 Type VI, respectively. In contrast, Class 2 Type II is characterized by Cas9, RuvC, and HNH nuclease domains, while Class 2 Type V uses a single Ruv [41]. Some of these effectors have experimented with some plant species.

The discovery of RNA-dependent RNase enzyme systems from Class 2 Type II (FnCas9) and Class 2 Type VI (C2c2) cleared the path for novel approaches to genome editing. The bacterium Leptotrichiashahii Class 2 Type II C2c2 is directed by a single crRNA and may be trained to cleave any ssRNA with corresponding protospacers. These effectors, which are composed of two HEPN domains containing catalytic residues, preferentially cleave ssRNAs at varying distances from the crRNA binding site rather than adenine targets. C2c2 binding is controlled by a crRNA secondary structure with at least one 24-nt stem-loop structure and a 22–28-nt complementary sequence to these RNA protospacers. The latter must be flanked at the 3'end by a mononucleotide protospacer-flanking site (PFS) comprised of adenine, uracil, or cysteine [41, 42]. Another RNase-based system was identified in 2013 [43] from microbe *Francisellanovicida* (*Fn*Cas9), which could target bacterial mRNA and lead to altered gene expression and is PAM independent. This enzyme successfully inhibited the hepatitis C virus (HCV) in Huh-7.5 cells by RNA inhibition method. This enzyme targets

both positive and negative strands of the virus, thus paralyzing RNA translation and replication. It was shown that mismatches up to three to six base pairs at 3'or 5′ end were tolerated by *Fn*Cas9 whereas more than six mismatches led to complete loss of activity. This enzyme is also capable of targeting DNA [44]. The above studies suggest the feasibility of developing viral infections resistant crops. The regulatory policies related to the usage of transgenic plants are still going very strong in many countries. To overcome this problem, smaller versions of genome editing enzymes are developed that can be used along with viral vectors to transform plants with desired traits. Virus vectors allow high and transient expression of heterologous genes for editing. This is proved in the case of targeted mutagenesis of *Nicotiana benthamiana* and *Petunia hybrida* using tobacco rattle virus (TRV) [45].

As *Sp*Cas9 is having a larger size (4.2 kb), the tobacco rattle virus cannot be used to express *Sp*Cas9 in plants. To resolve this problem, small genome editing enzymes were identified from different microbes such as *Staphylococcus aureus* (*Sa*Cas9, 3.2 kb), *Streptococcus thermophilus* (*St*1Cas9, 3.4 kb), and *Neisseria meningitidis* (*Nm*Cas9, 3.2 kb). These enzymes belong to the Class 2 Type II immune system and cleave double-stranded DNA using RuvC and HNH domains. Moreover, this group of enzymes cuts DNA at a specific target region, usually 21- to 24-nt long near 5′-NNGRRT-3′ or 5′-NNNRRT-3′ 5′-NNAGAAW-3′and 5′-NNNNGMTT-3′ PAM motifs, respectively. Here, in the PAM sequence, N signifies any nucleotide, R signifies A or G, M signifies A or C, and W signifies A or T [46–49]. In addition, research suggest that while using *Sa*Cas9, a greater rate of mutation (80%) was obtained by targeting the 5′-NNNGGT-3′ PAM sequence and induced homologous recombination in the selected lines. The above enzymes target a much longer PAM sequence for genome editing purposes. As an alternative, a new set of single crRNA-guided DNase enzymes with shorter PAM motifs have been recently identified again from the microbial community. This also belongs to Class 2 Type V CRISPR effectors Cpf1 from *Francisellanovicida* U112 (*Fn*Cpf1), *Acidaminococcus* sp. (*As*Cpf1), and *Lachnospiraceae* bacterium (*Lb*Cpf1) and have been successfully tried in rice and tobacco. *Fn*Cpf1 uses a single short RNA guide molecule, 42- to 44-ntcrRNA, which begins with 19 nt of the direct repeat followed by 23–25 nt of the spacer sequence. *Fn*Cpf1 identifies5′-TTN-3′region, a short T- rich PAM upstream of the 5′end. Further, it cuts the double-stranded DNA in a staggered way after the 18th base on the nontargeted (+) strand and after the 23rd base on the targeted (-) strand [50]. Targeted mutations were observed in *NtPDS* and *NtSTF1* of *N. benthamiana* and *Os*DL, *Os*ALS, *Os*NCED1–3, and *Os*AO1–5 loci of *Oryzasativa* when codon-optimized *Fn*Cpf1 and crRNA were expressed in rice and tobacco. Interestingly, deletions were observed in both the transgenic plants as well as in transformed progenies, and mutation efficiency in rice and tobacco was around 47.2 and 28.2%, respectively [50].

Many new versions of *Sp*Cas9 have been developed with the core objective to enhance their specificity. One of the limitations that have come across was off-targeting, which will cause undesired mutations in the target. The next drawback is that some plant species have larger genomes with many duplicate genes, making genome editing technology less precise. The first *Sp*Cas9 variant that was obtained by mutating one of its domains (HNH or RuvC) was single-stranded DNA cleavage *Sp*Cas9-nickases [35, 51]. SpCas9-nickases are employed in pairs to carry out nonhomologous repair of double-stranded breaks (DSBs) using properly offset (>100 bp long) guide RNAs [47]. This strategy decreases off-target mutagenesis by extending the recognized DNA target area from 23 to 2 9 23 bp while

maintaining an on-target cleavage rate comparable to that of wild-type SpCas9 [47]. In *Arabidopsis*, a single SpCas9 D10A nickase was equally efficient at initiating homologous recombination as a nuclease or homing endonuclease I, SceI, [52]. On the other hand, coupled SpCas9 nickases generated alterations comparable to those induced by SpCas9 nuclease. Furthermore, deletions were detected, not insertions, which occurred at a lower frequency [53]. Slaymaker et al. enhanced the specificity of SpCas9 by decreasing its helicase activity and created an improved form of SpCas9 (eSpCas9) [54]. Wright et al. created a split-SpCas9 system, a binary SpCas9 system, to enhance SpCas9 specificity. This was accomplished by overexpressing the nuclease and a-helical lobes in *Escherichia coli* as distinct polypeptides [55]. Komor et al. used a different approach to enhance the specificity of SpCas9, combining SpCas9-nickase with cytidine deaminase to create SpCas9-CD. While all other SpCas9 variations cause deletions or insertions in the DNA sequence, this variant enables the direct conversion of cytidine to uridine, which has the same base-pairing properties as thymine [56].

Another variant of *Sp*Cas9 is termed dead Cas9 (d*Sp*Cas9), which is developed by mutating both cleavage domains of *Sp*Cas9, and this enzyme is an RNA-guided DNA binding protein without cleavage activity [14]. In addition, it is fused with fluorescent or other types of markers and can be used in several biotechnological applications. This kind of fusion creates catalytically inactive and dead *Sp*Cas9 having the FokI nuclease domain at the N-terminus [57]. Compared to monomeric *Sp*Cas9, homodimer FokI enzymes are more precise in cleaving the target genome and can induce lesser off-targets. Piatek et al. demonstrated the fusion of synthetic transcriptional activators with the C terminus of d*Sp*Cas9 to the EDLL domain or the TAL activation domain. They developed d*Sp*Cas9—EDLL and d*Sp*Cas9—TAD synthetic transcriptional activators. This effector, guide RNA, and target molecules were transformed to *tobacco* through the agroinfiltration method. Though there were no stably transformed lines, the strong transcriptional activity of EDLL and TAD was proved in transgenic plants [58]. Fusion of d*Sp*Cas9 with methylated or demethylated promoters can lead to activation or inactivation of a gene. Some of the examples of this type of fused protein are d*Sp*Cas9-Tet1 and d*Sp*Cas9-Dnmt3a [59]. The deletion and insertion of methylases using CRISPR/Cas9 technology will allow modifications at the genetic level in living organisms [1].

4. Applications

CRISPR/Cas9-based genome editing has contributed a lot in improving various traits of crops for the past years. Still, new challenges are being faced by the scientific community for the enhancement of the quality of various edible plants. There are numerous applications of CRISPR/Cas-based gene editing, namely enhancement of yield, improvement of resistance to pathogens, diseases, resistance to herbicides, and improvement of stress tolerance. In this chapter, details of studies related to the applications mentioned above of CRISPR are given and summarized in **Table 1**. Most of the CRISPR-related work in vegetables is done in tomatoes. The earliest report based on CRISPR-based genome editing was done by editing the *ARGONAUTE7* (*SlAGO7*) gene which is involved in the development of leaves. Moreover, mutated *SlAGO7* caused a change in the morphology of leaves which turned them into wiry leaves [77]. Another interesting work is to edit *SELF PRUNING 5G (Sp5G)* and *SELF PRUNING (Sp)* genes that caused early flowering [78]. Similar editing in developmental genes was also carried out

Name of the gene	Crop	Function	References
LOGL5	Rice	Increased yield	[60]
CKX	Wheat	Increased yield	[61]
Amino acid permease	Rice	Increased yield	[62]
GS3, GW2, *GW2,* and *GW5*	Rice	Increased yield	[63]
GBBS1	Maize	Decreased low amylose	[64]
SWEET	Rice	Increased resistance to bacterial blight	[65, 66]
LOB1	Sweet orange	Resistance to *Xanthomonas citri*	[67]
EDR1	Wheat	Resistance to *Blumeria graminis*	[68]
MLO1	Tomato	Resistance to *Oidiumneo lycopersici*	[69]
ALS	Rice	Herbicide resistance	[70, 71]
ACCase	Rice	Herbicide resistance	[72]
ACCase	Wheat	Herbicide resistance	[73]
OsMPK5	Rice	Increased abiotic and biotic stress	[74]
MAPKs	Tomato	Increased resistance to drought stress	[75]
GmF3H1, *GmF3H2* and *GmFNSII-1*	Soybean	Isoflavone synthesis	[76]

Table 1.
Applications of CRISPR/Cas9 genome editing in various crops for improved traits.

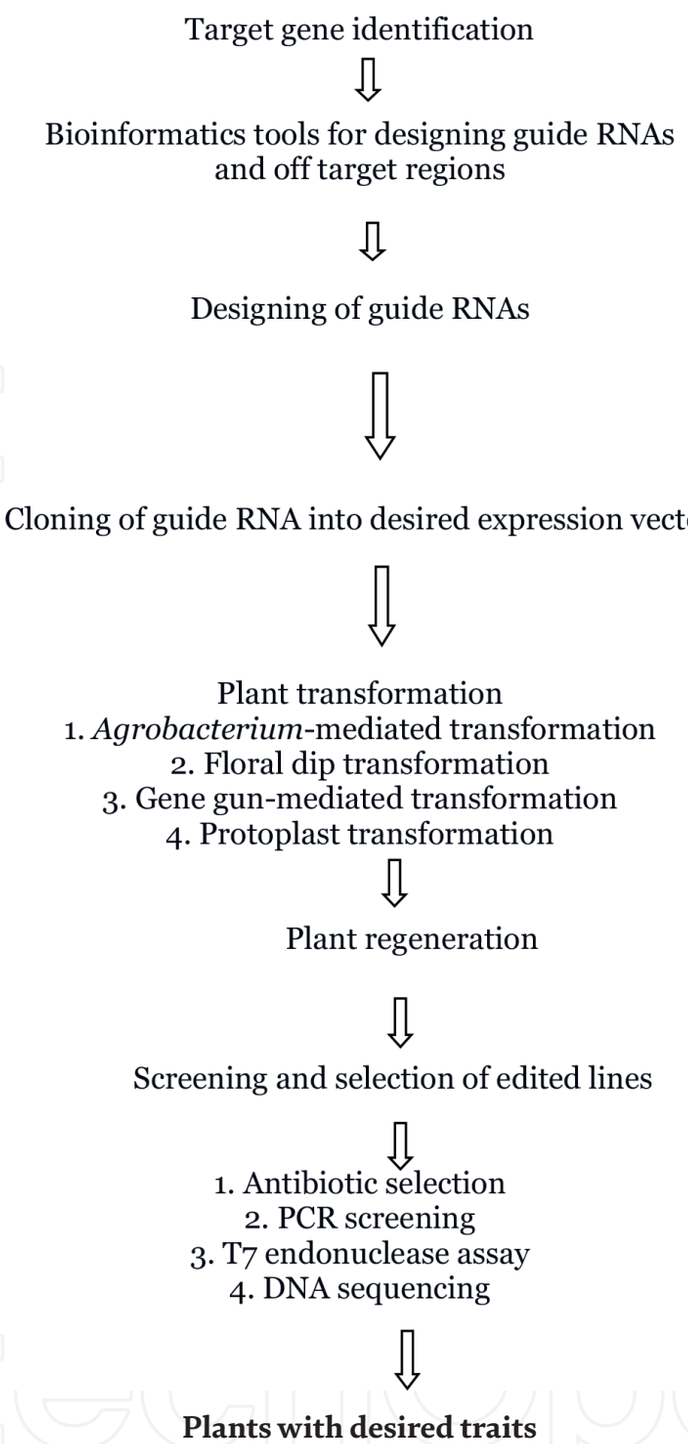
Brassica oleracea [79] and *Lactuca sativa* [80]. Many genes involved in the biosynthesis of carotenoids such as *Anthocyanin 1* (*ANT1*) are involved in anthocyanin biosynthesis [81], *Phytoene desaturase* (*SlPDS*), *Phytochrome interacting factor* (*SlPIF4*) [82], and *Phytoene synthase* (*PSY1*) [83]. Mutant tomato plants with the knockout of the *Slagamous-like 6* (*SlAGL6*) gene produced parthenocarpic fruits under heat stress conditions that otherwise rigorously hinder fertilization-dependent fruit set [84]. Silencing the *eIF4E* gene in tomatoes and melons has attained resistance to RNA virus [85, 86]. Granule bound starch synthase (*GBSS*) gene using CRISPR/Cas9 that produced amylopectin and lacks amylose [87]. CRISPR/Cas9 mediated mutagenesis of genes involved in starch biosynthesis in sweet potato (*Ipomoea batatas*) was done for improving the quality of starch [88]. CRISPR/Cas9 induced mutation of CaERF28 conferred resistance to anthracnose in chili pepper (*Capsicum annuum* L.) [89].

Regulating homeostasis is a reliable way to enhance the yield of cereal. Genome editing of the C terminus of *LOGL5* in rice which codes for the cytokinin-activation enzyme increases the yield in rice during all ecological conditions [90]. Production of a high-yielding wheat variety was done by editing the gene that encodes cytokinin oxidase/dehydrogenase (*CKX*) by a knock-out mechanism [91]. Similarly, knocking out another gene that codes for amino acid permease in rice which is actively involved in nutrient portioning, led to an increase in yield [60]. In addition, some genes in rice (*PIN5b, GS3, GW2*, *GW2*, and *GW5*) have been edited based on CRISPR/Cas9 technology, which has led to an increase in yield [61, 62]. To increase their yield, scientists have successfully edited genes in fruits such as *CLV* [63] and *ENO* [92]. The nutritional content of crops is an essential trait

for adding their commercial value to local and global markets. Genome editing of granule-bound starch synthase 1 *GBBS1* gene in maize produced low amylose content variety [93]. Gluten causes celiac disease in susceptible individuals and CRISPR/Cas9 editing techniques have successfully edited the conserved region in the loci of wheat, leading to 85% loss in immunoreaction [94]. Bacterial blight caused by *Xanthomonas oryzae* is a severe threat to rice production, and genome editing of *SWEET* genes using the CRISPR technique imparted high resistance to bacterial blight [64, 95]. In addition, editing of the *LOB1* gene in *Citrus sinensis* conferred resistance to *Xanthomonas citri* [65]. Powdery mildew caused by *Blumeri agraminis* fungi also leads to various growth defects in wheat plants. Targeting three wheat homologous genes (*EDR1*) by CRISPR/Cas9 editing improved resistance to these fungi [66]. Similarly, the tomato was also conferred resistance to *Oidium neolycopersici* that also causes powdery mildew, by mutating *Solanum lycopersicum MLO1* [67].

CRISPR/Cas-based genome editing technology has to produce double-stranded breaks efficiently in the genome. Hence, scientific communities have also very well utilized their ability to edit plant viruses that attack plants and cause various diseases by employing various RNA-targeting versions of Cas enzymes such as Cas13a, Cas13b, Cas13d, and *Fn*Cas9 [68, 69]. Another problem that CRISPR has solved is to produce herbicide-resistant crops for controlling weeds that adversely affect crops' growth and decrease soil fertility and directly affect the yield from various crops. Acetolactate synthase (*ALS*) is a critical enzyme in producing branched-chain amino acids that are specifically targeted by herbicides such as sulfonylurea and imidazolinone. Using cytosine base editing, we impart herbicide resistance to *Oryza sativa ALS* by creating base transitions [70, 71]. Additionally, acetyl coenzyme A carboxylase (*ACCase*) is a critical enzyme in the production of lipids and a valuable target for genome editing with herbicides. Adenine base editing of the rice ACCase gene resulted in establishing a C2186R substitution, resulting in a mutant rice strain that is tolerant to haloxyfop-R-methyl [72]. Similarly, quizalofop-resistant wheat has been developed by adding an A1992V mutation into wheat ACCase [73]. Additionally, it has been demonstrated that altering *EPS* [96], *PPO* [97], *TubA2* [98], and *SF3B1* [99] mediates resistance to glyphosate, butafenacil, trifluralin, and herboxidiene (GEX1A). In addition to their numerous agricultural applications, these herbicide-resistant alleles can be used as selective markers to enhance gene editing processes [70]. CRISPR-based technology has been very effective in tackling stress conditions in plants and many literature surveys define the higher rate of success based on CRISPR genome editing. Xie and Yang [74] demonstrated targeted mutagenesis of the *Oryza sativa* gene (*OsMPK5*), which negatively regulates both biotic and abiotic stresses in rice.

Moreover, minimum low off-target efficiency was proved using specific guide RNAs and two appropriate vectors pRGE3 and pRGE6 [74]. Mitogen-activated protein kinases (MAPKs) are important signaling molecules that respond to drought stress in tomatoes by defending the cell membrane against oxidative damage and regulating the transcription of drought-stress-related genes. Slmapk3 mutants produced by CRISPR editing are fewer droughts tolerant and display increased wilting symptoms [75]. CRISPR/Cas9 genome editing has been used to manipulate the synthesis of secondary metabolites in plants. Zhang et al. recently showed multiplex CRISPR genome editing to enhance the isoflavone content of soybean and strengthen its resistance to soybean mosaic virus by altering the *GmF3H1*, *GmF3H2*, and *GmFNSII-1* genes [76].



Flowchart showing the production of plants with the desired trait.

5. Safety regulations in genome editing

Plant genome editing methods are critical for developing crops that can withstand biotic, abiotic, and extreme climatic changes and resolving global policy and governance issues. Apart from remarks on principle-based biotechnology adoption and the ethical, social, and biological issues raised by the CRISPR/Cas system, the current state of agriculture is examined. As a result of the technology's limitations, moral concerns about CRISPR have developed, necessitating intra and international attention to discover solutions that benefit the broader public interest. However, there is a public debate regarding the direction and control of new methods' utility inside the industrialized world [100]. Governments in developing

nations expect to give an alternative that promotes the alleviation of famine and poverty [101]. However, the uncertainty of agricultural biotechnology safety leads to suspicion of the regulatory framework and implies that the biological safety regulations [102] to preserve biological variety, including environmental protection and health safety, must be rejected. The unpredictability of agricultural biotechnology safety results in mistrust of the regulatory framework and implies that biosafety regulation should be rejected. Global hunger and poverty have been significantly reduced because of the green revolution. Malnutrition and the occurrence of certain chronic illnesses among otherwise healthy people have been exacerbated by inadequate amounts of specific amino acids, minerals, vitamins, and lipid acids in staple crops, as well as animal diets derived from them (the so-called diseases of civilization). The green revolution has made major contributions to the decrease of world hunger and poverty. Certain amino acids, minerals, vitamins, and fatty acids are deficient in staple crops and animal diets generated from them, contributing to malnutrition and the growing incidence of certain chronic illnesses in otherwise well-nourished people (the so-called diseases of civilization). Nevertheless, a recent trend demonstrates according to research conducted by the United Nations Food and Agricultural Organization (FAO), worldwide hunger and malnutrition have increased since 2014, with an estimated 821 million people lacking access to sufficient food in 2017 [103]. The advent of transgenic plants with insect-resistant, herbicide-tolerant, and nutritional characteristics has led to an explosion in the number of genetically modified crops grown on a wide scale. According to the latest report, 18 million farmers planted GMO crops on 181.5 million hectares in 28 countries in 2014, representing a 3–4% increase over the 2013 figures [104]. Recent marketed genetically modified crops include tomato, corn, soybean, cotton, canola, rice, potato, squash, melon, and papaya, with soybean, corn, and cotton being the most important because of their widespread cultivation critical role in the agricultural economy in many nations. America, Argentina, and Canada lead the world in producing and exporting genetically modified goods [105]. A good portion of the countries battling against GMOs in Africa are the same ones that struggled with them in Europe. Only local civil society organizations and a few NGOs have stood in the way of Uganda's effort to combat banana leaf wilt with its genetically modified bananas. All GMO-related activities in India must be regulated by the Environmental Protection Act (EPA) [106], which was passed in 1986. The Ministry of Environment, Forests and Climate Change (MoEF&CC) is responsible for this regulation [107]. Indian genetically modified crops are subject to a multitiered regulatory system controlled by the Ministry for Environment and Forest and Department of Biotechnology, which is part of the Ministry of Science and Technology. Six competent authorities comprise this system—the Recombinant DNA Advisory Committee (RDAC), the Review Committee on Genetic Manipulation (RCGM), the Genetic Engineering Appraisal Committee (GEAC), the Institutional Biosafety Committees (IBSC), the State Biotechnology Coordination Committees (SBCC), and District Level Committees (DLC). All the various committees' tasks and responsibilities are defined in the Rules 1989 [108]. Genetic Engineering Appraisal Committee (GEAC) is a board within the Ministry of Environment, Forests and Climate Change that regulates the manufacture, import, export, and storage of hazardous microorganisms and genetically engineered organisms or cells under the Environment Protection Act 1986. Indian lawmakers have charged the Government Environmental Assessment Council (GEAC) with performing environmental evaluations of operations using GMOs and their products in research, industrial manufacturing, field application, and environmental discharge. For the production, environmental release, and

marketing of GM crops, the Indian Parliament passed three essential laws. Included in these laws were the Environment Protection Act of 1986, which the Ministry of Environment manages, Forests and Climate Change (MoEF&CC), the Seeds Act of 1966, and the Seeds (Control) Order, which is administered by the Ministry of Agriculture [109]. It is generally recognized that transgenic crops offer significant advantages to society in solving concerns of food and nutrition security. Adding to the benefits of improved nutrition, herbicide tolerance, viral resistance, and tolerance to different environmental stressors, many fruits will last longer on the shelf, helping farmers in the marketplace. To accomplish its goal of food and nutrition security, India must continue its research into genetically modified crops. Despite the lack of concrete proof against the safety of genetically modified foods, the argument over whether or not they are safe will continue. Even while few public sector intuitions share worries about genetically modified foods, it is surprising that the same is true in regards to genetically modified animals. Government of India–funded intuitions should follow the same general principles as the Indian government, demonstrating their value by cooperating with the government to battle poverty and hunger. The members of the Technical Expert Committee constituted by the Supreme Court of India for Safety and Guidelines for genetically modified agricultural research debate the argument presented here, with some believing that it is significant and others seeing it as unimportant. However, while India has the inadequate infrastructure and lacks standards for genetically modified agricultural research and risk assessment, the initiative is of utmost importance given India's dire need. To prepare for future deregulation, India must continue researching genetically modified crops and construct basic infrastructural facilities while developing strict marketing and biosafety rules. Although portals such as the GEAC, the IGMORIS, and Biosafety Clearing-House play a role in biosafety assessment and regulations on GM plants, there is an urgent need to develop a single-window system and an online portal for the assessment, control, regulation, and approval of GM plants. The government should require every firm and public institution to register with this platform before starting any transgenic event or field testing, regardless of whether or not they are seeking clearance. To commercialize transgenic products, each new genetically modified organism (GMO) being developed must have a registration number, and registration date displayed online on a site or portal particularly intended for approval in any nation. This portal should include a publishing list that documents any genetically modified crop development activity so that any person interested in any genetically modified crop development event may access the entire information in one location. A gateway of this sort will be invaluable and broadly accessible for the public good by promoting the beneficial benefits of genetically modified food research, food safety, and the current state of genetically modified foods.

6. Demonstration for CRISPR-CHOPCHOP for sgRNA designing

In just 3 years, CRISPR genome editing dramatically changed biology, but also its acceptance and utility continue to expand. New CRISPR mechanisms and criteria for choosing ideal targets are being published all the time, highlighting the need for computerized CRISPR targeting tools to combine these guidelines to make target appropriate selection quicker. Among the most popular website software for genome editing with CRISPR and TALEN is CHOPCHOP (https://chopchop.cbu.uib.no/). It provides a user-friendly online environment for target selection, primer development, and restriction site identification, all based on the most

recent large-scale investigations. In both protein-coding and noncoding genes, CHOPCHOP allows accurate localization of subsections, including coding regions, UTRs, splice sites, and particular exons. For all sgRNAs, the program detects probable off-target sites, generates primers for target sites automatically, and shows all elements in a variable graphical interface that incorporates restriction relevant details for subsequent confirmation [109].

1. Go to https://chopchop.cbu.uib.no/
2. Enter gene of interest in target field (for example *Arabidopsis thaliana* Leucoanthocyanidin dioxygenase (LDOX), NM_001341563.1)
3. Choose the genome of interest in the ln field.
4. Choose the CRISPR/CAS9 in the using field.
5. Choose the knockout in the field.
6. Press find the target site [110].

6.1 Results interpretations

- In that result window, the green color represents no off-targets, the orange color represents minimal off-targets, and the red color represents more off-targets.
- NOTE: For designing of suitable sgRNA, needs no off-targets.
- Click any green color symbol, which indicates the next level of target identification like percentage of GC content, off-target levels, and primer sequences.
- The violet color indicates primer pairs. Green and red boxes indicate the respective restriction sites (Ex. HindIII).
- Note: The sequence should include the PAM sequence.
- Any possibility of off-targets are listed in the below table with the location of the genome, possible off-target mismatches are represented in red.
- Note: Since there is no off-target it's mentioned as "there are no off targets".
- Once the target is fixed copy the target sequence with their respective primers from the web page.

7. Future prospective

To improve vegetables, certain factors must be considered such as an increase in production and insect resistance; abiotic stress tolerability; improved shelf life; processing quality; and improved nutritional contents, to name a few. In traditional breeding, it is difficult and time-consuming to achieve the stated traits. A novel genome editing technology, CRISPR/Cas9 technology can alter a plant genome resulting in several mutations. By putting the Cas9 gene into sgRNA specific viral

DNA, the plant has evolved virus resistance through CRISPR/Cas9 driven mechanisms [111]. A broad range of viral resistance plants was created by introducing several sgRNAs that target genomic areas of the whole virus into plants. CRISPR/Cas9 can therefore be used to enhance metabolic engineering of horticultural crops by providing health-promoting factors.

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