CRISPR Genome Editing Brings Global Food Security into the First Lane: Enhancing Nutrition and Stress Resilience in Crops

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Abstract

Crop yield is adversely being influenced very frequently due to biotic and abiotic stresses, globally. The insufficient yield and low nutritional value of the crops are due to the numerous ambient stresses. Which has challenged the nutritional security of people in developing and underdeveloped nations that are already been malnourished. The tremendously changing global climate and the ever-increasing world population are the principal apprehensions guiding towards the adaptation of a neoteric technique that can aid in achieving the sustainable development of agriculture with enriched nutritional value, plant resilience, and improved yield potential. The clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas) protein-based genome-editing (CRISPR-Cas) tool is the most valuable technique to boon the modern world and offers an edge over to meganucleases, zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs) by being its tremendous potency, accuracy, ease of use, and versatility. Here, we have highlighted the neoteric advancements of the CRISPR-Cas-based approaches that have revolutionized the way of food production in the agriculture industry. It has paved the way for food security by modifying crucial crop attributes by introducing desirable characteristics that employ knockout and/or knockin of targeted genes to generate resistant crop plants with enriched nutritional quality, yield enhancement, and stress resilience. In addition, we have also shed light on different mechanisms, challenges, approaches for the minimization of off-target effects, and future possibilities of these neoteric genome-editing tools.

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Furthermore, with the advent of the CRISPR-based platform, the numerous emerging biotechnologies have broadened the basic crop research toolbox and synthetic biotechnology via the incorporation of artificial intelligence (AI) and various bioinformatics frameworks. Eventually, the current global regulatory stratagems and social approval of CRISPR-Cas-based crop trait enhancement have been explored.

Keywords

Climate change · CRISPR-Cas · Genome editing · Nutrition · Stress resilience

9.1 Introduction

9.1.1 Genome Editing: An Introduction to the Plethora of Tools in the Arsenal of Gene Editing

Genetic modification for the development of desired traits in plants utilized for food began before the end of the Pleistocene era about 12,000–11,000 years ago (Larson et al. 2014). Since then, numerous changes have happened due to natural evolutionary processes, which resulted in new crop species that are now genetically different from their ancestors. After establishing Mendel's 'principle of genetics' in 1865, actual genetic modification was started. Plant genetic engineering has designed to generate plants with neoteric attributes that could conquer sustainability goals. Hence, it necessitates introducing advanced genetic engineering strategies, for instance, mutagenesis, transgenic approach, RNAi approaches, genome editing (GEd) via ZFNs, TALENS, CRISPR-Cas approaches. GEd was promoted by introducing double-strand breaks (DSBs) at the targeted locus, which relies on sequence-specific nucleases (SSNs). Recently, the toolkit of GEd comprises four classes of SSNs: meganucleases, ZFNs, TALEN, and CRISPR-Cas systems.

Meganucleases are the naturally available endonucleases, also familiar as homing endonuclease. It is the first-generation SSN and came into the limelight as a self-splicing component of mitochondrial large ribosomal DNA (mtLrDNA) introns of *Saccharomyces cerevisiae* (Colleaux et al. 1988). This can identify a wide range of DNA (14–40 bp) (Orlowski et al. 2007). Due to variation in target recognition and cleavage site, these SSNs can be grouped into six major families, for instance, His-Cys Box, LAGLIDADG, HNH, EDxHD, GIY-YIG, and PD-(D/E)xK (Belfort et al. 2014). I-SecI is the most frequently utilized meganucleases and was first utilized in tobacco plant (Puchta 1999), since then it was being used by plant biologists for GEd. D'Halluin et al. (2007) reported the utilization of meganucleases in maize. Nevertheless, the lack of editing capability of broad target sequences via protein redesign mightily narrows this SSN's applications (Rosen et al. 2006).

The re-programmability lacking of meganucleases was solved with ZFNs. ZFNs comprise multiple zinc finger domain harbouring proteins. Those protein domains are generated from the typical Cys-2-His 2-zinc finger domain (Gaj et al. 2013); after

recognition of specific sequences, those protein motifs were fastened to DNA in a sequence-specific manner (Weeks et al. 2016). The C-terminal part of each ZFN motif is responsible for targeted sequence recognition. The composition of each ZFN motif binds a 3-bp DNA sequence and is made up of almost 30 amino acids (Maeder et al. 2008). Therefore, unlike meganucleases, these separate domain arrangements made ZFN simpler. Nevertheless, due to lack of endonuclease activity, they require to be fused with Fok I endonuclease domain for cleavage of DNA at the target site (Kaul et al. 2019). The efficiency of ZFNs-mediated gene editing was first successfully employed in *Arabidopsis (*Lloyd et al. 2005). Similarly, Dicer-like DCL4a and DCL4b gene in soybean was successfully edited utilizing ZFNs (Curtin et al. 2011). ZFN is also used for HDR-mediated gene editing, for instance, the amino acid substitution of SuRa and SuRb gene in tobacco, which conferred resistance to sulphonylurea herbicide (Townsend et al. 2009). However, two separate ZFN motifs target two proximal sites and double the construct size, which may complicate the design of this SSN.

TALENs are specific DNA-binding proteins, large sequences (>30 bp) targets make them more precise (Miller et al. 2011). The TALEs proteins were identified from plant pathogen *Xanthomonas* sp. Unlike ZFNs, these proteins have DNA-binding modular domains, specifically recognizing one single base instead of three (Moscou and Bogdanove 2009). Additionally, like ZFNs, this single DNA identification modules must be fused with Fok I endonuclease domain (Mahfouz et al. 2014). The possibility of TALEN-based editing was realized in 2009, wherein successful gene editing was first reported in yeast (Christian et al. 2013). Over the past few years, TALENs have emerged as a choice of GEd in plants. It has been successfully employed in a variety of crops, for instance, tobacco (Moore et al. 2014), barley (Budhagatapalli et al. 2015), tomato Čermák et al. 2015), *Arabidopsis* (Forner et al. 2015). Genome modification via TALENs is handy in comparison to ZFNs, due to its simplicity in using TALEs repeats for each of the DNA nucleotide recognition.

Amongst all the approaches, recently discovered CRISPR-Cas9-based GEd tools have replaced the ZFNs and TALENs and opened the way to modify plant's genomes with unprecedented precision. This GEd system is revolutionizing the field of plant biology due to its efficiency, specificity, unparalleled flexibility, and target design simplicity. Apart from these, CRISPR-Cas9 has additional advantages over ZFNs and TALENs, including target specificity design, efficiency in incorporating the guide RNA (gRNA), and the RNAs guided Cas9 protein and the ability of multiplexing in a single event. Compared to the previously available techniques, designing a CRISPR-Cas9 vector is easy and efficient with the availability and accessibility of enhanced bioinformatics tools, which can be utilized to find the most selective sequences for designing gRNAs, eliminating the potential for screening libraries to find the most effective target. This technology has been rapidly and widely adopted for a range of applications for instance, multiplex gene knockout, targeted sequence insertion, base editing, prime editing, and so on. A variety of strategies have been developed for optimizing the CRISPR-Cas9 reagents and their delivery systems. This chapter tries to compile a detailed review of the existing GEd

approaches, emphasizing the CRISPR-Cas9 technique. We also shed light on a glimpse of information about novel breakthrough and milestone achievements of CRISPR-Cas9 systems and the impact of this system as the next gene tool for crop improvement.

9.2 Era of CRISPR-Cas-Based Genome Editing

The invention of the CRISPR-Cas microbial self-defense mechanism and its ongoing achievement as a genome-editing tool represents the findings of numerous researchers all over the world. Our concise historical era will represent the contributions of different scientists who pushed this GEd field forward from the initial discovery. The clusters of repeats which are separated by spacers were first observed in 1987 during the study of *E. coli* harbouring *jap* gene (Ishino et al. 1987). In 1989, the structure of the CRISPR array was defined but without its functional mechanism (Nakata et al. 1989). Interestingly, similar structures were identified later in numerous bacteria and archaea (Hermans et al. 1991; Mojica et al. 1995; Bult et al. 1996). Francisco Mojica characterizes those sequences for the first time in 1993, what is now known as CRISPR locus, and the potentiality of this locus was shown in 2000 (Mojica et al. 2000). Simultaneously, 45 protein families were identified with clusters of CRISPR-associated genes (Haft et al. 2005). After increasing the volume of prokaryotic sequence data, the crucial breakthrough happened in 2005. It was reported that identified CRISPR sequences showed similarity with some bacteriophage and led to immunity against those infectious bacteriophages (Mojica et al. 2005; Pourcel et al. 2005). Bolotin revealed some anomaly in the CRISPR locus and found a large protein with nuclease activity, which is now known as Cas9 (Bolotin et al. 2005). Although found some viral genes resemble sequences at one end, those are the PAM sequence. In the same year 2005, Jennifer Doudna and Jillian Banfield started their investigation on CRISPR and their functions. In 2006, the hypothetical scheme of the CRISPR-Cas adaptive immunity mechanism was proposed by Koonim (Makarova *et al.* 2006). Later on, this hypothesis was confirmed by Barrangou et al. (2007). After that, scientists started to report the CRISPR-Cas action that how this RNA-mediated system interferes with the invading phage DNA (Brouns *et al.* 2008). It was also demonstrated that this system could target both DNA (Marraffini and Sontheimer 2008) and RNA (Hale et al. 2009). Alongside, PAM sequences are also essential for some systems described simultaneously (Mojica et al. 2009). Details about the transcription mechanisms of crRNAs were also revealed in 2010 (Haurwitz et al. 2010). The classification of the CRISPR-Cas systems was demonstrated in 2011 (Makarova *et al.* 2011). In the same year, 2011, Emmanuelle Charpentier and Jenifer Dounda conjointly started to study the CRISPR-Cas mechanism, and they discovered the function of tracrRNA for the Cas9 system. Moreover, the role of RNase III in pre-crRNA and tracrRNA processing was characterized in 2011 (Deltcheva et al. 2011). In 2012, Siksnys and his team mechanically characterized the mode of adaptation of Cas9 via understanding the cell infection kinetics with the CRISPR-Cas system (Datsenko

et al. 2012; Gasiunas et al. 2012). At the same time, in 2012, similar findings were reported by Jennifer Doudna in collaboration with Emmanuelle Charpentier. They demonstrated that synthetic gRNAs could be generated via fusion of the crRNA and the tracrRNA (Jinek et al. 2012; Qi et al. 2021). Finally, the newly invented CRISPR-Cas system was led to use for targeted genome modification in bacteria (Gasiunas et al. 2012), yeast (DiCarlo et al. 2013), human (Cong et al. 2013a, b; Jinek et al. 2013; Mali et al. 2013a, b). In 2013, CRISPR-Cas machinery was successfully employed to engineer plant genomes (Shan et al. 2013). In 2014, CRISPR-Cas9 was demonstrated in primates (Cas9 mRNA and sgRNAs were coinjected in monkey embryo); therefore, Cas9/sgRNA screens were established as a tool for genetic analysis in mammalian cells (Shen 2014). In 2015, US and UK research scientist, Medical Research Council (MRC) declared their support for using GEd strategy for human cells (Charo 2015). After that, the International Summit on human gene editing was met to discuss about the medical and ethical issues (Charo 2016). New protein-Cpf1 was invented in the same year, which made gene editing become simpler (Koonin et al. 2017). Moreover, US scientists reported about the modified CRISPR-Cas9 technique with fewer off-target effects. The first clinical trial of the genetically modified human embryo was approved in 2016 (Cyranoski 2016; Reardon 2016). A new base editing technique was discovered in 2016 by US scientists, offered a new approach where any gene can modify without cleavage of double-stranded DNA as well as without donor DNA template (Rees and Liu 2018; Porto et al. 2020; Bharat et al. 2020). For RNA editing, a new CRISPR approach was identified in 2017 (Cox et al. 2017; Adli 2018). In 2018, Weissman's lab created a new GEd strategy called CRISPRa (for 'activation'), which activate the gene expression, and they also made CRISPRi (for 'interference') technology (Kampmann 2018). In 2018, a group of scientists identified pre-existing Cas9 antibodies in cells, leading to immune issues during gene therapy employing CRISPR-Cas9 (Crudele and Chamberlain 2018; Wagner et al. 2021). Same year another Cas variant Cas14 (a-c) was identified (Harrington et al. 2018) In 2019, Chinese researchers declared their first gene-edited human baby (Wang et al. 2020). Newly developed 'search and replace' tool for GEd known as prime editing was discovered in 2020 (Hampton 2020). In the same year 2020, a Chinese researcher was convicted for employing CRISPR-Cas9 in a human baby (Cyranoski 2020). Moreover, in 2020 for the first time, one patient received gene editing therapy employing the CRISPR-Cas9 approach (He 2020; Ledford 2020). In early 2020, a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreaks rapidly evolved into a global pandemic. For the detection and quantification of SARS-CoV-2 RNA, a CRISPR-Cas13-based approach was employed (Konwarh 2020; Kumar et al. 2020). In 2020, Jennifer Doudna and Emmanuelle Charpentier were jointly awarded the Nobel Prize in Chemistry for the identification of an efficient method in GEd known as the CRISPR-Cas9 technique (Ledford and Callaway 2020). The CRISPR 'on-off switch'- a new genome-editing approach was discovered by MIT and UCSF researchers in 2021, successfully implicated in Alzheimer disease (James et al. 2021). Any part of the targeted genome can be silent via controlling the gene's expression without altering DNA sequences. Therefore,

unlike first-generation GEd tool, the CRISPR-Cas9 technology has empowered researchers with an unprecedented toolbox via breakthrough discoveries and methodological advancements in science.

9.3 CRISPR-Cas: New-Fangled Dawn in Genome Editing

CRISPR-Cas is the most efficacious and ease-to-design editing tool, which generate a buzz in the field of research in current times. This is one of the crucial tools in an endless arms race between bacterial and archaeal hosts and viruses (Newsom et al. 2021). The CRISPR immunity gets triggered when a virus' foreign genetic material (DNA/RNA) is introduced into bacterial cells. The bacterial cell effectively produced specialized molecules (Cas protein) that can recognize the past similarity of foreign DNA and destroy them as antibodies work. The defense mechanism of this system comprises into three-stage process, i.e. (i) Adaptation: small DNA sequences (protospacers) of foreign plasmid are chosen and incorporated into the particular CRISPR locus of the host genome; (ii) Biogenesis of crRNA: multiple gRNA spacers and their repeats are transcribed into a precursor RNA and processed into mature gRNAs. Targeting complexes are produced via fastening of gRNAs with the Cas enzyme, which contain a distinctive spacer sequence resembling foreign target DNA; and (iii) Interference: Cas nuclease starts searching the unique sequences complementary to the gRNA. Cas nucleases fasten up to the gRNA resemble target foreign DNA site via complementary base pairing and cleave the targeted DNA sequences. By utilizing this machinery, bacteria generated the ability to avoid transcribing the matching targeted viral DNA, making its genome resistant to viral invasion. As research gains grounded, numerous CRISPR-Cas systems have been identified for GEd. All these systems have their own attributes, for instance, variation at PAM regions, varying sizes of Cas protein, and different cleavage sites. Amongst all, the type II CRISPR-Cas9 system provided the most simple, versatile precision editing in crop plants. This system required only two key molecules, i.e. Cas9 endonuclease and gRNA: fusion of CRISPR RNA (crRNA, a 20-nucleotide sequence complementary to the target DNA) and trans-activating crRNA (tracrRNA, acts as a binding scaffold for the Cas9 endonuclease). It also should be noted that the gRNA can be expressed as synthetic sgRNA, where the crRNA and tracrRNA are fused into one molecule for ease of expression (Fig. 9.1). Widely accepted *Sp*Cas9 (*Streptococcus pyogenes*) comprises a conserved core with two major big globular recognition lobe, for instance, REC (recognition) and a NUC (nuclease) lobe for nucleic acid binding. Wherein, the REC (functional domain of Cas9) contains bi-partite domain, for instance, REC1 & REC2 and bridge helix cd domain. It was revealed that base pairing between the ligand DNA strand and the seed region of gRNA (up to 8–12 bp) triggers the development of RNA–DNA heteroduplex, which occupied by both NUC and REC lobe (Anders et al. 2014). The small NUC nuclease lobe comprises a highly conserved RuvC- & HNH- and PI-domain (arginine-rich alpha-helical bridge helix) (Hsu et al. 2014). Simultaneously, RuvC and HNH nick the complimentary and non-complimentary strand in the target

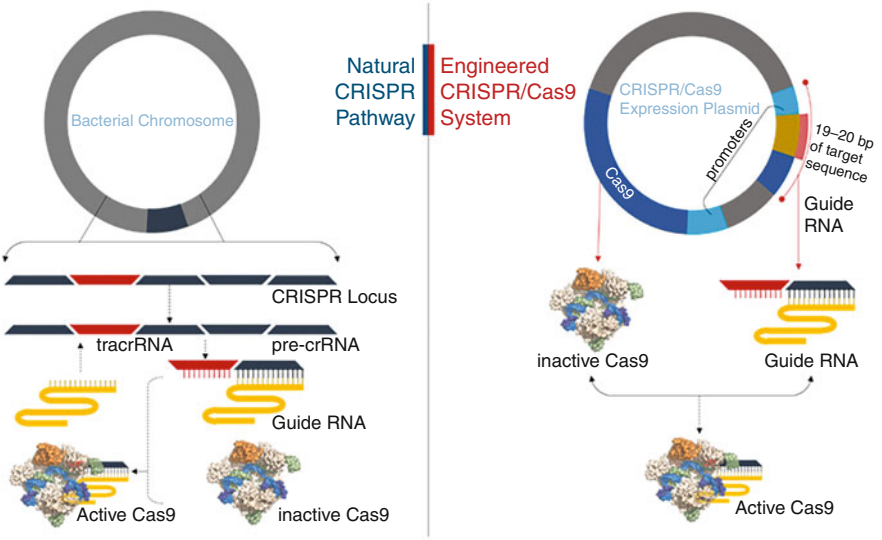

Fig. 9.1 CRISPR mechanism in action: Natural vs Engineered CRISPR system. This system required only two key molecules, i.e. Cas9 endonuclease and gRNA. gRNA is fusion of CRISPR RNA (crRNA, a 20-nucleotide sequence complementary to the target DNA) and trans-activating crRNA (tracrRNA, acts as a binding scaffold for the Cas9 endonuclease). Inactive Cas9 is become active when bind with gRNA. In synthetic sgRNA, the crRNA and tracrRNA are fused into one molecule for ease of expression

sequence, introducing double-strand breaks (DSBs) (Nishimasu et al. 2014). According to previous studies, the PI domain plays a crucial role in the PAM site (5′-NGG-3′) recognition because of having a tryptophan-rich flexible loop (Jinek et al. 2014). At 3 bp prior to PAM sites, the assembled CRISPR-Cas complex created DSBs. DSBs can be repaired at defined positions by integrating numerous alterations utilizing DNA repairing machinery, i.e. HDR and NHEJ (Fig. 9.2).

NHEJ is the primary DSB fixing pathway in plant cells and is comparatively effortless to exploit for GEd (Lieber 2010; Pannunzio et al. 2017). This error-prone pathway generally introduces indel mutations (insertions and/or deletions) by disrupting the targeted DNA, resulting in gene knockout (KO). The CRISPR-Cas9-based KO is utilized in gene function study, and modifying a variety of beneficial traits, for instance, stress resistance (Singh et al. 2020); disease resistance (Schenke and Cai 2020); higher yield (Huang et al. 2018; Ma et al. 2019; Liu et al. 2021; Tabassum et al. 2021); nutritional enhancement (Zhang et al. 2018a; Sanchez-Leon et al. 2018; Ku and Ha 2020; Dong et al. 2020; Huang et al. 2020; Dong et al. 2019; Xu et al. 2020; Yang et al. 2020; Zeng et al. 2020; Kaul et al. 2020a, b; Sashidhar et al. 2020; Tiwari et al. 2020); and male sterility (Chen et al. 2021). For the achievement of successful KO, it is recommended to target early exon because functional activities of a gene will be less if indel mutation is generated in either 3′ end of exon sequences or intron region. Nevertheless, due to alternative splicing if

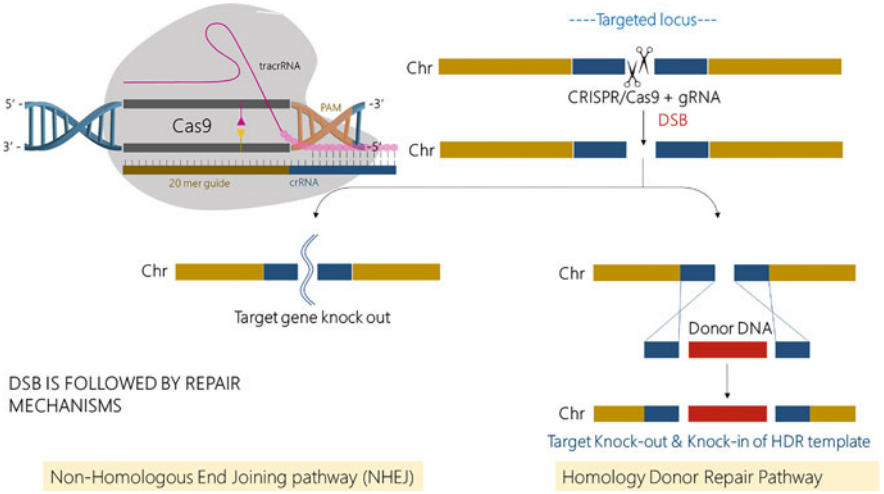

Fig. 9.2 Schematic representation of CRISPR/Cas9-based DSBs repair mechanism, including NHEJ and HDR-mediated repair pathways. The CRISPR-associated endonuclease Cas9 generated DSBs in the target DNA. NHEJ pathway results in random indels via gene disruption at the target site. HDR pathway uses homologous donor DNA sequences for accurate insertions or base substitutions between DSB sites. *DSB* double-strand break; *NHEJ* non-homologous end joining, *HDR* homology donor repair, *Indels* insertions and deletions

target gene enciphers various proteins, then frameshift mutation or stop codon introduction in early exon may not reveal gene KO. In this situation, complete gene deletion can be possible by utilizing the multiplex CRISPR-Cas9 KO strategy by targeting the gene's 3′ and 5′ end. For example, (115–245) kb in size chromosomal deletions were generated via gene cluster deletion in rice (Zhou et al. 2014) employing multiplex CRISPR-based KO strategy. Recently, multiplex CRISPR-Cas9 system utilized for simultaneous KO of multiple genes and revealed de novo domestication of wild tomato (Zsögön et al. 2018; Xie and Liu 2021). Gene KO is extremely difficult in polyploidy species due to its gene functional redundancy. It was successfully utilized in hexaploid wheat to develop fungal-resistant wheat by KO of disease susceptible S-gene (Wang et al. 2014; Wang et al. 2018a). Corteva Agriscience generated amylopectin rich (waxy) corn via KO of *Wx1* gene (DuPont Pioneer 2016). Similarly, two japonica rice varieties (glutinous sticky) were achieved through Waxy (*OsWx*) gene KO (Yunyan et al. 2019). Moreover, amylose-rich rice grain was revealed via targeted modification of the *SBEIIb* gene (Sun et al. 2017). Gaoneng et al. (2017) developed fragrance enriched rice via targeted KO of the *BADH2* gene (negative regulator for aroma production). Additionally, KO of *OsERF922* gene generated blast-resistant rice lines was reported by Wang et al. (2016). Edited rice lines with pale green colour in leaf were generated via KO of chlorophyll biosynthesis regulated gene *OsCAO1* gene (Jung et al. 2021). Interestingly, *OsHOL1* plays a major role in the production of methyl iodide, and KO of this gene abolished methyl iodide emissions from rice plants (Carlessi et al.

2021). Targeted KO of TERMINAL FLOWER 1 (*TF1*) gene in *Brassica napus* altered the flowering time and plant architecture (Sriboon et al. 2020). Targeted KO mutations of *HvHPT* and *HvHGGT* gene rendered a high level of vitamin E (tocopherol) in barley (Zeng et al. 2020). According to Li et al. (2019), KO of numerous genes, i.e. *SVP, AP1*, and *TFL* elicited floral features advancement in *Arabidopsis*. High-oleic acid content was generated in allotetraploid cotton (*Gossypium hirsutum* L.) (Chen et al. 2021) and tobacco (Tian et al. 2020) via KO of *GhFAD2* and *NtFAD2–2* genes, respectively, as well as Monounsaturated Fatty Acid (MUFAs) contents enhancement in Hexaploid *Camelina sativa* seed oil was generated through *FAD2* Gene KO using CRISPR-Cas9 (Lee et al. 2021). Functional KO of *StDND1*, *StCHL1,* and *StDMR6–1* generated potatoes highly resistance against late blight disease (Kieu et al. 2021).

On the other hand, HDR mechanism introduces specific base pair substitution point mutations via target DNA recombination with complementary HDR template (Reis et al. 2014; Sander and Joung 2014). Knocking-in of targeted and precise sequences has been more challenging. Repairing Cas9-induced DSBs or nicks using HDR-mediated pathway makes GEd more accurate. Thus, unlike NHEJ, in case of knockin, the incision must be embedded precisely, without extra insertions/deletions (indel) mutation. Unfortunately, GEd frequency employing HDR mechanism is relatively low in plants in comparison to NHEJ. Amongst numerous approaches to recurrence, the HDR efficiency in plants, the utilization of mastrevirus (Geminiviridae) vectors for delivery of donor template is the most successful generated so far. This method was first demonstrated in tobacco to develop bean yellow dwarf virus-resistant (Baltes et al. 2014). The HDR template frequency was increased dramatically in nucleus due to replication of donor template, revealing a high editing frequency. Later on, it was employed in tomato ANT1 gene to precisely insert a promoter upstream of the gene, resulted in pigment accumulation in foliage, flowers, and fruits via controlling anthocyanin biosynthesis (Čermák et al. 2015). Moreover, point mutation was introduced in the potato *ALS*1 gene, conferring herbicide resistance (Butler et al. 2016). A viable alternative method is delivering a large copy of the donor template into plant genome employing biolistic approach. This approach was successfully utilized in rice and maize (Baltes et al. 2015; Gil-Humanes et al. 2017; Wang et al. 2017) resulted in higher precise edits. In line with this, numerous advancements had been developed, for instance, in *Arabidopsis* the absence of a repair protein, KU70/80 may lead to a 5–16 fold enhancement in HDR editing frequency via suppressing the NHEJ repair pathway (Endo et al. 2016). Moreover, Lu et al. (2020) discovered a tandem repeat-HDR (TR-HDR) approach for high frequency targeted sequence replacement, wherein the precise editing frequencies ranged from 3.4 to 11.4%. According to Shi et al. (2017), the promoter swapping, for instance, GOS2 promoter by the native ARGOS8 promoter employing CRISPR-Cas based GEd via HDR approach generated drought tolerance in maize. Tomato lines with higher self-life were generated via T317A substitution in the *ALC* gene (Yu et al. 2017). Newly developed RNA-mediated CRISPR/Cpf1-based approach also rendered efficient, targeted gene insertion in tomatoes (Vu et al. 2020). Therefore conjointly, CRISPR-Cas9 and CRISPR/Cpf1 may overcome all

difficulties for precise gene knockin via HDR mechanism for crop plant enhancement.

9.4 Novel Technical Breakthrough of Genome Editing in Plants

The necessity to genetically improve crop varieties became the reason for the discovery of target-specific endonucleases (TSENs) and since 2005 there has been a significant improvement and addition of new tools/techniques in the GEd toolkit. The genetic engineering field experienced another boost with the discovery of the CRISPR-Cas9 system which back in 1987 was recognized as the bacterial immune system (Ishino et al. 1987). The last decade has witnessed the evolution of this technique to reduce the bottleneck in terms of efficacy, efficiency, applicability, and other already discussed shortcomings. Substantial diverseness in genes, loci configuration, and action mechanisms of CRISPR-Cas approach made their classification a formidable task. An updated classification was reported by MaKarova et al. (2020), which include 2 classes, 6 types, and 33 subtypes; they identified novel class 2 CRISPR-Cas systems including 3 types and 17 subtypes (Table 9.1). Class 1 systems contain ~90% of all discovered CRISPR-Cas loci constituting type I, III, and IV (Makarova et al. 2015). The Class 2 system contains 10% of CRISPR-Cas loci (Makarova et al. 2015) and clearly differentiating into type II, V, and VI (Makarova et al. 2020). Numerous Cas9 variants are identified in recent years to broaden the opportunity of genome alteration. Thus to greatly expand the range of targets, different orthologs of Cas9 were reviewed, and VQR (5′-NGA-3′) and VRER (5′- NGCG-3′) variants of Cas9 were developed for plants (Hua et al. 2016). In the same line of study, an ortholog from *Francisella novicida* (Fncas9) was engineered to recognize 5′-YG-3′ PAM. FnCas9 is also known to target the RNA substrate, consequently it can be utilized to gain viral resistance in plants (Zhang et al. 2018b). Later to increase the penetrability of the Fn Cas9, proximal CRISPR (proxy-CRISPR) was developed (Chen et al. 2017). To increase the target specificity and reduce the off-target effect, Cas9 nickases mutants (nCas9) came into the picture by introducing point mutations like D10A in RuvC (Jinek et al. 2012) and

Table 9.1 Classification of CRISPR-Cas system

Class	Type	Subtype	Spacer acquisition	crRNA biogenesis	Interference crRNP	Type of nucleic acid targets
1	I	A-G	Cas1, Cas2, Cas4	Cas6/ Cas5d	Cascade	DNA
	III	A-F	Cas1, Cas2	Cas6	Csm/Cmr	DNA/RNA
	IV	A-C	Csf5	Csf	Unknown	DNA
2	II	A-C	Cas1, Cas2, Cas4/Csn2, Cas9	RNase III Cas9	Cas9	DNA
	V	A-I, K	Cas1, Cas2, Cas4	Cas12	Cas12	DNA
	VI	A-D	Cas1, Cas2	Cas13	Cas13	RNA

N863A or H840A in HNH domains (Nishimasu et al. 2014). Along the same line of work, Satomura et al. (2017) designed a 'CRISPR Nickase system' (CNS) to target sequences that were non-editable with the conventional CRISPR-Cas9 tool. Furthermore, the inducible Cas9 or split Cas9 can be used for temporally and spatially restricted Cas9 expression (Zhou et al. 2018; Carlson-Stevermer et al. 2020).

The continuous endeavour led to the discovery of class II type V CRISPR from *Prevotella* and *Francisella* 1- Cpf1/Cas12a was a potential alternative to Cas9 primarily because it could target AT-rich (5′-TTTN-3′) PAM instead of GC rich PAM (Doudna and Charpentier 2014). Besides *cis*-cleavage of the target double strand, it can also cleave non-specific ssDNA in *trans* (Swarts and Jinek 2019) which contributed to the invention of a sensitive nucleic acid detection technique, i.e. DETECTOR (Li et al. 2018a). Recently, Zhang et al. (2021) have contributed exceptionally with the discovery of six highly efficient orthologs (ErCas12a, Lb5Cas12a, BsCas12a, Mb2Cas12a, TsCas12a, and MbCas12a) of Cas12a. Similarly, a related enzymatic activity harbouring Cas12b (C2c1) from *Alicyclobacillus acidiphilus,* i.e. AaCas12b was prospected as a potential add-on to the tool kit. Another class II type VI-A Cas protein, i.e. Cas13a (C2c2) is an effective tool that possesses RNA-guided RNase activity (Abudayyeh et al. 2016). Single strand RNA (ssRNA) targeting LshCas13a (*Leptotrichia shahii*) and other orthologs (b,c,d) have two HEPN (Higher Eukaryotes and Prokaryotes Nucleotide-binding) domain with no requirement of PAM (Bandaru et al. 2020). Cas13a has been utilized for RNA/transcript knockdown and RNA editing (REPAIR and RESCUE; Cox et al. 2017; Abudayyeh et al. 2019). Moreover, Cas13a has been utilized for SHERLOCK, PAC-MAN, and SARS/Covid 19 detection kits (Gootenberg et al. 2018; Joung et al. 2020; Zhang et al. 2020). In plants, it can be utilized to gain viral resistance against specific viral pathogens (Abudayyeh et al. 2017). Another class II type V effector, i.e. the Cas14 family (Cas14a-c: 400–700 amino acids) present in archaea came as a significant discovery (Harrington et al. 2018; Savage 2019). Cas14s can target both ssDNA and dsDNA with no PAM or AT-rich PAM (5′-TTAT-3′) requirement (Karvelis et al. 2019). Due to sensitivity towards mismatching, it can be used for high precision SNP genotyping and because of trans cleavage activity it can be used as a Cas14-DETECTOR and to gain viral resistance in plants (Aquino-Jarquin 2019; Khan et al. 2019a). With continued hustle to discover better alternatives for GEd, the database mining led to the discovery of smaller Cas proteins such as Cas12f and the features closely related to the previously known Cas 14s (Karvelis et al. 2020). The recent classification thus unifies these proteins together Cas12f1 (Cas14a and type V-U3), Cas12f2 (Cas14b), and Cas12f3 (Cas14c, type V-U2 and U4) and expands the utility tools in the GEd artillery (Makarova et al. 2020).

Further expanding the smaller type V effectors family, DpbCasX (Deltaproteobacteria) is one such mini (~980 aa) novel protein (Liu et al. 2019a). CasX (alias Cas12e) is a dual RNA (crRNA and tracrRNA) guided protein (naturally combined into single-guide RNA; sgRNA) targeting dsDNA adjacent to 5′-TTCN-3′ PAM to generate 10 nt staggered break (Yang and Patel 2019). However, it shows the nominal *trans* activity as compared to other type V effectors which highlight structural differences between Cas X and other enzymes. Recently, in Doudna's lab

a supercompact CRISPR-CasΦ system encoded by bacteriophage genome has been discovered, where a bacteriophage uses the system to target other competing phages. CasΦ (Cas12j) also has a C-terminal RuvC domain but shares no similarity (<7% amino acid identity) with type V effectors, rather it is remotely related to the TnpB enzymes. The CasΦ locus lacks the spacer acquisition enzymes such as Cas1, 2, and 4 which results in a really compact CRISPR array and the locus also lacks the presence of tracrRNA. CasΦ represents the consolidated form of the CRISPR-Cas system and thus can be utilized to its full potential for genome manipulation (Pausch et al. 2020). With the discovery of such versatile, flexible, and miniature (400–1093 amino acids) effectors, the Cas12 family is expanding and till date, there are 11 subtypes of type V which has been reported, namely Cas12a to k (Li et al. 2021) and a subtype V-U which is more closely related to transposon TnpB. Cas12a, Cas12b, Cas12e, Cas12h, and Cas12i specifically target dsDNA with PAM assisted unwinding (Yan et al. 2019). However, an ortholog Cas12g (thermostable) was reported to initially target ssRNA and then indiscriminately degrade both ssDNA and ssRNA (Chen et al. 2018). Till now, class 2 effectors have dominated the terrain of GEd primarily because it utilizes single subunit protein effectors, whereas class 1 CRISPR-Cas system utilizes multiple subunit protein effectors (Makarova et al. 2018). Type III effectors of class 1 are known to target RNA substrates and hold the potential to be developed into diagnostic tools or to attain tolerance against viruses or mobile genetic elements (MGE) (Samai et al. 2015; Staals et al. 2014) in any system. Type III effectors are divided into III-A (Csm), III-B (Cmr), III-C, III-D subtypes, and the common feature between these subtypes is the presence of Cas10 (Csm1 or Cmr2) (Burmistrz et al. 2020) in the complex. Cas10 predominantly has two domains (Makarova et al. 2018) notably the palm domain (the cyclase activity of palm domain is absent in type III-C effectors) and a nuclease HD-type (unavailable in type III-D effectors) domain (Zhu et al. 2018). Typically, this multi-subunit complex protein effector is composed of two parallel filaments from which the first filament is generally made of six subunits of Cas7 protein and the other is made up of three subunits of Cas11 homolog (Csm2 or Cmr5) protein. The crRNA is stretched in between these filaments (Lintner et al. 2011) and the 5′- end having the handle derived from repeat is capped by Cas10 and Cas5 (Csm4 or Csm3) proteins (Staals et al. 2014), whereas the maturation of crRNA from pre-crRNA is catalysed by the Cas6 (Nickel et al. 2018) protein. Cas7 as a family of protein (members like *Thermofilum pendens* Csc2 protein) has members in both type I-D (commonly present in Archaea and Cyanobacteria) and type III branches of classification (Staals and Brouns 2013; Cai et al. 2013). Moreover, the protein organization in CASCADE (CRISPR-associated interference complex type I) and type III complex is similar, and proteins of type I-D have HD domain fused to the Cas10 (type III protein) and thus Cas7 is considered as the evolutionary link between type I and type III CRISPR-Cas system (Hrle et al. 2014). Exceptionally, the type III system has three different nuclease activities, and primarily it possesses sequence-specific RNase activity where acidic residues of the RNA-recognition motif (RRM) of Cas7 targets a specific RNA sequence (Estrella et al. 2016). The CRISPR-associated Rossman fold (CARF) located at the N-terminal of Csm6 sense the presence of the cyclic

oligoadenylate while the C-terminally located higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domain non-specifically cleaves the ssRNA (Niewoehner and Jinek 2016) molecule. Considering the potency of Csm6 protein, it has been included in the SHERLOCKv2 and this resulted in the three fold increase in the efficiency of the technique by improving the reporter signal (Gootenberg et al. 2017; Kellner et al. 2019). With continued exploration and screening over 11 billion protein sequences revealed the existence of a single-protein effector under type III-D2 CRISPR-Cas system, referred as Cas7x3 which have three Cas7 protein fused into a single protein (Özcan et al. 2021). In consonance, a novel breakthrough has resulted in the discovery of a programmable type III RNA targeting single-protein effector termed as Cas7–11, structurally having four Cas7 proteins fused to a putative Cas11 protein (Makarova et al. 2020). *Di*Cas7–11 from *Desulfonema ishimotonii* is a programmable RNase with no reported collateral activity. The discovery of this protein further expands the classification nomenclature by adding a type III-E subtype to the previously known subtypes. Both Cas7x3 and Cas7–11 process their own pre-crRNA into mature crRNA for targeting specific sequence template and not even display any toxic effect in mammalian cells (Özcan et al. 2021). However, they still need to be developed into programmable CRISPR-Cas tools to utilize their full potential for GEd across different systems including plants.

Introducing foreign DNA and generating of DSBs in any system for GEd raised some regulatory concerns which led to the evolution of the DNA-free GEd strategy. Under this, the ribonucleoproteins (RNP) which are pre-assembled Cas nucleases with the target-specific gRNA are delivered into the target system to achieve the desired GEd in plant and animal systems (Woo et al. 2015; Wu et al. 2020). The use of CRISPR-Cas tool in prokaryotic (Qi et al. 2013) and eukaryotic (Gilbert et al. 2013) systems introducing DSB leads to unexpected changes and toxicity. Precision transcriptional regulation without the introduction of any DSBs, i.e. without changing the underlying DNA sequence, with the strategies like CRISPRi and CRISPRa has revolutionized the field of genetic engineering (Liu et al. 2019b). In CRISPRi and CRISPRa, a dCas9 is fused with transcriptional effector to either repress (repressor like Kruppel associated box, or KRAB) or activate (activators like VP64 and p65) the gene expression (Lawhorn et al. 2014; Mali et al. 2013a, b). The newly developed customizable epigenome memory writer 'CRIPSR on-off' technique can alter gene expression by generating heritable epigenome modification. CRISPRoff is a fusion protein with dCas9 with DNA methyltransferase (DNMT1) and KRAB domains to silence the gene expression. However, the modifications are specific, tunable, and reversible as the methylation can be removed (inhibitor of DNMT1, i.e. 5-aza-2′-deoxycytidine (5-aza-dC)) by CRISPRon, and gene expression can be activated via recruitment of the transcriptional machinery. Genome-wide screen helped to find the targetable genes and showed that genes lacking the CpG islands can also be silenced with the CRISPR-off technique (Nunez et al. 2021). 'CRISPR on-off' is a complementary technique to the already existing CRIPSRi, CRISPRa, and CRISPR nuclear approaches.

Base editors (BEs) in conjunction with the CRISPR-Cas tool have been used for precise, specific single base modification with no induction of DSB and as an

alternative to HDR-based GEd (Komor et al. 2016). dCas9 or any inactive RNA-guided Cas protein with cytidine base editor (CBE; cytidine deaminase) can catalyse target specific C-to-U (Uracil recognized as T) base substitution which results in C-G to T-A base pair conversion (Rees and Liu 2018) and with adenine base editor (ABE; deoxyadenosine deaminase), it can catalyse the A-to-I (Inosine; recognized as G) base substitution which results in A-T to G-C base pair conversion (Gaudelli et al. 2017), respectively. CRISPR-BE has gone under severe optimization and development and in a recent generation a D10A nCas9 (to induce nick in unedited strand) fused with cytidine deaminase enzyme, i.e. rAPOBEC1 (rat apolipoprotein B mRNA editing enzyme) or to Lamprey cytidine deaminase (pmCDA1) for activation-induced cytidine deaminase (AID) at N-terminal, and two copies of uracil DNA glycosylase inhibitor (UGI) at C-terminal are used for base editing. The fusion of UGI increases the efficiency of editing in the case of C-to-U conversion as it helps in retaining the U in the target sequence till the next cycle of replication by inhibiting the inherent conversion of U-to-C again by uracil DNA glycosylase (UDG) (Abdullaha et al. 2020). Whereas, nCas9 (D10A) was also utilized in conjunction with the TadA (tRNA adenosine deaminase) and TadA* (modified at K157N, I156F, E155V, R152P, D147Y, S146C, H123Y, D108 N, A106V, L84F, R51L, P48A, H36L, W23R) domains connected via varying linker length, for ABE optimization and development (Bharat et al. 2020). Along with DNA base editing, RNA base editing can be achieved with the RNA directed RNA targeting dCas13. RNA editing comprises REPAIR (RNA editing for programmable A-to-I (G) replacement; catalysed by ADARs) and RESCUE (RNA editing for specific C-to-U exchange; catalysed by cytidine deaminase) techniques in plants (Abudayyeh et al. 2019). Although the base editing approach has faced few challenges in terms of off-target, range of editing, and bystander editing (Jeong et al. 2020). These shortcomings have led to the revolutionary discovery of prime editing (PE) which is based on the search and replace ideology and is a template free strategy (Anzalone et al. 2019). PE2 system is dependent on an amalgamation of the nCas9 (H840A), reverse transcriptase (RT; M-MLV from mouse-murine leukaemia virus), and the prime guide RNA (pegRNA). pegRNA have a primer binding site (PBS) sharing sequence complementarity to the sequence of the nicked DNA strand upstream of PAM and a reverse transcriptase template strand (RT strand). The 3′ flap is utilized as the primer to transcribe the desired sequence (written in the RT template), whereas the 5′ flap is cleaved via structure-specific host endogenous flap endonuclease (FEN1; Flap endonuclease *Homo sapien*s). Later the edited strand is ligated after 5′ flap digestion forming a heteroduplex of edited and unedited strands co-exist (Anzalone et al. 2019). The induction of a second nick on the unedited strand 10–12 nt away from the original pegRNA cut on the edited strand resulted in the development of the PE3 system. In PE3 when the unedited strand is repaired after induction of the second nick, it leads to the formation of the homoduplex of the edited dsDNA (Anzalone et al. 2020). In order to avoid incorporation of indel mutation by PE3 while repairing, in PE3b the second nick was introduced after successful completion of the flap resolution and editing (Kantor et al. 2020). PE till now displays really low events of off-target in any system

(Scholefield and Harrison 2021). In a study by Lin et al. (2020), the efficiency of the plant PE system increased at some locus by using the PPE-Ribozyme (PPE-R) system where the PE protein transcript is expressed by Polymerase II (Pol II) and pegRNA is processed by the ribozyme. Prime editing has come as a boon in the field of GEd and has expanded the toolbox for deep genome modification with enhanced efficiency, specificity, and tenacity even in polyploidy genomes such as wheat, as well (Lin et al. 2020).

9.5 Revisiting Challenges and Impediments of CRISPR-Based Approach for Precise Genome Editing

CRISPR is regularly portrayed as 'cut and paste' approach for genes, but the actual procedure is not that easy. However, further research is needed to gain a deeper understanding of the CRISPR-Cas process and its neoteric uses in plants. To date, researchers face umpteen obstacles related to utilizing the CRISPR approach in plant research, including hurdles in GMO regulation. Recently, researchers have achieved huge achievements utilizing CRISPR in its native and closely related organisms. But, employing CRISPR into bigger genomes containing complex organisms has accompanied its own set of difficulties. Some plants have multiple copies of each chromosome, for instance, hexaploid wheat (6 copies), strawberries (up to 10 copies), which is become strenuous to engineer compared to humans and animals. Subsequently, the probability of getting target gene editing in each copy decreases as the quantity of chromosome copies increases (Yang et al. 2020). Scientists are improving traditional CRISPR-Cas workflow by employing varying modifications so that multiple copies of the identical gene can be altered at once (Wilson et al. 2019; Lin et al. 2020; Jouanin et al. 2020; Smedley et al. 2021). Lamentably, this type of alteration sometimes create off-target mutation/s. Screening of accurate mutation and potential off-target sites is a very sensitive and significant challenge in the field of gene editing.

Earlier PCR/RE strategy was utilized to screen mutation in edited plants (Shan et al. 2014). The T7 endonuclease I (*T7E1*) assay was employed to detect off-target mutations; however, it is neither feasible nor cost-effective for large-scale screening due to its deprived sensitivity. Therefore, RNA-guided endonucleases, i.e. SpCas9- or FnCpf1- based PCR/RNP method for identifying indel/s, overcome the PCR/RE strategy (Liang et al. 2018). Unlike *T7EI*, this PCR/RNP-based technique can differentiate the mutant types, i.e. homozygous, heterozygous, bi-allelic, and mosaic mutants. It is also a SNPs independent mutation detection method essential for polyploidy plants like wheat (Liang et al. 2018). Numerous web-based approaches, for instance, deep sequencing (mutation detection range: 0.01–0.1%), genome-wide, unbiased identification of DSBs facilitated by sequencing (GUIDE-seq), RNA-guided endonucleases (RGEN), had been widely adapted (Wu et al. 2014; Zhang et al. 2015; Tsai and Joung 2016; Kosicki et al. 2018). Consequently, different bioinformatics-based programs (TALE-NT, CAS-OFF Finder, PROGNOS) have been developed to profile off-target mutations via CRISPR-Cas

nucleases (Fine et al. 2013; Listgarten et al. 2018; Minkenberg et al. 2019). Recently, genome-wide off-target edit frequencies were identified using the whole-genome resequencing (WGRS) approach in rice, maize, cotton (Tang et al. 2018; Lee et al. 2019; Li et al. 2019).

In addition, the plant regeneration and transformation approach is quintessential for delivering the editing reagents into plant cells for genome editing. Wherein, genotype-dependency is one of the major bottlenecks in completely appearing the incredible capability of genome altering in plant species (Alpeter et al. 2016). The development regulator (DR) genes of maize: *Baby Boom (Bbm)* and *Wuschel2 (Wus2)* in combination with phytohormones lead to enhance the transformation efficiency in plants (Lowe et al. 2016; Maher et al. 2020). Moreover, *Agrobacterium*-mediated transformation is frequently restricted due to the narrow range of genotypes within a species. As well as plant growth conditions, co-incubation time & temperature, pre-treatment with phytohormones, variability of *Agrobacterium tumefaciens* are well-known factors to affect transformation efficiency (Zambre et al. 2003; Gelvin 2006). However, these shortcomings can be overcome by utilizing the biolistic transformation approach due to its efficient and potent high transformation efficiency (Wu et al. 2015; Li et al. 2019; Kaul et al. 2021). The CRISPR-Cas-based genome editing in crop plants can only be manifested by fine-tuning the targeted gene or genetic elements (Kwon et al. 2019; Oliva et al. 2019).

Identifying the targets (quantity) due to an inadequate understanding of biological networks and their interactions with environmental factors is another critical obstacle for CRISPR-Cas-based plant genome editing. Applications of multidisciplinary strategy, for instance, genome-wide, and high-throughput functional genomics strategy for identification of beneficial agronomic traits harbouring targets in both the model and non-model crop plants are crucial for genome editing (Lu et al. 2017; Meng et al. 2017; Araus et al. 2018). Alongside, the achievement of high base substitution efficiency via fragment knockout and knockin of homology donor repair (HDR) is an important implication for crop enhancement. However, precision editing in plants employing an HDR-based approach is a significant challenge due to its lower editing potential. Optimizing the optimal quantity and the effective delivery methods of the donor DNA template might ease the base substitution editing approach (Kaul et al. 2020a, b).

The presence of protein inhibitors of CRISPR-Cas systems, known as anti-CRISPR (Acr) proteins, enables the generation of more precision in CRISPR-Cas-based GEd. More than 50 Acr proteins are currently shown to interact with CRISPR-Cas variants, for instance, Cascade-Cas3, Cas9, Cas12, and Cas13 (Dolgin 2019; Marino et al. 2020). The functional mechanism of ACr proteins is one of three ways: firstly, prevention of DNA binding: Acr either blocks or reduced Cas9's interaction with the PAM recognition site; secondly, prevention of crRNA loading: the interaction of Cas9 may disrupt or prevents the proper integration of the crRNA-Cas complex; and thirdly, and blocking of DNA extraction: Acr binds with HNH endonuclease domain of Cas9 and inhibits its activity (Dong et al. 2017; Zhu et al. 2019). However, Acrs can be used to eliminate allergies in unidentified areas

(Aschenbrenner et al. 2020; Shin et al. 2017), unwanted mutations in unintentional cell types or tissues (Hirosawa et al. 2019; Hoffmann et al. 2020). In addition, Acrs (AcrII4s) can be employed as a ligand biosensor to detect and measure CRISPR-Cas9 RNP affinity reagents (Johnston et al. 2019). Similarly, other alternative approaches also being developed to prevent Cas9 activity, for instance, nucleic acid-base inhibitors and (Barkau et al. 2021) and smaller molecules of inhibitors (Maji et al. 2019). Despite precision genome alteration, Acrs provide a prospect to exploit their ability to inhibit Cas9 and to address other engineering limitations of the Cas9 genome.

Comparative genomic analysis revealed that CRISPR and its associated proteins, especially Cas9, were present in umpteen bacterial phylogenetic groups (Lillestøl et al. 2006; Makarova et al. 2006). Cas9 from *S. pyogenes* showed 23 to 58% and 35% similarity to Cas9 proteins from *Streptococcus thermophilus and Lactobacillus plantarum*, respectively. Those organisms were utilized for various human edible food processing, for instance, yoghurt, cheese, kefir, fermented drinks, and so many (Settachaimongkon et al. 2014; Sidira et al. 2017; Behera et al. 2018). Thus, humans were exposed to Cas9 protein in their diet long before the development of CRISPR-Cas9 genome editing. Additionally, Cas9 from *S. pyogenes* showed 80% sequence similarities with a variety of gram-positive and negative bacteria that present in human body (Qin et al. 2010; Louwen et al. 2014). The above-mentioned findings do not imply that human exposure to Cas9 used in genome-editing planning is insignificant (Pineda et al. 2019). However, the biosafety risk assessment regarding human exposure to Cas9 after consuming GEd plants product requires further testing.

Another hindrance is the adoption of edited crop plants success in natural field conditions. An enormous number of researches on genome editing reported so far, but the majority is only about proof of concepts in the greenhouse environment. The performances uncertainties of the edited plants are still existed due to the lack of field trials. Despite all these challenges and impediments, the CRISPR-Cas9 approach is considered the most promising tool due to its precision editing. This approach incorporates numerous heritable traits in plants, which may produce modified plants similar to those developed through conventional breeding. CRISPR-Cas9 strategy leads towards a progressive change via high yielding crop plant production to meet food security globally.

9.6 Overcoming Challenges for 'Off-Target' Mutations

Alteration of plant genome employing the CRISPR-Cas approach sometimes resulted in off-target effects (alteration of the additional region beyond the target region of the genome), which is a pivotal impediment of this application. However, numerous strategies can be employed to minimize off-target mutations. Till date, above than 30 plant varieties (~100 attribute traits) have been edited successfully employing the CRISPR-Cas9system. The precession binding of Cas9 depends on the 7–8 nucleotides seed sequence and the existence of the PAM close to the target sequence, but unwanted insertions/deletions could happen in the genome

(Hajiahmadi et al. 2019). To improve genome-editing efficiency, scientists devised in vivo/vitro biological analysis and algorithm-based computational methods to uncover and increase gene editing efficiency. Promoters and target genes are essential elements involved in the regulation of gene expression by modifying transcription factors (TFs) via RNA polymerase recognition. The specificity of a promoter is essential for controlling transgenic expression in target tissues or throughout the plant. Over the last few years, constitutive promoters like the cauliflower mosaic virus (CaMV35S) promoter (Paparini and Romano-Spica 2006; McCaw et al. 2021) and the maize ubiquitin (pZmUbi) promoter (Xu et al. 2018; Samalov and Moore 2021) have already been used. In dicot plants, the CaMV35S driven promoter showed a high level of expression, in contrast, monocot plants employ pZmUbi promoter more effectively. In *Arabidopsis*, the promoters of (rd29A and rd29B) genes showed well performance to a variety of stress stimuli, such as salinity and drought (Bihmidine et al. 2013). Salt induces activity in the BADH promoter from *Suaeda liaotungensis* (Zhang et al. 2008). The Rab16A promoter might up-regulate GUS expression in transgenic rice under salt stress (Rai et al. 2009). The TsVP1 promoter from *Thellungiella halophila* is effective in almost all tissues except the seeds, and salt stress in leaves and roots, particularly root tips (Sun et al. 2010). DREB2 coordinated expression of transcription factors will generate successful regulatory activity; thus, monocotyledonous plant promoters' operations are higher in monocots as in dicots. Heat-shock protein 17.5E (Hsp17.5E) gene promoter from soybean (*Glycine max*) has been utilized to direct Cas9 expression in rice for genome editing. Several methods have been described to reduce off-target mutations; primarily, the effect can be minimized by using a highly specific Cas nuclease or a stringent sgRNA design that differs from the other genomic regions by three mismatches, in addition to one mismatch in the PAM proximal region. The designed sgRNAs determine the occurrence of a 'off-target' effect; sgRNAs with more than 50% GC content are competent enough to promote on-target mutagenesis due to strong binding to the target sites (Kim et al. 2015; Ren et al. 2014). Precisely designed sgRNAs enable specific targeting, even if so many homologous loci are present in the studied genome (Baysal et al. 2016). Many recently introduced computer-based innovations, i.e. Cas-OFF Finder that identifies the unique target sequences and possible off-target sites in the genomes of various species minimizes the off-target sites (Cong et al. 2013a, b; Hsu et al. 2013). CRISPR-P enables gRNA design for substantially all plant species with accessible genome sequences, as well as off-target site and restriction enzyme sequence analyses (Lei et al. 2014). Subsequently, CRISPR-PLANT has used a genome-wide platform of highly comprehensive RNAs in more than eight plant species and favours restriction endonuclease analysis of target sites. Various guidelines for sgRNAs design to lower the potential off-target effects which can be beneficial for various crop species have been documented in recent articles. It is critical to avoid using sgRNAs with seeds that are homologous to various other genome loci in order to minimize off-target mutations. Indispensable components of CRISPR-Cas9, the PAM and seed sequence, need to be carefully designed.

The sites cleaved with the genome-editing tool CRISPR-Cas9 system can be both on-target and off-target sites and that need to appropriately balance according to the different experimental purposes. To avoid these events, bioinformatics tools, for instance, E-CRISPR and Cas OT, can promote sgRNA design concerning whole-genome sequence information. A vector performs as a vehicle for delivering an element of interest. The vector only needs two components: the single-guide RNA (sgRNA) sequence and the Cas9 gene, both of which may be expressed from a single vector system. A variable crRNA (approximately 20 bp) and a constant tracrRNA make up the sgRNA. To boost performance and eliminate off-target impacts, various target sequences of the same gene might be introduced. The Cas9 gene encompasses multiple nuclear localization signals (NLS) for nuclear targeting, and Cas9 can be defined in a variety of ways (Heintze et al. 2013). In addition to this, various delivery methods such as agrobacterium-mediated, bombardment or biolistic approach, PEG-mediated protoplast, and floral-dip are widely used in plants to regulate genes properly (Table 9.2). There are widely used transformation mechanisms, but the agrobacterium-mediated method is extensively used for the delivery of various Cas enzymes (Ali et al. 2015). The RNP strategy is another important way to reduce the intended effect when sgRNA and RNP nuclease processes are introduced by biolistic and electroporation into plant protoplasts, showing a small frequency of target changes and successfully reported on various plants such as maize (*Zea mays*), rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), and many others (Woo et al. 2015). Nanoparticle-mediated RNP delivery systems have been successfully adopted in plant species due to the reduction of unwanted changes via the potentiality of RNP. The recommended system is time-effective, affordable, species-independent, and equipment-independent CRISPR-Cas9 vector or ribonucleoprotein complexes. Consequently, the specificity of CRISPR-Cas9 is influenced by several parameters, including the aggregation of the Cas9/sgRNA complex and the characteristics of the off-target sites.

Off-target mutation is a major apprehension in the emergence of the CRISPR-Cas9 system in plants used whole genome sequencing WGS and deep sequencing, respectively, to investigate CRISPR-Cas9 specificity in *Arabidopsis thaliana*. According to their findings, CRISPR-Cas9 is highly specific in plants owing to low Cas9 protein expression levels, which resulted in undetectable levels of off-target alterations. Most CRISPR-Cas9 investigations in plants have reported a low frequency of off-target mutation, which could be attributed to its occurrence in non-coding areas and, as a result, the inability to detect off-target implications (Zhang et al. 2018a). CRISPR-PLANT v2 is a popular tool for predicting off-target mutations in plants. This software has the highest sensitivity of among all off-target prediction tool and can be utilized in the genomes of seven plants, including *Sorghum bicolor, Arabidopsis thaliana*, *Oryza sativa, Medicago truncatula, Solanum lycopersicum, Glycine max, and Brachypodium distachyon*. However, in eukaryotes, several strategies for off-target recognition have been introduced, including deep sequencing and online prediction software. Although in vitro approaches for investigating potential off-target sites have been established, exact prophecies of the prevalence of undesired mutations in vivo are difficult to

Table 9.2 Novel delivery approaches of CRISPR-Cas based genome editing in agronomically important crop plants

Crop plant	CRISPR-Cas9 ribonucleoprotein complexes (RNP)-based vector	Targeted genes	Delivery method	References
Apple (*Malus domestica*)	Cas9-sgRNA ribonucleoprotein complexes	DIPM-1, 2, 4	PEG-mediated CRISPR-Cas9 components delivery	Malnoy et al. (2016)
Soybean (*Glycine max*)	pCas9-GmU6-sgRNA, pCas9AtU6 sgRNA	Glyma08g02290, Glyma12g37050, Glyma06g14180	PEG-mediated CRISPR-Cas9 components delivery	Sun et al. (2015)
	QC810 and RTW830, QC799 and RTW831	DD20, DD43	Particle bombardment method for CRISPR-Cas9 component delivery	Li et al. (2015)
	p201N Cas9	GFP transgene	*Agrobacterium*-mediated delivery of CRISPR-Cas9 components	Jacobs et al. (2015)
Rice (*Oryza sativum*)	pRGE3, pRGE6, pUC19-*Os*Cas9, pJIT163-2NLSCas9	*Os*MPK5, *Os*SWEET14, *Os*SWEET11, *Os*PDS, *Os*BADH2, crtI, OsPDS1, OsPDS, OsBADH2, OsPDS, OsDEP1	PEG-mediated CRISPR-Cas9 components delivery	Jiang et al. (2013)
	pCam1300-CRISPR-B CRISPR-RNP complex pJIT163-2NLSCas9 p*Os*U3-sgRNA, pJIT163-2NLSCas9, VK005	crtI,,*Os*PDS1, *Os*PDS1, *Os*DEP1	Particle bombardment method for CRISPR-Cas9 component delivery	Banakar et al. (2019)
	VK005	ISA1	*Agrobacterium*-mediated delivery of CRISPR-Cas9 components	Shufen et al. (2019)
	Cas9-sgRNA Ribonuclease	PhACO1	PEG-mediated CRISPR-Cas9	Xu et al. (2020)

(continued)

Table 9.2 (continued)

Crop plant	CRISPR-Cas9 ribonucleoprotein complexes (RNP)-based vector	Targeted genes	Delivery method	References
Petunia (*Petunia hybrida)*	protein complexes (RNPs)		components delivery	
Wheat (*Triticum aestivum*)	pCR8-U6-gRNA	*Ta*EPSPS	PEG-mediated CRISPR-Cas9 components delivery	Arndell et al. (2019)
	pJIT163-ubi	TaMLO-A1, TaMLO B1, TaMLO-D1	Particle bombardment method for CRISPR-Cas9 component delivery	Wang et al. (2014)
	pBI121	Inox, PDS	*Agrobacterium*-mediated delivery of CRISPR-Cas9 components	Upadhyay et al. (2013)
Maize (*Zea mays*)	pZmU3-gRNA, T-nCas9	*Zm*IPK, *Zm*ALS1, *Zm*ALS2	PEG-mediated CRISPR-Cas9 components delivery	Svitashev et al. (2015)
	pSB11-ubi:Cas9	LIG1, Ms26, Ms45, ALS1, ALS2	Particle bombardment method for CRISPR-Cas9 component delivery	Liang et al. (2014)
	pMCG1005	Argonaute 18, Dihydroflavonol-4-reductase Strain- EHA101	*Agrobacterium*-mediated delivery of CRISPR-Cas9 components	Char et al. (2017)
Barley (*Hordeum vulgare*)	pCas9:sgRNA	ENGase	PEG-mediated CRISPR-Cas9 components delivery	Kapusi et al. (2017)
Arabidopsis (*Arabidopsis thaliana*)	pCAMBIA1300	AtPDS3, AtFLS2, RACK1b, RACK1c, BRI1, GAI, JAZ1	*Agrobacterium*-mediated delivery of CRISPR-Cas9 components	Feng et al. (2013)
Tomato (*Solanum lycopersicum*)	pYLCRISPR-Cas9	SGR1, LCY-E, Blc, LCY-B1, LCY-B2	1.1. *Agrobacterium*-mediated	Li et al. (2018b)
		SlCCD8		

(continued)

Table 9.2 (continued)

Crop plant	CRISPR-Cas9 ribonucleoprotein complexes (RNP)-based vector	Targeted genes	Delivery method	References
	pENTR-sgRNA: pMR290/Cas9		delivery of CRISPR-Cas9 components	Bari et al. (2019)
	pMDC32	StALS1		
	Cas9-sgRNA ribonucleoprotein complexes (RNPs)	GBSS(GT4)	PEG-mediated CRISPR-Cas9 components delivery	

acquire. Digenome-seq, SITE-seq, and CIRCLE-seq are the most used in vitro genome-wide detection systems and quantifying off-target effects (Cameron et al. 2017). Digested genome sequencing (Digenome-seq) is a reliable, delicate (~ 0.1%), and frequently used for detecting Cas9 and other nucleases for off-target effects in genome-wide. The most prominent strategies established to solve the Digenome-seq difficulties are selective enrichment and identification of tagged genomic DNA ends by sequencing (SITE-Seq), followed by circularization for in vitro reporting of cleavage effects by sequencing (CIRCLE–seq). The SITE-Seq approach could map all of the Cas9 cleavage sites in a genome (Naeem et al. 2020). This study employed sgRNA and Cas9 RNPs in a cell-free environment to cleave purified genomic DNA. Afterwards, both (on- and off-target) cleavage fragments are tagged, and off-target sites are detected using next-generation sequencing (NGS). The total amount of off-target sites has a considerable impact on nuclease concentration. RNPs (low to high) were employed as variable concentrations to recover off-target locations with low and high cleavage sensitivity. When low doses of RNPs are subjected to cell identification, they exhibit a significant proclivity for off-target alterations. SITE-Seq also requires less NGS read depth than Digenome-seq, with some procedural modifications; CIRCLE-Seq has a similar concept. In CIRCLE-Seq, the DNA is first trimmed, then circularized, and finally destroyed. Prior to treatment with (Cas9–sgRNA) RNPs, the degradation phase practically eliminates high background DNA to boost sensitivity, condensing NGS read space that would otherwise be squandered on random reads. Following that, DNA is linearized using Cas9 and then exposed to NGS for off-target detection. CIRCLE-Seq, like SITE-Seq, could be employed in a reference-independent manner to discover off-target cleavage sites, for organisms whose genome sequences are less well-characterized and/or show considerable genetic variability. Several approaches were proposed, including bioinformatics tools for in silico detection of off-target mutations and increased on-target efficiency to mitigate off-target impacts. However, off-target effects might have happened, yet the alterations will be lower than those developed via conventional breeding. Thus, GEd employing the CRISPR-

Cas approach produces a far less off-target effect in comparison to the traditional crop enhancement strategy.

9.7 CRISPR Implementation in Sustainable Agriculture: Climate-Smart and Nutritionally Secure Crops

The global population is assumed to increase 9.2 billion in 2050, and so agronomic production needs to rise by about 70% from existing levels to encounter the increased demand of food, as predicted by Food and Agriculture Organization (accessed on 1 February 2021). Cereal crops such as rice, wheat, and maize are the world's most important sources of energies intended for humans, livestock feed for animals, and raw material for biofuel. Therefore, improving cereal-crop-grain production is critical to meet further demand. For most cereal crops, the annual yield relates to grain production. Until the last decade, the core crop improvement strategies banked upon chemical mutations, hybrids, and expression of trans gene/s (Chari et al. 2017). The shift from the conventional breeding approach, which relied on the occurrence of the naturally relevant variations to the molecular breeding approach, has alleviated some barriers attached with the conventional methods. Now, the targeted traits can be swiftly incorporated into the plant system to generate a new plant variety for food as well as nutritional security. The gradual increase in human population, deteriorating arable land conditions, the drastic climatic changes through uplifted temperature, and escalated pollutants by excessive emission of greenhouse gases (GHG) causes threat to agriculture and food security (Asseng et al. 2014). Therefore, to develop climate-smart crops via sustainable agriculture, the need of the hour is to achieve a 'triple win' by targeting enhanced productivity, improved adaptivity, and GHG mitigation. Targeted GEd made the revolution in molecular biology by discovering programmable SSNs (Chandrasegaran and Carroll 2016). CRISPR-Cas-based GEd has become an essential tool that has effectively caused enormous ripple effects in plant research. Throughout the last decade, we have seen fast development in numerous fields, including plant functional genomics and crop enhancement (>45 genera of plants) in a manner that straightforwardly benefits consumers (Shan et al. 2020). In plant species, the practice of CRISPR-Cas9-mediated genome alteration in diverse crops was successful, for instance, in maize, rice, wheat, maize, and cotton. In 2015, the fourth quarter experienced the employment of DNA-free, pre-assembled RNP complex of CRISPR-Cas9 for genome alteration in model plants such as *Arabidopsis*, rice, lettuce, tobacco, wheat, maize, and so on (Woo et al. 2015). An extremely systematic transgene integration free GEd and most importantly callus-based methodology were introduced for wheat pertaining transitory expression of CRISPR-Cas9 in the form of DNA or RNA (dubbed TECCDNA or TECCRNA, respectively), the technique had the potential to be applied in different crops (Zhang et al. 2016). The crops developed via RNP complex mediated and TECCRNA-based editing techniques are foreign gene integration free, thus they could be spared from GMO regulatory concerns. In a recent study, by altering the sequence of a S gene, namely

SIAGAMOUS-LIKE 6 (SIAGL6) which is linked to enhanced fruit setting even under heat stress, tolerance towards high temperature was attained in tomato (Klap et al. 2017). Further, optimization of method for targeting multiple genes via CRISPR-Cas9 in a single organism was done for numerous crops which include rice, cotton, maize, and wheat (Miao et al. 2013; Gao et al. 2017; Char et al. 2017; Wang et al. 2018b). Thus, CRISPR-Cas9 is a remarkable technique, which is potent enough to develop crop with multiple stress tolerance by choosing concurrently different S genes as a target in exclusively high productive but sensitive cultivars.

The growth of plants is linked to diverse developmental and environmental cues. Plants receive and respond to those cues via cellular signaling cascades, which regulate gene expression at the pre-mRNA level by tuning splicing patterns and controlling the transcript abundance at mature-mRNA level. Alternatively, spliced pre-mRNA represents the genome's coding potential for multi-exon genes and synchronizes gene expression by different mechanisms. In *Solanum tuberosum* (potato), vegetative reproduction (tuberization) is regulated via photoperiod, for example, flowering controlling transcription factor- *StCDF1* (CYCLING DOF FACTOR 1), which regulate the antisense transcript of *StFLORE* to gain drought tolerance. Loss of function mutation in promoter of this *StFLORE* via CRISPR-Cas9 revealed drought tolerance via stomatal size and number regulation (Gonzales et al. 2020).

In agriculture, weed control is critical for a high yield of crop production, which can reduce the phytotoxicity of herbicides to crops, cut off the cost of the weeding, and upgrade the efficiency of the chemical weeding. Consequently, substantial attempts to develop herbicide -resistant crop varieties have been undertaken to contribute the frugal and economic tools to serve farmers for clean and effortless weed management. To develop robust herbicide-resistant crop plants, endogenous genes like cellulose synthase A catalytic subunit 3 (*CESA3*), splicing factor 3B subunit 1 (*SF3B1*) and more commonly acetolactate synthase (*ALS*), 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) are targeted for CRISPR-Cas9-mediated gene editing. The crucial amino acid substitution in *EPSPS* and *ALS* genes in rice employing CRISPR-Cas9 HDR-mediated machinery conferred resistance to glyphosate and sulfonylurea herbicide, respectively (Li et al. 2015; Sun et al. 2016). Similarly, T102I/P106S and T102I/P106A substitution were introduced in *EPSPS* gene of flax (Sauer et al. 2016) and cassava plant (Hummel et al. 2017). To acquire effective gene replacement, CRISPR-Cas9 is employed with target sequence-specific sgRNAs directing the CRISPR-associated RNA endoribonuclease csy4 from *Pseudomonas aeruginosa,* for sequence-specific induction of DSBs (Wang et al. 2021). Till now among the developed crop germplasm specifically resistant to herbicides, crops only resistance towards *ALS*-inhibiting herbicides, ACCase-inhibiting herbicides, and glyphosate has been successfully established. One of the greatest important applications intended for gene editing in agriculture is biotic stress resistance. The genetic mechanisms of the agents that cause biotic stressors in plants can be examined in order to overcome these stresses by GEd (Yin and Qiu 2019; Zafar et al. 2020; Pak et al. 2020). In addition to some crop species like rice, a CRISPR-Cas9 targeted mutation in the ethylene responsive factor,

OsERF922, has been effectively established to improve resistance to *Magnaporthe oryzae* blast disease (Wang et al. 2016). Similarly, *OsMPK5*, a negative regulator of biotic and abiotic stressors in rice, was identified for targeted mutagenesis in rice protoplasts utilizing three gRNAs by using a more precise gRNA design strategy with a low level of off-targets (Xie and Yang 2013). By producing genetically modified resistant crop varieties, which have proven to be a significant effort to fight against biotic stressors. Despite CRISPR-Cas9 inimitable accomplishment, there are substantial trials in incorporating this technology into agricultural research, especially with transformation-resistant crops reproduced asexually. Several projects are presently in progress to fine-tune CRISPR-Cas9-based technologies for precise editing in the plant genome of the target locus.

Consequently, crop improvement now targets not only improving quantity (yield), but quality (nutrition) of the crop product as well. Great quality food grains have a critical and direct impact on human health and well-being, as plants produce numerous molecules with anti-inflammatory, anti-cancerous, and anti-oxidation properties (Liu et al. 2021) that have beneficial effects on human health. Thus, plants are the major source of nutrients and natural dietary products and are considered as 'dietary doctors' as they can cure the prevalent undernourishment (FAO 2020). Thus, crops biofortified with micronutrients and minerals such as iron, zinc, selenium, and iodine can curb the nutrient deficiency in addition making anti-nutrient, such as heavy metals, phytate, and gluten, devoid crops can make the unavailable nutrient available for absorption in human body and protect humans from developing allergies, metabolic disorders, and chronic ailments. Conventional breeding accompanied by technology has saved humanity from the food crisis in the past but now these approaches culminate into no added benefit in enhancing the productivity, whereas new techniques like CRISPR-Cas hold the potential to drive the way towards sustainable food security. Recent breakthroughs (Table 9.3) have paved the way to introduce or manipulate the inherent genes to improve the quality of the majorly consumed crops. Alteration of genes for crop biofortification as well as for removing anti-nutrients have the potential to provide macro and micronutrients and alleviate the 'hidden hunger' (Majumder et al. 2019) condition as well as to cure and prevent the non-infectious, lifestyle related chronic ailments in humans. Although the CRISPR-Cas system is still developing and evolving, the latent potential of this technique has resulted in some benchmark studies, and it will continue to bestow the field of genetic engineering with more novel breakthroughs.

9.8 Amalgamation of MI and CRISPR-Based Genome Editing

Despite being one of the common genetic engineering techniques, CRISPR-Cas9 GEd relies on the accuracy of well-designed guide RNAs as it is an essential aspect of successful target gene editing (Cox et al. 2015). In recent years, various algorithms have been generated for assessing CRISPR activity (on-target) and specificity (off-target) as well as web-based tools for in silico gRNA designing (Henry et al. 2014; Zhu 2015). Machine learning (ML) and Artificial Intelligence

Table 9.3 Recent breakthroughs of CRISPR-Cas based approaches for the quality improvement of majorly consumed crops

Crop	Targeted gene	Gene function	Editing technique	Associated trait	References
Biofortification					
Rice	Starch-branching enzyme I (OsBEI) and starch-branching enzyme IIb (OsBEIIb)	OsBEI is expressed in all tissue, whereas OsBEIIb is expressed in the endosperm. Both genes are involved in the starch synthesis. Downregulation of these genes will result in the pathway direction towards amylose formation	Cas9: Knockout	High amylose content	Sun et al. (2017)
	Phytoene synthase (PSY), carotene desaturase (CrtI)	PSY converts geranylgeranyl-diphosphate (GGPP) to phytoene, and CrtI catalyses the desaturation reaction and introduces four double bond in phytoene and results in the formation of ζ-carotene and ultimately lycopene	Cas9: Gene insertion	High β-carotene content	Dong et al. (2020)
	Glutamate decarboxylase 3 (OsGAD3)	GAD is known to catalyse the conversion of L-glutamate to gamma-aminobutyric acid (GABA). OsGAD3 one of the five GAD genes present in rice is predominantly expressed in the seeds	Cas9: Deletion of C-terminal calmodulin binding domain of OsGAD3	High GABA content	Akama et al. (2020)
	Vacuolar iron transporter (OsVIT2)	VIT is an iron transporter which is expressed in various tissue and downregulation of this transporter can reduce Fe allocation to leaf sheath, nodes, and aleurone and in contrast increase allocation to leaf	Cas9: Knockout	High iron in rice grain	Che et al. (2021)

		blade and grains. High Fe in grains increase the bioavailability of Fe for absorption in human gut			
	Arsenite tolerant 1 (ASTOL1)	ASTOL1 encodes the chloroplast-localized O-acetylserine (thiol)lyase (OAS-TL) which catalyses the condensation of O-acetylserine (OAS) and sulphide to form cysteine (Cys). OAS in turn is produced by the action of serine acetyltransferase (SAT) on serine and acetyl CoA. SAT and OAS-TL form the hetero-oligomeric cysteine synthase complex (CSC)	Cas9: Gene correction	High selenium content	Sun et al. (2021)
	Omega-6 fatty acid desaturase (OsFAD2–1)	FAD2–1 is highly expressed in seeds and catalyses the desaturation of the C18:1 to C18: 2 at position sn-2 and conversion of oleic acid to linoleic acid	Cas9: Knockout	High-oleic acid proportion and reduced linolenic acid content	Abe et al. (2018)
	Phospholipase D gene (OsPLDα1)	It converts phosphatidylcholine (PC) to phosphatidic acid (PA) and changes the flux from the biosynthesis of lysophospholipid (LPL) to the accumulation of phytic acid	Cas9: Knockout	Increased LPL content	Khan et al. (2019b)
Banana	Lycopene ε-cyclase (MaLCY-E)	LCY-E catalyses the cyclization of the lycopene to produce α-carotene	Cas9: Knockout	High β-carotene content	Kaur et al. (2020)
Tomato		SlGAD2 and SlGAD3 primarily express at the time of fruit		High GABA content	

(continued)

Table 9.3 (continued)

Crop	Targeted gene	Gene function	Editing technique	Associated trait	References
	Glutamate decarboxylase 2 (SlGAD2), glutamate decarboxylase 3 (SlGAD3)	development and are key enzymes for the biosynthesis of GABA in tomato fruits	Cas9: Introduction of stop codon before autoinhibitory domain		Nonaka et al. (2017)
	Stay-green 1 (SGR1), phytoene desaturase (slyPDS), lycopene β-cyclase (slLCY-B1 & 2), lycopene ε-cyclase (slLCY-E)	SGR1 directly interacts with phytoene synthase 1 gene (PSY1) to regulate lycopene accumulation in ripening fruits. PDS catalyses the desaturation to convert the phytoene to ζ-carotene which further converts to the lycopene, whereas LCY-B1 & 2 and LCY-E catalyses the cyclization of the lycopene to produce β-carotene and α-carotene, respectively	Cas9: Gene replacement of PDS and knockout of SGR1, LCY-B, and LCY-E	Increased lycopene content	Li et al. (2018b)
Rapeseed	Fatty acid desaturase (BnFAD2)	FAD catalyses the desaturation of oleic acid to linoleic acid	Cas9: Knockout	High-oleic acid proportion	Okuzaki et al. (2018)
	BnTT8 (transcription factor involved in flavonoid pathway; TT comes from transparent testa mutant)	Play a role in flavonoid biosynthesis pathway in the seed coat and lead to the accumulation of oxidized form of flavonoids known as proanthocyanidins (PA; condensed tannins) in the endothelial layer of the inner integument which gives dark colour to the seed coat. It also play a role in the fatty acid synthesis pathway	Cas9: Knockout	High oil production and GPC	Zhai et al. (2020)
Camelina	Fatty acid desaturase (CsFAD2)		Cas9: Knockout		

		FAD catalyses the desaturation of oleic acid to linoleic acid		High-oleic acid proportion	Jiang et al. (2017)
Potato	Starch-branching enzyme 1 (StSBE1), starch-branching enzyme 2 (StSBE2)	SBE's genes are involved in the starch synthesisDown regulation of these genes will result in the pathway direction towards amylose formation. It basically reduces the branching frequency which results in compact starch formation	Cas9: Knockout	High amylose content	Tuncel et al. (2019)
Sweet potato	Granule-bound starch synthase I (IbGBSSI), starch-branching enzyme II (IbSBEII)	GBSSI is involved in amylose biosynthesis, and SBEII is involved in amylopectin biosynthesis	Cas9: Knockout	High amylose content	Wang et al. (2019)
Anti-nutrients					
Rapeseed	Inositol tetrakisphosphate kinase (BnITPK)	ITPK catalyses the penultimate step of phytate synthesis in rapeseed	Cas9: Knockout	Low phytic acid content	Sashidhar et al. (2020)
Wheat	α-gliadin genes	α-type gliadin is encoded by Gli-2 locus and is responsible for the gliadin, i.e. gluten protein in the wheat flour. Although there are numerous copies that code for gluten protein thus by targeting conserved region of α-gliadin genes, gluten content can be reduced	Cas9: Knockout	Low gluten content	Sanchez-Leon et al. (2018)
	Inositol 1,3,4,5,6-pentakisphosphate 2-kinase 1 (TaIPK1)	Catalyses the final step of PA biosynthesis by phosphorylating inositol pentaphosphate (IP5) to	Cas9: Knockout	Low phytic acid content and improved accumulation of Fe and Zn	Ibrahim et al. (2021)

(continued)

Table 9.3 (continued)

Crop	Targeted gene	Gene function	Editing technique	Associated trait	References
		phytate (inositol hexakisphosphate/IP6)			
Rice	Natural resistance- associated macrophage protein 5 (OsNramp5)	OsNramp5 located on the exo- and endo-dermis of root and is the major transporter for cd influx in plants	Cas9: Knockout	Low cd accumulations	Tang et al. (2017)
	Low affinity cation transporter (OsLCT1)	LCT regulates the cd transport in rice	Cas9: Knockout	Low cd accumulations	
	Phospholipase D gene (OsPLDα1)	From multidisciplinary actions of OsPLDα1, it has been highlighted that it is involved in lipid-dependent phytic acid biosynthesis pathway by converting phosphatidylcholine (PC) to phosphatidic acid (PA) and changing the flux from the biosynthesis of lysophospholipid (LPL) to the accumulation of phytic acid	Cas9: Knockout	Low phytic acid content	Khan et al. (2019b)
	Inositol 1,3,4-trisphosphate 5/6-kinase (OsITPK6)	IPTK is encoded by six genes in rice, and OsIPTK6 knockout significantly reduces phytic acid content in rice grains. By phosphorylating inositol triphosphate (IP3) at 5 and sixth position, ITPK plays a key role in phytic acid synthesis	Cas9: Knockout	Low phytic acid content	Jiang et al. (2019)
	Arsenite tolerant 1 (ASTOL1)	ASTOL1 encodes the chloroplast-localized O-acetylserine	Cas9: Gene correction	Low arsenic content	Sun et al. (2021)

		(thiol)lyase (OAS-TL) which catalyses the condensation of O-acetylserine (OAS) and sulphide to form cysteine (Cys). OAS in turn is produced by the action of serine acetyltransferase (SAT) on serine and acetyl CoA. SAT and OAS-TL form the hetero-oligomeric cysteine synthase complex (CSC). Cys is the key regulator of production of glutathione (GSH) and phytochelatins (PCs). GSH and PCs are responsible of sequestration of Ar into vacuoles and thus restricting its distribution to grains			
Grape	L-idonate dehydrogenase gene (ldnDH)	IdnDH controls the biosynthesis of tartaric acid (TA) in grape	Cas9: Knockout	Low tartaric acid	Ren et al. (2016)

(AI) offer revolutionary approaches for utilizing the CRISPR-Cas9 technology to analyse edited crop lines with better features, for example, higher nutrient value, palatability, modified root, flower architectures, stress tolerance, and so on. Some examples of CRISPR-based design tools are described in Table 9.4. All of these gRNA design tools, off- and on-target prediction tools have contributed to the success and application of CRISPR genome technology.

Several functions have been shown to be important for target gRNA activity, including secondary structure, sequence composition, thermodynamics, and physicochemical characteristic, but for off-target predictions, this is the size, composition, and combination of discrepancies. Many machines and deep learning methods have been established to represent the activity of CRISPR, which can be broadly divided into two types. (1) Machine learning based, which includes CRISPRscan, sgRNA Scorer, SSC, sgRNA Designer, and CRISPRater. CRISPRScan, CRISPRater, and SSC are trained using simple linear models, and Azimuth2.0 and TUSCAN are trained using general linear models that are logistic regression and random forests, respectively (Listgarten et al. 2018). (2) Deep learning based. CNN_std, DeepCas9, DeepCRISPR, CRISPRpred, and DeepCpf1 predict sgRNA activity builds on automatic recognition of sequence characters using a Convolutional Nuclear Network (CNN). MIT server estimates off-targets based on the distance and number between unpaired nucleotides (Hsu et al. 2013). Subsequently, a cutting frequency determination (CFD) score was developed that predicts off-target scores by reproducing the frequency of bases in gRNA spacer sequence (Doench et al. 2016). Synergizing CRISPR combines the projection results of five different models (CCTop, CFD, CROPIT, MIT, and MIT website) into an input function based on hypothetical and statistical methods (Dobson et al. 2015; Singh et al. 2015). There are currently numerous procedures available to generate accurate sgRNAs using basic rules. Here, a new algorithm called CRISPR target estimation (CRISTA) was introduced as part of ML, which performed the important task of identifying specific genomic regions to be accurately removed via given sgRNAs. The CRISTA predictions have been proven to be more accurate than previously predicted thresholds (Abadi et al. 2017). However, identifying prospective off-target sites required the recognition of short sequence motifs up to 20 bp, besides the PAM with frequent mismatches. In most cases, the aligners first match the seed sequence and extend the seed sequence in a specific direction and then check for a match. Therefore, ML and AI analysed possible regression points that may converge or deviate from on-target and off-target specificity charts.

The precision of these tools for predicting gRNA activity in different species and cell types remains unclear (Chuai et al. 2017). Large variations between species have led to the development of species-specific software (e.g. CRISPR-P for plants, flyCRISPR for fruit flies, CRISPRscan for zebrafish, and EuPaGDT for pathogens). Of these, only CRISPRscan was generated based on ML, and the rest were theoretical software. Since organisms cannot rapidly limit the previous off-target scoring process, researchers wanted to create a new procedure for assessing off-target action called CASPER (Mendoza and Trinh 2018). Although these tools can be selected for prior study when performing experiments by editing them in corresponding species,

Table 9.4 Different types of CRISPR-based designing tools

Tool	Input	PAM	Website	References
Azimuth2.0	DNA sequence	NGG	https://github.com/maximilianh/crisporWebsite/tree/master/bin/Azimuth-2.0	Doench et al. (2016)
Benchling CRISPR gRNA design	Gene ID/genome coordinates	User customizable	https://benchling.com/crispr	Doench et al. (2016)
Cas-designer	DNA sequence	NGG,NRG, NNAGAAW, NNNNGMTT	www.rgenome.net/cas-designer	Park et al. (2015)
Cas-OFFinder	crRNA sequence	20 PAMs (NGG, NRG, NNAGAAW, . . .)	http://www.rgenome.net/cas-offinder/	Bae et al. (2014), Baltes et al. (2014)
CasOT	DNA sequence	NGG, NAG, NNGG	http://eendb.zfgenetics.org/casot/	Xiao et al. (2014)
CASPER	DNA sequence	TTTN, NGG, NGCG	https://github.com/TrinhLab/CASPER	Mendoza and Trinh (2018)
CCTop	DNA sequence	NGG, NRG, NNGRRT, NNNNGATT, NNAGAAW, NAAAAC	https://crispr.cos.uni-heidelberg.de/	Stemmer et al. (2015)
CFD	DNA sequence	NGG, NAG, NCG, NGA	https://broadinstitute.org/rnai/public/software/index	Doench et al. (2016)
ChopChop	RefSeq, genomic region, gene ID	NGG, NGA, NAG, NRG, NNNNGANN, . . .), user customizable	https://chopchop.cbu.uib.no/	Montague et al. (2014)
ChopChop v2	RefSeq gene ID genomic region	User customizable	http://chopchop.cbu.uib.no/	Labun et al. (2016, 2019)
CINDEL	DNA sequence	TTTN, TTTA, TTTC, TTTG, TTTT, TTTV	http://big.hanyang.ac.kr/cindel	Kim et al. (2017)
CNN_std	DNA sequence	NAG, NGT, NTG, NGC, NGA, NGG, NAA, NCG	https://github.com/MichaelLinn/off_target_prediction	Lin and Wong (2018)
COD				

(continued)

Table 9.4 (continued)

Tool	Input	PAM	Website	References
	DNA sequence	NGG, NRG NNAGAAW NNNNGMTT NNGRRT	http://cas9.wicp.netsgRNAcas9	Park et al. (2015)
CrisFlash	DNA sequence	NGG	https://github.com/crisflash	Jacquin et al. (2019)
CRISPick	DNA sequence	NGG, CGGH, CGGT, TGGG	https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design	Doench et al. (2014)
CRISPOR	DNA sequence/ genomic region	NGG, NGA, NGCG, NGGNG, NNAGAA, NNGRRT, NNNRRT, NNNNACA, NNNNGMTT, TTTN	http://crispor.tefor.net	Haeussler et al. (2016)
CRISPR finder	DNA sequence	NGG/user customizable	www.crispr.u-psud.fr/server	Kurtz (2003), Doench et al. (2014)
CRISPR MultiTargeter	DNA sequence/ gene ID	NGG, user customizable	http://www.multicrispr.net/	Prykhozhij et al. (2015)
CRISPR primer designer	DNA sequence	NGG	http://www.plantsignal.cn/	Yan et al. (2015)
CRISPR-ERA	DNA sequence	NGG	www.CRISPR-ERA.stanford.edu	Liu et al. (2015)
CRISPR-GE	DNA sequence/ gene ID	NGG, TTN, TTTN, user customizable	http://skl.scau.edu.cn/	Xie et al. (2017)
CRISPR-P	DNA sequence/ gene locus/ genome coordinates	NGG, NAG	http://crispr.hzau.edu.cn/cgi-bin/CRISPR/CRISPR	Lei et al. (2014)
CRISPR-P 2.0	DNA sequence/ gene locus/ genome coordinates	14 PAMs (NGG, NNAGAAW, NNNNGMTT, TTTN, . . .)	http://crispr.hzau.edu.cn/CRISPR2/	Liu et al. (2017)
Crispr-plant	Gene locus/ genome coordinates	NGG	https://www.genome.arizona.edu/crispr/	Minkenberg et al. (2019)
CRISPRater		NGG		

(continued)

Table 9.4 (continued)

Tool	Input	PAM	Website	References
	DNA sequence		https://crispr.cos.uni-heidelberg.de/	Labuhn et al. (2018)
CRISPRdirect	DNA sequence genome coordinates	NNN, user customizable	http://crispr.dbcls.jp/	Naito et al. (2015)
CRISPRoff	DNA sequence	NGG, NAG, NGA	https://rth.dk/resources/crispr/	Alkan et al. (2018)
CRISPRpred	DNA sequence	NGG	https://github.com/khaled-buet/CRISPRpred	Rahman and Rahman (2017)
CRISPRscan	DNA sequence	NGG	www.crisprscan.org	Moreno-Mateos et al. (2015)
CRISPRseek	DNA sequence	NRG, NGG, user customizable	http://www.bioconductor.org/packages/release/bioc/html/CRISPRseek.html	Zhu et al. (2014)
CRISTA	DNA sequence	NGG	http://crista.tau.ac.il/pair_score.html	Abadi et al. (2017)
CROPIT	DNA sequence	NGG, NNG, GGG	http://cheetah.bioch.virginia.edu/AdliLab/CROP-IT/homepage.html	Singh et al. (2015)
CT-finder	DNA sequence	NGG	http://bioinfolab.miamioh.edu/ct-finder	Zhu et al. (2016)
DeepCas9	DNA sequence	NGG	https://github.com/lje00006/DeepCas9	Xue et al. (2019)
DeepCpf1	DNA sequence	TTTN	http://deepcrispr.info/	Luo et al. (2019)
DeepCRISPR	sgRNA sequence	NGG, NGT, NGA, NAG, NGC, NCG, NTG, NAA	http://www.deepcrispr.net/	Chuai et al. (2018)
E-CRISP	Gene ID/DNA sequence	NGG, user customizable	http://www.e-crisp.org/E-CRISP/	Heigwer et al. (2014), MacPherson and Scherf (2015)
Elevation	Gene ID transcript		https://crispr.ml/	Listgarten et al. (2018)

(continued)

Table 9.4 (continued)

Tool	Input	PAM	Website	References
	ID genomic region	NAG, NGA, NCG, NGC, NGG, NTG, NGT		
Elevation-search/ dsNickFury	DNA sequence	NGG, NCG, NAG, NGA, NGG, NGC, NTG, NGT	https://github.com/michael-weinstein/dsNickFury3PlusOrchid	Listgarten et al. (2018)
EuPaGDT	DNA sequence	NGG, NAG, NGA	http://grna.ctegd.uga.edu/	Peng and Tarleton (2015)
FlashFry	DNA sequence	NGG	http://aaronmck.github.io/FlashFry/	McKenna and Shendure (2018)
FlyCRISPR	DNA sequence	NGG	www.tools.flycrispr.molbio.wisc.edu/targetFinder	Gratz et al. (2014)
Ge-CRISPR	DNA sequence	NGG	http://bioinfo.imtech.res.in/manojk/gecrispr/	Kaur et al. (2016)
GT-scan	DNA sequence	User customizable	https://gt-scan.csiro.au/	O'Brien and Bailey (2014)
Off-spotter	DNA sequence	NGG, NAG, NNGRRT, NNNNACA (R is Aor G)	https://cm.jefferson.edu/Off-Spotter/	Pliatsika and Rigoutsos (2015)
Optimized CRISPR design	DNA sequence	NGG, NAG	https://crispr.mit.edu	Hsu et al. (2013)
Predict CRISPR	DNA sequence	NGG	https://github.com/penn-hui/OfftargetPredic	Peng et al. (2018)
Protospacer workbench	Gene ID/DNA sequence	NGG	www.protospacer.com	MacPherson and Scherf (2015)
sgRNA designer	DNA sequence, gene ID, transcript ID	NGG	https://portals.broadinstitute.org/gpp/public/analysistools/sgrna-design	Doench et al. (2014)
sgRNA scorer	DNA sequence	NGG, NAG, NNNNGMTT, NNAGAAW	https://crispr.med.harvard.edu/sgRNAScorerV2/	Chari et al. 92,015)
sgRNAcas9	DNA sequence	NGG, NAG	www.biootools.com	Xie et al. (2014)
SSC		NGG		

(continued)

Table 9.4 (continued)

Tool	Input	PAM	Website	References
	DNA sequence		www.crispr.dfci.harvard.edu/SSC/	Xu et al. (2015)
SSFinder	DNA sequence	NGG	https://code.google.com/archive/p/ssfinder/	Upadhyay and Sharma (2014)
SynergizingCRISPR	DNA sequence	NGG	https://github.com/Alexzsx/CRISPR	Zhang et al. (2019)
Synthego design tool	DNA sequence	NGG	https://design.synthego.com/#/	Roginsky (2018)
TUSCAN	DNA sequence	NGG	https://github.com/BauerLab/TUSCAN	Wu et al. (2014)
uCRISPR	DNA sequence	NGG, NAG, NGA	https://github.com/Vfold-RNA/uCRISPR	Zhang et al. (2019)
WGE	DNA sequence	NGG	www.sanger.ac.uk/htgt/wge	Hodgkins et al. (2015)
WU-CRISPR	RNA sequence	NGG	http://crispr.wustl.edu/	Wong et al. (2015)
ZiFiT	DNA sequence	NGG	http://zifit.partners.org/ZiFiT	Sander et al. (2010)

their prediction of sgRNA efficiency and target in various cell types is debatable. However, these tools have been proved in the laboratory using mouse cell lines, and human or both, major and cross-species variations have not yet been testified. Therefore, ML-based learning approaches can effectively predict lethal sgRNA interactions and characterize target regions in specific gene combinations. However, there is a large amount of work to be tested and optimized for utilizing CRISPR-Cas9 gene editing in plant systems. In the future, genome-wide engineering crops will include trained data sets, including variants and orthologs.

9.9 Regulatory Aspects of Genome Edited Crops

GEd technology has proved its potential uses in a broad array of industries, notably human and animal health, food, agriculture, and others, in a relatively short period of time. GEd innovations, the same as any other new technology, have dual-use prospective and so raise both safety and security concerns. Novel GEd techniques, particularly CRISPR-Cas9, have a unified mechanism for the insertion of elite attributes in crops plants, allowing unconstrained base substitutions, additions, deletions, and gene introduction or replacement. The offspring produced are similar to those produced by random mutagenesis, natural genetic variants, and traditional

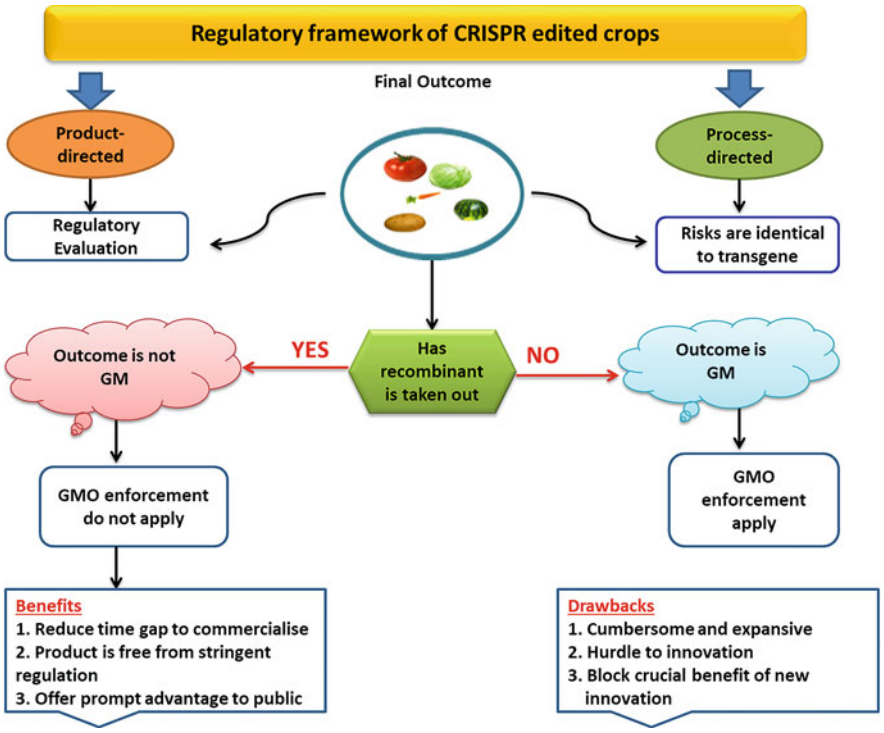

Fig. 9.3 Illustrates the regulatory roadmap for the CRISPR/Cas-based genome editing (GEd), including genome edited crops. Here, showing product/process based regulatory policy for GEd crops. *GM* genetically modified, *GMO* genetically modified organism

breeding. The Cartagena Protocol governs the regulation of genetically modified organisms (GMOs), which is part of the worldwide regulatory framework for living modified organisms (LMOs). LMOs, according to their definition, are living organisms with a unique combination of genetic material that has been improved via the use of contemporary technological methods. In contrast to GMO, the integration site is pre-decided, precise and without an insertion of foreign DNA in GEd organisms. The Cartagena Protocol is based on international terms and conditions that each state and its government must adhere to when enacting biosafety legislation. In addition, the lack of clear perception mentioned in the protocol has been a subject of an argument to date.

Universally, there are differing perspectives on how to harmonize genome edited product/process-based policy in every region (Fig. 9.3). One argument is that GEd species do not need to be regulated because there is no trace of genetic engineering in particular categories, and they resemble organisms that have evolved naturally. The opposing point of view is that GEd organisms must be regulated, but they do not have to go through the same stringent biosafety regulatory process as all GMOs/LMOs. Such divergent viewpoints reflect the rules and regulations that govern the

regulation of GE organisms and products in each country. The insertion of considerable modifications to the genomes of GE crop plants generated using gene editing or Site-Directed Nuclease (SDN) technologies showed genetic differences. There are three types of SDN technology: SDN-1: These were made by cleaving double-stranded DNA in the existing genome without involving of foreign DNA particles, as a result the end products characteristics are almost similar to what arose from natural plant mechanisms and or artificial mutation . SDN-2: involves a short homologous DNA fragment that contains few base pair different from the targeted DNA template. Double strand cut is recognized by the host repair system and simultaneously repaired with the help of donor DNA fragment and introduces predetermined mutations. Lastly, SDN-3: requires a DNA repair donor template longer than 20 bp for incorporation into the target area, which is accomplished by a DSBs nick in the gene that is accomplished by a fragment carrying a gene or other genetic material template. The first and second SDN approaches lack foreign DNA insertions or recombinant DNA because they do not produce new plant varieties. SDN-3, on the other hand, would be subject to GMO regulation if newly created plant types comprised more than 20 bp foreign DNA insertions, showing the same outcome as the classic recombinant DNA technique (Pauwels et al. 2014). Mutation breeding (induced random mutagenesis) or CRISPR-Cas9 (gene editing technology) can be used to create crop features with similar phenotypes, and they will fall into the same category. The change to genetic modifications is appealing due to the possibility for developers to use SDN technology to build superior crops that could bypass the cumbersome regulatory assessments associated with GE crop adoption (Arora and Narula 2017; Yin et al. 2017; Pacher and Puchta 2017; Kumlehn et al. 2018; Sedeek et al. 2019). Policymaker laws that facilitate the commercialization of gene-edited crops could reduce the time between the lab and the farmer even more. Globally, the countries that have welcomed GM crop production and export policy have a planned structure that is quick, simple to comprehend and follow, and enforced (Levin 1994). Notwithstanding their various process or product-based techniques, Argentina, Brazil, Chile, Costa Rica, Honduras, Mexico, and Uruguay were among the first Latin American countries to give GM agricultural permits (Ishii and Araki 2017; Rosado and Craig 2017). This day, these nations are fast forward in cultivating biotech crops and thus, their economic success could be explained by something other than the GMO framework (Table 9.5) (Rosado and Craig 2017). SDN-1 products are almost universally regarded as non-GMO, and the final product would go through the same legislative framework as classically produced plant species (Schmidt et al. 2020).

Divergence re-emerges, however, when it comes to SDN-2 techniques: Australia and Japan have taken a cautious approach, determining that organisms modified with the SDN-2 technology will be classified as GMOs (Thygesen 2019; Tsuda et al. 2019). Plants that have undergone a genetic modification requiring an initial assessment on the basis of their creation using NBTs are characterized as gene-edited organisms, with the exception of those that have been modified without a template or with a modest template. This is not always a negative attitude; in fact, it is one of the key causes driving the formation of biosafety regulation in the first place: it upholds

Table 9.5 Worldwide regulation status of genome edited crops

Country	Regulation status	Remarks
Australia	Deregulated	Edited crops are deregulated when modification occurs via NHEJ-mediated repair pathway (SDN-1), wherein regulated, if donor template or foreign genetic material inserted for alteration of genes
USA	Deregulated	Edited crops cannot be considered as GM crops when any foreign DNA is absent there
Europe	Regulated	Genome edited crops must have regulated via assessment rules designed for the GM crops release
Japan	Deregulated	Edited crops can be reassessed any time, if insufficient information is provided
Brazil	Under existing GMO regulations	Case to case assessment of edited crops, crops are deregulated if they don't carry any transgene
India	Regulation guidelines released	Department of Biotechnology (DBT) under the ministry of science and technology released the much-awaited regulatory guidelines for GE organisms. Edited (SDN-1)/ KO crops are in the pipeline of deregulation.
Canada	Deregulated	Edited crops are deregulated, those are regarded as fast version of conventional breeding
Chile	Under existing GMO regulations	Edited crops are deregulated if they have not any transgene
New Zealand	Regulated	Genome edited crops must have defined regulated policy as designed for the release GM crops
Argentina	Under existing GMO regulations	Edited crops become deregulated due to absence of any transgene

societal ideals of risk assessment and risk management with the ultimate goal of safeguarding human, animal, and environmental health.

9.10 Conclusion

Implementation of Noble Prize winner CRISPR-Cas GEd technique for plant GEd and regulation has revolutionized the field of genetic engineering and advanced the plant molecular breeding aspect for crop improvement. Recent advances in genome sequencing (reading) and DNA editing or engineering (writing) techniques have led to an era where we can read and write or even re-write the complex genome of plants. With novel breakthroughs of CRISPR-Cas system, we have witnessed the rise of genetic engineering 2.0 which has contributed enormously to the development of practical, valuable, applicable, and multifaceted tools. These tools are the arsenal for future gene editing, genome modification, metabolic engineering avenues via gene knockout, knockin, replacement, point mutations, fine-tuning of gene regulation, and other modifications at any gene locus. This comprehensive review highlights the successful implementation of the CRISPR-Cas system in plant GEd as well as aids in the documentation of some novel events in the field of plant genetic engineering. In

addition, it addresses the technical limitations & shortcomings of the tools, and how to overcome those challenges. Additionally, grieve regulatory concerns and applicability of the machine learning approach i achieve the next-generation engineering or breeding technique. However, it encourages the utilization of the new addition of the CRISPR tool kit for their development into programmable nucleases for efficient, precise, and easy to achieve plant GEd tools. While public acceptance will always be a great concern but there has been a shift in the general notion of disapproval of the genome edited crops however not completely. But even a pitch positive turn with the subsisting endeavours of the scientific community and government ministries of INDIA will be a great achievement, and a way forward to the release of the CRISPR generated new robust variety in the global market.

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