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Review

Revisiting CRISPR/Cas-mediated crop improvement: Special focus on nutrition

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Genome editing (GE) technology has emerged as a multifaceted strategy that instantaneously popularised the mechanism to modify the genetic constitution of an organism. The clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas) protein-based genome editing (CRISPR/Cas) approach has huge potential for efficacious editing of genomes of numerous organisms. This framework has demonstrated to be more economical in contrast to mega-nucleases, zinc-finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs) for its flexibility, versatility, and potency. The advent of sequence-specific nucleases (SSNs) allowed the precise induction of double-strand breaks (DSBs) into the genome, ensuring desired alterations through non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathways. Researchers have utilized CRISPR/Cas-mediated genome alterations across crop varieties to generate desirable characteristics for yield enhancement, enriched nutritional quality, and stress-resistance. Here, we highlighted the recent progress in the area of nutritional improvement of crops via the CRISPR/Cas-based tools for fundamental plant research and crop genetic advancements. Application of this genome editing aids in unraveling the basic biology facts in plants supplemented by the incorporation of genome-wide association studies, artificial intelligence, and various bioinformatic frameworks, thereby providing futuristic model studies and their affirmations. Strategies for reducing the 'off-target' effects and the societal approval of genome-modified crops developed via this modern biotechnological approach have been reviewed.

Keywords. CRISPR/Cas9; crop improvement; crops; genome editing; nutrition; off-target

1. CRISPR/Cas system: a prospective strategy for crop improvement

Genome editing (GE) is an efficient toolkit for altering a target genome, which turns out to be explicitly effective, and targeted change from an individual nucleotide to a broad-range sequence alteration. This task is usually performed with numerous endonucleases that cleave a specific gene at a distinct locus. Clustered regularly interspaced short palindromic repeats (CRISPR) or CRISPR associated (Cas) proteins perform as a molecular cleaving tool-kit in an agreement with the cell's endogenous restoration system, intended to cut and modify DNA at a particular point within the genome. CRISPR/Cas mechanism is comprised of a CRISPR arsenal harbouring many Cas nucleases (Cas9, Cpf1, Cas13a, Cas14a) (table 1). This

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Table 1. Characteristics features of different CRISPR-Cas (Class 2) system

Features	Cas9	Cpf1	Cas13a	Cas14a
CRISPR subtype	(II)	(V-A)	(VI-A)	(V-F)
Target substrate	dsDNA	dsDNA	ssRNA	ssDNA & dsDNA
Target sequence length (bp)	20–22	36–39	20	20–25
PAM	3′-GG	5′-TT(T)	3′-H	5′-TT(T)
gRNA characteristic	crRNA & tracrRNA	crRNA	crRNA	crRNA & tracrRNA
Cleavage pattern	5′ Overhangs	5′ Overhangs	gRNA dependent RNA cut	gRNA dependent ssDNA & dsDNA cut
Protein size (kb)	2.9–4.9	(High) 3.6–3.9	4.1	1.2–2.1

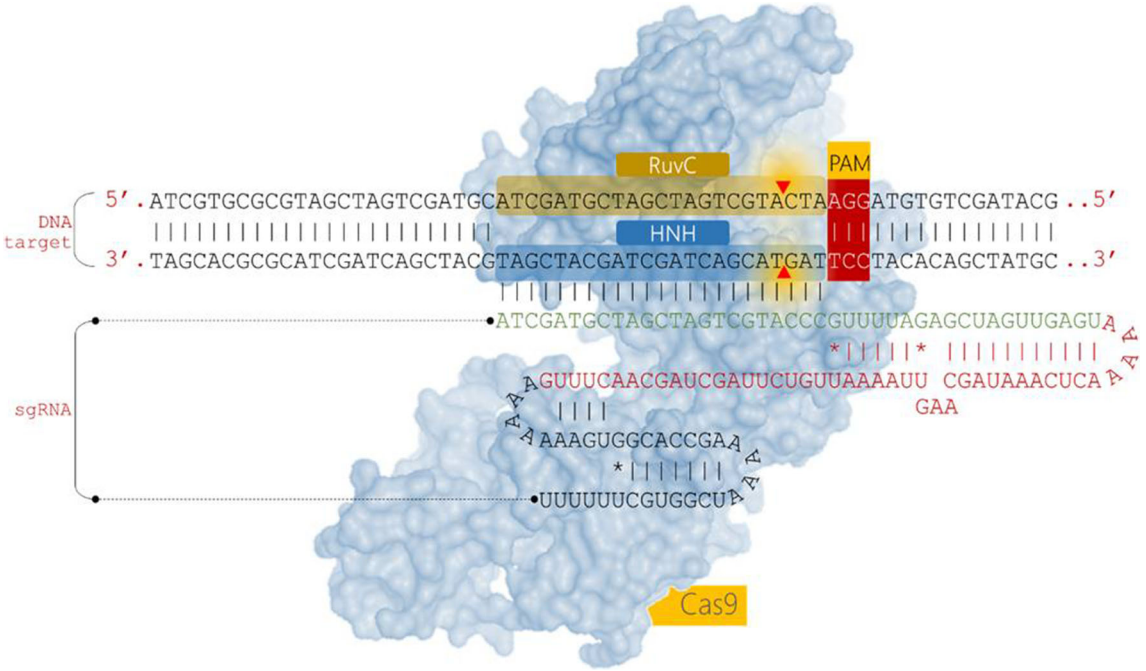

Figure 1. Overview of CRISPR/Cas9-sgRNA targeted genome editing. sgRNA complexes with Cas9 nuclease on the targeted genomic site containing an adjacent PAM sequence. Nucleotide hybridization of sgRNA-Cas9 complex to targeted loci generated a conformational change that activates Cas9 nuclease activity, resulting in DNA double-strand breaks.

technique utilizes a single effector Cas endonuclease with two RNAs, i.e., CRISPR RNA (crRNA) that contains 20-nucleotide target-specific sequence and partial alternative trans-activating crRNA (tracrRNA) to fulfill its function. The assembled CRISPR/Cas complex fastens to the target recognition position upstream of the protospacer adjacent motif (PAM) and introduces a double-strand break (DSB) 3 bp prior to the PAM (Jinek *et al.* 2014) (figure 1). Recently, a different hypercompact CRISPR/Cas system identified in numerous bacteriophages harboured the CasΦ enzyme that has magnified the genome editing toolbox in both human and plant systems. It invariably exhibited higher target recognition capabilities in contrast to other CRISPR/Cas proteins, i.e., Cas9, and Cas12a and possessed half their molecular weight. CasΦ enzyme utilized an identical active site for processing mature CRISPR RNA (crRNA), that is responsible for cleavage of foreign nucleic acids (Pausch *et al.* 2020). According to phylogenetic classification, CRISPR/Cas system may be categorized into 2 classes, namely Class 1 that included types I, III, and IV, whereas, Class 2 comprised of types II, V, and VI that employs a particular protein to target overwhelming genetic materials (Makarova and Koonin 2015; Kaul *et al.* 2020). Among all the type II proteins, Cas9

has been universally accepted as an innovative tool-kit for genome altering functions. It revealed that Cas9 endonuclease consisted of a conserved core, bilobed structure juxtaposed to an active site with two major nucleic acid binding grooves (Jinek *et al.* 2014). The bilobed structure has a big globular recognition lobe known as the REC lobe and is connected with the small nuclease lobe (NUC). The determinate functional domain of Cas9 of REC-lobe has bi-partite domains, i.e., REC1 and REC2; and bridge helix cd domain (arginine-rich alpha-helical domain). NUC lobe acts as a seat for two nuclease domains; RUV C & HNH and, PI domain (PAM interacting domain) (Jinek *et al.* 2014; Hsu *et al.* 2014). Among two nucleic acid binding grooves, the primary large groove is positioned within the REC lobe and the exiguous major groove is positioned within the NUC lobe (Jinek *et al.* 2014; Nishimasu *et al.* 2014). Without the ligand DNA molecule, Cas9 behaves in an auto-inhibited confirmatory state and converts into the active state when loaded on the ligand DNA molecule via the guide RNA. This leads to the induction of conformational change in the NUC and REC lobes of Cas9 and creates a proper channel for the generation of a DNA-RNA heteroduplex complex (Jinek *et al.* 2014). The primary interaction of guide RNA with the REC lobe of Cas9 leads to the synthesis of the bicameral sgRNA-Cas9 complex. This binary complex examines the PAM site on the sense strand of the DNA double helix (Jinek *et al.* 2014; Sternberg *et al.* 2014). Various studies revealed that the PI domain of the NUC lobe of Cas9 plays an essential role in the recognition of the PAM site via its unsaturated tryptophan-rich flexible loop (Jinek *et al.* 2014). Due to the energy-independent helicase activity of Cas9, the PAM recognition triggers the destabilization of DNA sequences that are juxtaposed to the PAM site resulting in the formation of R-loop (Nishimasu *et al.* 2014; Sternberg *et al.* 2014). Several reports also concluded that the two arginine residues within the major groove of the DNA binding domain of Cas9 (R13.33 and R13.35) recognize the guanine dinucleotide of the PAM site on the non-complementary strand of a ligand DNA molecule. Besides, the two amino acid residues within the minor groove of DNA binding domain, lysine (K1107) and arginine (S1109) interact with the PAM sequences' phosphate group (position +1) in the complementary strand of ligand DNA molecule in the form of a loop known as phosphate lock loop, which in turn plays an important role in the correct orientation for the hybridization between complementary DNA strand and guide RNA, may lead to the unwinding of DNA double helix (Sternberg *et al.* 2014). It was revealed through experimentation that the base pairing between the ligand DNA strand and the seed region of guide RNA (up to 8–12 bp) triggers the simultaneous stepwise destabilization of DNA duplex and the development of RNA-DNA heteroduplex. The RNA-DNA heteroduplex was occupied by NUC and REC lobe of Cas9, which resulted in a four-way node that ascended the arginine-rich alpha-helical bridge helix (Anders *et al.* 2014). As a result of the two domains of the NUC lobe, first is mobile HNH domain, always ready to cleave the complementary ligand DNA strand of tertiary complex, and the second is Ruv C domain which nicks the non-complementary strand, hence produces the double-strand breaks (Nishimasu *et al.* 2014). However, it is still obscure by what means recycling of Cas9 and its dissociation from sg-RNA occurs (Sternberg *et al.* 2014). The CRISPR/Cas system led to DNA double-strand breaks (DSBs) at defined positions that in turn introduced numerous genomic alterations employing DNA repair pathways i.e., HDR and NHEJ (Symington and Gautier 2011). Numerous customized engineered nucleases have been employed for targeted genome modifications, for instances, disruptions, additions and/or substitutions within genes, and over-expression of the gene (Xie and Yang 2013; Kim and Kim 2014; Sedeek *et al.* 2019; Chen *et al.* 2019; Hua *et al.* 2019a; Kaul *et al.* 2020). Various Cas9 and gRNA variants are accessible, which might be used for neoteric purposes, especially in the enhancement of traits within crops, such as, the increment of stress resistance level, crop turnout, and particularly nutritional up-gradation of crops.

2. Recent progress of genome editing tools in crop improvement

GE tools developed rapidly during the previous decade are being reliably utilized by researchers for crop improvement, globally. They offer extensive applications with a massive impact on life sciences and agriculture. The CRISPR/Cas system has already created a prominent position in several disciplines of basic and applied biology, genetic engineering and biotechnology because of its ease and robustness in modifying genomes, in order to comprehend the underlying regulatory processes of gene expression, transcriptional activation and repression, promoter activity and, so on. Here, we have compiled the numerous tools available for GE (figure 2), highlighting their robust implications

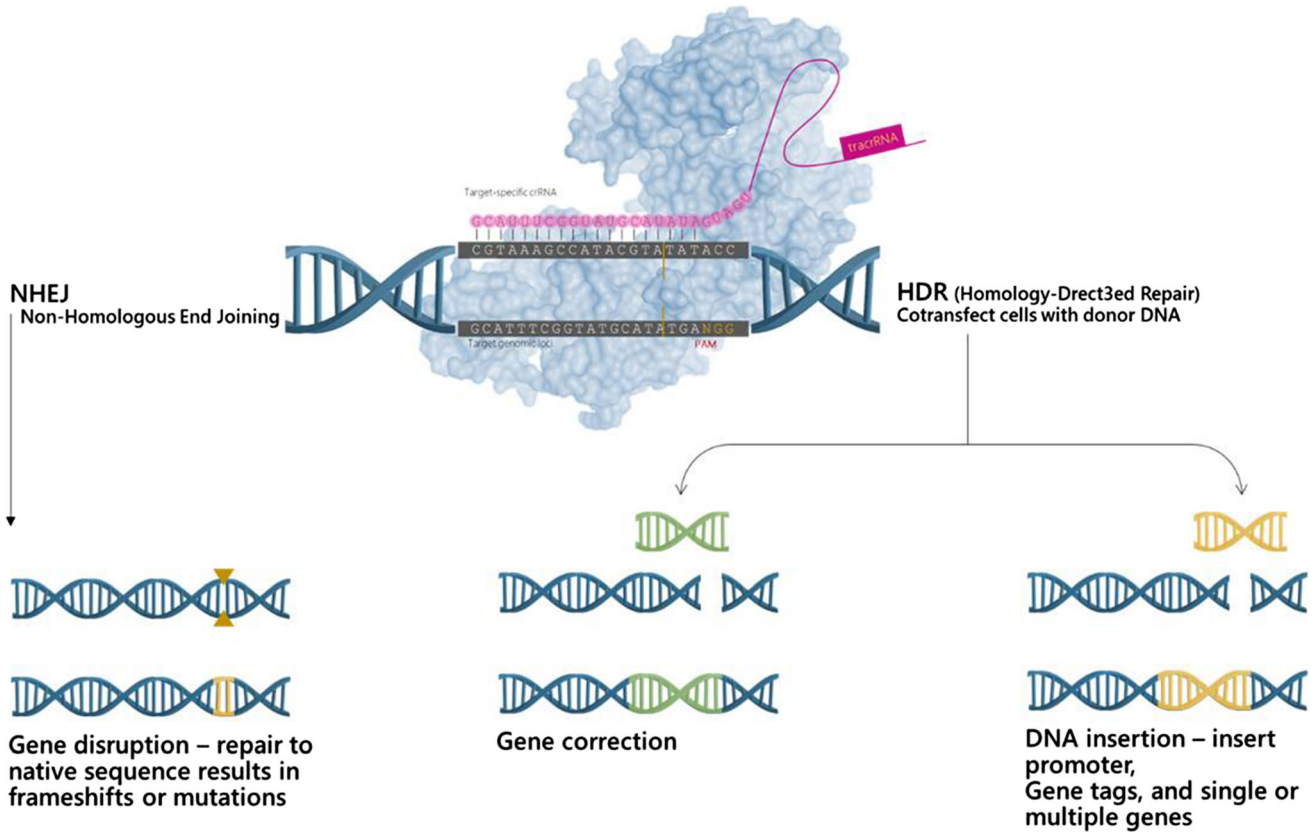

Figure 2. Potential genome editing outcome of CRISPR/Cas9-based technique based on NHEJ and HDR-mediated repair pathways. In NHEJ, random nucleotide insertions and/or deletions occur as the cell ligates the DSB within DNA, resulting in gene disruption. In HDR, the DSB is repaired by using an externally supplied homologous donor repair template resulting in directed precision repairing.

on advancements in the area of crop improvement and the regulatory concerns related to their viability.

2.1 *NHEJ-mediated knockout of genes*

Creation of double-strand breaks (DSBs) led to the generation of gene knockout/s (KO) by means of fragment excision or deletions/insertions of few base pairs of a gene from a crop genome and ensuing repair by NHEJ, in this manner incorporating a mutation within the gene that modifies the expression of the targeted gene. CRISPR/Cas-based gene KO strategy has been utilized to interrogate the functional significance of a particular gene/s of interest. As of now, these tools have been efficiently set up in numerous crop species, for instance, S-gene (disease susceptible genes) KO strategy was successfully employed in hexaploid bread wheat that generated fungal-resistant wheat (Wang *et al.* 2014; Wang *et al.* 2018a). DuPont Pioneer (2016) revealed that CRISPR/Cas-based KO of corn Wx1 gene (waxy gene), produced amylopectin rich (waxy) corn grains by Corteva Agriscience. Wx1 gene that encoded the granule-bound starch synthase (GBSS), protein stimulated the development of amylopectin enriched corn. The development of two best glutinous sticky *japonica* rice varieties was achieved by the Waxy (*OsWx*) gene KO (Yunyan *et al.* 2019). Reports revealed targeted modification of starch branching enzyme SBEIIb in rice induced high amylose content in grains (Sun *et al.* 2017). Moreover, blast-resistant rice lines were generated by the *OsERF922* gene KO (Wang *et al.* 2016). CRISPR/Cas-based KO of genes associated with floral development, i.e., *AP1, SVP*, and *TFL* evoked the advancement of floral attributes in Arabidopsis (Liu *et al.* 2019). According to Gaoneng *et al.* (2017), rice betaine aldehyde dehydrogenase (BADH2) gene acts as a negative regulator of the aroma production in rice. Targeted KO of the *BADH2* gene generated fragrance enriched rice. Similarly, the use of CRISPR/Cas-based frameworks for the introduction of explicit hereditary

changes in plant genomes prompted the rectification of complex metabolic mechanistics that further offers tremendous scope for the generation of newfangled crop germplasm with desired agronomic attributes.

2.2 *HDR-mediated gene replacement*

The robust CRISPR/Cas9, gene editing system is eminent for its stringent editing efficiency and user-friendliness. A new array of genome editing techniques have been developed for accurately and efficiently inserting desired mutations into a gene. Knocking-in of desired nucleotide sequences has been more challenging than knocking them out. Knocking out involved insertion of CRISPR/Cas9-sgRNA into a cell that targets the gene of interest, followed by fixing the cut (DSBs) using a cell's regular DNA repair mechanism or NHEJ repair pathway. However, in case of knocking a gene in, the cuts must be fixed accurately, with no additional insertions/deletions, which needs harnessing of a second alternative DNA repair approach known as Homology-Directed Repair (HDR). Many agronomic traits may be conferred by gene expression changes via single-nucleotide insertions/substitutions or the expansion of novel gene activities. Meticulous gene alteration via knock-in and replacement may boost plant breeding by the introduction of new alleles without producing allelic variants, which do not occur normally (Chen *et al.* 2019). Unfortunately, HDR-mediated editing frequency is quite low and its utilization in trait advancement has hitherto been constrained. Shi *et al.* (2017) employed the CRISPR/Cas-based genome altering technique via swapping of the GOS2 promoter by the native ARGOS8 promoter through HDR-mediated genome editing for enhanced drought tolerance in maize. Yu *et al.* (2017) also generated edited tomato lines with extended shelf life via T317A substitution in the ALC gene. Geminivirus-mediated-DNA replicon has been utilized for enhanced gene-targeting efficiency in various crops, for instance, tomato (Cermak *et al.* 2015; Dahan-Meir *et al.* 2018), potato (Butler *et al.* 2016), wheat (Gil-Humanes *et al.* 2017), rice (Wang *et al.* 2017), cassava (Hummel *et al.* 2017) via increased copy number of repair templates. The replacement of principle amino acids via HDR-mediated pathway in the endogenous genes, for instance, acetolactate synthase (*ALS*) and 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) bestowed resistance to non-selective, systemic herbicides, i.e., sulfonylurea and glyphosate, respectively (Li *et al.* 2015; Sun *et al.* 2016). Correspondingly, double amino acid T102I/P106S (TIPS) and T102I/P106A replacements were incorporated into the *EPSPS* gene the flax plant (*Linum usitatissimum*) (Sauer *et al.* 2016) and cassava plant (Hummel *et al.* 2017). Another new RNA-mediated genome editing technique CRISPR/Cpf1 effectuated efficient and targeted gene insertions, in contrast to the HR-guided recombination. Therefore, the CRISPR/Cpf1 in conjunction with the CRISPR/Cas9 system may indomitably activate the focused gene knock-in mechanism for crop advancement.

2.3 *Gene targeting*

CRISPR/Cas technique is an efficacious strategy for the transmutation of crop genomes. In the light of homologous recombination (HR), a gene fragment substitution may be obtained via gene targeting (Capecchi 2005). To beat the low rate of HR frequency in crops, DSBs in DNA has been achieved at the defined loci (Steinert *et al.* 2016). Besides, co-delivery of the SSN altering system and an HDR template into the plant cell facilitated gene targeting in a specific way (Li *et al.* 2013). The rearranging of intrinsic TAL effector-binding sites in the promoters of R genes bestowed resistance transversely to various *Xanthomonas* sp. strains (Romer *et al.* 2009). To upgrade the negligible HR frequencies of somatic cells, Cas9 expression riveted to the young embryo and egg cells conferred a higher gene targeting potency in *Arabidopsis* (Miki *et al.* 2018; Wolter *et al.* 2018). As reported by Li *et al.* (2019), *in vivo* fabrication of RNA transcripts existing within the nucleus, assisted as repair templates for transcript-template HDR (TT-HDR)-based explicit gene-altering manifested an optimistic mutation frequency in rice. Although genome editing propounds differentially useful aspects, nonetheless complicated alleles demanded high accuracy. CRISPR/Cas system enforces HDR-based gene targeting to overcome such limitations. Nevertheless, HDR repair pathway potency in a plant cell is still inadequate and that may be ascribed to the inadequacy of efficacious delivery techniques for DNA repair templates (Steinert *et al.* 2016; Yin *et al.* 2017; Sedeek *et al.* 2019; Chen *et al.* 2019). In line with this, numerous advancements had been developed, for instance, increasing the quantity of donor DNA (Baltes *et al.* 2014); also a repair protein, KU70/80, and LIG4 may have suppressed NHEJ repair pathway (Endo *et al.* 2016). Therefore, the CRISPR/Cas system

represents a better frequency of HDR-mediated gene targeting in plants.

2.4 *Base editing with programmable nucleases*

Precise genome editing is troublesome to attain with NHEJ- and HDR-mediated correction because of its lower editing frequency. Recently, an alternative tool, base editors or programmable deaminases, were developed to beat those limitations. Base editing is a novel genome modifying technique that empowers the irretrievable change of one DNA base to another at target position without DSBs as in the case of HDR (Komor *et al.* 2016; Nishida *et al.* 2016; Eid *et al.* 2018; Li *et al.* 2018a; Moon *et al.* 2019). DNA base editors were divided into two categories: adenine base editors (ABEs) and cytosine base editors (CBEs), wherein, ABEs sustain the switching of A•T pairs into G•C base pairs and CBEs interchanging the C•G into T•A base pairs. Both base editors (BEs) utilize nickase Cas9 (nCas9) and catalytically inactivate Cas9 (dCas9), and then fused to deaminases, thus hydrolyzes the amine group of A (Gaudelli *et al.* 2017) and C (Komor *et al.* 2016). In this methodology, nucleoside deaminases (cytidine, adenine) may be superintended by the sgRNA-Cas9 loaded complex to a target position, that culminated in the reversal of adenine or cytosine into inosine or uracil by hydrolysis. Eventually, following rectification or DNA replication, the edited strand had thymine or guanine instead of cytosine or adenine (i.e., single nucleotide bubble generated by Cas9n) (Li *et al.* 2017a; Hua *et al.* 2018; Ren *et al.* 2018; Kang *et al.* 2018). In cytosine base editors, a uracil glycosylase inhibitor is also employed in combination with Cas9n for expanded accuracy and potency of base-editing technique (Komor *et al.* 2016; Ren *et al.* 2018; Wu *et al.* 2020). dCas9 conjointly with cytidine deaminase enzyme may prompt the immediate change of cytidine to uridine. So, by following replication of the duplex a C -to- G was changed into T- to -A. It was reported that for catalyzing the first 'A (adenine) to I (inosine)' *E. coli* tRNA adenosine deaminase enzyme (TadA) was deployed for ABEs. The created I is recognized as G by DNA polymerase. Both CBEs and ABEs were employed earlier in numerous plant species, which required no double-stranded DNA breaks or HDR templates. Gao *et al.* 2017 demonstrated the base editing potency using BE3 in rice, wheat, and maize were up to 43% with an indel frequency of 0.01–0.22%. Each base editing tool offered various editing choices depending upon the deaminase, type of nuclease, and gRNA (Rees and Liu 2018). Various rice genes, for instance, *OsSLR1, OsPDS, OsSBEIIb,* and *OsNRT1*.1B modified with the aid of the BE3 technique effectuated an altering potency up to 20% (Komor *et al.* 2016; Nishida *et al.* 2016; Ren *et al.* 2018; Zhu *et al.* 2019). The earliest narrated CBEs that encouraged the C-to-T changes were generated via a combination of Cas9n with activation-induced cytidine deaminase orthologs PmCDA1 or rat cytidine deaminase (rAPOVEC1) (Li *et al.* 2017a; Lu and Zhu 2017; Ren *et al.* 2018; Shimatani *et al.* 2017; Zong *et al.* 2017; Zhong *et al.* 2018; Zuo *et al.* 2019; Endo *et al.* 2019; Kaul *et al.* 2020). Wu *et al.* (2019) further extended their applications by widening the editing frame of numerous base pairs from the canonical PAM (NGG) sequence. They combined cytidine deaminase1 from *Petromyzon marinus*, i.e., PmCDA1 with Cas9 D10A nickase of numerous SpCas9 variants, likewise as uracil DNA glycosylase inhibitor (UGI), and thereby generated two novel potent PmCDA1-based cytosine base editors (pBEs). Using this methodology, they broadened the base modifying efficiencies (4–90%) in T_0 lines that commensurated to expanded base editing efficiencies from 1.3 to 7.6 fold (Wu *et al.* 2019; Wang *et al.* 2019a). Various engineered Cas9 variants that recognized non-canonical PAM sequences have been utilized with rAPOVEC1 or activation-induced cytidine deaminase to generate novel CBEs in rice (Negishi *et al.* 2019; Jin *et al.* 2019). Additionally, Zhou *et al.* (2019) tentatively assessed RNA single nucleotide variations (SNVs) that were acquired via either CBEs or ABEs and highlighted that both CBEs and ABEs produced numerous off-target RNA SNVs. Umpteen off-target effects in the DNA altering system may be erased through such robust engineering of deaminases. Gene alterations and write over employing mitochondrial DNA (mtDNA) have emerged as an encouraging option to orthodox therapy for mitochondrial dysfunction that may culminate in a broad range of ailments and inadequacy of organ and tissue functions. Recently, CRISPR-free DddA-derived cytosine base editors (DdCBEs) have been employed to target the alterations in human mtDNA, which proved significantly crucial for the therapeutic research related to mitochondrial diseases (Mok *et al.* 2020).

2.5 *'Search-and-replace' genome editing or prime editing*

CRISPR-mediated genome editing may alter any base within the entire genome with utmost precision.

However, despite the breakneck advancements in the genome editing machinery, most of the known human genetic variants (>75,000) related to diseases remains problematic to address or introduce in the majority of therapeutically vital cell types. One amongst the several reasons that may be caused by imperfect genome editing systems is the off-target effect. Even the most effective available CRISPR-based editing system that employed HDR may introduce unwanted mutations. Whereas escalating the potency and preciseness of DSBs-mediated editing is still the fundamental target, these difficulties may motivate the investigation of other precise genome editing techniques. A new genome altering tool referred to as prime editing may render the CRISPR system more precise than existing genome editing approaches in both animal and plant systems (Lin *et al.* 2020). Prime editors are often considered as word processors that are competent at searching precise DNA sequences and replacing them (Cohen 2019). It's a fusion between Cas9n and reverse transcriptase (RT), which has similar effectiveness to CRISPR/Cas9 but showed less off-target effects. It used the same Cas9 nuclease but combined the enzyme with two new reagents: a longer-than-usual guide RNA referred as prime editing guide RNA (pegRNA) and a fusion protein consisting of Cas9 H840A nickase combined to a specially designed RT enzyme that initiated the addition of a new sequence or base into the genome. Primer binding site (PBS) sequence complementary to the nicked genomic DNA hybridized to the target site and acted as a template for reverse transcription. Host primer binding the RT stimulated the incorporation of a novel sequence or base into the genome, the new genetic material integrated into the cleaved strand of DNA, and the prime editor nicked the unedited strand, directing the cell to reconstruct by matching with the edited strand. Liu and his team (Anzalone *et al.* 2019) used prime editing to target genes underlying Tay-Sachs disease and RBC anemia. Prime editors (PEs), in the first instance represented by PE1, where an RT combined with a nickase Cas9 or RNA-guided nickase (Cas9n) and a pegRNA directly duplicated heritable data from the appended region on the pegRNA into the target genomic site. Whereas, PE2 exploited an assembled RT to regulate altering potencies, while PE3 nicked the non-edited strand, induced its substitution and augmented modifying potency, usually from 20–50% and 1–10% indel development in human HEK293T cells (Anzalone *et al.* 2019). Prime editing generated fewer by-products and effectuated similar potency in comparison to Cas9-initiated HDR as well as corresponding virtues and flaws in comparison to base editors. Prime editing is less repressed by the location of the PAM sequence and has immense potential towards the advancement of genome editing technology via rectification of most pathogenic alleles.

2.6 *Single-cell genome engineering*

Generating the whole plant from a single cell remains challenging because of the genotype-dependent low regeneration frequency and lack of elite germplasm for use in agriculture (Altpeter *et al.* 2016). Therefore, an efficient single-cell genome engineering evoked significant accomplishment in plant biotechnology by embarking research on numerous forefronts. Recent, endeavours employing protoplast transformations in lettuce and tobacco highlighted the transfer of CRISPR/Cas9 constructs in the form of RNP (Ribonucleoprotein)-complexes (Woo *et al.* 2015; Kim *et al.* 2017; Lee *et al.* 2018a). In many plant species, the regeneration frequency was quite low (<2%) from protoplasts after transformation, thus different morphogenic parameters have been utilized to boost the regeneration efficiencies (Altpeter *et al.* 2016; Lowe *et al.* 2016). Temporal expression of shoot-related transcription elements employing protoplast single-cell transformation, recognizing efficient procedures to pump up regeneration competency of the CRISPR/Cas modified recalcitrant cells and/or equating cells of germplasm with robust regeneration potency to those that are recalcitrant for screening regeneration amplifiers are approaches that may fire-up the capability of regenerating plants from single cells. In this manner, the single-cell genome engineering approach may be significantly effective in boosting up the CRISPR/Cas genome editing system.

2.7 *CRISPR/Cas9-based germline engineering*

Plant genome editing endeavours are generally regulated via conventional tissue culture, as well as transgenesis techniques. However, all these classical strategies confined the repurposing of the CRISPR/Cas machinery in numerous crop plants, in particular, those which were recalcitrant to regeneration or *Agrobacterium*-mediated transformation (Yadava *et al.* 2017; Agarwal *et al.* 2018; Sandhya *et al.* 2020). So, an alternative technique needs to be developed, which is independent of the classical tissue culture system. Germline cells are the precise target cell kinds for this

technique, where transport of DNA or protein required for CRISPR/Cas9 methodology can permanently modify the genotype. Foreign DNA free RNP-mediated genome modifying of germline cells would be an ideal approach for germline engineering. Several approaches may be used including, polyethylene glycol (PEG)-mediated transfection, biolistic gene gun, microinjection, magnetofection, or electroporation that are suitable to few germline cells corresponding to the developmental stage of plant and plant species (Mao *et al.* 2016; Mohanty *et al.* 2016). Improving delivery methods may ensure the amplification of the programmes in plant genome engineering. Some viral systems may also be employed to supply sgRNAs to germ cells (Sedeek *et al.* 2019).

2.8 *Genome editing for a single trait*

CRISPR/Cas9-based genome modification has crucially empowered crop breeding via targeted genome editing of numerous agronomically significant traits (Chen *et al.* 2019; Schindele *et al.* 2020). Agronomically significant trait regulated by only one gene is termed as a single-gene trait, for instance, several qualitative traits, i.e., oil quality, rice grain aroma. Mutations of this single gene generally do not affect other important agronomic traits but the targeted one. For instance, CRISPR/Cas-based KO of the rice metal transporter gene *OsNRAMP5* culminated into low Cd accretion in grains without influencing other significant agronomic characters (Tang *et al.* 2017a). Editing a single gene *ZmLG1* led to erect architecture in corn and these plants showed enhanced density in the field (Li *et al.* 2017b; Tian *et al.* 2019). Additionally, umpteen single gene controlling traits, for instance, *OsWAXY* gene that controlled the rice amylose content (Sun *et al.* 2017); *OsBADH2* gene that codes for rice grain aroma (Shan *et al.* 2015); *FT2* gene, i.e., *GmFT2a* and *GmFT5a* that regulated the photoperiod in soybean (Cai *et al.* 2018, 2020) and phytate in corn (Liang *et al.* 2014) have been controlled by directed mutagenesis employing CRISPR/Cas9 editing.

2.9 *Multiplexing of genes for trait stacking*

Installation of some agronomic traits may be dependent upon the precise engineering of complex metabolic pathways that require a conjunct expression of multiple genes which in turn often needed multiple loci editing. Hence, a molecular tool-kit with an aptitude to alter numerous genes concurrently, pose immense potential to interrogate gene functions, and introduce quantitative traits. CRISPR/Cas-based genome modification offers robust multiplexing abilities (Adiego-Pérez *et al.* 2019). Multiple sgRNAs were assembled into a single sgRNA-Cas9 expression vector utilizing the Golden Gate Cloning/Gibson Assembly method (Xie *et al.* 2015; Silva and Patron 2017). The endogenic tRNA-processing technique was also engineered by scientists as a platform for increased targeting via the multiplexing capacity of the CRISPR/Cas9/Cpf1 system (Ding *et al.* 2018). Wang *et al.* (2017) reported the feasibility of multiplexing gene modification. Further, several gRNAs might be tailored to target a unique gene for increased crop editing rates with meagre trans-modification efficiencies by the customization of numerous sgRNAs, for instance in rice, engineering CRISPR/Cpf1 with a crisp DR-guide assembly (Hua *et al.* 2019b; Wang *et al.* 2019c). Lately, plant MGE has shown promising results via enabling modified homeoalleles/numerous alleles or concurrent incorporation of around 107 genes of a single gene family, to create umpteen target sites within a gene, to target numerous genes, and to generate discrete gene variants (Kannan *et al.* 2018). Despite, genome editing capabilities and benefits of ZFNs and TALENs, the CRISPR/Cas9 framework provided far more adaptability for the modification of dissimilar targets due to its simplicity, precision, and relative cost-effectiveness to deliver multiple sgRNAs (Xing *et al.* 2014; Ma *et al.* 2015; Kaul *et al.* 2020). Recently, it was revealed that two gRNAs were concurrently employed to change two targets lying in the conserved domain of endogenous acetolactate synthase gene (*ALS1*) in rice that resulted in amino acid replacements, i.e., W548L and S627I (Sun *et al.* 2016). The MGE machinery has proved efficacious in numerous practical applications with different forms. MGE randomly stimulated sizable fragment deletions and/or insertions in the non-coding regions that generated a vast range of genetic variants with differential expressions (Najera *et al.* 2019). Targeting various positions of a 2 kb fragment upstream of the *CLAVATA3* gene in tomato via MGE mechanism generated heterozygous loss-of-function in terms of variations in fruit dimensions and architecture of the inflorescence (Rodriguez-Leal *et al.* 2017). Lately, the optimization of the MGE framework in *Arabidopsis thaliana* generated concurrent mutations in the genes via excision of 450 bp controlling regions inside the second intron of *AGAMOUS* gene that regulated flowering (Yan *et al.* 2016). In addition, alterations in genes of different hormone biosynthesis pathways within umpteen number of crops were achieved employing MGE. For instance, mutants

with excessive accumulation of gibberellic acid were obtained by simultaneous KO of four paralogs of rapeseed RGA family utilizing two gRNAs (Yang *et al.* 2017). Similarly, simultaneous mutation of two paralogs of the barley *HvPM19* gene hindered the gibberellic acid biosynthesis pathway (Lawrenson *et al.* 2015). In addition, the re-modeling of plant developmental features was attempted in numerous crop species. This may be enumerated as follows: (a) various seed storage proteins were effectively altered utilizing the MGE of paralogous genes; (b) In sorghum, the simultaneous modification of numerous genes from the KIC family employed a unique gRNA that introduced multiple mutations (Li *et al.* 2018b). (c) MAP kinase signaling pathway was targeted to determine the role of partially inessential genes, i.e., *MPK1, MPK2, MPK5*, and *MPK6* that produced double (45%) and quadruple (86%) mutants in rice (Minkenberg *et al.* 2017). The CRISPR/Cas-based method has been comprehensively used for the promotion of enormous technological breakthroughs in other crops, which was not accomplished, formerly.

2.10 *Molecular farming in plants*

Molecular farming is a biotechnological programme that features the genetic alteration of agricultural merchandise, manufacture chemicals and proteins for pharmaceutical, more so for commercial purposes by enhancing crop quality. In line with this, field plants may pose as beneficial model systems over alternative mammalian systems (Buyel 2019). The CRISPR/Cas system provided a far better way to generate biopharmaceuticals in plants by altering the salient features of a recombinant macromolecule. The alteration of plant metabolism sans the synthesis of glycans is a notable example of molecular farming (Ma *et al.* 2003). Generation of human therapeutic proteins in plants many a time import a plant-like glycan instead of human-like that may be viewed as unwanted as they may influence numerous biological functions, immunogenicity, and protein stability. KO of the desired gene, which encoded the catalysts, i.e., as b(1,2) xylosyltransferase (XylT) and a(1,3)-fucosyltransferase (FucT) generated preferred recombinant protein instead of plant glycan synthesis. These two catalysts were responsible for the generation of principle glycan impressions in crop proteins that are distinct than those generated in mammals. Reports revealed the KO of two genes that encoded for 2 XylT and 4 FucT enzymes led to the production of a recombinant antibody that lacked synthesis of plant glycans in *N. benthamiana* mutant (Jansing *et al.* 2019). On similar lines, CRISPR/Cas-based KO of two genes encoding enzyme XylT and FucT in tobacco, cv Bright Yellow 2 (BY2) cell suspensions generated plant glycan-deficient recombinant proteins. Subsequently, the MGE method provided a brilliant forum for the development of important biopharmaceutical products (Mercx *et al.* 2017; Hanania *et al.* 2017). This may offer an enormous probability of enhanced credible target for genome engineering.

2.11 *CRISPR and plant domestication*

Modern crops have been specifically bred for several centuries for the incorporation of significant attributes in order to develop nutrient-rich, better-quality foodstuffs. Thus, this method has culminated into reduced diversity amongst crops that may have an effect on human fitness (Østerberg *et al.* 2019). Currently, tools for editing genomes may be used to re-design and reconstitute genetic variations in distinct genes offering an efficient solution for current demands for food. Combined with classical breeding, CRISPR/Cas-based editing of genomes may accelerate the fusion of favourable attributes, thereby minimizing the expenses efficiently. Subsequently, the genetic components incorporated may be removed from the genome via breeding or developing segregating lines that resulted in null segregates (Mao *et al.* 2019). The effectiveness of targeting traits that are controlled by a single gene employing CRISPR/Cas-based editing of genomes has been validated in several crops, for instance, enriched amylose content in rice and oil quality of soybean (Haun *et al.* 2014; Zhang *et al.* 2017a). However, reports showed that quantitative traits were controlled by numerous QTLs, many of which have been screened, mapped, and cloned (Zuo and Li 2014; Xing and Zhang 2010) and editing of such QTLs may lead to negative effects. CRISPR/Cas breeding has emerged as an innovative and efficacious approach for plant domestication, thereby ensuring increased crop diversity and agricultural sustainability. To exemplify, CRISPR/Cas-empowered aping of domestication events in wild or semi-domesticated plants resulted in the development of crops harbouring neoteric traits and generated enormous sources of germplasm for breeding, thereby creating a new sort of evolution. Improvement of potato has faced enormous difficulties using the conventional breeding process. Recently, a self-compatible diploid potato has been re-domesticated via disruption of the self-incompatibility

S-RNase gene employing the CRISPR/Cas9 technique (Ye *et al.* 2018). Wild tomato plants, those that are naturally biotic/abiotic stress-resistant may emerge as a starting point for *de novo* domestication. Based on this wild tomato was domesticated without losing the stress resistance capability of the first wild germplasm (Li *et al.* 2018c; Zsögön *et al.* 2018). One group of scientists revealed that a wild relative of tomato referred to as husk tomato (*Physalis pruinosa*) was domesticated with higher yield and larger fruit (Lemmon *et al.* 2018). Novel domesticated crops amoured with increased tolerance to a variety of harsh environments would augur agricultural diversity and resolve several issues related to agricultural sustainability in the future.

2.12 *Metabolic engineering via CRISPR*

Metabolic engineering through the CRISPR/Cas system is an evolutionary approach for modulating metabolism, wherever plant cells are focused to generate desired metabolites (Lau *et al.* 2014). A regulatory gene, O-methyltransferase (OMT2) facilitated the biosynthesis of different metabolites (noscapine, papaverine, codeine, and morphine) through benzylisoquinoline alkaloids (BIAs) pathway. Alagoz *et al.* (2016) altered the biosynthesis of BIAs in *Papaver somniferum* by KO of 30 *OMT2* genes employing the CRISPR/Cas9 mechanism. Moreover, GABA shunt metabolic pathway regulating genes, i.e., SSADH, CAT9, GABA-TP1, -TP2, and -TP3 were modified employing six-gRNAs that led to increased GABA levels by 19-fold as compared to wild type (Li *et al.* 2018d). Li *et al.* (2017c), targeted the SmCPS1 (diterpene synthase) gene from *Salvia miltiorrhiza* involved in tanshinone (well-known for antiarrhythmic and vasorelaxation effects) biosynthesis. With the aid of the CRISPR/Cas9 technique, eight chimeric and three homozygous mutants were generated from twenty-six distinct transgenic hairy root lines of *Salvia*. The chimeric mutants exhibited reduced accumulation of tanshinone, whereas the homozygous mutants showed no accumulation. Therefore, such a genome editing technique may be employed for transforming plants to biofactories for the generation of desired metabolites, just by altering gene sequences in a targeted manner. Thus, the new advanced tools in the CRISPR/Cas genome editing tool-kit have expanded the avenues to design different strategies for the development of preferable agronomically significant attributes in crops of interest.

3. Novel approaches in genome editing for precision crop improvement

CRISPR/Cas genome editing platform has emerged as a multifunctional tool-kit for genetic improvement of important traits, thereby conferring a revolutionary effect on plant research and crop breeding. Mainly three novel genome modification methods may be recruited for defined desirable mutational events in crop genomes i.e., (i) Loss of function mutants (ii) Gain/change of function mutants and, (iii) Epigenemone modified mutants: fine-tuning gene regulation

3.1 *Loss of function mutants*

Over the past decade, loss-of-function mutations were studied extensively with the development of RNAi technology for knock-down of the desired gene. Due to incomplete loss-of-function and a high percentage of off-target effects, the RNAi technique is less effective than the CRISPR/Cas system (Xu *et al.* 2006; Barrangou *et al.* 2015). The loss-of-function mutation by the CRISPR-based system is the simplest mechanism for a much better understanding of gene function. Desired loss of function may be accomplished by DSB to the targeted loci, which then would be repaired by the NHEJ-mediated repair pathway. Alterations within the genome may be attained via frame-shift mutations. In frameshift mutations, indels are produced by ZFNs/TALENs/Cas9 rendering loss of function alleles. Due to its efficacious and versatile nature, indels developed by this approach have evolved as a distinctive technique to analyze the functions of uncharacterized genes in plants (Chen *et al.* 2018). Numerous economically important agronomic traits have been developed via site-directed mutagenesis, for instance, 41.2% LbCpf1-derived mutations were recognized via KO of the bentazon sensitive lethal (OsBEL) gene (Xu *et al.* 2017). Shan *et al.* (2013) revealed that disruption of the *OsPDS* gene using CRISPR/Cas9-assisted mutagenesis resulted in expected phenotypes in rice. Similar results had been highlighted in *Arabidopsis* and tobacco (Li *et al.* 2013). The CRISPR/Cas-mediated KO of *ZmIPK* in *Zea mays* protoplasts revealed 13.1% mutation efficiency (Liang *et al.* 2014) along with the reduction in phytic acid and simultaneous increment in inorganic phosphate levels in maize seeds (Shi *et al.* 2003; Liang *et al.* 2014). sgRNA-Cas9-effectuated loss of function mutation in the promoters of two genes (*OsSWEET14* and *OsSWEET11*) resulted in bacterial blight resistance

in rice (Jiang *et al.* 2013; Xu *et al.* 2019). Soyk *et al.* (2016) reported that mutations in the self-pruning 5G (*SP5G*) gene enabled the development of early-yielding varieties of tomato.

3.2 *Gain/change of function mutants*

Besides loss-of-function analysis, the CRISPR/Cas9 machinery may also be utilized for gain or change of function mutations by knock-in of desired sequences via HDR-mediated repair mechanism. HDR or homology donor repair involved an exogenously supplied DNA donor template with ends homologous to the targeted DNA, accurately modified the genes thereby creating a new phenotype or function. HDR-mediated knock-in has been achieved in different plants, for instance, tobacco and rice by using protoplast transformation (Shan *et al.* 2013; Li *et al.* 2013) and in *Arabidopsis* using *Agrobacterium*-mediated transformation (Schiml *et al.* 2014). Zhao *et al.* (2016) effectively standardized a gene deletion/replacement method to develop stably transformed Arabidopsis plants that may be employed to incorporate desired traits for prospective precision-targeted crop enhancement. They developed a vector carrying the dual-sgRNA-Cas9 constructs that efficaciously deleted miRNA gene regions from *MIR169a*, *MIR827a* and highlighted deletion efficiencies for *MIR169a* (20%) and *MIR827a* (24%) loci, respectively. Subsequently, they created another construct that comprised of sites homologous to *Arabidopsis TERMINAL FLOWER 1* (*TFL1*) to enable HDR with sequences identical to the two sgRNAs. The co-transformation of the two constructs into *Arabidopsis* plants led to both precision deletion and donor guided repair for desired gene substitution. Results exhibited that 0.8% of the transformed *Arabidopsis* plants harboured the swapped templates. They also incorporated an eGFP expression cassette flanked by left and right homologous arms of the AtTFL1 (*A. thaliana* terminal flower 1) gene into a targeted site within this gene via CRISPR/Cas9-mediated gene replacement. Further, it has been noted that amino acid substitutions via the base editing approach may generate partial gain or change of functional alleles. Active sites within distinctly-characterized proteins might be targeted by base editors (ABE/CBE), to evoke specific amino acid alterations for the creation of loss or gain-of-function alleles (Nishida *et al.* 2016; Shimatani *et al.* 2017; Zhong *et al.* 2018; Li et al 2018a). Different herbicide-resistant soybean (Li *et al.* 2015), maize (Svitashev *et al.* 2016), and rice (Endo *et al.* 2016; Sun *et al.* 2016; Li *et al.* 2016; Butt *et al.* 2017) plants were developed via HDR-guided base transversions.

3.3 *Epigenemone modified mutants: fine-tuning gene regulation*

Beyond gene disruption via knock-out/knock-in, genome modification tools may control the expression of genes. Gene regulation may be attained by a fusion of transcriptional repressors or activators to the DNA-binding domains thereby directing gene activation or repression (Qi *et al.* 2016; Kaul *et al.* 2020). Expression of genes may be influenced at different levels, such as transcription, mRNA maturation, and translation. Portions of promoter regions of desired genes may serve as potential target sites for CRISPR/Cas-based genome modification. Non-specific disruption in the *cis*-elements modulated the expression of the desired gene, in a dose-dependent way (Birchler 2017). Besides, the non-specific mutations, the targeted disjunction of the *cis*-elements may regulate the gene transcription. Insertion/s of a transposon in promoters or upstream of the promoter sequences might modify the genes' epigenetic status. dCas9-equipped epigenome modifying tools may regulate gene expressions via epigenomic modulations. The idea of controlling the epigenetic status of the promoter for the modulation of gene transcription has been utilized in plant systems for the advancement in crop breeding (Hilton 2015; Liu *et al.* 2016; Dominguez *et al.* 2016; Hua *et al.* 2019a). In addition to targeting the promoter regions, gene expression may be regulated via spatio-temporal targeting of the gene. In this strategy, the dCas9 can be fused to any activator such as VP16 and VP64 (Gilbert *et al.* 2013; Moradpour and Abdulah 2020) or repressor such as Kruppel-associated box (KRAB) (Lawhorn *et al.* 2014; Li *et al.* 2017c) and thereby, activating (CRISPRa) or inhibiting (CRISPRi) the gene expression, respectively. Gene expression may be regulated even at the post-transcriptional level. By far microRNAs (miRNAs) regulated the gene expression at the post-transcriptional level by a translational blockage of the coding mRNA. Gene expression may be regulated by interfering with miRNA-mRNA binding through the introduction of a point mutation in the miRNA-binding site of the target gene that may in turn be achieved without disruption in the amino acid sequence of the protein. The position and frequency of the mismatches in the miRNA-mRNA pair, guide the efficacy of miRNA directed downgradation of the mRNA, wherein, both CBEs and ABEs may be used; thus

effectuating the post-transcriptional expression of the gene in plants (Hua *et al.* 2019a). The regulation of gene expression through CRISPR/Cas tools played a potent role at the gene translational level also. In plants, numerous transcripts harbour upstream open reading frames (uORFs) in conjuction with the standard open reading frame. CRISPR/Cas9-based hindrance of the uORFs' translation due to the inception of mutation/s in the start codon of the uORF consequently accelerated protein translation downstream of the standard open reading frame (Zhang *et al.* 2018). Conjointly, the incorporation of an NHEJ or HDR guided translational enhancer in the 5' UTR region increased the expression of the target gene (Hua *et al.* 2019a), although this technique has not been employed in crop plants yet. Neoteric approaches employing CRISPR/Cas-mediated genome editing tools have ushered in an era of crop trait improvements, such as enhanced yield, upgraded nutritional traits, resistance/tolerance to biotic/abiotic factors, and so on.

4. Strategies alleviating off-target effects

Despite many advantages of CRISPR/Cas technology, it still faces various challenges. One of the potent features that scientists require to appropriately analyze is its off-target impacts in the CRISPR/Cas modified genomes. Generation of unbidden mutations that may arise due to off-targets at random sites in the target gene sequence or at any other site within the genome may hamper precise genome modifications. Nevertheless, accuracy and efficacy of the CRISPR/Cas machineries may be influenced by numerous parameters, for instance, target site recognition and designing of sgRNAs, the frequency of HDR-mediated repair, different delivery techniques, and Cas9 inactivity induced by anti-CRISPR proteins, etc. These themes have been discussed in a nutshell in the following sections.

4.1 *Stringency in sgRNA designing*

Various approaches have been proposed to alleviate off-target mutation/s. Firstly, the effect may be reduced by the use of highly specific Cas nuclease or stringent sgRNA design, which is different from the other genomic sites by three mismatches in combination with one mismatch present in PAM proximal region. The success of any CRISPR experiment relies enormously on the specificity of the guide RNA (sgRNA). The sgRNAs with more than 50% GC content were sufficient to promote on-target mutagenesis due to their enhanced target-site binding capability (Kim *et al.* 2015). Efficient designing of sgRNAs enabled the targeted mutagenesis, regardless of whether several homologous loci existed in the genome that are being interrogated (Ren *et al.* 2014; Baysal *et al.* 2016; Mao *et al.* 2016).

Currently, different computer algorithms are freely available, for instance, Cas-OFFinder (*http://www.rgenome.net/casoffinder*) that identifies unique target sequences and possible off-target sites in the genomes of targeted species and organisms (Cong *et al.* 2013; Hsu *et al.* 2013; Gerashchenkov *et al.* 2020). Among several bioinformatic tools, CRISPR-P and CRISPR-PLANT are explicitly structured for Cas9-based genome modifications. CRISPR-P facilitated gRNA designing of practically all plant species whose genome sequences are accessible, and furthermore provided both off-target- and restriction-site analysis (Lei *et al.* 2014). Consequently, CRISPR-PLANT has provided a genome-wide analysis of extremely specific RNAs in eight or more plant species and also facilitated restriction enzyme analyses of target sites (Xie *et al.* 2015). Moreover, many improved bioinformatic software tools are also available for designing gRNAs, for instance, CRISPR Design (*http://crispr.mit.edu*), which provided a scoring procedure for selecting sgRNAs with least off-target sites. sgRNA designing tools, for instance, Chop-Chop (*http://chopchop.re.fas.harvard.edu*) and sgDESIGNER, a freely accessible application (*http://crispr.wustl.edu*) provided an easy-to-use sgRNA designing interface (Hiranniramol *et al.* 2020). The four distinct forms of sgRNA may generate maximum on-target and minimum off-target effects. Modified sgRNAs may decrease off-target mutations with increased efficacy without renouncing mutation efficiencies at an on-target site. Lin *et al.* (2014) described various guidelines to design sgRNAs for reducing the off-target effects in numerous crop species. Results revealed that sgRNA bulges to a maximum of 4 bp can be sustained by the CRISPR/Cas9 machinery. The correspondence between cleavage function and position of DNA or sgRNA bulges in correlation to the PAM seems to be loci- and sequence-dependent. Firstly, (i) target sequences should be selected strictly with GC level (~40%–60%), (Hiranniramol *et al.* 2020) (ii) target DNA sequences should be distally placed from either NGG- or NAG-PAMs with ≤3 mismatches at 5′- and 3′- ends of DNA bulges or around 7–10 bp upstream of PAM, (iii) moreover, efficient sgRNA bulges should persist within 12 bp from PAM (Lin *et al.* 2014). The sgRNAs with minimum mismatches beyond seed sequences may prompt

off-target changes (Shan *et al.* 2013). Few mismatches may be tolerated by the sgRNA-Cas9 complex, with mismatches being furthermore endured within regions downstream of the seed sequence (Cong *et al.* 2013; Lino *et al.* 2018). To avoid off-target effects, important components of CRISPR/Cas9, the PAM, and seed sequences require designing with utmost precision. For enhanced targeted cleavage efficiency, the designed sgRNAs should be complemented with the proper selection of promoters and terminators. Undesired cleavage of DNA target sites may arise due to promiscuous PAM regions. To avoid these events, bioinformatic tools, for instance, E-CRISPR (Heigwer *et al.* 2014) and CasOT (Xiao *et al.* 2014) may be employed to ensure sgRNAs designing based on whole-genome sequence information. Therefore, the specific designing of the sgRNAs fostered targeted mutation efficacies and alleviated unintended off-target mutation/s, which has been accounted for in numerous plants, for instance, *A. thaliana* (Peterson *et al.* 2016), *Oryza sativa* (Feng *et al.* 2014) and many more.

4.2 *Factors affecting RNA efficacy*

Length, mismatches, and GC content of gRNAs are important factors that regulate off-target effects. The distinctive target sequence choice that may vary from any other regions within the genome by a minimum of two or three nucleotides in a 20-nt sequence is a significant factor for reducing off-target effects (Cho *et al.* 2014). Cas9 off-target efficiency may raise significant concerns as it can re-write a target DNA harbouring five mismatches to its sgRNAs (Cradick *et al.* 2013; Fu *et al.* 2013) and alters the fidelity of Cas9-gRNAs. Generally, Cas9-gRNA was unable to recognize a DNA site that harboured greater than three mismatches close to a PAM (within10–12 bp). Therefore, the length, mismatches, and GC content of sgRNAs may influence the rate of targeted mutations. Sugano *et al.* (2014), described the impact of sgRNA nucleotide lengths (∼16, 17, 18, and 20 bp) on genome editing efficacy and off-target mutation frequency. It was revealed that sgRNA sequences with 18 bp nucleotides reduced the off-target mutation efficiency. Doench *et al.* (2016) re-inforced that CRISPR-gRNA sequences directly influenced the on-target DNA cleavage efficiency, thereby avoiding unintended off-target binding and cleavage (Doench *et al.* 2016). Li *et al.* (2017a), assessed the effect of off-target mutations utilizing four sgRNAs in rice. Tang *et al.* (2018) also revealed that greater than two mismatches in sgRNAs on the target position may avoid unexpected mutations in rice and many other crops.

4.3 *Concentration of sgRNA-Cas9 and Promoter selection*

Another crucial parameter that modulates off-target effects is the concentration of sgRNA-Cas9, which may be controlled by titrating the amount of sgRNAs and Cas9. Lower concentrations of the sgRNA-Cas9 complex led to reduced off-target mutations. This strategy effectuated high-efficiency rates of on-target mutagenesis in targeted gene/s within plant species (Jia and Wang 2014). The choice of the promoter is a crucial step towards the regulation of Cas9 expression. Wang *et al.* (2015a) revealed that employing the egg cell-specific promoter in place of a constitutive CaMV35S promoter led to stronger expression of the Cas9 gene that in turn resulted in higher genome editing efficiency in *A. thaliana*. Contrastingly, on the utilization of high amounts of sgRNAs, lower editing efficiencies of the CRISPR/Cas9 system were reported in *A. thaliana* and tomato plants. Zhang *et al.* (2017b) demonstrated that the use of YAO like embryo-specific promoters played a crucial role in the *Citrus sinensis* genome via enhancement of the sgRNA-Cas9 expression. In few monocots, the expression of Cas9 controlled by their endogenous promoters resulted in higher on-target mutations than the CaMV35S promoter (Shan *et al.* 2015). Moreover, it has been investigated by Sun *et al.* (2015) that the U6-10 promoter-based on-target mutation frequency increased by 2–4 fold when compared to the AtUbi promoter in case of *Glycine max*. Nevertheless, the AtUbi and 2×CaMV35S promoters' mediated expression of Cas9 generated similar on-target mutation efficiencies in tomato plants (Pan *et al.* 2016). Numerous studies conducted showed enhanced on-target mutations, for instance, Xu *et al.* (2015) showed that segregation of T_1 rice lines harbouring sgRNA-Cas9 exhibited lower off-target mutations. An appropriately balanced ratio of sgRNA:Cas9 led to an increase or decrease of on- and off-target variation rates, respectively. Li *et al.* (2013) investigated the effect of sgRNA:Cas9 ratios (1:1, 19:1, and 20:1) on the mutation rate in *A. thaliana* and revealed that sgRNA:Cas9 (1:1) resulted in targeted mutations in the desired genes AtPDS3 (5.6%) and AtFLS2 (1.1%). Further, they compared two methods, firstly, the cgRNA-Cas9 ratio (chimeric guide RNAs) at 1:1 and secondly, the cgRNA-Cas9 co-delivery into cells for exploring mutagenesis efficiency in *Nicotiana benthamiana*. They demonstrated that the gene modification frequency of the co-delivered Cas9 and sgRNA was higher (12.7%) than in the case of the mixed ratio of (1:1) cgRNA:Cas9 (1.8%). This suggested that the

presence of Cas9 and cgRNA in a single construct and their efficient co-delivery led to higher on-target mutation efficiency than employing a ratio of cgRNA:Cas9 at 1:1. It has been evaluated by Malnoy *et al.* (2016) that the mutation efficiency varied with the targeted gene locus and the ratio of sgRNA and Cas9. The best ratio of sgRNA-Cas9 as well as the use of RNP (ribonucleoprotein) complexes contributed to maximum mutation efficiencies in apple and grape. A few reports suggested that high level of sgRNAs led to low editing potentials, therefore, it was concluded that selection of suitable CRISPR/Cas9 edited plants wherein the CRISPR components were segregated, conferred low or no off-target mutations. Likewise, different ratios of sgRNA-Cas9 may increase or decrease on- and off-target mutations frequencies in various crops.

4.4 *Novel players in genome editing: Cas9 orthologs and Cpf1 variants*

Interestingly, Cas9 proteins reported from different organisms, including *Neisseria meningitides* (NmCas9) (Hou *et al.* 2013); *Staphylococcus aureus* (SaCas9) (Ran *et al.* 2013) and *S. thermophiles* (StCas9) (Kleinstiver *et al.* 2015) were employed for gene editing. These Cas9 variants identified distinct PAM sequences and displayed differential potencies. However, the choice of an optimal and specific ortholog of Cas9 may ensure enhanced gene editing efficacy for a target sequence. Recently, two important SpCas9 variants, for instance, engineered high accuracy xCas9 and Cas9-NG (targets non-canonical PAM) were identified. Amongst them, xCas9 variant revealed nearly indistinguishable editing efficiency as compared to wild-type Cas9 at almost every canonical PAM (NGG) site, while it revealed reduced activity at a non-canonical PAM site NGH (H = A, C, T). In the case of CBE systems, xCas9 at canonical PAM (NGG) sites resulted in higher editing efficiency. Contradictorily, Cas9-NG variants revealed increased editing efficiencies exclusively at non-canonical AT-rich PAM sites (GAT, CAA, and GAA) as compared to xCas9 variants. However, Cas9-NG variants showed significantly diminished activity at the sites harbouring canonical PAM (Hua *et al.* 2019b; Ren *et al.* 2019; Zhong *et al.* 2019). Furthermore, the efficiency of certain variants to act upon non-canonical NGCG, NG, and NGA PAMs has broadened the editable range of ABEs (Jeong *et al.* 2019). Besides, both the variants xCas9 and Cas9-NG, engineered SpCas9 (XNG-Cas9) also efficiently mutagenized constitutive target sites with GAA, NG, GAG, and GAT like PAMs in the genomes of *Arabidopsis* and tomato (Nishimasu *et al.* 2019). Further, other identified nucleases such as AsCpf1 (BV3L6) and LbCpf1 (ND2006) displayed restricted accessibility of relevant target loci, which reduced their functional utility. Cpf1 commands a definite prerequisite of a PAM (TTTV) to be present in their DNA substrates, where V might be A, C, or G. Two variants of AsCpf1 protein engineered to harbour mutations of S542R/K548V/N552R and, S542R/K607R accepted TATV and TYCV as PAMs, respectively, which enhanced their target sequence recognition specificities via the reduction in off-target effects. In addition, the introduction of a subsidiary non-PAM-interacting mutation further increased their efficacies. Similarly, the variant of LbCpf further showed increased target specificity and recognized altered PAM sites (Gao *et al.* 2017). Recently, FnCpf1-RR and FnCpf1-RVR, the two variants of FnCpf1 recognized canonical PAMs and exhibited increased target specificity in rice protoplasts than their wild type (FnCpf1) (Zhong *et al.* 2018).

4.5 *Anti-CRISPRs*

Anti-CRISPR (Acr) proteins referred to as natural 'off switches' de-activate the Cas9 protein by directly disarming its activity. The protein sequences and the functional mechanisms of these anti-CRISPRs vary widely and potentially interrupted several stages of the CRISPR/Cas machinery that include, crRNA maturation including processing and assembly, spacer acquisition, Cas protein expression, target DNA recognition and binding, and cleavage of target DNA (Marino *et al.* 2018). Initially identified Acrs were able to silence type I-E and I-F systems in phages that effectively infected *Pseudomonas aeruginosa* in combination with active type I CRISPR/Cas machinery and complementary CRISPR spacer (Bondy-Denomy *et al.* 2013). Eventually, numerous other Acr proteins were discovered and some of them actively inhibited types I-D, I-C, II-C, II-A, and V CRISPR/Cas machineries (Rauch *et al.* 2017; Marino *et al.* 2018; Lee *et al.* 2018b; Watters *et al.* 2018). Presently, more than 50 Acr proteins have been characterized that serve as inactivators of CRISPR's molecular scissors by inactivating the cut-and-paste features of CRISPR machineries (Dolgin 2019). AcrIIA4Lmo (type II-A) actively blocked the nuclease activity of the most frequently used SpCas9 by counteracting Cas9-DNA interaction by sterically

occupying the PAM-interacting domain (PID) and rendering the RuvC nuclease domain inaccessible, in part via DNA mimicry (Dong *et al.* 2017; Shin *et al.* 2017; Yang and Patel 2017). It has been revealed that type II and V Acrs drastically reduced the activity of Cas9 and Cas12a-mediated applications. Furthermore, sixteen families of the type I Acr proteins were routinely encoded next to the putative transcriptional regulatory genes but did not inherit nucleotide sequences or common structural resemblances to that of anti-CRISPR-associated (A*ca*) genes (Marino *et al.* 2018; Pawluk *et al.* 2018). Till now, the important functions of Aca proteins remain ambiguous. Recently, it was deciphered that Aca2 acted as an auto regulator, which suppressed the anti-CRISPR–aca2 operon. Helix-turn-helix domain of Aca2 protein generated a homodimer, which through interaction with dual inverted repeats in the promoter region of the anti-CRISPR gene inhibited DNA binding. Thus, it was highlighted that auto regulator Aca2-mediated the anti-CRISPR suppression (Pawluk *et al.* 2016; Birkholz *et al.* 2019). Recently, scientists incorporated Acr proteins into numerous tools, for instance, biosensors where Acrs may track the activity of a therapeutic gene editor inside the cells, and their optogenetic control strategies. It was revealed that if Cas9 remains active for long spans, it may lead to enhanced peril of unwarranted edits in the genome. Incidentally, Acr proteins may overcome this problem by blocking the activity of the CRISPR system (Dolgin 2019). Therefore, Acr proteins may limit the incidence of the undesired effects especially the off-targets within genomes.

4.6 *Genome-wide mutant library screening*

Mutant libraries employing whole-genomes pose as a significant functional genomic analysis approach for genetic improvement of crop traits. The CRISPR/Cas9 machineries are efficacious tools for genome-wide screening, that offered an innovative 'gene discovery platform' due to the simplicity of designing and precision mutagenesis enabled via sgRNA-Cas9 complex for modifying target gene/s expression (Sharma and Petsalaki 2018). To identify desirable traits, for instance, screening of biotic and/or abiotic stress tolerance, nutritional improvement, and other attributes may be performed in the expressed progeny. In the past genetic improvement of crop traits was time-intensive and tedious, which required consistent mapping endeavours. Large scale CRISPR/Cas9-mediated KO mutant libraries were constructed by two major groups, that covered the majority of the rice genes. Lu *et al.* (2017), have identified 34,234 genes and developed over 90,000 transgenic plants. Similarly, Meng *et al.* (2017) screened 12,802 genes and developed greater than 14,000 transgenic lines. For characterizing the unknown proteins, key functional residues of those proteins may be characterized by functional screening using pooled libraries of tiling array of sgRNAs by BEs or Cas9. In case of proteins that are uncharacterized, the crucial functional residues may be scanned via functional evaluation and screening employing methods for the transformation of pooled libraries of a tiling microarray of sgRNAs (utilizing either Cas9, CBE or ABE). The tiling microarray of sgRNAs was devised to comprise several hundreds of sgRNAs that spanned the complete exon of the targeted gene to be edited. In order to screen beneficial alleles, the tiling microarray of sgRNAs may be merged for the development of vector and plant transformation (Hua *et al.* 2019a; Wang *et al.* 2018b). With the emergence of the CRISPR framework, the breeder's toolkit would be updated towards yield improvement and other trait improvements by the introduction of desired mutations in crops.

4.7 *Enhancing HDR-mediated knock-in efficiency*

Amongst two DNA repair pathways, the frequency of HDR-mediated precision DNA repair of DSB is typically lower than the error-prone NHEJ repair pathway. However, several approaches have been attempted for the enhancement of HDR efficiency by minimizing the frequency of NHEJ pathway, including synchronization of the cell cycle (Lin *et al.* 2014), utilization of minute molecular inhibitors of NHEJ repair pathway (Vartak and Raghavan 2015; Yu *et al.* 2015), silencing of the targeted gene (Chu *et al.* 2015) and, employment of NHEJ deficient cell lines (Weinstock and Jasin 2006). The CRISPR/Cas-based DSB repair efficacy employing the HDR pathway enhanced 19-fold by inhibition of DNA ligase IV enzyme of the NHEJ pathway by Scr7 inhibitor (Srivastava *et al.* 2012; Chu *et al.* 2015; Vartak and Raghavan 2015). A dominant-negative mutant of 53BP1, DN1S, when combined to Cas9 nucleases led to DN1S-Cas9 fusion protein that efficiently enhanced HDR frequency (86%) in K562 cells through masking off the NHEJ episodes precisely at Cas9 cleavage loci (Jayavaradhan *et al.* 2019).

4.8 *CRISPR/Cas delivery systems*

Diverse gene-modifying reagents have been transferred into plant cells, employing varied methods, i.e., transformations via protoplast, *Agrobacterium*, and particle bombardment (Zhang *et al.* 2016; Liang *et al.* 2018). Regardless of the delivery techniques, genome-modified cells must be regenerated into whole plants, which employs time-consuming and tedious tissue culture protocols. Majority of crops, lack a distinct and standardized tissue culture-based transformation systems, and developing one may be tedious, time-intensive, and infeasible (Altpeter *et al.* 2016). Even for crops with standardized transformation protocols, several new varieties remain recalcitrant to transformation owing to meagre regeneration capacity, for instance, several cereals (Altpeter *et al.* 2016). A recent report revealed scientific breakthrough employing ectopic production of morphogenic regulators, i.e., Baby Boom and WUSCHEL through *Agrobacterium*-based transformation, which significantly increased the regeneration frequency of various explants, for instance, mature seeds and leaf segments (maize) (Agarwal *et al.* 2018); immature embryos (sorghum, sugarcane, indica rice) (Lowe *et al.* 2016).

Plant viruses lead to enormous losses in yield, globally. The CRISPR/Cas machinery may be repurposed to function as defense mechanism effectuating cleavage of both DNA and RNA viruses, in order to generate virus-resistant plants. Numerous geminiviral replicons have been recommended as transport vehicles, wherein REP (replication initiation protein) gene is co-delivered with sgRNA-Cas9 constructs in to plants (Baltes *et al.* 2014). Yin *et al.* (2015) reported that a geminivirus, i.e., Cabbage Leaf Curl virus (CaLCuV) is capable to transfer gRNA and can be employed to accurately target genomic sites to induce systemic gene mutations in plants. Tripathi *et al.* (2019) generated streak virus-resistant banana. Similarly, targeting the tomato yellow leaf curl virus (TYLCV) encoded coat protein sequences or replicase ensured robust virus interference, exhibited by a significant reduction of TYLCV disease in transgenic tomato plants (Tashkandi *et al.* 2018). As opposed to the majority of Cas proteins, single-stranded (SS) RNAs were cleaved by Cas13a (C2c2) that interceded with turnip mosaic virus (TuMV) replication in plants. Employing viral promoters for driving Cas9 expression minimized off-target effects to an unassuming level (Chen *et al.* 2018).

The choice of a transformation method posed as a crucial factor for increasing on-target efficiency. Generally, gRNAs and Cas9 were incorporated into plant cells either via *Agrobacterium*-based transformation of T-DNA regions or physical delivery systems such as PEG-mediated transformation (protoplasts), biolistic approach (callus). *Agrobacterium*-mediated transformation has emerged as a popular strategy harnessed for transmission of various Cas nucleases (Ali *et al.* 2015). Nonetheless, geminiviral DNA replicons enhanced gene targeting efficiencies by one to two-fold, in contrast to traditional *Agrobacterium*-mediated T-DNA transformation. Recently highlighted TRV or Tobacco Rattle Virus-a RNA virus that replicated within the cell's cytoplasm efficiently transported sgRNAs into Cas9 expressing tobacco lines, in order to accomplish systemic editing of genome. However, on similar lines DNA-virus-based vector delivery system is elusive for CRISPR/Cas genome modification. TRV-assisted CRISPR/Cas system is a speedy, affordable, and effective approach that may infect desired plant species in a comprehensive manner. The development of a virus-mediated genome editing system led to the stable transmission of targeted changes to the next generation.

RNP strategy has arisen as another innovative method to reduce the off-target effects, wherein, the sgRNAs and RNP complexes were delivered either via biolistic or electroporation methods into the protoplast of plants that revealed minimum off-target mutation/s. This system was successfully reported in various plant species, for instance, rice, maize, tomato, and many others (Woo *et al.* 2015; Lee *et al.* 2018a). However, *Agrobacterium*- and biolistic-based delivery methods may be used for stable transformation of sgRNA-Cas9-RNP complexes. Newly developed gesicle technology is a potent tool-kit for reducing off-target impacts with the delivery of active Cas9 protein complexed with sgRNAs by using nanocarriers. Guide-it CRISPR/Cas9 gesicle system is a novel technique for the delivery of active sgRNA-Cas9-RNP complexes to target cells. Cong *et al.* (2013) revealed that the delivery of active RNP complexes in this manner arrested both the genomic integration and overexpression. Integration of these active RNP complexes via gesicles resulted in reduced off-target effects with increased editing efficiency at targeted loci (Hsu *et al.* 2013). Nanoparticle-mediated RNP delivery system has been successfully adopted in plants by decreasing the unwanted changes. Numerous CRISPR/Cas delivery systems in different crop species are mentioned in table 2. The recommended CRISPR/Cas delivery and transformation systems are affordable, cost-effective, and species-independent and led to the generation of efficient genome edited crops harbouring desired trait/s.

Table 2. Different CRISPR-Cas delivery systems in crops

Crop/species	Delivery method	Target tissue	Target gene	Target trait	References
Maize (*Zea mays*)	Biolistic	Embryonic cells or immature embryo	LIG gene, male fertility gene (MS26 &MS45), Liguleless1, acetolactate synthase gene (ALS2)	Stress tolerance	Svitashev *et al.* (2015)
Potato (*Solanum tuberosum*)	Particle Bombardment (gold nano particle and infiltration of RNP Complexes)	Plant protoplasts	Colin	Defence response to biotic and abiotic stress	Anderson *et al.* (2017)
Tomato (*Solanum lycopersicon*)	*Agrobacterium*-mediated transformation	Callus plantlets	RIN	Regulating fruit ripening	Ito *et al.* (2015)
Tomato (*Solanum pimpinellifolium*)	*Agrobacterium*-mediated transformation	Callus plantlets	SP, OVATE, CyCB, FN2	Morphology, fruit no., domestication traits and nutritional improvement	Zsögön *et al.* (2018)
Kiwi fruit (*Actinidia deliciosa*)	*Agrobacterium*-mediated transformation	Callus derived plantlets	PDS	Decrease of carotenoid content and Albino phenotype	Naim *et al.* (2018)
Apple (*Malus prunifolia*)	Direct delivery of purified CRISPR-Cas9 RNP to the protoplast	Plant protoplast	DIPM-1, DIPM-2 DIPM-3	Resistance to fire blight disease	Malnoy *et al.* (2016)
Grapevine (*Vitris vinifera*)	Direct delivery of purified CRISPR-Cas9 RNP to protoplast region	Plant protoplast	ML07	Susceptible gene to powdery mildew	Malnoy *et al.* (2016)
Grapevine (*Vitis vinifera*)	*Agrobacterium*-mediated transformation	Embryonic cells	ldnDH	Biosynthesis of tartaric acid (TCA)	Ren *et al.* (2016)
Banana (*Banana* cv. *rasthall*)	*Agrobacterium*-mediated delivery of Cas9-sgRNA	Plant protoplast	PDS	Albino phenotype and decrease of carotenoid content	Kaur *et al.* (2018)
Sweet orange (*Citrus sinensis*)	XCC-facilitated Agro infiltration delivery	Plant protoplast	PDS	Albino phenotype and decrease of carotenoid content	Jia and Wang (2014)

5. CRISPR achievements: Improving the nutritional content in crop plants

Providing adequate nutritious food emerges as a major challenge for agriculture. Plants are the primary source of the required nutrients for humans and livestock. During the last few decades, improvement of crop quality was achieved via conventional breeding and transgenic approaches, however, recently gene editing tools, viz., mega-nucleases, TALENs and ZFNs have been used. To hasten this improvement technique, an unconventional, efficient, and precise strategy was required (Turcotte *et al.* 2017). More recently, the trend of using modern genome editing strategies like the CRISPR/Cas technique progressively focussed on genes for the improvement of crop quality (Wada *et al.* 2020). The CRISPR/Cas9 framework creates permanent heritable modifications without influencing the agronomic features of the prevailing valuable traits (Feng *et al.* 2014; Pan *et al.* 2016). CRISPR/Cas9 offered a potent mechanism with comparatively higher editing potential in contrast to ZFNs and TALENs (Johnson *et al.* 2015; Gaj *et al.* 2013). Here, we have highlighted the progress and perspectives of

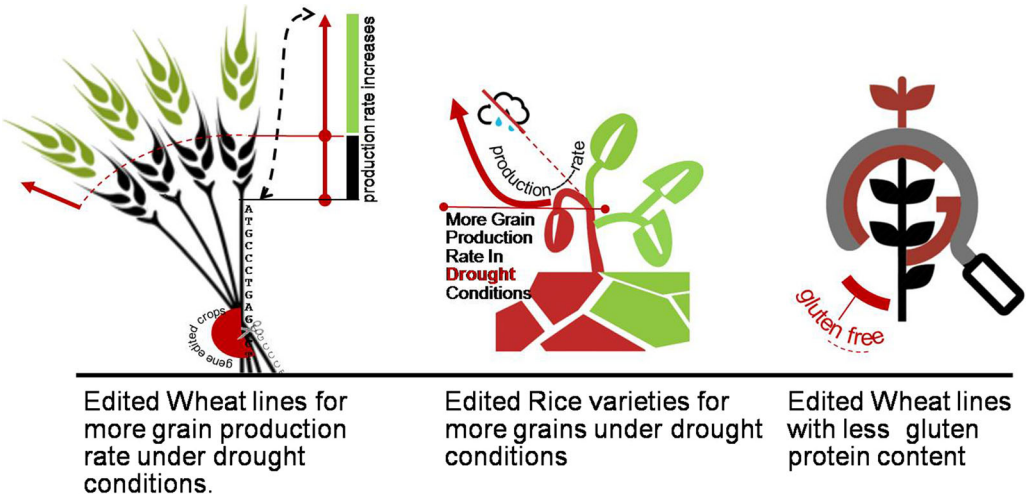

Figure 3. Gene editing in primary crops such as rice and wheat that constitute over 80% source of world's nutritive index. This illustration shows different approaches that can be incorporated by plant breeders that target yield under normal, drought conditions and result in seeds with high nutritive quotient.

nutritionally enhanced food crops developed via CRISPR/Cas-mediated genome engineering (figure 3).

This potent editing innovation has already been enforced in several plant species for its explicit genome modifying capacities. The implementation of the CRISPR/Cas9 machinery in major food crops, for instance, rice and wheat were initially reported by Shan *et al.* (2013). Wang *et al.* (2014) reported the sgRNA-Cas9-mediated precise genome altering in sweet orange. Florida's Institute of Food and Agricultural Sciences (UF/IFAS) generated sweeter strawberries employing the CRISPR/Cas technique (Seonghee *et al.* 2018). Researchers in the USA had engineered anti-browning mushrooms utilizing the CRISPR/Cas9 technique, wherein minimization of browning in white truffles led to extension of their life span, thereby offering a business advantage. A minor deletion in the polyphenol oxidase gene via genome editing encouraged the oxidation of polyphenol when exposed to air, bringing about an appetizing and attractive appearance in the mushrooms (Waltz *et al.* 2016). Halterman *et al.* (2015) reported the classic work of the development of acrylamide free potatoes. Starch present in potato tubers degrades into glucose and fructose, during cold storage time. Cold storage potatoes revealed a reduced amount of sugars transforming into dark-brown pigments that led to generation of a strong carcinogen 'acrylamide'. During the processing of potato chips or french fries at high temperatures, this acrylamide created a major problem. On the contrary, at high-temperatures, the genome edited potatoes during cold storage revealed hardly any brown pigments and negligible production of acrylamides as compared to wild-type. Similarly, non-browning apples have been developed via mutating the gene that encoded the polyphenol oxidase (PPO) enzyme (Nishitani *et al.* 2016). Liang *et al.* (2014) developed edited maize lines with low phytic acid content. Maize/corn grains contain significant levels of phosphorous. However, this phosphorous was stored in the form of phytate, which was ineffectively digested by humans. Employing the CRISPR/Cas9 approach, the reduction of phytic acid/phytate (an anti-nutritional compound) in maize grains was performed by targeting two key enzyme coding genes of its biosynthetic pathway (Liang *et al.* 2014). Rice being the principle food crop has been the primary target for quality enhancement. Amylose is regarded as an important nutrient quality parameter in rice grains. Targeting the starch branching enzymes, resulted in lower amylose content of rice grains (Sun *et al.* 2017). Another report revealed, amylose content was increased via the editing of *OsWaxy* gene, whereas the targeted genome editing in the *OsBADH2* enhanced the aroma in rice grains, without affecting the yield (Shan *et al.* 2015; Sun *et al.* 2017; Zhang *et al.* 2018). The Cd content in rice grains was diminished, via the CRISPR/Cas12a-based KO of a Cd transporter gene *OsNRAM5* (Tang *et al.* 2017a). Recently, two key sweet potato genes (*IbGBSSI* and *IbSBEII*) of its starch biosynthesis pathway were KO independently via the CRISPR/Cas9 approach, which revealed mutants with an increased and decreased amounts of amylose content, respectively (Wang *et al.* 2019b). Miyamoto *et al.* (2019) generated lignin enriched rice lines by CRISPR/Cas9-mediated site-directed mutagenesis of the transcriptional repressor *OsMYB108* that enhanced the

levels of p-coumaroylated and tricin lignin units in culm cell walls of rice. Incontestibly, high concentrations of α- linolenic and linoleic acids were the vital reasons for the oxidative instability of soybean oil and the foodstuffs thereby prepared. Soybean omega-6 desaturase (*GmFAD2*) genes perform a significant role in the conversion of oleic to linoleic acids. On similar lines, targeted modification of the *GmFAD2* gene via the CRISPR/Cas9 method resulted in the increased accumulation of oleic acids via a reduction in the linoleic and α- linolenic acids (Amin *et al.* 2019). Isoflavonoids enacted an important function in plant-environment interactions, being widely present in pulses. Of late, the gene modification technique has uncovered considerable accomplishments in the soybean isoflavone pathway via concurrent targeting of three genes. Implementation of this GE framework led to augmentation of isoflavones in soybean seeds and leaves, consequently elevating the protection against soybean mosaic virus (Zhang *et al.* 2019). A similar CRISPR/Cas-based approach targeting two iron sensing genes to increase the contents of requisite micronutrients, i.e., Fe and Zn in the endosperms and one Cd transporter gene to decrease the accumulation of Cd in white-rice grain has been achieved in our nutritional improvement of crops (NIC) laboratory at ICGEB. Crops such as, grain amaranths of *Amaranthus* sp. and *Vigna umbellata* (rice-bean) have emerged as superfoods possessing remarkably high health benefits with tremendous nutritional value, including a vast amount of essential amino acids and minerals in contrast to umpteen number of cultivated crops. Moreover, they hold enormous potential for the reduction of micronutrient malnutrition in populations, globally. The NIC group at ICGEB has spearheaded the whole genome sequencing (NCBI SRA-SRP-132447) as well as transcriptome analyses to expedite the trait advancements (palatability, flowering, and habit) of the underutilized rice-bean crop (Kaul *et al.* 2019a).

6. CRISPR: a fast forward way for global food security

Conventional plant breeding techniques use naturally accessible resources harbouring desirable traits for crop improvement; however, this is a less effective approach due to its repercussions, i.e., loss of genetic diversity and stress susceptibility. Across the years, several biotic and abiotic cues adversely affected crop plantations thereby threatening global food security. In contrast to TALENS for targeted DNA cleavage, CRISPR/Cas9 emerged as a highly economical strategy that facilitated efficacious genome modifications, in order to upgrade food and feed crops, thereby sustaining food security (table 3). Preliminary studies demonstrated the potential genome editing method in the first generation and robust transformations with targeted site analysis in subsequent plant generations. Twelve different target regions in seven genes were examined in plants for biallelic, chimeric, heterozygous, or homozygous mutations in three generations, respectively. Nowadays, molecular biologists have exploited the CRISPR/Cas technique from bacteria and repurposed this system for functional studies in animals and plants. Biotechnologists have highlighted its crucial practical implications while transfecting into the model plants like *Nicotiana tabacum* (Gao *et al.* 2014), *Nicotiana benthamiana* (Li *et al.* 2013; Nekrasov *et al.* 2013; Jiang *et al.* 2013; Belhaj *et al.* 2013), *Arabidopsis* (Li *et al.* 2013; Jiang *et al.* 2013; Feng *et al.* 2013; Mao *et al.* 2013) and numerous crops, for instance, wheat (Upadhyay *et al.* 2013; Shan *et al.* 2013); rice (Feng *et al.* 2013; Mao et al 2013; Shan *et al.* 2013); sorghum (Jiang *et al.* 2013); sweet orange (Jia and Wang 2014); and maize (Liang *et al.* 2014). Two genes *NtPDS* and *NtPDR6* were mutated in *N. tabacum* via the CRISPR/Cas system employing transient editing assays in tobacco protoplast via the transfection of sgRNA-Cas9 that led to a mutation frequency rate of 16.2–20.3% (Gao *et al.* 2014). The gRNA-Cas9-mediated mutations of *NtPDS* and *NtPDR6* obtained in the transgenic *tobacco* plants were gRNA specific and exhibited a mutation ratio of 81.8% for *NtPDS* gRNA4 and 87.5% for *NtPDR6* gRNA2. A clear phenotypic effect was found in both the mutants, i.e., etiolated leaves in the PDS mutant, and higher number of branches in the PDR6 mutant. Results indicated a biallelic mutation in both transgenic lines (Gao *et al.* 2014). In addition to CRISPR/Cas-based protoplast transient assays, *agro*-mediated transformations of sgRNA-Cas9 and non-functional green flurescence protein (*GFP*) into modern plants like *Arabidopsis* and *tobacco* offered an effective alternative approach.

Cleavage of the target site present at the 5′ coding region of the non functional *GFP* via sgRNA-Cas9 was followed by NHEJ repair that resulted in functional mutations within *GFP* genes (Jiang *et al.* 2013). The chimeric guide RNA (cgRNA) with Cas9 endonuclease enact a crucial function in generating the deletion mutation by targeting single or multiple sites in the target gene (Upadhyay *et al.* 2013). Ron *et al.* (2014) revealed the conserved nature of SHORT-ROOT and SCARECROW gene functions in *Arabidopsis* and

Table 3. Comparison of characteristics of different plant genome editing tools

Features	ZFNs	TALENs	CRISPR/Cas9
Custom Design and assembly/construction	Technically challenging and complex technique; require expertise in protein engineering. Require a new target site for redesigning	Comparatively easy procedure; redesigning for each new target site required	Quick and easy procedure; redesigning for each new target site required
Target sequence length (bp)	18–24	24–59	20–22
Mismatch tolerance at target site	Moderate	Low	Relatively high
Cleavage specificity	High	High	High with multiplexing capacity
Target recognition efficiency	High	High	High
Gene multiplexing ability	Unattainable	Very difficult to obtain multiplexed genes: require separate dimeric proteins specific for each target	High, Several genes can be edited at the same time
Off-target effects	Fewer off-target effect	Unavoidable off-target effect or Higher potential for off-target effects	Moderate, effects can be minimized by selecting a unique crRNA sequence
Cleavage module	Fok1 nuclease domain	Fok1 nuclease domain	Cas9 protein
Specificity module	Zinc finger domain	TALE domain	crRNA
Repair mechanism	NHEJ and HR	NHEJ and HR	NHEJ and HR
Transgene free in offspring	Yes	Yes	Yes

tomato via the transfection of the CRISPR/Cas9 machinery. Liang *et al.* (2014) reported the CRISPR/Cas-mediated KO of ZmIPK gene in *maize* protoplasts with an efficiency rate of 13.1% that led to the reduction of phytic acid content, and accumulation of inorganic phosphate in the seeds, which was similar to the results found by Shi *et al.* (2003) in case of maize IPK mutants developed using mutator insertion KO technology. The table 4 represented the editing efficiencies of target genes in different crops via the CRISPR/Cas system. However, numerous agriculturally significant traits (yield, crop quality, resistance to abiotic and biotic cues and male sterility) of wheat were altered by means of gene editing (Borisjuk *et al.* 2019). The CRISPR/Cas9 approach was successfully employed in wheat to alter of a powdery mildew-resistance gene *TaMLO* (Shan *et al.* 2013). In another effort, the CRISPR/Cas9 machinery has been successfully implemented in order to target a homolog of TaCe (ECERIFERUM) to induce drought tolerance trait in wheat (Liang *et al.* 2018). In addition, numerous genes, for instance, *TaGASR*, *TaGW*, and *TaDEP* have been modified via the CRISPR/Cas9 approach for the enhancement of yield characteristics (Zhang *et al.* 2016). Recently, Lyzenga *et al.* (2019) targeted the homologs of CRUCIFERIN C (CsCRUC) genes of *Camelina sativa*, which led to an altered seed storage protein profile. The knockout profile of seeds revealed modified fatty acid content and proportionate profusion of all saturated fatty acids. In recent years, modification of TALE responsive promoter elements of two susceptible genes, i.e., OsSWEET11 (*PthXo1*) and OsSWEET14 (*PthXo3/AvrXa7*), in rice cv. Kitaake, a recessive stress tolerant allele of Xa25/OsSWEET13 enhanced resistance to bacterial blight disease (Xu *et al.* 2019; Oliva *et al.* 2019). Enhanced repair ratio and non-transformational approaches for plants with precise alterations may be generated via the use of geminiviruses for the delivery of templates and constructs to all parts of the plant (Ali *et al.* 2015). Exploration of direct delivery of viruses initiated in 2015 demonstrated the acheivements of virus-mediated sgRNA-Cas9 delivery (Yin *et al.* 2015). In another report, the foxtail mosaic virus (FoMV) derived viral vectors have been used in monocots for virus-enabled gene editing (VEdGE), virus-based over-expression (VOX) and virus-induced gene silencing (VIGS). Furthermore, these vectors were employed for temporal expression of genes, employing single sgRNA-Cas9-based gene modifications in *N. benthamiana, Setaria*

Table 4. CRISPR-Cas-mediated gene targeting in different crop species

Crops	Target genes	Gene knockout via Cas9 sgRNA	Cas9-sgRNA assisted multiplexing	HR assisted gene insertion/replacement via Cas9-sgRNA &donor template	Gene knockout (KO) or editing with Cas9 paired nickase/sgRNA	References
Potato (*Solanum tuberosum*)	StIAA2, StALS1	Yes	No	No	No	Wang *et al.* (2015b) and Butler *et al.* (2015)
Chinese white-poplar (*Populus tomentosa*)	PtoPDS, 4CL, PtPDS	Yes	No	No	No	Fan *et al.* (2015), Zhou *et al.* (2015b) and Tingting *et al.* (2015)
Soybean (*Glycine max*)	Glyma06g14180, Glyma08g02290, Glyma12g37050, Glyma18g04660, Glyma20g38560, GmFEI2, GmSHR	Yes	No	No	No	Sun *et al.* (2015), Michno *et al.* (2015), Jacobs *et al.* (2015) and Cai *et al.* (2018)
Maize (*Zea mays*)	UBI:MoPAT	No	No	Yes	No	Svitashev *et al.* (2015)
Chinese white-poplar (*Populus tomentosa*)	PtPDS1 and PtPDS2	No	Yes	No	No	Tingting *et al.* (2015)
Soybean (*Glycine max*)	01gDDM1, 11gDDM1, 01gC11gDDM1, GmFEI2, and GmSHR	No	Yes	No	No	Jacobs *et al.* (2015) and Cai *et al.* (2018)
Rice (*Oryza sativa*)	CDKB1, CDKA1, MPK1/2/5/6, PDSOsFTL	No	Yes	No	No	Ma *et al.* (2015) and Xie *et al.* (2015)
Barrel medic (*Medicago trancatula*)	GUS	Yes	No	No	No	Michno *et al.* (2015)
Sweet orange (*Citrus sinensis*)	CsPDS	Yes	No	No	No	Jia and Wang (2014)
Liver wort (*Marchantia polymorpha*)	MpARF1	Yes	No	No	No	Sugano *et al.* (2014)

Table 4 (continued)

Crops	Target genes	Gene knockout via Cas9 sgRNA	Cas9-sgRNA assisted multiplexing	HR assisted gene insertion/replacement via Cas9-sgRNA &donor template	Gene knockout (KO) or editing with Cas9 paired nickase/sgRNA	References
Mouseear cress (*Arabidopsis thaliana*)	*Thaliana YFFP*, GU.US, DGU.US-GUS functional, ADH1	No	No	Yes	No	Fauser *et al.* (2014) and Schiml *et al.* (2014)
Common wheat (*Triticum aestivum*)	TaMLO-A1CTaMLO-B1CTaMLO-D1	No	Yes	No	No	Wang *et al.* (2014)
Maize (*Zea mays*)	ZmHKT1	No	Yes	No	No	Xing *et al.* (2014)
Tomato (*Solanum lycopersicum*)	Solyc07g021170 + Solyc12g044760	No	Yes	No	No	Brooks *et al.* (2014)
Tabacco (*Nicotiana tabacum*)	NtPDS + NtPDR6	No	Yes	No	No	Gao *et al.* (2014)
Mouseear cress (*Arabidopsis thaliana*)	RTEL1, ADH1, TT4	No	No	No	Yes	Fauser *et al.* (2014) and Schiml *et al.* (2014)
Sorghum (*Sorghum bicolor*)	DsRED2	Yes	No	No	No	Jiang *et al.* (2013)
Common wheat (*Triticum aestivum*)	TaMLO, INOX, PDS, TaMLO-A1	Yes	No	No	No	Upadhyay *et al.* (2013)
Rice (*Oryza sativa*)	OsPDS, OsBADH2	No	No	Yes	No	Shan *et al.* (2013)
Tobacco (*Nicotiana benthamiana*)	NbPDS	No	No	Yes	No	Li *et al.* (2013)
Tobacco (*Nicotiana benthamiana*)	NbPDS, PDS, NbPDS, NbPDS3, NbIspH	Yes	No	No	No	Nekrasov *et al.* (2013), Li *et al.* (2013), Upadhyay *et al.* (2013) and Jiang *et al.* (2013)
Mouseear cress (*Arabidopsis thaliana*)	AtRACK1bCAtRACK1c, CHLI1CCHLI2, ETC2, CPC, TRY, PYL1-6, and At5g55580	No	Yes	No	No	Li *et al.* (2013) and Mao *et al.* (2013)

viridis, and *Zea mays* (Mei *et al.* 2019). CRISPR interference (CRISPRi) exhibited RNA-guided, systematic, stable regulation of transcription by fusion of inactivated dCas9 to effector domains. Combining of dCas9 C-terminus to the EDLL and SRDX domains during alteration of the native tobacco PDS gene effectuated activation and repression of PDS gene, respectively (Piatek *et al.* 2015). Genome-scale CRISPRi and CRISPRa libraries may be used to comprehend the intricate stress signaling pathways for interrogation of gene functions (Liu *et al.* 2015). Bacterial blight resistant indica rice (IR24) was generated via utilization of the CRISPR/Cas9-based toolkit to target *OsSWEET13* promoter regions (Zhou *et al.* 2015a). Furthermore, a targeted mutation in the ethylene-responsive factor, *OsERF922* led to generation of rice varieties that were tolerant to blast disease (Liu *et al.* 2012). KO of Annexin gene (*OsAnn3*) employing CRISPR/Cas9 machinery conferred cold stress-resistance in rice (Shen *et al.* 2017). Rice yield may be enhanced by single or complex genome modifications of numerous QTLs. Shen *et al.* (2018) revealed the function of numerous QTLs that were crucial for grain size (GS3) and number (Gn1a) in rice crops via the CRISPR-mediated QTL altering strategy. CRISPR/Cas-mediated engineering of herbicide-tolerant crop varieties facilitated the utilization of non-selective herbicides to target diversified weed populations without hampering the crop. The NIC Group at ICGEB has established the effectual genome editing of the PEP-binding active site in *EPSPS* genes of maize, rice, and pigeonpea via CRISPR-mediated knock-out of a conserved region and homology donor repair via knock-in of a desired mutated fragment to achieve tolerance to glyphosate-a non-selective herbicide. *EPSPS* gene edited lines of maize and rice led to prominently amplified aromatic amino acid (Phe, Tyr, and Trp) content in contrast to the wild progeny. This approach may be extrapolated to other crops, for example, wheat, soybean, onion, and those crops that face weed constraints.

Along with the CRISPR/Cas9 approach, CRISPR-Cpf1 has emerged as a significantly flexible editing system for genome alterations in crops (Zetsche *et al.* 2017). Recruiting CRISPR-Cpf1 employing the Pol III driven ribozyme-fused with crRNA showed 100% mutation (biallelic) frequency in rice (Tang *et al.* 2017b). Enzymatic activity of the CRISPR/Cas13a empowered efficacious RNAi activity in the crops, in the face of diseases due to RNA viruses and also transformed lengthy pre-crRNA copies into active crRNAs. CRISPR/Cas13a approaches led to RNAi via Turnip Mosaic Virus (TuMV) in tobacco (Aman *et al.* 2018). Transient assays employing CRISPR/Cas13a created interference in GFP-expressing TuMV that in turn led to stable overexpression in tobacco. crRNAs targeted to HC-Pro and GFP sequences revealed improved interference than those targeted to regions for instance; coat protein (CP) sequence. CRISPR/Cas13a approach magnified research intercessions in crops and other eukaryotic organisms using viral RNAi. The CRISPR/Cas14a approach expanded genome modifications of both plant ssDNA and dsDNA viral replicates (Ali *et al.* 2015; Watters *et al.* 2018). Besides crop trait advancements, fermentation activities of microorganisms belonging to the *Lactobacillus* family also profited from gene editing. Genome alterations of microorganisms empowered by CRISPR-based approaches pose huge potential, predominantly as probiotics and psychobiotics in the food sector (Misra and Mohanty 2017) and remarkably enhanced their tolerance to stress (Ismail *et al.* 2019). Kaul *et al.* (2019b) and Raman *et al.* (2019) demonstrated a comprehensive assorted encapsulation of multifarious perks employing neoteric CRISPR associated proteins for instance; Cas9/12/13, outlined for wheat, maize, rice and other crucial crops. Therefore, the recent quantum leap in the genome modifying strategies, if integrated would transform the field of precision breeding by elevating its stature to precision CRISPR/Cas breeding. Recently, compendious and robustious testaments have endorsed the applications of the CRISPR/Cas9 approaches in crops for the generation of agronomically improved varieties.

7. Machine learning and CRISPR/Cas framework

The CRISPR/Cas9 genome editing has emerged as a pivotal mechanism in the governance of the genes, by employing a complementary sequence as the single guide RNA. However, it is impractical to have an exact homology sequence at the target site, so there is a possibility of cleaving fortuitous off-targets. Machine learning (ML) and Artificial Intelligence (AI) propound neoteric approaches to utilize the CRISPR/Cas9 technology for the assessment of altered plant lines empowered with superior attributes for example, abiotic and/or biotic stress tolerance, altered flower and root architectures, higher nutritional values, palatability, etc. Alteration of both genomic regions and target genes remarkably depends upon pre-determination of sgRNA target positions, nevertheless, the efficacy of genome editing may be not elucidated as sgRNAs may

lead to extensive off-target effects. Currently, umpteen procedures available may enable designing of accurate sgRNAs, recruiting elementary rules. Here, the two novel algorithms known as CRISPR target assessment (CRISTA) and Elevation were introduced within the framework of machine learning, which performed a significant task in the determination of a particular genomic site to be precisely cleaved via given sgRNAs. The CRISTA and Elevation assisted predictions were found to be more accurate than the conventionally predictable thresholds (Abadi *et al.* 2017; Listgarten *et al.* 2018). Furthermore, to the forecast off-target sites, these inferred novel algorithms hold significance for instance, prediction of gRNA secondary structure, genome location, and prediction of GC-rich sites, which were hypersensitive to DNase I enzyme. Target site prediction played a significant role in the differentiation between the wobble pairing assisted mismatches and the mismatches caused through DNA/RNA bulges that in turn had structural impacts (Abadi *et al.* 2017; Listgarten *et al.* 2018). Moreover, different experimental designs had a distinctive prediction model that differed in the rules, which governed the activity of CRISPR/Cas9. It has been found that the Bowtie and Burrows-wheeler Alignment (BWA) were the two important bioinformatic tools that were used to achieve the arrangement of short target sequences in comparison to the BLAST-like traditional tools. However, the identification of feasible off-target positions required the perquisition of short sequence motifs up to 20 bp, in addition to the PAM with frequent mismatches. However, only three to five mismatches were allowed by the Bowtie and BWA alignment, respectively, which led to omission of highly mismatched off-targets (Tsai *et al.* 2015; Doench *et al.* 2016). To overcome this problem, the new bidirectional alignment like methods were implemented, which played a crucial role in the accurate identification of all potential off-targets (Canzar and Salzberg 2017). In most cases, the aligners first performed the matching of seed sequence and extended the seed sequence in a direction and then tested the match. However, in bidirectional alignment, the aligners lengthened the seed sequence in both the directions. Among all, the bidirectional alignment tool was the most powerful one for the identification of all potential off-targets. The MIT broad score and the cutting frequency determination (CFD) score (Hsu *et al.* 2013; Doench *et al.* 2016) were the two most prominent scoring methods based on the synthetic datasets. In these two algorithm-based scoring methods, the mutation in the gRNAs target sequence took place via a specific dataset represented as one, two or three mismatch combinations. The capability of gRNA-assisted target site cleavage frequencies was quantified and the outcome was utilized to design a linear regression algorithm to point out the off-target sites. Athough, both scoring algorithms were based on similar hypotheses however, they differed at the level of definite model development. The MIT broad algorithm assessed up to 20 bp target sequences excluding PAMs, while the CFD score algorithm assessed target sequences including PAM sequences but passively scored the non-canonical PAM harbouring target sequences. In a bid to accurately predict off-target sites through distinct experimental datasets, a comparison between these two scoring algorithms proved that the CFD score algorithm executed the best results as compared to MIT broad score algorithm (Haeussler *et al.* 2016). In addition, common off-target prediction tools have been mentioned in table 5. Thus, ML and AI analyzed the probable regression point, which may either converge or diverge the plot of both off-target and on-target specificities.

8. Regulatory concerns related to genome edited (GE) crops

Engineering of genomes employing CRISPR/Cas machinery enabled a synchronized approach for the integration of elite traits in crops via base substitutions, additions, deletions, and gene integrations or re-in-statements in an arbitrary manner, resulting in characteristics that were identical to those generated employing induced mutations, natural genetic variations and breeding techniques. Regulation of genetically modified organisms (GMOs) is in line with the Cartagena Protocol, which is under the control of international trade of living modified organisms (LMOs). In line with this protocol, LMOs encompassed an altered genetic material modified via modern molecular biology techniques. However, the regulation of this protocol has been a controversial topic over the years. The societal approval to accommodate genome edited crop varieties as non-GMOs requires a re-categorization of the regulatory framework. A fundamental feature of existing regulation is either based on the end product or the process involved (Araki and Ishii 2015). Globally, CRISPR/Cas framework debatably extended from the present GMO laws, however, new specialized difficulties and government methodologies are yet the greatest issues in the method of social endorsement of genome modified crops (Araki and Ishii 2015; Jones 2015; Kanchiswamy *et al.* 2015). One of the most

Table 5. Different bioinformatics tools use in CRISPR-Cas system for off-target detection

Program name/software	Off-target detection method	Off-target scoring method	Notes/features	Available web-app/ Links	References
Elevation	Custom alignment tool	Custom model based on the number, position and type (wobble vs bulge) of mismatches	Prediction of: gRNA secondary structure, genome location and hypersensitive sites of DNase I enzymes	Available as a web-app (crispr.ml). The scoring method is available as a stand-alone tool	Listgarten *et al.* (2018)
CRISTA	NA	Custom model based on the number, position and type (wobble vs. bulge) of mismatches	Prediction of: gRNA secondary structure, genome location and hypersensitive sites of DNase I enzymes	Available as a stand-alone tool	Abadi *et al.* (2017)
CRISPOR	Alignment with BWA	MIT-Broad score	Allows the selection of various canonical PAMs	*http://crispor.tefor.net/*	Haeussler *et al.* (2016)
CROP-IT	Alignment with PATMAN	Custom model based on position of mismatches and also weather the potential off-target falls within a DNase-sensitive region	Allows the selection between NGG or NNG PAMs	*http://cheetah.bioch.virginia.edu/AdliLab/CROP-IT/homepage.html*	Singh *et al.* (2015)
CCTop	Alignment with Bowtie	Custom model based on presence of mismatches and weather they fall within seed region	Allows the selection of various canonical PAMs and some sequence limitations	*https://crispr.cos.uni-heidelberg.de/*	Stemmer *et al.* (2015)
GT-Scan	Alignment with Bowtie2	NA	Allows user to identify the required sequence rules (e.g., what PAMs are acceptable, how large a target site)	*https://www.gt-scan.net/*	O'Brien and Bailey (2014)

extraordinary features of genome-edited organisms remains that it is without an exogenous gene fragment/s, in contrast to GMOs. Genome modified crops may be socially more admissible than plants that have transgene insertions in their genomes. On these lines, the USDA and FDA have acknowledged genome edited plants (mushrooms and corn), which have transgene-free DNA and are considered as equivalent to products generated from conventional plant breeding methods. These altered food products do not fall into the category of GMOs and would follow deregulation from conventional GMOs policy and regulation (Waltz 2016). Conversely, the European Court of Justice (EU) has cognominated to consider GE crops as equivalent to GMOs, and the commercialization of GE crops has been restricted in the European countries (Callaway 2018). This might represent a significant issue for intercontinental business between such nations, including India. The upsides of GE innovation for the world's food security might be retained as a result of public opinion and the current success of plant breeding (Wolt *et al.* 2016). To surmount these difficulties, straightforwardness in decision-making is quintessential for GE employment.

9. Perspectives on nation-wide policies regulating GE crops

GE crops generated by gene-editing, gene replacement, or Site-Directed Nuclease (SDN) technology showcased hereditary variations by the introduction of significant alterations to their genomes. The prominent features of the SDN system comprises the integration of unique and minute modifications at the DSB loci by the utilization of the host's inherent repair machinery, which has evolved as a potent tool-kit for acquiring desired traits via targeted modifications. Targeted genome modifications enabled nucleotide additions and/or deletions that procreated neoteric desirable

characteristics, for instance, enhanced nutrient content, or reduced generation of allergens. SDN approaches have been typecasted into three classes; firstly, Class-I/SDN-1 created via a DSB within the host cell of a genome without any transgene insertions, and spontaneously reconstructed by a inherent repair machinery, which resulted in nucleotide deletion/s and/or insertion/s (indels), mutation/s, gene repression, removal of a gene. Secondly, Class-II/SDN-2 system that is identified and reorganized by the cell's inherent repair machinery via a short DNA fragment possessing the beneficial mutations that exhibited sequence complementarity to the targeted area in the genome. Finally, Class-III/SDN-3 approach involved creation of a DSB in the DNA followed by the re-instatements of a fragment harbouring a gene or any other genomic sequences. Interestingly, the opinions concerning organisms that resulted from SDN-1 and SDN-2 approaches were overlapping for both the National Technical Working Group (NTWG) and the German Central Commission of Biological Safety (ZKBS). The subsequent organisms carried mutations, which originated from the inherent DNA repair mechanisms either by NHEJ and/or HDR. According to Jones (2015) SDN-1 may be contemplated as a mutant derived from mutagenic agents like chemicals or radiation utilized in conventional mutational breeding. New plant varieties generated by mutations induced employing SDN-1 and SDN-2 approaches were exempted from the GMO regulations as no external DNA remained in the genome. However, SDN-3 approaches possess a stable insertion of foreign DNA and the developed crop varieties contain $\geq$ 20 base pair fragments of DNA integration and would be regulated as the conventional GMOs (Lusser *et al.* 2011; ZKBS 2012).

Normally, two categories of regulatory frameworks are present; few nations regulate the process, while others follow the product. Whereas, guidelines under the systematized framework is often named as product-based, the legislative framework of genome edited crops showed an existing process-based spark in umpteen number of illustrative cases (Wolt 2017). Small and Medium Enterprises (SMEs) that formed Europe's unprecedented plant breeding area would especially be profited from SDN (site-directed nuclease) framework to co-ordinate market requests and create eco-accommodating and highly sustainable advanced varieties exhibiting enhanced productivity. Although, this unpredictability may be addressed only if the EU clears its vision towards existing SDN innovations. We comprehend that every existing regulation has both pros and cons. In addition, no existing approach has fully comprehended how GE technology works that involved clean deletions, insertions, or base-pair swapping. Henceforth, the regulating legislations should be re-designed for the aforementioned products akin to the products arising from conventional breeding methods. Few countries like South and North America have welcomed the commercialization of GE crops by removing regulatory constraints. Moreover, Japan and Australia, have recently revisited and revised their normative approval procedures for GE organisms/products that involved SDN-1 type modification. A phenomenal degree of uncertainity related to regulatory stature of GE crops, yet remains in most Asian and European nations (Wolt 2017).

10. Conclusions

Genome editing via CRISPR/Cas based system offers applications involving alteration and regulation of genomes for crop advancement through gene KO, knock-in, point mutations, nucleotide substitution, and alterations at any gene locus. The latest developments in CRISPR/Cas technologies for instance prime editing and so on, enabled expansion of genome editing capabilities to encompass base substitutions, precision gene targeting, and manipulation of gene expression. Incidentally, such advancements have elaborated an arrangement of crop enhancement tools accessible to agronomists. Nevertheless, the successful implementation of any genetic method for crop betterment is reliant on its complete genome sequence and functional genomics data including the genetic networks governing crucial agronomic traits. In numerous crop species, such information is still elusive. The emergence of innovative and comprehensive DNA sequencing know-how and the inception of umpteen 'omics' databases will be instrumental in the screening of significantly novel targets for genome editing in plants, for instance, negative regulators of positive traits. Genome editing innovation, especially the CRISPR/Cas frameworks have changed the plant science in a gigantic way because of their approach capacity, simplicity, and capacity to concurrently modify numerous attributes in various organisms, including plants. Employing CRISPR/Cas innovation in plants to augment high yields, disease resistance, and nutrition content improvement has proved to be propitious in contrast to genome engineering technologies. The agricultural sector has bloomed in a rapid manner owing to the CRISPR/Cas system that has fostered the functional genomics research to provide nutritionally enhanced,

disease-resistant, high yielding crop lines, in order to produce more food with good nutritional value, thereby ensuring global food and nutritional security. Additionally, it has been effectively utilized for the development of comprehensive mutant libraries and antiviral breeding strategy. Eventhough, striking progress has been achieved in streamlining genome editing, the efficacy of novel genome editing systems would depend on pre-requisites, for instance, genotype-independent delivery techniques, functional genomics, and minimal off-target effects. Prospects of genome editing techniques, especially the CRISPR/Cas systems, have shown multi-fold progression over the years, but these are still not acceptable to the public yet. Presently, genome editing being at its inception, scientists should practice self-restraint and forbearance while implementing gene drives, until legislative policies and public acceptance for GE approaches are in place. Synergistic application of functional genomics in consonance with robustious GE methodologies and NGS, alongside synthetic- and systems-biology approaches may be utilized for the production of crops exhibiting improved qualitative and quantitative traits. Re-addressing the issue of synchronization of crops that are generated via genome editing techniques, in general. Thus far, tracing the footprints of techno-savvy nations for instance, Japan, Australia, and Vietnam, India is on the verge of GE crops' approval with the representative quintessential regulatory machinery being fine-tuned by the Ministry of Science and Technology and its sister concern, the Department of Biotechnology.

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