CRISPR / CAS: A NEW HORIZON IN CROP IMPROVEMENT

Abstract

Human population has grown exponentially over the last several decades, making the problem of feeding an ever-growing population a huge concern. In such situations, augmentation in agricultural production is the utmost need for supplying nutrition along with food to the mankind. Moreover, conventional breeding methods are not enough to meet this demand due to its high time-consumption and labour-intensive nature. Therefore, to vanquish these circumstances, CRISPR-Cas comes into the field of molecular approach to provide an alternative to the conventional procedures. CRISPR has played a crucial role in the progress in genome editing studies since last few decades. Base editing, knock out or knock in of desirable genes, fine tuning of several genes, promotion of antiviral defense, and alteration of various demanding biochemical pathways through this specific gene editing system has proven its ability to spread new wings for accelerating crop advancement in last few years. In a nutshell, this chapter mainly aims at the execution of CRISPR/Cas system towards the revolutionary approaches of future plant breeding, along with a short overview and future endeavour.

Keywords: CRISPR / Cas; crop improvement; double-stranded break; future breeding; genome editing; hybrid breeding

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I INTRODUCTION

Classical or conventional approach was proven extremely if successful Over times and again in plant breeding. Most of the high yielding varieties (HYVs) and hybrids available today are the resultant of the same. Throughout the years, it played a major role in crop improvement [1, 2]. But to substantiate this rapidly growing human population these days in the diverse situation of changing climate coupled with scarcity of available water and arable land resources, some sorts of innovative breeding techniques were required for accelerating genetic gain, productivity as well as sustainability in agriculture. The inability of conventional breeding to meet today's demands for increasing crop production due to its highly time-consuming nature and laborious procedures highlighted hybridization, mutagenesis, and transgenic breeding as the leading strategies [3, 4]. Transgenic breeding generates desired traits by transferring foreign genes into the background of well-known mega-varieties [5]. Although genetically modified (GM) crops encompass the capability to overcome the bottleneck of reproductive isolation, their commercialization are still restricted by stringent Government guidelines considering future concerns [5, 6]. Therefore, developing a novel variety through precise genome editing is a revolutionary approach for rapid advancement of agricultural crops.

Zinc finger nucleases (ZFN) were first discovered in 2005 while experimenting with tobacco plant [7], and five years later, transcription activator-like effector nucleases (TALENs) surfaced as a genome-editing tool in the field of plant biotechnology [8]. In 2013, three independent groups of researchers discovered the CRISPR/Cas9 system for using in wheat (*Triticum aestivum*), rice (*Oryza sativa*), rockcress (*Arabidopsis thaliana),* and benthi (*Nicotiana benthamiana*), respectively [9, 3]. Later, this has been introduced as an ingenious technique for rapid crop improvement due to its ability to create variation by introducing targeted sequence at specific sites. Apart from ease of hybrid breeding, this approach can also knock in desired traits or knockout unwanted traits into elite varieties. Its multiplexing nature helps to modify multiple targets expeditiously, and therefore, multiple traits can be pyramided into an elite background within a single generation. Unprecedentedly, this system paves the way of creating genetic diversity too. High-sugar content tomatoes are very expensive because of its lengthy tedious procedure of production, though this reduces the size. Kawaguchi et al. [10] implemented gene editing to modify cell wall invertase inhibitor gene to accumulate higher quantity of sugar in the fruit. Digestion of the inhibitor through gene editing technology leads to 30 percent increase of sugar content than usual, without even effecting fruit size [10]. Sanatech Seed in Japan first launched the genome-edited directly consumable tomato [11] CRISPR/Cas mediated gene editing was used to produce "Sicilian Rouge", which was a γ-aminobutyric acid (GABA) rich (five times higher than the regular one) tomato. GABA, an inhibitory neurotransmitter, reported helps in controlling blood pressure and diabetes [11]. The ministry of Japan withdrew the rules and regulations of GM products for this GABA rich tomato [11]. Thus, for sustainable agriculture, the power of CRISPR and its variants remains undeniable.

Currently, CRISPR technology, with its discrete features and simplicity of usage, is widely applied in genetic modifications throughout the world. Consequently, diverse facets of biology, especially molecular biology is being glorified with its profound executions. Apart from transcriptional control, molecular editing, promoting various biosynthetic as well as

biochemical pathways in order to enhance the desired characters, this tool keeps going ahead with its potential in epigenome editing, DNA labelling etc. Further, in plant models, its implementation in synthetic biology, precision editing, acceleration of domestication processes, control of enormous prey species and many more can light up the upcoming pathways of future biotechnology for crop advancement and eventually, human welfare.

II CRISPR/CAS: A PLANT GENOME EDITING TOOL

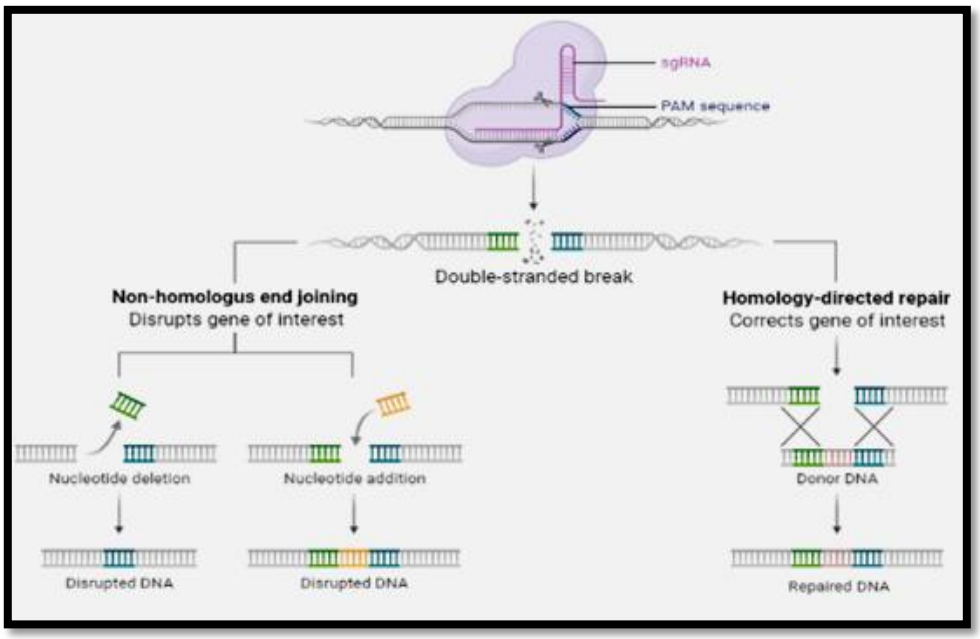
1. **An overview of CRISPR/ Cas system:** CRISPR/Cas system is an innate prokaryotic immune system inside the bacterial cells to provide protection against phages. CRISPR refers to Clustered Regularly Interspaced Short Palindromic Repeats and Cas represents CRISPR-associated protein, which is a kind of endonuclease. This system is known to provide a certain form of acquired immunity to the prokaryotes [3].

   As bacteria struggles from infection of bacteriophage for their destruction, they evolved a protective layer of immune system called CRISPR, in response to those. The CRISPR array, a short DNA stretch inside bacteria, is composed of a repeated sequence altered by target specific spacer. These spacers contain DNA fragments of previously invading viruses. The CRISPR array keeps growing with the newly added spacer segment after every virus attack. Identification of the viral DNA by a protein complex, namely Cas1-Cas2, initiates removal of a specific segment of DNA, *i.e.* protospacer. These protospacer, after addition with the CRISPR array, reserves a memory of that particular phage infection, so that the bacteria can easily and immediately defend the next attack. The transcription of long CRISPR-RNA (pre-crRNA) starts the defense process. Later, transacting or spacer RNA, pairs bases with the crRNA. The protein Cas9 binds on the dual RNA in order to trim them. Upon matching of the sequences of both crRNA and the viral DNA, Cas9 creates a double stranded cut on the viral DNA at precise position in order to destroy it. Further to mention, the juxta-positional orientation of PAM (Protospacer Adjacent Motif) nearby protospacer in viral DNA helps Cas9 to distinguish despite their sequence similarities [3].

   Six distinct types of bacterial CRISPR systems (type I-VI) have been discovered in different types of bacteria, so far, among which CRISPR/Cas9 belongs to type II. Another popular system type V where protein cpf1 can be found [3].

   Recently, a multipurpose efficient mini-CRISPR genome editing tool was developed by Stanford University, which acts like a ‘molecular swiss knife’. Its compactness makes it fifty-percent of the conventional Cas proteins *viz.* Cas9 and Cas12a, in size. CasMINI, a powerful dwarf among the CRISPR giants, can efficiently activate, delete, and edit genetic code, just like Cas12a. Now-a-days, this tool becomes very popular for *in vivo* and *ex vivo* gene therapy as well as cell engineering because of its robust nature [12].

2. **CRISPR-induced genome editing by DNA double-strand breaks (DSBs):** Creation of double stranded breaks (DSBs) at target loci is the basic feature of CRISPR/ Cas. It generally undergoes either of the two principal DNA repairing pathways, *i.e.* ‘non-homologous end joining’ (NHEJ), and ‘homology-directed repair’ (HDR) in order to introduce genomic modifications (Figure 1; Table 1) [13].



(Source: https://biorender.com)

Figure 1: Non-homologous end joining (NHEJ) and Homology dependent repair (HDR)-mediated double stranded break repair in DNA

The former one, without the requirement of homologous repair templates, can be implemented as a productive strategy for insertions of donor DNA sequences following a homology-independent manner, and therefore, can be utilized for crop improvement through gene pyramiding [3]. Although, NHEJ is well recommended for knockout studies at a large scale and it can be initiated in any cell cycle stages, the lack of precision creates a barrier towards sophisticated genome engineering. On the other hand, the latter one possesses the capability to add or replace the sequence of interest into the targeted place of DNA. Further to mention, HDR can be initiated in the second and last phase of interphase *viz*. S and G_2, respectively. An exogenous template or sister chromatid can serve as a template for repairing DSB due to its homology of sequences [14].

CRISPR is not limited to the formation of DSBs, sequence-oriented gene regulation, genome imaging, and epigenome editing [3]. dCas9 can also provide a unique stage for proteins recruitment. dCas9, fused with transcriptional repressor (e.g., the SRDX and KRAB domain) or transcriptional activators domains (*e.g.*, p65AD, VP64, and VPR), has the ability to regulate gene expression [15]. Post recruiting epigenetic effectors such as the histone demethylase LSD1, histone acetyl transferase (HAT) p300, and ten-eleven translocation (TET) proteins, dCas9 brings about some epigenetic changes at either DNA or histone level. This leads to the alteration of chromatin remodeling, which eventually results in change in various biological events *i.e.* gene expression, cell differentiation, etc. [16].

Beyond DSB-mediated genome editing, these systems, independent of HDR or donor DNA, can introduce specific base changes. As instance, cytosine base-editor (CBE) system makes a targeted transition (C→U) in genomic DNA [17]. Subsequently, Gaudelli et al. [18] discovered adenine base editors (ABEs) for converting A to G in genomic DNA. Higher efficiency, generation of a very few numbers of non-specific products, lesser chromosomal aberrations etc. make base-editing systems more advantageous over the DSB mediated one [3].

III APPLICATIONS OF CRISPR/CAS IN PLANT BREEDING

1. **Knockout-controlled trait development:** Elimination of negative elements has been reported as one of most promising approaches for genetic advancement. Therefore, CRISPR/Cas9 can be commonly applied to knock out undesirable genes from the genetic background (Figure 2a). Till date, it successfully improved several quantitative traits like quality, resistance to abiotic and biotic stresses, and overall yield. Rather, various breeding techniques like hybrid-breeding techniques and many more have also been enhanced, as reported [3].

   - **Yield improvement:** Yield is a dependent polygenic complex trait. Several traits such as grain number (*OsGn1a*), grain size (*OsGS3*), grain weight (*OsGW5*, *TaGW2*, *TaGASR7*, *or OsGLW2*), panicle size (*TaDEP1*, *OsDEP1*), and tiller number (*OsAAP3*) have negative impact on grain yield. In such circumstances, knocking out those traits using CRISPR/Cas9 was proven to be a proficient tool for improving yield through creation of loss-of-function mutation of respective genes [19, 20, 21, 22, 23, 24]. Simultaneous knockout of three grain-weight related genes in rice (*GW2*, *GW5*, and *TGW6*) generated trait pyramiding for significant enhancement of grain weight as per the published report [12].

   - **Quality improvements:** Quality characters depend on the specific breeding pre-requisites. Now-a-days, genome editing have impacted various quality parameters in several crops *i.e.* amylose content, aroma, nutritional value, duration and condition of storage etc. Improvement in amylose content in rice for better cooking and eating quality was induced by *Waxy* gene knockout by CRISPR/Cas9 [25]. By this knockout procedure, line of waxy corn coupled with higher yielding ability was developed by DuPont Pioneer for commercial purpose [26]. CRISPR/Cas9 also produced high-amylose containing rice through mutation of *SBEIIb*, *a* starch branching enzyme gene [27]. The study also suggested that consumption of enhanced amylose containing foods should strengthen the dietary habit of the patients of various chronic non-infectious diseases [27].

     2-acetyl-1-pyrroline (2-AP) is the principal volatile compound behind the aroma in rice, which leads to increase in consumer demand as well as market value. A mutation in betaine aldehyde dehydrogenase 2 (*BADH2*) gene causes its biosynthesis. An aromatic rice line with a nearly comparable 2-AP content (0.35–0.75 mg/kg) like a natural mutant aromatic rice variety was developed by executing TALEN-induced disruption of *OsBADH2* [28]. With evolution of CRISPR/Cas9 techniques, aromatic trait has been incorporated into almost 30 elite rice genotypes in China [3].

Almost 7% of the population in western countries suffers from celiac disease, which is triggered by gluten protein in cereals. The major gluten-encoding gene family in wheat is the α-gliadin gene family, which consists of around 100 genes and/ or pseudo-genes. Researchers already developed low gluten wheat through CRISPR/Cas9 editing technology by simultaneous knocking out of most conserved domains of that particular α-gliadin gene family [29]. Other than wheat, CRISPR/Cas9 editing also created seeds of *Brassica napus* [30] and *Camelina sativa* [31, 32] with increased oleic acid oil, longer shelf life in tomatoes [33, 34], high-value tomatoes with elevated lycopene [35] or GABA content [36, 37], and potato containing reduced levels of steroidal toxic glycoalkaloids [38].

- **Biotic-and abiotic-stress resistance:** Several plants with developed biotic-stress resistance have been achieved *via* CRISPR/Cas9 knockout. For example, TALEN along with CRISPR/Cas9, knocked out all six *TaMLO* alleles for developing increased resistance against powdery mildew in wheat [39]. Similarly, Nekrasov et al. [40] reported that knockout of *MLO via* CRISPR/Cas9 leads to develop powdery mildew resistance in tomato. Knockout of *OsERF922* and deletion of the *OsSWEET13* resulted in blast- and bacterial leaf blight (BLB) resistant rice, respectively [41, 42]. CRISPR/Cas9 also efficiently offered rice tungro virus resistant *eif4g* rice [43], potyvirus–resistant *eif4e* cucumber [44], and resistant *clcud* cotton for cotton leaf curl virus [45].

  On the other hand, amongst all the abiotic factors, contamination of arable lands has prompted the need to prevent hazardous heavy metal accumulation in crops [3]. Breeders already developed rice variants with low amounts of heavy metal likes cadmium (Cd), arsenic (As), and radioactive caesium (Cs), by knocking down OsARM1, OsNramp5, and OsHAK1, respectively [46, 47, 48]. In addition, pyl1/4/6 triple knockout rice generated by CRISPR/Cas9 editing leads to higher yield, higher temperature tolerance, and lower pre-harvest sprouting than the wild type [49].

- **Accelerating hybrid breeding:** For developing high quality hybrid, the pre-requisite is to development of male sterile lines. Development of such thermo-sensitive male-sterile *tms5* lines in rice [50] and maize [51], photosensitive genetic male-sterile (GMS) *csa* rice [52], and *ms45* wheat [53], etc. are some instances of the progress in male sterile line development through knocking out genes by CRISPR/Cas system. Moreover, to overcome hybrid sterility in *japonica-indica* hybrids, *SaF/SaM*, and *OgTPR1* at the sterility locus *Sa,* and *S1* were disrupted, respectively [54, 55]. Knockout of *Sc-I* allele in *Sc* gene in *indica* allele was reported to rescue male fertility in the aforesaid hybrids [56].

2. **Improvement through knock-in and/ or replacement:** Several yield-attributing traits are conferred by addition, substitution, or change of expression of several nucleotides or genes. Knock-in or replacement of specific alleles facilitates breeding excluding linkage drag or non-existing allelic variants (Figure 2b) [57]. Moreover, it could also alter multiple elite traits through gene pyramiding in a singular variety [3].

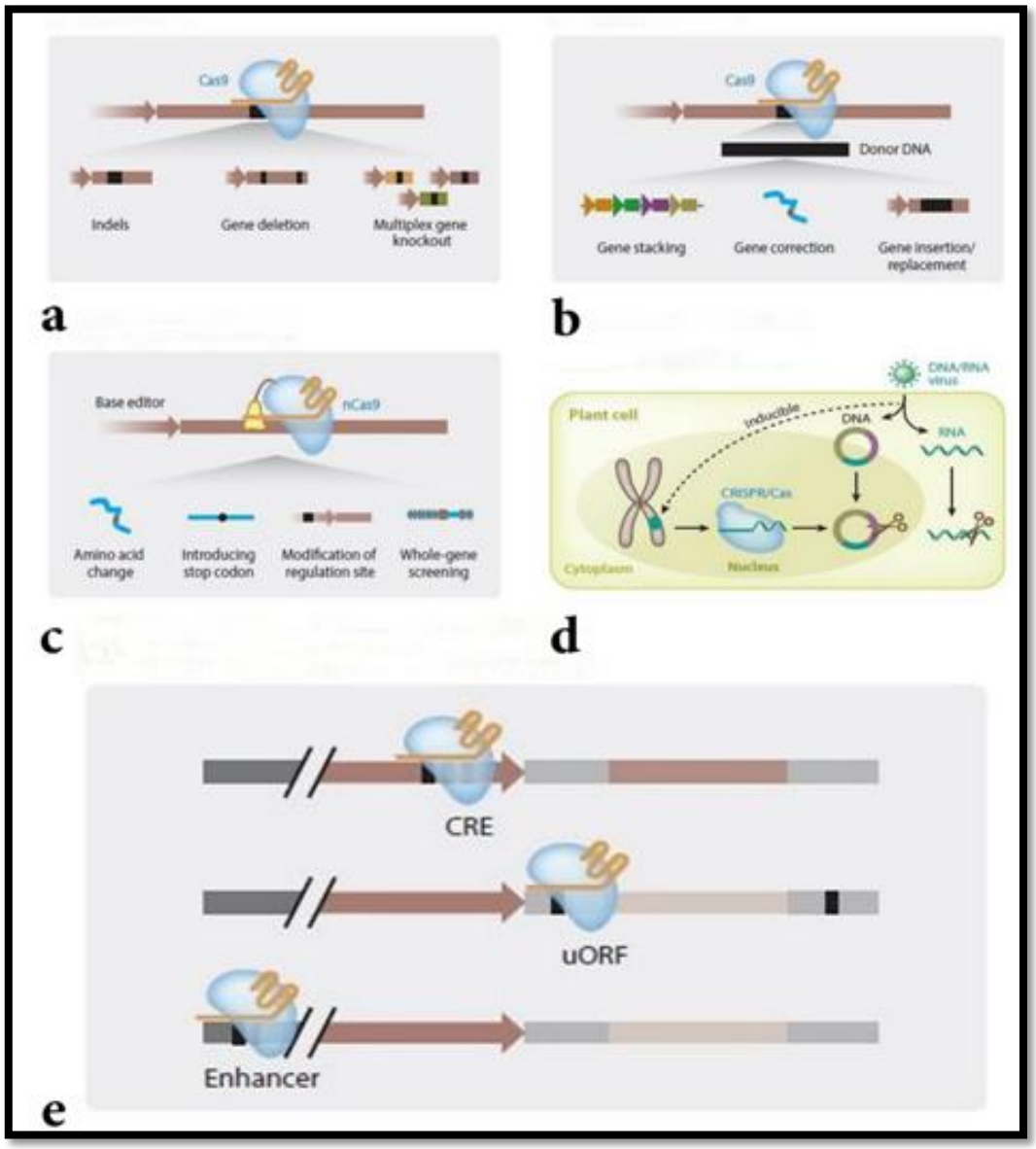


Figure 2: Applications of CRISPR/Cas-Based Gene Editing Methodologies

a Gene knockout, **b** Gene knock-in/ replacement, **c** Base editing, **d** Antiviral resistance in plant breeding, **e** Fine tuning gene regulation. (Adapted from [3])

She et al. [58] enhanced the expression of ARGOS8 by knocking in the GOS2 promoter instead of the regular ARGOS8 promoter through HDR. Under drought stress, the altered ARGOS8 variants possessed high level of transcription as well as yield. In another study, replacement of T317A into the ALC gene resulted in longer shelf life in the created tomato line [59]. On the other hand, the use of Gemini virus replicons reportedly increased repair template numbers to enhance gene-targeting efficiency in various crops [3]. It efficiently elevated the insertion frequency of Cauliflower mosaic virus (CaMV) 35S constitutive promoter upstream to *ANT1* gene, which was constitutively expressed in tomato in order to increase the anthocyanin content [60]. Moreover, substitution in the conserved domains of various principal enzymes likes endogenous 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) and acetolactate

synthase (*ALS*) genes, the key target for gene editing, conferred resistance against several herbicides like glyphosate or those based on sulfonylurea. As example, the substitution of double amino acid (T102I/P106S and T102I/P106A) in flax [61] and cassava [62] gave rise to HDR-mediated glyphosate resistance.

3. **Applications of base editors in plants:** Base editing at coding sequences is one of the major achievements for developing herbicide resistance (Figure 2c). Imidazolinone or sulfonylurea-resistant rice [63], wheat [64], *Arabidopsis* [65], and watermelon [66] have been created by targeting *ALS* with a plant cytidine base editor, and plant adenine base editor targeted acetyl-coA carboxylase *ACCase* gene to generate haloxyfop-R-methyl resistant rice [67,68]. On the other hand, in RNA splicing processes, most eukaryotic mRNA follows the systematic GU/AG rule, where introns possess a splice donor site (GU), and a splice acceptor site (AG) at 5' and 3' end, respectively. At consensus sequences, base editing can induce point mutation, which leads to the loss or alteration of particular splice forms. Xue et al. [69], in their study, performed transition (G→A) in the splice donor site, which not only causes hypersensitivity to abscisic acid (ABA) but also constitutively retain an intron of *AtHAB*. Kang et al. [70] distorted the splicing acceptor site by following the opposite conversion, which ultimately leads to change in splicing pattern of *AtPDS* mRNA.

4. **Antiviral plant breeding strategies:** CRISPR/Cas system offers a defense mechanism to cleave viruses and plasmids, that attack the primitive creatures *i.e.* Archaea and bacteria (Figure 2d). Besides, it is well-known to provide virus resistance in various plant species too. As instance, continuous over expression of Cas9 and sgRNAs specifically target the Gemini virus genome in order to inhibit its replication [71, 72, 73]. For developing Gemini virus-resistant plants, the intragenic sequences required for replication initiation was the ideal target for this system [73]. Further to indicate, the main constrains of this antiviral system was the constitutive expression of Cas9/sgRNA, which led to off-target mutations, although a viral promoter with the ability to drive Cas9 expression could reportedly check this [74].

5. **Fine-tuning gene regulation in plants:** Modulation of gene expression for assessing gene functions at transcriptional, post-transcriptional or translational level can greatly facilitate plant breeding apart from creation of mutations (Figure 2e). An array of genome-edited cis-regulatory elements generally controls these processes. Till date, genome editing for altering gene expression in plants has predominantly focused on either promoter replacement or deletion of *cis*-regulatory elements [75, 76]. Rodriguez-Leal et al. [77] edited the promoter regions of quantitative trait–associated genes *viz. SlS*, *SlCLV3,* and *SlSP*. These eventually led to continuous variation as well as the selection of mutated alleles with enhanced yield.

Table 1: CRISPR/ Cas-Mediated Gene Editing in Various Plants/ Crops

Plants/ crops utilized	Targeted genes	Method of gene editing	References
Arabidopsis thaliana	*BRI1, JAZ1, GAI*	NHEJ	[78]
	GFP	NHEJ	[79]
	CHLI1, CHLI2, TT4	NHEJ	[80]
	ADH1, TT4, RTEL1	NHEJ	[81]
	ADH1	NHEJ, HDR	[82]
	TRY, CPC, ETC2	NHEJ	[83]
	FT, SPL4	NHEJ	[84]
	AtCRU3	NHEJ	[85]
	At1g16210, At1g56650, At5g55580	NHEJ	[86]
Nicotiana benthamianum	*NbPDS*	NHEJ	[87]
	NbFLS2, NbBAK1	NHEJ	[88]
	Nbpds	NHEJ	[89]
	NbPDS, NbIspH	NHEJ	[90]
	XT	NHEJ	[91]
Oryza sativa	*ROC5, SPP, YSA*	NHEJ	[78]
	OsSWEET11, OsSWEET14	NHEJ	[79]
	OsMYB1	NHEJ	[80]
	CAO1, LAZY1	NHEJ	[92]
	OsMPK5	NHEJ	[93]
	OsPDS, OsDEP1	NHEJ, HDR	[94]
	OsBEL	NHEJ	[95]
	OsPMS3, OsPDS, OsEPSPS,	NHEJ	[96]
	SWEET1b, SWEET1a, SWEET11	NHEJ	[97]
	ALS	HDR	[98]
	CDKA2, CDKA1, CDKB1,	NHEJ	[99]
	YSA, CDKB2	NHEJ	[100]
Solanum lycopersicum	*SlAGO7*	NHEJ	[101]
	SHR, SCR	NHEJ	[102]
	RIN	NHEJ	[33]
	SlPDS, SlPIF4	NHEJ	[103]
S. tuberosum	*StALS1*	NHEJ	[104]
	StIAA2	NHEJ	[105]
Triticum aestivum	*TaMLO*	NHEJ	[106]
	Tainox, Tapds	NHEJ	[89]
	TaLOX2	NHEJ	[94]
	TaMLOA1, TaMLOB1, TaMLOD1	NHEJ	[39]
Zea mays	*ZmIPK*	NHEJ	[107]
	LIG, MS26, MS45	NHEJ	[108]
	Zmzb7	NHEJ	[109]
	PSY1	NHEJ	[110]

Abbreviations: NHEJ: non-homologous end joining; HDR: homology-directed repair.
(Table adapted from [111])

IV FUTURE PROSPECTS

1. **Potential utilization of plant synthetic biology:** Now in plant biotechnology, an emerging field is synthetic biology, which will play a major role in crop improvement through development of different novel bio-production processes [112]. In 1982, the discovery of the first transgenic plants indicated the beginning of designer plants with novel functionalities. The CRISPR/Cas possess the tremendous potential not only to improve plant architecture but also the synthetic biology. Several DNA sequences (*viz*. promoters, enhancers, repressors, transcriptional regulatory elements, etc.) can be incorporated into the genome of interest to alter the behaviour for generating their novel functions. For example, this CRISPR/Cas system can be deployed to transfer the DNA sequences involving nod factor signaling pathways from legumes to cereals for atmospheric nitrogen fixation. Additionally, synthetic biology also aims to build regulatory circuits for manipulating vegetative behavior or to generate novel traits for improving various economically important traits in plants [113].

   Multiplex gene modification and epigenome editing *via* dCas9-controlled gene regulation provides the plausibility to design various synthetic transcription factors. Those factors could be used to build efficiently programmable complex gene circuits. Finely tuned gene expression in the C4 cycle was essential for increasing the carbon fixation efficiency through optimization of protein levels in C4 rice project [114].

2. **Speeding up the domestication of wild plants:** Domestication mainly comprises of some modifications in genetic level of various wild species, which undergoes selection for meeting the demands of human being [115]. While investigating the domestication process, it illustrated the history of selection and improvement from the ancestral era. These selective forces imposed by humans led to the changes in various traits, which is now referred as “domestication syndrome traits”. These traits can easily distinguish between the cultivated species and its wild progenitor. The selection of these traits enriched the process of domestication and crop improvement, subsequently. Noteworthy to mention, various major crop families, particularly ‘poaceae’, conform to this classical 'domestication syndrome'.

   These domestication events are mainly related to various modifications, especially mutation in those genes. These mutational events brought about some marked changes on key phenotypes of several cereals. Their changes become favorable for humankind leads to selection of those traits. Among them, six-rowed spike 1 (*vrs1*) was responsible for increasing spike number in barley [116]; *tga1* conferred naked kernels in maize [117]; and *Rc*, *Sh4*, *PROG1*, and *LABA1* leads to formation of white pericarp, non-shattering rachis, erect growth, and barbless awns, respectively, in rice [118]. At very first, domestication was experimented on the winter annual plant field pennycress (*Thlaspi arvense* L., Brassicaceae). Modifications of genes by genome editing are controlling seed dormancy (*DOG1*), glucosinolate accumulation (*HAG1*, and *GTR2*), oil quality (*FAE1*, and *FAE2*), and oil content (*DGAT*). They significantly aid in the creation of exceptional domesticated forms of the aforesaid species [119]. Secondly, the modern tomato which bred through intensive inbreeding programme, was suffering from various biotic and abiotic stresses. Naturally available stress tolerant genes in wild tomato could be the

perfect resource for *de novo* domestication *via* precise genome editing technique. This technique was recently used in two separate investigations to accelerate the domestication process of wild tomato regarding flower and fruit production, growth habit, and nutritional traits with intact stress tolerance [120, 121]. Lemmon et al. [122] edited *Physalis pruinosa* (ground cherry), a wild relative of tomato, to produce high yielding tomato with larger fruit size. In near future, newly domesticated crops with increased tolerance to an array of challenging environmental condition will promote crop diversity and help solving many issues regarding sustainable agriculture.

3. **Enhancing the precision of CRISPR / Cas systems:** There is an ongoing debate on the extent of off-target changes through CRISPR/Cas-mediated gene editing in plant genomes and whether these need to be entirely fixed before being applied for trait development or not. Generally, a typical seed multiplication process undergoes outcrossing of numerous varieties. In comparison to the conventional cross-breeding procedures, it permits the removal of potential off-target impacts with timescales that are indeed significantly shortened. Whole genome sequencing was examined in various studies to detect the off-target site cleavage by Cas9 or Cpf1 nucleases in transgenic *Arabidopsis* [123], cotton [124], and rice [125]. These studies revealed high specificity of both Cas9 and Cpf1. These studies also suggested that by designing highly specific sgRNAs, low-level off-targeting could be eluded. Numerous strategies have been implemented to increase the specificity of Cas9-associated base editors *viz.* sgRNA guide sequences extension, Cas9-HF1-connected APOBEC1, along with base editor delivery *via* RNP (ribonucleoprotein) [126, 127].

4. **Precise gene editing *via* HDR:** The need of simultaneous development of DSBs as well as location-specific repair template delivery within the genome was proven to be the major constrain of HDR-mediated gene editing. Several potential ways like manipulation of DNA repair pathways increased the HDR frequency in plant cells. Heterologous expression of several key proteins could enhance HDR efficiency, *i.e.* proteins involved in homologous pairing and nuclear strand exchange like RAD52, RAD54, and RPA, resection protein RecQL4 (helicase), Spo11 and Exo1, etc. [128]. It is interesting to note that the expression of the mutant RAD18-ΔSAP conferred a significant enhancement of CRISPR-mediated HDR as compared to the regular one. In this variant, HDR stimulation occurred due to the inhibition of 53BP1 localization to DSBs.

   Furthermore, HDR-associated genome editing could be enhanced through some plant delivery methods which prioritized donor delivery. In case of *Agrobacterium* sp., it used type IV secretion system for transferring virulence effector proteins [129]. The covalent bond formation between VirD2 and single-stranded T-DNA promotes its transfer process *via* protein transfer mechanism. This mechanism has the ability to transfer CRISPR DNA or RNP accompanied by the donor templates by triggering HDR-liaise genome editing. The integration between CRISPR/Cas with the non-integrating Gemini virus replicon system is able to increase the copy number of donor templates [3].

5. **Restricting invasive species by gene drives:** Gene drives, through CRISPR/Cas genome-editing system, offer not only a strong practice for efficient proliferation of heritable elements throughout the populations *via* sexual reproduction but also the speeding up in this process. These gene drives could eliminate or suppress the community

of prey organisms including pests and weeds, altering the virulence of pathogen, and in order to introduce novel characters into the present population [3].

V CONCLUSION

The unprecedented capability of generating genome-wide sequence-defined diversity in plants through genome editing has unlocked a new horizon in crop improvement programme. The simplistic, versatile, and robust nature of CRISPR/Cas makes it a powerful tool. It can improve crop genetic structure by precise point mutations, fine tuning of gene regulatory elements, and many other changes at any specific location. Additionally, it helps to construct the mutant library and had tremendous potential in the antiviral breeding. However, quick detection of the genetic bases of traits of interest, improved efficiency of gene targeting (gene insertion and/ or replacement), successful transfer of CRISPR/Cas reagents to plant cells and succeeding plant regeneration irrespective of tissue culture, and the accessibility of base editors with enhanced range and frequency of targets are required for efficient transfer technologies from the *in vitro* to *ex vitro*. Despite enormous social and ethical issues, this technology owns immense potential for futuristic approach in agriculture.

Now there is a need to reexamine the laws governing genome-edited crops in various countries. Recently, on 30thMarch, 2022, the Indian Ministry of Environment, Forest and Climate Change has announced that genome-edited crops without exogenous DNA are free from being classified as transgenic crops. There are three categories of gene-editing: SDN1, SDN2 and SDN3. The first two, involves "knocking off" or "over expressing" certain traits in a genome without any insertion of foreign gene. The third one, will be treated as GMO due to it involve insertion of foreign genes. The Memorandum states, "SDN1 and SDN2 genome-edited products free from exogenous introduced DNA be exempted from biosafety assessment in pursuance of rule 20 of the Manufacture, Use, Import, Export and Storage of Hazardous Microorganisms/Genetically engineered Organisms or Cells Rules 1989."

Farming might be revolutionized by the development of climate-smart cultivars and increased nutritional value and productivity employing CRISPR-Cas technology. This can improve the genetic gain to meet the food demand of an increasing world population and fed the humankind. Specificity and less time consumption make CRISPR-Cas advantageous over other approaches. Therefore, this is the need of the hour as time is going to be most determining factor in future breeding programmes.

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Authors' contribution: PS conceived the idea and collected data; PS and BP wrote and edited and the manuscript; BP did some addition and correction to the manuscript; PS prepared the table; BP added the pictorial diagrams; SD revised the manuscript. All authors read, finalized and approved the manuscript.

