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CRISPR-Cas Mediated Genome Editing: A Paradigm Shift towards Sustainable Agriculture and Biotechnology

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Review Article

ABSTRACT

CRISPR–Cas genome editing technology developed from prokaryotes has transformed the molecular biology of plants past all assumptions. CRISPR–Cas, which is distinguished by its resilience, relatively high specificity, and easy implementation, enables specific genetic modification of crops, allowing for the creation of germplasms with favorable characters and the development of innovative, highly efficient agricultural systems. Moreover, many new biotechnologies in the framework of CRISPR–Cas platforms have bolstered basic research as well as synthetic biology toolkit of plants. In this article, initially, we provide a brief overview of CRISPR–Cas gene editing, emphasis on the modern, most specific gene-editing techniques, such as prime and base editing. Following that, the major role of CRISPR–Cas in plants in enhancing pesticide and disease resistance, quality, yield, breeding, and faster domestication are next discussed. In this review, we discuss the current advancements in plant biotechnology linked to CRISPR–Cas, such as CRISPR–Cas gene control, reagent conveyance, multiplexed gene editing, directed evolution, and mutagenesis. In the end, we talk about how this innovative technology may be used in the future.

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1. INTRODUCTION

Unexpected difficulties are confronting the world's agricultural productivity. By 2050, the population of the world is expected to reach 9.6 billion, and the requirement for staple food crops is expected to exceed 60% [1]. Plant production has been continuously decreasing, and environmental issues are predicted to further restrict plant productivity. Thus, new cultivars that can withstand harsh circumstances and provide higher output and better quality are needed. Traditional approaches to crop breeding are inefficient, time-consuming, and complex, necessitating the development of more efficient breeding methods that must save time [2].

An ever-enhancing number of plant species now have genomic information accessible, and genome editing methods allow scientists to change genes precisely, opening up new possibilities for crop enhancement. Nucleases that target particular DNA sequences are used to create DSBs at a specific target location. The error-prone non-homologous end joining (NHEJ) network or homology-directed repair (HDR) network induces the DSB as well as various forms of gene modification. Plant meganucleases [3], effector nucleases [4], and zinc-finger-based nucleases [5] are considered efficient in plant genetic modification. However, their creation involves complicated protein transformation restricting their usefulness in the field of genetic modification.

CRISPR–Cas is an evolutionary phage defense mechanism in Archaea and Bacteria. CRISPR–Cas9 and other CRISPR–Cas pathways are simply organized to induce DSB at a specific target location at the lowest possible cost [5,6]. It has been used for genetic modification in plants since 2013 [7–9], providing valuable agricultural characteristics to several crop species [10]. CRISPR–Cas technology has the potential to increase plant yield as it can alter the nucleotide. It should be noted, however, that CRISPR–Cas technology not only has the ability to alter particular loci to boost plant yield but also has an important influence on agriculture. Recently developed several revolutionary plant biotechnologies have the potential to facilitate protein transformation and gene control while also being cost-effective. These technologies have already influenced basic biological research, and the potential for wider application has been boosted as a result of their development.

The CRISPR–Cas molecular technologies for efficient genetic modification are initially described in this review. CRISPR–Cas is now being used in wild species domestication, yield enhancement, and crop breeding, as well as in the development of genetically modified crops. Innovative delivery mechanisms, in situ directed evolution, multiplexed high-throughput genetic manipulation, and modulation of gene-expression are all discussed concerning CRISPR–Cas in plants. In this paper, we aim to present a complete overview of the recent breakthroughs in CRISPR–Cas methods in plants, as well as an assessment of their future potential.

2. PRECISE GENOME EDITING IN PLANTS

The method involving the targeting of plant genes is based on HDR, which allows for accurate genome modification via nucleotide substitutions, insertions, and sequence replacements [11]. However, due to HDR's poor editing efficiency, its use in plants has been limited [11,12]. Alternative genome editing technologies include reverse transcriptase-mediated prime editing and deaminase-mediated base editing; because they exclude the use of donor DNA and the formation of DSBs [12]. These tools are more efficient and cause accurate sequence editing in plants when compared to HDR. After the invention of the adenine base editor (ABE) and the cytosine base editor (CBE) in mammalian cells, the development of base-editing-mediated DNA deletion techniques and dual base editors in plants were the initial steps toward the application of these technologies. The recently established CRISPR–Cas9 techniques, which accurately modify plant genomes, are briefly described in this section T1.

2.1 Cytosine Base Editing

Cytosine base editing is made up of an nCas9, i.e., Cas9 nickase with the mutation of D10A, which inhibits RuvC, coupled with two different protein molecules: a uracil DNA glycosylase (UNG/UDG) inhibitor (UGI) and a cytidine deaminase (CDA). Cytosine base editing (CBE)

incorporates C:G→T:A base substitution into DNA locations that are specifically targeted by sgRNA, i.e., single-guide RNA [13]. The UGI inhibits UDG in the conversion of cytidines to apyrimidinic sites, whereas CDA converts cytidines into uridines within the non-target single-strand DNA section of the R-loop formed by the nCas9–sgRNA network. When nCas9 causes a snip upon the target single-strand DNA, the contradictory repair network of DNA is triggered resolving the U:G mismatch favorably into the required U:A and, after the replication of DNA, a T:A product, resulting in a C:G→T:A base transformation.

CBE systems were developed and refined in numerous species of plants because this technology delivers a great efficiency of precision editing [14-16]. Plant CBEs have included a number of CDA orthologues with various genome editing characteristics (Table 1). CBEs derived from rat APOBEC1 modify cytosines inside editing pathways of about six nucleotides from position 4-9 in the protospacer and prefer TC over GC, depending on a sequence motif to do this. In contrast, CBEs based on human activation-induced cytidine deaminase and *Petromyzon marinus* cytidine deaminase 1 have significantly higher effectiveness in GC motifs in *Oryza sativa* and do not appear to contain a stronger motif priority [13,15]. Human APOBEC3A (hAPOBEC3A)-based CBEs, like human AID-based and *P. marinus* CDA1-based CDEs, show the high efficiency of base editing without a preference of motif, with the base editing pathways ranging from a position 2 to 17 in the protospacer [17]. Two new CBEs established upon coherently engineered truncated human APOBEC3B (hAPOBEC3B) demonstrated remarkable accuracy in the rice plant [18]. Lastly, Cas9 orthologues and Cas9 reliant on the PAM, i.e., protospacer adjacent motif, have been created to overcome restrictions of targeting imposed by the classical PAM (NGG) in rice [18].

2.2 Adenine Base Editing

Adenine base editing is being used to broaden base editing in order to provide A:T→G:C transitions utilizing adenosine deaminase (ADA) combined with nCas9 having a mutation of D10A [19,20]. During DNA replication and repair, ADA forms inosines by deaminating adenosines, which are identified as guanosines by the enzyme DNA polymerase [21]. Despite the fact that there is no known natural ADA capable of deaminating ssDNA, an enzyme for this purpose has been developed by modifying the ecTadA, i.e., *Escherichia coli* tRNA-specific adenosine deaminase [21].

Adenine base editing centered upon modified ecTadA mutants, also known as ecTadA*, have recently been created in *A. thaliana*, rapeseed, rice, and wheat [21-24] (Table 1). Numerous ABE8 variants developed lately for mammalian cells might be beneficial for enhancing the effectiveness of A→G base transitioning in crops [19]. ABEs are, however, ineffective at particular sites, and for this reason, numerous techniques have been employed to improve their efficacy for monocots, like creating improved sgRNAs by the modification of sgRNA scaffold, utilizing a simplified monomer version of ecTadA*, and incorporating three additional SV40 nuclear localization signals (NLS) to the nCas9s C-terminus [22,23]. In rapeseed and *A. thaliana* genome, the ribosomal protein subunit 5a

Table 1. CRISPR-Cas mediated genome editing to induce tolerance to various stress factors in crop plants

Crop specie(s)	Impact	Targeted gene (s)	Reference
Tomato	Drought tolerance	*SlNPR1*	[17]
Wheat	Drought tolerance	*TaNAC071-A*	[13]
Maize	Drought tolerance	*ARGOS8*	[9]
Maize	Drought tolerance	*ZmSRL5*	[15]
Rice	Cold tolerance	OsPIN5b, GS3, and OsMYB30	[4]
Rice	Cold tolerance	OsAnn3	[18]
Soybean	Heat tolerance	GmHsp90A2	[12]
Cotton and *Arabidopsis*	Drought and salt tolerance	GhHB12	[7]
Tomato	Heat tolerance	SlMAPK3	[11]
Arabidopsis	Cauliflower mosaic virus (CMV) resistance	CaMV CP	[10]

(RPS5a) gene promoter, which drives the plant ABEs expression, is more effective than the egg-cell specific *YAO* promoter or 35S promoter [24]. PAM variants have also been used to extend the ABEs scope of editing, although they are less effective than the original SpCas9 or SaCas9 variants [17,23].

2.3 Dual Base Editing

Dual base editing makes use of an adenosine deaminase, a cytidine deaminase, a UGI fusion, and nCas9 (D10A), and is named as “saturated targeted endogenous mutagenesis editor” (STEME). An adenine and cytosine dual-deaminase base editor, which has recently been developed, can perform simultaneous C:G→T:A and A:T→G:C modification in plants utilizing a molecule of sgRNA [25,26] (Table 1). The STEMEs deaminate adenosines to inosines and cytidines to uridine, which are then subsequently duplicated by DNA replication and repair, producing dual C:G→T:A and A:T→G:C replacements. A variant of SpCas9–NG PAM [27], that identifies NG PAMs, has been utilized to broaden the editing prospects and to improve the potential to edit the maximum number of targets as practically feasible. This method makes possible the in situ directed evolution of internal plant genomes. Saturated targeted endogenous mutagenesis editor may be utilized for modification of *cis*-regulatory elements (CREs) and high-throughput screening (HTS) in crops.

2.4 CBE-directed DNA Deletion

In cytosine base editing, cytidine deamination produces uridine that is retained by the UGI, which reduces the function of cellular UDG [28]. In a different scenario where UDG is overexpressed, base excision repair must be activated, leading to the removal of uridines and the production of AP target positions that is cut by the enzyme AP lyases. It should be possible to achieve a highly precise deletion of DNA between the Cas9 cleavage site and deaminated cytidine by combining this cut with the adjacent generation of a DSB by the Cas9 enzyme. Based on that logic, tools and techniques for creating accurate and specific multinucleotide deletions, including a UDG, Cas9, cytidine deaminase, and AP lyase — dubbed “APOBEC–Cas9 fusion-induced deletion systems” (AFIDs) — were designed to stimulate particular deletions inside the protospacer [Table 1). AFIDs have utilized two CDAs, i.e., hAPOBEC3Bctd and hAPOBEC3A. hAPOBEC3Bctd produces an accurate DNA deletion spanning from the TC-preference motif to the Cas9-directed DSB, while hAPOBEC3A produces foreseeable DNA deletions spanning from the targeted cytidine to the Cas9-directed DSB; these deletions make sure for further consistent outputs. APOBEC–Cas9 fusion-induced deletion systems may be used to interfere with regulatory elements of DNA, modify microRNAs, and create in-frame deletions [22].

2.5 Prime Editing

ABE and CBE can induce accurate base transitions, however, the techniques for producing base transversions are restricted. It was solved in 2019 by a groundbreaking genome editing approach named "Prime editor" which can synthesize in mammalian cells all the 12 types of base transitions, accurate insertions and deletions of up to 43 and 80 base pairs (bp) respectively, and integration of these alterations [29]. An nCas9 with a mutation of H840A fused to a modified reverse transcriptase (RT) and a multifunctional pegRNA are the two components of the prime editor. The pegRNA is made up of an RT template and a primer-binding site (PBS) at the sgRNA's 3′ end [24,27]. The RT template carries the genetic code for the required variations, and the PBS pairs with the nCas9 (H840A)-nicked ssDNA strand, therefore priming the process of reverse transcription and inserting the genetic code from the RT template into the genome [28]. Later comes the balance between the 3′ flap and 5′ flap, ligation, and repair, that results in the creation of the required edit. Because the prime editor creates base replacements as well as short insertions and deletions at a rather broad number of sites, it is not significantly restricted by its PAM [29].

This robust and smart technique has been shown to produce and fix mutations in the human cell that cause genetic disorders [30–32]. Afterward, prime editing systems were built and examined in wheat and rice, and it was discovered that they could make multiple base substitutions at the same time, with having the ability to substitute all 12 bases, as well as insertions and deletions in wheat and rice. However, despite the use of relevant approaches such as using RT orthologues with differing catalytic activities, using ribozymes to generate accurate pegRNAs, increasing the culture temperature to enhance the catalytic rate, incorporating improved sgRNA scaffold alterations into pegRNA to enhance

Cas9 binding activity, and manipulating selective markers for the development of modified cells, the prime editor editing effectiveness in plants remains restricted [29,31]. It is worth noting that the potential of this technology to cause exact nicks has only been observed in wheat, rice, and maize; its functionality in different crops has yet to be studied. In addition, the potential of primary editor to make bigger genetic mutations and its selectivity have yet been shown in neither plant nor mammalian cells. Therefore, more effort is required to enhance and broaden this advanced technology.

3. SIGNIFICANCE OF CRISPR-CAS IN PLANT BREEDING

CRISPR–Cas appears to be a promising technique in agriculture because of its unique capacity to properly modify plant genetics. As a result, not only has it been working to create new kinds with desired characteristics, but it has changed the present breeding methods. Furthermore, CRISPR–Cas has opened the way to the domestication of formerly wild organisms. In a brief period, the vast majority of studies reviewed indicated that SpCas9 was employed for genetic modification in this area.

3.1 Applications in Crop Improvement

It is possible to rapidly create perfect germplasm using the CRISPR–Cas technique rather than traditional breeding methods by eliminating undesirable genetic elements or inserting gain-of-function alterations in the genome. According to the examples provided (Table 1), it is observed that during the last two years, the usage of CRISPR–Cas has enhanced various agricultural traits, comprising quality disease resistance and production, and weed control [24,31,32].

3.1.1 Increasing yield

Cereal yield can be increased by realistically regulating cytokinin regulation. Enhancing paddy grain yields by modifying the C terminus of *Oryza sativa LOGL5*, the cytokinin-activation enzyme, was possible in several different atmospheric conditions [33]. A similar high-yielding trait was seen in wheat when the gene encoding the cytokinin oxidase/dehydrogenase (CKX) was knocked out [34]. By mutating the gene that encodes amino acid permease 3, which is important in nutrient division, paddy cultivar was produced with higher yields and improved grain quality [35-39]. It has been shown that CRISPR–Cas-induced modification of additional genes, such as *Triticum aestivum G2* (controlling grain weight), *O. sativa GS3* (grain size regulation), and *O. sativa PIN5b* (controlling the size of panicle), has also resulted in plants with improved production. *ENO* [40] and *CLV* [41], which influence meristems growth, have also been modified by researchers to boost fruit crop yields.

3.1.2 Improving quality

Other aspects of a crop's performance, beyond yield, are crucial to agricultural productivity. Consumption and cooking quality of grain with a lower amylose level is superior, and it is widely used in textiles as well as the adhesives market. Starch linked to granules of amylose production is dependent on the activity of synthase 1 (GBSS1). Twelve superior inbred lines produced waxy maize variants using CRISPR–Cas9 to alter GBSS1 [42], GBSS1's amino acid sequence was altered with a CBE [43], resulting in rice varieties with a range of amylose content. It is possible to use these techniques with a wide range of different crops (Table 1). Low amylose content, on the other hand, isn't necessarily a good thing, since amylose-rich cereal crops are advantageous to human health. It was possible to generate rice varieties with increased amylose concentrations by selectively inhibiting the amylopectin production pathway enzyme [44]. Coeliac disease is brought on vulnerable individuals by gluten proteins found in wheat grains. Traditional breeding strategies are unable to significantly reduce gluten concentration in wheat due to the genome's 100 loci encoding gluten proteins. Low-gluten *Triticum aestivum* varieties with up to 88% reduction of immunoreactivity were produced [45] using CRISPR–Cas technology to target the conserved area of gluten genes. Carotenoid enrichment [46-48], phytic acid reduction [49], and higher oleic acid content [50] have been made possible because of CRISPR–Cas technology.

3.1.3 Disease resistance

Interrupting host vulnerability variables using CRISPR–Cas seems to be a more effective strategy for protecting plants from biotic stress than adding dominating resistance genes, which may encourage the mutual evolution of resistance in pathogens. As a result of the destructive bacterial disease *Xanthomonas*

oryzae pv. *oryzae*, the world's rice crop is at risk. SWEET genes, which are essential for disease susceptibility, may be activated by a collection of bacterial agents during an infection. Researchers have created rice varieties resistant to *X. oryzae* pv. *oryzae* [51, 52] used CRISPR–Cas to alter the promoter region of *O. sativa SWEET14*, *O. sativa SWEET13*, and *O. sativa SWEET11*. *Citrus × Sinensis LOB1* may also provide resistance to *Xanthomonas citri* subsp. *citri* by targeting its promoter region [53].

Powdery mildew in wheat may be caused by *Blumeria graminis*, a biotrophic fungus [54]. Plants with increased tolerance to *B. graminis* were created by introducing CRISPR–Cas gene editing into three wheat *EDR1* homologs at the same time using the MAPK kinase gene *EDR1*. *Mildew resistance locus O* (*MLO*) homologs were also simultaneously mutated in wheat to produce a wheat variety with wide-ranging tolerance to powdery mildew, and in tomato CRISPR–Cas targeted *Solanum lycopersicum MLO1* was adapted for resistance to *Oidiumneo lycopersici*, the fungus that causes powdery mildew in *Solanum lycopersicum* [55,56].

CRISPR–Cas9 can be trained to fragment plant DNA viruses' genomes and give viral resistance because of its capacity to create DSBs. For geminivirus [57] and caulimovirus [58], researchers have developed plant immunity systems using this strategy. Additionally, the RNA-targeting Cas13a, Cas13b, Cas13d, and *Francisella novicida* Cas9 have been used to generate a defencing system against a variety of RNA viruses [59,60]. Defeating wide-ranging virus resistance can also be achieved, by deleting plant susceptibility genes. A category of plant RNA viruses known as potyviruses intercepts the host factor “eukaryotic translation initiation factor” (ETIF) 4E (eIF4E) and its identical form to begin the translation of their own. Encoding potyvirus resistance in cucumber by altering the *Cucumis sativus* gene called eIF4E, provided wide-ranging immunity without affecting cucumber's physical performance [61].

3.1.4 Herbicide resistance

Weedicide resistant germplasms are an efficient way to maintain high yields and prevent soil degradation as weed problems increase worldwide. Genetically modified methods are conventionally used to insert foreign herbicide resistance genes such as the *bar*. The *bar* gene encodes for modifying of crops with phosphinothricin *N*-acetyltransferase herbicide-resistant genes that can be reprogrammed using CRISPR–Cas technology. CRISPR–Cas is enticing because of its attractiveness, flexibility, and the absence of transgenes. Nature-based research shows that the ALS gene's point mutations have indicated certain benefits that ALS-related amino acid replacements can provide weedicide tolerance [62]. The introduction of the specific base is transformed into *O. sativa ALS* using means of Rice was able to resist herbicides to use of CBEs keeping ALS activity [14,40].

ALS mutations have also been utilized in other species to impart herbicide resistance using HDR [63]. Coenzyme acetyl-CoA Carboxylase (ACCase) is an essential enzyme in lipid production and a promising herbicide target. Rice strains resistant to haloxyfop-R-methyl [22] were created by introducing an adenine base editor into the *O. Sativa ACCase* gene and replacing the C2186R codon with a C2186R. Similar to the development of quizalofop-resistant wheat, *T. aestivum* ACCase [64] was engineered to induce an A1992V substitution for the enzyme. CRISPR has shown that W2125C and P1927F induce haloxyfop tolerance in *Oryza sativa* [21,55]. Glyphosate resistance is conferred by altering *TubA2* [54], *PPO* [55], *EPSPS* and *SF3B1* [65-68], as well as resistance to herboxidiene, butafenacil, and trifluralin. They may be utilized as selective markers to enhance genome-editing steps [24, 44], in addition to their utility in agriculture. The United States Department of Agriculture has identified over 100 plant varieties generated by genetic modifications as not regulated, enabling commercial cultivation in the United States of America [69].

3.2 Applications in Breeding Technologies

However, using CRISPR–Cas with traditional breeding techniques will significantly boost agricultural production. There are a variety of new breeding methods that use CRISPR–Cas to target reproduction-related genes that gained prominence in the recent past.

3.2.1 Haploid induction

Compared to the six to eight generations of selfing required by conventional methods, doubling haploid technology may balance the genomic basis of hybrid lines within next two generations. Haploid maize embryos may be

formed by frameshift alteration in *MATRILINEAL* (*MTL*) that express pollen-specific phospholipases A1 (PLA1), a gene that codes the zygote's paternal DNA [70]. CRISPR–mediated mutagenesis of *MTL* [71,72] resulted in the creation of wheat and rice haploid induction lines. Many genes, for example, *DMP* [73] and *CENH3* [74,75], have been successfully edited by CRISPR–Cas to promote haploidization, as shown in this study.

3.2.2 Generating male sterile lines

Breeders have relied heavily on hybrid vigor to boost yields while also enhancing product characteristics in agricultural breeding. To prevent the production of homozygous seeds, the self-pollination of the female parent must be prohibited when commercially producing hybrid seeds. The most efficient and practical solution to this challenge has been the establishment of male sterility in maternal lines. A few male-sterile lines of diverse crops have been described, but it is often time-consuming and labor-intensive to transfer male sterility into other genetic characteristics. Male sterility may be established in reconfigurable lines using CRISPR–Cas genetic modification [76,77]. Researchers induced male sterility into *Triticum aestivum* varieties by targeting *Ms45* and *Ms1*, that codes a strictosidine synthase-like enzyme [37] and a glycosylphophatidylinositol-based lipid transport protein [47]. A putative strictosidine synthase gene was mutated to produce a male-sterile tomato line [78]. Some species have been able to benefit from these tactics as well. They have also been developed in rice and maize thermo- and photoperiod-vulnerable *genic male sterile* lines that are efficient and easier to employ by interruption of *carbon deprived anther* [79] and *thermosensitive genic male-sterile 5* [80].

3.2.3 Fixation of hybrid vigour

A well-established approach for producing hybrid seeds from male-sterile lines is expensive and tedious in certain crops. Alternatively, inducing apomixis, a naturally occurring asexual reproduction route, might fix elite hybrid backgrounds. Researchers found that CRISPR–Cas-induced triple mutations in *PAIR1*, *REC8*, and *OSD1* in rice and *A. thaliana* result in clonal tetraploid seeds and diploid gametes. Parthenogenesis may be induced by the deranged expression of *BABY BOOM 1*, that stimulates embryogenesis in *MiMe* rice egg cells [81,82]. Similarly, rice clonal diploid embryos were created by disrupting *MTL* and *MiMe* genes. Artificial-apomictic germplasms may be utilized directly in crops like vegetables and pastures where seed yield is least important. They cannot yet be utilized to mass-produce hybrids.

3.2.4 Manipulating self-incompatibility

The absence of inbred lines in crops like potatoes has hindered genetic potential because of the inherent self-incompatibility. *S-RNase*, the co-dominant gene responsible for the gametophytic SI, is altered by CRISPR–Cas because of Solanaceae gametophytic self-incompatibility; there are now potato lines that are self-contained [83]. Additionally, sporophytic self-incompatibility has been shown to be overpowered by the removal of the *M-locus* protein kinase (oilseed rape) inhibitor [84] and *S-receptor* kinase (Cabbage) [85], respectively. Additionally, this reduces heterozygosity, interspecific reproductive barriers, and reducing the requirement for pollinators may overcome by this method as well in fruit trees. Additionally, in genetic mutations such as *farnesyl pyrophosphate synthase 2*, it is possible to use CRISPR–Cas because of its self-incompatibility restoration ability [83], as well as in the development of a more efficient hybrid breeding system, the development of seedless fruit production techniques, and the creation of inducing parthenocarpy in citrus.

3.2.5 Other breeding technologies

Because of the detrimental genetic interactions between divergent alleles, cross-breeding between distant lines leads to acute hybrid sterility. It is possible to restore male fertility in *O. sativa japonica-indica* hybrids by selectively eliminating portions of the *Sc-i* allele that restrict the expression of the pollen-essential *Sc-j* allele [86]. There have also been hybrid-compatible African–Asian rice lines developed by the mutation of *Oryza glaberrima TPR1* [87]. Targeting a single parental allele by CRISPR–Cas may lead to meiotic homologous recombination at precise places during meiosis, which is unusual [88]. It is also possible to induce reciprocal translocations between two chromosomes by introducing two double-stranded breaks (DSB) [89]. To stack favorable alleles, disrupt unwanted genetic links, and swiftly construct near-isogenic lines, such procedures might be applied to the lines that are very close to becoming isogenic.

3.3 CRISPR–Cas-accelerated Domestication

As far back as 10,000 years ago, cultivating crops has required artificial selection for desired qualities including high yield, nutritional density, and harvestability. Genetic heterogeneity and resistance to abiotic and biotic stresses are both reduced as a consequence of this productivity-focused breeding strategy [90]. Only 14 out of a total of 28,000 edible plant species are thought to provide 72% of the energy required by humans [91]. Nature has given us a vast genetic resource that we have yet to tap into: wild species and orphan crops typically have favorable nutritional qualities or stress tolerance and are better suited to native conditions, compared to established crops. This makes it possible to supply the ever-increasing need for food by domesticating wild creatures or using semi-domesticated crops. There are numerous factors that go into traditional domestication, but only a few of them are critical in achieving the intended result [92]. Crop domestication might be sped up significantly given the precision that CRISPR–Cas offers in genomic editing.

Accelerated domestication has previously been the subject of many groundbreaking investigations. *Solanum pimpinellifolium*, a possible predecessor of the tomato, is more resistant to environmental stressors than modern tomatoes. It is resistant to both bacterial spot disease and salt in the environment. As a result of these undesired characteristics, *S. pimpinellifolium* cannot be developed into an economically viable crop and the sensitivity to day-duration must be altered. Scientists have employed multiplexed CRISPR–Cas systems in an effort to better understand these characteristics concurrently edit genes linked to each other, such as *SP* (plant growth habit), *SP5G* (induction of floral behavior), *CLV3*, and *WUS* (fruit size), *MULT* (fruit count), and *OVATE* (fruit shape), *CycB* ((lycopene content)) and *GGP1*(high in vitamin C), and escorted *S. pimpinellifolium* a level near to become best cultivar of *Solanum lycopersicum* [93,94]. These are very important in domesticated plants; a high level of resistance was preserved. Pathogenic microorganisms and salt may damage *S. pimpinellifolium*. The adaptation of ground cherry, is another example of an orphan Solanaceae member, three genes were disrupted, *SP*, to produce the crop more blooms and bigger fruits. These strains were shorter than the parent strains, *SP5G* and *CLV1* [95]. Studies intended to domesticated African rice (*O. glaberrima*) [96] have also been implemented. These investigations which paved the way for adaptation has been sped up.

Other species may potentially be suitable for agricultural use. *Thinopyrum intermedium* is a perennial cousin of wheat that needs less labor and absorbs water and nutrients better than wheat [97]. However, seed shattering and poor yielding traits limit its cultivation. *Chenopodium quinoa* is suited for domestication owing to its high nutritional content and great resistance to abiotic stress, but its short day length and heat sensitivity need adjustment. Other crops, like lupin, alfalfa, and pennycress (*Thlaspi arvense*), lupin (*Lupinus* spp.) [98] has exceptional qualities. It should be feasible to improve upon existing strains by modifying their genes using CRISPR–Cas.

Although CRISPR–Cas rapid domestication has considerable potential, the process is nevertheless plagued by several bottlenecks. Further research is necessary to get fundamental genetic information of wild species and to locate domestication genes. Moreover, to domesticate wild species that are resistant to regeneration, effective transformation methods must be created. To produce a perfect cultivar, the multiplexed genome editing approaches must be developed that are more efficient.

4. CRISPR-CAS-RELATED PLANT BIOTECHNOLOGY

Besides considerably aiding agricultural advancements, plant biotechnology linked to CRISPR–Cas have recently been introduced. For instance, when the absence of strong delivery mechanisms became a barrier in the development of plants gene editing, various unique techniques for creating edited plants were designed that enable genome modification without the need of foreign DNA. Research on gene expression regulation has made extensive use of CRISPR–Cas later on. Moreover, due to its clarity and strong orthogonal characteristics, CRISPR–Cas has been modified to conduct high-throughput and multiplexed genome editing, as well as to serve as a dynamic tool in synthetic biology of plants [73,91].

4.1 Incorporation of CRISPR-Cas Reagents in Plant Cells

Nevertheless, there are several drawbacks to both of the commonly utilized delivery systems,

i.e., biolistic bombardment and *Agrobacterium*-mediated delivery, on which plant transformations has mostly depended for decades. Biolistics, short for "biological ballistics", is a type of bombardment which can drive genetic matter through cell walls and membranes using a gene gun, but it is inefficient and can disrupt genomic sequences [99]. While *Agrobacterium* can colonize a wide variety of plants, exogenous DNA integration is inevitable, and recipient genotype affects transformation effectiveness, particularly in monocots. For CRISPR–Cas9 to be used effectively in plants, a reliable and ubiquitous method for CRISPR–Cas reagents delivery into plant cells is required (Fig. 1). Moreover, not one of these traditional approaches can eliminate the need for time-consuming tissue culture operations. As a result, a new generation of delivery techniques is urgently required.

4.1.1 De novo meristem induction

A significant tool for allowing CRISPR–Cas–induced genome modification in plants is emerging from the regeneration-enhancing actions of morphogenetic regulators. Beyond aiding in the transition of resistant cultivars, morphogenetic regulators can also be designed and manufactured to instigate de novo meristems on different plant species, eliminating the requirement for tissue culture. Not long ago, genetically modified plants were procured immediately from the newly formed shoots of a Cas9-overexpressing *Nicotiana benthamiana* by injecting *Agrobacterium tumefaciens* having the morphogenetic regulators STM AND IPT, WUS2, and sgRNA cassettes inside the pruned areas which were made free of meristems, and the instigated alterations were also seen in the next progeny (Fig. 1). The approach also was applied, in that same research, on grapes, potatoes, and tomatoes [100]. This outstanding study gives a generalizable in planta delivering method that cuts the time which is necessary to generate gene-edited *N. benthamiana* in half, and if the methodology is extended to different species, it would considerably improve the future research on plants.

4.1.2 Virus-assisted gene editing

It is possible to get genetically modified plants without the requirement of tissue culture by manipulating plant viruses [101]. This is an impressive idea because a virus replicates and moves around in planta, virus-assisted gene editing is very effective and may be used to achieve a comprehensive gene mutation (Fig. 1). In the previous decade, positive-strand RNA (+ssRNA) viruses, such as the tobacco mosaic virus [102], the tobacco rattle virus [103], barley stripe mosaic virus [96], pea early browning virus [104], beet necrotic yellow vein virus [105], and foxtail mosaic virus [106], as well as the ssDNA cabbage leaf curl virus, were established for delivery of sgRNA in plants having up to 80% editing efficiency [107]. But, due to their cargo capacity limitations, these viruses cannot co-encode Cas9 with sgRNA, hence already present Cas9-overexpressing plant lines are needed. To find a solution, two teams simultaneously introduced sgRNA cassettes and Cas9 into the genetic structure of sonchus yellow net rhabdovirus [108] and barley yellow striate mosaic virus [109], two negative-strand RNA (-ssRNA) viruses with remarkable delivery capacity and genome stability and accomplished extensive genome modification in *N. benthamiana*. A further obstacle to virus-assisted genome editing is that intact viruses are unable to penetrate the reproductive tissue or meristem, preventing the transmission of mutations caused by the virus [110]. To overcome this constraint, researchers inserted sgRNAs containing RNA mobile elements in tobacco rattle virus (TRV, genus Tobravirus) RNA2. With *Agrobacterium*-induced penetration, the mobile elements guided them towards cells of shoot apical meristem, resulting in mutations that were heritable with rates of up to 100% in the next generations [87] (Fig. 1).

4.1.3 Role of haploid inducers in gene editing

Many plants still restrict genome editing to specific genotypes, severely restricting breeding operations. In order to tackle this issue, two new delivery pathways have been created, which are referred to as “haploid-inducer mediated genome editing” [111] and “haploid induction edit” [112] (Figure 1). Both approaches use haploid inducer lines with CRISPR–Cas systems to fertilize elite maize lines. When haploid inducer lines are used to fertilize maize, the paternal DNA causes mutations in the maternal genetic makeup, which is then removed from the zygote, resulting in genetically modified maize haploids having maternal backgrounds. Likewise, two genes of wheat were effectively modified by pollinating wheat varieties with elite maize lines representing CRISPR–Cas9 stably [105]. Edited haploid lines may have their chromosomes doubled either naturally or intentionally by the administration of mitotic inhibitors. These approaches not just successfully overcome the

intractable transformation barrier, but they also result in the production of homozygous transgene-free genetically modified crops [113].

4.2 Gene Regulation using CRISPR-Cas

Instead of utilizing CRISPR– Cas to induce deadly mutations in plants caused by gene knockout, a heritable and programmable transition of gene expression offers a more adjustable and versatile technique to modify phenotypes allowing us to develop elite features without altering coding sequences of proteins [103,105].

4.2.1 Transcription modulation

However, even though catalytically dead Cas9 (dCas9) lacks DNA cleaving function, they do possess sequence-specific DNA-binding activity mediated by the sgRNA. dCas9 may inhibit transcription by docking at certain genomic locations and preventing the attachment process of transcriptional machinery or blocking the pathway of RNA polymerases [114,115]. Another advantage of dCas9 is that they may be used to precisely control gene expression by fusing with epigenetic modulators [116-118] or transcription regulators [119,120]. With the help of multiple effectors linked together, this gene control can also be improved [116,119,121]. Cas9 may also be used to change chromatin structure, which can then be used to influence gene expression by either increasing or suppressing interactions of enhancer-promoter [120].

This method is strong, but in order to maintain stable gene regulation, dCas9-fusion proteins as well as sgRNA sequences must be incorporated inside the genome continuously. When it comes to producing dCas9 fusion proteins, editing of *cis*-regulatory elements is a viable alternate method of doing so. For instance, Tomato alleles with diverse genotypical and phenotypic traits were generated by targeting *cis*-regulatory elements in the promoter site of tomato *CLV3* with eight gRNAs [40]. *O. sativa TB1*, a gene related to the yield of rice, was also edited using six gRNAs to change its expression level [122]. Additionally, since many genes have many regulatory mechanisms, just controlling the transcription process may not provide the targeted phenotype, while altering cis-regulatory elements may affect gene expression in a stage-specific way, perhaps responding to external stimuli [40,51,52,123,124]. As a result, this strategy would allow for the development of multidimensional features as well as the discovery of DNA patterns that respond to certain signals.

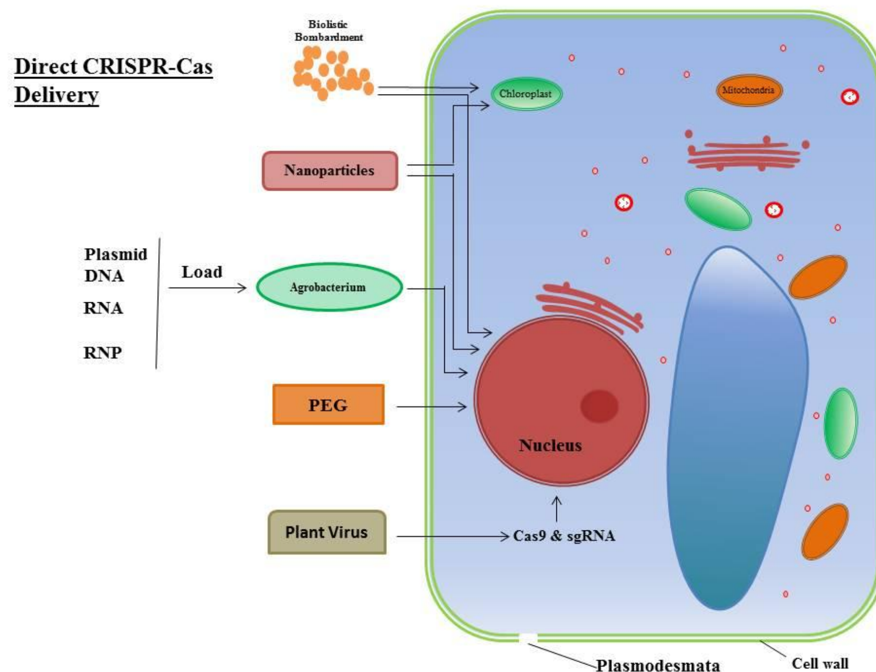

Fig. 1. Strategy for CRISPR-Cas delivery

4.2.2 Targeting RNA

In recent years, many RNA-targeting systems of CRISPR–Cas, including Cas13a and Cas13b, have been successfully developed in crops. The cleavage and degradation of the targeted RNA allow these systems to suppress single transcripts with more accuracy than the commonly utilized RNAi approach [125]. Additionally, CRISPR–Cas is also utilized to influence pre-mRNA splicing. Since splicing depends on the conventional GU–AG principle for most pre-mRNA splicing, altering splicing-related motifs may affect gene activity [23,126]. Furthermore, because essential introns can encourage gene expression through a badly understood system known as "intron-mediated enhancement" (IME), altering the intronic splicing region in the 5′ untranslated area of rice GBSS1 resulted in a reduction in the expression of the gene and the development of waxy rice varieties [34]. Aside from integral splicing, several genes produce various mRNA isoforms by alternative splicing [127]. Using a CBE, researchers perturbed the auxiliary splicing of genes HAB1.1 and RS31A of *Arabidopsis*, resulting in crops that were insensitive to mitomycin C and hypersensitive to abscisic acid [128].

4.2.3 Upstream open reading frames

The "Upstream open reading frames" (uORFs), are well-researched regulatory pathways found in the 5′ untranslated sites among several plant mRNAs that inhibit translation of the downstream, primary ORF (pORF) and increase mRNA degradation. Thus, altering uORFs can be a useful way to enhance gene expression. The increased translation of three genes in lettuce and *Arabidopsis* was achieved by eliminating the primary codons of uORFs, resulting in a high ascorbate content germplasm in lettuce [129]. A CBE was also used in a diploid strawberry to modulate the uORFs of the *Fragaria vesca bZIP1.1* gene which resulted in an increased translation of the pORF and an increase in the sweetness of strawberry [130]. Since around 35% of plant genes include upstream open reading frames that can be changed by CRISPR–Cas, the new genotypes might be passed down through generations by asexual reproduction. This method can be used for translational control of gene expression. Several other common genetic elements, including substitutive transcription primary sites, polyadenylation signals, and promoters, play key functions in controlling gene expression in plants and are potential contenders for gene-editing techniques. Since several important plant genes are tightly regulated and knocking them out or abnormally overexpressing them could have a negative impact on fitness, genome editing which disrupts or artificially creates regulatory elements, offers significant potential for precise gene expression and development of crops having high diversity with the least possible vigor risks.

4.3 Conditional CRISPR-Cas systems

It is estimated that around 10% of the genes responsible for protein-coding in plants are essential for survival and that their impairment has pleiotropic impacts or results in a fatality [131]. Alternatives like gene knockout through CRISPR–Cas-based regulation or RNAi are often ineffective. To tackle this serious setback, conditional CRISPR–Cas systems have recently been created. Gene editing may be targeted to certain tissues by using promoters which are specific to the desired tissues that control Cas9 expression in various cell types [132]. In order to better understand gene activity in the lateral roots, root cap, and stomatal lineage, researchers have used this method [133]. Additionally, this method can also be used in conjunction with inducible expression networks. Utilizing inducible promoters which are cell-specific, altering genes may also be limited to certain tissues and regulated by external promoters [134]. A conditional system may also coordinate the expression of Cas9 with the congregation of the donor template and therefore improve the effectiveness of gene targeting [134,135]. In the current plant genetic studies, the use of conditional CRISPR–Cas systems may become more widespread because of their high versatility and compatibility.

4.4 Bi-directional Genome Editing

Bi-directional genome editing regulates traits, regulatory pathways, and gene expression, allowing for crop improvement, breeding, and domestication which was previously described [123,125].

4.4.1 Multiplexed sgRNA expression systems

Multiplexed methods for CRISPR–Cas9 have recently been designed in plants, which are both efficient and convenient. The classic approach for Pol III promoter-regulated pathways in plants, for example, employs several Pol III promoters

(U3 and U6) to express numerous sgRNAs in a single construct [132,134]. Furthermore, by using cellular RNase P and RNase Z to activate pre-tRNAs, which act as spacers interposed in-between the numerous sgRNAs of a polycistronic tRNA–sgRNA transcript, multiple sgRNAs may be transcribed with surrounding tRNA sequences under the regulation of a singular Pol III promoter [136,137]. Another example is Pol II promoter-regulated pathways that use poly-sgRNA-containing transcripts to express several sgRNAs concurrently, including ribozyme sequences surrounding the sgRNAs [138], polycistronic tRNA–sgRNA transcripts incorporated into introns [139], and 6-bp or 12-bp linkers flanking the sgRNAs [140]. Also, in plants, the Pol II promoter was used to drive a more productive CRISPR system yersinia 4 (Csy4) processing mechanism, that can cut specified 20-nucleotide sequences surrounding the sgRNAs [141]. Lastly, multiplexed editing utilizing CRISPR RNA arrays is now more versatile because of the class II type V CRISPR–Cas12a, that can develop its own CRISPR RNA by refining pre-crRNAs which are split by direct repetitions [142]. Nevertheless, techniques for expressing randomized sgRNAs in multiplex, which would allow for high-throughput sequencing, have yet to be established.

4.4.2 Multiplex gene editing

Most instances of editing based on the multiplex system in plants have employed a single kind of editor that combines one CRISPR–Cas system with several sgRNAs [143] (Fig. 2). Meanwhile, multiplexed orthogonal genome editing requires more than one kind of scRNA or Cas protein. Various methods have been established for the application of multiplexed orthogonal genome editing in mammalian cells. For instance, one method is to combine dCas9 and multiple scRNAs, each of which contains an array of RNA aptamers capable of attracting various transcription activators and repressors (such as VP64 and KRAB, respectively) [144] (Fig. 2). Other approaches use an sgRNA with a full-length protospacer to target DSBs and therefore gene knockouts, in conjunction with a second sgRNA with a truncated protospacer to target a different gene by Cas9 [145], Cas12a-[activator] or Cas12a-[repressor] [146] (Fig. 2). It is possible to do multiplexed gene deletion and transcription control using the pairing of Cas orthologues, which makes it easier to analyze complicated gene networks (Fig. 2). All the three above mentioned multiplexed orthogonal genome editing techniques have been applied to plants [147] (Fig. 2).

The modern breakthrough of CRISPR-directed SWISS, i.e., immediate and broad editing generated by an individual system, allows for the orthogonal and multiplexed generation of concurrent base alterations and gene deletions in *Oryza sativa* [147,148]. In this system, the RNA aptamers included inside the designed scRNAs attract their corresponding binding proteins. These are then fused with both an adenosine deaminase (ADA) and a cytidine deaminase (CDA) enzyme to simultaneously generate ABE and CBE edits on sites specified by Cas9 [148]. In addition, the introduction of a pair of single-guide RNAs enables nCas9 to insert a third style of edit, known as indels. Another research used a dual-function framework consisting of a truncated protospacer and a full-length protospacer to regulate the function of an improved specificity SpCas9 variant 1.1-based CBE to induce an indel and C:G→T:A base alterations in plants [149]. Multiplexed orthogonal genome editing in rice was achieved by combining the CBE and ABE based on SpCas9 and SaCas9, respectively [20,150]. The development of these multiplexed orthogonal editing techniques opens the door to the possibility of modifying the genome of living organisms synthetically.

4.5 Mutagenesis and Directed Evolution

The CRISPR–Cas technology is capable of doing multiplexed genome editing as well as high-throughput genetic research. Because the main variable of programmed gene editing is the spacer sequence, technologies based on CRISPR–Cas may be simply upscaled to utilize sgRNA pools and are potential techniques for high-throughput genetic studies and guided plant evolution [145-147].

4.5.1 Genome screening with the help of CRISPR-Cas technique

Genome screening is an effective method for finding genes involved for certain phenotypes. CRISPR–Cas, due to its configurable and resilient features, enables high-throughput screening on a genomic scale in plants within a single progeny. A CRISPR library including 25,505 pooled sgRNAs covering 12,703 genes was devised and built, and more than 15,000 separate T0 lines demonstrating a high rate of modifications were reproduced [151]. The sgRNA

spacer sequencing revealed the genotypes of 54 out of the 200 examined lines with changed morphological traits. Likewise, a library of 88,541 sgRNAs was used to construct 91,004 rice mutants [150,151]. Genomic screening utilizing sgRNA libraries may also be used to aid in the validation of functional genes. Genes associated with agronomic quality were precisely mapped in maize by the screening of 1,244 contender loci utilizing high-throughput genome editing [152]. There have been similar studies in soybean [153] and tomato [154], which will no doubt lead to further refinement of these techniques. A di- or tri-sgRNA library is more suitable to study phenotypic alterations caused by numerous mutations in non-coding areas than mono-sgRNA libraries. Furthermore, CRISPR–Cas-based transcription regulation platforms might be utilized to search for abnormalities linked with high subtle variations in the expression of genes.

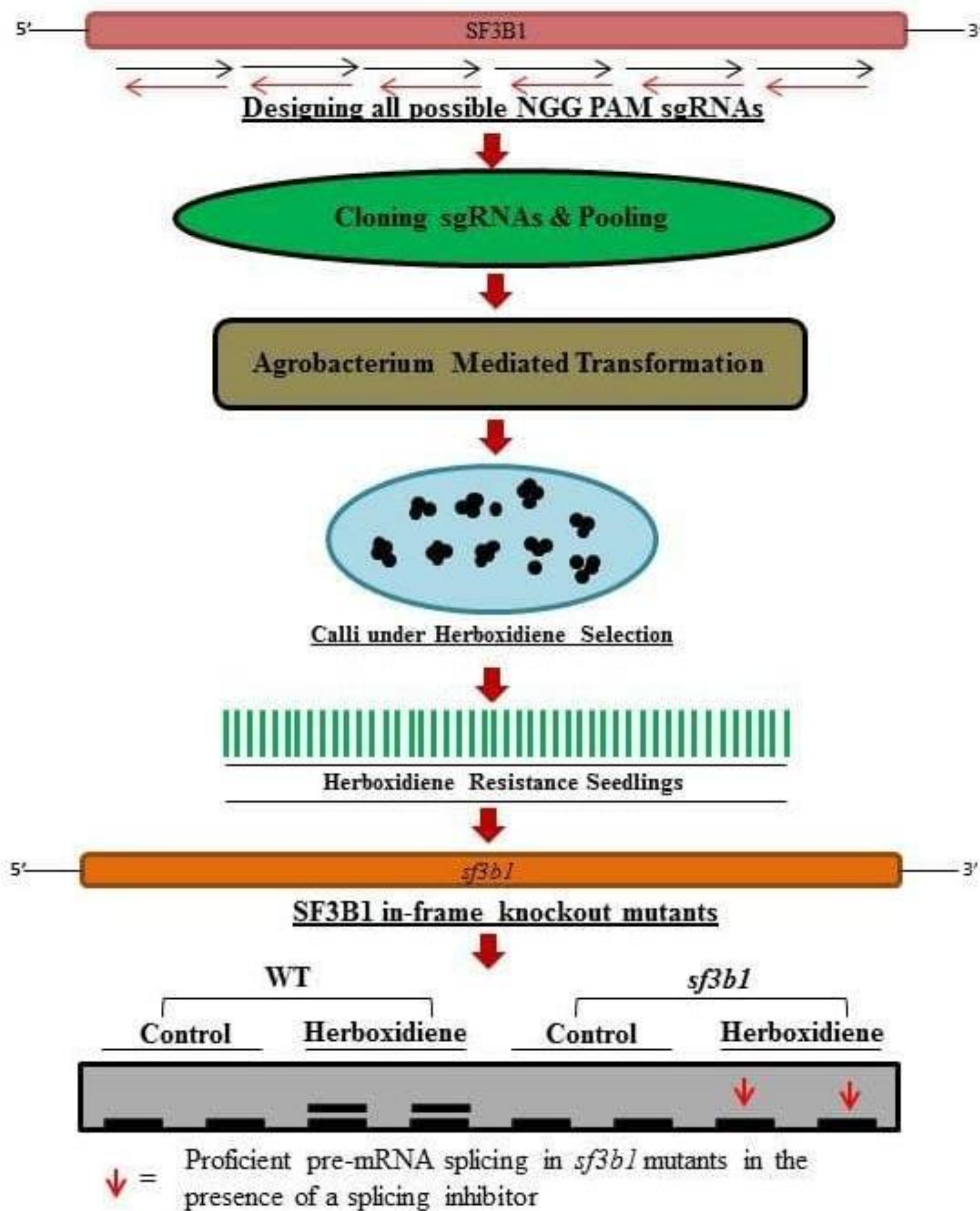

Fig. 2. Directed evolution by CRISPR-Cas

4.5.2 CRISPR-Cas-directed evolution

With the emergence of directed evolution, it is now possible to improve or create new features for desired genes of interest. Despite the fact that a variety of technologies for directed evolution were created in microbes, due to the dissimilarities in the cellular structure and environment, these systems may not behave the same way in plants [121,137]. As a result, techniques of directed evolution would be very beneficial if they can be successfully implemented in plant systems. Directed evolution systems are made up of two subsystems: mutagenesis, in order to create different genotypes, and selection, for the enrichment of desirable genotypes. As a result, directed evolution systems would be very beneficial if they can be successfully implemented in plants. As opposed to the widely used error-prone approaches are used in directed evolution, the use of a CRISPR–Cas sgRNA library in conjunction with the selection of desired traits, for instance, allows for high-throughput sustained mutagenesis inside the genes of interest in living organisms.

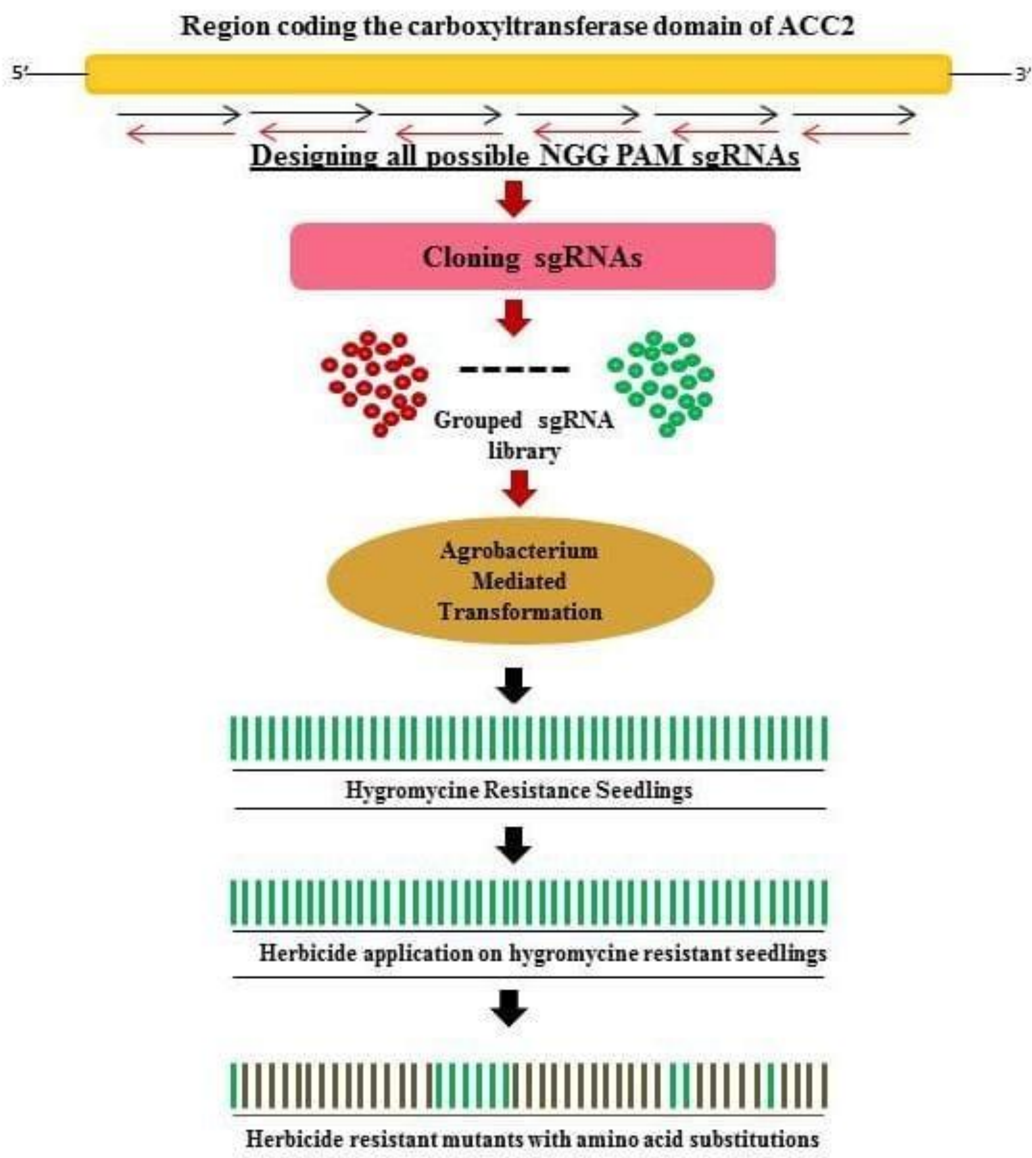

Fig. 3. Directed evolution by STEME dual base editors

CRISPR–Cas-directed evolution in crops has been established in a number of high-quality studies. GEX1A has been developed to suppress the rice splicing factor 3B subunit 1, which is encoded by the *O. sativa SF3B1* gene [68]. Of 16,000 transformants, five had in-frame knockout mutations that conferred tolerance to GEX1A, and no fitness loss was found as compared to wild-type *O. sativa* (Fig. 3). Other herbicide-resistant ACC2 variants were discovered in another work that used C→T and A→G dual base editors (STEME-1 and STEME-NG) to execute saturation mutagenesis on the carboxyltransferase domain coding area of *O. sativa* acetyl-CoA carboxylase 2 (ACC2) [19] (Fig. 3). CBE and ABE have also been used to accomplish directed evolution on the *O. sativa ALS1* [155] and *O. sativa ACC2* genes [156]. Weed management might benefit greatly from these newly acquired herbicide-resistant mutant plants since they could be employed to improve food production.

Still, in its infancy, the use of CRISPR–Cas-directed evolution strategies, for the time being, the only genes of interest that can be evolved are those that are resistant to herbicides. As a result, complex genetic circuits combining genotypes with readily observable phenotypes must be devised in order to generate new genes of interest. The generation of genotypes with many mutations and the reduction of labor both need repetitive mutagenesis and selection platforms. Aside from identifying gene functions, we believe these approaches will also help expand the plant synthetic biology toolbox and generate significant genes for agriculture.

5. CONCLUSION AND FUTURE PERSPECTIVES

Basic and applied plant research have both benefited greatly from the advent of CRISPR–Cas. Additionally, a variety of CRISPR–Cas-based editors have been developed that can induce precise genome alterations, in addition to the indel mutations generated by the nuclease. These tools, which are unmatched in their capacity to modify genes, have aided in the development of hundreds of better agronomic crop varieties and transformed breeding techniques.

Using CRISPR–Cas, orphan crops and wild animals may be swiftly tamed, reducing food shortages and poverty. High-throughput and multiplexed gene editing systems have allowed genetic modifications at various loci, functional genomics screening, and plant-directed adaptation. More research is required to apply CRISPR–Cas in plants because certain agricultural features are the product of several quantitative trait loci, so it would be useful to establish effective CRISPR–Cas-derived selective insertion and chromosomal rearranging technologies. The ability to regulate gene expression and perform precise genome editing will need to be improved in the future to fine-tune gene activity with more efficacy and accuracy.

The direct transformation of some foreign proteins into plants could be difficult, if not impossible, using current CRISPR–Cas platforms. Developing new delivery systems for CRISPR–Cas agents to plant genetic modifications is essential; nanomaterials (such as carbon nanotube) [157-159], DNA-nanostructure [160], and cell-penetrating peptides [161] are good vehicles for delivering CRISPR–Cas agents in different forms as they can dissipate into walled plant cells without mechanical help and without having caused tissue dams. To discover genes linked to certain desirable agronomic characteristics, advancements in fundamental genetic research are also necessary.

Other potential uses for CRISPR–Cas include changing the mitochondrial and chloroplast genomes, locating cell lineage to better understand plant development patterns, creating genetic circuitry to merge and transduce signals, and creating plant biosensors to recognize signals internally and externally. These and other potential uses for CRISPR–Cas are just a few of the many possibilities. Overall, CRISPR–Cas technology has and will continue to change both agricultural practices and plant biotechnological advancements.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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