Tapping into the Unsung Potential of CRISPR/CAS Technology in Agriculture

ABSTRACT

The application of clustered regularly interspaced short palindromic repeats (CRISPR) for genetic manipulation has revolutionized life science over the past few years. CRISPR was first discovered as an adaptive immune system in bacteria and archaea, and then engineered to generate targeted DNA breaks in living cells and organisms. During the cellular DNA repair process, various DNA changes can be introduced. The diverse and expanding CRISPR toolbox allows programmable genome editing, epigenome editing and transcriptome regulation in plants. However, challenges in plant genome editing need to be fully appreciated and solutions explored. This Review intends to provide an informative summary of the latest developments and breakthroughs of CRISPR technology, with a focus on achievements and potential utility in plant biology. Ultimately, CRISPR will not only facilitate basic research, but also accelerate plant breeding and germplasm development. The application of CRISPR to improve germplasm is particularly important in the context of global climate change as well as in the face of current agricultural, environmental and ecological challenges.

Keywords: CRISPR-SpCas9; CRISPR-Cas12a; CRISPR nucleases; epigenome editing; transcription regulation; base editors; germline editing; gene insertion

1. INTRODUCTION

Creating targeted genetic modifications in living cells and organisms has long been a tough challenge in many species. The most common method for introducing genetic changes is to utilize a sequence-specific nuclease (SSN) to induce double-strand breaks (DSBs) at the targeted chromosomal site. The nonhomologous end-joining (NHEJ) pathway is primarily responsible for DSB repair in higher eukaryotes, resulting in indels. Homology-directed repair is a secondary mechanism that contributes to achieving precise genomic changes.

To assess specificity, early SSNs such as zinc finger nucleases (ZFNs) [1] and transcription activator-like effector nucleases (TALENs) [2] depend on challenging to build and multiplex protein–DNA interactions [3]. The following year, CRISPR genetic editing was successful in mammalian systems [4,5]. Clustered regularly interspaced short palindromic repeats has dominated the genome editing business as a versatile, simple, and cost-effective technique for genetic modification [4,5].

Since the first demonstration of CRISPR in plant genome editing in 2013 [6–8], there has been much progress in fundamental crop research and plant improvement [9,10]. Plant genetic engineering has been achieved through the development of a variety of molecular techniques and platforms, including targeted mutagenesis (TM) [11–13], base editing (BE) [14], precision editing by high-density recombination (HDR) [15], and transcriptional regulation [16]. In this

review, we summarize the most recent breakthroughs in CRISPR methods that have been used in plants or are actively being researched. Recent breakthroughs in plant breeding that utilize CRISPR with other modern techniques are also explored. CRISPR-based plant genome editing will undoubtedly open up new possibilities. This review, we believe, provides a comprehensive review of CRISPR technology in plants, as well as prospective future paths.

2. THE EVER-EXPANDING ARRAY OF CRISPR TECHNIQUES

CRISPR arrays, which are made up of repeats and unique spacer sequences, are discovered in bacteria and archaea DNA sequences [17]. Tiny clusters of Cas proteins-encoding genes surround CRISPR arrays. Within every class in Cas, there are several types depending on the signature proteins of the corresponding classes: type I, III, IV, and V included in class 1, whereas type II, type V, and type VI are included in class 2 [18–23]. The presence of Cas proteins at CRISPR loci and the operon structure allow subtypes of CRISPR systems to be identified. Even though the categorization of CRISPR system is continuously developing, this established technique will help us better understand CRISPR systems and discover novel Cas proteins.

2.1 The genome editing method CRISPR–SpCas9

A type II CRISPR system (Cas9), has been utilized widely to change the genomes of the vast majority of organisms, including humans [5,24,25]. Cas9 must be constructed with sgRNA, then recognise and adhere to the relevant DNA sequences before cleaving the whole genome (PAM) [26-28]. When D10A in the RuvCl system or H830A in the HNH system (nCas9, respectively) is added into the Cas9 enzyme, the enzyme only cuts targeted or non-targeted DNA [4,29,30]. Cas9 (dCas9) is a catalytically inactive or dead protein [31].

2.2 Orthologues and variants of Cas9 are employed to broaden target range

Because it requires PAM to work, SpCas9 can only target a tiny region of a genome. Mutations with altered PAM needs can be acquired in the PAM-interacting (PI) domain by rational design and controlled evolution (Table 1). Each VRER, SpCas9 VQR, and EQR variant can recognise NGA PAMs [32]. The QQR1 variant, on the other hand, was exceptionally engineered to bind to the NAAG PAM [33]. It has been developed by phage assisted continuous evolution (PACE) to recognise the PAM sites in the NG, GAA, and GAT regions of the genome [34,35]. In sites with NGH-PAM, rationally built SpCas9-NG has benefits, as does the capacity to recognise relaxed NG-PAM [36,37]. The VQR and VRER SpCas9 variants in rice (*Oryza sativa*) and *Arabidopsis* [38–46] have been discovered as SpCas9-NG and xCas9-NG variants. SpCas9-NG outperforms xCas9 at target sites with NG PAM and AT-rich PAM sites [39,40,46]. Although some Cas9 variations may not perform well in crops, additional development may assist to restore editing activities at plant cell level to achieve successful genes editing [36,37].

It is possible that Cas9 orthologues from several prokaryotic species will be used to identify PAM in a different way (Table 1). SaCas9 is the preferred choice for virus-based delivery because of its smaller size when compared to SpCas9. To broaden the system's use, nicking enzymes [47], SaCas9 [48], and a KKJ variants [49] with a loosen PAM condition have been created. This enzyme has been discovered in various plants, including tobacco [50], *O. sativa* [51], and *A. thaliana* [52]. *Streptococcus thermophilus* Cas9 (St1Cas9) and St3Cas9 (ScCas9) are two additional Cas9 orthologues employed in human systems for genome editing [53], as are NmCas9 [54,55], FnCas9 and its RHA modifications [56] in *Treponema* bacteria [57,58]. The Cas9 strain identified in *Brevibacillus laterosporus* (BlatCas9) may alter the maize genome [59]. Cas9 orthologues distinct sgRNA structures enable them to be used for orthogonal genome editing [60].

2.3 CRISPR-Cas12a is a distinct CRISPR system

A class 2 type V endonuclease, Cas12a is a genome editing mechanism separate from Cas9 [61,62]. Cas12a may target T-rich areas because of the PAM requirement [63,64]. Cas12a only needs a short crRNA (40 nt), making it simple to synthesis, multiplex, and engineer Cas12a crRNA [65,66]. Cas12a has RNase activity along with DNA nuclease activities, allowing it to handle a CRISPR arrays for multiplexed genetic editing [66,67]. NHEJ-based gene insertion may also be promoted by Cas12a, which forms a DSB with staggered ends that are distant to the PAM site [68-70]. In some biological systems, Cas12a is thought to be more specific than wildtype SpCas9 [71-76].

Mutations in the RuvC domain of Cas12a's catalytic residues, on the other hand, prevent both DNA strands from being cleaved at target locations [69,70,73,77]. Mutations in the wedge and PI domains of Cas12a have been introduced to generate RR and RVR variants to broaden the target ranges [78,79] (Table 1). Orthologues from various bacteria species have been investigated in order to improve the applicability of Cas12a in gene editing [80,81] (Table 1).

2.4 DNA-targeting CRISPR nucleases

Cas12b, also known as C2c1, is a type V-B endonuclease of class 2. Cas12b creates staggered DSBs with 7-nt 5′ overhangs when it detects target sequences with a distal 5′-T-rich PAM sequence [82-84]. Cas12b, like Cas9, recognizes targets using crRNA and tracrRNA, which may be created as a sgRNA21. Cas12b, like Cas12a, has a REC lobe and a NUC lobe but no HNH domain [85]. *Alicyclobacillus acidoterrestris* Cas12b (AacCas12b) and *Bacillus thermoamylovorans* Cas12b (BthCas12b) were the first Cas12b nucleases to display editing ability in vitro, with a propensity for higher temperatures (50–52 °C) [21], which is not suitable for mammalian and plant genome editing. After investigating a number of Cas12b orthologues and demonstrating excellent editing activity throughout a wide temperature range, *Alicyclobacillus acidiphilus* Cas12b (AaCas12b) was discovered [86]. Later, *Bacillus hisashii* Cas12b was chosen from a pool of Cas12b orthologues and tweaked to increase activity at lower temperatures [87]. Cas12b orthologues with lower-temperature nuclease activity might be employed to change plant genomes.

Table 1. Variants and orthologues of Cas9 and Cas12a

Species	Cas	Size (amino acid)	PAM	Mutations	Features	Reference(s)
Rice	SpCas9 VQR	1372	NGA	D1135V/R1335Q/T1337R	Altered PAM	[31,32]
Arabidopsis and Rice	eSpCas9 (1.0)	1424	NGG	K810A/K1003A/R1060A	Enhanced specificity	[36,47]
Rice	eHypa-Cas9	1368	NGG	N692A/M694A/Q695A/H698A/K848A/K1003A/R1060A	Enhanced specificity	[78,79]
Arabidopsis, rice and citrus	SaCas9	1053	NNGRRT	-	Altered PAM and enhanced specificity	[80,81]
Arabidopsis	St1Cas9	1122	NNAGAAW	-	Altered PAM and enhanced specificity	[67]
Soybean, maize	LbCas12a	1228	TTTV	-	Altered PAM and enhanced specificity	[71,77]
Rice	LbCas12a RR	1228	LbCas12a RR	G532R/K595R	Altered PAM	[72]
Rice and Tobacco	FnCas12a	1300	TTV, TTTV and KYTV	-	Altered PAM	[82]

The CasX protein identified in hitherto uncultivated ambient microbial populations, targets double-stranded DNA with a 5′-TTNC PAM and is a member of the Cas family [18,20]. CasX is a Cas protein discovered in previously unknown environmental microbial species. CasX requires a guide RNA (gRNA) and a trace RNA (tracrRNA) to cleave, resulting in a spaced break with a 10-nt overhang [20]. CasX genes from *Deltaproteobacteria* (DpbCasX) and *Planctomycetes* (PlmCasX) have been shown to modify *E. coli* and human cells, respectively [20]. CasX20 can be silenced by making changes to the RuvC domain. CasY (also known as Cas12d) is a CRISPR system that has been found but is still poorly understood. If CasY recognises a 5′-TA PAM, it can cleave dsDNA [88]. Cas14 belongs to the nuclease family [19], and it can cleave single-stranded DNA (ssDNA) without PAM. According to some researchers [20], Cas14 can identify illnesses [19] and may also be used to interact with ssDNA viruses in plants.

2.5 CRISPR is being repurposed as a recruiting platform

It is possible to engineer DNA linkage in CRISPR systems, and this feature offers a robust foundation for recruiting functional domains to specific sections of the genome through protein synthesis or sgRNA–protein associations (Figure 1). Various functional domains could be introduced to the N or C terminals of the Cas protein, allowing it to perform a variety of functions [89]. SunTag exploits GCN4 epitopes coupled to dCas9 that are recognised by scFv antibodies tied to effectors in order to boost the concentration of functional domains.

Protein recruitment can also be accomplished by using self-complementing split green fluorescent protein (GFP) [90-93] (Figure 1a.). The recruitment of functional domains via RNA–protein interactions is a technique that varies from the protein fusion strategy (Figure 1b). Because Cas9 sgRNA is adaptable, structural modules such as the upper stem, 1st and 2nd hairpins, and 3'-end of the sgRNA may be modified without decreasing binding efficacy [94-96]. In the most widely used MCP– MS2 method, the 3′-end of sgRNA have been reported to be ornamented with up to 16 MS2 loops [97,98]. The CRISPR–Sirius system consists of an octet array of MS2 linked to the top stem of the sgRNA to produce the CRISPR–Sirius system, resulting in more stable secondary structures of the sgRNA [99,100]. We will further discuss the consequences of genome engineering in plants, including transcriptional control and base editing.

2.6 RNA-targeting CRISPR nucleases

Cas13 is a type VI CRISPR system that targets RNA [101] and the Cas systems that target DNA mentioned above. Unlike LshCas13a, LwaCas13a, and PspCas13b [102,103], no specific protospacer flanking sequence (PFS) requirement for *Leptotrichia wadei* Cas13a has been discovered (LwaCas13a). Cas13a, like Cas12, possesses RNase activity and can process crRNA arrays, which can be utilized to target several RNAs simultaneously [101,104]. When Cas13a demonstrates nonspecific RNase activity in vitro and bacteria, cleaving nontarget RNA after initially adhering to its target RNA [104], it piques our interest. The introduction of an alanine substitution into any of the five HENP catalytic sites may produce catalytically deactivated Cas13 or dCas13 [101]. Cas13 has been utilized in vivo to detect RNA102 and change base sequences [103]. It has also been used in the development of programmable RNA binding proteins. The LwaCas13a protein has been found to suppress gene expression in rice protoplasts [105]. LshCas13a has been shown in both [106] and *Arabidopsis* [107] to interfere with RNA virus replication.

3. FLEXIBLE AND MULTIPLEXED CRISPR EXPRESSION SYSTEMS

In plants expression of the two CRISPR components, Cas protein and gRNA, there are typically four choices to choose from, each with its own set of advantages and disadvantages. In response to the limitations of Pol III promoters, an alternative promoter system comprised of two Pol II promoters was developed [66,108,109]. This technique has resulted in a high amount of gRNA transcription. Pol II promoters may control both the spatial and temporal expression of a gene, and they frequently surpass Pol III promoters in terms of producing long transcripts with multiplexed gRNAs.

It is conceivable to achieve even higher simplifications by controlling Cas protein and gRNA with a single promoter [110–113]. During splicing, the intron region of a compact system that yields gRNAs is removed [113,114]. The gRNAs are expressed in a similar tight arrangement beyond the intron region. A bidirectional promoter with separate 3′-untranslated regions can be used to regulate Cas and gRNA synthesis independently of one another. Hopefully, these four expression methods will be studied further and applied to specific plant purposes in the future.

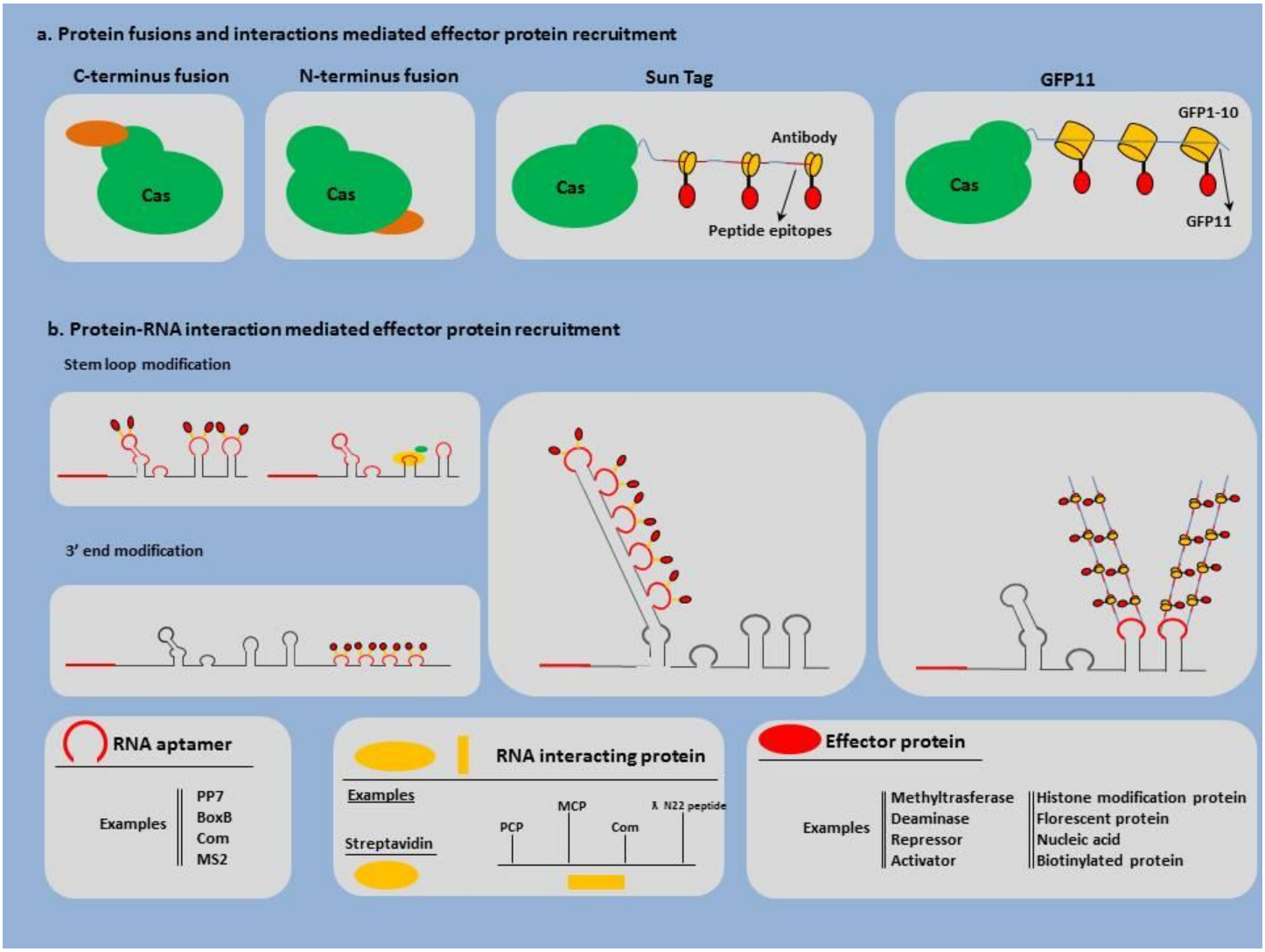

Fig. 1. Repurposing CRISPR as a recruiting platform

Another essential aspect of the CRISPR system is its flexibility to multiplexing, which is commonly done by delivering and producing many gRNAs in a single cell simultaneously. Using ribonucleoproteins (RNPs) or particle bombardment methods, it is possible to disseminate many gRNAs. When it comes to gRNA expression units, the simple method is to heap several units of expression, each of which consists of a promoter and a terminator [24,115–117]. This approach may produce large constructs with a high number of repeating components, increasing the complexity of cloning while decreasing transformation efficiency. As a result of this advancement, numerous alternative approaches for multiplexed genome editing in plants have been developed. Using the inherent CRISPR array expression mechanism [62,118] is one way to do this. This is the most straightforward conceivable expression system, and it is the most successful in plants using Cas9 [112,119] and Cas12a [70]. It is worth noting that CRISPR arrays can comprise multiple sgRNAs [112,119] or only crRNA, with the tracrRNA synthesised independently [4]. The ribozyme-gRNA-ribozyme system is another technique,

in which the gRNA is flanked by two different ribozyme sequences, one from the hammerhead virus (HH) and the other from the Hepatitis delta virus (HDV) [108].

When the RGR units are stacked in parallel, the synthesis of a single transcript proceeded by the accurate processing of every unit is conceivable, as confirmed in *O. sativa* [120]. Endogenous RNaseZ and RNaseP enzymes recognise and break off T-RNAs during transcription in the polycistronic T-RNA–gRNA system, allowing the synthesis of each mature gRNA [121]. Many species have profited from the use of this T-RNA system in combination with Cas9, including *Oryza sativa* [122], wheat [123], and *Zea mays* [124]. Using the efficient multiplexing technologies described earlier in the review, it is possible to target multiple genes and gene families at the same time [125].

4. ROLE OF BASE EDITORS IN PRECISE GENOME EDITING

Base editors contain cytidine and adenine base editors. The first generation of CBE was developed using the rAPOBEC1 enzyme [126,127], which was created by combining cytidine deaminase with the DNA editing enzyme dCas9 [128,129]. Cytosine deamination converts cytosine to uracil, which the cell replication machinery recognises as thymine, carrying in the C-T conversion [126]. Cytosine (C) deamination is a reaction that converts C to uracil (U). Apurinic/apyrimidinic (AP) sites are converted into uracil DNA glycosylase (UDG) sites, allowing C to U conversion to be reversed [130].

The second generation of CBE inhibits UDG by inserting a (U) DNA glycosylase inhibitor (UGI) to the first generation of CBE [126,131], resulting in a shift to the mismatch repair (MMR) pathway, which improves purity and editing efficiency. The employment of additional free or fused UGIs in the editing process may increase editing quality and efficiency, showing the utility of this method [132,133]. To boost base editing even further, the *Mubacteriophage Gam* protein has been linked to Gam, a protein that shields double-strand breaks from degradation and lowers indel generation during base editing [132]. Linker modification codon optimisation [134] and the inclusion of different nuclear localization sequences [135] have all been shown to increase base editing efficiency.

Many other forms of cytidine deaminases are used in research, including APOBEC1, AID, PmCDA1, and APOBEC3A [136,137]. APOBEC1, AID, and PmCDA1 are the most often used cytidine deaminases. Because no naturally occurring enzymes exist to deaminate adenine in DNA, the *E. coli* TadA, gene was modified and engineered to catalyze adenine deamination in soluble ssDNA [128].

Additionally, various versions of CBE and ABE have been designed to address the short-comings of modern base editors, hence increasing the toolbox available for base editing. A complete review of a wide range of basic editors, including their compositions, editing windows, types, and efficiency, was recently published [138,139]. The optimization and application of base editors have helped many plant species, with the cytidine base editor BE3 [140–144] presently being the most widely used [140–144]. In addition to these, several other basic editing approaches in plants have been demonstrated. When the APOBEC3A gene has been codon optimized for the cereal plant, wheat, rice, and potato can convert C to T successfully [145]. Furthermore, SaCas9 and SpCas9 variations are included in base editors to increase the targeting breadth in crops [55,140].

It has been used effectively used for base editing in rice, both through and without a UGI [146]. Target-AID, a CBE based on PmCDA1, was successfully edited in rice and tomato [147]. According to the findings of a different study, PmCDA1 demonstrated more significant rice base editing then rAPOBEC1111. ABEs have been found in protoplasts *of O. sativa* [140,148], *Arabidopsis*, and *B. napus* [149]. Aside from DNA base editing, RNA base editing is being investigated as a potential tool for posttranscriptional regulation. Antisense or Cas13-guided RNA base editors are currently in use [150], which employ the ADAR enzyme [151] or the Cas13 system [102,103] to convert A to inosine (I). In order to reduce off-target impacts [152], to allow additional flexible targeting areas, and to increase the amount of editing activity conducted [153], it is indeed necessary to improve the base editing optimization.

5. GENE INSERTION AND REPLACEMENT

Base editing does not allow for gene substitutions or targeted gene insertions, despite nucleotide conversions being achievable inside narrow areas of plant genetics. The most often used technique for adding foreign genes or modifying gene content is HDR, that is also referred to gene targeting (GT) [15]. The introduction of SSNs has made gene targeting much easier in higher plants than it was before [25,154,155]. DNA segments with specific sequences may be utilized as donor templates to repair DSBs caused by SSNs in plants. Following DSB introduction, gene replacement and insertion can be carried out by any of three ways: cNHEJ, MMEJ, or HDR [156]. Precision genome editing by HDR may be accomplished using Cas9 and Cas12a by generating DSBs and co-introducing repair templates. Because genome editing using cNHEJ and MMEJ for gene insertions and deletions is challenging, significant efforts have been made to improve HDR.

5.1 Improving HDR through donor design

With a donor format, you may have more control over the HDR process and your final edits. A variety of dsDNA delivery methods are available for donor templates, including PCR products, linearized or non-linearized plasmids, or even short strands of ssDNA. When it comes to knocking efficiency, PCR products and short-stranded DNA (ssDNA) beat out supercoiled plasmids in mammalian systems, whereas ssDNA causes less off-target integration than dsDNA [157]. Asymmetric Cas9 dissociation may be responsible for a bias toward using donor DNA that is complementary to the nontarget strand in Cas9-induced DSB repair by HDR with ssODN. Longer homology arms are often better for HDR [158]. If the donor template is symmetric or asymmetric, the knock-in efficiency may be affected in various ways. The effectiveness and zygosity of HDR outcomes may be affected by the gRNA-to-mutation distance, which is interesting [159]. To enhance HDR in plant cells, it will be good to evaluate donor types and the lengths and placements of homology arms.

Increasing the quantity of donor DNA supplied to the cell is another way to boost donor availability. Using *Agrobacterium*-mediated transformation, donor DNA fragments may be flanked by target sequences such that even though the donors

are incorporated into the genome, the nuclease can free them [160,161]. Using a circular plasmid is thought to be less efficient than this technique [162]. Particle bombardment may be used to supply additional free dsDNA or ssDNA donors together with CRISPR reagents [155,163]. Plant cells may be amplified using a geminivirus-based replicon system. Rolling-circle replication of the donor is possible using the geminivirus system, which was first given by *Agrobacterium* [154,164–166]. To distribute vast amounts of DNA donors, CRISPR delivery technologies will allow the use of nanoparticles [167,168].

HDR may be improved by recruiting donor DNA directly to the place of interest. One way to get the donor sequence into the nucleus is to make it easier for it to get there. The connection between a peptide nucleic acid and RNA adaptor allows for the addition of a NLS peptide to donors 5′ ends, increasing HDR efficiency and donor potency [169]. Streptavidin–biotin interactions may also be used to recruit biotinylated donor DNA to target locations, in which case an D1m aptamer is introduced to the sgRNA stem loop to attract streptavidin [97]. Another way to increase local donor availability is to combine avidin with Cas9, which will attract biotinylated donor DNA [170]. An improved approach for covalently attaching Cas9 to HUH endonucleases has recently been devised [171]. Covalently binding the ssODN to the HUH endonucleases may boost HDR efficiency by up to 40 times [171]. Although no equivalent experiments have been conducted in plant cells, these tactics for recruiting focused donors provide hope for plants to achieve high HDR efficiency. Though their efficiency is poor, it is interesting to note that RNA molecules strongly produced from a DNA template may also act as donors for HDR in yeast [172] and plants [173], prompting additional investigation into the use of RNA donors to enhance HDR in plants.

5.2 Improving HDR by modifying repair pathways

Key enzymes associated with this system might be used to improve HDR. RS-1 therapy and RAD51 mRNA insertion increased Cas9 and TALEN knock-in effectiveness in mouse embryos [174,175]. If RAD51 expression in plants can be altered, it may enhance the risk of DSB-induced HDR. Other DNA repair enzymes' activity can be altered to improve DSB-induced HDR efficiency [176,177].

Nickase-induced single-strand breaks promote HDR, and nCas9 [158] may be used to accelerate this process. There are no DSB-activated NHEJ pathways that might serve as nick creation substrates. Transpaired nicking is a novel approach that enables more effective homology-guided gene insertion than Cas9 nuclease [178], allowing for nicking of both the targeted genomic DNA and the donor plasmid. HDR generated by nCas9 has also been seen in plants [25].

Genetic or pharmacological reasons can potentially disrupt NHEJ mechanisms. RNA interference, SCR7, and viral proteins have all been shown to damage DNA LIGASE 4 in human cells, enhancing HDR efficiency [179,180]. NHEJ-mediated [181] repair was revealed to be hampered by DNA-dependent protein kinase catalytic subunit inhibitors, while HDR-mediated repair was increased. HDR increase in plants has been demonstrated in *Arabidopsis* ku70 and lig4 mutations [182] and *O. sativa* lig4 mutants [183]. Although utilizing NHEJ mutants is not optimal, increasing HDR by simultaneously inhibiting NHEJ genes via RNA interference or CRISPRi (CRISPR interference) is possible.

6. EPIGENOME EDITING AND TRANSCRIPTION REGULATION

CRISPR–Cas9 gene-editing technique has the prospective to be utilized to regulate gene expression by changing regulatory regions such as promoters, transcription factors, and enhancers. Editing regulatory elements, for example, may result in a broad change in the expression levels of a gene of interest in order to find and select new alleles, uncover the workings of gene regulatory elements, and modify QTLs [184,185]. Modifying splicing sites may also change the creation of distinct gene isoforms, allowing for the identification of splice variants and splicing processes [149,186,187] and the discovery of novel gene isoforms.

Furthermore, CRISPR–Cas might be programmed to regulate transcription. CRISPR–dCas9 gene suppression was first accomplished by reducing transcription machinery initial binding when targeting the promoter region or limiting RNA polymerase elongation when targeting the coding region [31]. It is feasible to increase the efficacy of repression in plant cells by utilising CRISPR to recruit transcriptional repressors such as SRDX [115,188]. The combination of these approaches is referred to as CRISPRi. Endogenous genes can be overexpressed in their natural environment due to CRISPR activator recruitment, a process known as "CRISPR activation."

It is feasible to significantly enhance transcriptional regulation by attaching several types of effector proteins to the target site using previously established protein recruitment strategies (Figure 1). VPR [189], synergistic activation mediator (SAM) [91,190], TREE systems [100,191], SunTag [89,192,193], to mention a few, have all been proven to be effective in activating VP64-p65AD-Rta [190]. The location of the target site also influences gene regulation [194]. Another method for further controlling expression is to use many gRNAs to target a single gene, therefore increasing the number of effector proteins accessible [195,196].

For the first time [197], gene suppression and activation may be accomplished simultaneously by utilising dead Cas activators that target various areas, allowing for the manipulation of more sophisticated pathways. As an extra benefit, because shorter gRNA may be bound by Cas9 or Cas12a without being cut, modifying the length of the gRNA provides a simple way to switch transcriptional regulation using Cas9 [198] or Cas12a [199].

In addition to editing epigenetic markers, transcription may be influenced by editing epigenetic markers. In order to modulate the epigenetic modifications associated with regulation, methyltransferases and acetylases may be utilized [200–203]. Plants have benefited from the usage of SunTag systems, which have been shown to successfully mutate and upregulate gene expression via DNA methylation [193] and demethylation [204].

It has been demonstrated that genome-wide hypermethylation may occur when utilising CRISPR-directed methyltransferases in crops [193] and mammalian systems [205], suggesting a significant difficulty when using this approach to modify the epigenome. CRISPR systems that target RNA, such as Cas13 and RNA-targeting Cas9s, can change gene expression levels after transcription. SpCas9 has also been found to target RNA when provided with a PAM sequence on a separate oligonucleotide in conjunction with a gRNA [206]. Including SpCas9, dCas9 in combination with

an RNase has been proven to degrade RNA [207]. Furthermore, RNA base editing allows for exact changes to RNA sequences [103]. Plant genome editing presents several challenges as well as opportunities [102].

7. PLANT GENOME EDITING CHALLENGES AND OPPORTUNITIES

The extensive CRISPR toolset, as well as sophisticated technologies, offer excellent platforms for the editing of plant genomes [66,115,188]. However, there are still obstacles to overcome in the deployment of genome editing technologies in plants. This is an issue that might be addressed with the advancement of new technology and scientific understanding.

7.1 Temperature sensitivity of Cas9 and Cas12a

In mammalian cells [208], *Danio rerio* [209], and crops [210,211], Cas12a and Cas9 need elevated temperatures to attain optimum editing efficiency. When repeated high temperature treatments are applied to *Arabidopsis*, the efficiency of Cas9 is dramatically increased [212]. Similarly, heat treatments in *Arabidopsis* (29 degrees Celsius) and maize (28 degrees Celsius) result in effective Cas12a editing [211]. Consequently, when using these CRISPR–Cas systems in plants, it is critical to consider the temperature of the environment.

7.2 Generation of transgene-free edited plants

When CRISPR is utilized for crop development, the lack of transgenes in final crops is critical to reducing regulatory load, promoting public acceptance, and reducing any ecological ramifications. Cross-pollination of CRISPR-encoding genes with herbicide sensitivity may aid in deleting CRISPR transgenes [213, 214]. Cross-pollination of CRISPR-encoding genes with herbicide sensitivity may also aid in eliminating CRISPR transgenes. Plants that are incompatible with themselves or are polyploid may find it much more challenging.

Transgene-free genome editing can also be achieved by creating mutant plants that have not been genetically manipulated with transgenes [215,216]. Plants can be genetically changed by inserting DNA or RNA encoding the CRISPR machinery and producing it transiently to effect modifications [217,218]. The regeneration of modified crops from protoplasts [74,219] on the other hand, poses a considerable obstacle and has the potential to induce unwanted somaclonal modifications as a result of the time-consuming tissue culture technique [220]. It has also been demonstrated that using particle bombardment to transfer RNPs within plant cells, which is commonly used for plant regeneration, is efficient [221,222]. RNPs may be inserted into plant zygotes generated by in-vitro fertilization and then used to regenerate adult plants without the requirement for selection [223]. A breakthrough in developing a transgenic killer CRISPR system that links suicide transposons to CRISPR constructs, therefore destroying all transgenes in the genome of modified T1 rice plants, has recently occurred [224].

7.3 Genome editing of polyploid plants

Multiallelic genetic editings have been accomplished in a variety of polyploid crop species, as well as model systems [225,226] and plants [221,227–236] among others. Enhanced oil quality [230], disease tolerance [233,237], and other beneficial agronomic features have been successfully introduced into crops by successful gene editing. The development of transgene-free modified polyploid crops via RNP injection [221] and selection-free techniques [234] have both been attempted, but both have been unsuccessful so far. Even though both techniques are labour-intensive and time-taking, they both have the prospective to speed applied research in crop breeding and breeding crops. Additionally, to gene knockout, precise gene editing using HDR in stable transgenic lines of polyploid plants remains a difficult task. Genome editing in polyploid plants, in addition to crop improvement, provides a platform for gene dosage experiments, in which altered lines with different copy numbers of functional genes may be generated in a single cycle of transformation and plant regeneration [225,232]. In this way, the link between genotype and phenotype may be studied in more detail quantitatively.

7.4 Germline editing by floral dip transformation

It is important to develop germline-edited plants in order to conduct downstream genetic and trait assessments. The delivery of CRISPR transgenes in plants, even in those that use the typical *Agrobacterium*-mediated floral dip method, such as *Arabidopsis*, remains difficult, not because of limited editing effectiveness in germlines but also due to the disconnection of editing effects in somatic and germinal cells [24,238-240]. *Agrobacterium*, which is assumed to be responsible for this delivery, is thought to carry T-DNA containing CRISPR into egg cells. Only if CRISPR edits the genome after *Agrobacterium* infestation but before the first embryogenic cell division will you be able to make germline changes [241–245]. A progressive transformation strategy was employed in conjunction with carefully picked transgenic lines generating large quantities of Cas9 in the germline to produce HDR in *Arabidopsis* with great success [246,247]. While the new oil crop camelina has similar obstacles to *Arabidopsis,* it is uncertain whether any of the strategies outlined above will enhance genome editing in this species [230].

7.5 Off-target effects in plants

The off-target effect of Cas proteins in gene therapy is a significant concern for CRISPR technology. Cas9-induced DSB creation has the potential to cause massive genomic alterations [248]. *Arabidopsis*, rice, cotton, and other plants whole-genome sequencing has been extensively utilized to find off-target effects [220,249,250]. Somaclonal variations are the most prevalent source of genomic alterations found in plants edited by Cas9 and Cas12a in their wild-type forms. CBEs, rather than ABEs, have been demonstrated to have significant genome-wide off-target effects in *O. sativa* [251-254], indicating that further screening and purifying selection may be necessary before they can be employed. Off-target effects

on transcriptome-wide RNA in human cells have also been demonstrated in recent studies [255,256] for both CBEs and ABEs. Base editors may be tailored to considerably decrease their RNA editing activity [257,258] (Table 1). In rice, the T-RNA–sgRNA processing system allows eSpCas9 and SpCas9–HF1 to preserve their on-target editing activity with increased specificity [259]. Rice was also utilized to demonstrate genome editing using two Cas9 variants, eHF1-Cas9 and eHypa-Cas9 [260]. When it comes to rice, xCas9 has recently been more specific than wild-type Cas9 [40]. However, because many high-fidelity SpCas9s in plants have fundamentally inferior nuclease capabilities, they may not be widely employed for plant genome editing until they are refined further. Off-target effects can also be reduced by generating more precise gRNA sequences. Off-target activity can be decreased by restricting the genome's exposure to CRISPR reagents, such as via transient expression and RNP transformation [261,262].

7.6 CRISPR and other innovative technologies are revolutionizing breeding

The utilization of tissue culture-based embryogenesis or organogenesis is required to create uniform plants with CRISPR-induced mutations in several major crops. CRISPR cannot be employed on plant varieties that are resistant to transformation [123,263].

One solution to this challenge is to express genes that enhance plant growth in order to increase plant regeneration and transformation. BBM and WUS2 transcription factors were overexpressed in resistant *Z. mays* lines, *S. bicolor*, *S. officinarum*, and *indica*, significantly improving transformation efficiency [264-267]. A unique and successful strategy to genome editing in many problematic plant species is the use of BBM, WUS, or similar factors (whether generated or provided ectopically) in conjunction with genome editing reagent administration to promote meristem tissue creation from somatic tissues.

In plant breeding, a desirable trait should be able to be handed on from generation to generation. Seed production will eventually lead to segregation in self-incompatible and hybrid crops that benefit from heterosis. CRISPR has enabled asexual seed propagation by creating the genotype by mitosis rather than meiosis and introducing haploid seeds [268,269].

In the future, CRISPR technology will greatly influence plant breeding. CRISPR has recently been demonstrated to be an effective method for producing novel plant varieties [270–272]. When paired with new ideas, CRISPR will accelerate plant breeding in the future. Dead Cas can be employed to mobilize Spo11, which causes DSBs for meiotic recombination and aids in the reduction of linkage drag during breeding [273]. Furthermore, CRISPR may be used to promote chromosomal shuffling in order to stack numerous elite alleles into one tightly related location, hence reducing elite allele segregation throughout the breeding process. Plant breeding will enter a new era thanks to CRISPR genome editing techniques, which cannot be accomplished by traditional breeding or genetic engineering.

8. CONCLUDING REMARKS AND FUTURE DIRECTIONS

CRISPR technology's fast progress will allow for a wide range of applications in plant biology. For example, the utilization of gRNA libraries will allow for large-scale screening of genes and regulatory elements to determine their activities. Pooled CRISPR libraries were utilized to create mutants for tomato gene families [274] and virtually all rice coding genes [275,276] using *Agrobacterium*-based T-DNA delivery, offering rich genetic resources for fundamental research and breeding. One gene at a time may be targeted by targeting regulatory regions to induce quantitative trait variation [184] or tilling the coding sequence for in planta gene evolution [277]. However, delivering CRISPR libraries to plants for use in the future is labor-intensive and time-consuming. We hope to be able to transport gRNA libraries directly into plant cells (such as protoplasts) in the future, which will allow us to conduct cell-based tests. This approach will allow for high-throughput genotype-phenotype correlation. Plants employ two organelles to store genetic information: mitochondria and plastids. Gene editing in organelles might alter metabolic pathways to obtain desired phenotypes. Mitochondria utilize both HDR and NHEJ pathways to repair DSBs [278].

Because mitochondria cannot repair double-strand breaks (DSBs) efficiently, mitochondrial DNA (mtDNA) is damaged and the heteroplasmic ratio shifts in mammalian cells [279,280]. TALENs and ZFNs have been employed to change the genome in mammalian mitochondria [281]. In the effort to install CRISPR in mitochondria [282], RNA import into mitochondria has been attempted multiple times but has met with limited success [283]. Cas9 and gRNA RNPs were shown to be preassembled in mitochondria before being delivered into mitochondria in one study [284]. Plastids, such as chloroplasts, are thought to be deficient in the NHEJ repair pathway. SSNs, such as CRISPR, might improve HDR in plastids [278] by introducing targeted DSBs, even though HDR in chloroplasts can be adjusted without the introduction of any SSNs [285]. Using any SSN, including CRISPR, it cannot change the genome in plant mitochondria or plastids.

CRISPR-created *Arabidopsis* mutants have opened up new avenues of investigation in this fascinating subject [286]. DNA repair choices, which are essential to the short-term effectiveness of genome editing reagents, are not yet strictly regulated. The target sequence context and the broken ends created by SSNs are strongly linked to the edits done by the cNHEJ and MMEJ pathways [287,288] (Figure 1). Machine learning and modelling approaches and probability may be used to anticipate deletion and insertion patterns. Prediction models and algorithms established in human cells may not be useful to plants because of the variations in DNA repair routes and preferences [289,290]. In order to better anticipate and regulate editing results, future research should investigate the DNA repair processes in plants [291-295].

New germplasm and perhaps new crops might be developed using CRISPR technology in the future, which would speed up research in the field of plant biology Food security will be improved as a result of this initiative's contribution to resolving agricultural, environmental, and ecological challenges.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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