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REVIEW

Recent advancements in CRISPR/Cas technology for accelerated crop improvement

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Abstract

Main conclusion **Precise genome engineering approaches could be perceived as a second paradigm for targeted trait improvement in crop plants, with the potential to overcome the constraints imposed by conventional CRISPR/Cas technology.**

Abstract The likelihood of reduced agricultural production due to highly turbulent climatic conditions increases as the global population expands. The second paradigm of stress-resilient crops with enhanced tolerance and increased productivity against various stresses is paramount to support global production and consumption equilibrium. Although traditional breeding approaches have substantially increased crop production and yield, effective strategies are anticipated to restore crop productivity even further in meeting the world's increasing food demands. CRISPR/Cas, which originated in prokaryotes, has surfaced as a coveted genome editing tool in recent decades, reshaping plant molecular biology in unprecedented ways and paving the way for engineering stress-tolerant crops. CRISPR/Cas is distinguished by its efficiency, high target specificity, and modularity, enables precise genetic modification of crop plants, allowing for the creation of allelic variations in the germplasm and the development of novel and more productive agricultural practices. Additionally, a slew of advanced biotechnologies premised on the CRISPR/Cas methodologies have augmented fundamental research and plant synthetic biology toolkits. Here, we describe gene editing tools, including CRISPR/Cas and its imitative tools, such as base and prime editing, multiplex genome editing, chromosome engineering followed by their implications in crop genetic improvement. Further, we comprehensively discuss the latest developments of CRISPR/Cas technology including CRISPR-mediated gene drive, tissue-specific genome editing, dCas9 mediated epigenetic modification and programmed self-elimination of transgenes in plants. Finally, we highlight the applicability and scope of advanced CRISPR-based techniques in crop genetic improvement.

Keywords Base editing · CRISPR/Cas · CRISPR-TSKO · Crop improvement · Gene drive · Prime editing · Transgene free

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Abbreviations

CRISPR/Cas9	Clustered regularly interspaced short palindromic repeat/CRISPR associated protein 9
gRNA	Guide RNA
ZFNs	Zinc finger nucleases
TALENs	Transcription activator like effector nucleases
PAM	Protospacer adjacent motif
DSBs	Double stranded breaks
Cpf1	CRISPR from *Prevotella* and *Francisella* 1
HDR	Homology directed repair
NHEJ	Non homologous end joining
crRNA	CRISPR RNA

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tracrRNA	Trans-activating crRNA
DSBs	Double-stranded breaks
InDels	Insertion and deletions
UDG	Uridine DNA glycosylase
CBE	Cytosine base editor
ABE	Adenine base editor
ecTadA	*Escherichia coli* TRNA-specific adenosine deaminase
UGI	UDG inhibitor
AP site	Apyrimidinic site
pegRNA	Prime editing guide RNA
MMLV	Moloney murine leukemia virus
ngRNA	Nicked guide RNA
PBS	Primer binding site
dCas9	Nuclease-dead Cas9
S gene	Susceptibility gene
ALS	Acetolactate synthase
nif	Nitrogen fixation genes
CRISPR-TSKO	CRISPR/Cas mediated tissue-specific knockout
DMC1	Disruption of meiotic control 1
EC promoter	Egg cell-specific promoter
CMS	Cytoplasmic male sterility
RNP complex	Ribonucleoprotein complex
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
TFs	Transcription factors

Introduction

Globally, agricultural production encounters unprecedented challenges. The world's population has soared to an astounding level, and it is projected to grow by 25% in the next 30 years, hitting 10 billion people (Hickey et al. 2019; Ray et al. 2012). In the backdrop of devastating environmental circumstances, dwindling farmlands and ground water depletion, plant-breeding technological breakthroughs are deemed necessary to enhance agricultural output and expedite sustainable agricultural development to support, nurture, and feed a burgeoning population. Crop improvement strategies include hybridization, mutation breeding, and transgenic breeding, which play a significant role in today's agriculture. However, introducing beneficial alleles via hybridization and expanding polymorphism via genetic recombination takes several years (Scheben et al. 2017). Due to decades of directed evolution through plant breeding, the natural diversity of many essential crops has been greatly reduced, making it more challenging to improve a range of important traits. Mutation breeding has expanded genetic diversity by creating arbitrary mutations using chemical mutagens (ethyl methanesulfonate, methyl methanesulfonate, sodium azide) or physical radiation (X-ray, gamma-ray, ion beams) (Pacher and Puchta, 2017). However, such approaches are constrained in terms of producing and screening huge numbers of mutants due to their probabilistic nature. Transgenic breeding, which introduces desired traits to elite cultivars by inserting foreign genes, has successfully bypassed the reproductive barrier. Unfortunately, the adoption of genetically modified crops is limited by time-consuming and expensive governmental approval processes raised concerns (Prado et al. 2014). As a result, the advantages of genetically modified traits are restricted to a few crop plants.

Since the first customized plant genome-editing experiment in tobacco (*Nicotiana tabacum*) protoplast (Paszkowski et al. 1988) and the revelation in 1993 that DNA double-strand breaks (DSBs) bolster gene targeting efficiency, scientists have focused on approaches to customize plant genome editing (Puchta et al. 1993). Zinc finger nucleases (ZFNs) were developed for application in tobacco in 2005 and used to augment a few plants' traits (Wright et al. 2005). The plant genome-editing toolkit was expanded in 2010 with transcription activator-like effector nucleases (TALENs) (Christian et al. 2010). While incorporating both devices has resulted in significant advances, each has its limitations and is not used in plants regularly. In 2012, Charpentier and Doudna reported in a study "that the Cas9 endonuclease can be programmed with guide RNA engineered as a single transcript to cleave any double-stranded DNA sequence" (Jinek et al. 2012). They received "The Nobel Prize in Chemistry, 2020" for discovering the CRISPR/Cas9 genetic scissors (Nobel prize 2020), which was a phenomenal scientific quantum leap that changed both fundamental and translational research in a variety of species, including plants (Nadakuduti and Enciso-Rodríguez 2021). Three distinct groups of researchers adapted the CRISPR methodology in 2013 for promising applications in the model (tobacco and *Arabidopsis thaliana*) and crop plants like wheat (*Triticum aestivum*) and rice (*Oryza sativa* L.) (Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013). In due course of time, continual advancements in CRISPR/Cas platforms, including CRISPR/Cpf1 or Cas12a (Zetsche et al. 2015) and nucleotide swapping methods for base editing (Zong et al. 2017; Shimatani et al. 2017), have rendered genome editing a highly embraced, cost-effective, and straightforward tailored genetic modification technique applicable to a wide range of crops. Productivity, quality, and various abiotic and biotic stress resilience are among the traits that have been improved via genome editing. In this review, we comprehensively discuss CRISPR/Cas technology, precision genome editing methodologies and their potential use in crop genetic improvement and latest applications of CRISPR technology such as tissue-specific genome editing, CRISPR mediated gene drive for crop plants, weed and pest control, transgene-free genome editing approaches, and promoter editing for controlled activation of target genes.

Plant genome editing with CRISPR technology

An overview of CRISPR/Cas genome editing

Depending on the structural and functional attributes of the Cas protein, the CRISPR/Cas system has been categorised into two classes (class I and class II), which are further subdivided into six types (types I–VI) and 33 subtypes (Yan et al. 2019; Makarova et al. 2020; Koonin et al. 2022). Class I comprises type I, III, and IV, whereas Class II encompasses type II, V, and VI (Mohanraju et al. 2016). Type I, II, and V systems recognize and cut DNA, while type VI and III tweak both DNA and RNA (Liu et al. 2020). Type IV CRISPR/Cas systems are distinguishable from other CRISPR/Cas systems by the presence of a discrete cas7-like gene (*csf2*), the absence of adaptation genes, the absence of an apparent nuclease, and the fact that they are typically found on plasmid (Koonin and Makarova 2017, 2019; Pinilla-Redondo et al. 2019). Because of these distinguishing features, it is difficult to predict how type IV systems would function (Taylor et al. 2021). Class 1 effector components are made up of numerous Cas proteins, including some that form crRNA-binding complexes (similar to the Cascade complex in type I systems) that facilitate pre-crRNA processing and interference with the involvement of other Cas proteins. Class 2 systems, on the other hand, include a single multidomain crRNA-binding protein (like Cas9 in type II systems) that integrates all interference-related functions as well as pre-crRNA processing in certain variants (Makarova et al. 2020). Because of the simple fundamental structure of effector complexes, Class 2 CRISPR/Cas systems have proved useful for creating a wide variety of genome editing devices.

The ease, efficiency, and sturdiness of the CRISPR/Cas9 methodology are ascribed to its extensive application in the field of genome editing. The CRISPR/Cas9 toolkit is characterized by two building blocks that may be delivered as a single plasmid: a bacterial Cas9 endonuclease protein and a specially engineered gRNA scaffold with a 20-bp sequence complementary to the target DNA sequence to be edited (called protospacer). The availability of the invariant protospacer adjacent motif (PAM) sequence 5′-NGG-3′ (Jinek et al. 2012) or 5′-NAG-3′ (Hsu et al. 2013) is essential for cleaving of the target DNA. Notably, numerous guide RNA (gRNA) spanning multiple genomic regions could be used concurrently to accomplish increased levels of multiplex genome editing without the requirement for other Cas9 proteins (Cong et al. 2013). Additionally, earlier in vitro and in vivo findings revealed that DNA CpG islands (CGIs) had no impact on Cas9 endonuclease activity (Hsu et al. 2013). Nevertheless, gRNA selectively binds to open chromatin regions and even off-target positions (Wu et al. 2014; Kuscu et al. 2014).

The most extensively utilized Cas nuclease is derived from the *Streptococcus pyogenes* (SpCas9) type II (class 2) CRISPR/Cas9 system. Furthermore, Cas9 orthologues from several species of bacteria have proven to be effective in augmenting the present CRISPR/Cas9 system. For example, the Cpf1 (CRISPR from *Prevotella* and *Francisella* 1) system (often referred as Cas12a), a new class II type V endonuclease, exhibits distinct biochemical characteristics that make it an enticing tool for genome engineering (Zetsche et al. 2015). The CRISPR/Cas12a system has steadily gained traction as a better alternative to CRISPR/Cas9 and a more flexible and potent genome-editing tool (Binmoon et al. 2018). Because Cpf1 endonuclease is smaller than Cas9, it requires shorter CRISPR RNA (crRNA) to work effectively. Cas12a makes use of the 5′-TTN/TTTN/TTTV-3′ (N = A/T/C/G; V = A/C/G) PAM motif, which extends the diversity of protospacers (Bandyopadhyay et al. 2020). Cas12a typically cleaves the target DNA region 18–23 bases downstream from the PAM sequence, resulting in 5–8 nucleotide 5′-overhangs as opposed to the blunt ends produced by Cas9 (Zetsche et al. 2015). Using the homology directed repair (HDR) process, these cohesive DNA ends can improve adding and replacing a specific DNA sequence at the cleaved region (Fonfara et al. 2016; Bandyopadhyay et al. 2020; Van Eck, 2020). During the processing of Cas12a-associated CRISPR repeats to mature crRNAs, the CRISPR/Cas12a system does not require trans-activating crRNA (tracrRNA) (Zetsche et al. 2015; Zhang et al. 2017). Because the CRISPR/Cas12a system has a shorter crRNA and a smaller Cas protein, it can accommodate larger vector loads and is, therefore, more appropriate for multiplexing (Zetsche et al. 2015, 2017; Wang et al. 2017). Unlike the CRISPR/Cas9 system, CRISPR/Cas12a is a single RNA-guided endonuclease that utilizes only one crRNA and no additional tracrRNA (Zetsche et al. 2015; Zaidi et al. 2017a, b; Mahfouz et al. 2017). It improves gene introduction at targeted locations in the genome, providing a highly desired alternative that is otherwise difficult to achieve in plants (Zaidi et al. 2017a, b). Cas12a has a longer protospacer unlike Cas9, rendering it more precise (Kleinstiver et al. 2016).

The major obstacle to employing the CRISPR/Cas9 system for genome editing is the likelihood of unanticipated off-target modifications, which has been described in multiple studies (Cong et al. 2013; Pattanayak et al. 2013; Hsu et al. 2013). Because of the short length of the sgRNA guide sequence (20 nt), targeting efficiency has become a major issue when using CRISPR/Cas9, and the system's off-target impacts have been systematically investigated (Pattanayak et al. 2013). The seed sequence, which is 8–12 nucleotides

proximal to the PAM sequence, governs targeting precision by connecting with the arginine-rich bridge helix (BH) within the Cas9 protein's recognition (REC) lobe; thus, selecting sites that are expected to be the most specific seed regions with the least number off-target disparities could be vital to enhancing on-target accuracy (Liu et al. 2016). Distal sequence from the PAM, on the other hand, is regarded to be less important for specificity, therefore changes are more likely to be accepted therein.

To modulate the precision of CRISPR/Cas9, a variety of approaches have been devised, where the design of the gRNA is one of the most significant and straightforward. Several techniques and systems biology tools have been developed to enable researchers to choose unique target sites in species with high-quality whole genome sequences (Belhaj et al, 2013; Doench et al. 2014). Using a 17-bp truncated gRNA or an extended gRNA with two additional guanidine residues at the 5′ end, non-target modifications could be minimized (Fu et al. 2014; Cho et al. 2014).

Mutation induction through CRIPSR/Cas mediated DNA double-strand breaks (DSBs)

The generation of DSBs at targeted regions is a defining hallmark of CRISPR/Cas genome editing, which may be used to initiate a multitude of genomic alterations via one of two primary DNA repair pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Symington and Gautier, 2011).

Repair of DSBs through non-homologous end joining (NHEJ) mechanism

A homologous repair template is unnecessary for the NHEJ repair pathway, which is triggered in most cell cycle events. As a result, introducing small indels at appropriate locations in target genes has become a common method of disrupting genes. NHEJ may also be leveraged to incorporate donor DNA sequences with little concern for homology, making it an effective gene stacking strategy for crop improvement. Like many other eukaryotes, plants undergo the NHEJ through two distinct routes (Salomon and Puchta 1998). Classical NHEJ (cNHEJ) entails simply end-to-end rejoining of DSBs, resulting in small Indels or exact restoration without the involvement of homologous sequences (Chang et al. 2017). In cNHEJ, the Ku70-Ku80 hetero-dimer detects and firmly attaches to the DSBs. Following that, multiple cNHEJ factors, namely DNA-PKcs, XRCC4-ligase IV-XLF (XRCC4-like factor, often termed as Cernunnos), and Artemis nuclease, are mobilized to the fragmented terminals, alongside DNA μ and γ, to accomplish the end processing and ligation (Chang et al. 2017; Ceccaldi et al. 2016).

The second pathway of NHEJ is known as alternative end joining (altEJ). PolyADP-ribose (PARP) proteins bind to the DSBs in this mechanism. When PARP attaches to the fragmented ends, the MRE11-RAD50-NBS1 (MRN) complex is recruited to commence end excision, which enables the formation of microhomology (MH) between the two strands of DNA having free ends (Chang et al. 2017; Ceccaldi et al. 2016). Finally, this process uses the short microhomologies (MHs) and the protein polymerase ɵ, to extensively eliminate and thereafter extend the fragmented DNA ends, culminating in substantial deletions and insertion of filler sequences, which occasionally give rise to chromosomal rearrangement and translocation (Chang et al. 2017; Ceccaldi et al. 2016; Schimmel et al. 2017; Wang and Xu, 2017).

Repair of DSBs through homology-directed repair (HDR) mechanism

In contrast to animal counterparts, the efficacy of HDR in plant somatic cells is remarkably limited (Szostak et al. 1983; Puchta et al. 1996), and NHEJ is strongly favored. Homology-directed repair (HDR), which primarily uses template DNA, is the second DNA repair mechanism. In somatic cells, homologous recombination (HR) is often used to restore DSBs, and in meiotically dividing cells, it is exploited to interchange genetic material between parental chromosomes (Malzahn et al. 2017). The synthesis-dependent strand annealing (SDSA) process (Steinert et al. 2016; Puchta, 2005) is the most prevalent HDR strategy in plants, and it fixes approximately all DSBs in somatic cells. As a consequence of a DSB, 3′ overhangs elongate from the breakage site. A D-loop is formed when a 5′ terminus invades the homologous strand. The outcome is a template or "donor" DNA strand that is perfectly inserted (Malzahn et al. 2017). Template DNA, which may obstruct HDRs, is not always accessible as sister chromatids or homologous chromosomes. Nevertheless, synthetic template DNA may be given artificially and deployed for inserting genes, substitution, or epitope/fluorescent labelling. HDR has a wide range of intriguing applications in fundamental and applied research. For instance, CRISPR/Cas mediated resistance (*R*) gene insertion in monocot plants through HDR has been reported by Vu and colleagues (Vu et al. 2020).

Novel technical breakthroughs in the field of CRISPR/Cas system

Base editing definition and types

Base editing is a genome engineering technique that produces accurate point mutations in DNA template or cellular RNA without introducing DSBs, involving a donor

DNA strand, or depending on cellular HDR (Komor et al. 2016; Gaudelli et al. 2017). Because base editors seldom induce DSBs, the generation of DSB-related byproducts is minimized (Gaudelli et al. 2017; Komor et al. 2017). Base editors (BEs), on the other hand, are a composition of a catalytically defective Cas nuclease and a deaminase that operates on single-stranded DNA (ssDNA) but never double-stranded DNA (dsDNA). When the gRNA binds to its targeted site in DNA, base coupling between the gRNA and target DNA sequence produces a tiny stretch of ssDNA to be displaced in a "R-loop" (Nishimasu et al. 2014). The deaminase enzymatic activity modifies the nucleotide bases within that ssDNA bubble. To boost efficacy in eukaryotes, the catalytically crippled nuclease creates a snip in the unmodified strand of DNA, compelling cells to resolve it utilizing the modified strand as a template (Komor et al. 2016; Gaudelli et al. 2017). Thus far, two types of base editors have been created: cytosine base editors (CBEs) and adenine base editors (ABEs), which are described in depth below.

Cytosine base editor (CBE)

CBE is comprised of a D10A-mutated Cas9 nickase (nCas9), which specifically inhibits RuvC (another of the two Cas9 nuclease subunits) and two enzymes: a uridine DNA glycosylase (UDG) inhibitor (UGI) and a cytidine deaminase. CBE inserts C:G > T:A base conversions into DNA locations that are identified by guide RNA (gRNA) (Komor et al. 2016) (Fig. 1 top panel). In the non-target strand, which is the single-strand DNA (ssDNA) segment of the r-loop formed by the nCas9 (D10A)-gRNA pair, the deaminase converts cytidines to uridines. At the same time, the UGI precludes UDG from deaminating cytidines to apyrimidinic (AP) sites. Once nCas9 (D10A) generates a cut on the target strand, the DNA mismatch repair mechanism (or other DNA repair processes) is prompted, which selectively converts the U:G discrepancy into the expected U:A, followed by a T:A product during DNA replication, resulting in a C:G > T:A base conversion. CBE techniques have been fine-tuned and improved in numerous plant species because this base editing technique delivers high-efficiency accurate modifications (Shimatani et al. 2017; Zong et al. 2018, 2017). Plant CBEs have been engineered to encompass many cytidine deaminase orthologues with distinct base editing characteristics. CBEs modelled on rat APOBEC1 modify cytosines in editing frames of about 7 nucleotides from position-3 to position-9 in the protospacer, typically favor TC over GC based on the sequence motif. CBEs designed on the cytidine deaminase 1 (CDA1) of *Petromyzon marinus* (lamprey eel) and activation-induced cytidine deaminase (AID) of humans, on the other hand, are significantly more effective in GC motifs in rice and do not appear to have a distinct motif selectivity (Shimatani et al. 2017; Ren et al. 2018). Human APOBEC3A (hAPOBEC3A)-based CBEs, like *P. marinus* CDA1-based and human AID-based CDEs, demonstrate significant base editing efficiency without motif bias, having base editing frames ranging from position 1 to position 17 within the protospacer (Zong et al. 2018). In rice seedlings, two new CBEs based on strategically engineered truncated human APOBEC3B (hAPOBEC3B) have recently exhibited incredible selectivity and precision (Jin et al. 2020). Eventually, Cas9 and Cas9 orthologues based on variations of the PAM sequence have been designed to overcome the targeting constraint imposed by the existing traditional PAM (NGG; where N can be any nucleotide) and augment the editing coverage of CBE processes in plants (Li et al. 2020a, b, c, d; Hua et al. 2019).

Adenine base editor (ABE)

ABEs contain adenosine deaminase as an effector that is combined with nCas9 (D10A), to widen base editing to incorporate A:T > G:C substitutions (Gaudelli et al. 2017). During DNA repair and replication, adenosine deaminase converts adenosines to inosines, which DNA polymerase recognises as guanosines (Fig. 1 bottom panel). Even while no native adenosine deaminase (adA) for deaminating ssDNA has been reported, one derived from *Escherichia coli* tRNA-specific adenosine deaminase (ecTadA) has been presented (Gaudelli et al. 2017).

In wheat, rice, rapeseed and *A. thaliana*, ABEs based on evolving ecTadA versions (ecTadA*) have been created (Li et al. 2018a, b; Kang et al. 2018). Nevertheless, they are ineffective at certain targets, and numerous approaches were being used to improve their editing performance in monocots, like incorporating three SV40 nuclear localization sequences to the C terminal of nCas9, creating improved gRNAs by customizing the gRNA scaffold (scRNA), and using a streamlined ecTadA* monomer version (Li et al. 2018a, b; Hua et al. 2020a, b). The RPS5A gene promoter, which drives the transcription of plant ABEs in *A. thaliana* and rapeseed, is highly effective than the constitutive 35S promoter or the YAO promoter (expressed at early developmental stage) (Li et al. 2010) utilized for Agrobacterium-mediated genetic transformation (Kang et al. 2018). Both the availability of a unique PAM sequence (NGG PAM for SpCas9) and the targeted base being within a restricted base editing range are required for effective base editing (Gaudelli et al. 2017; Komor et al. 2016). This specific PAM requirement is a major hurdle to base editing efficiency in plants. Nonetheless, numerous Cas proteins, as well as modified Cas9 proteins, have been shown to recognise various PAMs and can be leveraged as base editors (BEs) (Bharat et al. 2019). A number of recent studies have substantially broadened the PAM specificity of BEs using various modified Cas9 proteins; these systems are not limited by the presence

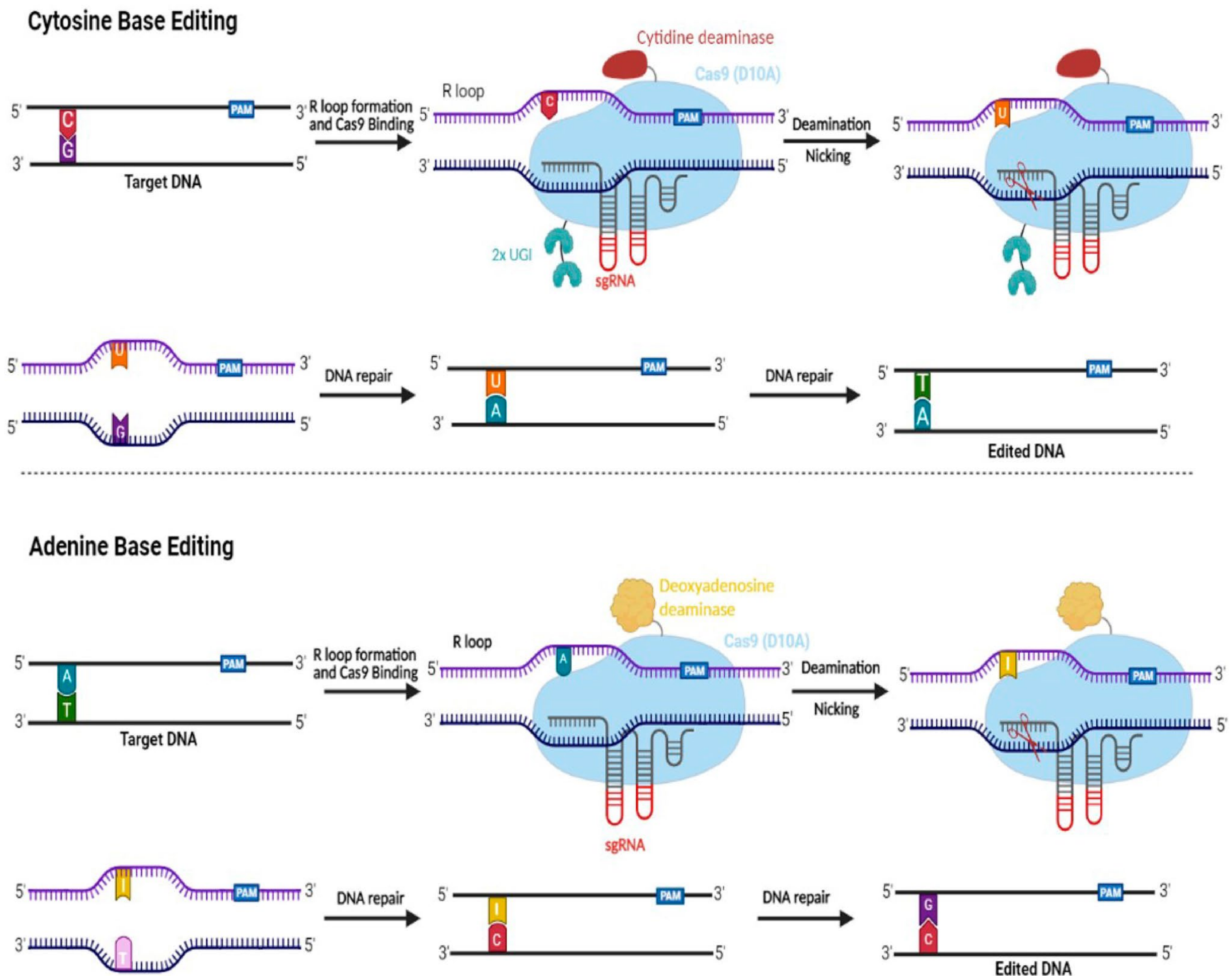

Fig. 1 Base Editing System. Cytosine base editing (top panel): a ssDNA-specific dCas9/nCas9 protein is attached to a cytidine deaminase that converts cytosine to uracil. An ssDNA R-loop is formed when Cas binds to a designed gRNA, and the linked cytidine deaminase converts accessible cytosines to uracil. Cas nicking of the unedited strand biases DNA repair toward restoring the unedited strand, whereas conjoined uracil glycosylase inhibitor (UGI, 1 or 2) obstructs uracil base excision repair, facilitating C•G-to-U•A modifications, which are changed to T•A base pairs after replication or additional DNA repair. Adenine base editing (bottom panel): a TadA monomer is attached to a dCas9/nCas9 protein. A customized gRNA binds to Cas, forming a ssDNA R-loop in which the linked TadA monomer converts accessible adenosines to inosine. Cas nicking of the unedited strand biases DNA repair toward fixing the unedited strand, encouraging A•T-to-I•C conversions, which are then transformed to G•C base pairs following replication or further DNA repair. A wild-type TadA monomer could also be fused, which can boost editing efficiency by increasing the production of dimeric TadA

of NGG PAMs, but can also recognise NG, GAG, CAG, AGG, GAA, GAT, NGA, and NGCG PAMs (Jin et al. 2019; Endo et al. 2019; Hua et al. 2019; Li et al. 2020a, b, c, d). Kleinstiver et al. (2015) modified CRISPR/Cas9 PAM specificity by introducing mutations into SpCas9 protein. Furthermore, Kim et al. 2017 used engineered SpCas9 proteins to create a series of BEs (VRER-BE3, VQR-BE3, SaKKH-BE3, and EQR-BE3) that could recognise NGCG, NGAN, NNNRRT, and NGAG PAMs, respectively. These tailored BEs can increase the effectiveness of base editing while also widening its scope to target other genomic locations in crop plants. Numerous ABE8 versions developed recently for human cells might significantly improve A > G base editing efficacy in plants (Richter et al. 2020; Gaudelli et al. 2020).

Cytosine-dependent DNA manipulation

In the cytosine base editing strategy, the UGI (uridine DNA glycosylase inhibitor) preserves uridine synthesized by deaminating cytidines and decreases the functioning of cellular uridine DNA glycosylase (UDG). On the other hand, over-expression of UDG activates base excision repair pathways, which result in the removal of uridine bases and the formation of apyrimidinic (AP) sites,

which are subsequently nicked by AP lyase. The uridine DNA glycosylase inhibitor (UGI) in CBEs preserves uridine synthesized by deaminating cytidines, inhibiting the functioning of cellular UDG. Cas9-generated nicks and DSBs would drive tailored deletion between deaminated cytidine and the Cas9 cleaving site. Based on this hypothesis, the APOBEC Cas9 fusion-mediated deletion system (AFIDs) consists of cytidine deaminase, UDG, Cas9, and AP lyase and results in selective removal of certain nucleotides-has been designed to orchestrate tailored deletions inside the protospacer. hAPOBEC3A and hAPOBEC3B are two different types of cytidine deaminase that have been used with AFIDS. hAPOBEC3A induces targeted DNA deletion from the deaminated cytidine base to the DSBs generated by the Cas9 protein.

Prime editing

Prime editing (PE), invariably known as the "search and replace" method, is a significant breakthrough in genome editing that has picked up steam in CRISPR research since 2019. This method makes it feasible to incorporate tailored insertions, deletions, and transversion of all four bases in DNA without inflicting double-strand breaks. A

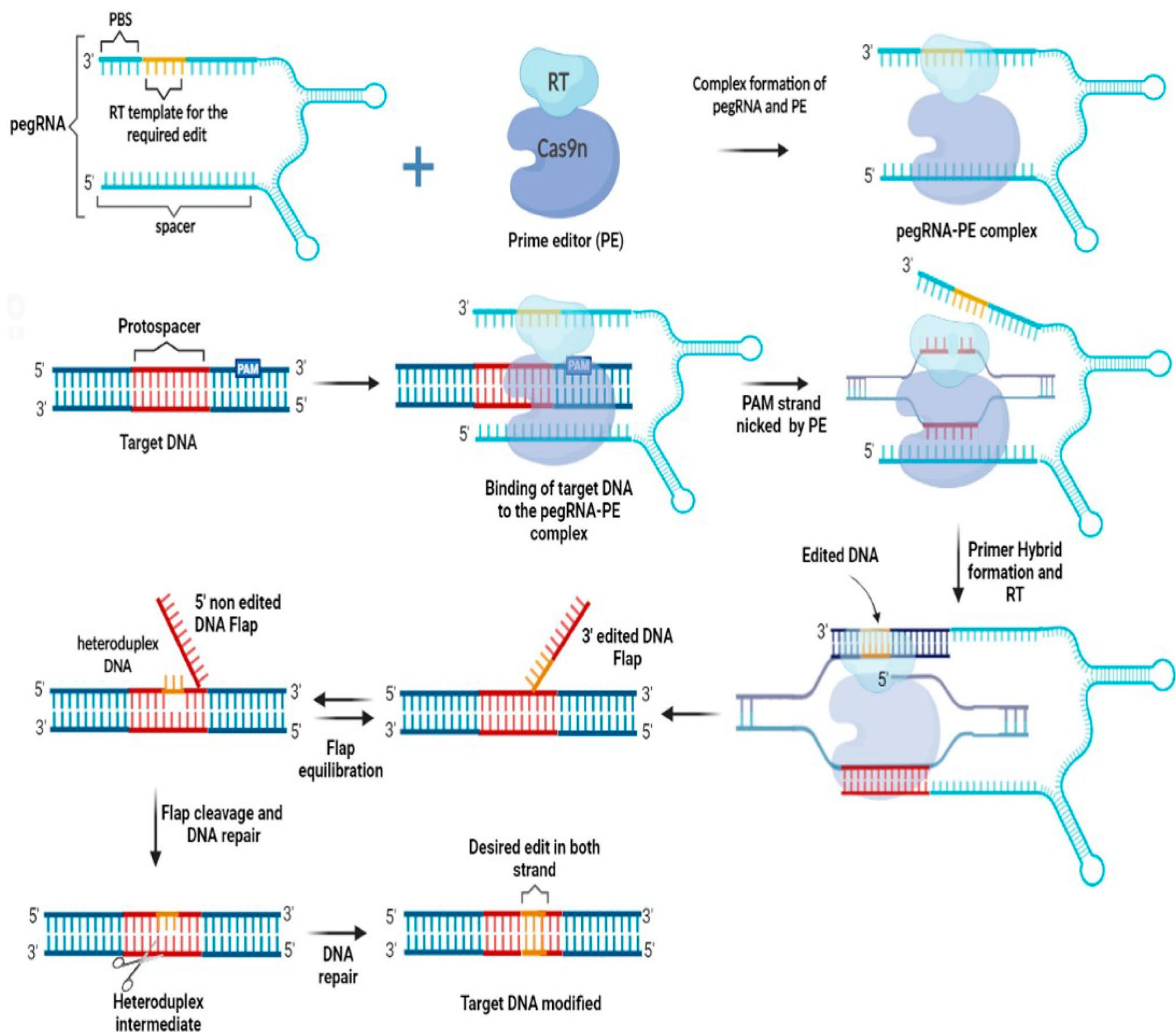

Fig. 2 Prime editing technology. A combination of nCas9 and reverse transcriptase, as well as a prime editing guide RNA, makes up the prime editor tool (pegRNA). At the 3′ end of the reverse transcriptase template, the pegRNA carries the required modifications. The primer-binding site (PBS) attaches to the nicked DNA strand, allowing the template to be reverse-transcribed into the appropriate DNA sequence. The altered nucleotides are then precisely put into the target location. The protospacer adjacent motif is abbreviated as PAM

customized prime editing guide RNA (pegRNA) and a prime editor (PE) make up the prime editing system. PE is often regarded as word processors capable of searching precise gene sequences and supplanting them (Fig. 2). In this approach, the gRNA scaffold is supplanted with a longer-than-usual gRNA known as pegRNA and a fusion protein composed of Cas9 H840A nickase, along with a specially designed RT enzyme that mediates the incorporation of new target sequences and bases into the genome. pegRNA is distinguished by the inclusion of a primer binding site (PBS) (~ 8–16 nucleotide), a reverse transcriptase (RT) template that encompasses the appropriate editing sequence to be reverse-transcribed into the designated target in the genome, and a spacer region complementary to one strand of DNA (Anzalone et al. 2019). PE harbors a modified Cas9 protein termed as Cas9 nickase (Cas9n), which can only cleave one strand of DNA. PE and pegRNA conjure up a complex that proceeds to the targeted DNA region steered by pegRNA upon continuous or transient expression of the prime editing construct. Cas9n produces a flap by nicking one DNA strand, which carries the PAM sequence at the specified location, and then binding the PBS of pegRNA to the snipped strand. The sequence details from the pegRNA are thereafter used by RT, an RNA-dependent DNA polymerase, to lengthen the snipped DNA strand, enabling the integration of the intended modification in one strand of the DNA. The clipped strand of DNA hooks onto the PBS and works as a primer to trigger reverse transcription, with the required changes from the RT template sequence being added to the strand of DNA that contains PAM. Upon RT-mediated incorporation of the defined modifications in the clipped DNA strand, the editing area comprises two duplicated single-stranded DNA flaps, 5′ DNA flap (does not include edits) and 3′ DNA flap (does contain edits). The cellular DNA repair mechanism performs the single-stranded DNA flaps are subsequently repaired and anchored into the genome. By the completion of the editing process, the clipped strand of DNA is swapped with the corrected strand by funneling the nucleotide sequence from the pegRNA, leading to the creation of heteroduplexes, which encompass one processed and one unprocessed DNA strand. The gRNA generates a second snip in the unprocessed DNA strand, which is subsequently corrected by extracting sequence details from the modified strand, culminating in incorporating of essential alterations in both strands of DNA.

In terms of editing efficiency, there have been four versions of PE [i.e. prime editing system 1 (PE1), PE2, PE3, and PE3b] created and classified thus far. Anzalone et al. (2019) used different PBS lengths (8–15 nt) to design the first-generation of PE (PE1) by fusing the WT Moloney murine leukemia virus (MMLV) RT to the C-terminus of the Cas9 (H840A) nickase. PE1 adeptly incorporated transversion point mutations with efficiency of up to 5.5% at five distinct genomic loci and tailored insertions/deletions with 4–17% magnitude. Different versions of M-MLV RT with alterations influencing DNA-RNA substrate affinity, processivity, thermostability and RNaseH activity have improved PE efficiency. Escalated RT activity at high temperatures was achieved by incorporating three modifications (D200N, L603W, and T330P), which further enhanced the frequency of transversion mutations (~ 6.8-fold compared to WT RT). Two further changes (T306K and W313) enhanced RT thermostability and binding to the template-PBS complex, culminating in a 1.3–3.0 fold improvement in editing frequency. Eventually, a second version PE (PE2) was designed as a pentamutant RT tethered to the nickase (Cas9(H840A)-MMLV RT (D200N/T306/W313F/T330P/L603W), which demonstrates 1.6- to 5.1-fold increase in performance of induced point mutations in contrast to PE1. Two important determinants have primarily governed the editing efficiency of PE2: first, which single-stranded DNA flap (edited or non-edited) would be paired with the unmodified DNA strand, and second, which single-stranded DNA flaps (edited or non-edited) would end up serving as a template for ensuring DNA repair or replacement. Although PE2's editing efficiency has substantially increased over PE1, the bases inserted by PE2 may still be erased owing to mismatch repair of the edited DNA strand. An ancillary gRNA is inserted to alleviate this problem during DNA-RNA heteroduplex resolution. The gRNA is introduced precisely to recognize the altered sequence integrated by pegRNA. In both animal and plant systems, it was reported that a nick in the unmodified strand of DNA increases the base editing efficiency (Komor et al. 2016; Lu and Zhu, 2017). To implement this strategy a nickase enzyme was leveraged which was guided by a gRNA with spacer sequences having similarity with only the edited strand of DNA. This modified PE system was denoted as PE3. PE3 demonstrated threefold enhancement in introducing point mutations (Anzalone et al. 2019). Subsequently, with a frequency of up to 33% (~ 7.9%), all the 12 possible transition and transversion point mutations were created, which is equivalent to the editing efficiency of existing base editing techniques such as cytidine and adenine base editing. Even after using the same protospacer sequence, the off-target frequency of PEs was noticeably reduced than that of the Cas9 enzyme. PE3 exclusively mutated three of the 16 reported Cas9 off-target regions for the HEK3, HEK4, EMX1, and FANFC loci in human cells (Anzalone et al. 2019). The following three phases of DNA hybridization occur during prime editing instances: between target DNA and pegRNA's spacer; between target DNA and pegRNA's PBS; and between target DNA and the altered DNA flap account for the enhanced selectivity. In the typical Cas9 mechanism, simply hybridization of target DNA and the protospacer from gRNA takes place. A nicked guide RNA (ngRNA) spacer is engineered to complement

the edited sequences in the PE3b mechanism so that it exclusively attaches to the altered DNA sequences, substantially improving editing fidelity (Anzalone et al. 2019).

A comparison among CRISPR-mediated HDR mechanism, base editing, and prime editing is shown in Table 1.

Multiplexing

Many gRNAs are delivered synchronously within a single CRISPR/Cas complex for multiplex genome engineering. The significance of delivering numerous gRNAs would be that the remodelling of various intended or unintended genomic locations might well be addressed at the same time via a single CRISPR complex delivered into plant cells. Architecting constructs that are an array of interlinked gene cassettes is being leveraged to achieve this kind of manipulation (Xie et al. 2015). Nonetheless, compared to those presented mostly during initial periods of CRISPR/Cas-driven genetic manipulation, there are more streamlined vector construction strategies available that were especially intended to minimise the sophistication of construct designing to target many regions in the genome (Cermak et al. 2017).

Unlike the CRISPR/Cas approach, which requires a number of components, each of which includes a polymerase III promoter, like U3 and U6, gRNA, and terminator, the streamlined multiplexing systems speed up the construction of gene editing structures to target different regions in the genome (Xie et al. 2015). All of this is on top of the fact that selectable marker and Cas genes require their respective plasmids.This form of assembly could be difficult based on number of intended sites chosen and, therefore, may result in hefty plasmids that are difficult to mobilize into plant cells using gene delivery techniques.

Several researchers overhauled the design fixate on single gRNA expression cassettes, resulting in substantial multiplex editing possibilities. Xie et al. (2015) and Cermak et al. (2017) described two initial multiplex editing vector frameworks. Xie et al. created a strategy that involved the transcription of several gRNAs from a single transcript. They exploited endogenous tRNA (transfer RNA) processing to execute this method, which involves cutting both terminals of tRNA precursors.They proposed that placing many tRNA-gRNAs structures in a vector's polycistronic expression module in a sequential order delivered gRNAs with the intended in vivo target sequences, culminating in Cas9 cleavage of diverse chromosomal targets in rice. In stable rice transgenic plants, authors recorded an efficiency of 100%. Cermak et al.'s approach further streamlined design for targeting numerous sites since both gRNAs and Cas9 are expressed by the same kind of polymerase promoter, polymerase II, which is more well defined than polymerase III promoters prevalent in plants.With these initial research, various multiplex editing vector strategies are being developed to edit several genes at the same time (Zhang et al. 2019a, b; Debbarma et al. 2019).

Using multiple gRNAs on the same or another T-DNA construct is easy and straightforward. Different promoters could be used to perform multiplex genomeediting, albeit an unique promoter is advantageous in expressing each gRNA and integrate the final construct into smaller vectors, as long as the mutations' efficiency is upheld. The gRNAs were spaced with tRNA genes, Cys4 recognition domains, and ribozyme sites via a polycistronic gene (Lowder et al. 2016). In *A. thaliana* and *Saccharomyces cerevisiae*(yeast), CRISPR/Cas9-mediated tailored modifications by the ribozyme-gRNA-ribozyme (RGR) mechanism has been described (Gao et al. 2014). In animal cells, the RGR system was utilised to characterise single promoter-regulated CRISPR/Cas9 induced modification (Gao et al. 2014; Yoshioka et al. 2015). It was reportedly shown that the hammerhead (HH) ribozyme, an RNA processing enzyme, may be used to create efficient gRNAs from CRISPR/Cas constructs in plants (Tang et al. 2016). For effective CRISPR/Cas9-driven tailored mutagenesis in plants, the Cys4 processing approach, which harnesses the CRISPR type III RNase, Cys4, to cleave the 20-bp sequences spanning the

Table 1 Comparison among CRISPR/Cas mediated HDR, base editing and prime editing technology

Characteristics	CRISPR-mediated HDR	Base editing	Prime editing
Constituents	sgRNA + Cas protein + template DNA	Base editor (nCas9 or dCas9 + deaminase) + sgRNA	nCas9 + reverse transcrites + pegRNA
Probable modifications	Capable of editing small target sequences or a few point mutations	Transition mutations (no transversion, insertion or deletions)	Capable of introducing All 12 types of point mutation into the genome
Design complexity	Editing efficiency greatly decreases with distance between PAM and targeted mutation site	Efficient for mutation 15 bases ± 2 from PAM only	Efficient for mutation 1 base to ≥ 30 bases from PAM
Pros	Biallellic changes obtained with efficiency	Higher editing efficiency, fewer indels byproduct	More targeting flexibility, greater editing precision
Cons	Chromatin structure affects efficiency	Bystander editing, genome wide off-targets	Potential transcriptional dysregulation

gRNAs, can be employed (Cermak et al. 2017). In comparison to gRNAs generated from distinct Pol promoters, many studies imply that tRNA and Cys4 approaches employing the cestrum yellow leaf curling virus (*CmYLCV*) promoter are unparalleled in generating mutations (Cermak et al. 2017). In tetraploid wheat genomes, multiplexed genome targeting in many loci has indeed been accomplished via homologous recombination (HR) exploiting virus-based CRISPR/Cas apparatus. To accomplish multiplex gene editing, researchers used the tobacco rattle virus (TRV) to transform tobacco plants with RNA and introduce numerous gRNAs expressing Cas9 (Ali et al. 2015). Multiplex genome editing in *A. thaliana*, maize (*Zea mays*), wheat (*T. aestivum*), and tobacco (*Nicotiana benthamiana*) utilising the CRISPR approach has highlighted the major benefits of RGENs over other configurable endonuclease. Furthermore, Cas12a's ability to manipulate its very own crRNA makes it an effective tool for engineering multiplex crop genomes.

Chromosome engineering

CRISPR/Cas approaches have predominantly been adopted to configure single or many genes by modifying open reading frames, regulatory domains, or remodelling genomes and epigenome architecture utilizing Cas-mediated systems (Schindele et al. 2020). With the recent advancement and creation of more robust gene editing, the attention has switched to more audacious strategies like manipulating meiotic recombination or large-scale chromosomal reorganisation. These developments might serve as a stepping stone for potential synthetic plant biology techniques. In 2018, for instance, CRISPR/Cas-driven chromosomal rearrangement was effectively employed to produce singleand two-chromosome yeast (Shao et al. 2018; Luo et al. 2018), respectively.

The initiation of crossing overs (COs) between homologous chromosomes, that intends to modulate meiotic recombination in particular genomic areas, is an apparent method for using tailored chromosomal structural changes in plant genetics.This method focuses on regulating CO rates and density.Homologous recombination (HR) unites homologous chromosomes for consecutive COs unless they are being parted again during meiosis (Lambing et al. 2017). Meiotic recombination makes it possible for unique allelic variations, which is why this phase is important in crop improvement.As a result, manipulating the frequency and distribution of COs has already been a paramount concern for plant breeders.Through anchoring the Spo11 protein, a member of the meiosis initiating machinery, to site specific DNA-binding domains, meiotic COs was triggered at typically low-recombination locations in the yeast *S.cerevisiae* in a unique manner (Sarno et al. 2017).Those comprised ZFNs, TALENs, and catalytically inactive Cas9 (dCas9) scaffolds.All of the methods were capable of increasing CO rates in typically low-recombination areas, yet only to a limited extent.Certain areas in the homologous chromosome were remained unreachable for selective DSB induction, suggesting that reliance on proteins from the normal meiosis machinery for DSB formation had limitations.Plant reproductive cells differentiates later in development, unlike other eukaryotic organisms, allowing for the transmission of somatically accumulated alterations (Wang and Ma, 2011). Filler Hayut et al. (2017) reported that leveraging a homologous chromosome as a template, localized DSBs may generate somatic HR.In their work, they used a visual marker gene (PSY1) and single-nucleotide polymorphisms (SNPs) to establish a selection strategy in tomato hybrids to determine HR between homologous chromosomes. Filler Hayut et al. harnessed CRISPR/Cas9 to trigger allele-specific DSBs to locate somatic HR incidences, notably gene conversions and a potential CO event. Nonetheless, these occurrences are unlikely to be passed down to the subsequent generations. This work proved that 'controlled COs' may be delivered using DSB-induced somatic HR.

Chromosome reorganisation may also be induced using the CRISPR/Cas system.Regulating these sorts of reconfigurations can be viewed as the next stage in plant breeding, since it will aid in the removal of current obstacles. When two DSBs are formed on the given chromosome at the same time, inversions or deletions may occur.If the DSBs are generated on two independent chromosomes, they can potentially cause translocations. Until now, most of these reconfigurations have been achieved on a limited scale, such as by flipping specific genes. In *A. thaliana*, CRISPR/Cas-driven rearrangement of up to 18 kb have been described using the Cas9 enzyme from *Staphylococcus aureus* in conjunction with an egg cell-specific promoter (Schmidt et al. 2019). In individual T2 lines, editing efficiency of up to 10% were achieved.Lately, in a follow-up investigation to the initially achieved 18 kb inversion (Schmidt et al. 2020), the heterochromatic knob in *A. thaliana* genotype Columbia has been adeptly inverted, utilising the same pairing of *S. aureus* Cas9 with an egg cell-specific promoter that had previously demonstrated toward being effective in creating heritable inversions (Schmidt et al. 2019). For the very first time, inversions in the Mb range could be generated and reliably transmitted in plants utilising the CRISPR/Cas mechanism.

There had previously been no evidence of CRISPR/Cas-mediated translocations in plants, as opposed to CRISPR/Cas-driven inversions.They were previously only been identified as a byproduct of CRISPR/Cas genome engineering (Peterson et al. 2016). CRISPR/Cas-mediated reciprocal chromosomal translocations in *A. thaliana* have been reportedly accomplished in a landmark work (Beying et al. 2020). The researchers have been able to create anapproach for creating heritable tailored translocations.Reciprocal translocations have been elicited between chromosome 1 and 2, as

well as chromosome 1 and 5, overall sizes of approximately 1 Mb and 0.5 Mb, correspondingly.Translocations, like inversions, contribute to diversification and genetic evolution (Lysak et al. 2006; Gabur et al. 2019). The deliberate induction of translocations presents additional possibility for genetic linkage disruption or fixing.Not only may harmful genes be dissociated in this fashion, but beneficial features can also be irreversibly appended to ensure widespread transmission.

Potential applications of CRISPR/Cas technologies in crop improvement

Application of base editing approaches in crop improvement

Crop improvement using traditional breeding or transgenic technologies does not compensate for climate change losses or fulfil global food demand. In addition to such strategies more advanced procedures are required at this time. Base editing offers the ability to enhance crop varieties in this respect by precisely targeting the single-nucleotide mutations that affect several critical agronomic parameters (Azameti and Dauda 2021). Base editing is employed for crop improvement because of its accurate targeting, convenience, and flexibility to multiplex. The numerous applications of base editing for crop improvement have gained a lot of consideration in recent years. In this paper, we also discuss how base editing may be used to fill in the gaps left by traditional genome editing for crop improvement. The application of the base editing technique for targeted trait improvement in different crop species is enlisted in Table 2.

Herbicide resistance

One of the most significant goals for modern agriculture is the establishment of herbicide-tolerant crops. *Acetolactate synthase* (*ALS*), a major enzyme in branched-chain amino acid biosynthesis, is a possible herbicide tolerance target. For weed management in rice, corn, wheat, and cotton crops, most commercial herbicides target the *Acetolactate synthase* (*ALS*) gene (Garcia et al. 2017). Herbicide resistance has been linked to a large number of SNPs thus far. Nucleotide changes in such SNPs might be employed in newly established CRISPR-mediated base editing techniques to create genetic variations in the crop germplasms with enhanced herbicide resistance.

Rice (O. sativa L.) It is advantageous to establish gain of function genome modified plants using base editing rather than producing indels since off-target mutations are linked with undesirable phenotypic alterations. Base editing made efforts were made in rice to generate plants with herbicide resistance. Numerous herbicide-resistant rice lines were created using the cytidine-base multiplex editing approach and the *ALS* gene as a target gene (Shimatani et al. 2017). A fusion construct consisting of dCas9 coupled to cytidine deaminase of *Petromyzon marinus* (*Pm*CDA1)1 and gRNAs of CRISPR/Cas9 and activation-induced cytidine deaminase (Target-AID) at specified genomic areas via gRNAs was utilised to target the *ALS* gene. The ALS gene's targeted codon was C287, where C287T base editing resulted in an A96V amino acid change that conferred resistance to the herbicide imazamox.The base-editing-mediated gene evolution (BEMGE) approach was used to modify the *OsALS1* gene (Kuang et al. 2020). For genome editing at the targeted region, the BEMGE approach employs cytidine and adenosine base editors, as well as a gRNA library. Base editing was prominent in the target gene, with a bias for C > T and G > A transitions directed by a pool of gRNAs rather than one gRNA. The altered plants demonstrated varying degrees of resistance to the herbicide bispyribac-sodium. In rice, the *OsALS* gene with the P171F mutation demonstrated high resistance to herbicide treatment. This principle may be applied to any endogenous gene. Another study employed a cytidine-base editor to generate various missense mutations in the P171 and/or G628 codons of the *OsALS* gene in rice for herbicide tolerance (Zhang et al. 2021a, b). In rice, the sites P171 and G628 were selected based on the amino acid sequence alignment of *OsALS* and *AtALS*. In contrast, in Arabidopsis, mutations P197 and G654 provide resistance to SU and IMI herbicides, respectively. The four types of missense mutations that demonstrated varying herbicide tolerance levels at the P171 codon in rice were P171S, P171A, P171Y, and P171F.

Wheat (T. aestivum) Due to the polyploidy of wheat, inducing point mutations for conferring herbicide resistance through traditional breeding is complex. Transgene-free wheat lines with herbicide resistance were created (Zhang et al. 2019a, b). *Acetolactate synthase 1* (*ALS1*) and *acetyl-coenzyme A carboxylase* (*ACC*) were the genes targeted to develop herbicide resistance. The wheat lines created were resistant to various herbicides, including nicosulfuron, an SU herbicide.

Maize (Z. mays) Svitashev et al. (2015) found that replacing P165 with Ser in maize *ALS2* using single-stranded oligonucleotides or double-stranded DNA plasmids as repair templates culminated in the establishment of maize plants resistant to chlorsulfuron.

Soybean (Glycine max) CRISPR/Cas9-driven gene editing has been leveraged to effectively insert a P178S mutation in *Acetolactate Synthase 1* (*ALS1*), a crucial enzyme

Table 2 List of genes targeted by base editing techniques in different crop species

Crops	Target gene	Findings	References
Cytosine base editing			
Arabidopsis (*Arabidopsis thaliana*)	*eIF4E1*	Resistance to potyviruses	Bastet et al.(2019)
Watermelon (*Citrullus lanatus*)	*Acetolactate synthase (ALS)*	Herbicide resistance	Tian et al. (2018)
Rice (*Oryza sativa* L.)	*C287*	Herbicide resistance	Shimatani et al. (2017)
	Pi-d2	Blast resistance	Ren et al. (2018)
	OsPDS, OsSBEIIb	Nutritional improvement	Li et al. (2017a, b)
	NRT1.1B and *SLR1*	Enhanced nitrogen use efficiency	Lu and Zhu (2017)
	OsRLCK185, *OsCERK1*	Defence response	Ren et al. (2018)
	SNB, *PMS3*	For functional genomics research and molecular breeding in crops	Hua et al.(2019)
Rapeseed (*Brassica napus)*	*ALS*, *RGA* and *IAA7*	Herbicide resistance	Cheng et al.(2021)
Cotton (*Gossypium hirsutum)*	*GhCLA* and *GhPEBP*	For functional genomics research and molecular breeding in crops	Qin et al.(2020)
Maize (*Zea mays*)	*ZmCENH3* CBE	Chromosomal segregation	Zong et al. (2017)
	Acetolactate synthase (ALS)	Herbicide resistance	Li et al. (2020a, b, c, d)
Potato (*Solanum tuberosum*)	*StALS*, *StGBSS*	Herbicide resistance, Starch synthesis	Zong et al. (2018)
	SLALS1	Herbicide resistance	Veillet et al. (2019)
Tomato (*Solanum lycopersicum*)	*SLALS1*	Herbicide resistance	Veillet et al. (2019)
Wheat (*Triticum aestivum*)	*TaLOX2*	Lipid metabolism	Zong et al. (2017)
Adenine base editing			
Arabidopsis (*Arabidopsis thaliana*)	*AtALS, AtPDS, AtFT, AtLFY*	Plant ABE application	Kang et al.(2018)
Rice (*Oryza sativa* L.)	*OsACC-T1*	Herbicide resistance	Li et al. (2018a, b)
	SLR1; *OsSPL14*	Della protein for plant height; ABE plant architecture and grain yield	Hua et al. (2018)
	OsMPK6	Pathogen-responsive gene	Yan et al. (2018)
	Wx ABE; *GL2*/*OsGRF4*, *OsGRF3*	Rice amylose synthesis; Grain size and yield	Hao et al. (2019)
	OsWaxy	Testing of multiple cas9 Variants	Zeng et al. (2020)
	Multiple genes	For functional genomics research and molecular breeding in crops	Hua et al. (2020a, b)
	OsALS1,*OsGS1OsTubA2* and *OsACC*	Herbicide resistance and Multiplex gene editing	Yan et al. (2021)
	OsTubA2	Herbicide resistance	Liu et al. (2021)
	Multiple genes	For functional genomics research and molecular breeding in crops	Hua et al. (2019)
	Waxy (*Wx*)	Lowering Amylose content	Monsur et al. (2021)
Wheat (*Triticum aestivum*)	*TaDEP1*, *TaGW2*, *TaEPSPS*	Panicle length, grain weight and Herbicide resistance	Li et al. (2018a, b)
Rapeseed (*Brassicca napus*)	*BnALS*, *BnPDS*	Plant ABE application	Kang et al. (2018)

in the biosynthetic pathways of branched-chain amino acids and a primary target for important herbicides like chlorsulfuron and bispyribac sodium (BS) (Mazur et al. 1987).

Plant architecture

The newly designed base editors allow for the conversion of cytidine (C) to thymidine (T) or guanine (G) to adenine (A) with no need for DSB or HDR (First developed by David

R. Liu and his colleagues). ABE-P1 (adenine base editor plant version 1) was utilised to target the *OsSPL14* gene in rice for optimal plant architecture and increased grain yield (Hua et al. 2018). ABE-P1 is a highly effective base editor made up of bacterial tRNA adenine deaminase TadA and DNA as a substrate for programmed A.T to G.C conversion. The ABE-P1 induced a point mutation at the binding site of the microRNA *OsmiR156*, which increased grain yield. The Adenine-base editor technology outperformed the Cytidine-base editor or HDR-mediated targeted genome editing systems. Furthermore, this technique might be employed for multiplexing with excellent efficiency, allowing for many genes to be edited to regulate diverse agronomic parameters.

Nutritional improvement

The rat cytidine deaminase enzyme APOBEC1 was used to create a cytidine-base editor for manipulating two agronomically significant genes in rice, *NRT1.1B* and *SLR1* (Lu and Zhu 2017). The two amino acids were targeted because the C to T alteration (Thr327Met) in the nitrogen transporter gene *NRT1.1B* results in increased nitrogen use efficiency, whilst the base replacement (Ser97Leu) in the TVHYNP motif results in decreased plant height (Hu et al. 2015; Ikeda et al. 2001; Asano et al. 2009). A dominant mutation in the *SRL1* gene resulted in dwarf plants, whereas a C to G substitution in the *NRT1.1B* gene resulted in a chimeric mutant. These findings indicated the possibility of employing the technique in additional crop species for improvement.

Application of prime editing for crop improvement

Prime editing is a cutting-edge genome editing approach created in human cells (Anzalone et al. 2019) that potentially insert indels and all twelve conceivable base-to-base conversions, encompassing transitions and transversions. This system has now been exploited to generate desirable mutations in plant cells, including rice, wheat, tomato, potato, etc. Although prime editing is still in its nascent stages, it is a speedy and powerful technique for precision plant genome editing. Several researchers have applied this robust technology to bring desirable mutations in targeted genes in crop plants.

Tang et al. (2020) demonstrated the use of three versions of plant prime edits (PEs) viz., PE2 (a second gRNA is not used to nick the non-edited strand), PE3 (the ngRNA nicks the non-editing strand within 100 bp from the editing site) and PE3b (gRNA was designed to match only the edited strand and not the wild-type sequence) for precise editing at many endogenous genes in rice (*OsALS, OsKO2, OsDEP1,* and *OsPDS*). SNPs and indels have been successfully introduced at these sites at variable frequencies. Similarly, the plant prime editing system was established in rice in a study by Li et al. (2020a, b, c, d).They successfully achieved homozygous and heterozygous stable lines with the desired edits in exogenous *hptII* gene (confer hygromycin resistance) and endogenous genes, *5-enolpyruvylshikimate-3-phosphate synthase* gene (*OsEPSPS*). Lin et al. (2020) reported that prime editing could efficiently produce a range of edits at genomic sites in rice and wheat through transversions, point mutations, insertions, mixtures of different substitutions and deletions. After optimizing of codon, promoter, and editing-condition, a frequency of up to 21.8% of regenerated prime-edited rice plants was obtained. Xu et al. (2020a) demonstrated the use of prime editing in T_0 lines of rice by introducing a varied number of single or multiple nucleotide edits at several target sites of three rice genes viz.*OsALS1* and *OsACC* which confers herbicide tolerance and *OsDEP1* that is involved in nitrogen-use efficiency. The prime-edited rice plants were obtained at frequencies of about 26% through different targets showed a variation in editing efficiency. Xu et al. (2020b) used the plant prime editor 2 (pPE2) system to induce targeted editing at different genome sites of rice. They targeted the *ACC1*, *PDS1* and *WX1* genes in rice and the generated mutants were obtained with 0%-31.3% frequency. This was conclusive of the fact that the editing efficiency may vary in different genomic sites and is dependent on the structure of prime-editing guide RNAs. Hua et al. (2020a, b) used the prime editing tool to precisely edit the transgenic reporter 'EGFP' and endogenous gene *ALS* and *APO1* in rice, although no mutation was reported at the latter gene. A similar study was carried out by Butt et al. (2020) to engineer herbicide resistance in rice through prime editing of the *acetolactate synthase* (*OsALS1*) gene. They also targeted two transcription factors *OsIPA* (*IDEAL PLANT ARCHITECTURE 1*) and *OsTB1* (*TEOSINTE BRANCHED 1*) using the PE3 strategy. The prime-editing efficiencies in the studies mentioned above varied from 2.22% to 31.3%. However, Jiang et al. (2020) achieved a much higher prime-editing efficiency in maize transgenic lines. The S621I and W542L mutations in *ZmALS1* and/or *ZmALS2* exhibited an editing efficiency of 53.2% and 6.5% respectively in their attempt to generate maize lines resistant to herbicide.

Although prime editor seems efficient and well-adapted in monocot plants (Lin et al. 2020), its application in dicotyledonous plants is still limited and has immense scope for further improvement. A successful application of prime editors through codon and promoter optimization was reported in tomato. Lu et al. (2021) optimized a primer editor pCXPE03 and used it to edit three endogenous genes in tomato viz. *GAI*, *ALS2* and *PDS1*. However, sequencing results detected the desired edits at only two genes, multi-nucleotide substitutions in *ALS2* and CG-insertion in *PDS1*. Their editing results proposed the possibility of using pCXPE03 for prime editing in

tomato. In yet another study, prime editing was also successfully demonstrated by Veillet et al. (2020) in tetraploid potato (*Solanum tuberosum*) which is highly heterozygous in nature. They used a dicot codon-optimized prime editor to simultaneously introduce nucleotide transitions and transversions in the *StALS1* gene. Though they used both the PPE2 and PPE3 strategy to induce mutation, Sanger analysis exhibited three expected substitutions at the *StALS1* target locus using the PPE2 approach. Further improvement in the prime editing technology, through an increased variation in the targeted base substitutions, can have immense potential for precision breeding in polyploid and vegetatively propagated crops.

The prime editing system is in its early stage of development and its application for accelerating crop improvement through genetic manipulation has immense potential. Precision crop breeding through prime editing can provide new opportunities for development of cultivars that are resilient to various biotic and abiotic stresses. Sometimes missense mutations that arise due to single nucleotide polymorphism in different alleles result in pseudogenes (Yin and Qiu. 2019), which can render plants susceptible to various diseases. Similarly, plants' susceptibility (*S*) genes are potential targets for phytopathogens for their proliferation (Zaidi et al. 2018). Recovering the functions of pseudogenes and inactivation of the *S* genes through prime editing can consequently provide specific disease resistance. In response to invading pathogens, the plant defence employ intracellular nucleotide-binding leucine-rich repeat (NLR) immune receptors that play critical roles in effector-triggered immunity (ETI) (Seo et al. 2016). Manipulation in such kind of receptor genes via prime editing can generate plants with enhanced disease resistance. Moreover, the plant-microbe interaction is a crucial phenomenon that governs overall plant health. Targeting the genes that regulate the signaling pathways and are beneficial to the plants, during plant-microbe interaction can be achieved through prime editing.

Apart from biotic stress tolerance, prime editing could also be used to generate abiotic stress tolerance (Veillet et al. 2020; Xu et al. 2020a; Lu et al. 2021). However, there is tremendous scope of improvement in targeting cis-regulatory elements including transcription factors and other regulatory proteins via prime editing (Hassan et al. 2020) for combating stress like drought, salinity, temperature extremes, etc.

The scientific community will likely witness rapid progress in exploiting this novel technology for crop improvement. However, many challenges need to be addressed, such as low editing efficiency, species-specificity for pegRNA designing, and limited editing window to utilize the potential of prime editing for crop improvement.

Applications of CRISPR/Cas technology in crop breeding

While CRISPR/Cas technology has demonstrated a high potential to strengthen crops, embedding it with traditional breeding procedures might promote agricultural productivity even more. CRISPR/Cas has lately been exploited in a wide range of innovative breeding techniques that address reproduction-related genetic traits.

Heterosis

Despite the fact that approaches for producing hybrid seeds based on male-sterile lines are extensively documented, they are nonetheless costly and time-consuming in certain crops. For superior hybrid backgrounds, triggering apomixis, a naturally arising asexual reproduction process, could be an alternate method. Triple mutants of the *PAIR1*, *REC8*, and *OSD1* (genes that culminate in the abrogation of meiotic recombination, dissociation of sister chromatids in the very first meiotic division, as well as avoiding the second meiotic division, respectively) produced by CRISPR/Cas technique in mitosis instead of meiosis genotypes (*MiMe*) of rice and *Arabidopsis* resulted in the generation of clonal diploid gametes and tetraploid seeds. Parthenogenesis could be elicited by aberrant expression of *BABY BOOM 1 (BBM1)* that stimulates embryogenesis in *MiMe* rice egg cells, resulting in offspring's genetic makeup similar to the maternal progenitors. Even if the low apomixis induction percentage and limited fertilization of these synthetic apomictic breeding lines make them unsuitable for mass-production of hybrid varieties, they can be employed deliberately in plants such as vegetables and meadows where seed dissemination is minimal.

Male sterility

Hybrid vigor has indeed been exhaustively applied in crop genetic improvement to maximize production and improve quality. Nonetheless, to minimize homozygous seeds, the self-pollination of the female progenitor must be bypassed when producing hybrid seeds economically. The most prolific and pragmatic solution to this hurdle would be to generate male sterility in maternal lines. CRISPR/Cas-mediated genetic engineering allows transformable lines to develop male sterility rapidly. Male sterility was achieved in wheat (*T. aestivum*) cultivars by modifying the genes *male sterility 1* (*Ms1*) and *Ms45*, which encode a glycosylphophatidylinositol-anchored lipid transfer protein and a strictosidine synthase-like enzyme, respectively (Khanday et al. 2019; Wang et al. 2019). Modifying of a potential strictosidine synthase gene resulted in creating a male-sterile tomato genotype (Du et al. 2020). Such approaches have

also been extrapolated to other organisms as well. In addition, temperature-sensitive and photoperiod-sensitive genic male sterile genotypes have already been generated in rice and maize, accordingly, by deleting *thermosensitive genic male-sterile 5* (Li et al. 2017a, b) and *carbon starved anther* (Gu et al. 2019).

Self-incompatibility

The paucity of inbred lines in certain crops, like potato, has impeded genetic advancement owing to its inherent self-incompatibility. Self-compatible potato cultivars were developed via CRISPR/Cas-mediated editing of *S-RNase*, a co-dominant gene essential for gametophytic self-incompatibility in the Solanaceae (Qin et al. 2018). Inhibition of M-locus kinase activity and S-receptor kinase, respectively, in rapeseed (Chen et al. 2019) and cabbage (Ma et al. 2019), has also been demonstrated to surpass sporophytic self-incompatibility. This technique pledges to surmount interspecific reproductive obstacles and minimize the requirement for pollinizers in fruit trees, in addition to lowering heterozygosity. Furthermore, by causing mutations in genes like farnesyl pyrophosphate synthase 2, CRISPR/Cas can be employed to garner highly successful hybrid breeding techniques and induce parthenocarpy in citrus crops, recovering self-incompatibility (Qin et al. 2018).

Next generation CRISPR-mediated genome editing

Tissue-specific CRISPR genome editing

Since its advent, CRISPR/Cas technique has been increasingly leveraged to survey gene function by producing long-lasting and heritable alterations. Nevertheless, because 10% of *A. thaliana* genes (out of approximately 25,000) are necessary for growth and development, this technique gives rise in significant pleiotropic mutations or even mortality (Lloyd et al. 2015). This presents substantial impediments to the functional study of essential genes. As a result, a toolset that can be fully exploited for functional analysis of gene without dropping dead or significantly impacting the plant is anticipated. Such constraint can be subverted with the recently entrenched CRISPR-TSKO (tissue-specific knockout) technology (Decaestecker et al. 2019). Grounded on updated GreenGate vectors and Golden Gate cloning Decaestecker et al. (2019), designed a scalable, robust, and low-cost vector toolbox. The versatility allows for the use of several Cas9 or nuclease, each regulated by a unique promoter. Researchers can use this toolset to generate tissue-specific mutations in a number of important genes without compromising plant development. For example, via CRISPR-TSKO, the stomatal gene *PHYTOENE DESATURASE* (*PDS3*) was modified, culminating in viable plants with no chloroplasts in the stomatal cells (Decaestecker et al. 2019). The stomata-specific promoters *pTMM* (*TOO MANY MOUTHS*) and *pFAMA* were used to regulate Cas9 expression. These plants could be utilized to better understand the significance of chloroplasts in stomatal functionality. Cas9 controlled by particular promoters was used to evaluate CRISPR-TSKO for cell-, tissue-, and organ-specific expression and gene mutation. Aside from native promoters, synthetic promoters could be engineered to be more precise and/or stronger than their biological versions in plants (Liu and Stewart 2016). Formerly, genetically modified tobacco and Arabidopsis plants with disease-inducible synthetic promoters were produced for possible application as phytosensors (Liu et al. 2013). A promoter like this may be leveraged to make gRNA that is only synthesized in response to defense-related signals or pathogen invasion. The breadth of CRISPR/Cas-mediated multiplex genome engineering may be significantly enhanced by integrating tissue-specific and inducible promoters for gRNA synthesis. Additionally, this might enable Cas9 to be linked with a diverse array of base editors and transcriptional modulators. The progress of CRISPR-TSKO has indeed provided a potential direction for scientists to uncover the significance of crucial genes in various cell, tissue, and organ formation phases of *A. thaliana* and other species of plants. Because CRISPR-TSKO is exclusively based on promoters, a paucity of knowledge on promoters might preclude its applicability. Tissue-specific Cas9 expression, nevertheless, could be challenging to attain since promoters can trigger faulty expression in adjacent cells and tissue (Ali et al. 2020).

Tissue-specific promoter-mediated CRISPR/Cas technology was apparently exploited in *A. thaliana* to examine accurate spatially modulated genetic mutations at the cellular level by GFP-aided live imaging (Li et al. 2019a, b). Li et al. 2019a developed transgenic *A. thaliana* plants expressing GFP-tagged genes like *H2B* and *PIN1* that were regulated by tissue-specific promoters. This method served as a proof-of-concept for live imaging, allowing researchers to evaluate the consequence of mechanical stress variations in the epidermal layers on *PIN1* gene function. Such novel techniques using CRISPR-TSKO would enhance domain-specific attributes while also providing a basic comprehension of in vivo gene activity in plants' post-embryonic developmental processes. A similar strategy may be adopted to effectively modify the genomes of cereal grains. Our group has reviewed very recently a detailed overview of harnessing tissue-specific genome editing in plants through CRISPR/Cas system (Singha et al. 2022).

CRISPR mediated gene drive for plant improvement

Engineered gene drives are cutting-edge genetic modification technologies that sidestep nature's classical hereditary principles. A given gene in a diploid organism has a 50% probability of being transferred to every individual offspring in the scenario of natural inheritance. On the other hand, CRISPR/Cas9-driven gene drives significantly enhance this possibility to 100%, nevertheless in actuality, concerns like efficiency and rigidity may constrain such enhancements.

The CRISPR-gene drive design should preferably be placed into the specific genomic site of the chromosome that would be cleaved by Cas9 nucleases to adequately propagate the intended trait through successive generations. When the cassette is incorporated into a specific genomic region, the chromosome becomes impervious to digestion. As a result, a heterozygote cell for gene drive has one allele amenable to cleavage (wild type; WT) and one refractory to digestion (gene drive allele) (Teem et al. 2020). When the haploid with the gene drive element for the target trait mates with WT haploids in the presence of Cas9, cleaving and eventual replenishment or disruption of WT creates gene drive diploids that generate primarily gene drive offspring following meiosis (DiCarlo et al. 2015). The introduction of a gene drive cassette in a heterozygote cell would trigger DSB in the chromosome of the WT allele. The DSBs that result from this process are refurbished by the HDR mechanism, which use the gene drive allele as a repair template. A mechanism known as "homing" transforms the WT allele to the gene drive allele, rendering the cell homozygous for the gene drive allele (**Fig. 3**). The "homing" mechanism may be localized to only gametic cells (egg/sperm), resulting in selective homozygosity of germline cells (the somatic cells can still be in heterozygote state) (Teem et al. 2020). The "homing" process can plausibly be constrained to the zygotic cell (one-cell embryo). The "homing" process will ascertain that the gene drive cassette is espoused in all alleles and is passed down to all offspring. The gene drive component would gradually transmit across the targeted population over multiple generations. Though the inheritance of gene drive element occurs in a Super Mendelian fashion (up to 100%), any increase above normal Mendelian frequency (50%) would promote multiplication of the gene drive element throughout the entire population. Gene drives have previously been acknowledged for their promising implications, and mechanisms have been established to operate well in insects. Researchers have previously embraced for the employment of gene drive in plants (Neve, 2018; Zhang et al. 2021a, b); however, because of the dearth of an impactful HDR mechanism in plants, this has been a pipe dream. To rectify a character and establish homozygous lines, conventional breeding is a painstaking and time-intensive procedure. CRISPR/Cas9-mediated gene drive has the potential to significantly streamline the approach of fixing traits as well as establishing viable breeding lines.

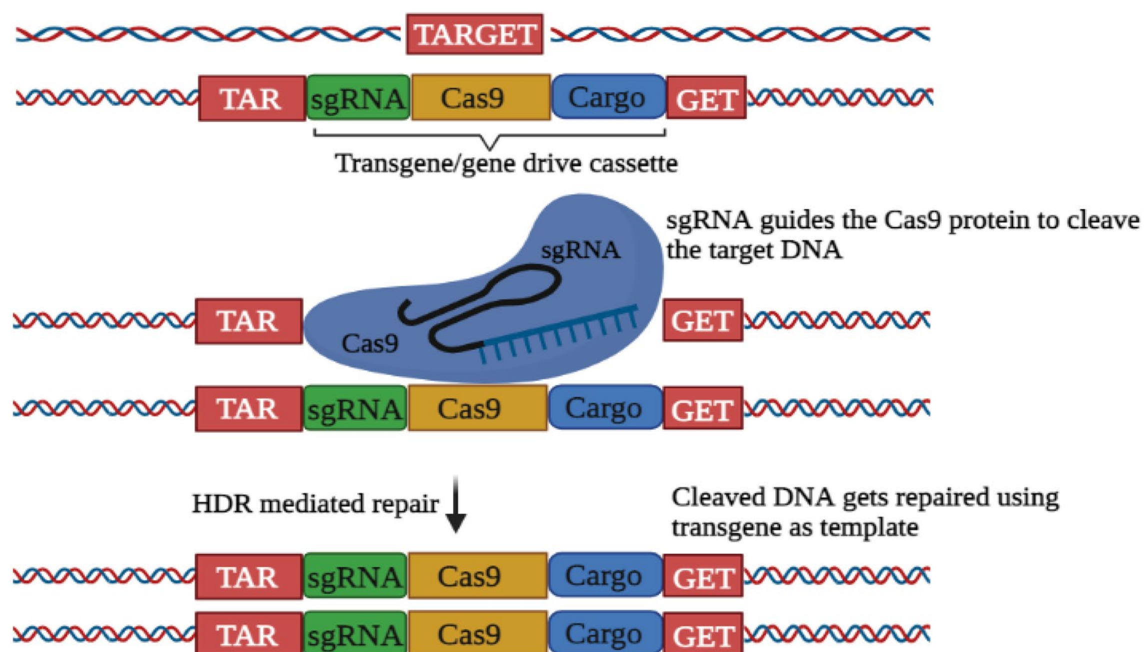

Fig. 3 A synthetic gene drive construct. Cas9 is a nuclease shreds DNA and inserts the pertinent genetic material. When all three components are present in a gene drive cassette (gRNA, Cas protein and cargo/payload), each chromosome will possess the appropriate payload and would be transmitted by the following generation, propagating the gene drive

For the very first time, Zhang and co-workers have demonstrated a successful CRISPR/Cas9-mediated gene drive experiment in *A. thaliana*, yielding in F_1 homozygous plants via zygotic switchover (Zhang et al. 2021a, b). Furthermore, the findings suggested that using a non-autonomous trans-acting gene drive mechanism, endogenous locus that are not driven by the gene drive might even be rendered homozygous. An effective HDR process, instead of the NHEJ pathway, as the primary mode of DNA restoration in higher plants, is a required for a prolific gene drive study. Interestingly, it was reported that the timing of the DSB and the abundance of Cas9 within the cell, have a crucial sway in ascertaining if HDR occurs (Miki et al. 2018). Zhang et al. (2021a, b) utilized already established transgenic Arabidopsis plants for the Cas9 protein, with Cas9 gene expression controlled by two distinct promoters, *Disruption of meiotic control 1* (*DMC1*) and *DD45/EC1.2* egg cell-specific (*EC*). They started the experiment by integrating two Cas9-expressing gene drive constructs into the Arabidopsis *CRYPTOCHROME 1* (*CRY1*) gene's second exon. *CRY1* encodes a blue light photoreceptor that, when produced in a homozygous defective form, culminates in the production of long hypocotyls when grown under light, which could be advantageous in identifying genetic mutations. Cas9 transcription in one gene the drive construct was regulated by the *DMC1* promoter and the *DD45/EC1.2* egg cell-specific (*EC*) promoter in the other. Cas9 production was controlled by *DMC1* and *EC1.2* promoter during meiosis and early embryogenesis, respectively. Zhang et al. anticipated that delivering an adequate level of CRISPR/Cas9 complex in early embryonic embryo phases, together with a higher expression level of Cas9 protein, would lead to significant zygotic conversion efficiency. To address this hypothesis, they crossed EC-Cas9/DD45 (EC-Cas9 from the gene drive cassette and DD45 from the background) and DMC1-Cas9/DD45 (male, ♂) with the Landsberg (Ler) ecotype (female, ♀). Natural Mendelian heritability and outcrossing would have resulted in only drive heterozygotes from that cross; nevertheless, the findings suggest that PCR profiling and later sequence corroboration divulged that 8.10% of the F_1 plants from EC-Cas9/DD45 were homozygous (zygotic conversions), in contrast to 3.08% from DMC1-Cas9/DD45. HDR activity significantly augmented when an egg cell-specific promoter was included in the constructs, and the background of the Arabidopsis lines being used transformation. CRISPR/Cas9 genetic manipulation inevitably leads in variegated plants (He et al. 2018). The *CRY1* phenotype was witnessed in each of the examined light-grown F_2 seedlings, which was corroborated by PCR-based genomic data, demonstrating the precise homozygosity of F_1 plants just at loci of concern. The appearance of a hybrid of both parental genotypes was verified by PCR sequencing of DNA regions flanking the target sites, as envisaged considering sexual reproduction.

CRISPR/Cas9-mediated gene drives have already been competently employed in insects like mosquitoes (to prevent vector-borne diseases), Drosophila, and mouse; however, it has remained problematic to execute in plants (Hammond et al. 2016; Grunwald et al. 2019). Zhang and colleagues' accomplishment of gene drive in *Arabidopsis* has garnered considerable attention for deploying CRISPR/Cas9-mediated gene drive in plants, which might be valuable in expediting breeding and impeding or alleviating weed populations, particularly invasive alien weed species. By permitting modified alleles to co-transmit with gene drives over generations with absolute homozygosity in all progeny of an edited allele, CRISPR/Cas9-mediated gene drives, in tandem with genome editing, might be useful in restoring quantitative attributes in grain crops. Historically, crop improvement has been performed via a series of inbreeding and selfing processes to develop a homozygous line, which is thereafter subjected to experimental verification, seed production, and distribution to the farming community. CRISPR-based gene drives can significantly shorten the number of generations required to achieve homozygosity and trait fixation, thereby streamlining breeding methodology. Generating modifications in polyploid species has always been challenging, but gene drives could be a valuable tool to drive modifications across the homologous alleles in polyploid species (Barrett et al. 2019).

Controlling weeds and invasive plant species might also be one of the primary implications of CRISPR/Cas9-gene drives in plants. Weeds are a severe agricultural problem, and a developing challenge is the emergence of weed resilience to herbicides (Gould et al. 2018). Gene drives might be used to eradicate these weeds in a variety of ways. Firstly, the species might be restrained by spreading detrimental genes that reduce weed viability. Secondly, population sensitizing-drives would further debilitate the population by restoring herbicide-resistant weeds' vulnerability, making them susceptible to routinely used herbicides (Esvelt et al. 2014).

While the method detailed was compelling as a concept design, it might be optimized in a number of different ways. For instance, in order to have it be appropriate as a crop improvement tool for establishing homozygosity, the percentage of zygotic conversion would have to be enhanced above the current 8%. Moreover, in the perspective of employing gene drives as a population containment strategy (for instance, weeds), it would be appropriate to configure the methodology whereby the skewing phase took place in the germline and less on the zygote since it would culminate in rapid population expansion. Controlling Cas9 gene transcription/translation and/or limiting it to genetically engineered plants' germline cells would be a practical alternative to these issues. Concerns, such as the development of resistance and unexpected spreading, might have significant

ramifications and must be considered before deploying gene drives in the area of research. On the other hand, gene drives could be an appropriate and expedient technique for uplifting agricultural production, nutrition sustainability, and weed management if we have a better knowledge of the cell repair mechanisms in plants through which HDR could be primarily harnessed.

Transgene free editing

Plant genome engineering without foreign DNA has a number of advantages over traditional genome editing, including less off-target effects and fewer regulatory issues. While crossing can wipe away genome-integrated exogenous DNA sequence, if any, undetectable vector components may persist in the genome and crossing is therefore not sufficient for asexually reproduced organisms. Excluding the Cas9 protein from genome-edited plants would preclude mutating unintended locus. Multiple exemplary research employing CRISPR/Cas9 ribonucleoprotein or mRNA have shown that genome engineering may be done without involving DNA in wheat (Liang et al. 2017; Zhang et al. 2016), rice (Woo et al. 2015) and lettuce (Woo et al. 2015). Nonetheless, because of its limited efficacy and complexity with integrating external selection markers, DNA-free genome editing is not widely used. The use of endogenous selection markers is one strategy to overcome these issues. Because herbicide tolerance is imparted by precise amino acid replacement in herbicide-targeted genes like the *ALS1* gene, co-targeting a gene-of-interest and ALS using gRNAs and opting for herbicide resistance might dramatically raise the count of plants with the intended gene-of-interest modification (Shimatani et al. 2017; Zhang et al. 2019a, b). If all components were provided as RNA or protein, this selectable co-editing technique might help with DNA-free editing. Another option for generating DNA-free gene editing would be to utilize morphogenetic regulators (MRs) to optimize editing outcomes by providing them with DNA-free CRISPR/Cas reagents, because MRs might allow transformants to regenerate more quickly (Ali et al. 2018; Lowe et al. 2018). DNA-free editing might become the standard methodology of plant genome editing in the coming years as technology advances.

The programmed self-elimination technique has been successfully utilized to extinguish the presence of transgenes such as Cas9 or any other vector sequence from the genome engineered plant. In rice, two suicidal genes (*BARNASE* and *CMS*) have been unearthed to be prolific in establishing transgene-free null segregants (He et al. 2018). *BARNASE* encodes a toxic nuclease, while *CMS* is a male gametophyte-specific lethal protein. In the CRISPR expression vector, the *BARNASE* gene was modulated by the early embryo-specific promoter (*REG2*), whereas the *CMS* gene was controlled by the *35S* constitutive promoter, presuming that transgenic plants carrying the Cas9 protein and the two suicide genes would be removed in a single generation. Using this method, He and colleagues seamlessly regenerated transgene-free plants (He et al. 2018). Nonetheless, this method might only be adopted in plant species that are amenable to tissue culture and seed propagation.

Transient expression of CRISPR/Cas9 DNA or mRNA could also be exploited to produce genome-modified plants that are devoid of transgenes (Saradadevi et al. 2021). Agrobacterium-mediated genetic manipulation introduces specific genes into plant genomes. Agrobacterium (*Agrobacterium tumefaciens*) is also well recognized for mediating transient transgene expression in plant cells (Krenek et al. 2015). This mechanism may be harnessed to transiently generate Cas9 and gRNA in plants for genetic manipulation without the transgenes getting incorporated into the genome. Chen and co-workers adeptly engineered the tobacco *PHYTOENE DESATURASE* (*PDS3*) gene using this method (Chen et al. 2018). Chen et al. did not use antibiotic screening to permit transiently modified cells to survive, which was a fundamental distinction between their approach and the typical Agrobacterium-mediated transformation technique. Chen et al. obtained around 10% transgene-free and modified tobacco seedlings. One more major benefit of transient approaches is that they do not require sexual segregation for transgene expulsion.

Ribonucleoproteins (RNPs)-based genome editing can also result in transgene-free genome engineered plants. In contexts when controlled mutagenesis without donor DNA templates is sought, a ribonucleoprotein (RNP) comprising Cas9 protein and gRNA can be employed. In plants, the RNP complexes can be introduced using a gene gun or other techniques (Woo et al. 2015). When compared to plasmid DNA-based systems that rely on intracellular machinery for Cas9 and gRNA synthesis, RNP strategies provide several advantages. Certain types of cells may be unable to express sufficient CRISPR components. More crucially, no transgenes are used in the RNP approach. As a result, the modified plants may unambiguously be categorized as non-transgenic plants, making regulatory permission easier to obtain. Commonly, particle bombardment or transformation have been used to deliver the RNP component into protoplasts, immature embryos, or calli (Liang et al. 2017; Svitashev et al. 2016; Woo et al. 2015; Toda et al. 2019). Woo et al. (2015) exemplified for the first time that Arabidopsis, tobacco, lettuce, and rice plant protoplasts could be efficiently supplied with the modular Cas9-gRNA RNP component. The researchers established genome-edited plants with a frequency of 8.4–44%, and the alterations were persistent and passed on to the offspring. Several experiments have employed protoplasts as the plant material for the delivery of RNP complexes (Malnoy et al. 2016; Subburaj et al. 2016; Liang et al. 2017; Murovec et al. 2018). Although

protoplast transformation is relatively easy, regeneration of a plant from a single protoplast is challenging, and only a few plant species are responsive to such a procedure (Lin et al. 2018). Genome editing could also be accomplished by bombarding RNP components into immature embryos or calli. In contrast to plasmid transformation, which confers antibiotic-resistant traits, the RNP component does not transfer any selection markers if the genome-edited plants do not exhibit a detectable characteristic.

CRISPR/Cas genome editing beyond generating DSBs

Site-specific gene modulation is attainable by designing Cas9 like a DNA recognizing complex instead of a tailored nuclease, in addition to gene editing through the creation of DNA breaks (Thakore et al. 2016). Cas9's enzymatic action is impaired by alterations in the RuvC (D10A) and HNH (H840A) nuclease domains, but it tends to uphold its RNA-guided DNA targeting function (Jinek et al. 2012; Qi et al. 2013). By combining dead Cas9 (dCas9) with a variety of effectors like transcription repressors or activators, chromatin modifiers, and fluorophores, the CRISPR/Cas repertoire has indeed been strengthened.

When dCas9 attached to DNA elements, the RNA polymerase enzyme is spatially inhibited, which might impede transcription in the instance of CRISPR interference (CRISPRi) (Qi et al. 2013). CRISPRi is a dCas9-driven spatial interference mechanism that functions well in bacteria but not in eukaryotes (Qi et al. 2013; Larson et al. 2013). To improve CRISPR repression in multicellular organisms, dCas9 has been attached to transcriptional repressor domains like Kruppel-associated box (KRAB1) (Gilbert et al. 2013) present in several native zinc-finger TFs (Margolin et al. 1994). Heterochromatin formation is reported to be triggered by KRAB, and chromatin structural alterations frequently follow dCas9-KRAB-targeted transcriptional repression (Kearns et al. 2014). In mammalian cells, dCas9-KRAB is a vital technique for silencing single transcripts and noncoding RNAs by controlling promoters, 5' untranslated regions (5'UTRs), and distal and proximal enhancer regions (Gilbert et al. 2013; Gao and Zhao 2014; Kearns et al. 2014). The transcription regulation domains of KRAB and methyl-CpG-binding protein were coupled to dCas9 for enhanced inhibitory activities (Yeo et al. 2018). The ability of dCas9-KRAB to repress transcription by persecuting genes as well as gene-regulatory areas exemplifies its adaptability. The 12 amino acid SRDX domain, also referred as an ERF-associated amphiphilic repression (EAR)-motif present in various transcriptional repressors, represents one such repressor that has been exploited in plant research (Lowder et al. 2015; Hiratsu et al. 2003).

Alternatively, dCas9 might be attached with activator effectors for CRISPR activation (CRISPRa), which is a sort of programmable transcription activation. In eukaryotic species, dCas9 attached to the transcriptional activation motifs of the NF-B transactivating subunit (p65) or even to VP64 (modular repeats of the herpes simplex activation domain) might activate both reporter and endogenous genes (Perez-Pinera et al. 2013; Maeder et al. 2013; Farzadfard et al. 2013). By delivering several gRNAs to a regulatory regions (promoters), all such synthetic transcription factors have recently been reported to activate genes in a synergistic manner (Perez-Pinera et al. 2013; Maeder et al. 2013). Furthermore, by fusing various activation domains, synergistic interaction can be established (Cheng et al. 2013; Konermann et al. 2015; Chavez et al. 2015). Reprogrammable endogenous gene expression could also be leveraged for cellular remodelling. CRISPR, especially RNA-guided CRISPR activation (CRISPRa) mechanisms, have earlier proven to be highly capable of activating genes in plants. Coordinated activation of several genes, on the other hand, continues to be a challenge. Recently, scientists at the University of Maryland (UMD) intended to accentuate this facet of CRISPR toolkit. CRISPR-Act3.0, a redesigned and more effective CRISPR system, was introduced into plants to accomplish this objective. The emphasis of this third-generation CRISPR technology is combinatorial activation of genes, which boosts the activity of several genes at the same time (Pan et al. 2021).

Furthermore, DNA methylation is a type of epigenetic change that contributes to gene silencing. DNA methylation changes can be transmitted down across generations, resulting in persistent epialleles. The loss of cytosine methylation (5mC) in the promoter region of the *FLOWERING WAGENINGEN* (*FWA*) gene gives rise in *fwa* mutant, a well-studied example of a stable epiallele in plant that typically results in *FWA* overexpression and a heritable late flowering phenotype. Gallego-Bartolome et al. (2018) reported that combining the human demethylase TEN-ELEVEN TRANSLOCATION1 (TET1cd) with an artificial zinc finger (ZF) that targets the FWA promoter can culminate in extremely effective targeted demethylation, *FWA* up-regulation, and a genetically inherited late flowering characteristic. Analogously, the CRISPR/dCas9 SunTag system has indeed been reported to be very successful in targeting DNA demethylation in plants. Precise DNA demethylation and accompanying fluctuations in gene expression can be successfully accomplished throughout the targeted areas using the ZF and SunTag systems, with no direct impact on genome-wide methylation or gene expression (Gallego et al. 2018).

Promoter editing

Taking into account the CRISPR/Cas system's propensity to incorporate Indels in the targeted sequences, strategies have been undertaken to manipulate the regulatory domain in the promoter region of several important genes to modulate transcript dynamics. Indels introduced within the critical motif in the promoter region interfered with the binding of RNA polymerase/accessory protein, prompting mRNA copies of the pertinent gene to be induced or suppressed. This method was reportedly exploited to create multiple alleles of the tomato self-pruning gene (Rodriguez-Leal et al. 2017). *Solanum pimpinellifolium*, a wild relative of tomato resistant to drought and salinity stress, bacterial blight was domesticated through promoter editing of several genes controlling completely distinct characteristics in ingenious research (Li et al. 2018a, b). Analogously, Huang et al. (2018) used the CRISPR/Cas9 mediated promoter editing technique to create multiple alleles of the *Wx* gene that regulate amylose synthesis in the endosperm to improve the cooking quality of rice. In a similar line, attempts have been made to edit the promoter element of the *Xa13* gene to create a number of alleles showing diversified bacterial blight resistance ability in rice (Li et al. 2020a, b, c, d). Creating diverse alleles through promoter editing, rather than directly targeting the coding region of genes that mostly result in knock-out mutation, appears to be a suitable strategy for controlling the degree of expression of many pluripotent genes having a role in a variety of gene expression of the developmental process.

Exploring the potency of plant synthetic biology

Plant synthetic biology is a nascent discipline that incorporates plant science and engineering perspectives to develop novel devices with predetermined behaviors. This discipline would be significant in traditional crop improvement and, therefore, may facilitate the introduction of innovative bioproduction strategies (Nemhauser and Torii 2016). Plants generate a vast array of beneficial secondary metabolites for pharmaceutical and commercial implications, as well as the primary metabolites that support the globe (viz. carbohydrates, fatty acids and proteins). Well over 30 years ago, the first genetically modified plant was produced, ushering in a new paradigm of plant engineering with unique characteristics. The CRISPR/Cas technology has a lot of promise for plant genetic engineering and synthetic biology. Scientists have been able to reroute implicit metabolic networks or create new pathways in plants by modifying endogenous genes or incorporating foreign genes encoding various enzymes or signaling pathway elements. This has resulted in foods supplemented in the intended natural or artificial substances.

For crop growth and development, nitrogen is a significant constraining nutrient. The majority of nitrogen fixation (*nif*) genes in leguminous crops have been explored, as well as their environment-specific expression profiles (Temme et al. 2012). The CRISPR/Cas system might be leveraged to transfer genetic components of the Nod factor signaling pathway from legumes to grains like wheat, enabling the crop to fix atmospheric nitrogen and reducing our reliance on inorganic fertilizers. Synthetic biology also aims to create regulatory circuits that may be used to modify plant behavior, resulting in novel attributes and boost agricultural output (Jusiak et al. 2016). The ability to generate synthetic transcription factors (TFs) using dCas9-driven gene modulation by multiplex transcriptional activation, inhibition, and epigenome modification opens up new possibilities for creating highly complicated, configurable, and efficient gene circuits. Photosynthetic activities in plants, for instance, are beyond ideal because Rubisco, the primary enzyme involved in the photosynthetic cycle, is poor at CO_2 fixation and is hindered by photorespiration, resulting in massive losses of carbon, nitrogen, and energy. Plant photosynthesis and biomass might be enhanced by introducing elements that explicitly sidestep photorespiration (South et al. 2019) or reprogramming Rubisco (Gunn et al. 2020) by CRISPR-mediated DNA introduction. Additional areas of plant synthetic biology, like developing plant biosensors to measure sub-cellular transmissions or plant bio-recorders to sense external stimulus, might be augmented by genome engineering.

Conclusions

For both fundamental and translational plant biotechnology, CRISPR/Cas has shown to be a game-changer. Other than the indel mutations created by the CRISPR/Cas nuclease, a multitude of CRISPR/Cas-based editors are being exploited to achieve targeted genome editing. Because of their extraordinary ability to manipulate genes, these technologies have substantially diversified breeding procedures and contributed to the production of hundreds of crop varieties with outstanding agronomic performance. Base editing (CBEs and ABEs), prime editing, and other CRISPR/Cas-based novel approaches have emerged in the past 10 years, facilitating researchers to incorporate desired alterations (down to the level of individual bases) in the genome. A wide range of plant biotechnologies pertaining to CRISPR/Cas have been developed or improved, including transgene-free plant genome editing, use of morphogenetic regulators, strategies for targeted regulation of gene expression at various stages of plant growth and development, and multiplexed and/or precise gene-editing methodologies that have permitted functional genomics studies. Nevertheless, these techniques have not yet surpassed all of the criteria for modifying plant genomes, and further discoveries are necessary before CRISPR/Cas can be extensively employed in

plants. Because some agricultural attributes are the outcome of multiple quantitative trait loci (QTL) and changing specific genes may not produce enough phenotypic difference, developing efficient CRISPR/Cas-mediated tailored insertion and chromosomal rearrangement techniques to combine or "stack" genetic mutation alleles would be preferable. Since manipulating individual genes may compromise plant fitness, further advances in spatiotemporal gene expression regulation and precision genome editing are anticipated to successfully and effectively fine-tune gene activity. In this review, we have thoroughly explored current breakthroughs in the field of CRISPR/Cas and the possible uses of such approaches in crop improvement. We also addressed and emphasized how CRISPR-TSKO and CRISPR-mediated gene drive might be used to achieve tissue-specific genome editing and homozygous plants for the designated features in the F_1 generation.

Author contribution statement CC and DD: conceptualized the idea, prepared the background information, literature survey and critically evaluated the manuscript. DD, DLS, RRP, NC, MS, PSR and CC: prepared the manuscript. All authors read the final version of the manuscript, provided necessary suggestions and approved it for publication.

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Data availability Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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