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REVIEW

Can genetic engineering-based methods for gene function identification be eclipsed by genome editing in plants? A comparison of methodologies

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Abstract
Finding and explaining the functions of genes in plants have promising applications in crop improvement and bioprospecting and hence, it is important to compare various techniques available for gene function identification in plants. Today, the most popular technology among researchers to identify the functions of genes is the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9)-based genome editing method. But by no means can we say that CRISPR/Cas9 is the go-to method for all purposes. It comes with its own baggage. Researchers will agree and have lived through at least seven more technologies deployed to find the functions of genes, which come under three umbrellas: 1. genetic engineering, 2. transient expression, and 3. chemical/physical mutagenesis. Each of the methods evolved when the previous one ran into an insurmountable problem. In this review, we compare the eight technologies against one another on 14 parameters. This review lays bare the pros and cons, and similarities and dissimilarities of various methods. Every method comes with its advantages and disadvantages. For example, the CRISPR/Cas9-based genome editing is an excellent method for modifying gene sequences, creating allelic versions of genes, thereby aiding the understanding of gene function. But it comes with the baggage of unwanted or off-target mutations. Then, we have methods based on random or targeted knockout of the gene, knockdown, and overexpression of the gene. Targeted disruption of genes is required for complete knockout of gene function, which may not be accomplished by editing. We have also discussed the strategies to overcome the shortcomings of the targeted gene-knockout and the CRISPR/Cas9-based methods. This review serves as a comprehensive guide towards the understanding and comparison of various technologies available for gene function identification in plants and hence, it will find application for crop improvement and bioprospecting related research.

Keywords Plant genetic engineering · Genome editing · Clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) · Off-target mutations · Gene targeting · Homologous recombination · Zinc finger nuclease · Tal effector nuclease

Genetic engineering is a method used to bring changes in the genome. In plants, genetic engineering is important because of its wide range of applications in crop improvement, bioprospecting, and understanding the functions of genes and regulatory sequences. In plants, conventional gene function identification was based on the random introduction of foreign DNA into the genome and analyzing related changes occurring in the organism. Plant genetic engineering paved way to several other techniques including targeted knockout, RNA interference, micro RNA-based gene silencing, and genome editing based on using zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR)/Cas9). In parallel to genetic engineering, methods of chemical and physical mutagenesis evolved which also lead to the understanding of the functions of many genes and their allelic versions. Choice of these techniques depends on the required outcome, nature of the plant or plant part selected for transformation and mode of transformation. Examples of individual strategies are described previously in many other reviews

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(Mansoor et al. 2006; Razzaq et al. 2019; Stanford et al. 2001). Here, we have charted out the differences and similarities between various technologies used for gene function identification in plants and, described their advantages and disadvantages. This review explains why many of the older technologies cannot be completely masked by the recent CRISPR/Cas9-based genome editing. Recent advancements in CRISPR/Cas9 technique have been summarized and modifications required in the existing methods of genome editing and targeted knockout have been suggested. This review will aid in the understanding and selection of appropriate tool for gene function identification in plants.

Plant genetic engineering and the evolution of its applications for functional genomics

Plant genetic engineering is the making of changes in plant genome via insertion of an external DNA. This can be performed using *Agrobacterium*-mediated or direct gene transfer methods (Hansen and Wright 1999). Prior to the CRISPR/Cas9 era, plant genetic engineering created a revolution due to its plethora of applications. Any DNA segment, from any source, with desirable function, could be incorporated into the plant genome. This technology created large number of transgenic crops with agricultural traits like enhanced yield, nutrition, and disease resistance (Singh et al. 2006). Identification of gene functions was one of the major off-shoots of plant genetic engineering.

Using the genetic engineering methodology, constructs for creating targeted knockouts could be conveniently transferred into the genome (Terada et al. 2002). Targeted knocking out of genes was done using double homologous recombination (HR) where, a chunk of DNA was inserted into the target gene, rendering it non-functional. In plants, random T-DNA insertion prevailed over HR and in this method it was avoided using negative selectable markers (NSMs), flanking the regions of homology (Fig. 1). Knockouts were created to study gene functions based on the phenotypic alterations that follow the gene knockout. These also had potential application in removing undesirable traits (example allergens and toxins) from crop plants. Similar to targeted knockout, the targeted knockin method, which relies on HR for insertion of a DNA segment, with the difference that it is a gain-of-function method unlike the former method. Here, the insertion can be in promoter of coding region, with the intension to modify the existing protein's expression, quantitatively or qualitatively (Yamauchi et al. 2009). However, reports on targeted knockouts and knockins in plants were more of a proof-of-concept experiments due to the low efficiency (1–2%) of HR (Terada et al. 2002, 2007).

Interestingly, random knockouts paved way for plant functional genomics study much before the development of targeted knockout methods (Koncz et al. 1992). These were created when T-DNA, which randomly integrated into the genome, often got inserted into important genes, there by leading to altered phenotypes (Alonso et al. 2003; Lijsebettens et al. 1991; Majhi et al. 2014b). Since the efficiency of generating targeted knockouts was very low in contrast to random integration, most of the plant gene function identification relied on the T-DNA insertion mutant plants. Extremely huge collections of T-DNA insertion mutants of plants including *Arabidopsis* (Alonso et al. 2003) and rice (Zhang et al. 2006) were generated and the information was made globally available in databases such as The *Arabidopsis* Information Resource (TAIR), the *Arabidopsis* Biological Resource Center (ABRC) and Rice Mutant Database (RMD) (Garcia-Hernandez et al. 2002; Rhee et al. 2003; Zhang et al. 2006). A vast majority of plant gene functions identified till this date are from T-DNA insertion mutants. Like T-DNA insertion mutants, transposon insertions were also used to create random knockouts. For example, Tos17 of rice and activator/dissociation (AC/Ds) elements of maize have generated many random insertion mutants of their native carrier plants as well as heterologous plants (Hirochika 2001; Walbot 1992). Transposon-tagged lines of many genes are available for various plants such as *Arabidopsis*, rice, and dioecious plants in the databases such as TAIR,

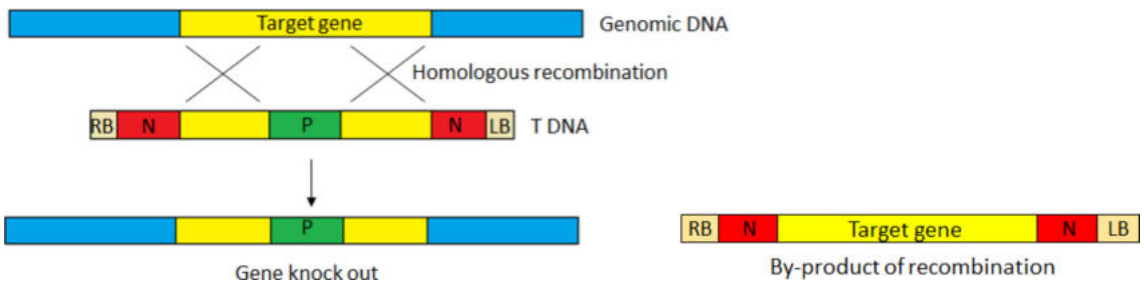

Fig. 1 Illustration of homologous recombination (HR)-mediated gene targeting. Right border (RB), left border (LB), positive selection marker (P), negative selection marker (N). HR at the target site results incorporation of positive selection marker. Negative selection marker for the removal of ectopic recombinants

RiceGE/SIGnAL, RiTE (Rice TE Database), and DPTEdb (dioecious plant transposable element database).

Other methods of studying gene function, which depended on foreign DNA integration, were based on gene overexpression. Here, phenotypic changes were studied by overexpression of certain gene. One method of achieving this was by inserting additional copies of the gene in to the plant genome (Van der Krol et al. 1990). Other method comprised of random insertion of enhancer elements. This also led to gene overexpression if, by chance, the T-DNA harboring the enhancer element integrated in the promoter vicinity of a gene with less/no expression, a strategy known as 'activation tagging' (Hayashi et al. 1992).

Discovery of a novel promoter could be done by random integration of promoter-less reporter gene down stream of promoter, a strategy known as 'promoter trap' (Nakayama et al. 2005; Springer 2000). Since such an integration of reporter often lead to the expression of a fusion protein with the reporter, protein localization could also be studied, thereby giving deeper understanding of the gene function. Hence, this method is also known as gene trap. Similarly, random integration of reporter gene with a weak promoter often led to the discovery of novel enhancer elements, a strategy known as 'enhancer trap' (Page and Grossniklaus 2002; Springer 2000).

Due to low efficiency of targeted knockouts and, due to the unpredictable DNA insertion behavior in above genetic engineering-based strategies, RNA interference (RNAi) was also chosen as a method to identify gene function by silencing a trait (McGinnis 2010; Wesley et al. 2001). Construct for RNAi could be either integrated into the genome or, could be expressed transiently (discussed below) (McGinnis 2010). This method, though was highly efficient, it came with its own baggage of disadvantages such as inconsistency in the phenotypic alteration and silencing of non-target genes (Small 2007). RNAi involving micro RNAs (miRNAs) have been used for gene functional analysis (Djami-Tchatchou et al. 2017). miRNAs are short RNAs, about 19–24 nucleotides long, evolutionarily conserved sequences formed from long Pol II transcript with specific stem-loop structures that are recognized by Dicer-like (DCL) 1 protein (Narjala et al. 2020). miRNAs are involved in post-transcriptional regulation of target sequence (mRNA) by targeted RNA degradation. Plants possess natural miRNA of less sequence complementarity with the target mRNA, which results reduced post-translational regulations. As a solution for this problem artificial miRNA (amiRNA) is introduced in plant, with high target specificity and the specificity can be manipulated based on the selected gene (Schwab et al. 2006). amiRNA-mediated post-translational gene silencing has great agronomic value, which can be used for crop improvement, gene validation and comparative functional genomics studies of different varieties (Warthmann et al. 2008). amiRNA can target multiple genes from a gene family (Schwab et al. 2006). Thus, many plant species, even those which are recalcitrant to T-DNA integration, can be made amenable to RNAi-mediated gene silencing using alternative viral vectors.

In plants, genetic engineering is the stepping stone for CRISPR/Cas9-mediated genome editing. Though CRISPR/Cas9-based genome editing is known as a non-transgenic method, it is important to note that in case of plants, most efficient method to cargo the genome editing reagents is via T-DNA (Char et al. 2017). Since the T-DNA often ends up in integrating itself into the host genome, the CRISPR/Cas9 construct is retained in the genome even after the completion of editing. This construct has to be segregated out in the subsequent generations.

Transient expression of genes in plant and its applications

In contrast to the stable expression, if an external gene is able to express in the plant cell without integrating itself in to the plant genome, it is known as transient expression. This is generally achieved using viral vectors as they can efficiently sail into the plant system, expressing without integration (Scholthof and Scholthof 1996). Viral vector-based transient expression is desirable for expressing RNAi constructs (Kurth et al. 2012) and the recent CRISPR/Cas9 constructs (discussed in the subsequent section; Zaidi and Mansoor 2017). Transient expression can also be achieved using protoplast transformation (Zhang et al. 2016). This is similar to the animal counterpart system. Animal cell is not surrounded by cell wall and hence, genome editing via transient expression can be easily achieved. Plant protoplast transformation is possible but there are few reports adopting this method probably due to the difficulty in regeneration from protoplast (Abel and Theologis 1994). It is to be noted that, as the name indicates, the expression is only 'transient' and hence, the same cannot be achieved in subsequent generation and the phenotypic change is reverted in the subsequent generation. Fortunately, in case of CRISPR/Cas9-based genome editing, only the expression of the construct is 'transient' and not its job. Hence, once the genome is edited by the CRISPR/Cas9 construct, this edited version is stably transferred to subsequent generations (Zhang et al. 2016).

Genome editing in plants

There is no foreign DNA integration in this method and, as the name indicates, it involves amendments in the innate genome. Prior to the emergence of CRISPR/Cas9, genome editing was done using chemical or physical mutagens and

the mutants were screened using targeting induced local lesions in genomes (TILLING) (Kumawat et al. 2019; Li et al. 2019; McCallum et al. 2000). These methods involve treating plants with chemical mutagens like ethyl methyl sulfonate (EMS) and methyl methyl sulfonate (MMS) or physical mutagens such as fast neutron radiation (FNR), gamma-ray, and carbon-ion irradiation (Bae et al. 2009; Belfield et al. 2012; Kazama et al. 2011). These mutagens cause random mutations and a large number of progenies obtained from the plants generated after seed/meristem treatment are screened for the mutant version of a particular gene using TILLING (Colbert et al. 2001; McCallum et al. 2000). Disadvantage of this method was that the mutagenesis was random. One of the advantages of this method is that any plant is amenable to chemical/physical mutagenesis, unlike the former two methods where, certain plants exhibited recalcitrancy to factors like the vector used (*Agrobacterium* or virus) and/or the in vitro regeneration. Another big advantage of TILLING was that often multiple alleles of the same gene could be generated.

Later more efficient genome editing methods such as those involving ZFN and TALENs evolved, which could also be used to create gene knockouts. ZFN is a hybrid protein which acts as a dimer at the target site. ZFN is composed of a DNA binding domain known as zinc finger motif [Cysteine2 Histidine2 (Cys2His2)] and a restriction endonuclease called *Fok*I. The nuclease *Fok*I is a bacterial type II restriction endonuclease. Zinc finger motif interacts with DNA through the major groove by inserting at the α-helix that specifically identifies 3 base pairs (3 bps). The availability of ZFN specific to 64 triplet codons made genome editing possible at any site (Beumer et al. 2006). Binding of zinc finger motifs on both strands of the DNA duplex leads to dimerization of endonuclease *Fok*I which results double strand breaks (DSB) at the target site (Mani et al. 2005). The introduced DSB concealed either by following template independent non-homologous end joining (NHEJ) (Curtin et al. 2011) or by a template dependent HR repair pathway (Wright et al. 2005). Repair using HR leads to targeted insertion. TALENs, another artificial nuclease, consist of transcription activator-like effector (TAL Effector) with a central domain of nearly identical tandem repeats. Each repeat unit is 33–35 amino acids long with in which the repeat variable diresidues (RVDs) are located at the 12th and 13th position. The repeat unit ends with 20 amino acids long truncated 'half repeat'. About 90% of these RVD repeats constitute six residues, histidine aspartic acid (HD), asparagine glycine (NG), asparagine isoleucine (NI), asparagine asparagine (NN), asparagine serine (NS) and histidine glycine (HG). These six RVDs specify nucleotides C, T, A, G/A, A/C/T/G, and T, respectively. RVDs are the ciphers on the TAL effectors that specifically bind on target site (Boch et al. 2009; Christian et al. 2010). Similar as in ZFN, *FokI* is the nuclease part of TALENs. TALENs also act as dimers at the target site, resulting in the dimerization of *FokI* there by introduction of DSB. Even though this two site-specific nucleases (SSNs) have different DNA binding motifs, their activities are the same. Thus ZFN and TALENs act on the target site as dimers, they work with high efficiency and specificity. However, since the customized making of both ZFN and TALENS was laborious and expensive, reports involving genome editing and genetic engineering using these enzymes were minimal.

Most recent and efficient method of genome editing is the CRISPR/Cas9 technique (Arora and Nerula 2017; McCarty et al. 2020). CRISPR/Cas9 complex is an acquired adaptive immune machinery in bacteria and archaea. Microbes use this machinery for defending the cellular entry of foreign DNA particles derived from phages and plasmids (Terns and Terns 2014). This mechanism is adopted in plants for inducing genetic alterations by introducing DSB. For recognizing the target site, this method requires a customizable single guide RNA (sgRNA) of about 20 nucleotides in length and a conserved tandem guanosine nucleotides (NGG) sequence motif termed as proto-spacer adjacent motif (PAM), always located downstream of the target sequence (Fig. 2). In plant genome, the expressed CRISPR/Cas9 cassette generates DSB at target site by the complementary binding of sgRNA based on the former essential requirements, and the binding leads the associated Cas9 protein to introduce DSB by its two nuclease domains called HNH and RuvC (Jinek et al. 2012). Subsequently, introduced DSB triggers NHEJ repair (Bortesi and Fischer 2015). In the presence of an external DNA fragment with sequence homology, the DSB

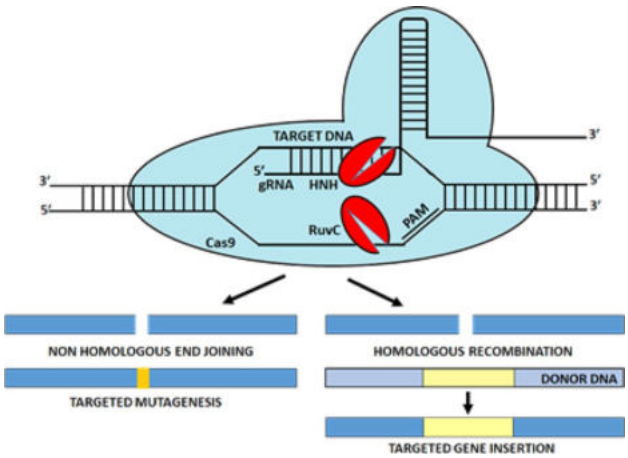

Fig. 2 Illustration of CRISPR/Cas9-mediated targeted gene modification. CRISPR/Cas9 complex, sgRNA binds complementarily on one strand. Double strand break followed by non-homologous end joining results targeted mutagenesis either by insertion or deletion. Introduction of donor DNA facilitate homologous recombination at the target site results incorporation of foreign DNA fragment. Proto-spacer adjacent motif (PAM)

gets concealed by HR at the target site (Zhao et al. 2016). Based on the elementary features such as high efficiency (in contrast to targeted knockout), stability (in contrast to RNAi), flexibility of editing the desired location of one particular desired gene (in contrast to chemical mutagenesis and T-DNA insertion mutagenesis) or, a family of genes or, creating allelic versions of the same gene or, even multiple genes, CRISPR/Cas9 emerged as one of the most promising method. An additional elementary feature of CRISPR/Cas9 system is multiplexing, the simultaneous targeting of multiple genes using single molecular construct and, this is also the major advantage of CRISPR/Cas9 system over all other above techniques (Peterson et al. 2016). Multiplexing is very useful in improving traits that are controlled by multiple genes and also for studying the functions of genes involved in a biological pathway. In rice, the quantitative trait-grain weight was effectively increased by the efficient multiplexing of CRISPR/Cas9 tool (Xu et al. 2016). Also in rice, the multiplexing was performed by developing a new strategy called simplified single transcriptional unit (SSTU) in which the crRNA array and the nuclease are co-expressed under the same promoter (Wang et al. 2018). Many CRISPR/Cas9-mediated multiple gene editing experiments have also reported in *Arabidopsis* (Zhang et al. 2015a, b; Peterson et al. 2016; Lowder et al. 2015).

Whether genome editing via genetic engineering or transient expression?

Genome editing of plants can be accomplished either by *Agrobacterium*-mediated T-DNA insertion or by transient expression. The advantage of the former method is that many plants are amenable to *Agrobacterium*-mediated transformation. Transporting the genome editing construct via any of the plant genetic engineering method though assures genome editing, integration of this construct into the T_0 genome is often unavoidable. This construct has to be segregated out in the T_1 generation (Hensel et al. 2012). Hence, in plants, genome editing is accompanied by genetic engineering during the T_0 generation. In T_1 generation, those individuals which do not carry the T-DNA insertion and have their genome edited as well, have to be selected by appropriate screening methods involving southern hybridization, PCR, and sequencing. Along with these techniques, incorporation of reporter genes in the T-DNA make faster selection of T-DNA free mutants (Castel et al. 2019). Transient expression is the best method to accomplish the job of genome editing. It is not only sufficient, it is suitable as well because, transient DNA has a lesser viability and this prevents continuous expression of the genes in the construct (like Cas9). It is found that CRISPR/Cas9 has the potential of inducing off-target mutations (Zhang et al. 2015a, b) and hence, its continuous expression is undesirable. However, transient expression using viral vectors has host limitations and, protoplast regeneration is not easy.

Regeneration involving tissue culture is often required for *Agrobacterium*-mediated transformation of many crop plants. Tissue culture, when combined with transformation, often results in low regeneration efficiency. Also, plant tissue cultures are time consuming and can induce unintended somaclonal variations. To overcome these hurdles, recently, genome edited plants have been successfully raised using meristem transformation. Plant growth regulators and *Agrobacterium* carrying the genome editing reagents when applied to young seedlings generated new meristems with genome edited shoots (Maher et al. 2020). Interestingly, this method does not need the sterile lab facility and hence is economical and can be easily used for commercial purpose. Recently, to overcome the low regeneration problem, efficient genome editing was achieved in transformable wheat genotypes (Debernardi et al. 2020). These transgenic wheat plants expressed a fusion protein comprising of wheat GROWTH-REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1) that resulted in high regeneration. This fusion protein seems to work in a wide host range as it increased regeneration efficiency in citrus as well (Debernardi et al. 2020). Nevertheless, since both these recent reports involve stable transformation, they cannot escape the disadvantages of T-DNA segregation and off-target mutations.

Other fine delivery methods used to transfer genome editing reagents include floral dip method (Yan et al. 2015), electroporation (Castel et al. 2019), poly ethylene glycol (PEG) -mediated protoplast transformation (Sant'Ana et al. 2020), bombardment or biolistic method (Zhang et al. 2016). Floral dip method involves *Agrobacterium* and is routinely used in *Arabidopsis* transformation, which leads to the integration of transgene into the genome and has to be segregated out in the progenies. The remaining three methods are deployed to deliver Cas9/gRNA ribonucleoproteins and not the transgene, thereby providing excellent platform for transgene integration-free genome editing. However, these three methods are based on protoplast regeneration, which is a difficult task to achieve. Two other methods which can be used for transgene integration-free genome editing include pollen magnetofection-mediated delivery and nanoparticle-mediated delivery as they have the potential to facilitate transient expression (Sandhya et al. 2020).

Which technology leads to what?

All the technologies by which changes can be brought in to plant genomes, for gene overexpression or for functional identification, come under either of the three

umbrella-methods—genetic engineering, transient expression, and TILLING (Fig. 3).

Critical analysis of the cluster of applications under plant genetic engineering and genome editing (Table 1) clearly displays that all these tools have their own advantages and disadvantages. Appropriate method has to be decided based on the decisions like the requirement of foreign gene integration or editing of the innate genome (Fig. 4). Certain traits in plants can be modified by minor tweaking of the genome and these can efficiently be achieved using genome editing. There could be certain other traits that are absent in the host and can be provided only from a foreign source, by genetic engineering. It is extremely essential to consider factors like off-target mutations which are cumbersome and misleading (discussed subsequently; Shelake et al. 2019). Hence, if the intension is functional analysis of a gene using knockout method, though genome editing can be adopted and this does give high frequency of knockouts, we suggest HR-based plant genetic engineering methods. Also, if the intention is mere expression of the introduced construct (with either foreign gene or for genome editing) and not stable integration, viral vector-mediated method should be used where ever possible. Though plant genetic engineering is the stepping stone for plant genome editing, it looks like the outburst of reports on plant genome editing are slowly eclipsing plant genetic engineering and allied functional genomics methods like HR-based gene targeting. The comparison of various aspects of CRISPR/Cas9-based plant genome editing, plant genetic engineering, transient expression, and genetic engineering-based knockouts convey that there are subsets of applications that actually demands plant genetic engineering not editing and vice versa.

Off-target mutations—the undesirable co-passengers

While targeting a gene for editing using CRISPR/Cas9, off-target mutations are often observed. These are unwanted mutations that occur at random in the genome other than the target site. These mutations are undesirable as they lead to misleading interpretations (Nekrasov et al. 2013). Little mismatches in the guide RNA cause the CRISPR/Cas9 to break the genome at non-specific site (Zheng et al. 2017). The frequency of off-target mutations is high in plants because of the stable expression of the genome editing construct in T_0 plants. Since the edited plants are segregated out only in the T_1 generation, CRISPR/Cas9 gets ample time in the T_0 generation to create off-target mutations (Brooks et al. 2014). Screening out such off-target mutations is done in the T_1 generation and this involves extensive whole genome sequence analysis (Chen et al. 2018). Though the shelf life of the editing construct within the plant can be reduced by transient expression, this method has its own limitations as not all plants are amenable to protoplast regeneration or viral infection. Off-target mutations in methods deploying ZFN and TALENs are comparatively less as these enzymes interact with the target site as dimers of which, each monomer binds to each DNA strand, thereby increasing the target specificity. Even though the negligible size of DNA binding motif in SSNs (ZFN and TALENs) and sgRNA in CRISPR/Cas9 increases the chance of off-target effect. Off-target mutations due to random integration (also known as ectopic integration) of the targeting construct were initially reported during the homology-based gene targeting of plants (Hanin et al. 2001). These random integrations were successfully eliminated using the positive–negative selection (PNS) method (Fig. 1) (Terada et al. 2002). Here, the positive

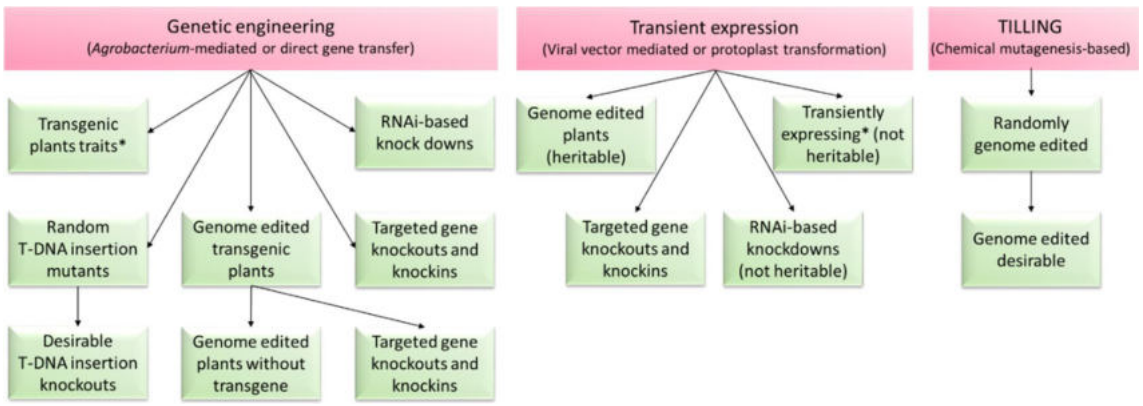

Fig. 3 Classification of methods used for plant genome modification or editing based on the mode of transformation or mutagenesis. Targeting induced local lesions in genomes (TILLING). *All desirable events inclusive of overexpression and gene identification tools such as gene trap, enhancer trap, and activation tagging

Table 1 Applications, advantages and disadvantages of different plant genome modifying, RNAi and editing methods

Methods	HR-based knockout involving PNS	T-DNA insertion mutants	RNAi expressed from T-DNA insertion	Transiently expressed RNAi	Knockout involving ZFN or TALENs	Chemical/physical mutagenesis and TILLING	CRISPR/Cas9-based (*Agrobacterium*-mediated or DGT method)	CRISPR/Cas9-based (transient expression method)
Queries								
Whether knockout possible?	Yes	Yes	Yes[a]	No	Yes	Yes[b]	Yes	Yes
Whether knockout is targeted?	Yes	No	No	NA	Yes	No	Yes	Yes
Whether RI-based?	No	Yes	Yes	NA	No	NA	No	No
Whether unwanted RI possible?	No[c]	Yes	Yes	NA	Yes[d]	NA	Yes[e]	NA
Whether genome editing possible?	No	No	No	No	Yes	Yes	Yes	Yes
Whether off-target editing is possible?	NA	NA	NA	NA	Yes	Yes	Yes	Yes
Is extensive WGS and/or screening required to rule out off-target editing?	NA	NA	NA	NA	Yes	Yes	Yes	Yes
Can an existing trait be modified?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Can a foreign trait be inserted/expressed?	Yes	Yes	Yes[f]	No	Yes	No	Yes[g]	Yes[h]
Is the modification heritable?	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
Whether knockdown possible?	No	No [i]	Yes	Yes	No	No	No	No
Whether off target knockdown possible?	NA	NA	Yes	Yes	NA	NA	NA	NA
Can multiple alleles be generated?	No	No[j]	No	No	Yes	Yes	Yes	Yes
Is targeted knock-in possible?	Yes[k]	No	No	No	Yes	No	Yes	Yes

Table 1 (continued)

HR homologous recombination, *PNS* positive–negative selection, *ZFN* zinc finger nuclease, *TALENs* transcription activator-like effector nucleases, *TILLING* targeting induced local lesions in genomes, *DGT* direct gene transfer method, *RI* random insertion, *WGS* whole genome sequencing, *DSB* double strand break

[a]Due to random insertion of T-DNA

[b]Depends on the type of mutation, e.g., if a stop codon is generated

[c]RI possible if truncated T-DNA is integrated and can be screened out

[d]RI has to be segregated out

[e]RI has to be segregated out

[f]If required, in the same T-DNA as that of RNAi construct

[g]If required, in the same T-DNA

[h]If required using DSB-induced HR

[i]Except when RNAi construct is present in the T-DNA

[j]But multiple alleles can be inserted

[k]For positive selectable markers and reporter genes like GUS

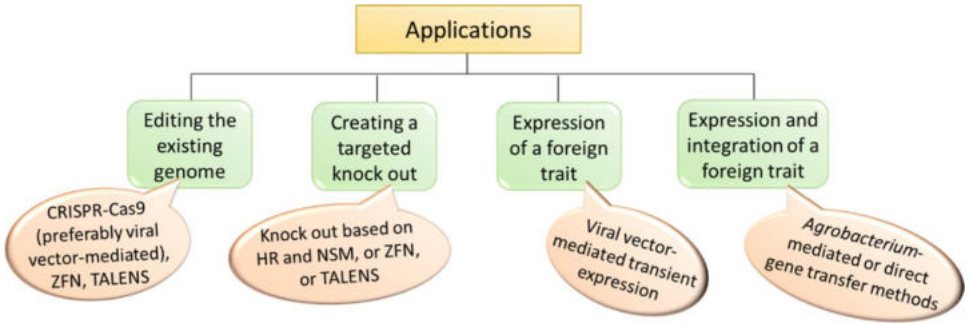

Fig. 4 Applications of different genome modifying methods. Homologous recombination (HR), negative selection marker (NSM), zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN)

selectable marker selected double HR events and, the ectopic integration events were eliminated using the NSMs.

Proposed strategies to reduce off-target mutations

Optimization of CRISPR/Cas9 for genome editing specificity is done using intense bioinformatic analysis, which involves designing of sgRNA having minimal homology with the genome other than the target site. Along with sgRNA specificity, PAM sequence and Cas9 activity determine the target gene editing and thus manipulating these three factors based on the need can improve the gene editing efficiency without off-target effect (Hajiahmadi et al. 2019). Alterations have been made in sgRNA by removing three nucleotides (nt) in the 5′end and target specificity was improved by adding two guanine nucleotides (GG) in the 5′ end (Cho et al. 2014; Fu et al. 2014). Controlled expression of Cas9 protein can limit its activity only on the target site. Use of egg cell-specific promoter-controlled CRISPR/Cas9 system showed reduced off-target effect in comparison to the *Cauliflower mosaic virus* (CaMV) 35S promoter (low efficiency promoter) -controlled CRISPR/Cas9 system in *Arabidopsis* (Wang et al. 2015). Similarly in *Citrus* plants, the *YAO* promotor-controlled Cas9 expression showed rapid and efficient genome editing (Zhang et al. 2017). Inducible promoters can also regulate Cas9 expression and this strategy has been widely used in many recent reports such as that in maize where a heat-shock-driven promoter controls Cas9 expression (Barone et al. 2020). Modified Cas9 protein also improved targeting efficiency. Use of Cas9 nickases (Cas9 protein in which one of the two nuclease domain is catalytically inactive), RNA-guided *Fok*I–dCas9 nuclease (RFNs) [catalytically inactive Cas9 (dCas9) fused with *Fok*I nuclease] and engineered Cas9 protein (SpCas9 with alanine substitution at 3rd and 4th residues) limited off-target mutations (Steinert et al. 2015; Tsai and Joung 2016). Both the Cas9 nickase and RFNs act as dimers at the target site so lengthy recognition site increase the target specificity. Though single nuclease domain is active in Cas9 nickase, it cleaves only one strand of the genome. Thus two CRISPR/Cas9 nickases are required for the targeted DSB induction. RFNs resemble SSNs ZFN and TALENs, because here *Fok*I cleaves genomic DNA which is fused with dead Cas9 (dCas9) by its amino terminal. Disadvantage of Cas9 nickase is the nuclease domain is always active whereas in RFNs the nuclease become functional only when they get dimerized.

Cas9 toxicity can be limited by controlling the expression of Cas9 gene by placing it under tissue-specific promotor (like callus, embryo, and anthers). Here, the type of promoter is decided based on the explant selected. Tissue specific expression of Cas9 reduce unwanted somatic mutations, which will reduce formation of chimeras in T_2 generation (Mao et al. 2016). Different versions Cas9 proteins can also be used to reduce off-target effect. Cas9 protein from *Francisella novicida* (FnCas9) is an example, which is highly specific to the target DNA with negligible binding to off targets (Acharya et al. 2019). This is one of the largest Cas9 protein functions efficiently as SpCas9. Unlike SpCas9, FnCas9 has a bilobed structure with a different sgRNA scaffold. FnCas9 retain higher intrinsic specificity, which can read more than two mismatches in the 5′ distal end of the PAM sequence, that results a conformational change in the HNH cleavage domain render it in inactive mode. Thus it is less tolerant to off-target effect. High rate of substrate recognition efficiency of this protein can bring precise heritable changes in the genome (Acharya et al. 2019). Thus introducing these Cas9 variant in plants can result an off-target free gene edits.

A noteworthy advancement in genome editing is the DNA-free genome editing. As the name indicates, the method does not involve DNA. Here, a preassembled ribonucleoprotein complex comprising of Cas9 protein and the guide RNA is transferred directly into the cell (Svitashev et al. 2016). This was successfully achieved by particle bombardment method in maize (Svitashev et al. 2016) or by protoplast transformation in potato (Andersson et al. 2018). Many more crop plants have been subjected to DNA-free genome editing to improvise various traits of agronomic importance (Metje-Sprink et al. 2019). The major advantage of this method is that it does not involve transgene integration and its subsequent segregation. As a result, the exposure time of the cell to Cas9 is minimum, thereby reducing the vulnerability of developing off-target mutations. Nevertheless, this method is not free of the baggage of off-target mutations (Andersson et al. 2018). There is a promising scope of improvising DNA-free genome editing by combining it with strategies like prime editing, which can still reduce off-target mutations (disused below). In plants, protoplast regeneration is difficult to achieve and most crops are more amenable to *Agrobacterium*-mediated transformation than other methods of direct transformation. Hence, DNA-free genome editing may be difficult in such cases (Metje-Sprink et al. 2019). We hereby propose an alternative strategy that can be combined along with the existing methods used to combat off-target mutations. This strategy relies on combining negative selection and transient positive selection (Fig. 5). Here, a conditional NSM (like *CodA*) (Majhi et al. 2014a) can be introduced into the genome editing construct having a positive selectable marker like hygromycin (Hyg) and the components for genome editing like sgRNA and Cas9, all under constitutive promoter. Conditional NSMs turn out to be lethal only in the presence of appropriate substrate (Shah and Veluthambi 2010). *CodA* is an *E. coli* gene encoding cytosine deaminase and, if the substrate 5-fluorocytosine (5 FC) is added to the media in which the transformed explant is grown, cells expressing *CodA* will be killed (Majhi et al. 2014a). T-DNA can be transiently expressed up to 2–4 weeks and hence, such cells can survive on antibiotic containing media, i.e., transient positive selection (RamanaRao and Veluthambi 2010). The substrate 5-fluorocytosine (5 FC) is to be added to the media after the transient positive selection period so that all cells with T-DNA will be killed. Hence, the plants that regenerate will be devoid of T-DNA integration and we expect the genome to be edited during the transient positive selection period. Alternatively, instead of using a conditional NSM, a non-conditional NSM like *DIANTHIN* (Shah and Veluthambi 2010) regulated by an inducible promoter such as *EKCCM* (Hou et al. 2012) can be used. However, it is important to choose an appropriate inducible promoter as most of such promoters in plants exhibit a very low basal expression that may result in lethality due to the expression of toxic protein from the NSM. We hope that this strategy should work because in a similar strategy using non-conditional negative selection and transient positive selection, RamanaRao and Veluthambi (2010) could eliminate T-DNAs and obtained marker-free transgenic plants with an efficiency of 4.4%. This method may drastically reduce mutations due to random integration of T-DNA, will save the time and effort of segregation in T_1 generation and, will give less time for Cas9 to cause off-target mutations. Though the above method does not guarantee complete elimination of off-target mutations, we believe that there will an appreciable decrease in these mutations.

Is there still a scope for improving HR-based targeted knockout?

Plants prefer template independent NHEJ than the template dependent HR-based repair mechanism (Puchta 2005). Due to the low efficiency, though HR-based gene targeting is considered just as a proof of concept, this method did fetch true gene targets without any off-target integration, when combined with positive–negative selection (Terada et al. 2007). For obtaining the quality results in HR-mediated gene targeting, many alternatives have been combined. One strategy reported previously was based on the expression of proteins that enhanced HR. Expression of yeast *Rad54*, a member of SWI2/SNF2 chromatin remodeling gene family, enhanced gene targeting efficiency in *Arabidopsis* plants (Shaked et al. 2005). Study on mammalian cells revealed that inactivation of genes *Ku70* and *Polϴ* in the canonical

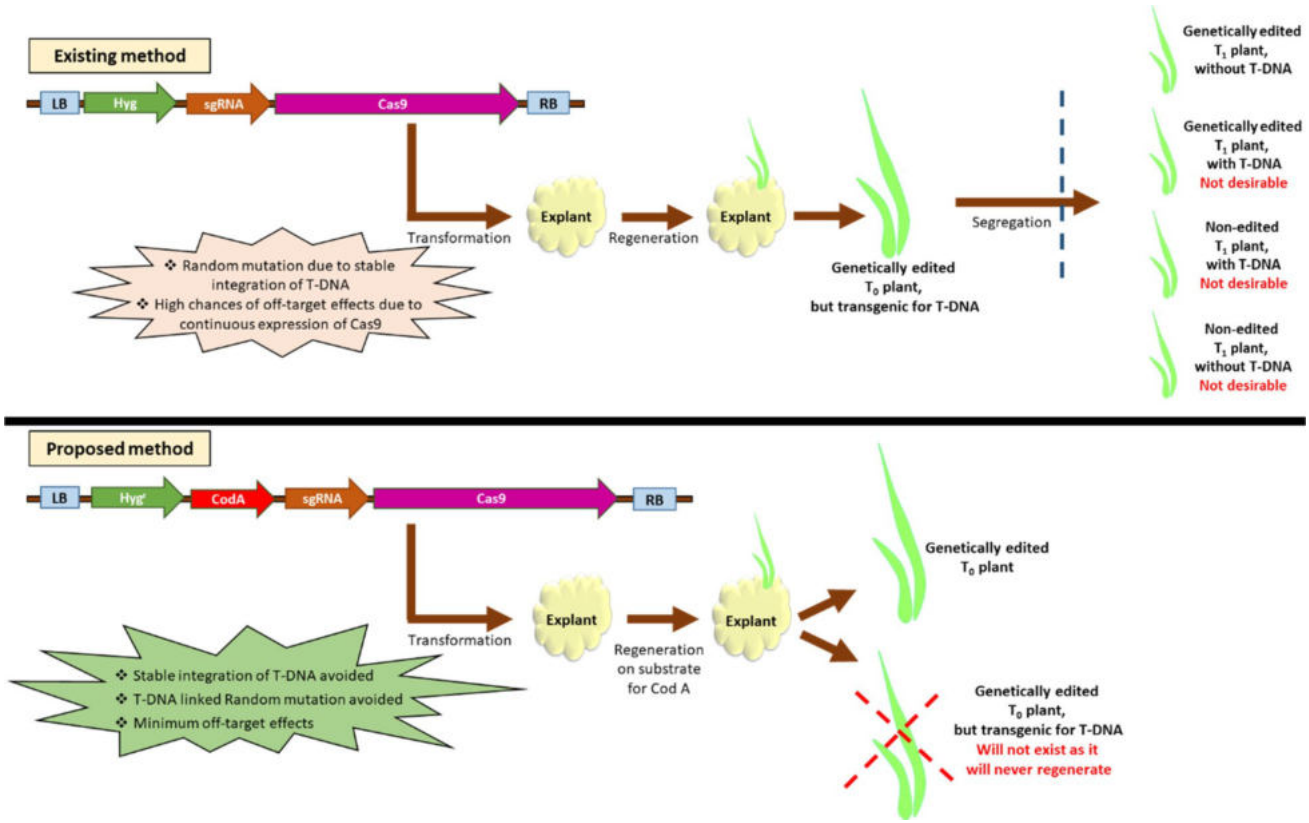

Fig. 5 Strategy proposed for controlling off-target effect of CRISPR/Cas9 complex by incorporating a conditional negative selection marker (*CodA*) and positive selection marker (Hyg^r) in the T-DNA construct. Left border (LB), right border (RB), hygromycin resistant gene (Hyg^r), and cytosine deaminase (*CodA*)

non-homologous end joining (C-NHEJ) and alternative end joining (Alt EJ) pathways, respectively, showed 100% HR without RI (Zelensky et al. 2017). Like *Ku70*, *Lig4* is an important gene involved in NHEJ pathway. In *Arabidopsis*, *Lig4* mutation increased the HR rate by 3–4-fold and *Ku70* mutation increased the HR rate by 5–16-fold (Qi et al. 2013). Inactivation of these genes favors homologous recombination in plants. Endo et al. (2016) also observed more gene targeted events for the *ALS synthase* loci in rice, if the plants were first targeted for the *Lig4* gene and then followed by GT for *ALS*.

Another strategy induced site-specific DSB in the genome, which impelled plants towards HR. For example, Wolter and Puchta (2019) obtained enhanced gene targeting by expressing an orthologue of Cas9 protein, the LbCas12a from *Lachnospiraceae bacterium ND2006,* in *Arabidopsis* plants. Efficient HR-based knockin was reported by Miki et al. (2018) where they followed the sequential transformation strategy. They first transformed *Arabidopsis* plants with Cas9 under egg cell-specific promoter and then followed transformation with construct for sgRNA-mediated targeted HR. *In planta* gene targeting is another method used in plants that involves stable integration of the T-DNA construct (construct carry HR donor sequence and Cas9 expression cassette) into the genome. As a result the targeted gene is modified in the germline cells and is transferred in to the next generation, hence progeny can be easily screened (Fauser et al. 2012). Wolter et al. (2018) and Hahn et al. (2018) efficiently performed *in planta* GT in *Arabidopsis* and targeted the genes *ALS* and *GLABROUS1 (GB1)*, respectively. There could still be a scope of improving GT efficiency if some of the above approaches are combined (Fig. 6). This could be achieved by co-transformation (as described in Jacob and Veluthambi 2002), involving both the cointegrate vector T-DNA and a binary vector T-DNA in single *Agrobacterium* strain. The single copy cointegrate vector T-DNA will harbor the sgRNA-encoding region, Cas9 gene with tissue-specific promoter (TSP) and a positive selectable marker, like that reported by Miki et al. (2018), with the difference that this construct can accommodate yeast *RAD54* (like in Shaked et al. 2005), as it will promote HR. The genotoxic effect of Cas9 protein can be controlled by an inducible or tissue-specific promoter. The high copy binary vector T-DNA will harbor the region of homology for gene targeting, flanked by non-conditional NSMs on both sides (like Terada et al. 2002). Ectopic recombinants will

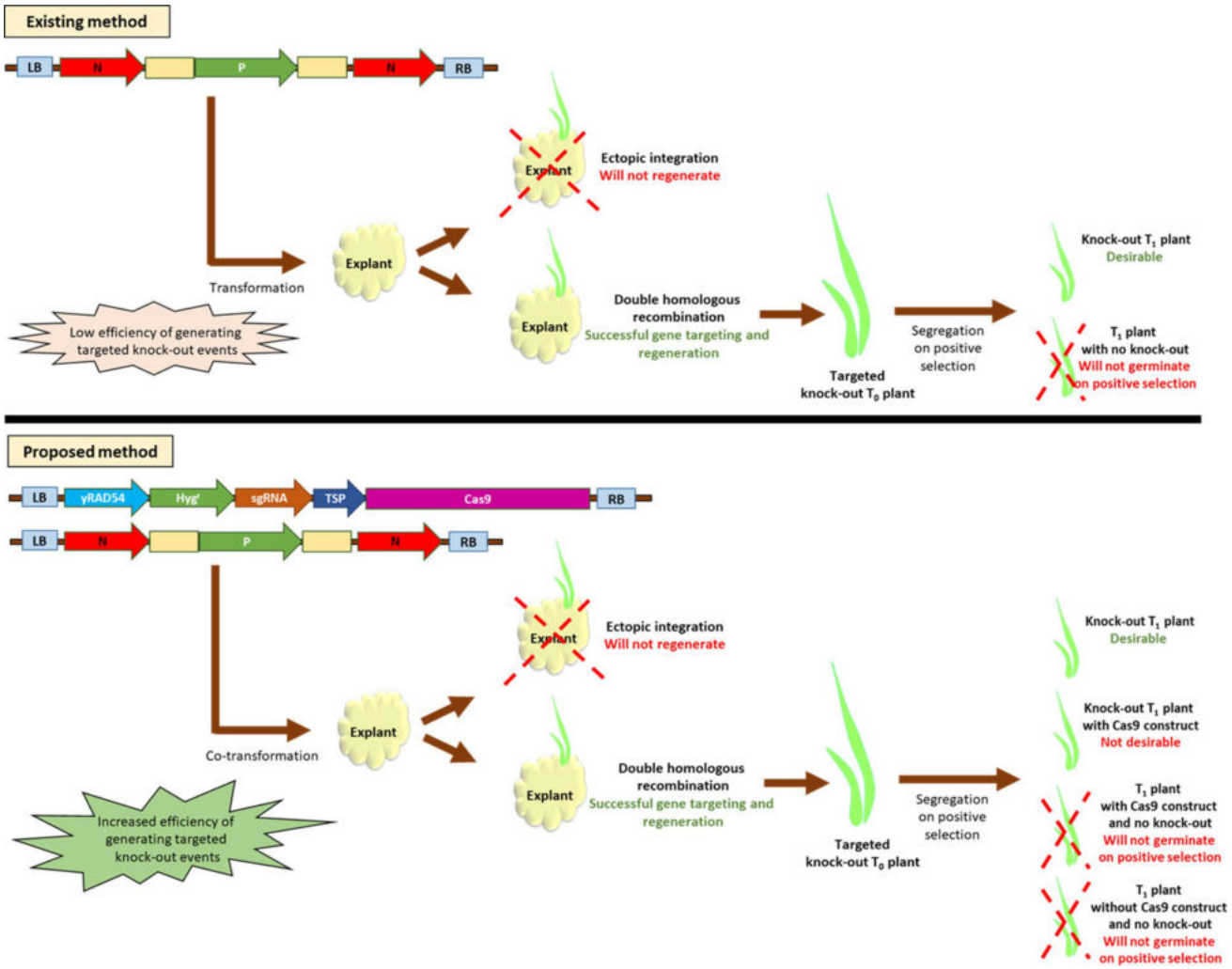

Fig. 6 Strategy proposed for gene targeting by co-transformation using T-DNA constructs with Cas9 and regions of homology. Left border (LB), right border (RB), N (non-conditional negative selection marker), P (positive selection marker but not the same used in the Cas9 construct), yeast-derived *RAD54* gene (*yRAD54*), hygromycin resistance gene (*Hyg*^{r}), tissue-specific promoter (TSP)

not survive due to the non-conditional NSM. This strategy can thus segregate out the Cas9 cassette carrying T-DNA in the T_1 progenies.

Genome editing—recent advancement

Owing to the unwanted off-target mutations, CRISPR/Cas9 method of genome editing remained more of a laboratory experiment rather than being used for applications such as treating genetic disorders or crop improvement. One of the main reasons for the off-target mutations is the DSBs caused by Cas9. These DSBs are repaired by the cell's DNA repair machinery and this process is often accompanied by micro-deletions and insertions, thereby leading to inadvertent mutations (Rees and Liu 2018). Towards this end, two new technologies namely base editing (Komor et al. 2016) and prime editing (Anzalone et al. 2019) have been developed, which are an upgradation to the existing CRISPR/Cas9 method. In both these methods, the CRISPR/Cas9 construct is modified such that it generates single strand breaks and not DSBs (Kantor et al. 2020). In base editing, as the name indicates, editing comprises of single base substitutions. The Cas9 for base editing is fused to an enzyme required for substitution such as cytidine deaminase that converts cytidine to uridine, thereby converting a C–G pair to T–A pair (Komor et al. 2016). Two categories of base editors, the cytosine base-editors (CBEs) and adenine base-editors (ABEs) have been used for base editing leading to any of the four possible transition mutations (Kantor et al. 2020). All types of base substitutions comprising of all transitions and transversions, as well as small deletions and insertions are possible with a more evolved technique, the prime editing (Kantor et al. 2020). Here, the Cas9 enzyme is fused

to a reverse transcriptase and a prime editing guide RNA (pegRNA) (Anzalone et al. 2019). Base editing and prime editing methods are of typical importance in treating genetic disorders due to alleles with point-mutations such as sickle cell anaemia and Tay–Sachs disease (Anzalone et al. 2019; Kantor et al. 2020). Prime editing has been successfully performed in crop plants such as rice and wheat (Lin et al. 2020), thereby promising it's potential to be used to make changes of agronomic importance. However, apart from DSB-induced mutations, occurrence of other off-target substitutions cannot be successfully ruled out by both these editing technologies and much research towards this end is to be performed (Kantor et al. 2020; Rees and Liu 2018).

Editions made in the genomes are mostly intended to bring about a variation in the expression of certain gene(s) and this is also analogous to the variations brought about by epigenetic factors (Papikian et al. 2019). Towards this end is the development of the concept of CRISPR/Cas9-based epigenome editing where, dCas9 (deactivated Cas9), which does not have its nuclease activity but can bind DNA, is deployed to regulate transcriptional expression if an activator or a repressor domain is fused with it (Tadic et al. 2019). In *Arabidopsis*, CRISPR/dCas9-containing modified p65-HSF-mediated transcriptional activation, increased the expression of *PAP1* and *AVP1* genes of about 2–3-fold and 2–5-fold, respectively (Park et al. 2017). Papikian et al. (2019) introduced a robust transcriptional activation system called CRISPR/dCas9 SunTag system which can specifically activate genes as well as transposable elements, if it was fused with a transcriptional activator VP64 that can target DNA methylation at specific loci when fused with a methyltransferase. In mammalian adipose cells, gene knockdown was achieved by targeted H3K27Me3 modification using CRISPR/dCas9 fused with histone methylation enzyme (EZH2) (Chen et al. 2019). Hence, CRISPR/dCas9-mediated epigenome modification can be a substitute for gene over expression as well as gene knock down, with an advantage that this tool can influence gene expression without altering the nucleotide sequences (Papikian et al. 2019; Park et al. 2017).

Conclusions

Whether to go for genetic engineering or genome editing is a decision that solely depends on the nature of application and this review has brought about a clear comparison of both these techniques that can aid in deciding on it. New techniques often emerge with a baggage of undesirable features which lead to the evolution of these techniques and, this review reveals how this rule holds true for genetic engineering and genome editing as well. Strategies need to be decided based on the amenability of individual crop type and the required improvement in it. With the logarithmic advancements in the genome editing such as base editing, prime editing, and epigenome editing, it is most likely that heritable and precise modifications devoid of undesirable off-target changes will soon lead to the development of non-transgenic crop types with improved qualities that can be brought to commercial use. Also to be considered is the point that genome editing is not the method of choice if the intensions are de novo over-expression of a transgene or complete knock-out of a gene. Interestingly, the genome editing reagents such as Cas9 can be used to enhance the efficiency of gene knock-out. Developments in various areas including CRISPR/Cas9-based genome editing, HR-based gene knock out and histone modification-based epigenetic modifications complement each other towards development of strategies for gene function identification, crop improvement and gene therapy.

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Author contributions JMS have conceived the idea of writing this manuscript and contributed much to design the content. She proposed the strategy to control the off-target effect of CRISPR/Cas9 complex. PPA has performed the literature search and contributed to writing this manuscript. She proposed the strategy for improving efficiency of homologous recombination based targeted knock out. Both the authors contributed to final review and acceptance of the manuscript.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

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