



RNA-guided multiplex genome engineering using *cas9* nucleases for crop improvement: A review

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ABSTRACT

Crop improvement through genome editing techniques has aroused substantial excitement among agricultural scientists as it offers opportunities for precise and predictable modification directly in elite cultivars or accessions which are not possible by time-consuming conventional breeding schemes. CRISPR-Cas9, a recently evolved gRNA-guided nuclease technology, is crucial in plant genome modifications which enable us in carrying out rapid and extremely precise mutations at pre-determined genomic sites by using guide RNA which is complementary to the target locus. This targeted DNA is then cleaved by Cas9 nuclease to generate double-strand breaks (DSBs), followed by subsequent DNA repair via non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms leading to the disruption of gene functions in plants. Multiplexed CRISPR-Cas9 exploits multiple gRNA sequences for simultaneous editing of multiple sites within the same genome, demonstrating its efficiency over traditional gene pyramiding. CRISPR/Cas9 is relatively convenient and easier to implement technology that has proven to be extremely efficient and versatile as compared to previously developed tools like ZFNs and TALENs and hence, it has been considered as a revolutionary technique in crop genome editing. The technique is continuously evolving to produce variants with its application in enhancing the agricultural performance of most crops. Moreover, plants developed by this technique have been considered superior to transgenic plants due to non-involvement of foreign DNA, hence, alleviating safety issues associated with GM plants. The current review briefly emphasizes on the potentiality of this game-changing technology in revolutionizing the basic plant genetic research and plant breeding.

Key words: CRISPR/Cas9, DNA repair, DSBs, Genome editing, Guide RNA, Transgenic plants

Production of crop cultivars with improved quality is very much necessary to encounter the potential demands of the exponentially growing world population. Success of classical plant breeding rests on the availability of natural genetic variation and introgression of any trait into an elite genetic background but non-availability of beneficial alleles in nature consequently limits the speed of improving crop performance. Acquainting new worthy alleles is possible by arbitrary mutagenesis, but is costly, inefficient along with the laborious screening of bulk populations for desirable phenotypes. Countless generations of making selections among mutants went in advancing nearly every “traditional” crop. To enhance the frequency of genetic modifications, various strategies have been developed in the recent years. Availability of gene and genomic sequence information of important crops has entirely transformed our vision and interpretation of their genomic organizations and evolution patterns and hence, various genome based tools could be

designed according to plant breeders’ need of revolutionizing crop improvement. Substantial development of molecular techniques such as molecular-based marker aided selection, antisense RNA (Mol *et al.* 1990), virus-induced gene silencing (Baulcombe 1999), RNA interference (Smith *et al.* 2000b), transposon/T-DNA tagging and targeting induced local lesions in genomes (TILLING) have possibly opened the way of manipulating metabolic pathways in crops by targeting a specific gene sequence. However, such achievements are not feasible by conventional breeding.

Precise and straightforward method of modifying the function of target gene directly in the endogenous framework of virtually any organism of choice has become rather easy by means of genome editing tools. Genome editing or genome editing with engineered nucleases (GEEN), literally denotes to the modification of targeted nucleotide sequences of a genome with engineered nucleases in cultured cells or plants for functional genomics and crop improvement. Targeted gene editing for either correcting or improving traits of economically important crops can significantly help in achieving global food security. Transgenic or genetically modified (GM) crops with improved characteristics also hold the promise of food safety but unfortunately pose unsubstantiated concerns over health and environmental

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safety issues however, crops modified by genome editing are considered as non-transgenic and hence proves its enormous potentiality for coming future.

Previously, artificial nucleases based targeted genome editing approaches, viz. zinc-finger nucleases (ZFNs; Kim *et al.* 1996), transcription activator-like effector nucleases (TALENs, Christian *et al.* 2010) were known but, then the groundbreaking technology for highly targeted genome editing based on the RNA-guided engineered nucleases, CRISPR/Cas acronym for clustered regularly interspersed short palindromic repeats, was emerged in 2013, holding a potential of speeding up basic research as well as crop improvement programs. This has been found more excellent owing to its simplicity, efficiency, and versatility in several biological research fields.

CRISPR/Cas9: from adaptive immunity to genome editing

To accomplish efficient genome editing by incorporating site specific DSBs, different classes of DNA binding proteins, viz. meganucleases from microbial mobile genetic elements (Smith *et al.* 2006), zinc finger nucleases (ZFNs) (Urnov *et al.* 2005), transcription activator-like effectors nucleases (TALENs) from *Xanthomonas* bacteria (Christian *et al.* 2010) and lastly, the gRNA-based CRISPR/Cas9 (Cong *et al.* 2013, Mali *et al.* 2013a) have been developed so far. CRISPR/Cas arrays were primarily discovered as a sort of type II adaptive immunity mechanisms against invading pathogens in *Escherichia coli* bacteria in 1987 (Ishino *et al.* 1987) later, RNA-based mechanism of this immune system was established by Brouns *et al.* (2008). In 2013, the CRISPR/Cas9 was demonstrated to have potential of reprogramming the target DNA sequence by several researchers (Feng *et al.* 2013, Li *et al.* 2013, Nekrasov *et al.* 2013, Shan *et al.* 2013). CRISPR/Cas9 comprises small guide RNA (gRNA) sequence (usually 20 nucleotides) formed by merging trans-activator crRNA (tracrRNA) with crRNA (Jinek *et al.* 2012) which distinguishes the target loci in the genome based on gRNA:DNA complementation rule. Cas9 endonuclease act upon target loci of the genome for precise cleavage resulting in site specific DSBs.

Subsequently, such DSBs are restored through error-prone DNA repair mechanisms like NHEJ or HDR *in vivo*, to drive site-specific DNA sequence modifications involving either insertion, deletion or some mutations (Fig 1). RNA-guided DNA endonuclease Cas9 (CRISPR/Cas9) is a simple and multipurpose tool that has resulted in groundswell of genome editing research and hence, recently become known as the 'CRISPR craze' in the world of biological research (Pennisi 2013). More interestingly, the multiple gRNAs could also be used for high-efficiency multiplexing in order to perform multiple cleavage events simultaneously (Cong *et al.* 2013, Mali *et al.* 2013, Bortesi and Fischer 2015).

A comparison of CRISPR/Cas9 with other techniques

The emerging techniques of targeted genome editing offer exciting opportunities for all aspects of biology. The CRISPR/Cas9 systems supersede all previously known nuclease based techniques such as meganuclease, ZFNs and TALENs owing to much easier genome manipulation and wider applications. Meganuclease, ZFNs and TALENs necessarily recognize specific DNA sequences through protein-DNA interactions followed by DNA cleavage. The meganuclease have not been extensively adopted as a genome editing tool due to poor interaction of meganuclease protein residues with the target DNA whereas, in case of ZFNs, constructing ZFNs are little costly and involving complex zinc fingers-DNA interactions which reduce context-dependent specificity (Sander *et al.* 2011) and thus, hindering their utility in several plants (Sanjana *et al.* 2012). On the other hand, assembling TALENs is comparatively easier due to one-to-one recognition rules among polypeptide repeats and nucleotide sequences which encourage homologous recombination *in vivo* (Holkers *et al.* 2013) and hence, TALENs provide a hope of easier genome manipulation but at the same time face challenge of utilizing complex repetitive polypeptide domains in TAL proteins. Thus, owing to the technical hitches of sophisticated protein engineering for every site, context-dependent specificity necessities and increased costs, the utility of meganuclease, ZFNs and TALENs technologies remains very restricted in

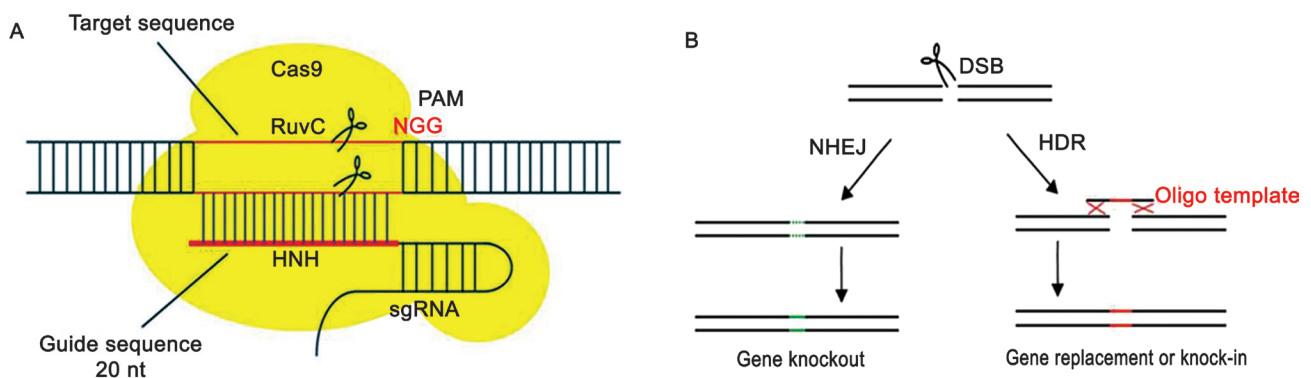


Fig 1 A sketch of CRISPR/Cas9 system. (A) sgRNA for identification of the target gene, Cas9 domains for cleaving the target sequence, (B) DSB repair through imprecise NHEJ resulting in a gene knockout mutation and through HDR resulting in gene replacement or knock-in. (PAM-protospacer adjacent motif; sgRNA-single guideRNA; DSB-double-strand break; NHEJ-non-homologous end-joining; HDR-homology-directed repair).

Table 1 Comparison of CRISPR/Cas9 with ZFNs and TALENs

Features	ZFNs	TALENs	CRISPR/Cas9
Site recognition depends on	Protein and DNA recognition	Protein and DNA recognition	RNA and DNA recognition
Components involved	ZFN and FokI nuclease	TALE and FokI nuclease	gRNA and Cas9 protein
Efficiency in genome editing	Complex with limited utility	High but variable	Simple, accessible and versatile
Endonuclease involved	<i>FokI</i>	<i>FokI</i>	<i>Cas9</i> protein
Target nucleotide seq. length	18-36 bp	30-40 bp	20 bp
Mutation frequency (%)	10	10-20	>20
off-targeting frequency	High	High	low
Multiplexing	No	No	Common
Cost incurred	Costly	Costly	Cost effective

ZFNs- Zinc-finger nucleases; TALENs- transcription activator-like effector nucleases; CRISPR/Cas- clustered regularly interspersed short palindromic repeats.

plant genome editing (Voytas 2013). In contrast, CRISPR/Cas9 has evolved as technically more simple, accessible, cost effective and versatile tool for genome manipulation. It involves a single monomeric protein and a 20-nt long gRNA sequence for conferring targeted DNA sites (Jinek *et al.* 2012) followed by Cas9 protein mediated cleavage. CRISPR/Cas9 is generally more efficient in inducing high frequency of targeted mutations. The most practical achievement of CRISPR/Cas9 over meganuclease, ZFNs and TALENs is the ease of multiplexing, i.e. modifying multiple traits simultaneously, besides the ability to differentially target either DNA or RNA (Table 1).

Validation of newly established CRISPR/Cas9 vector system

In case of monocot and dicot plants, T-DNAs having both Cas9 and sgRNA expression cassettes are incorporated into the plant genomes using *Agrobacterium*-mediated transfections of callus, immature embryos, or other tissues whereas, *Agrobacterium*-mediated floral dip transformation is quite commonly used for genome editing in *Arabidopsis*

species. Once the transformation is completed, the editing efficiency of CRISPR/Cas9 has to be validated which requires detection of targeted mutations followed by sequencing of the mutated sites (Fig 2). Strategies like use of reporter genes, such as those encoding β -glucuronidase or fluorescent proteins (Mao *et al.* 2013), obtaining mutated sequences based on cleavage pattern of restriction sites by Cas9/sgRNAs (Voytas 2013), use of high-throughput sequencing of the whole genome especially for identifying possible off-target mutations (Fauser *et al.* 2014), detection of differential migration rates through single-strand conformation polymorphism (SSCP) method (Zheng *et al.* 2016) and using the restriction enzyme digestion method (Shan *et al.* 2013) can be followed to detect mutations induced by CRISPR/Cas9.

Applications in crop genetic improvement

The fluctuating climate and rising global population has challenged our ability to produce ample food. Advancing breeding techniques for novel traits with respect to the improved quality and adaptation is obligatory to safeguard

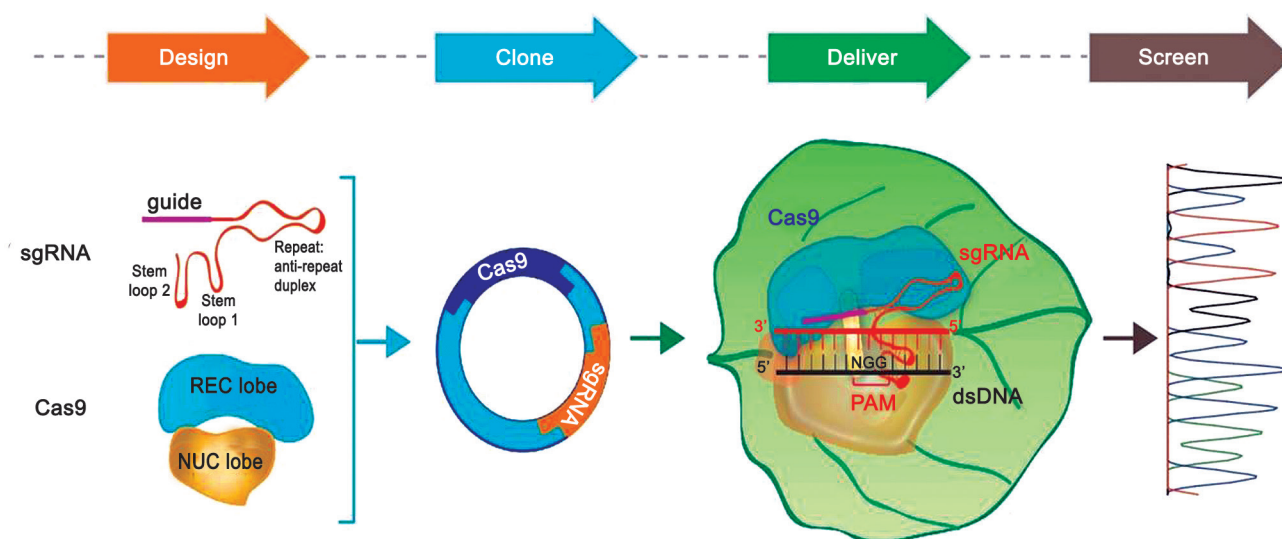


Fig 2 Schematic diagram representing basic steps of carrying out CRISPR/Cas9 based genome editing.

sustained food production. Acquaintance with reverse genetics and bioinformatics tools can quicken the genomics based improvement of crop plants. CRISPR/Cas9, due to its high efficiency and simplicity, is being widely explored by introducing precise and predictable gene mutations, up regulating or down regulating the gene expressions directly in the leading genetic background of several important plant species (Fig 3). In addition, it has accessibility for gene correction and gene knockouts for elucidating the function of single or multiple gene targets in plants as it can modify genomes in an exact and expectable fashion. It also facilitates suppression of viral infections in plants, for many of which there is no worthy method of control. Moreover, multiplexing ability of CRISPR/Cas9 makes possible simultaneous accumulation of multiple traits into a single crop with a low risk of segregation, which is quite difficult to achieve using classical breeding or conventional genetic engineering.

CRISPR/Cas9 has been successfully employed in wide array of monocot and dicots plant species such as, *Sorghum bicolor*, *Zea mays*, *Oryza sativa*, *Glycine max*, *Brassica oleracea*, *Citrus sinensis*, *Nicotiana tabacum* and *Triticum aestivum* for improving various traits (Table 2).

In *Sorghum bicolor*, a gene (*DsRED2*) was targeted with 33% mutation rate using *Agrobacterium*-mediated transformation (Jiang *et al.* 2013). A locus *ZmIPK*, encoding for antinutritional phytic acid biosynthesis, was mutated with a rate of 16.4-19.1% using *CaMV 35S* as Cas9 and *ZmU3* as sgRNA promoter (Liang *et al.* 2014) whereas, loci *LIG1*, *MS26*, *MS45*, and *ALS1* were targeted with

0.13–3.9% mutation with the CRISPR/Cas9 system in *Zea mays* (Svitashev 2015). Xie and Yang (2013) transiently co-expressed Cas9 and gRNA in *Oryza sativa* to target the *OsMPK5* gene encoding for stress-responsive rice mitogen-activated protein kinase and estimated a mutation efficiency of 3-8% using *Agrobacterium* based RNA-guided genome-editing. Li *et al.* (2015) have mutagenized *DD20* and *DD43* loci of *Glycine max* with 59% and 76% frequencies respectively, using *GmEF1A2* and *GmU6* promoters delivered by particle bombardment whereas, Du *et al.* (2015) targeted *GmPDS11* and *GmPDS18* genes (hairy root) with 11.7–18.1% rate by *Agrobacterium*-mediated transformation, both justifying potentiality of CRISPR/Cas9 system for precise gene targeting in soybean. Lawrenson *et al.* (2015) edited *BolC.GA4.a* locus (dwarf phenotype), an ortholog of *Arabidopsis* GA4a gene with indel frequency of 10% in *Brassica oleracea*. Jia and Wang (2014) successfully investigated transient expression targeting *CsPDS* (*Phytoene desaturase*) locus with a mutation frequency of 3.9% via *Xanthomonas citri* subsp. *citri* mediated agro-infection in *Citrus sinensis* with no off-target effects. Yin *et al.* (2015) used CRISPR/Cas9 based virus-mediated genome editing (VIGE) for transient expression targeting *NbPDS3* and *NbIspH* genes responsible for photo-bleaching phenotype in *Nicotiana tabacum*. Three homoeoalleles, viz. *TaMLO-A*, *TaMLO-B*, *TaMLO-D* conferring resistance to powdery mildew have been targeted simultaneously with 23-38% mutation rate using Cas9/sgRNA vectors delivered by particle bombardment to protoplasts in hexaploid *Triticum aestivum* (Wang *et al.* 2014). These successful studies

Table 2 Applications of the CRISPR/Cas9 system in plants

Plant species	Targeted gene	Cas9 promoter	gRNA promoter	Mutation rate (%)	References
<i>Arabidopsis thaliana</i>	<i>ADHI</i>	<i>PcUbi4-2</i>	<i>AtU6-26</i>	42.8	Schim <i>et al.</i> (2014)
	<i>TRY, CPC, ETC2</i>	$2 \times 35S$	<i>U6-26, U6-29</i>	42-90	Xing <i>et al.</i> (2014)
<i>Brassica oleracea</i>	<i>BolC.GA4.a</i>	<i>35S</i>	<i>U6-26</i>	10	Lawrenson <i>et al.</i> (2015)
<i>Citrus sinensis</i>	<i>CsPDS</i>	<i>CaMV 35S</i>	<i>CaMV 35 S</i>	3.2-3.9	Jia and Wang (2014)
<i>Glycine max</i>	<i>GmDD20, GmDD43</i>	<i>GmEF1A2</i>	<i>GmU6</i>	59-76	Li <i>et al.</i> (2015)
	<i>GmPDS11 and GmPDS18</i>	<i>ZmUbi</i>	<i>AtU6, GmU6</i>	11.7–18.1	Du <i>et al.</i> (2015)
<i>Hordeum vulgare</i>	<i>HvPM19</i>	<i>35S</i>	<i>U6-26</i>	10–23	Lawrenson <i>et al.</i> (2015)
<i>Nicotiana tabacum</i>	<i>NtPDS, NtPDR6</i>	$2 \times 35S$	<i>AtU6-26</i>	81.8–87.5	Gao <i>et al.</i> (2015)
<i>Oryza sativa</i>	<i>OsMPK5</i>	<i>CaMV 35S</i>	<i>OsU6-2</i>	3-8	Xie <i>et al.</i> (2013)
	<i>OsSWEET11, OsSWEET14</i>	<i>CaMV 35S</i>	<i>OsU6</i>	N/A	Jiang <i>et al.</i> (2013)
	<i>OsBEL</i>	$2 \times 35S$	<i>AtU6-26</i>	2-16	Xu <i>et al.</i> (2014)
	<i>OsROC5, OsSPP, OsYSA</i>	<i>CaMV 35S</i>	<i>AtU6</i>	4.8–75	Feng <i>et al.</i> (2013)
	<i>OsPDS, OsDEP1</i>	$2 \times 35S$	<i>OsU3</i>	33-38	Shan <i>et al.</i> (2014)
<i>Sorghum bicolor</i>	<i>DsRED2</i>	Rice Actin 1	<i>OsU6</i>	33	Jiang <i>et al.</i> (2013)
<i>Triticum aestivum</i>	<i>TaMLO-A, TaMLO-B, TaMLO-D</i>	<i>Ubi</i>	<i>TaU6</i>	23-38	Wang <i>et al.</i> (2014)
	<i>TaLOX2</i>	$2 \times 35S$	<i>TaU6</i>	45	Shan <i>et al.</i> (2014)
<i>Vitis vinifera</i>	<i>IdnDH</i>	<i>35S</i>	<i>AtU6</i>	Upto 100	Ren <i>et al.</i> (2016)
<i>Zea mays</i>	<i>ZmIPK</i>	$2 \times 35S$	<i>ZmU3</i>	16.4-19.1	Liang <i>et al.</i> (2014)
	<i>PSY1</i>	<i>ZmUbi2</i>	<i>ZmU6</i>	0.18–78.83	Zhu <i>et al.</i> (2016)
	<i>LIG1, MS26, MS45, ALS1</i>	<i>Ubi</i>	<i>ZmU6</i>	0.13–3.9	Svitashev (2015)

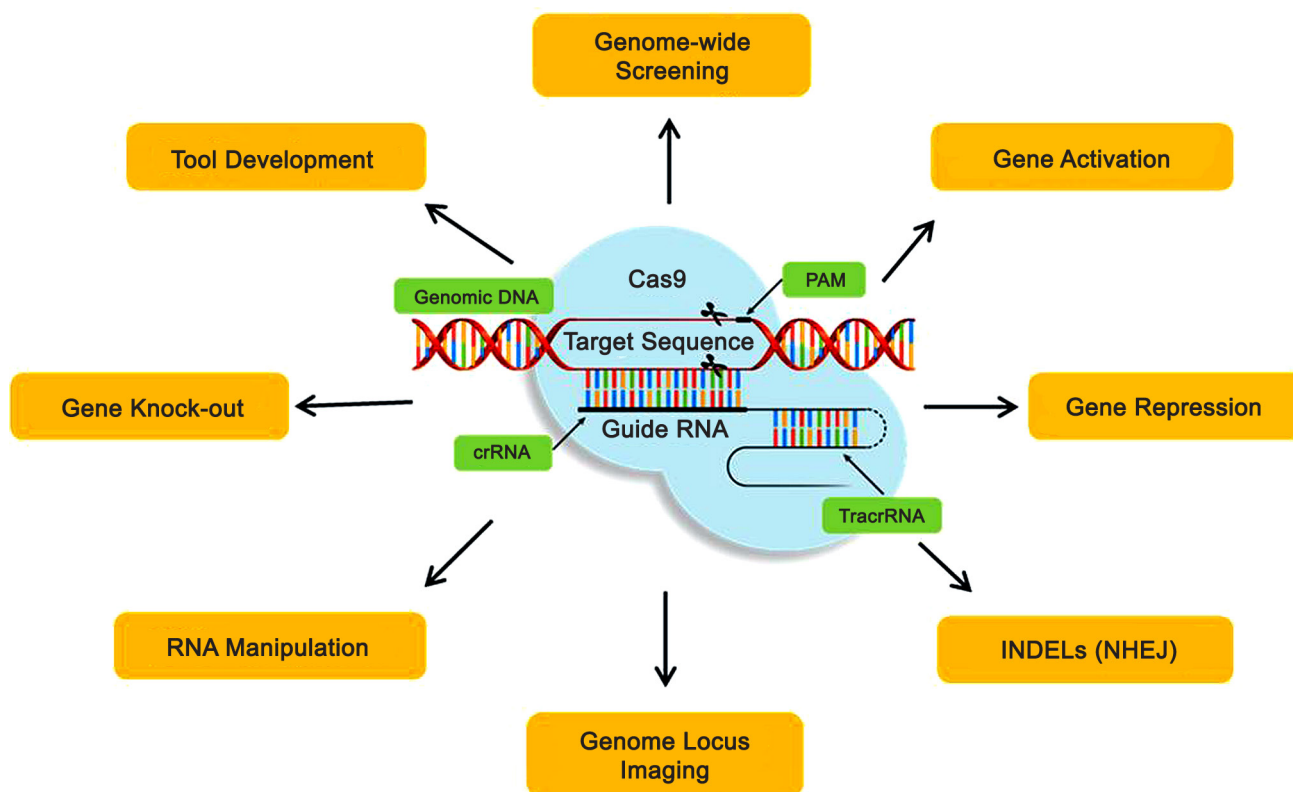


Fig 3 Potential applications of CRISPR/Cas9 systems in crop improvement.

undoubtedly demonstrate that the efficiency of CRISPR/Cas9 system in creating precise and heritable modifications in several plant species for faster crop improvement.

Technical pitfalls in using CRISPR/Cas9 system

Genetically engineered (GE) crops provide an opportunity for crop improvement but on the other hand face severe GM regulations. USDA has arbitrated that plants produced by genome editing technology will not be considered as GM (Waltz 2016a). For example, genetically modified mushrooms (a fungus, not a plant) with less browning tendency were developed through CRISPR-based mutation of a polyphenol oxidase gene and thus judged not to require GM regulation (Waltz 2016b). CRISPR/Cas9 is a revolutionary genome editing technology holding outstanding potential of fastening the crop improvement programs but also faces some of criticisms like appearance of unpredicted off-target mutations as reported in ZFNs and TALENs techniques. The presence of inappropriate ratio of Cas9 to sgRNA concentrations could be one of the reasons (Hsu *et al.* 2013, Pattanayak *et al.* 2013) for such unexpected mutations. Li *et al.* (2013) addressed this problem and optimized such mutations in *Arabidopsis* by trying 1:1 ratio of Cas9:sgRNA using *AtPDS3* and *AtFLS2* genes. Promiscuous PAM sites were also reported to have off-target effects (Sternberg *et al.* 2014) which could possibly be reduced by engineering Cas9 proteins with longer PAMs as well as by means of bioinformatics tools like E-CRISPR and CasOT for whole genome sequence information (Xie *et al.* 2015). Careful selection

of appropriate promoters for Cas9 as well as sgRNAs is essential for minimizing the risk of unwanted genome modification. For instance, 35S promoter of CaMV in dicots and both 35S and *Ubi* in monocots are preferred for Cas9 expression, whereas *U6*, *OsU6* and *TaU6* promoters are considered superior for sgRNA (Shan *et al.* 2014, Mikami *et al.* 2015). However, further information is required before drawing any conclusions about the off-target mutation rates in plants.

Conclusions and future prospects

CRISPR-Cas9 has triggered a revolution in biological research across the world likely due to the simplicity, high efficiency and versatility of the system. It is being extensively exploited as a powerful technique for determining gene functions and genetic modification in economically important plant species. It has outstanding potential of manipulating large genomic regions, transgene-free genetic modification, and diversification of breeding resources and discovery of gene functions. The rapid progress in developing *Cas9* into a set of tools for genome editing and gene-targeting has been remarkable. In coming future, the usefulness of CRISPR/Cas9 can also be expected in the commercial production of specific metabolites or proteins via site-targeted insertion of transgenes in the fields of metabolic engineering and molecular farming. Though the CRISPR/Cas9 is excellent in site-specific genome editing, there are still certain challenges like minimizing the frequency of off-target mutations and elucidating its precise mechanism and further improvement in our understanding

towards the system in order to diversify its applications, can be certainly expected to be addressed in near future.

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