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REVIEW ARTICLE

Genome editing for targeted improvement of plants

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Abstract Recent advances in gene/genome editing technologies, such as engineered meganucleases (EMNs), zinc finger nucleases (ZFNs), transcription activator-like effector nuclease (TALENs) and clustered regularly interspaced palindromic repeats (CRISPR/Cas9) allowed researchers to precisely modify or mutate genes. These genome editing tools make double-strand breaks (DSB) in DNA and then repair it by employing error-prone non-homologous end joining (NHEJ) or homology directed repair (HDR) mechanism which leads to mutation in specific location in genome. Since these editing techniques are simple to use, highly efficient and specific as compared to earlier mutation methods, their use in plant biology research is increasing rapidly to enhance biotic and abiotic stress tolerance, increased nutritional value and new trait development. Here, we review the applications of EMNs, ZFNs, TALENs and CRISPR/Cas9 in various plants (cereals, vegetable, oil crops and fruits), comparison of genome editing methods and their biosafety regulations.

Keywords ZFN · TALEN · CRISPR/Cas9 · Meganuclease · Biosafety

Introduction

The ability to modify genomes in a site-specific manner has the potential to transform plant biological research by addressing the basic questions relating to plant growth, development and responses to the environment. Therefore, continuous efforts were being taken for getting desired mutants and various methods have been evolved with the time. First generation mutagenesis methods were non-specific and cause random mutations in genome. The random mutagenesis was achieved with the help of mutation breeding, direct gene transfer, transposons and *Agrobacterium* mediated genetic modifications. Use of physical and chemical mutagenesis in breeding is characterised by large population size for screening, very low efficiency, time consumption and hazardous nature of mutagens. Further screening of mutation is also cumbersome process that requires sequencing, tilling, denaturing high pressure liquid chromatography, etc. (McCallum et al. 2000; Caldwell et al. 2004). For transposons and T-DNA insertion, target DNA sequence with high homology is required so that homologous recombination mechanism works well. T-DNA insertion lines of many crop plants thus produced help us to study and understand various mechanism and functions in plant biology. It is also accompanied by some limitations, such as multiple insertions, vector sequences insertion, and rearrangement in chromosome. (De Buck et al. 1999; Tax and Vernon 2001). Second generation mutagenesis methods were more specific and efficient. They are based on the recombinase (Cre-LoxP and Flp-FRT systems), rare cutting restriction nucleases such as homing/meganuclease based methods. Application of Cre-lox system for gene insertion and excision was reported way back in 1990s (Russell et al. 1992; Albert et al. 1995). They largely used in production of marker free transgenic plants, gene stacking and gene cassette replacement (Nanto et al. 2009; Petolino et al. 2010; D'Halluin et al. 2013). The popular reverse genetic techniques; micro-RNAs and RNA interference (*RNAi*) has been commonly used for gene functional studies in plants (Reddy et al. 2012; Khandagale

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et al. 2016). At the same time it has some limitations, such as variation in silencing level, not stable over several generations and the off-target effect.

Meanwhile, through next generation sequencing, sequence information of large number of plants became available. This DNA sequence information is used for the development of newer and more precise and efficient genome editing tools compared to the traditional methods. Third generation of mutagenesis was characterised by very rapid and precise genome editing tools which includes zinc finger nuclease (ZFN) (Porteus and Carroll 2005), transcription activator-like effector nucleases (TALEN) (Li et al. 2011) and clustered regularly interspaced palindromic repeats/CRISPR-associated system (CRISPR)/(Cas) (Barrangou 2012). These targeted genome editing is characterised by creation of a double-stranded breaks (DSBs) at specific position of target locus to be mutated (Carroll 2011). These DSBs are highly prone to the genetic mutations during repair process. Non-homologous end joining (NHEJ) and homology directed repair (HDR) pathways are involved in the repair of these DSBs in DNA. NHEJ led to random indels of varying lengths which finally cause frame shift mutations in coding as well as regulatory sequences of DNA (Cristea et al. 2013). In case of HDR, sequence homologous to the DSB must be available which can be exogenously supplied by donor DNA (Bortesi and Fischer 2015). Therefore, these repair pathways exploited for precise gene editing or targeted genome editing. Thus, creation of DSBs is perquisite and essential step in gene editing approach. With the discovery of TALEN and CRISPR/cas9 system, there was explosion of research papers describing the application of precise gene editing from last 2 to 3 years. Though precise gene editing is a new field, few review papers have been published describing the molecular mechanism and potential applications of various gene editing tools in plants such as ZFNs (Weinthal et al. 2010), TALEN (Wright et al. 2014), CRISPR/Cas9 (Bortesi and Fischer 2015). Various steps involved in the targeted genome engineering, viz. selection of a target nucleotide sequence in the genome; generation of a nuclease construct aimed at the selected target; delivery of this construct to the cell nucleus; and analysis of produced mutations were elaborated by Sander and Joung (2014). Various software and online resources are available for designing of these gene editing tools were depicted in Table 1.

As very little or no foreign DNA is inserted during gene editing process, scientists are claiming that it may not come under traditional definition of transgenic plant, and hence there is no need of stringent biosafety regulations for release of gene edited plants (Woo et al. 2015). Thus, biosafety regulation has become a matter of debate in case of genome edited plants. Recently, Swedish Board of Agriculture stated that CRISPR/Cas9 modified plants does not come under the EU definition of genetically modified organism (GMO). Hartung and Schiemann (2014) and Wolt et al. (2015) surveyed the status of biosafety regulation of plants modified using gene editing techniques and emphasized on development of regulatory policy for genome edited crops.

Present review will discuss the use of genome editing tools for highly precise and desired modifications in plants, comparison of various genome editing tools as well as biosafety concerns associated with the genome editing technology.

Tools used in precise genome editing

Homing/meganuclease

Meganucleases are kind of endonucleases which are characterised by their large recognition site of about 12–40 bp long. Such a long recognition site makes them most specific restriction enzyme. They are also called as homing endonucleases. Among the various families of meganuclease LAGLIDADG family is well understood and widely exploited for gene editing purpose. *I-SceI* (*Saccharomyces cerevisiae*), *I-CreI* (*Chlamydomonas reinhardtii*) *and I-DmoI* (*Desulfurococcus mobilis*) are a few well characterised meganucleases of LAGLIDADG family. They either form homodimers of two identical subunits (each 160–200 amino acid) or two tandem monomers joined by a linker to form single peptide (Stoddard 2011). α/β fold within structure of meganuclease could be modified to generate engineered meganuclease with desired specificity (Silva et al. 2011). The molecular structure of *I–SceI* is a complex thus makes it more challenging for re-engineering for customized applications, where as *I–CreI* is found to be more suitable for customized protein engineering (Arnould et al. 2007) (Fig. 1). Puchta et al. (1993), Salomon and Puchta (1998) pioneered the use of meganuclease in the study of DNA repair and gene integration. Further, Kirik et al. (2000) demonstrated that DSB repair by NHEJ led to knock out of genes in *Arabidopsis* and tobacco. To edit the gene of interest, we need to engineer the DNA recognition sites of the meganucleases. But DNA-binding domains and catalytic domain of meganucleases are not separated from each other, and therefore it becomes difficult to re-engineer the meganucleases compared to other genome targeting tools (Taylor et al. 2012). Continuous efforts of protein engineer resulted in a few successful attempts of gene editing in plants using engineered meganucleases such as *Arabidopsis* (Roth et al. 2012), Cotton (D'Halluin et al. 2013) and Maize (Djukanovic et al. 2013). Due to difficulty in manipulation of meganucleases, researchers were

Table 1 Web resources available for designing of targeted genome editing tools

No.	Database	Purpose	Website
ZFNs			
1	ZFNGenome v2.0	A comprehensive resource for locating ZFN target sites	http://bindr.gdcb.iastate.edu/ZFNGenome/
2	ZifBASE	Provides an extensive collection of various natural and engineered ZFP	http://omictools.com/zifbase-tool
3	Zinc Finger Database (ZiFDB)	Serves as a resource engineering ZFAs for use as sequence-specific DNA-binding reagents	http://omictools.com/zinc-finger-database-tool
4	Zinc Finger Tool	Design and target selection for ZFN	http://www.scripps.edu/barbas/zfdesign/zfdesignhome.php
5	EENdb	A database and knowledge base of ZFNs and TALENs for endonuclease engineering	http://eendb.zfgenetics.org/
TALENs			
6	E-TALEN	A web tool to design TALENs for genome engineering	http://www.e-talen.org/E-TALEN/
7	TALEN Library	A library of TAL effector nucleases spanning the human genome	http://www.talenlibrary.net/
8	TALENoffer	Genome-wide TALEN off-target prediction	http://galaxy2.informatik.uni-halle.de:8976/
9	SPATA	Allows researchers to design highly active TALEs and TALENs to a target DNA sequence	http://bao.rice.edu/Research/BioinformaticTools/TAL_targeter.html
10	TALE-NT 2.0	Provide software, protocols, tools for designing, evaluating, and assembly custom TALE constructs	https://tale-nt.cac.cornell.edu/
CRISPR			
11	CHOP CHOP	A CRISPR/Cas9 and TALEN web tool for genome editing	https://chopchop.rc.fas.harvard.edu/
12	CRISPRs web server	A gateway to publicly accessible CRISPRs database and software	http://crispr.u-psud.fr/
13	Crass: The CRISPR Assembler	A program that searches through raw metagenomic reads for CRISPRs	http://ctskennerton.github.io/crass/
14	CRISPRTarget	Predicts the most likely targets of CRISPR RNAs	http://bioanalysis.otago.ac.nz/CRISPRTarget
15	CRISPRdirect	Prediction of off-targeting	http://crispr.dbcls.jp/
16	CRISPR-P	A web tool for gRNA design of CRISPR-system in plants	http://cbi.hzau.edu.cn/crispr/
17	CRISPR-RGEN	Provide tools such as RGENs, Cas-OFFinder, Microhomology-Predictor, Cas-Designer and library: Cas-Database	http://www.rgenome.net/
18	CRISPR Multitargeter	Find unique target sequence from multiple genes	www.multicrispr.net/
19	sgRNA Designer	It ranks and picks candidate sgRNA sequences for the targets provided, and thus maximizes on target activity	www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design
20	CRISPRseek	Genome wide search for off-targets, score, rank sequence and specify target and off-targets	www.bioconductor.org/packages/release/bioc/html/CRISPRseek.html

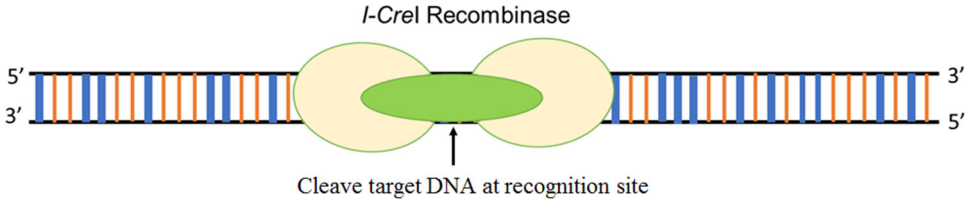

Fig. 1 Schematic representation of *I*-CreI meganuclease inducing break in double-stranded target DNA. *I*-CreI recombinase attaches to its target DNA at long recognition site and catalytic domain (shown in *green colour*) cleaves the DNA at specific target. Finally, mutations occur during the endogenous DNA repair process

working on other simple, efficient and precise alternatives for the gene editing which gave rise to ZFN, TALEN and CRISPR.

ZFNs

Engineered Zinc finger nuclease has become one of the powerful tools for targeted genome editing by making double-stranded break in genome. Zinc fingers are the first DNA-binding proteins which were engineered to target specific DNA sequence to make double-stranded breaks with the help of *Fok*1 nuclease (Porteus and Carroll 2005). NHEJ pathway is used to repair DSBs which is error prone leads in insertion and deletions (indels) during repair process. Structurally ZFNs monomer consists of two domains: *DNA-binding domain*, typically contain between three and six individual zinc finger repeats and each zinc finger repeat can recognize between nine and 18 base pairs, and *DNA-cleavage domain,* it is non-specific cleavage domain of type IIs restriction endonuclease *Fok*1, it is typically used as the cleavage domain in ZFNs. Two ZFN monomers attach their target sequences in reverse configuration flanking a 5–6 bp sequence of target DNA (Carroll et al. 2006). Dimerized *Fok*1 domains cut DNA within this flanking sequence (Fig. 2). DNA-binding domain of ZFNs generally recognizes sequence of 24–30 bp, and thus has unique or rare target sites in the genome. Therefore, specificity of ZFNs made their wide application in precision gene editing in variety of organisms. Modular assembly (Sander et al. 2007) and Oligomerized Pool ENgineering (OPEN) (Maeder et al. 2008) are two widely used methods for construction of engineered ZFNs. Use of ZFNs was initially demonstrated in model plants Arabidopsis (Lloyd et al. 2005) and tobacco (Wright et al. 2005) which ascertained scope of engineered nuclease in gene targeting and ignited the researchers for further application this technology in other crop plants. Few years later several reports had come describing the use ZFN in gene targeting in various model plants and crops plants: Arabidopsis (de Pater et al. 2013), tobacco (Townsend et al. 2009), maize (Shukla et al. 2009; Ainley et al. 2013), etc. Application of ZFN in gene targeting of different plants is described briefly in subsequent sections. ZFNs have limitation in binding to any DNA sequence due to context dependent nature of DNA as one zinc finger binds to 3 nucleotide in target DNA and off-target effect is also one limitation of ZFN.

TALEN

TALEN (transcription activator-like effector nucleases) system for precise genome editing is named as method of year by nature methods in 2011 (Baker Becker 2012). TALENs are transcription activator-like effectors (TALEs) fused to the non-specific cleavage domain of the *Fok*I endonuclease for binding target DNA. These TALEs are type III effector proteins secreted by phytopathogenic bacteria *Xanthomonas spp.* which attaches to DNA by the virtue of DNA-binding domain to alter the gene expression pattern in host plant (Boch et al. 2009). Here, DNA-binding domain has comprised conserved repeats of 34 amino acid and each repeat recognizes a single nucleotide in target DNA, whereas in case of ZFN, each repeat recognizes 3 nucleotides, and thus TALEN designing is comparatively easier and amenable to programming. Target recognition specificity of TALEs repeat is determined by the presence of repeat variable di-residue (RVD) at 12 and 13 positions of each repeat (Moscou and Bogdanove 2009). Various TALENs designing criteria and precautions for selection of target site, assembly of TALENs and their cloning were nicely elaborated by Bogdanove and Voytas (2011). With the available technology and kits one can assemble TALENs in less than 10 days. A dimerized *Fok*I cleavage domain is responsible to make DSB, therefore two TALENs were used to target the DNA strands in an opposite orientation with proper spacing of 12–30 bp (Li et al. 2011) (Fig. 3). *Fok*I domain self-dimerization is essential for that two separate TALE nucleases (TALEN)

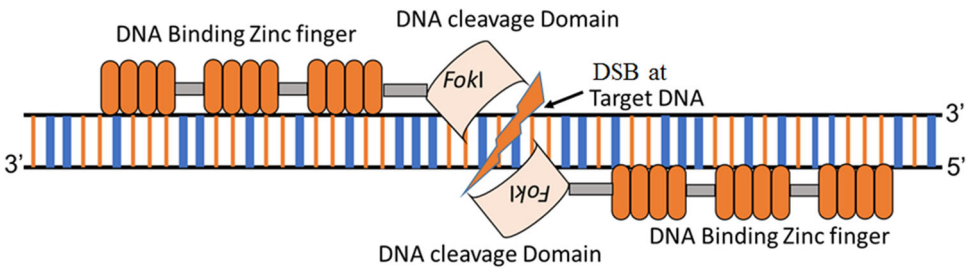

Fig. 2 Schematic representation of engineered zinc fingers fused with *Fok*1 restriction enzyme domain inducing DSBs in target DNA. Each zinc finger domain recognizes 3 nucleotides in target DNA, Fok1 nuclease is joined to C-terminal of three zinc finger domains to form a ZFN monomer. Dimerization of ZFN monomer essential for making DSBs by Fok1, thus two ZFN monomers bind to complementary strands of target DNA and cleave target DNA at the 6 bp spacer sequence in between recognition site of two monomers. Further, these DSBs are repaired through HDR or NHEJ DNA repair pathway

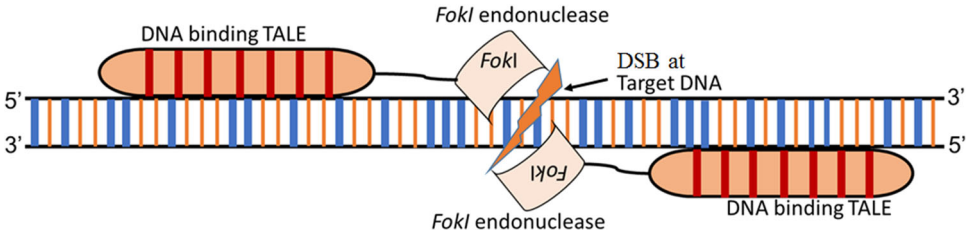

Fig. 3 Schematic representation of engineered TALEs fused with *Fok*1 restriction enzyme domain that induces DSBs in target DNA. TALENs target sequence is detected by two monomers of TALE repeats. There is nuclear localisation signal at N-terminal and C-terminal gets fused with Fok1 nuclease, dimerization of monomer is necessary to make DSBs in target DNA. Further, ultimately mutations were induced at targeted site during repair of DSBs by HDR or NHEJ repair pathway

need to be co-expressed, it may cause off-target effect (Aouida et al. 2014). Monomeric TAL-based nucleases (mTALENs) are improvement over dimeric TALEN and it requires single domain for the activity, and thus has potential to decrease off-target cleavage by around 50%. Use of mTALENs have some benefits, such as less efforts required to design compared to dimeric TALEN, high specificity, low off-target cleavage and can be used for targeted mutagenesis of variety of organisms (Mahfouz et al. 2014). TALENs ability to target a specific genomic sequence allows highly precise targeted mutagenesis. Several research papers have been published reporting the TALEN mediated gene editing in various plants traits. Disease resistance rice (Wang et al. 2014), aroma in rice (Shan et al. 2015), oil quality in soybean (Haun et al. 2014) and browning of in potato (Clasen et al. 2016) and many more genes were targeted in model as well as other crop plants for functional studies as a proof of concept. TALENs are preferred over ZFN due to its comparative simplicity and cost effective design, and considerably less off-targeting. Though TALEN is convenient, efficient and highly specific tool for DNA editing, it is technically challenging due to need of protein engineering to alter the binding specificity of TALEs to suit our purpose or gene of interest and difficulty in multiplexing. TALEN is sensitive to methylation, and thus not able to edit methylated target (Bultmann et al. 2012). Continuous thrust of genomic craftsman for novel, easier and advanced tools resulted in the discovery of revolutionary gene editing tool called CRISPR/Cas9.

CRISPR/Cas9

Clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated system (Cas9) is recently emerged as a genome editing tool across eukaryotic species (Barrangou 2012). Unique CRISPR like repeats were initially discovered way back in 1987 in Eubacteria (Ishino et al. 1987) and in Archaea (Mojica et al. 1995) but later on in 2002 the term 'CRISPR' was coined for such repeats by Jansen et al. (2002). Earlier actual function of CRISPR was unknown, which was later experimentally proved as an adaptive immune system that protects bacteria and *archaea* from attack of invading bacteriophages (Barrangou et al. 2007; Wiedenheft et al. 2012). Initially it was assumed that CRISPR acts on RNA as in case RNA interference, but Marraffini and Sontheimer (2008) demonstrated that CRISPR targets DNA and it could be programmed. A few years later, Sapranauskas et al. (2011) demonstrated that CRSIPR system in one organism can be reconstituted in another distant organism. Further, Jinek et al. (2012) elucidated the molecular mechanism of CRISPR/Cas9 system in *Streptococcus pyogenes*. CRISPR/Cas9 system is composed of Cas9 endonuclease and two RNAs, viz. CRISPR RNA (crRNA) and *trans*-activating CRISPR RNA (tracrRNA), which guides the Cas9 endonuclease to its target DNA sequence flanked by a protospacer-adjacent motif (PAM) (Jinek et al. 2012) (Fig. 4). Cas9 has two cleavage domains which show the homology with RuvC and HNH nucleases. crRNA and tracrRNA are engineered to combine in one guide RNA (gRNA) which guides Cas9 nuclease to its target (Cong et al. 2013). From 2013, there is an outbreak of research on CRISPR technology from microbes, plants to human and is still continued. Many researchers demonstrated the use of CRISPR/Cas9 in plant genome editing (Shan et al. 2013; Li et al. 2013; Jiang et al. 2013; Feng et al. 2014; Ron et al. 2014). Further, Liang et al. (2016) demonstrated the rapid process for construction of CRISPR/Cas9 for multiplex editing of plant genes and parameters for most efficient gRNAs. Cas9 nuclease from *Streptococcus pyogenes* was most widely used in CRISPR mutagenesis but recently, Cas9 orthologues from *Streptococcus thermophilus* and *Staphylococcus aureus* were also found to be efficient in genome targeting (Steinert et al. 2015). Specificity of CRISPR is govern by small gRNA, and thus it is easy to design such small gRNA specific to particular target sequence, therefore within very short time CRISPR/Cas9 became method of choice for genome editing. Though it is simple and easy to develop and use, is accompanied limitations such as off-target effects and need of PAM sequence near the target. To minimise off-targeting, double-nicking strategy was

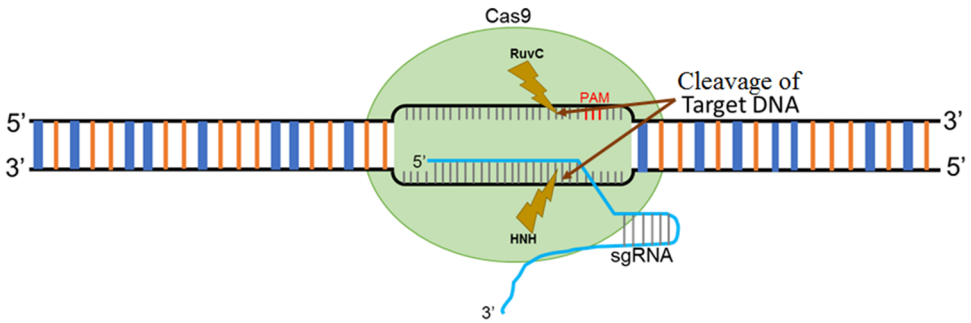

Fig. 4 Schematic representation of CRISPR/Cas9 genome editing tool. sgRNA (20 nucleotide long complementary to target site) guides the Cas9 to the target DNA sequence. PAM (proto spacer adjacent motif) is needed to cut the DNA by cleavage domain of Cas9 (RuvC and HNH) to make DSBs. Thus, resulted DSBs are repaired by HDR or NHEJ to induce desired mutations (insertion, deletion, substitution) at targeted site

developed, which uses mutant Cas9 enzyme (D10A) called DNA nickase. Mutations in RuvC domain converts Cas9 enzyme into DNA nickase (Cong et al. 2013). $Cas9^{D10A}$ which was used with a pair of equally efficient guide RNAs to reduce off-target effect by 50- to 1000-folds (Ran et al. 2013). Paired nickase mediated highly precise and efficient editing was performed in *Arabidopsis* (Fauser et al. 2014; Schiml et al. 2014). RNA guide Fok1 nuclease is another modification in CRISPR/Cas9 system, where fok1 nuclease was fused with catalytically inactive cas9 protein (Tsai et al. Tsai and Xue 2015). CRISPR technology can be used for gene expression modulation like RNAi and could be called as CRISPR interference (CRISPRi) (Qi et al. Qi et al. 2013a). Further length of sgRNA could be manipulated to overcome the problem of off-targeting (Cho et al. 2014). Thus, efficiency of CRISPR/Cas9 system is governed by the activity of Cas9 enzyme, designing of sgRNA, selection of target site and delivery methods used.

Applications of genome editing technologies in plants

With the advancement of precise genome editing technologies, their use in plants gene and genome modifications also increased in model plants as well as other crop plants. Initially, we discussed the genome editing in model plants, such as *Arabidopsis*, tobacco and rice, and then in general we described the application of genome editing in broadly in vegetable, oil crops, cereals and fruits.

Arabidopsis

It is model plant for genomic studies as lot of information available in public domain, so many researchers chose *Arabidopsis* for genome editing experiments. Efficiency of ZFN mutagenesis was assessed in *Arabidopsis* and analysis of mutants found that 78% were simple deletions of 1–52 bp, 13% were simple insertions of 1–4 bp, and 8% were deletions accompanied by insertions (Lloyd et al. 2005). ZFNs were designed using OPEN technique to target *Alcohol dehydrogenase* 1 (*ADH1*) and *transparent testa 4* (*TT4*) genes under the oestrogen-inducible promoter resulted in 7 or 16% mutation frequencies, respectively (Zhang et al. 2010). Similarly, *ABA-Insensitive4* (*ABI4*) (Osakabe et al. 2010) and *protoporphyrinogen oxidase* (de Pater et al. 2013) genes were targeted under heat shock promoter. Further, ZFNs were expressed under the egg apparatus-specific enhancer to introduce mutations in the germ line cells (Even-Faitelson et al. 2011). Use of engineered ZFNs in editing of tandemly arrayed genes was successfully demonstrated by editing the genes from the well-known *RPP4 (Recognition of Peronospora Parasitica 4)* resistance gene cluster, which contains eight genes. Mutation frequency was found to be 1% and deletions ranged from a few kb to 55 kb (Qi et al. 2013b).

Method and reagents for assembling of TALENs and guidelines for TAL effector and their binding sites were described by Cermak et al. (2011). Five *Arabidopsis* genes viz*; ADH1, TT4, map kinase (MAPKKK1), ubiquitin receptor protein (DSK2B)*, and ATP-binding transport protein (*NATA2)* were targeted for TALEN induced mutagenesis. Mutation frequency was ranged from 41 to 73% of which 1.5–12% of mutations were inherited in next generation (Christian et al. 2013). Forner et al. (2015) expressed TALEN under the meristem specific promoter targeting the *Clavata 3 (CLV3)*, few of the mutants showed loss of function of the *CLV3* and slight off-target effect was seen upon re-sequencing.

Multiplex and homologous recombination-mediated editing of *Arabidopsis Phytoene Desaturase* (*PDS3)* gene with mutagenesis frequency was demonstrated by Li et al. (2013). Similarly, Zhang et al. (2015) designed a multiplex CRISPR/Cas9 platform for fast and efficient editing and demonstrated editing of six PYL families of ABA receptor genes in a single transformation experiment with 13–93%

mutation frequency in T_1 generation. *Brassinosteroid Insensitive 1* (*BRI1*), *Jasmonate-Zim-Domain Protein 1* (*JAZ1*) and *Gibberellic Acid Insensitive* (*GAI)* genes were precisely mutated using CRISPR/Cas9 system with 26–84% mutation frequency (Feng et al. 2013). *Flowering Locus T* (*FT*) and *Squamosa Promoter Binding Protein-Like 4* were targeted using CRISPR/Cas9 and found that about 90% plants carrying mutation in T1 generation showed late flowering (Hyun et al. 2015). Similarly, CRISPR/Cas9 mediated gene targeting has been demonstrated at *ADH1* locus (Schiml et al. 2014), *GFP* gene (Jiang et al. 2014), while Feng et al. (2014) analysed the patterns, efficiency, specificity and heritability of CRISPR/Cas-induced gene mutations in Arabidopsis without off-target effect. Ning et al. (2015) generated CRISPR-CAS9 mediated double mutant of NAC transcription factors (*nac050/052),* mutant plant flowered early which is similar to *histone demethylase (jmj14)* mutant; these results indicated the functional association between NAC050, NAC052 and *JMJ14*. CRISPR-CAS9 mediated gene targeting of auxin binding protein 1 (ABP1) revealed that ABP1 is not a key component in auxin signalling as mutants did not show any obvious developmental impairments (Gao et al. Gao et al. 2015a). Methylation of DNA makes TALEN and ZFN useless for genome targeting, but CRISPR/Cas9 is not affected by DNA modifications. *AtFIS2* is imprinted in vegetative tissues by DNA methylation of promoter (Zemach et al. 2013); targeted editing of methylated *CpG* island in the *AtFIS2* promoter resulted in significant activation of *AtFIS2* transcription. Therefore, Cas9 system could act as transcriptional activator to activate silenced or imprinted genes in plants (Lowder et al. 2015).

Tobacco

Zinc finger nuclease mediated genome editing was performed in tobacco at the *GFP:NPT* (*neomycin phosphotransferase*) reporter transgene (Wright et al. 2005; Cai et al. 2009) and *acetohydroxyacid synthase* (*SuRA* and *SuRB*) genes (Maeder et al. 2008; Townsend et al. 2009) which showed the potential of ZFN as a genome editing tool. Mutation in *SuRA* and *SuRB* led to induce herbicidal resistance against the imidazolinone and sulfonylurea. A rapid, publicly available 'OPEN' strategy was used for construction of ZFN; found more efficient than modular assembly method. Petolino et al. (2010) deleted the transgene from the stably transformed plant using the ZFN. Transgene GFP-PAT construct was flanked by ZFN cleavage sites was transformed in tobacco, this transgenic plant was crossed with another tobacco plant expressing the corresponding ZFN gene and resultant progeny had a complete 4.3 kb deletion of transgene. Use of geminivirus based replicons for transient expression of zinc finger nucleases has been demonstrated by Baltes et al. (2014).

Precise genome modification has been successfully attempted in tobacco using TALEN (Mahfouz et al. 2011; Cermak et al. 2011). Further, Zhang et al. (2015) targeted *acetolactate synthase* (*ALS*) gene in tobacco protoplasts, 30% of transformed cells were mutated at *ALS* locus with 14% approximated gene insertion frequencies.

Targeted mutagenesis of *GFP* in *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease has been demonstrated (Nekrasov et al. 2013). Similarly, Li et al. (2013) reported multiplex and homologous recombination-mediated genome editing in tobacco. Further, two genes of tobacco: *NtPDS* and *NtPDR6* (*pleiotropic drug resistance*) were targeted with CRISPR/Cas9 and observed indel frequencies of 16.2–20.3% in protoplasts. Transgenic plants had 81.8 and 87.5% mutation in (*NtPDS*) and (*NtPDR6*), respectively, with no significant off-target effect (Gao et al. 2015b). Comparative study of different designed nucleases showed that cleavage of luciferase gene by CRISPR-Cas9 was more efficient than by TALENs, and also emphasized on optimization of Cas9 nuclease to make sure maximum cleavage efficiency (Johnson et al. 2015). Ali et al. (2015a) demonstrated *tobacco rattle virus* (TRV) mediated delivery of CRISPR/Cas9 without any off-target effect thus opened avenues for discovery of alternate methods for delivery of nucleases for genome editing. Moreover, Cas9 was delivered via TRV to manipulate *in planta* gene regulation by editing of transcriptional factors (Piatek et al. 2015). Marta et al. (2016) improvised the CRISPR/Cas9 by adapting the RNA-guided Cas9 system with a modular DNA construction framework (GoldenBraid). They developed a new software assembly for CRISPR/Cas9 with GoldenBraid. Feasibility and efficiency of this new approach was proved in *N. benthamiana,* therefore it can be extrapolated to other species for gene editing.

Rice

Sites for gene integration in chromosome are called as safe harbour loci. DNA damage and repair property of ZFN was exploited to locate these safe harbour loci in rice genome using different ZFN constructs with *β-glucuronidase (GUS)* as a reporter gene. 28 regions were identified for safe integration after DNA sequencing of which single is located in a non-coding region (Cantos et al. 2014).

Protocol for TALEN mediated mutagenesis was described by Chen et al. (2014) which consisted of constructing TALEN vectors, nuclease activity assay, and transformation of TALENs in callus or protoplast and identification and analysis of mutants. Using their protocol, one can generate TALEN mutant in T_0 rice plants within 4–5 months. Similarly, Li et al. (2014) reported the

procedure for TALEN mutagenesis in rice which included construction of modularly assembled TALENs, transformation, and screening for mutants. TALEN mediated gene editing led to generate blast resistance in rice. *OsSWEET14* gene which codes for sucrose-efflux transporter family was taken over by *X. oryzae* pv. *oryzae*, using its endogenous TAL effectors thus re-route sugars from the plant cell to pathogen. Due to TALEN-based disruption of *OsSWEET14*, pathogens TAL effector unable to bind *OsSWEET14* and ultimately resulted in disease resistance (Li et al. 2012). Similarly, Zhou et al. (Zhou et al. 2015a) edited *OsSWEET13* and resultant mutants were resistant to bacterial blight. TALEN with chimeric RNA/DNA oligonucleotides was used to substitute a single base in the rice *OsEPSPS* (*5-enolpyruvylshikimate-3-phosphate (EPSP) synthase*) gene. Mutant plants were more glyphosate sensitive and seed setting was also less as compared to wild-type plants (Wang et al. 2015). 2-acetyl-1-pyrroline (2AP) is a major aroma compound in the aromatic rice and presence of nonfunctional *betaine aldehyde dehydrogenase2* (*BADH2*) leads to synthesize 2AP and ultimately aroma in rice (Hinge et al. 2015). Disruption of BADH2 with TALEN increased grain 2AP content from 0 to 0.35–0.75 mg/kg, which was similar to the positive control aromatic variety (Shan et al. 2015). Yokoi et al. (2015) investigated classical non-homologous end joining and alternative-NHEJ repair mechanism in TALEN mediated targeted gene editing. For this they used DNA ligase4 (Lig4) mutants of rice and targeted waxy gene in rice. It was found that mutations frequency in lig4 null mutant was higher than lig4 heterozygous mutant or wild-type. Similar results were reported by Endo et al. (2015) with Cas9 mediated targeting of *acetolactate synthase (ALS)* gene in lig4 mutant rice calli.

Genome editing with CRISPR/Cas9 has been demonstrated in rice (Miao et al. 2013; Jiang et al. 2013; Xie and Yang 2013; Xu et al. 2014). The CRISPR/Cas9 system generated precise and homozygous gene editing in single generation in rice (Zhang et al. 2014). Zhou et al. (2014) reported large chromosomal deletions in gene cluster and heritable small genetic changes during CRISPR/Cas9 editing in rice and found 87–100% genome editing in T_0 plants. Endo et al. (2016) exploited the off-target effect of CRISPR/Cas9 system to edit the multigene clusters in rice. A single guide RNA (sgRNA) was designed to recognize 20 bp sequences of *cyclin-dependent kinase B2* (*CDKB2*). Sequence analysis of mutant revealed that single, double and triple mutants of *CDKA2, CDKB1* and *CDKB2* produced by a single sgRNA. Ma et al. (2015) reported a robust, convenient, efficient and multiplex CRISPR/Cas9 system for targeted genome modification in monocot and dicot plants using golden gate ligation or Gibson assembly. With this system, 46 target sites in genome were mutated with 85.4% average mutations. CRISPR/Cas9 mediated mutagenesis was demonstrated in alternative oxidase genes *(OsAox1a, OsAox1b, OsAox1c)* and *OsBEL* genes of rice, and also reported the transgene clean genome modification that inherited stably in next generation (Xu et al. 2015). Similarly, *Young Seedling Albino* (*OsYSA)* and *OsROC5* genes were precisely and simultaneously targeted and sequencing of T_0 plants revealed 33.3–53.3% mutation frequencies and most mutations were bi-allelic in nature (Lowder et al. 2015). *OsSWEET13* is the bacterial blight susceptibility gene as it is a target for TAL effector of pathogen *Xanthomonas oryzae pv. oryzae*. Modification in *OsSWEET13* with CRISPR/Cas9 resulted in blight resistance rice (Zhou et al. 2015a). Similarly, Wang et al. (2016) edited *OsERF922* gene which encodes for ERF transcription factor to develop resistant to rice blast disease. Li et al. (2016) targeted *carbon starved anther (csa)* gene in rice and the resultant mutant line showed photosensitive genic male sterility. Parameters affecting frequency of CRISPR/Cas9 targeted mutagenesis in rice was investigated. A positive correlation was found between mutagenesis frequencies and level of Cas9 expression and extended culture period of rice calli (Mikami et al. 2015a). Further, Mikami et al. (2015b) also studied the CRISPR/Cas9 mutagenesis with different Cas9 and gRNA expression cassettes. It was found that mutagenesis frequencies varied with the Cas9 expression cassette used, in addition to that superiority of *OsU6* promoter to *OsU3* promoter in gRNA expression was also reported.

Oil seeds

A ZFN mediated genome editing was performed in soybean targeting DICER-Like (DCL) genes. The mutation showed efficient heritable transmission of the ZFN-induced mutation. A context-dependent assembly platform, a rapid and open-source method was used for generating ZFN array (Curtin et al. 2011). Further, Curtin et al. (2013) described a method for targeted mutagenesis in the paleopolyploid soybean genome using ZFN that was capable for efficiently targeting either single or multicopy gene families. Zinc finger protein has been used for transcription activation of b-ketoacyl-ACP Synthase II in *Brasicca napus*. Mutant plants showed significant reduction in palmitic acid, increased total C_{18} and reduced total saturated fatty acid contents. Therefore, designed ZFP-TFs can play an important role in modification of endogenous genes to specifically modify agronomic trait (Gupta et al. 2012).

Soybean oil is often partially hydrogenated to increase its shelf life and improve oxidative stability due to polyunsaturated fats. Fatty acid desaturase 2 genes (FAD2-1A and FAD2-1B), which converts the monounsaturated oleic acid into polyunsaturated linoleic acid. TALENs were

employed to cleave conserved DNA sequences in both of these genes. In homozygous mutant plants, oleic acid increased from 20 to 80% and linoleic acid decreased from 50 to below 4% (Haun et al. 2014). Soybean genes *GmPDS11* and *GmPDS18* were targeted for modification using TALENs with efficiency of 17.5–21.1% under the *AtU6-26* promoter (Du et al. 2015).

Jacobs et al. (2015) demonstrated the CRISPR/Cas9 genome editing in soybean. GFP transgene and other nine endogenous loci were targeted using hairy roots and somatic embryos. A novel In-Fusion® cloning strategy was developed for easier design of CRISPR/Cas9 vectors, which should be applicable for targeting any gene in any organism. A transgene (*bar*) and different sites of two endogenous soybean genes (*GmFEI2* and *GmSHR*) were successfully targeted using CRISPR/Cas9 system (Cai et al. 2015). Cas9-guide RNA (gRNA) was applied for targeted mutagenesis, gene integration and gene editing of two genomic sites *DD*20 and *DD*43 on chromosome 4 of soybean with mutation frequency of 59 and 76%, respectively (Li et al. 2015). Du et al. (2015) compared the TALEN and CRISPR/Cas9 system in soybean *GmPDS11* and *GmPDS18* gene editing under *AtU6-26* and *GmU6-16 g-1* promoter. The mutation efficiency of CRISPR/Cas9 (11.7–18.1%) was slightly lower than the TALENs (17.5–21.1%) using the *AtU6-26* promoter but much higher (43.4–48.1%) under *GmU6-16 g-1* promoter. This suggested that Cas9/sgRNA system is more efficient for simultaneous editing of multiple homoeo alleles of *PDS* gene.

Cereals

Use of homing endonuclease derived from the *I-CreI* for specific genome editing was demonstrated in maize *liguleless* locus. 3% T_0 plants showed mutation at desired locus (Gao et al. 2010). Similarly, Djukanovi et al. (Djukanovic et al. 2013) re-designed the *I–CreI* homing endonuclease from *Chlamydomonas reinhardtii* to recognize a 22 bp target sequence of a maize fertility gene (*MS26*). The homozygous mutants were male sterile due to abnormal development of tapetum cells. Martin-Ortigosa et al. (2014) developed a mesoporous silicon nanoparticle (MSN) for the intracellular delivery of Cre protein for maize genome editing. ZFN mediated genome editing in maize endogenous loci was demonstrated by Shukla et al. (2009). Further, ZFN were used for *PAT* (*phosphinothricin acetyl transferase*) and *AAD1* (*aryloxyalkanoate dioxygenase*) herbicide resistance gene stacking at single locus in maize (Ainley et al. 2013).

Liang et al. (2014) targeted four genes viz; *ZmPDS, ZmIPK1A* (*Isopentenyl phosphate kinase*), *ZmIPK, ZmMRP4* (*multidrug resistance-associated proteins* 4) using TALEN in protoplast system. 23.1% of protoplasts were mutagenized whereas, about 13.3–39.1% of the transgenic plants were found to be mutated. Further, TALENs were used to generate stable, heritable mutations at the maize *glossy2 (gl2)* locus of maize with mutation frequency of about 10% (Char et al. 2015). TALENs were also employed in genome editing of barley (Wendt et al. 2013; Gurushidze et al. 2014).

CRISPR/Cas9 mediated targeted mutagenesis was employed to target the *ZmIPK* gene in maize protoplasts with mutagenesis efficiency of 13.1% (Liang et al. 2014). Similarly, Svitashev et al. (2015) attempted site-specific gene insertion in *liguleless1* (*LIG1*) gene, male fertility genes (*Ms26* and *Ms45*), and *acetolactate synthase* genes (*ALS1* and *ALS2*) of maize. Feng et al. (2016) demonstrated that gene present in heterochromatic region of the chromosome could be efficiently targeted using CRISPR/Cas9 technology. They targeted *Zmzb7* as a marker and mutant plant had an albino phenotype. Similarly, phytoene synthase gene (PSY1) in maize was targeted and the mutations thus obtained showed stable inheritance in next generation (Zhu et al. 2016). Moreover, *GFP* transgene in *Sorghum* was also edited using CRISPR/Cas9 (Jiang et al. 2013). TALEN and CRISPR-Cas9 technologies were used in hexaploid bread wheat to precisely mutate three homoeoalleles of *Mildew-Resistance Locus* (*MLO*) to achieve broad spectrum resistance against the powdery mildew disease (Wang et al. 2014). Similarly, CRISPR/Cas9 mutations were successfully targeted in the *inositol oxygenase* (*inox*) and *phytoene desaturase* (*pds*) genes of wheat in suspension culture (Upadhyay et al. 2013). Barley, *HvPM19* gene codes for ABA-inducible membrane protein which is involved in regulation of grain dormancy. Cas9-induced mutation in two copies *HvPM19* was attempted and found 23 and 10% precise mutant frequency. They also reported off-target effects, and thus showed limitations in targeting multicopy genes in crops (Lawrenson et al. 2015).

Vegetables

DELLA gene in tomato is called as *PROCERA* (*PRO*) which negatively regulates the signalling of gibberellic acid. Lor et al. (2014) mutated *PRO* gene in tomato using TALEN under the control of an oestrogen-inducible promoter and resultant mutant showed phenotypes consistent with increased GA response. A pipeline for tetraploid potato genome editing based on transient expression of TALEN in protoplasts has been developed. Transfection efficiency of protoplasts was 38–39%, whereas mutation frequency in calli was 11–13% (Nicolia et al. 2015). *Vacuolar invertase* gene (*VInv*) is responsible for accumulation of reducing sugars in potato tubers during cold storage. High temperature processing results in brown,

bitter-tasting products and elevated levels of acrylamide. TALENs were used to knockout *VInv* gene, *VInv*-knockout tubers had untraceable levels of reducing sugars, and reduced levels of acrylamide in chips and were lightly coloured (Clasen et al. 2016). RNAi approach was also used for the development of acrylamide free potatoes (Chawla et al. 2012).

Brooks et al. (2014) demonstrated the CRISPR/Cas9 mediated disruption of *ARGONAUTE*7 (*SlAGO*7) gene which results in needle like or wiry leaves. Functional conservation of *SHOR-TROOT* and *SCARECROW* genes between *Arabidopsis* and tomato was showed using CRISPR mutagenesis (Ron et al. 2014). MADS-box transcription factor encoding *RIN* gene regulates fruit ripening in tomato. CRISPR/Cas9 system was designed to target three regions within the gene, homozygous *RIN* mutant tomato plants exhibited incomplete ripening with reduction in red pigmentation than wild-type confirming the important role of *RIN* in ripening (Ito et al. 2015). Cermak et al. (2015) used geminivirus replicons as vector for delivery of CRISPR/Cas9 system. They reported overexpression and ectopic accumulation of anthocyanin in tissues upon insertion of strong promoter, and also found that more than 2/3 insertions were specific with no off-target effect. Geminivirus replicons were found to be more efficient than *Agrobacterium* for genome targeting. In *Arabidopsis,* GA4 knockouts exhibit dwarf phenotype due to inhibition of gibberellin biosynthesis pathway and reduced fruit dehiscence. GA4 orthologues in *B. oleracea, BolC.GA4*.a was targeted and reported 10% Cas9 induced mutations and also showed dwarf phenotype associated GA4 knockouts (Lawrenson et al. 2015).

Fruits and woody tree

ZFN mediated genome editing was demonstrated first time in perennial fruits, viz. Fig and Apple by targeted repairing of *uidA* gene under heat shock promoter. GUS staining and PCR product sequencing was employed to confirm the repaired *uidA* gene (Peer et al. 2015). Jia and Wang (2014) developed a novel *Xanthomonas citri* facilitated agro-infiltration technique to deliver CRISPR/Cas9 targeting the *CsPDS* gene, into sweet orange leaves. The mutation rate was approximately 3.2–3.9% without any off-target effect. Similarly, targeted editing of *phytoene desaturase (PDS)* gene in Apple was also demonstrated (Nishitani et al. 2016). Recently, Ren et al. (2016) demonstrated CRISPR mediated editing in Grapes cultivar 'Chardonnay', where they targeted *L-idonate dehydrogenase* (*IdnDH*) gene with 100% mutation frequency. There is a huge potential for gene editing in fruit crops to elucidate the mechanism behind flowering, ripening and shelf life, etc. This highly specific genome editing does not involve the introduction of foreign DNA so it will help in consumer acceptance of genome edited fruits. Kanchiswamy et al. (2015) and Xiong et al. (2015) nicely elaborated the potential application of genome editing in breeding of horticultural crops.

CRISPR/Cas9 mediated genome editing was also demonstrated in woody species like poplar (*Populus tomentosa* Carr). *Phytoene desaturase* 8 (*PtoPDS*) was targeted using four gRNAs. Transgenic poplar plants were albino in phenotype with mutation frequency of 51% (Fan et al. 2015). *4-coumarate:CoA ligase* genes (4CL1 and 4CL2) are involved in lignin and flavonoid synthesis in poplar. 4CL1 and 4CL2 were mutated using CRISPR/Cas9 system under *U6.6* promoter of *Medicago* with 100% efficiency having bi-allelic mutation. But another member of *4CL* family, *4CL5* was not mutated despite of 89% similarity with 4CL1 due to the presence of SNP in target site near PAM sequence (Zhou et al. 2015b). CRISPR/Cas9 system is highly specific and a single nucleotide change can affect the efficacy of genome editing. These findings suggested that we need to design gRNA carefully by considering the occurrence of SNP in out crossing species. Similarly Tsai et al. (2015) published commentary entitled 'CRISPRing into the woods' regarding the potential of genome editing in poplar and other woody trees. Breeding of fruits and woody perennial plants is difficult due to their long generation time. With the help of this, fast and specific genome editing tools we can generate homozygous mutation in very short time and ultimately can accelerate the breeding program in perennial plants.

Plant immunity against virus

Recently, CRISPR/Cas9 system has been used to increase the resistance of plants to geminiviruses (Ali et al. 2015b; Ji et al. 2015; Baltes et al. 2015). Ali et al. (2015b) engineered sgRNAs targeting open reading frames encoding viral genes such as *Rep*, coat proteins and conserved non-coding intergenic region (IR) were targeted. It was reported that conserved intergenic region was most effective target for reducing the virus titre of tomato yellow leaf curl virus (TYLCV). Further, simultaneous resistance to TYLCV, beet curly top virus (BCTV) and *Merremia* mosaic virus (MeMV) could be achieved with the use of sgRNA targeting conserved sequence from IR region. Similarly, Ji et al. (2015) and Baltes et al. (2015) targeted different region of geminivirus genome to develop virus resistance in plants. Cotton leaf curl disease is caused by begomoviruses and is one of the major diseases of cotton. A multiplex CRISPR editing technique was developed as a broad spectrum method for control of leaf curl diseases of cotton (Iqbal et al. 2016). Chandrasekaran et al. (2016) targeted *eIF4E* (*eukaryotic translation initiation factor* 4E)

gene in cucumber and the resultant homozygous mutant lines were immune to three different viruses, (Cucumber vein yellowing virus, Zucchini yellow mosaic virus and Papaya ring spot mosaic virus-W).

Chaparro-Garcia et al. (2015) postulated three mechanisms to explain reduction in virus titre and infection symptoms with the aid of CRISPR/Cas9 mediated genome editing: (1) blocking of replication due to binding of Cas9/sgRNA at the origin of replication, (2) replication may be affected due to fragmentation of viral genome by Cas9/sgRNA, and (3) non-homologous end joining (NHEJ) may incorporate error during DNA repair process.

Comparison of EMN, ZFN, TALEN and CRISPR/Cas9

Protein engineering in case of meganucleases is difficult for researchers as DNA-binding and cleavage domains are not separated (Silva et al. 2011). Engineering of DNA-binding domain is essential in case of TALEN and ZFN, and it is cost and labour intensive to have engineered protein for each gene of interest in different organism. TALENs are easier to design and engineer than ZFN, but have off-target effects whereas CRISPRs are easiest of all to design and to use and more efficient. ZFN and TALEN binding depends on protein-DNA interactions that might have repeat context dependence and methylation sensitive (Zemach et al. 2013), while CRISPR/Cas9 system depends on Watson–Crick base pairing, which is highly predictable and specific, therefore is a method of choice for genome editing (Ran et al. 2013). Similarly, dimerization of monomer is needed to make DSBs at target site in case of EMN, ZFN, TALEN, whereas single sgRNA is enough to edit multiple genes. Multiplex gene editing could be easily achieved using CRISPR to edit several genes at a time while studying gene families. With rapid progress and innovations in improvement of efficiency and preciseness of CRISPR/Cas9 editing system, it has clear edge over the other editing techniques (Khatodia et al. 2016). Nevertheless, there is no perfect answer to proceed with which of the genome editing systems and logically one needs to critically analyse different systems and choose the most appropriate for their scientific exploration (Table 2).

Biosafety regulations

ZFNs, TALENs and CRISPR/Cas9 systems are the robust genome editing techniques used for precise modification in many plant genome without or little foreign DNA insertion (Weeks et al. 2015). Woo et al. (2015) demonstrated the genome editing without insertion of foreign DNA with the help of preassembled CRISPR/Cas9 proteins and are similar to naturally occurring mutations. Kanchiswamy (2016) elaborated potential and importance of DNA-free genome editing of crops. Therefore, there is a debate in the scientific community and law makers whether genome edited plants come under the regulation of GMO or not. Scientists and policy makers have extensively discussed the issues regarding the regulation of genome edited plants. They considered various parameters related to genome editing; pathway of DNA repair (NHEJ/HDR), phenotype modified/developed, off-target effect and the available regulatory framework in various countries (European Food Safety Authority Panel on Genetically Modified Organisms 2012; Araki et al. 2014; Hartung and Schiemann 2014; Wolt et al. 2015; Araki and Ishii 2015; Swedish Board of Agriculture 2015). Several countries have already formed regulatory framework for genome editing technologies; product based and process based framework. Process based framework take into account the procedure and techniques used in genome editing as per the FAO guidelines and Cartagena protocol (Hartung and Schiemann 2014). Product based regulation gives less importance to process used, but emphasizes on public and environmental risk analysis of final product developed.

USDA in 2012 stated that plant edited using ZFNs without transgene insertions not come under regulation as in case of GMOs. Similarly in EU also, ZFN mediated genome edited crops were assessed under European Community regulations. Further, the New Zealand Environmental Protection Authority (EPA) committee announced that ZFN-1 and TALENs mediated editing of plants are not GMOs (The McGuinness Institute 2013). Recently, Swedish Board of Agriculture also stated that CRISPR-Cas9 does not fall under EU definition of GMO. Similarly, USDA also allowed cultivation and sell of CRISPR edited mushroom without passing through any regulation (Waltz 2016). Araki et al. (2014) emphasized on careful handling of genome edited crops to avoid misleading of society. Genome or transcriptome sequencing and other novel methods need to be employed for investigation of genome edited crops. Though some decisions were made in regulation of gene edited crops, still there is a need of international consensus for careful regulation of these technologies and risk management associated with them for better relationship between science/technology and the society.

Conclusion and future perspectives

Emergence of ZFNs, TALENs and CRISPR as a gene editing techniques has transformed the plant biology research as they have ability to generate highly specific and efficient mutations in short time span. These genome

Table 2 Comparison of EMN, ZFN, TALEN and CRISPR/Cas9 genome editing systems

Feature	EMNs	ZFNs	TALENs	CRISPR/Cas9
Mechanism	Make DSBs at target site with the help of catalytic domain	*Fok1*nuclease introduce DSBs in target DNA using zinc finger protein	*Fok1*nuclease introduce DSBs in target DNA using specificity of TALEs	Produce DSBs in target DNA by wtCas9 or single strand nicks by Cas9 nickase
Construction	Engineering of amino acids at the DNA-binding domain	*Fok*1 domain is attached Zinc finger monomers to target DNA	Engineering of the TALE at the DNA-binding domain and integration of *Fok*1	20 nucleotide sequence in small single guide RNA (gRNA) molecule
Efficiency of target recognition	Low	Medium	High	High
Off-target effects	Low off-target effect	Shows high off-target activities	Shows least off-target activities	Low off-target effects
Durability	Permanent and heritable change genome	Permanent and heritable change genome	Permanent and heritable change genome	Permanent and heritable change genome
Nature of effect	Knock out gene expression	Knocks out gene of interest	Knocks out gene of interest	Knocks out/in gene of interest
Site of action	Nucleus	Nucleus	Nucleus	Nucleus
Module	Protein based DNA targeting module	Protein based DNA targeting module	Protein based DNA targeting module	RNA based DNA targeting module
Target length (bp)	12–40	18–24	22–58	20–22
Ease of use	Technically complicated as DNA-binding and catalytic domains are not separated	Technically challenging in generation as requires protein engineering.	Technically challenging in generation as requires protein engineering	Requires more time and effort input than RNAi, but easier than TALEN
Ploidy level	Not studied	Not studied	Useful in case of polyploidy	Useful in case of polyploidy
Cost for development	High	High	Higher	Low

editing techniques are highly specific, rapid and cost effective, so can be used as support for the labour and time intensive classical plant breeding. This precise gene editing was successfully applied for functional genomics study, transcriptional regulation, disease and pest resistance and new trait development in model plants as well as cereals, vegetable, fruit crops. All the gene editing techniques have their own pros and cons, but it was seen that due to its simple, versatile nature and affordability, CRISPR/Cas9 has become a method of choice among the plant molecular biologist. It is proved by seeing the comparative number of publication churning out in plant genome editing (Fig. 5).

Though these techniques are highly specific, some degree of off-target effect has been reported but careful designing these tools and selection of target will reduce them completely. The degree of off-targeting can be overcome by designing and discovering highly specific nucleases as the development in these technologies is advancing rapidly. Zhang's group at Broad institute recently discovered Cpf1, an another nuclease for CRISPR editing which is smaller compare to earlier reported nucleases and require single RNA for activity (Zetsche et al. 2015). Such advances and discoveries of different

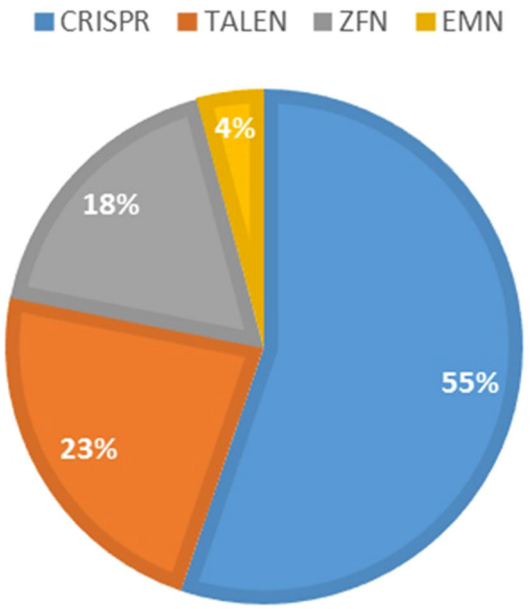

Fig. 5 Schematic representation of number of original research articles published reporting the use of targeted genome editing tools in plants. [Till August 2016 total 111 articles are published: CRISPR (61), TALENs (27), ZFNs (19) and EMNs (4)]

new nucleases have widened our choices for editing trait of our interest. Similarly, various modes of delivery of engineered nuclease into the cell were used and each one has its own properties, such as reduced off-targeting, efficiency and DNA-free editing. It was reported that ribonucleoprotein mediated delivery of nucleases reduced off-target effect and also resulted in DNA-free editing in plants (Woo et al. 2015). Still it is a challenge to completely reduce the off-targeting as well as accurate detection of off-targeting in whole genome. Further, genome editing tools enable researchers to study gene expression at various spatiotemporal stages of plant development by modulating the cis-acting element and transcription factors involved expression of trait of interest (Piatek et al. 2015; Chandrasekaran et al. 2016). It is also possible to edit or modify multiple genes at a time. Therefore, it can be a powerful tool for functional genomic analysis of plants. There is need of sgRNA libraries for genome wide detection of multiple mutations in a single experiment like animal system (Shalem et al. 2014). Such large sgRNA libraries for plant system will further improve the speed and accuracy of detection of mutation in coming days.

The increase in use of genome editing tools raised the regulatory issues as there is a debate on whether genome edited plant considered as GMO or not. There are differences among the regulatory bodies of the world on product based or process based approach to be used for regulation. So, there is a need of harmony at international level for regulation of genome editing crops to access the potential risk associated with them. Proactive discussion between researchers, public and regulatory institutions will be useful for regulation and as well as social acceptance of genome edited crops. Overall, these genome editing tools enhanced our ability to edit genome for better understanding of biological processes and development of desired traits in plant species.

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