Genome Editing for Stress Tolerance in Cereals: Methods, Opportunities, and Applications

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Abstract

Many advanced technologies were used along with conventional breeding to develop novel varieties, which increases the productivity of major cereal crops. Regardless of this progress, continuous increase in biotic and abiotic stresses imposes challenges for crop scientists to ensure the future food security to growing population. Recently, the availability of whole-genome sequence information and the advances in precise genome editing technology have revolutionized the crop breeding domain. The genome editing methods are becoming more accurate, simple, and highly efficient with time. The genome editing applications have been successfully proved in several cereals, viz., rice, wheat, maize, and barley, and produced various stress-tolerant crops. The current chapter compiles information on the advantages of using genome editing tools like zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated (Cas) (CRISPR/Cas9), and base editing and their application in cereals to enhance stress resilience. It also includes different steps involved in genome editing approaches in cereal crops. The emerging genome editing technologies can provide non-transgenic stress-resilient cultivars in less time to

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cope with rapidly changing climatic conditions. Furthermore, the ethical and regulatory policies to produce new cultivars through genome editing approaches are updated with respect to the global and national context.

Keywords

Genome editing · Cereals · Abiotic stress · Biotic stress · Crops

10.1 Introduction

Changing environment and increasing human population are the two major concerns that raised questions of worldwide food security, which enforce the present improvement of important crops to meet future requirement. Domestication and natural breeding processes have taken more than 10,000 years to produce varieties from landraces. To meet the human needs and to adapt local environment, modern crop varieties have various better agronomic traits. However, it takes a long time and a lot of effort to improve present elite germplasm. On the other hand, linkage drag and the transmission of detrimental genetic material associated to favorable features make it difficult to introduce helpful traits into an elite variety. However, introgression breeding also involves numerous rounds of backcrossing and selection to reestablish the elite genotypic background, which takes a long time and is inconvenient too.

Therefore, the slow pace of improvement via traditional breeding is assumed to be due to longer generation duration, random nature of recombination, and undirected mutagenesis of crop plants. The emergence of advanced breeding tools such as genome editing brought about a paradigm shift in biological and agricultural research, providing plant breeders with an open opportunity in de novo domestication to produce genetic variation for breeding in a unique approach to reduce generation time and develop elite varieties. Indeed, for improving characteristics in crop plants, new plant breeding techniques (NPBTs) have developed as alternative to traditional breeding and transgenic methods. The genome editing technique allows for the modification of endogenous genes in crops to improve the target qualities without having to allocate transgene crossway species boundaries. In particular, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) has emerged as the most popular technology for editing the genome of crops, with rapidly expanding agricultural applications in cereals such as rice, wheat, maize, and other food security crops. Cereals are staple food crops in our diet and provide ample primary sources of energy in the form of carbohydrates, minerals, fibers, niacin, riboflavin, thiamine, etc. Hence, cereals have huge significance for worldwide food security. Given its prominence, genome editing methods are commonly used in the genetic improvement of cereal crops to produce elite cultivars that are resistant to stress.

This chapter compiles the information on different genome editing tools—ZFN, TALENs, and CRISPR/Cas9—and also briefly sums up recent advancements in genome editing, i.e., base editing, that have revolutionized the crop improvement

program that allows effective and specific gene editing to single base level. Furthermore, various steps in genome editing are described, as well as current applications of genome editing in cereals, with a focus on its prospective for genetic enhancement of crops in terms of abiotic and biotic stress. Additionally, various aspects of challenges and opportunities in cereals and regulatory issues related to genome-edited crops are also discussed.

10.2 Types of Genome Editing Tools

ZFNs, TALENs, and CRISPR-cas9 are the keystones of gene editing tools, which are theoretically well-defined as deliberate alteration of gene sequences by means of molecular scissors by opening the novel way of targeted genome editing (Fig. 10.1). The introduction of double-stranded break (DSBs)at the target regions is the typical feature of these genome editing tools. Endogenous DNA repair mechanism such as non-homologous end joining (NHEJ) or by homology directed repair (HDR) (Gallagher and Haber 2018; Sander and Joung 2014) repairs these DSBs, resulting in DNA alteration such as insertion or deletions (indels) at the DSB sites. Indel frequency, on the other hand, has been used to assess the complete activity and preciseness (off-target) of genome editing tools.

10.2.1 Zinc Finger Nucleases (ZFNs)

The first genome editing tool utilized programmable nucleases, zinc finger nucleases (ZFNs), resulting in a breakthrough in genome engineering (Chandrasegaran and Carroll 2016). By taking the advantage of endogenous DNA repair mechanism, the reagents of the DNA repair mechanism can be utilized to accurately modify the genomes of higher species that lead to both targeted mutagenesis and gene replacement remarkably at higher frequency. ZFNs are the targetable DNA cleavage reagent made by fusing the DNA-binding zinc finger protein (ZFP) domain at the amino terminus with the *Fok I* nuclease cleavage domain at the carboxyl terminus, resulting in a target-specific desired sequence. ZF domain comprised of eukaryotic transcription factor and tandem array of Cys2His2 zinc finger in each unit of approximately 30 amino acids bound to a single atom of zinc that each recognizes 3 bp of DNA (Wolfe et al. 2000). To dimerize and cleave DNA, standard ZFNs merge the cleavage domain at the C terminus of every single zinc finger domain, and then two distinct ZFNs must bind opposite strands of DNA with their C termini at a particular distance away from each other. Zinc finger nucleases act as heterodimer because *Fok I* must dimerize to cut target DNA sequence (Bitinaite et al. 1998). However, monomeric is not active, so cleavage does not occur at single binding sites. Cytotoxicity will result from poor targeting and a high number of off-target effects. Thus, the usage of ZFNs is limited as compared to other programmable nucleases.

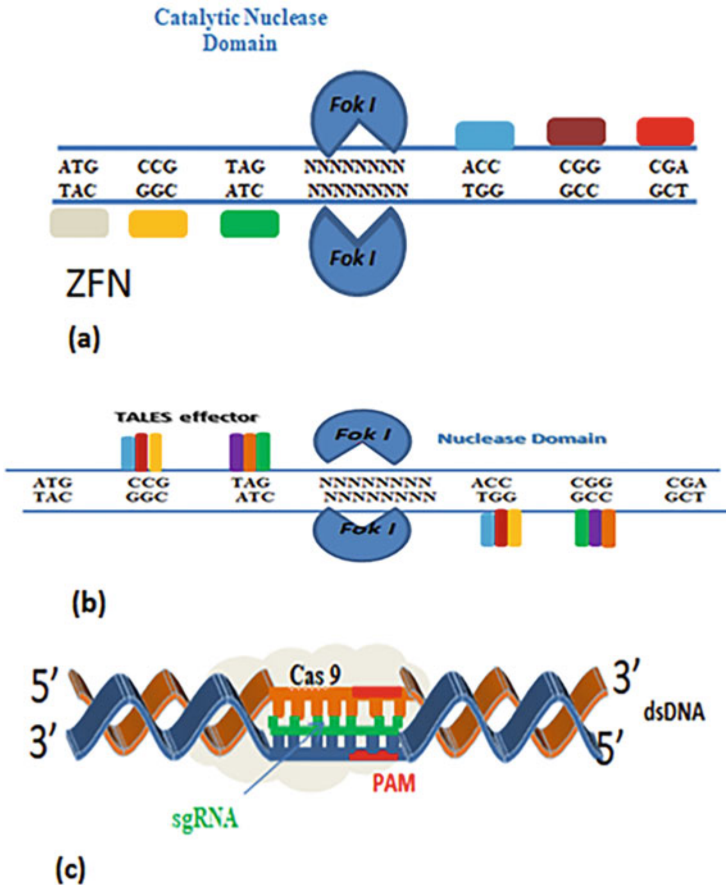

Fig. 10.1 Tools of genome editing: (**a**) zinc finger nucleases (ZFNs), (**b**) transcription activator-like effector nucleases (TALENs), (**c**) clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas)

10.2.2 Transcription Activator-Like Effector Nucleases (TALENs)

The next uprising in gene editing history is transcription activator-like effector nucleases (TALENs), generated by the fusion of DNA-binding domain protein called TALEs and deduced from transcription activators like effectors of *Xanthomonas* (Miller et al. 2011) to DNA cleavage *Fok* I nuclease domain. The TALEN DNA-binding domain is defined by order and number of four repeated domains, which has extremely preserved 33–34 amino acid (aa) repetitive domain with divergent 12th and 13th aa, well known as repeat variable di-residue (RVD): NG, HD, NI, and NN/NH/NK to mark T, C, A, G nucleotide, respectively. Selecting a combination of repeat segments including RVDs makes engineering a specific binding domain simple. Like ZFNs, *Fok I* function as a heterodimer with unique

DNA-binding domains for locations in the marked genome that are properly oriented and spaced. TALENs have a far more rigorous protein DNA coding for targeting, and it can also recognize a single base rather than triplet, giving it more versatility than ZFNs. Scientists all across the world are interested in TALENs because of its apparent advantages, such as greater precision and cleavage efficiency when introducing mutations over ZFN. However, as compared to ZFNs, the use of TALENs is limited due to higher amount of the encoding cDNA (3 kb).

10.2.3 Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated (CRISPR/Cas9)

In 2012, French and American scientists Emmanuelle Charpentier and Jennifer Doudna discovered CRISPR/Cas9, a third-generation genome editing tool. Due to its apparent benefits like more precision and cleavage efficiency to introduce mutation over ZFN and TALENs, CRISPR/Cas9 shows great attention of scientists around the world. The CRISPR/Cas9 system is a straightforward tool for site-specific mutagenesis, where genes can be knocked out or precisely altered by harnessing the different repair mechanisms. In bacteria and archea, CRISPR/Cas system is an RNA-mediated acquired immune response. It comprises CRISPR spacer arrays and Cas protein, which is naturally evolved to provide defense against phages. On the basis of Cas genes and interference complex, CRISPR/Cas system has been divided into two classes which have been further subdivided into six types. For interference, Class 1 CRISPR/Cas systems (types I, III, and IV) use multi-Cas protein complexes, whereas Class 2 systems (types II, V, and VI) use single effector proteins in complex with CRISPR RNAs (crRNAs) (Koonin et al. 2017).The *Streptococcus pyogenes* type II CRISPR/Cas9 was the first one to accurately break DNA in eukaryotic cells. It has two main components, namely, Cas9 nuclease and non-coding single guide RNA (sgRNA). Cas9 is a dual RNA-guided DNA endonuclease with 5′-NGG-3′ sequence as PAM (protospacer-adjacent motif) following the gRNA with 20 bp as target that creates blunt ends 3 nt upstream of the protospacer-adjacent motif. The Cas9 consists of HNH nuclease and RuV C domain, each cleaving one strand of the target. The other component is two short non-coding RNA that comprises of crRNA, which is composed of 20 nt target-specific sequence that establishes the uniqueness of this system and a *trans* activating crRNA (tracrRNA), which interacts with crRNA to mediate endonuclease activity of the CRISPR/Cas9 complex (Wiedenheft et al. 2012). When all of the components are carried to a target cell, three base-pair NGG (PAM) on the target DNA strand direct the Cas9 endonuclease to cut 3 bp upstream to PAM sequence (Jinek et al. 2013). As a result, the Cas9-gRNA complex images the protospacer-adjacent motif region and generates complementary base pairing with 20 nucleotides of the target DNA. This configuration allows the endonuclease to cut site-specific target DNA. Lastly, the cell repair DSB's internal DNA repair mechanism makes the appropriate alterations. Schunder et al. (2013) discovered another kind of Cas Cpf1 (also known as Cas12) in *Francisella* spp., which is a CRISPR type V endonuclease that identifies and

cleaves protospacer-adjacent motif 50-TTN, which will be more prevalent in the genome that cleaves the target DNA, creating 5 nt 50 overhang 18–23 bases away from protospacer-adjacent motif. There are many Cas9 variants developed, i.e., nickase Cas9 (nCas9) and dead Cas9 (dCas9), to overcome the limitations of Cas9, especially with respect to off-target mutations and indel formation (Certo et al. 2011; Brookhouser et al. 2017). As nCas9 produces single-stranded binding (SSBs), a pair of nCas9 can be used to produce paired nicks in its place of DSB, reducing off-target cleavage, whereas dCas9 functions as a site-specific DNA-binding vehicle that can combine with other effectors to modify target DNA sites with higher specificity and efficacy than nCas9 (Guilinger et al. 2014). In addition to mutagenesis, CRISPR/Cas9 can be used to repress or induce gene expression by combining repressor or transcriptional activator with a catalytically inactive Cas9 (dCas9) (Bortesi and Fischer 2015). As a result, it has the potential to replace standard traditional methods of gene overexpression and silencing. These advances have greatly aided to the broader adaptability of this technique among the eukaryotes.

10.2.4 Base Editing

To bypass the limitation of CRISPR/Cas, a modern evolution of a single base pair editing system has been devised based on CRISPR/Cas-based technologies. The base editing system directly creates point mutation in targeted DNA without inducing DSB. Furthermore, compared to non-DSB-mediated genome editing in plants, base editing approach improves gene modification efficiency by lowering off-target and random mutations in the DNA, multiplex, or whole-gene editing. This approach enables the programmed conversion of single bases into another (e.g., A/T to G/C, C/G to T/A) and allows four transitions. Aside from base editing (BE), prime editing (PE) allows for non-double-stranded break and template-free random sequence addition, removal, or nucleotide replacement. On the other hand, PE was created to allow base-to-base transitions, which facilitates targeted deletion and insertion.

Base editors are chimeric complexes that contain catalytically inactive CRISPR/Cas domain and cytosine or adenosine domain that creates desirable point alterations in the target region, allowing for precision genome editing. Cytosine base editors (CBEs) and adenine base editors (ABEs) are the two main types of DNA base editors that have been described (Fig. 10.2a, b). The two essential components of DNA base editors are a Cas enzyme for customizable DNA binding and a ssDNA-modifying enzyme for selective nucleotide alteration. The CBE systems consist of cytidine deaminase coupled to nCas9 and a uracil glycosylase inhibitor that converts targeted cytosine to uracil in genomic DNA (Komor et al. 2016). Cytosine (C) in DNA is converted to uracil (U), and subsequently U is replaced by T during DNA replication using cytidine deaminase. Through this process, uracil glycosylase inhibitors attach to and inhibit uracil DNA glycosylase, thus blocking uridine excision, resulting in the base excision repair pathway and enhanced base editing efficacy. The CBE system consists of a uracil glycosylase inhibitor and cytidine deaminase fused with

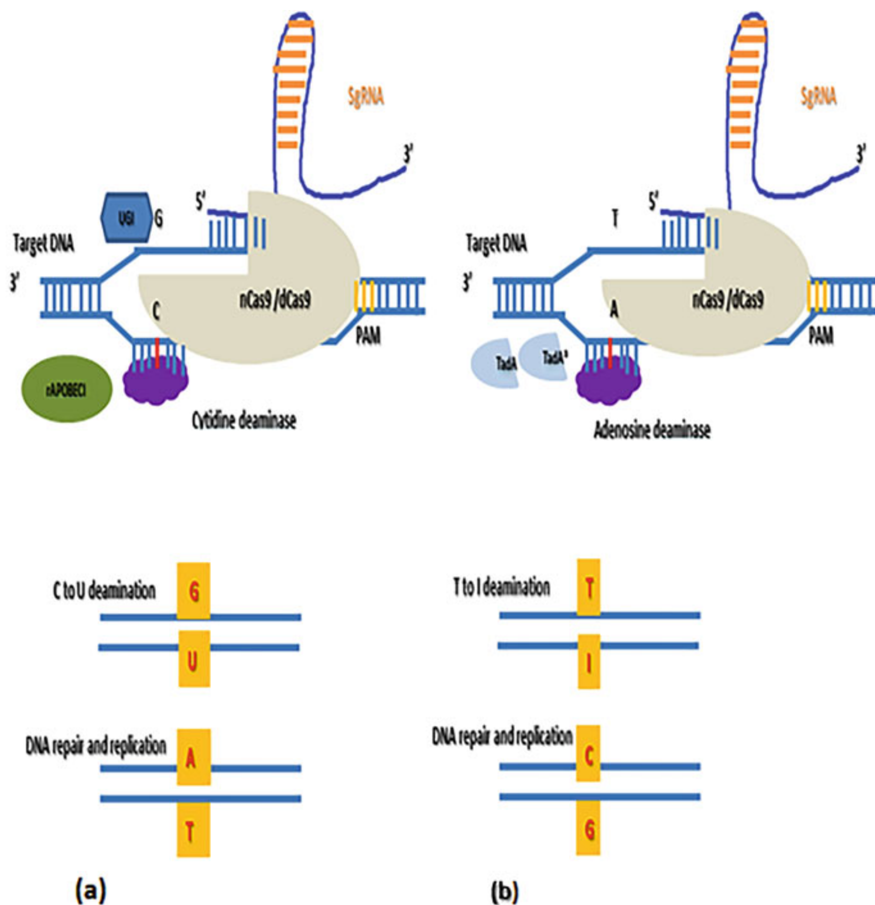

Fig. 10.2 Mechanism of DNA-based base editors: (**a**) cytosine base editing mechanism, conversion of C to T, (**b**) adenine base editing mechanism, conversion of A to G

Cas9, and changes targeted C to U in genomic DNA. The human APOBEC3A-based plant CBE has been employed in rice, wheat, and potato to efficiently convert Cs to Ts (Li et al. 2018; Zong et al. 2018).

Liu's group later produced ABEs, which used to facilitate the alteration of A to G in genomic DNA. In contrast to CBEs, ABEs do not require DNA glycosylase inhibitors. A deoxyadenosine deaminase (TadA*) and TadA-TadA* heterodimer was produced from modified *E. coli* transfer RNA adenosine deaminase (TadA) and linked with nCas9 (D10A) (Gaudelli et al. 2017). With excellent efficiency and product purity, the seventh-generation ABEs (7.10) were employed to convert A to G in extensive range of targets (Gaudelli et al. 2017). Rice and wheat ABE systems have also been optimized. In rice and wheat, the practice of improved sgRNAs [sgRNA(F + E)] in combination with three replicas of nuclear localization sequences

at the C terminus of nCas9 resulted in A to G conversion efficacies of up to 60% (Li et al. 2018).

10.3 Various Steps Involved in Genome Editing

For the successful genome editing in plant system, some of the sequential practices are as follows: identification and selection of target gene and designing of sgRNA, cloning of sgRNA into suitable vectors, delivery into plant system through various methods, selection of editing events in plants, and characterization of edited plants.

10.3.1 sgRNA Designing

sgRNA designing is the initial step for successful genome editing in cereals. The editing capability mainly depends upon sgRNA structure, GC contents, Cas9 codons, and targeted DNA. sgRNA acts as a functional guide in CRISPR-mediated editing and contains 20 nucleotide complementary sequences to the target site with a specific PAM site (5′-NGG-3′) at 3′ end. The expression of sgRNAs into plant system is commonly driven by small nuclear RNA gene promoters (U3 or U6). The transcription of sgRNAs is done through RNA polymerase III (Jiang et al. 2013). Before designing of target-specific sgRNA, the following important factors should be considered:

1. sgRNA size should be minimum (18–21 nucleotide length) at the target site.
2. Identify most common coding sequence of all isomers of a gene in the genome.
3. Preference should be given to the first exon of the targeted gene for sgRNA designing for loss of function mutation.
4. There should be a PAM site in the end of a target site.
5. There should be suitable restriction enzyme (RE) sites at each side of sgRNA for cloning work.
6. Designed sgRNAs should exhibit minimum off-target effects in the targeted organism.

The sgRNA designing can be done by using an online bioinformatics tool that allows identifying new target sites (Stemmer et al. 2015). Various online tools are now available with plant databases that will enable the identification of new target sites for sgRNA designing (Stemmer et al. 2015). The following were mainly used for many cereal crops: wheatCRISPR, CasOT, E-CRISP, biotools, Cas-OFFinder, CRISPRdirect, etc. In addition, a CRISPR Design tool was developed by Zhang and colleagues (http: //www.genome-engineering.org) and another by Xie and co-workers in 2014, CRISPR-PLANT, to get efficient sgRNA constructs which are used in genome editing events (Xie et al. 2014). Similarly, some novel web tools were developed for designing sgRNA for every plant whose genome sequence is available (Lei et al. 2014).

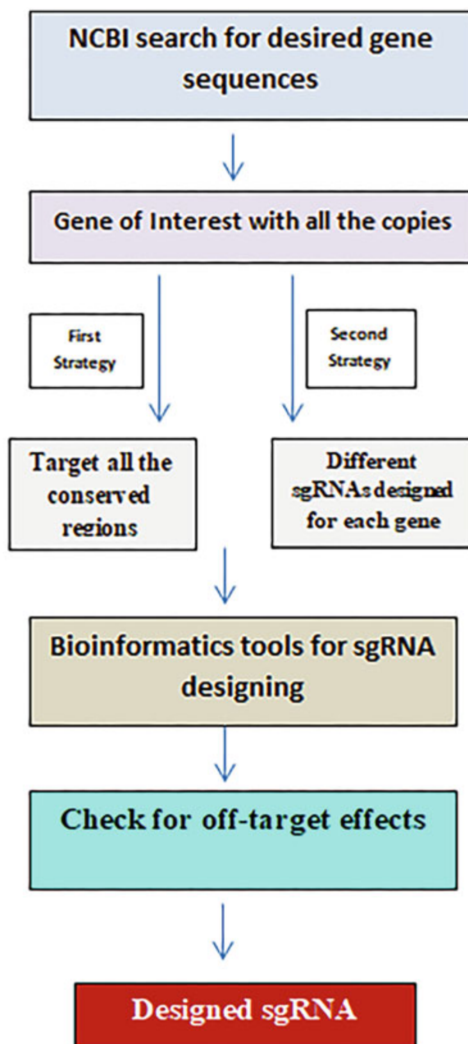

Fig. 10.3 Flow diagram depicting the different steps involved in sgRNA designing

The designing criteria for efficient sgRNA in plant systems are as follows:

(a) Designed sgRNA should show G/C content range between 30% and 80%.
(b) Designed sgRNA should contain intact secondary structures except for stem-loop 1.
(c) sgRNA contains not more than 12 total base pairs and no more than 7 consecutive base pairs between guide sequence and the other sequence.
(d) Not more than six internal base pairs (IBPs) (http://www.genome-engineering.org/). The complete process of sgRNA designing is shown in Fig. 10.3.

10.3.2 Cloning of sgRNA

The efficient genome editing in plants depends on the cloning of sgRNA through various vector systems. A binary vector system that utilizes features of two vectors in one and acts as a specific vector having several sgRNAs and cas9 proteins along with expression cassettes is used for successful genome editing events. In this vector system, for sgRNA expression, promoters (U6/U3) are designed (driven by RNA polymerase III), and CaMV35S and ubiquitin promoters are used by RNA polymerase II for Cas9 gene expression. The binary vectors utilized in cloning work used two types of basic structural units: (i) first type of structural unit is based on pGreen, and (ii) another type is based on pCAMBIA. The pGreen vectors were used owing to the small size of vectors and showed transient Cas9 and sgRNA expression in protoplasts to test the effectiveness. The vectors pCAMBIA1300/2300/3300 and their derivatives are the commonly used binary vectors for various plant species (Curtis and Grossniklaus 2003; Lee and Gelvin 2008). Improvement in the pCAMBIA backbone-derived vectors uses the BsaI site in the pVS1 region to assemble gRNA expression cassettes. However, for multiple sgRNA insertions into a single vector, 6gRNA module vectors are constructed, which consist of three designed for dicot species and three designed for monocot plants. More sgRNA expression cassettes are assembled into one vector either through Gibson assembly cloning or the Golden Gate cloning methods (Engler et al. 2008; Weber et al. 2011).

The traditional cloning method has several disadvantages, like the need for several rounds of cloning and being very time-consuming for the expression of few sgRNA cassettes into binary vectors. This cloning method is also called "regular cloning" (Fig. 10.4a). In this method, cloning of several sgRNA expression cassettes requires various restriction enzymes. The U3/U6 promoter (Pr)-driven sgRNA expression cassettes are organized in the middle vector and recovered by digestion with two respective restriction endonucleases. Generally in a binary vector, only three cassettes can be ligated together, but more than three fragments cause competitive self-ligation problems. The Golden Gate cloning method uses restriction enzymes (type II) to create non-palindromic sticky ends among multiple DNA fragments (Fig. 10.4b). This method can proficiently ligate several DNA fragments in a particular procedure (Engler et al. 2008). In the same method, two sets of vector systems have been developed in a single round of cloning to make CRISPR/Cas9 binary constructs with the help of PCR-amplified sgRNA expression cassettes (Ma et al. 2015; Xing et al. 2014). The expression cassettes are digested with a restriction endonuclease (RE type II)(BsaI) to create cohesive ends and joined all together to a binary vector for cloning (Ma et al. 2015). The Gibson cloning can capably join multiple DNA fragments.

The Gibson assembly technique can join several DNA fragments with homologous termini using the collective work of the Taq DNA ligase,T5 exonuclease, and the Phusion (DNA) polymerase (Gibson et al. 2009). The sgRNA expression cassettes ready in vitro by PCR and ligated to a binary vector shown (Fig. 10.4c) in the Gibson assembly method. Another strategy, the PTG which is polycistronic

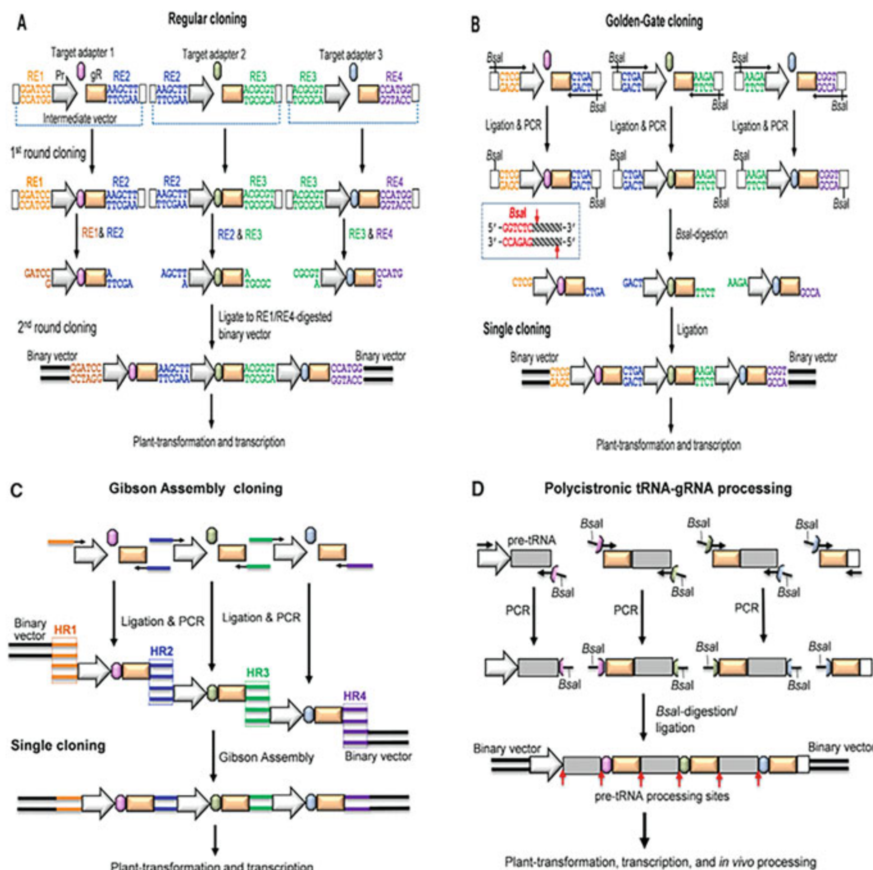

Fig. 10.4 Different methods of sgRNA cloning into a binary vector. (**a**) Regular cloning. (**b**) Golden Gate cloning. (**c**) Gibson assembly cloning. (**d**) Polycistronic tRNA-gRNA cloning. Adopted from Ma et al. (2016)

tRNA-gRNA system has been utilized by flanking the sgRNAs with a tRNA precursor sequence, and multiple sgRNAs are generated with different target sequences (Fig. 10.4d). The multiple sgRNAs with U3/U6 promoter is linked to pre-tRNA/sgRNA scaffolds using Golden Gate ligation.

10.3.3 Transformation into Plant System

Genetic transformation and regeneration processes are the major steps of gene editing. CRISPR/Cas9-mediated genome editing requires effective delivery of editing reagents, including sgRNAs and Cas9 nucleases that perform the actual targeted genome modification in most plant cells. The CRISPR/Cas system is able to make a cut in both strands of the DNA when the efficient transformation of Cas9

nuclease and single guide RNA into the plant system takes place. There are three major DNA transformation methods in plant system: *Agrobacterium*-mediated transformation, biolistic, and protoplast transfection method. *Agrobacterium* is the common method utilized for plant genetic transformation, where T-DNA transfer is accomplished with DNA to be delivered being incorporated within the plant genome and being stable transformed, which leads to transient gene expression (Krishna et al. 2016; Wang and Wang 2012). Particle bombardment using a gene gun is another method commonly used in monocot species. In this, CRISPR/Cas9 constructs are integrated at high speeds with carrier molecules into the target cells. Later, DNA dissociates from the microcarriers and integrates into the plant genome. The *Agrobacterium* and biolistic methods may also produce unwanted changes and off-target mutation. To overcome this drawback, CRISPR/Cas9 RNP-mediated transformation was established, which avoids transgene integration and decreased off-target mutations through preassembled CRISPR/Cas9 ribonucleoproteins (RNPs). In this method, protoplast with plasmids expressing the target sequence reagents or ribonucleoproteins was used. Protoplast facilitates direct delivery of DNA into cells with target sequence editing components, which leads to transient transformation and also retains their cell identities, which also can be regenerated into an entire plant. Protoplast has higher transformation efficiency as compared to other methods (Baltes et al. 2017). These RNA-guided endonuclease (RGENs) RNPs directly edit the targeted sequences just after transfection and are quickly degraded in the plant cells, thus leaving no traces of foreign DNA elements and minimum off-target effects (Kanchiswamy et al. 2017; Woo et al. 2015). It was already reported that mutated plants were successfully regenerated in lettuce, Arabidopsis, tobacco, potato, rice, wheat, and soybean using CRISPR/Cas9 or Cas12a RNP complex delivery into protoplast cells and is heritable (Andersson et al. 2018; Kim et al. 2017; Liang et al. 2017; Woo et al. 2015). The regenerated plants would likely be exempted from the regulatory process because of no integration of any foreign DNA into the targeted plants (Clasen et al. 2016; Haun et al. 2014). Another simplest and frequently used method in plant transformation is the floral dip using agroinfection, which replaces the lengthy tissue culture procedures (Zlobin et al. 2020).

10.3.4 Characterization of Edited Plants

The main practices used for screening CRISPR/Cas system-induced mutants include qPCR assay, surveyor nuclease (T7EI assays), high-resolution melting analysis (HRMA)-based assay, high-throughput tracking of mutations (Hi-TOM), and whole-genome sequencing (WGS) to detect it in targeted sequences. The qPCR is used to identify mutated DNA sequences by amplifying the locus and sequencing of PCR products, which is mainly used to differentiate between heterozygous and homozygous mutations. This method is widely used as it is a highly effective, rapid, and simple method to detect induced mutations and already validated in Arabidopsis, sorghum, maize, and rice (Peng et al. 2017).

The T7EI assays or surveyor nuclease are widely used and considered suitable for any target sequence. The CEL family of mismatch-specific nucleases includes surveyor nuclease. These nucleases recognize mismatches and create cut in heteroduplex DNA sequences. It targets mismatch sequences by cleaving both DNA and identifies mutations of up to 12 nt (Qiu et al. 2004). However, detection sensitivity of T7EI assays method is considerably less than PCR assays and are more labor- and time-consuming and cleave several double-stranded DNA molecules if their structure is curved and bend (Cong et al. 2013; Declais et al. 2006).

The high-resolution melting analysis (HRMA) technique entails DNA sequence amplification by qPCR covering (about 90–200 bp) genomic target, with fluorescent dye after that amplicon melt curve analysis (Wang et al. 2015). The nondestructive nature of this method requires less than 2 h for the whole procedure, from genomic DNA preparation to mutation detection. After that, amplicons could be studied using sequencing and gel electrophoresis. The advantage of this method is that they are a simple and more delicate technique and has a high-throughput screening format. However, this assay has some limitations: assay is not able to identify larger mutations and the cost is also higher for operating the assay. However, higher cost of this method can be decreased by coupling HRMA with online HRMA software (Talbot and Amacher 2014). The Hi-TOM assay is an online tool that can be used for quantitative and precise mutation detection induced by the CRISPR/Cas9 system, without any extra-complex parameter configuration and data analysis. The advantage of assay is it is easy and user-friendly, and does not require a skilled person for bioinformatics tools or next-generation sequencing (NGS). The Hi-TOM online tool has suitable high-throughput detection methodology for CRISPR system-induced mutations because of its convenience to use (Liu et al. 2018). A non-denaturing PAGE gel is also used for detecting the genome editing-induced mutations in the target genomes. In this method, variations in the single-stranded sequences can alter their conformations and show different migration rates in PAGE gel, and it is also called "single-strand conformation polymorphism" (SSCP) (Zheng et al. 2016). Sanger sequencing can be used to study the amplicon containing targeted sequences. The Sanger sequencing approach is suitable for identifying mutations; however, the real problem is that it is expensive and tedious.

10.4 Application of Genome Editing in Cereals for Abiotic and Biotic Stress Tolerance

The major losses in crop production are caused more by abiotic stresses, for instance, drought, salinity, and high temperature than by biotic stresses. The genome editing tools can contribute significantly toward creating novel plant types having tolerance to abiotic and biotic stresses in cereal crops as summarized in Table 10.1.

Table 10.1 Promising genes edited for abiotic/biotic stress tolerance in cereals

Crop	Gene	Stress	Reference
Abiotic stress			
Oryza sativa	*OsDST*	Drought and salt tolerance	Kumar et al. (2020)
Zea mays	*ARGOS8*	Drought tolerance	Shi et al. (2017)
Triticum aestivum	*TaDREB2, TaERF3*	Drought tolerance	Kim et al. (2018)
Triticum aestivum	*TaCer9*	Drought tolerance	Liang et al. (2018)
Oryza sativa	*OsNramp5*	Salinity tolerance	Tang et al. (2017)
Oryza sativa	*OsRR22*	Salinity tolerance	Zhang et al. (2019)
Zea mays	*ZmCLCg*	Salinity tolerance	Luo et al. (2021)
Biotic stress			
Triticum aestivum	*TaLpx-1, TaMLO*	*Fusarium graminearum* and powdery mildew	Wang et al. (2018)
Oryza sativa	*OsSEC3A*	Bacterial blast	Ma et al. (2017)
Triticum aestivum	*TaMLO-A1*	Resistance to powdery mildew	Wang et al. (2014)
Triticum aestivum	*TaLox2*	*Fusarium head blight*	Shan et al. (2013)
Oryza sativa	*OsERF922*	Blast resistance	Wang et al. (2016)
Oryza sativa	*eIF4G*	Rice tungro spherical virus resistance	Macovei et al. (2018)
Oryza sativa	*SWEET11, SWEET13, SWEET14*	Bacterial blight resistance	Oliva et al. (2019)
Triticum aestivum	*TaABCC6, TansLTP9.4, TaNFXL1*	Fusarium head blight (FHB) resistance	Cui et al. (2019)
Hordeum vulgare	*HvMORC1*	Resistant to powdery mildew and *Fusarium graminearum*	Kumar et al. (2018)
Triticum aestivum	*TaEDR1*	Powdery mildew resistance	Zhang et al. (2017)

10.4.1 Drought Stress

In the future, increase in frequency and severity in drought stress are expected in many regions. Hence, there is a need to develop genotypes tolerant to decreased precipitation and increased evaporation. The rice *OsDST* gene encodes a zinc finger transcription factor, and the loss of DST protein function in rice resulted in drought and salt tolerance (Huang et al. 2009). The mutation in the gene enhanced the leaf width, reduced stomatal density, and enhanced stomatal closure through modulation

of H_2O_2 homeostasis (Huang et al. 2009). TheCRISPR/Cas9-induced mutation in the DST gene of indica rice enhanced the leaf water retention under dehydration stress. In the seedling stage, the Cas9-free DST mutant showed moderate resistance to osmotic stress and excellent tolerance to salt stress (Kumar et al. 2020). Under drought conditions, targeted editing of two abiotic stress-responsive transcription factors in wheat, dehydration response element-binding protein 2 (TaDREB2), and ethylene responsive factor 3 (TaERF3) revealed increased expression of both genes in seedlings (Kim et al. 2018). The precise genomic DNA modification of a negative regulator of ethylene responses, *ARGOS8 (*auxin-regulated gene involved in organ size), *in* maize through CRISPR/Cas technology generated novel variants of *ARGOS8*, showing increased grain yield under drought stress at flowering stage (Shi et al. 2017).

10.4.2 Salt Stress

To improve salt tolerance in rice, CRISPR/Cas9 system has been used for target site genome editing. The salinity tolerance of T_2 homozygous mutant lines of *OsRR22*gene was engineered through a Cas9-OsRR22-gRNA expressing vector significantly enhanced as compared to wild-type plants (Zhang et al. 2019). CRISPR/Cas9 editing system was used to construct mutation alleles in drought and salt tolerance (DST) gene in indica rice cv. MTU1010. In the seedling stage, the Cas9-free DST mutant showed a high amount of salt stress (Kumar et al. 2020). Maize yield and quality is significantly affected by salt stress, and the genome-wide association analysis identified several QTLs for maize seedling salt tolerance. The functional validation of the candidate gene *ZmCLCg* in salt tolerance was done by generating gene knockout mutations through CRISPR/Cas9 technology. Under 100 mm NaCl treatment, three *ZmCLCg* mutants demonstrated higher decrease in root length, root fresh weight, shoot length, and shoot fresh weight than that of the wild type (Luo et al. 2021).

10.4.3 Biotic Stress

A major fungal disease powdery mildew is affected by *Blumeria graminis* f. sp. *tritici* in wheat and causes considerable yield losses. In bread wheat, different genome editing tools are utilized to introduce targeted mutations in the three homoeoalleles that encode mildew resistance locus (MLO) to provide powdery mildew resistance, a characteristic that is not seen in natural populations (Wang et al. 2014). The enhanced disease resistance1 (EDR1), a Raf-like mitogen-activated protein kinase kinase kinase (MAPKKK), reported as a negative regulator of powdery mildew resistance in *Arabidopsis thaliana* (Frye et al. 2001). The single guide RNA (T-EDR1) targeting a highly conserved region in the fourth exon was used in CRISPR/Cas9 technology to generate frameshift mutations for three homologs of wheat *EDR1*. The edited plants were resistant to powdery mildew and did not observe mildew-induced cell death (Zhang et al. 2017). The mutations were

introduced through CRISPR/Cas9-mediated genome editing in three sucrose transporter genes, namely, *SWEET11*, *SWEET13*, and *SWEET14* of rice. The mutations in the promoter regions of these genes resulted in broad-spectrum resistance to bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (Oliva et al. 2019). The confined screenhouse experiments indicated that most of the lines performed similarly to wild type for key agronomic characters such as plant height, panicle length, number of reproductive tillers, and fertility rate. Resistance to rice tungro spherical virus was acquired by a mutation in the eukaryotic translation initiation factor 4 gamma gene (eIF4G) in indica rice cv.IR64, a commonly planted variety across tropical Asia (Macovei et al. 2018).

The ethylene responsive factors are associated in imparting stress tolerance to abiotic and biotic stresses. The rice ethylene-responsive factor gene *OsERF922* is a negative regulator of blast disease produced by *Magnaporthe oryzae* in rice (Liu et al. 2012). The CRISPR/Cas9-targeted knockout of the ERF transcription factor gene *OsERF922* in a *japonica* rice variety improved the blast resistance. The mutant lines showed a significant reduction in blast lesion after pathogen infection. However, there were no significant variations in agronomic attributes between mutant lines and natural plants. Multiple sites within the *OsERF922* gene were also targeted to induce two or more mutations using Cas9/multi-target sgRNAs (Wang et al. 2016). Microrchidia (MORC) proteins act as negative regulators of immunity in barley. The barley genome contains seven MORC genes. *Hv*MORC1 was knocked out by *Streptococcus pyogenes* Cas9 (*Sp*Cas9) to generate loss-of-function alleles by targeting upstream of the ATPase domain of the gene. The hvmorc1-KO mutants showed increased resistance to fungal pathogens *Blumeria graminis* f. sp. *Hordei*, which bases powdery mildew and mycotoxin-producing fungus *Fusarium graminearum* (Kumar et al. 2018).

10.5 Challenges and Opportunities of Genome Editing in Cereals

The focus of genome editing is refining crops through improving yield and its associated traits. However, despite the significant attainments in crop improvement, there are few key obstacles that want to be focused while using genome editing technologies, such as polyploidy, off-target mutations, delivery methods, etc.

10.5.1 Polyploidy

Polyploidy is one of the biggest challenges to achieve the desired mutation, owing to additional complete chromosome sets within an organism. Due to the dosage impact of paralogous gene copies, this may not result in phenotypic alterations, especially during gene knockdown or knockout. However, occasionally desired trait alteration necessitates editing of all paralogs, which significantly reduces efficacy. In many polyploid crops such as sugarcane, wheat, etc., polyploidy is the major issue, as the

targeted locus is present in many copies. Therefore, it is more exciting for GE tools to achieve homozygous plant that contains multiple target loci in polyploid crops. The key success for editing the multigene family members is to avoid the conserved sequence and select a unique gene sequence. Another major problem in polyploid crops is to screen a large number of edited plant population. To overcome the current problem, high-throughput phenotyping facility development is considered as a potential strategy. But successful implementation of this strategy again requires special skills and high-tech instrumentation. So far, the CRISPR/Cas-mediated homology direct repair (HDR) has been reported in many plant species such as Arabidopsis, tomato, rice, and maize (Li et al. 2021). Furthermore, the prime plant editors were generated in wheat protoplasts to attain specific point mutations at seven exogenous gene targets with single-nucleotide substitutions; the frequency observed was 1.4% (Lin et al. 2020). This indicates that more efficient plant prime editors require further improvement, mainly in polyploid species (Li et al. 2020).

10.5.2 Transformation Methods

In CRISPR/Cas9, another greatest challenge is the effective delivery method to the proper tissue and subsequent regeneration or expression of viable plants. The main difficulties during transformation are the time-consuming process, lower transformation frequency, lower titer of DNA, less precision, many crops recalcitrant to regeneration, and random somatic mutations (Gao 2018).This challenge creates an urgent need to improve the delivery system to obtain high efficiency of genome editing by using regeneration, use of booster to enable tissue culture in recalcitrant crops, or direct delivery to apical meristem to get edited plants without tissue culture. With progression in ribonucleoproteins (RNPs), viral delivery and nanoparticle systems offer other transformation methods for a tissue culture-free GE system. These technologies not only enhance the efficiency of genome editing but also decrease the regeneration period of edited plants. This prime obstacle offers an opportunity to augment plant transformation and regeneration responses by targeting an extensive range of tissues and genotypes.

10.5.3 Off-Target Effect

Another major concern is an off-target effect that impedes the potential application of the CRISPR/Cas9 system, where Cas9 cleaves genomic DNA sites that are imperfect complements of sgRNA. RGEN (RNA-guided endonuclease)-induced mutations has high frequency in off-target activity ($\geq$50%) at sites other than the intended on-target site. Unwanted cleavage and undesired chromosomal rearrangements due to higher number of off-targets can induce cellular toxicity. Inversion, translocations, and deletions triggered by the repair of these off-target DSBs can be damaging to plants. Cas9's sensivity is related to the 20 nucleotide sgRNA guide sequences and the PAM sequence. Many studies have found off-target DNA cleavage in sgRNA sequences with 1–5 bp mismatches. It has also been

suggested the PAM sequence involved in the binding of Cas9. At the same time, 3′ end is essential for target identification, R-loop formation, and nuclease activation in Cas9 (Sander and Joung 2014). Potential off-target effects of the CRISPR/Cas system in the target sequence can be overcome by truncated Cas9 (Ran et al. 2013). An alternative approach is to use truncated gRNAs to minimize off-site targeting of the CRISPR/Cas system (Fu et al. 2014; Pattanayak et al. 2013). Further, ribonucleoprotein (RNP)-mediated genome editing has helped in the reduction of the off-target effects in wheat and maize (Liang et al. 2017; Svitashev et al. 2016). In silico prediction, HTGTS (high-throughput genome-wide translocation sequencing), ChIP-seq (chromatin immunoprecipitation), T7E1 (T7 endonuclease1) assay, fluorescence in situ hybridization, deep sequencing tools, etc. have been reported to examine off-target events. Off-target detection approaches like GUIDE-seq and Digenome-seq have been advanced with 0.1% sensitivity. The composition and structure of guide RNAs are the primary determinants of on-target and off-target cleavages. The off-target events can be reduced by manipulating the structure and composition of sgRNA (Manghwar et al. 2020). Another approach is using different Cas9 variants to reduce the off-target effects in various crops, and different cas9 variants are developed, merging dCas9 with FokI nuclease to develop fCas9 (Guilinger et al. 2014) and three to four amino acid replacements in Cas9, which lead to no detectable off-targets (Kleinstiver et al. 2016). However, various tools are being developed such as PEM-seq, CCTop, CHOPCHOP, CRISPR-PLANT v2, CT-Finder, CROP-IT, CFD (Cutting Frequency Determination) Score, CRISPOR, CRISPR-GE, etc. for sgRNA finding evaluation and predicting.

10.6 Regulatory Issues of Genome Edited Crops

Globally, the legal status of genome editing is not decided yet or is still under discussion. In Europe, the Court of Justice of the European Union (CJEU) rule on directed mutagenesis is that the genome edited products are subjected to current legal GMO framework without any exemptions, and genome editing are subject to the legal framework applicable to release, marketing, labelling, and traceability of GMOs (Menz et al. 2020). In Israel, during 2017, the National Committee for Transgenic Plants stated that genome-edited plants with no insertion of foreign DNA and with deletion of nucleotides will not be considered as transgenic and thus not to be subjected to the GE seed regulation. Moreover, the genome-edited plants with foreign DNA incorporated will be subjected to regulations and guidelines found in the GE seed regulation (USDA FAS 2020).

Different countries adopted different approaches of regulations to approve the genome editing crops. Mainly, two regulatory approaches are adopted by different countries, known as process-based and product-based regulations. The process-based regulations are adopted in the EU and Norway, and Canada is an example of product-based regulation. However, the United States is described as a combined regulatory approach (Ishii and Araki 2017; Zetterberg and Bjornberg 2017). The gene editing plants are considered under the GMO regulations in New Zealand and

European countries, while GMO regulations are removed by the United States from gene-edited plants (Gupta et al. 2021).

In India, the Department of Biotechnology under the Ministry of Science &Technology drafted guidelines after expert consultations and invited comments from researchers, institutions, and other stakeholders. According to draft guidelines, the GEd Group I (SDN-1, ODM) and GEd Group II (SDN-2) would be assessed mainly to confirm targeted edit(s) as well as an absence of any biologically significant off-target genomic changes. In addition, they would be subjected to phenotypic equivalence analysis. The GEd Group II would also be used for trait efficacy through appropriate contained and/or confined field trials. However, GEd Group III (SDN-3) harboring large or foreign DNA may represent similar biosafety concerns as that of genetically engineered (GE) organisms (DBT, India 2020).

10.7 Conclusion

The progress made through conventional breeding for food security is remarkable. However, climate change offers new challenges for further yield improvement in cereal crops. The genome editing tools offer a novel opportunity for designing the crop with the preferred trait(s). There is an urgent need for human resource development in these emerging technologies. The method also needs to be standardized for each crop for greater harvesting of products through genome editing in cereal improvement.

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