Chapter 12
Principles and Applications of RNA-Based Genome Editing for Crop Improvement

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Abstract Ever increasing population demands highly productive agriculture and nutritive crops. Precise genome editing serves as a promising tool to meet out those demands by rapid crop improvement. CRISPR/Cas system and its latest versions are exhibiting its potentiality on targeted editing, single base substitution, multiplex editing of key genes for accelerating plant growth and development. In this chapter, we review the basic principles of CRISPR/Cas9 system, modifications of Cas proteins, delivery methods and applications. We also addressed the applications of this technique for elevating crop yield and increasing biotic and abiotic stress resistance. Additionally, we summarized the regulatory aspects of genome-edited crops in India as well as in other countries. Although CRISPR/Cas is successful, it has some technical limitations which are mentioned at the end of this chapter. Altogether, this chapter could provide an overall picture about CRISPR/Cas systems and their influence in plant science research.

Keywords CRISPR/Cas9 · Genome editing · Base editing · Multiplexing · Cas9 engineering · Cpf1 · Cas13 · gRNA-Cas9 delivery · GE crops regulation

12.1 Introduction

Crop cultivation had been originated through the domestication of plants of interesting traits such as desirable architecture, altered plant height, increased number of tillers and/or seeds, modified fruit size and shifted flowering pattern. These traits were altogether hand-picked by farmers and being grown with the aim of getting higher returns from their fields. On the other end, plant breeders introduced the genetic improvement through interspecific and intraspecific crossing of sexually propagated plants and also through spontaneous mutations for asexually propagated plants by

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molecular breeding approaches (Sleper and Poehlman 2006). However, the mutations remained as the key to introduce genetic variability for aforementioned crop improvement processes. Random mutagenesis is the mother of all genome editing techniques as of till date but duration and efforts are the key limiting factors of developing mutated plants through natural mutagenesis. Generally, classical plant breeding strategies are time-consuming in terms of getting the best germplasm after crossing elite cultivars whereas genome editing snaps desired target as programmed and is efficient in terms of genome manipulation. So, the gear was shifted from molecular breeding to precision breeding in which targeted editing of genomes is the holy grail which allows rapid introduction of genetic diversity and accelerates variety development (Jansing et al. 2019b).

12.1.1 Genome Editing—As Molecular Scissors of Mutation

Genome editing is the targeted modification of gene(s) of interest through insertion, deletion, substitution in the genome to decipher gene functions. Site-specific double-strand breaks (DSBs) is the trigger for genome editing after which subsequent DNA repair is performed through error-prone non-homologous end joining (NHEJ) and error-free homology-directed repair pathways (HDR) (Zhang et al. 2017a). NHEJ is the most efficient and occurs in all stages of the cell cycle whereas HDR has low efficiency and occurs at S/G2 phases of the cell cycle (Mladenov and Iliakis 2011; Puchta 2005). HDR pathway is a highly precised technique for editing the target sites performed by utilizing a DNA template that is homologous to the upstream and downstream site of DSB (Jansing et al. 2019b). Unlike HDR, NHEJ causes rearrangement of chromosomes because of occurrence of DSB at multiple sites, followed by the fusion of inappropriate ends (Pacher et al. 2007). These mechanisms of DSB repair necessitate the way for targeted breaks at the specific sites for controlled manipulations of the genome. The advent of site-specific nucleases, as the programmable enzymes, solves the purpose of site-specific editing and the induction of DSBs (Jansing et al. 2019b).

12.1.2 Tools for Genome Editing

Genome editing approaches are quite relevant and having wide applications due to development and evolution of site-specific nucleases and precise editing of desirable targets. In general, the site-specific nucleases are composed of DNA binding domain which binds to the target and non-specific endonucleases which makes a snap at the target. These events initiate NHEJ or HDR pathway for DNA repair. NHEJ pathway for repair of DNA ends is non-specific due to the insertion or deletion of bases of different sizes whereas HDR remains specific because of the homology

template added as a donor DNA for the repair mechanism (Puchta 2005). Till date, the well-studied nucleases for genome editing are Zinc Finger Nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs), Clustered regularly interspaced short palindromic repeat—Cas9 (CRISPR/Cas9) and the multiple variants of CRISPR associated protein 9 (Cas9).

12.1.2.1 ZFN (Zinc Finger Nucleases)

ZFNs are the first artificial nucleases that are used for genome editing. They consist of a specific domain for DNA binding called Zinc Finger motif and non-specific FokI nucleases attached to the C- terminal of zinc finger motif. Zinc fingers are the series of three to six repeats of 3 bp DNA sequence, together called zinc finger array. Two zinc finger arrays are designed individually that bind to forward and reverse strands of the DNA leaving the gap of 5–7 bp in the target sequence. The two different zinc finger array (5' to 3' and 3' to 5' terminals) are necessary for leading the dimerization of FokI which is necessary for its nuclease activity (Lloyd et al. 2005; Zhang et al. 2017a). Fok1 is the type IIS restriction enzyme from *Flavobacterium okeanokoites* (Kim et al. 1996). Despite being used in various crops such as corn, soybean and Arabidopsis, ZFNs have key limitations in multiplex editing, target selection and laborious cost of assembly (Zhang et al. 2017a).

12.1.2.2 TALENs (Transcription Activator-Like Effector Nucleases)

TALENs are the second generation of artificial nucleases comprising of specific target binding TALE effector domain (Transcription activator like) and non-specific FokI nuclease domain (Mahfouz et al. 2011). TALE domain consists of 33–35 tandem repeats of amino acids which are conserved except two residues, called as RVD (Repeat Variable Di-residues) (Bogdanove et al. 2010). Like ZFNs, TALENs also contain FokI nuclease attached to the two monomers of TALE domains. The spacer length between TALEN monomer in the target is 15–20 bp that is higher than that of ZFN. The ease of making the assembly lets TALEN as a better tool for genome editing than ZFN. Similar to ZFN, TALEN also has a few limitations in terms of abundant targets, multiplexing and cost of assembly (Zhang et al. 2017a). Despite TALEN having better efficiency in comparison to ZFN, the number of reports of genome editing are very few for both classes of artificial nucleases. This could be due to a few reasons *viz.*, higher number of tandem repeats for binding with the target, experimental conditions and the choice of targets (Jansing et al. 2019b). The most common key limiting factor for both ZFN and TALEN is the stringent requirement of dimeric guide sequences of protein for a single target. This technique hampers the aim of doing multiplexing in which DSB occurs at multiple sites of the target (Kannan et al. 2018).

12.1.2.3 CRISPR/Cas System (Clustered Regularly Interspaced Short Palindromic Repeats)

CRISPR/Cas9 is the most flamboyant of the third generation of sequence-specific nucleases and is being extensively studied for enabling DSBs at desired site of target in the genome. CRISPR was first discovered in *Escherichia coli* as the DNA fragment with short repeats inter-twined with spacers derived from invading bacteriophages. Initial evidence of involvement of CRISPR in adaptive immunity was found out through the addition or deletion of these spacers in the bacterial system (Barrangou et al. 2007). Two or three nucleotides among the spacers acquired through phage invasion in bacteria served as a protospacer motif (PAM), crucial for the recognition of targets by the nucleases. Cas9 is the associated nuclease with CRISPR, located proximal to CRISPR locus (Ishino et al. 1987; Jansen et al. 2002). This system consists of a single effector Cas endonuclease and chimeric guide RNAs which together form ribonucleoprotein complex (RNP) to justify its role. Chimeric RNA acts as a single guide RNA (sgRNA) and consists of CRISPR RNA (crRNA), composed of target-dependent nucleotides, and trans-activating crRNA (tracrRNA), which interacts with both crRNA and Cas9 nuclease (Jinek et al. 2012). Cas nuclease contains HNH and RuvC-like domains. HNH binds to the complementary strand of guide RNA whereas RuvC-like domain binds to the non-complementary strand of the target. After binding, Cas9 endonuclease produces double-strand break (DSB) by blunt end cutting at upstream of PAM (Makarova et al. 2015; Jinek et al. 2012). CRISPR system is relatively easier and faster to design, as only sgRNA sequence has to be designed for pairing with the target gene and there is no requirement of any protein modifications like ZFNs and TALENs. Hence, these features basically make CRISPR/Cas system user-friendly technology in comparison to ZFNs and TALENs (Jinek et al. 2012; Bao et al. 2019).

After an initial report of CRISPR/Cas in plant system in 2013, CRISPR/Cas system has burgeoning fame and overwhelming output because of its applications in all areas of plant biology. Recent paper from Science reported that the number of publications and patents related to CRISPR are increasing steadily. Of which, the USA and China are the leading giants in the current scenario of blooming CRISPR applications in the field of mammalian and plant sciences. As of 2018, USA ranks top in terms of publishing papers (898) followed by China with a marginal difference (824). Japan, UK, Germany and Canada followed the consecutive places of publishing their CRISPR research (Cohen 2019). For more information on the chronological developments of CRISPR from its first report to the recent application, please refer to the review article by Razaaq et al. (2019). The detailed workflow of CRISPR is furnished in Fig. 12.1. The current chapter will focus on the multifaceted features of CRISPR technique and its development for tremendous success in the field of plant biology.

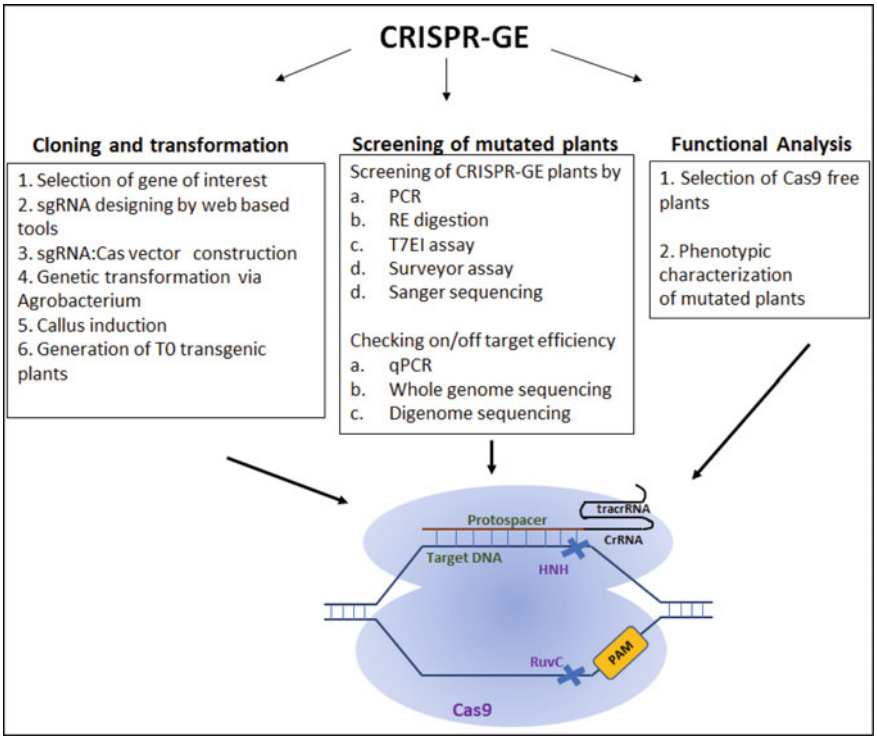

Fig. 12.1 Summary of workflow for generating CRISPR-GE (genome editing) crops. Three major steps are described here primarily involved in GE of crops. First, the workflow starts with construction of gRNA:Cas9 and transferring gRNA:Cas9 into plants. Second, confirmation of edited plants through various methods. Third, phenotypic analysis of gene edited plants

12.2 CRISPR/Cas System—A Wide Horizon of Genome Editing

12.2.1 CRISPR/Cas9

CRISPR is an adaptive defence system in prokaryotes to combat against foreign pathogens. CRISPR system has classified into six major types, *viz.*, type I to type VI. Each system has its own signature single Cas protein (in case of type II, V, VI) and multiple Cas proteins (in case of type I, III, IV) (Shmakov et al. 2017; Koonin et al. 2017). Of which, type II system encompasses the Cas9 protein from *Streptococcus pyrogenes* and SpCas9 is one of the mostly used nucleases for genome editing in plants (Makarova et al. 2015). As mentioned in the previous section (see also in 1.2.3), the CRISPR system is engineered based on type II Cas9 with tailored sgRNA comprised of fused crRNA and tracrRNA. SpCas9 is programmed to make DSBs at three bases upstream of PAM sequence of NGG in which N could be any one of the

four nucleotides preceded by N20 nucleotides of the gRNA sequence. Recently, it was found that SpCas9 engineered version, SpCas9-NGv1 could target NG PAMs in rice and Arabidopsis (Endo et al. 2019). The PAM sequence of Cas9 from *S. thermophiles* is 5'-NGGNG or 5'-NNAGAA whereas Cas9 from *Neisseria meningitidis* recognize the PAM of 5'-NNNNGATT (Garneau et al. 2010; Zhang et al. 2013; Gasiunas et al. 2012). Both sgRNA and Cas9 cassettes are introduced into plants by Agrobacterium-mediated transformation. Several features need to be considered for the successful genome editing of which the choice of promoters for gRNA and Cas9 is essential. Generally, gRNA is expressed by either U3 or U6 promoter-driven by RNA polymerase III whereas Cas9 is expressed by either ubiquitin or 35S promoter-driven by RNA polymerase II. The U6 and U3 promotors have definite transcription start nucleotides like G and A, respectively. So, the consensus guide sequences are G(N19)-PAM and A(N19)-PAM for U6 and U3 promoters, respectively (Nekrasov et al. 2013; Feng et al. 2013; Mao et al. 2013; Xie and Yang 2013; Miao et al. 2013; Jiang et al. 2013). Also, Cas9 with nuclear localization signal ensures the likely integration of the construct with the plant genome (Belhaj et al. 2013). Besides, the secondary structure of both gRNA and the target, the codon usage of Cas9 in plants, GC content of both gRNA and the target altogether influence the targeting efficiency of CRISPR/Cas9 in plants (Ma et al. 2015b).

During the initial stages, CRISPR applications had resulted in low efficiency in editing, therefore much improvements were continuously made later to improve its functionality through the selection of proper vector as well as the target, efficient construction of gRNA-Cas9 cassette, and improvement of delivery methods to plants. gRNA selection is one of the key steps for CRISPR-mediated genome editing, for which approximately 22 softwares have been developed within six years from 2013–2019 (Razzaq et al. 2019). Recent report (Gerashchenkov et al. 2020) has indicated the existence of 100 programmes to design gRNAs for CRISPR/Cas systems. Most of them are free to access and can also predict off-targets and secondary structures. In plants, for constructing efficient gRNA cassette, overlapping PCR or adapter ligation method has been used. For designing Cas9 cassette, plant-based codon should be used to improve the editing process in plants (Xie and Yang 2013; Fauser et al. 2014). Improved expression vectors have been developed by using single polymerase II and dual polymerase II driven gRNA: Cas9 cassettes. In the case of single pol II, both the guide RNA and Cas9 were expressed by a single promoter in the vector whereas, in dual pol II, different promoters drive their expression (Lowder et al. 2015). Owing to ease of developing CRISPR cassette, it has widespread applications in most of grain crops like rice, wheat, maize, sorghum, barley and fruits like tomato, sweet orange, apple and also in other crops like cotton, lettuce, soybean, citrus, lotus, petunia including mushroom and Arabidopsis. For all the references of above crops, please go through the review published in critical reviews in biotechnology (Bao et al. 2019).

12.2.2 CRISPR/Cas12a (Cpf1)

This is the next generation of Cas9 advancement into Cas12a, otherwise called Cpf1, the name was derived from *Prevotella* and *Francisella* bacteria. It is a monomeric protein belongs to the type V category of CRISPR system. It recognizes T rich PAM sequences like 5' TTTN 3' or 5' TTN 3' located at the 5' end of the target and makes a staggered cut with overhanging five nucleotides at 5' end of the PAM. This sticky cutting results in the loss of 6–13 bp, causing a larger deletion than Cas9 (Tang et al. 2017). In this system, crRNA (42 nucleotides) guides Cpf1 and cleaves the target without the need of tracrRNA as in sgRNA-Cas9 (Zetsche et al. 2015). Besides, Cpf1 has dual functions as a nuclease and as an RNAase where it cleaves at the target site and processes the pre-crRNA to mature crRNA, respectively (Dong et al. 2016; Fonfara et al. 2016). This dual role of Cpf1 has nodded off the usage of separate promoters for each gRNA while multiplexing. Another salient feature of Cpf1 is mainly its versatility by which it can be deployed in multiplexing, base editing and epigenetic modifications. The notable advantage is that Cpf1 generates low off-targets compared to Cas9 (Bayat et al. 2018; Zaidi et al. 2017). In plants, heritable mutations generated by Cas12a was first reported in rice and tobacco. Increased efficiencies of both FbCpf1 and LbCpf1 were observed in these studies (Endo et al. 2016; Xu et al. 2017). This could be mainly because of the reasons such as snapping by Cas12a leads to editing with HR because of the generation of overhangs away from PAM that promotes the repair preferably through HR than NHEJ (Begemann et al. 2017). The features of gRNA such as GC content, melting temperature, free energy and the attributes of the target significantly determine the efficiency of mutations generated by Cas12a (Safari et al. 2019). CRISPR-DT was the first web-based tool that helped to generate gRNA sequences for using Cas12a. CRISPR Inc is another web tool, works simple and rapid for gRNA designing, based on recent annotations and covers the pre-searched targets of Cpf1 in the complete genome of twelve organisms (Zhu and Liang 2019; Park and Bae 2017). As of now, there are three Cpf1 systems available for genome editing in plants *viz.*, FnCpf1, LbCpf1 and AsCpf1 (Tang et al. 2017; Xu et al. 2017; Wang et al. 2017b). A comparative diagram of Cpf1 and Cas9 functioning are furnished in Fig. 12.2.

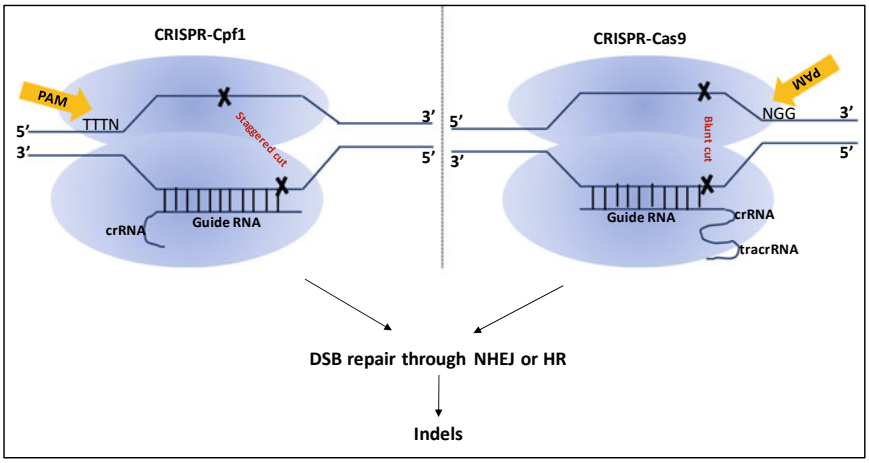

Fig. 12.2 Schematic diagram of two major CRISPR/Cas systems in plants. This figure depicts the key differences between Cas9 and Cpf1 system of gene editing. Cas9 makes blunt cut close to its PAM whereas Cpf1 makes staggered cut away from its PAM motif. It is also important to note the CRISPR RNA length is minimal in case of CRISPR/Cpf1 system

12.2.3 CRISPR/Cas13

Cas13 is the newest entry to CRISPR systems and it specifically targets cytoplasmic RNAs. This nuclease acts particularly on RNA through its catalytic activity of HEPN domain. Similar to Cas12, Cas13 also processes pre-crRNA, independent of tracrRNA, through its catalytic activity of Helical1 domain (Shmakov et al. 2015; Abudayyeh et al. 2016, 2017). In addition to mRNA, Cas13 also targets non-coding RNA which is very promising because of the key role of non-coding RNAs in gene regulation, protein translocation and splicing (Abudayyeh et al. 2017). These RNAs are the key targets of Cas13 *in vivo*, it provides ample chance of editing with inducible or tissue-specific promoters to avoid lethality due to the complete gene knock out (Schindele et al. 2018). Also, RNA virus infection and suppression were mitigated by exploiting the RNA targeting ability of Cas13 given the fact that RNA is the core of the majority of infective particles of the plant viruses. Since Cas13 targets mRNA, its likely applications in the field of post-transcriptional repression, mRNA transport, RNA binding proteins among others were evident (Abudayyeh et al. 2017).

12.2.4 Base Editing

The change in the single nucleotide base from the specific site of the genome without disruption of a gene, that leads to a notable phenotypic output is called base editing. This could be performed precisely through CRISPR/Cas system confining it to the

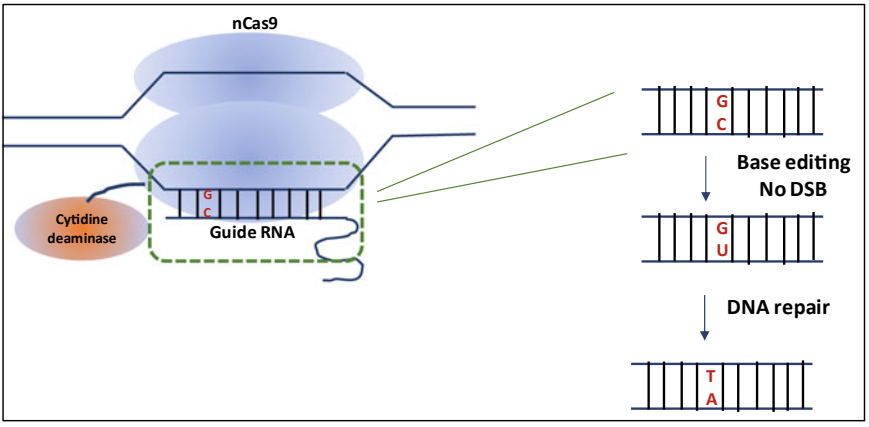

Fig. 12.3 Schematic diagram depicting the principle of base editing. An example of base editing in this figure shows that how the concept of single nucleotide change occurs through cytidine deaminase-fused nCas9 system

target in which the single base has to be edited. Base editing facilitates the direct change of nucleotides that are irreversible and causes promising genetic variants for crop improvement (Mishra et al. 2020). The limitations of double-strand break repair through NHEJ and low efficiency of HR in CRISPR raised the need of base editors to create point mutation at the desired target without double-strand break (Komor et al. 2016). Cytidine deaminase and nickase are the key components of the base editors in which the former changes the cytidine base to uridine and the latter changes Cas9 into nCas9 which is semi-active nuclease due to the mutation of D10A in one of its nuclease domains. Like CBE, ABE is designed to base edit the desired targets in plants by fusing adenosine deaminase with nCas9. The combination of these enzymes creates mismatch repair which resulted C: G altered to T: A base pair (Lu and Zhu 2017) (Fig. 12.3). Base editing has been standardized and employed in crops such as rice, wheat and maize (Zong et al. 2017). Interestingly, tRNA based adenosine deaminase (ABE) has been recently reported to change T: A to C: G which was difficult to alter previously. Through this adenosine deaminase application, 7.5% base editing in protoplasts and 59.1% base editing in stable transgenic rice plants were reported (Gaudelli et al. 2017; Li et al. 2018b). The readers are suggested to go through the information for the full list of base editors employed for crop improvement that has been updated in the review of Mishra et al. (2020). Recently, advanced base editor 3 was developed which has improved version of cytidine deaminase derived from rat (APOBEC1) and modified PAM sites to increase base editing efficiency (Hess et al. 2017). Similarly, APOBEC3A and AID from human and PmCDA1 from Lamprey were also fused with nCas9 and has been used in plants as base editors (Razzaq et al. 2019).

12.2.5 Multiplexing

Cellular development and growth in plants are usually governed by multiple genes. Also, few members of the gene families contribute to the important traits of interest in plants. Because of the natural phenomenon of association of several genes for expression of a single trait, more than one or many genes have to be edited or knocked out at multiple sites to study their association with the trait of interest. Multiplexing mediated through CRISPR/Cas9 involves the designing of multiple sgRNAs with single or multiple promoters expressed in a single vector system (Liu et al.2017a; Xing et al. 2014). Multiplexing technically can be categorized into different types based on the number of gRNA, promoters and the linkers attached in the cassette. Multiple gRNAs can be delivered as individual cassettes or as polycistronic cassettes by Cas9 nucleases or by its variants. However, multiple gRNAs with separate promoters is the typical multiplex which has been used so far. Some studies reported that multiple gRNA can be expressed as a single transcriptional unit in which functional gRNAs were individually generated by supplied ribozyme or by their own tRNA transfer system (Gao and Zhao 2014; Xie et al. 2015). Several approaches are used for multiplexing gRNAs by following one of the three methods *viz.*, golden gate assembly, polycistronic tRNA-gRNA system, ribozyme cleaving system and target-adaptor ligation (Lowder et al. 2015; Ma et al. 2015b). Instead of designing multiple cassettes of gRNAs for multiple editing, CRISPR-Cpf1 provides an easy platform for multiplexing. Cpf1 only needs single, direct short repeat spacer sequence (DR) which is processed by Cpf1 itself into functional crRNA units (Zhang et al. 2017a). Based on this strategy, Wang and the team have reported the efficacy of LbCpf1 and FnCpf1 in the editing of six different sites of three genes in rice such as Os*PDS*, Os*BEL* and Os*EPSPS*. Both variants of Cpf1 caused multiplex editing with their mature DR sequences and among them, LbCpf1 exhibited better editing than FnCpf1 (Wang et al. 2017b). Recently, SSTU (Simplified Single Transcriptional Unit) system was developed for multiplexing in rice to express FnCpf1 or LbCpf1 or Cas9 in which both the nuclease and crRNA are expressed under single Pol II promoter without any additional modules in the multiplex cassette (Wang et al. 2018a). Multiplexing was initially focussed for traits like yield increase and herbicide resistance but to date its application has been expanded from hormone perception to molecular farming (Najera et al. 2019).

12.2.6 CRISPR—Off-Targets

The specificity of CRISPR completely relies on 20 bp gRNA sequences complementary to the target of interest. Given the facts that the entire genome of the target is larger and Cas9 cleaves the target-like sequences instead of the right targets, leading to off-targeting of the CRISPR system, reported in several studies so far (Fu et al. 2013; Hsu et al. 2013; Pattanayak et al. 2013). Non-specificity arises mostly because

of the mismatches of 8–10 bp near to 3' end of gRNA (seed region) and mostly mismatches close to the 5' end are least bothered. Also, noted that PAM should follow immediately next to 20 bp of gRNA without any additional sequences (Hsu et al. 2013). This non-specific binding can mainly be avoided by designing the utmost specific gRNA sequence with the probability of fewer off-targets; identified by using web tools such as Cas-OFFinder, CRISPR-P, CRISPR-GE, sgRNAcas9, CRISPR design, E-CRISPR and CHOPCHOP. Additional strategies to avoid off-targeting are through using- truncated gRNAs with less than 20 bp, extra nucleotides like GG added to 5' end of gRNA sequence (Fu et al. 2014; Cho et al. 2014), and through using paired Cas9 nucleases, dCas9-*Fok1*, split Cas9, high fidelity Cas9 variants such as eSpCas9 1.0, eSpCas9 1.1 or using an engineered Cas9, SpCas9-HF1 (Komor et al. 2017; Kleinstiver et al. 2016a; Slaymaker et al. 2016). Despite, much attention has been given to minimize off-targeting in human genome editing for therapeutic applications, this is not worrisome in plants because the off-targets could be easily managed by segregation of allele through generations and getting rid of through the back crossing that will efficiently remove secondary mutations due to off-targeting (Schulman et al. 2020). It was also interesting to report that CRISPR/Cpf1 produces less off-targets compared to CRISPR/Cas9, which was consistent with the reports from animal studies (Kleinstiver et al. 2016b).

12.2.7 Delivery Methods of CRISPR Cassette

The delivering of CRISPR constructs into plant cells is as important as designing the construct for generating the edited plants, because the delivery method is one of the crucial deciding factors of CRISPR efficiency. CRISPR/Cas components are being transferred into plant cells mainly by *Agrobacterium*-mediated T-DNA transformation, particle bombardment and protoplast transfection methods. The first two methods are used for generating edited plants and the last one used for transient expression. *Agrobacterium* is the most commonly used tool for the transformation in general and also for editing constructs because of its high degree of T-DNA integration with the host genome. *Agrobacterium* is also used for the transient transformation of CRISPR constructs which resulted in low efficiency of both on and off-target mutations whereas stable transformations provided good expression of genome editing components and yielded a high frequency of on-target mutations (Jansing et al. 2019a; Chaparro-Garcia et al. 2015). The main lacuna of *Agrobacterium* transformation is the host specificity since some plant species, especially monocots that are recalcitrant to *Agrobacterium*-mediated transformation. Therefore, the second preferred method for transformation is the particle bombardment in which gold/tungsten particles are coated with DNA, RNA, protein, RNP and accelerated by gas pressure systems into plant cells (Sanford 1990). The quality and quantity of integration are much lower in this method compared to *Agrobacterium*-mediated transformation. However, it is advantageous that the broad host range of species are covered by particle bombardment (Verma et al. 2014).

Compared to plant systems, animal studies are using different methods for the delivery of CRISPR constructs because of the lack of cell wall in animal cells. If plant cell wall is removed, then multiple other methods for the delivery of CRISPR constructs are quite feasible in the plant system as well. Protoplast transformation is one of its kind to transform plants for genome editing with the help of PEG (polyethylene glycol) that helps the construct to permeabilize through plasma membrane (Darbani et al. 2008; Potrykus et al. 1998). Since protoplast transformation is a physical method, no specialized vectors are needed to transform the organism and also, multiple plasmids could be transformed at the same time resulting in transient or stable transformation with high frequencies (Baltes et al. 2017). Recently, by using magnetic field exposure, the construct coated with magnetic particles are directed to pollen grains for transformation. This could be applied for CRISPR constructs to increase their broad host range for transformation (Zhao et al. 2017). Recently, many variations in *in vitro* and *in planta* transformation of both crop plants and Arabidopsis with CRISPR-Cas constructs have been addressed in detail (Zlobin et al. 2020).

12.2.8 Engineered Cas9 Modifications

The generation of knock out mutants by CRISPR created a huge wave in the field of functional genomics (Decaestecker et al. 2019). However, this method has its limitations because of pleiotropic and lethal effects caused by loss-of-function of single gene. Although different plant species encode large number of genes, only small percentage of genes are important for the plant functions. For example, only 10% of ~25,000 genes in Arabidopsis are indispensable (Lloyd et al. 2015). Therefore, knock out of genes through CRISPR/Cas system should be customized based on the cell type, tissue type and organ type which is essential for reaping the complete benefits of this technology. Other than plant science, researchers working on mammalian systems have already demonstrated that modifications and/or fusion protein attachments of Cas9 have resulted in the tissue-specific knock out of gene of interest. One such example is that targeted knock out of *wingless* and *wntless* genes in Drosophila germ cells led to the generation of adult files whereas its non-specific overall knock out caused lethality (Port et al. 2014). Likewise, in plants, xylem specific promoter *NST3/SND1* was used to drive Cas9 expression in xylem cells to target *HCL* which resulted in decreased lignin content only in the specific cells (Liang et al. 2019).

2.8.1 CRISPR-TSKO (Tissue-Specific Knock out) is a new toolset that arrests gene activity in the tissues of interest leading to subset level genome editing (Ali et al. 2020). This technique is based on Golden gate and modified Green Gate vector technologies and designed for different cells, tissues and organ types for which Cas9 is driven by the respective tissue-specific promoter and attached with a fluorescent protein. By using this approach in Arabidopsis, nine different genes were targeted with four different tissue-specific promoters driving Cas9. Among the genes, *PDS3, YDA, CDKA1* are essential for plant growth and reproduction and whose ubiquitous knock out caused lethality whereas the TSKO approach yielded

viable plants from those mutants. This system allowed to study gene function in a spatial-temporal manner which was unlikely earlier due to the pleotropic effects of the loss of gene function (Decaestecker et al. 2019). Despite being useful for tissue-specific knock down of pleotropic genes, CRISPR-TSKO technique invariably depends on the promoters which may be leaky in neighbouring cells other than the targeted ones (Ali et al. 2020).

2.8.2 Cell type specific promoter—Genome editing in plants should be heritable in terms of the targeted mutation by CRISPR/Cas system. Most of the ubiquitous promoters such as 35S has been used to drive Cas9 expression, not providing good expression in meristematic and reproductive tissues (Ge et al. 2008). So it is necessary to use germ line-specific promoters such as egg cell-specific promoter EC1 and meristem-specific promoters such as CDC4 and, CLAVATA3 for heritable mutagenesis (Mao et al. 2016; Miki et al. 2018). Moreover, egg cell-specific promoters are preferred for DSB repair through HR due to the availability of donor template at higher concentration.

2.8.3 Cas9-PF—Generally, the stable integration of Cas9 and sgRNA have led to high-efficiency editing in plants. At the same time, retrieving edited plants free of Cas9 and gRNA is also important for CRISPR crops particularly for the concerns related to GMO regulations. The traditional methods of screening for Cas9-free plants require creation of T1 or T2 generation, and thereby is a time-consuming process. For generating edited plants without any background, Cas9-PF was developed in tobacco by Liu and team (Liu et al. 2019). In their work, they co-expressed *PAP1* (production of anthocyanin pigment 1) and *FT* (flowering locus T) in Cas9 cassette. *PAP1* served as a phenotypic marker to ensure the presence of CRISPR/Cas9 in T0 or T1 generation for selection. *FT* accelerated the breeding cycle for faster advancement into next generations. This PF cassette with Cas9 was used to target *E1F4E*, a recessive resistance gene to Potato virus Y in tobacco. Cas9-PF accelerated the process to get transgene free edited plants in a short time with increased efficiency.

2.8.4 Cas9-versions—Till now, many Cas9 variants and Cas9 from different bacterial sources are available for increasing the specificity as well as to enhance the editing efficiency. The best Cas9 for targeted knock out was studied by comparing the efficiency of different Cas9 on the same target. Cas9 from *Streptococcus pyogenes* (SpCas9), *Staphylococcus aureus* (SaCas9), *Francisella novicida* (FnCas12a), *Lachnospiraceae bacterium* (LbCas12a) and Cas9 engineered versions like eCas9 1.0, eCas9 1.1, eSaCas9 and xCas9 3.7 (Raitskin et al. 2019) in tobacco and Arabidopsis. The result indicated that SaCas9 has the highest editing efficiency. However, it may not be consistent because Cas9 activity depends on experimental conditions and its expression relies on promoters. Interestingly, temperature has been found to play a role in enhancing Cas12a activity (Moreno-Mateos et al. 2017; Schindele and Puchta 2020).

Besides its nuclease activity, Cas9 could be harnessed for different applications other than editing. The nuclease activity could be inactivated by introducing alanine substitutions in its catalytic sites at HNH and RuvC domains (Sapranauskas et al.

2011). Then the inactivated Cas9 variants (dCas9) have been used as a binding scaffold for attaching effector proteins for different purposes such as transcriptional activation, repression, histone methylation, and demethylation and mRNA transport and localization studies as well. For instance, in tobacco, *PDS* gene was transcriptionally tuned by fusing C terminus of dCas9 with the TAL domain or SRDX domain to activate or repress *PDS* expression, respectively (Piatek et al. 2015). Similarly, dCas9 is attached with fluorescent proteins to visualize the trajectory of target proteins and with demethylase to generate epigenomic modifications (Anton et al. 2014; Maeder et al. 2013). dCas12 and dCas13 were also used for multiple purposes, as mentioned above for inactivating RuvC domain of Cas12 and HEPN domain of Cas13, respectively (Platt et al. 2014; Liao et al. 2017). Hence, for successful use of CRISPR-based genome editing, a number of parameters need to be standardized. In Fig. 12.4, all essential factors for successful CRISPR are depicted.

2.8.5 Prime editing—This technique has recently taken genome editing to another level of success by introducing insertion, deletion and base to base conversion without the requirement of DSB and donor DNA template (Anzalone et al. 2019). In this novel approach, gRNA is replaced by pegRNA (prime editing gRNA) which drives

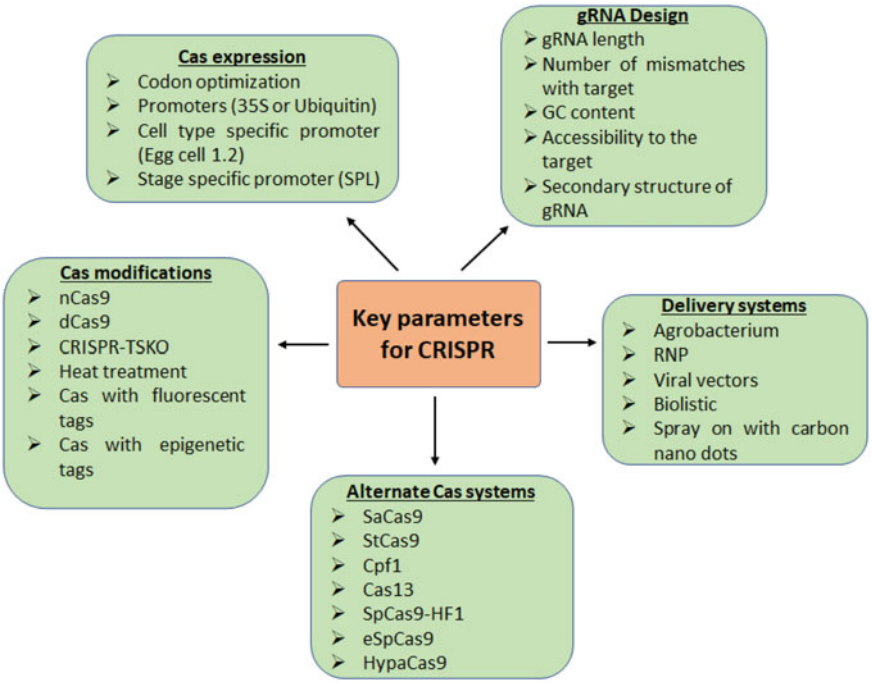

Fig. 12.4 Essential parameters for a successful CRISPR-GE experiment. This schematic diagram illustrates most of the key points for generating gene-edited plants through CRISPR system. It throws multitude of options from gRNA designing to delivery systems according to one's research directions

nCas9 fused with RT (reverse transcriptase). pegRNA contains PBS (primer binding region) which serves as template for RT to introduce mutations in the target. Lin et al. (2020) have firstly reported the prime editing application in plants by finding out the possibility of generating all types of base substitutions, insertions up to 15nt, deletions up to 40nt in the target genes of rice. Prime editing has been remained as more efficient and target-specific method than other editing technologies due to likely hybridization between the target and pegRNA which is probably higher at many places (Marzec and Henzel 2020).

12.3 CRISPR—For Revamping Plant Growth and Development

The advent and then development of CRISPR technologies has stirred basic plant biology research through its application towards crop improvement or new variety release. The molecular mechanisms CRISPR module have already been discussed in previous sections of this chapter, next let us see the variations of CRISPR modules and its applications in the following sections. Since its discovery in 2012, CRISPR application has been expanded to almost all of the major crops. Even, recalcitrant crop species which were difficult to transform, become amenable to gene editing because of the advancements in modulating Cas9 nuclease activity, its specificity and variants, base editing, and most importantly through novel delivery methods. In this section, we have attempted to uncover significant examples of applications of CRISPR in crop yield and resistance to biotic and abiotic stresses.

12.3.1 Yield and Grain Quality Enhancement

Yield is one of the key foci of plant science research as it is the ultimate contributor to sustained food security. Yield is a multi-genic quantitative trait, so knocking out single gene will not fetch desirable results. This creates the necessity to opt for multiple gene knockouts by CRISPR-based technologies. In other way around, knocking out negative regulators of yield through CRISPR, substantially contributed to yield advancement. Genes regulating tiller numbers, panicle size, grain size, grain weight in rice and wheat were targeted to increase the yield. Also, multiplex editing of three genes associated with grain size and weight in rice such as *GS3, GW2* and *GW5* has been achieved. Similarly, early heading genes, *Hd2, Hd4* and *Hd5* were targeted in rice since heading date is an important trait contributing to rice yield (Liu et al. 2016c, 2017b, c; Xu et al. 2016). CRISPR was also deployed in hybrid rice breeding as hybrid rice yield is 10–20% higher than inbred lines. Thermosensitive gene, *TMS5*, edited through CRISPR/Cas9 to develop 11 new lines of TGMS (thermosensitive genetic male sterile) *indica* rice. Likewise, PGMS lines are mutated by

targeting *CSA* gene in *japonica* rice (Zhou et al. 2016; Li et al. 2016b). Flowering is also a key trait directly associated with seed set and thereby yield. But this trait is mostly influenced by day length which is determined by its geographical distribution. In soybean and tomato, *FLOWERING LOCUS T* (*FT*) and *SELF PRUNING 5G* (*SP5G*) are mutated by CRISPR to alter day length-dependence and increase crop distribution in different areas (Soyk et al. 2017; Cai et al. 2018). In maize, narrowing the leaf angle affects the light-harvesting nature of maize leaves. *ZmLG1* gene was targeted by CRISPR/Cas9 and DTM line T0 (Desired Target mutator) and transferred to six different maize lines through hybridization to get LG1 edited maize plant. This study showed the application of genome editing in important crops like maize which needs a laborious method of tissue culture and plant transformation (Li et al. 2017a). Pedigree analysis and whole-genome sequencing enabled identification of around 57 yield-associated genes in IR8 rice variety and further, through Cas9 and dCas9 editing approaches, phenotypes of the most of the genes were found associated with the yield (Huang et al. 2018). These studies provided strong evidence of application of CRISPR in improving major yield traits of cultivated crops.

Quality, remains as an another economic aspect of crop plants and a key trait of focus in enhancing food production. As of now, several studies using CRISPR has shown improvement in terms of palatability, fragrance, storage and nutrition. For instance, in rice, eating and cooking quality increased by targeting *Waxy* gene. *SBEIIb* was mutated through CRISPR/Cas9 to reduce starch by enhancing amylose/amylopectin ratio. Likewise, starch in potato was reduced by targeted editing of *GBSS* (Zhang et al. 2018a; Andersson et al. 2017). Given that high concentration of PUFA (polyunsaturated fatty acid) decreases the oil quality and causes health concerns, *FAD2-1A* and *1B* were edited through CRISPR/Cpf1 to increase both yield and oil content in soybean (Kim et al. 2017). Gluten allergy is a serious concern causing celiac disease to the people who are dependent on wheat as their staple food. CRISPR/Cas9 alleviated the effect of gluten that is encoded by the α-gliadin gene family and produced low gluten wheat (Sánchez-León et al. 2018). So, the quality improvements in crops by CRISPR techniques keep continuing with the recent additions of high oleic acid content in *Brassica napus*, longer shelf life of tomato and higher level of lycopene in tomato (Okuzaki et al. 2018; Li et al. 2018c, e).

Fragrance in crops is one of the important qualities that has huge market demands. Fragrant rice was generated by targeting *OsBADH2* which produced 2AP (2-acetyl 1-pyrolline) compound, responsible for the fragrance. Both TALEN and CRISPR/Cas9 methods have been deployed to generate fragrance in Zhongua 11, from China (Shao et al. 2017). Seed longevity is an essential trait affected due to poor storage qualities in rice grains. *LOX* (lipoxygenase) genes are responsible for grain deterioration and act as negative regulators of seed longevity. TALEN approach mutated *LOX3* and caused an increase in the storage life of rice grains (Ma et al. 2015a).

Malnutrition is a seriously emerging problem arising due to the consumption of food deficient in essential proteins, energy, vitamins and minerals. It has been reported that 24,000 people died per day due to malnutrition (Potrykus 2008). So, it is the need of the hour to alleviate this problem through CRISPR. Predominantly, for more

than half of the world population, rice supplies most of the nutrients and calories. Despite being a rich source of carbohydrates, it lacks key essential amino acids such as lysine and tryptophan in its grains. This necessitates the need to improve the nutritional qualities of major crop plants. Several studies have reported the transfer of the seed storage and ferritin genes into rice by transgenic approaches but still this area is incomplete and needs lot of investigation. Interestingly, cadmium tolerant rice variety was generated through knocking out *OsNramp5*, a metal transporter, that controls the accumulation of cadmium in rice grains (Tang et al. 2017). Poor digestibility in sorghum is caused by the compound, Kafirin, which is encoded by *k1C* genes. This compound causes protein body accumulation and the grains become devoid of essential amino acid, lysine. *k1C* was targeted by CRISPR/Cas9 which increased the consumption and nutritive value of the crop. Similarly, polyphenol oxidase (*PPO*) gene in button mushroom (*Agaricus bisporus*) was edited to avoid browning, thereby increasing its market value (Li et al. 2018a; Waltz 2016).

12.3.2 Tackling Abiotic Stresses

Abiotic stress is one of the major limiting factors affecting plant growth and yield. Even though burgeoning publications have been out in the field of stress biology, the effective solution to the problems of drought, salt and cold stresses remains fragmentary. This could be mainly because stress tolerance is a complex trait regulated by a multitude of signaling pathways, regulated by several regulators. After the advent of CRISPR/Cas system, the development of efficient crops to withstand against adverse climatic conditions, is getting close to reality. For instance, ABA is a well-known stress hormone that controls several stress signaling pathways. It is perceived by PYL receptors, which is a 13 membered gene family in rice. Knocking out those 13 genes in rice by CRISPR/Cas9 resulted in increased rice productivity as well as plant growth. Among the mutated lines, the group I (edited *PYL1* to *PYL6* and *PYL12*) showed more plant growth while maintaining other traits (Miao et al. 2018). Similarly, *TaDREB2* and *TaDREB3* in wheat protoplasts were edited by CRISPR with almost 70% efficiency, demonstrating increased drought tolerance compared to wild type (Kim et al. 2018). *SAPK2*, one of the MAPK family members in rice and *SIMAPK3* in tomato were mutated by CRISPR/Cas9 which leads to increased tolerance against drought and salt stress (Lou et al. 2017; Wang et al. 2017a).

CBF genes impart tolerance to cold stress in plants, however it is a multi-membered gene family, for example 12 members in the case of rice and 3 in Arabidopsis. CRISPR/Cas9 was deployed to generate *cbf1cbf2* double mutant and *cbf1cpf2cpf3* triple mutant to study the significance of individual CBFs in cold stress tolerance (Jia et al. 2016). Regulation of stomatal density and stomatal index is an important trait for water use efficiency. CRISPR/Cpf1 was used to edit one of the regulators of stomatal density, *OsEPFL1*, which resulted in an improved stomatal patterning in rice under stress conditions (Yin et al. 2019). *SlNPR1* is

the ortholog of Arabidopsis *NPR1,* which regulates both abiotic and biotic resistance. The role of *SlNPR1* in drought has been found by editing through CRISPR and found that *slnpr1* mutant shows increased sensitivity to drought coupled with higher stomatal aperture and electrolyte leakage (Li et al. 2019b). Despite the above evidence were showing the potential of gene editing in generating stress tolerant crop plants, many stress-associated genes are difficult to be identified. Recently, in this context, targeting of structural, regulatory abiotic stress resistance genes and their cis-regulatory sequences through CRISPR/Cas was reported as one of the promising approaches for generating stress resilience crops (Zafar et al. 2019). Compiled information of a few CRISPR/Cas9-mediated stress resistance crops are furnished in Table 12.1. Readers are requested to go through recent reviews mentioned in this chapter for extensive examples on this aspect.

12.3.3 Defending Against Biotic Stressors

Crop yield reduction is fetched due to multiple biotic stress imposed by bacteria, fungi, viruses and nematodes. Transgenic plants expressing disease resistance genes, displayed enhanced tolerance against few pathogens, but this has also resulted in an upsurge of new strains of pathogens. To alleviate this problem, understanding of genes involved in plant-pathogen interaction is necessary. For example, S genes are the group of disease-causing genes in the plants. One of members is *OsERF922*, an ethylene responsive gene, whose knock out by CRISPR caused reduced blast infestation. Similarly, targeted editing of *OsSWEET13* gave rise to bacterial blight resistance in rice (Wang et al. 2016; Zhou et al. 2015). Targeted editing of effector binding elements in the promoter of *CsLOB1* in citrus increased disease resistance against *Xanthomonas citri* (Peng et al. 2017). Multiple gene editing of three homoeologs of *EDR1* in wheat, conferred resistance towards powdery mildew infection. Similarly, editing of *mlo* (mild resistance locus) alleles in Arabidopsis, wheat and barley resulted in resistance against *Blumeria graminins f.sp.tritici* (Wang et al. 2014). Similarly, viral diseases also cause huge yield losses unless they are controlled genetically. CRISPR techniques mutate the viral genome of pathogenic viruses in addition to controlling viral incidence in plants. For instance, FnCas9 driven by viral promoters, provided the viral resistance against TMV (tobacco mosaic virus) and CMV (cucumber mosaic virus) diseases (Zhang et al. 2017b, 2018b). Eukaryotic translation initiation factor *eIF4E* is the host factor essential for viral replication and mutation of this gene caused viral resistance in cucumbers (Chandrasekaran et al. 2016). Likewise, *eIF4G* is the negative regulator of viral resistance against rice tungro virus (RTV), and was knocked down by CRISPR to enhance disease resistance (Macovei et al. 2018). Wheat dwarf virus (WDV) is another serious threat in wheat and barley causing huge yield loss. Since there is a lack of natural resistance sources so far, CRISPR edited the conserved target site that has been discovered through mapping of WDV genome with PAM sequence and thus resulted in resistance to WDV (Kis et al. 2019). As we have seen before in abiotic stress

Table 12.1 A compiled information on stress resistance for major crops developed through CRISPR/Cas9

Crop	Target gene	Biotic stress	Abiotic stress	GE result	References
Rice	*eIF4G*	Tungro virus	–	Knock out	Macovei et al. (2018)
	OsERF922	Blast fungus	–	Knock out	F. Wang et al. (2016)
	OsSweet13	Bacterial leaf blight	–	Knock out	Zhou et al. (2015)
	OsNAC041	–	Salt	Knock out	Bo et al. (2019)
	OsOTS1	–	Salt	Knock out	Sadanandom et al. (2019)
	OsRR22	–	Salt	Knock out	A. Zhang et al. (2019)
	OsNAC14	–	Drought	Knock out	Shim et al. (2018)
	OsSAPK1&2	–	Salt	Knock out	Lou et al. (2018)
	OsAnn3	–	Cold	Knock out	Shen et al. (2017)
	SAPK2	–	Drought & Salt	Knock out	Lou et al. (2017)
	MPK2, PDS, BADH2	–	Multiple stress	Knock out	L. Wang et al. (2017)
Wheat	*EDR1*	Powdery Mildew	–	Knock out	Y. Zhang et al. (2017b)
	TaDREB2&3	–	Drought	Knock out	Kim et al. (2018)
Maize	*ARGOS8*	–	Drought	Knock out	Shi et al. (2017)
Cotton	*Gh14-3-3d*	Wilt	–	Knock in	Z. Zhang et al. (2018c)
Grapes	*VvWRKY52*	*Botrytis*	–	Knock out	X. Wang et al. (2018b)
Tomato	*SlJAZ2*	Bacterial Speck	–	Knock out	Ortigosa et al. (2019)
	CP & Rep sequences	Leaf curl virus	–	Knock out	Tashkandi et al. (2018)
	SlMlo1	Powdery mildew	–	Knock out	Nekrasov et al. (2017)
	SlNPR1	–	Drought	Knock out	Li et al. (2019)
	SlCBF1	–	Cold	Knock out	Li et al. (2018)

(continued)

Table 12.1 (continued)

Crop	Target gene	Biotic stress	Abiotic stress	GE result	References
	SlMAPK3	–	Drought	Knock out	L. Wang et al. (2017)

Source Razaaq et al. (2019). International Journal on Molecular sciences

section, there is a lot more to be explored for developing disease resistance with respect to CRISPR-based disease management strategies. In particular, the common hub of regulatory genes involved in the invasion by different pathogens have to be explored and could be edited through CRISPR technologies. Readers are requested to go through the review, published recently, for the updated information on crop-wise details for genome editing (Manghwar et al. 2019). Hereby, the compiled information of CRISPR/Cas9- mediated stress resistance crops are furnished in Table 12.1.

12.3.4 Other Key Applications of CRISPR

Mutant libraries are generated with the purpose of analysing gene functions systematically, through whole-genome mutagenesis or forward genetic screening. Also, the creation of mutant library is feasible for whole-genome sequenced plants such as rice, Arabidopsis, wheat. Since CRISPR/Cas system is a powerful tool for generating mutants, it was exploited to generate whole-genome mutant library. In the recent past, 12,802 genes were selected based on their high expression in rice shoot tissue and corresponding 25,604 sgRNAs were generated to create large-scale CRISPR mutant library (Meng et al. 2017). Similarly, Lu and his team generated 90,000 transgenic plants by targeting 34,234 genes in rice (Lu et al. 2017). In tomato, mutant library was generated by pooling sgRNA collections and large-scale mutant screening has also been carried out. From these mutants, alleles of leucine-rich repeat XII genes were identified which played a role in plant immunity (Jacobs et al. 2017). When these mutants were grown for screening, phenotype and genotype correlation was easily facilitated through sgRNAs (Bao et al. 2019). This evidence shows that CRISPR mutant libraries might play a crucial role in crop improvement in the coming decades.

Large deletion, translocation and inversion at genomic scale are some of the promising outputs of the CRISPR/Cas system. It drives the breeding approaches forward in terms of removal of the entire gene cluster, and establishing new linkages by translocation, transferring desirable traits from wild types by inversions of chromosomes (Puchta 2017). In rice, large-scale deletions of 245 kb and inversion of 300 bp by CRISPR/Cas9 has been demonstrated but their heritability has not been investigated so far (Zhou et al. 2014; Liang et al. 2016). Like deletion, targeted insertion of genomic fragments is quite possible through the HR-mediated pathway by providing a DNA repair template. In tomato, Geminivirus replicon was used to

supply both the repair template and CRISPR/Cas9-driven ANT1 construct- which led to dark purple coloration (Čermák et al. 2015). Biolistic transformation was also used to supply large fragments as the donor template. For instance, in maize, biolistic delivery of repair template targets *ALS2* and edited by HR resulted in resistance to chlorsulfuron (Svitashev et al. 2015). Alternatively, intron mediated gene replacement has been demonstrated for gene insertion through CRISPR/Cas9. In this method, sgRNA was designed to target two introns spanning the exon. Along with donor DNA template, Cas9 was supplied for targeted replacement (Li et al. 2016a).

Domestication is the process of generating modern crops through breeding by the introgression of desirable traits. Through this process, introgression of wild type traits into elite takes a long time. However, the process of domestication has been carried out only for major crops like rice, wheat and maize leaving out other important food crops. Now, CRISPR/Cas9 enabled the domestication process within a short time and produced elite crops from wild types with great agronomic traits through targeted mutations. For example, pennycress an important oil seed crop is improved with shorter life cycle, cold tolerance and increased oil production. Through CRISPR, its undesirable traits like seed dormancy (*DOG1*) and glucosinolate accumulation (*HAG1 and GTR2*) were modified to generate domesticated pennycress. Similarly, wild relative of tomato, ground cherry was modified by genome editing technologies to produce higher yield and larger fruits (Sedbrook et al. 2014; Lemmon et al. 2018).

Transgene free editing is one of the key concerns in creating CRISPR-edited crops for the public. Integration of Cas9 or vector backbone sequences makes it difficult to generate foreign DNA free plants. To circumvent this issue, DNA vectors and RNP complex approaches were executed (Li et al. 2019a). In the first approach, CRISPR/Cas9 delivery through *Agrobacterium*-mediated transformation allows the integration and editing of the construct at different chromosomes. Through segregation, transgene free edited plants were created in the next generation (Li et al. 2016a, 2017b; Wang et al. 2014). Also, transient transformation of CRISPR/Cas9 mediated by *Agrobacterium* have yielded transgene free edited plants in T0 generation in wheat and tobacco (Zhang et al. 2016; Chen et al. 2018). In recent past, TKC (transgenic killer CRISPR/Cas9) system was developed to self-eliminate transgenes through its suicide components like CMS2 and Barnase which kills the transgenes in the pollen and embryo, respectively. This led to transgene free T0 generations in rice (Rodríguez-Leal et al. 2017). In the second approach, Cas9 protein and sgRNA are assembled in vitro into an RNP complex and delivered to plants by biolistic transformation. After editing, RNP was degraded since it is devoid of foreign DNA, which eventually leads to transgene free plants. This technique was already deployed in rice, lettuce, tobacco and Arabidopsis. For instance, in maize, *als2* mutants were obtained through HR by the co-delivery of ALS2: RNP complex with single stranded DNA template by particle bombardment method (Svitashev et al. 2016).

12.4 Regulatory Aspects on CRISPR Plants

The potential of genome editing in crops has been getting enormous attention and growing tremendously. However, the discrepancies exist for gene edited (GE) crops in terms of its safety and adaptation. The regulatory networks for the approval of GE crops has some bottlenecks which hampers their development as well as marketability. Despite the commercialization of GMO crops since last several years, improper understanding and mistrust are still prevailing with public due to strict regulations by the Government. Basically, the major difference between genome editing crops with GMO, should be clarified to the regulatory bodies. Also, it is imperative to educate them about the recent progress in CRISPR techniques and improved delivery systems, which do not necessitate the insertion of foreign DNA into the crop plants. Besides, CRISPR-edited crops lead to rapid crop improvement, free of transgenes and produce genetic variability in a better way than transgenic and mutation breeding. In fact, the cost for producing genome-edited crops is around 30 US dollars which is surprisingly lesser compared to quarter of million US dollars for producing transgenic plants. In addition to saving money, it reduces laborious process, year-round field trails which altogether helpful for removing the barriers existing over GM crops (Baltes et al. 2015; Visser et al. 2001; Ledford 2015).

The assessment and acceptance of CRISPR-edited crops for its direct or long-term effect as food and as a feed varies from country to country. It affects the trading of genome-edited (GE) crops between two countries with different legislative procedures. So, it is important to consider the type of GE techniques used and their delivery methods (Jansing et al. 2019b). Till now, there is no international regulatory framework for GE crops worldwide. However, USA and Europe are the major stakeholders, having opposite legislation policies. GE crops have not been grouped under the category of GMO, and so granted permission to be developed and marketed in the US whereas in Europe, GE is included under GMO category with a notion that it could result in an unknown risk because of the genomic manipulation (Fears and Ter Meulen 2017). The consortium of research organizations in Europe, EPSO (The European Plant Science Organisation) have expressed their unpleasant opinion for the ban on genome editing. European government should focus on product-based research rather than method-based restrictions in scientific discoveries (Schulman et al. 2020). Unlike Europe, USDA in 2016, had ruled out the regulations for GE mushroom and corn and got them in the US markets. Along with USA, Canada, Brazil, Argentina, Chile, Australia have joined to draft new regulatory framework by discarding certain rules in order to absolve GE crops from GMO. Those new regulations are mainly concerned about developing GE crops free of transgenes, devoid of pest incidences and modification of traits (Razzaq et al. 2019). Interestingly, in Canada, PNT (plant with novel traits) regulations are followed for the crops attaining specific traits through traditional breeding, mutagenesis, genetic engineering and genome editing technologies (Smyth 2017). In Argentina, the legislation is cleared for any crops free of transgenes (null segregants) through drafting flexible assessment for developing GE crops. Despite transgenic techniques were used, the final

product should be free of foreign genes for the approval in Argentina (Whelan and Lema 2015).

In China, CRISPR is being used extensively in the field of agriculture and medicine and it has been featured as CRISPR revolution in China by Science journal recently (Cohen 2019), indicating that China is being the leading player of GE crop research. Also, an inventory analysis revealed that China is dominating among the 2000 patent applications dealt with CRISPR and USA is marginally ahead of China. Food security is considered as the major reason behind China's major interest towards CRISPR in academics and industries, in order to meet out its ever-expanding population. Also, 'China daily' paper reported that despite China showing great interest in GE crops, it lacks clear regulatory policies that arrest the development of CRISPR crops in the laboratory itself. An initiative was established recently in which twelve Chinese academicians were signed in the draft to look after the strict legislation on GE crops, which will probably increase the quality of outcome and competitiveness on GE technologies. Moreover, the recent findings followed by the accusation of 'CRISPR babies' in China have led to make strict regulations on genome editing, not only in mammalian research but also in the field of agriculture and pharmaceuticals.

In India, the regulation and bio safety evaluation of GMOs are under strict scrutiny and that framework has been formulated earlier along with other countries. The rules are governed under Environment Protection Act, 1996, by Ministry of Environment, Forest and Climate Change. From time to time, the policies are being upgraded depending on the new findings of the research (Choudhary et al. 2014; Warrier and Pande 2016).

Despite genome editing being more precise than natural mutagenesis, this technique is far from acceptance in many countries. This could be additionally due to lack of technical conveyance to the public and thought process of considering GE as a GMO. Although, CRISPR crops are accepted in few countries, co-ordination in the legal policy should be standardized at the global level to increase the marketability of CRISPR crops in future.

12.5 Conclusion with Perspectives

The range of applicability of CRIPSR has been recently burgeoning mainly because of its low cost, technical rapidity, easy execution and precised editing at genome level. The new developments such as prime editing, base editing, multiplexing, epigenome modifications will further help in increasing the horizons of CRISPR applications in various crops. In plant systems, CRISPR is being used effectively for the past five years to develop new varieties, with improved agronomic traits and resistance traits against biotic and abiotic stresses (Razzaq et al. 2019). However, those edited plants are confined in laboratory environments. Probably, in near future, more plants could

be bred with CRISPR technologies and get ready for the markets. Despite its significant progress, it has many rooms for improvements and technical challenges. HR-mediated genetic editing is one such challenge ahead for successful CRISPR application. Also, the effective delivery of donor template through Geminivirus vectors, utilization of NHEJ inhibitors and HR enhancers, have led to some output but remarkable progress is still under debate. Another priority of improvement lies in its efficient delivery method because vast number of major crop plants are lacking proper transformation methods and/or difficult to transform. Carbon nanotubes, silica nanoparticles and layered double hydroxides are some newly suggested delivery methods for precise GE. Also, the other concern is about the improvement in targeted editing rather than off-targeting. With the advent of improved CRISPR vectors, these issues could be overcome easily by the research community in near future. Despite excellent nuclease functionality, CRISPR efficiency basically depends on the target gene sequence, cell type and epigenetic state of the chromosome. Eventually, genome editing should be combined with other functional genomic approaches like next generation sequencing, synthetic and systems biology in order to reap the complete benefits of technologies for crop improvement in the current era of ever evolving climatic conditions.

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