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Preface

With the erratic changes in climate, crop plants are facing many forms of biotic stresses. Employing genetic resistance in their management is the most economical, effective, and eco-friendly approach. However, limited genetic variation in the gene pool is hindering the rapid progress in the field of plant genetic resistance. Moreover, major resistance genes are knocked-down due to continuous evolution of novel virulent races/biotypes. Therefore, the concept of durable resistance is ever lasting since ages in management of biotic stresses. Under natural conditions, plants face different biotic and abiotic stresses simultaneously. Therefore, broad spectrum resistance and resistance against multiple stress forms can be of prime focus to combat economic yield losses. When plants are under stress, among several gene families, regulatory genes play a vital role in signal transduction in modulating the expression of genes underpinning several defense pathways, and targeting regulatory proteins (viz, transcription factors (TFs)) can be the alternative. Transcription factors directly regulate the downstream R genes and are excellent candidates for disease resistance breeding. Till date, numerous transcription factors have been identified and characterized structurally and functionally. Of them, TF families, such as WRKY, NAC, Whirly, Apetala2 (AP2), and ethylene responsive elements (ERF), are found to be associated with transcriptional reprogramming of plant defense response. These TFs are responsive to the pathogen's PAMPs/DAMPs – host's PRR protein interactions, and specifically bind to the *cis*-elements of defense genes and regulate their expression. With this background, realizing the importance of TFs in resistance breeding, current book has been proposed.

This book provides an authoritative review account of different aspects and progress in the field that have been made in the recent past. Book includes chapters prepared by specialists and subject experts on different aspects of gene editing techniques, role of synthetic promoters and microbial bio-agents as elicitors in plant defense regulation, and role of TFs in disease resistance. The first chapter introduces various genome editing techniques, whereas six chapters deal with the role of TFs in biotic stresses in crops like wheat, sugarcane, maize, pearl millet, tomato, and potato. Three chapters are exclusively about the transcription factors associated with defense response against fungal biotrophs, necrotrophs, and viruses. One

chapter is exclusively about the synthetic promoters in regulating disease gene expression and one chapter about the role of microbial bio-agents as elicitors in plant defense regulation

The book provides state-of-the-art information on the potential of TFs in supplementing and complimenting the conventional methods of crop improvement against biotic stresses. We earnestly feel that this book will be highly useful for students, research scholars, and scientists working in the in the area of crop improvement and biotechnology at universities, research institutes, R&Ds of agricultural MNCs for conducting research, and various funding agencies for planning future strategies.

We are highly grateful to all learned contributors, each of whom has attempted to update scientific information of their respective area and expertise and has kindly spared valuable time and knowledge.

We apologize wholeheartedly for any mistakes, omissions, or failure to acknowledge fully.

We would like to thank our families (Sheikh Shazia and Muhammad Saad Wani (wife and son of SHW), Keerthi and Adhvay Rishi (wife and son of NV)) for their continuous support and encouragement throughout the completion of this book.

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Srinagar, India — Shabir Hussain Wani
Indore, India — Vennampally Nataraj
Karnal, India — Gyanendra Pratap Singh

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Targeted Genome-Editing Techniques in Plant Defense Regulation

Vineeta Dixit and Priti Upadhyay

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Keywords CRISPR · Meganuclease · Stress · TALENS · Zinc-finger nuclease

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1.1 Introduction

Domestication of crop plants coexisted with human civilisation. With the progress in the scientific arena, the skill to modify the plant characteristic sharpened, and various tools and techniques are searched and invented almost every decade to meet the nutraceutical, economical or agronomical needs. Improper selection method was successfully replaced by conventional breeding of distant crop population. While conventional breeding techniques depend on ambiguous needs of rigorous selection after successful crossing between likely related/distant species, advanced genetic engineering methods have the ability to modify the genome with stable integration of foreign desired genes. Narrow genetic base of plant species was broadened using mutagenetic tools, and later other modern techniques were used to create target-specific variations. Recombinases, transposons and TILLING technologies, in addition to chemical mutagens, were utilised in functional genomics and reverse genetic investigations. A special objective of molecular and plant biologists was/is induced variation at target locus. In the last few decades, considerable improvement has been observed in the field of targeted genome modifications. Diverse fields of genetics and life science including human genetics, clinical genetics, gene therapy, precision medicine, synthetic biology, drug development, plant biology and agricultural research have utilised them and produced the desired set of traits. Gen/Ed (gene/genome editing) tools at present are the most advanced and preferred applications that facilitate specific and efficient site-specific amendments in a chosen genome/organism. Gene editing utilising locus-specific nucleases enables for rapid and accurate reverse genetics, genome remodelling and targeted transgene insertion (Bortesi and Fischer 2015). Genome-edited GMO tagged crops are subjected to a variety of biosafety issues, and differences in regulatory legislation between countries provide significant impediments to the quick adoption of new GM features (Prado et al. 2014), limiting the benefits of GM traits to a small number of commercial crops. Targeted Gen/Ed produces sequence-specific nicks in the target DNA, and specific edits are incorporated during repair, and thus products of Gen/Ed can be designed for non-GMO tag (genetically modified organism). These approaches produce modifications that are only a few nucleotides long and mimic spontaneous mutation in the crop, implying that they potentially pose fewer risks than GMO crops (Voytas and Gao 2014). Thus, incorporating genome editing by Gen/Ed into contemporary breeding programmes would allow for expedited and accurate crop improvement, ensuring that future food demand is met and food security is assured. Plant breeding can employ a gene−/genome-editing system to make point mutations that mimic natural SNPs, integrate foreign genes, adjust gene function, gene pyramiding and knockout and inhibit or activate gene expression, as well as epigenetic editing (Kamburova et al. 2017). With advances in sequencing technology, genomic information on an increasing number of plant species is becoming

available, enabling genome-editing tools for precise gene editing in a wide range of crops and opening up new avenues for modern agriculture.

Gene editing (Gen/Ed) are broadly based on either DNA-guided editing and RNA-guided editing mechanism. The core technologies now most commonly used to facilitate DNA-guided genome editing are (1) meganucleases or homing endonucleases, (2) TALENs (transcription activator-like effector nucleases) and (3) ZFNs (zinc-finger nucleases). CRISPR (clustered regularly interspaced short palindromic repeats) and CRISPR-associated protein such as CRISPR/Cas 9 are solely based on RNA-guided editing mechanism. All the aforementioned Gen/Ed tools have the potential to catalyse the formation of double-strand breaks (DSBs) at the target DNA sequence, which activates cellular DNA repair mechanisms and enables the incorporation of site-specific genetic alterations (Rouet et al. 1994; Choulika et al. 1995). DNA repair can be achieved either through homologous recombination (HR) or non-homologous end joining (NHEJ). The artificial template provided by DSB-stimulated gene targeting is an exogenous template for a natural repair mechanism. The HR approach uses a homologous donor DNA segment as a template, and homologous recombination is employed to repair the DSB. This process might be used to perform precise gene changes or gene insertions. DSBs stimulate both mutagenesis and gene replacement locally in most organisms, including higher plants, even though the generation of breaks in both DNA strands induces recombination at specific genomic loci. In most organisms, including higher plants, NHEJ is the most common DSB repair process, whereas targeted integration by HR is significantly less common than random integration (Puchta 2005). In non-homologous end joining, broken ends are commonly joined erroneously, generating random indels (insertions or deletions) and substitutions at the break site. Thus, NHEJ is expected to cause frameshift mutations in the majority of cases and, if it happens in a gene's coding domain, can essentially result in a gene knockout. If overhangs are generated in the DSB, NHEJ can manage the targeted introduction of a DNA template with compatible overhangs efficiently (Cristea et al. 2013; Maresca et al. 2013). Other strategies, including the use of negative selection markers outside the homology region of the insertion cassette to avoid random integration events, or overexpressing proteins engaged in HR, can result in modest improvements in gene targeting efficiency (reviewed in Puchta and Fauser 2013). The design and cloning of targeted nucleases have become easier as a result of freely available software tools and knowledge, expanding the capacity of medium-funded laboratories. In addition to ZFNs, TALENs and CRISPR, other designed nucleases like homing endonucleases or meganucleases have been employed for targeted Gen/Ed (Roth et al. 2012), although their application is limited in contrast to the aforementioned nucleases. In this chapter, we first go through the many genome-editing techniques that are utilised for precise editing in plants, as well as their strengths and limitations. The possible uses of each technology for defensive regulation and resilient development in various plant species are then discussed.

1.2 Homing Endonucleases or Meganucleases

Homing endonucleases (HEs) or meganucleases are found in microbes that are enzymes that generate double-strand breaks at specified genomic invasion locations to mobilise their own reading frames (Fig. 1.1) and thus splitting DNA at particular sequence. HEs are molecular scissor proteins that display economies of size with an attribute to recognise long DNA sequences (typically 14–40 base pairs) (Belfort and Roberts 1997); hence, these are sequence-specific endonucleases (SSN) (Arbuthnot 2015). HEs may break double-stranded DNA at particular identified base pairs and have a broad range of precision at individual nucleotide sites having significant effect of host constraints on the targeted gene's coding sequence. These proteins' action creates recombination interactions that are very much site specific and it may produce DNA mutation having different mechanisms like insertion, deletion, etc. Researchers have been working on these proteins for over 15 years, and they have solved the crystal structure of various homing endonuclease families. Since the mechanism of creating variations by applying these enzymes is known and also that these cleave and create novel DNA targets, engineered homing endonuclease proteins are currently being employed in a number of biotech and medicinal applications to induce targeted genomic alterations.

Unlike restriction enzymes, which protect microbes from invasive DNA, HEs let genetic components to move around freely within an organism. HEs get their name from the process, which is known as "homing", a self-splicing mechanism where intervening sequence of group I or group II introns or inteins is precisely replicated into host gene receiver alleles that lack such a sequence (Belfort and Perlman 1995; Belfort and Roberts 1997; Chevalier and Stoddard 2001; Dujon 1989).

Homing endonuclease's (HE's) presence has been documented in all three biological kingdoms. Studies on budding yeast in the 1970s provided the first evidence of the presence of HEs (Belfort and Robert 1997). In another study in yeast, the transmission of the genetic marker omega (ω), that was reported as a group I intron of large ribosomal RNA, among yeast strains was proven (Chevalier and Stoddard 2001). The production of double-strand breaks (DSBs) at specific spots was used to

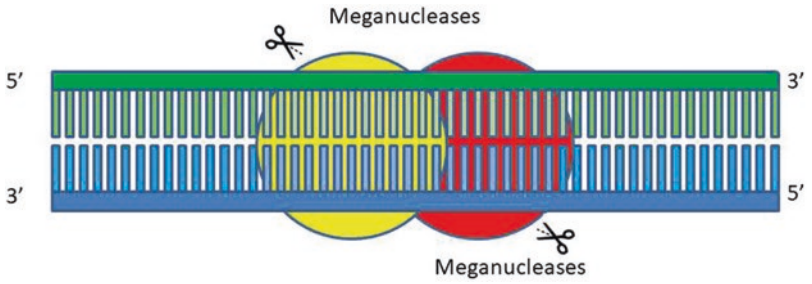

Fig. 1.1 Schematic representation to show mechanism of meganuclease gene-editing system. A meganuclease *has a homodimer structure.* Meganucleases are highly specific and easy to deliver to cells but difficult to redesign for new targets

transfer the genetic material, and the study discovered that the endonuclease responsible for the split was encoded by own DNA sequences of the group I intron. The first of numerous HEs to be characterised was I-SceI. At the target, cleft or break was followed by homology-directed repair which resulted in the intron sequence being introduced into the "intronless" target. In the target sites, there is some tolerance for sequence variation, which is considered to be crucial for homo endonucleases to accept a variation in the frequency of an existing gene variation in the population of a host organism (genetic drift). Degeneracy is tolerated at places that coincide with the wobble positions of protein-coding regions, which is an interesting coincidence.

It has wide application in targeted gene editing as it has an attribute of sequence specificity. The efficiency and success of sequence insertion mediated by homologous recombination employing homing endonucleases in maize were investigated by induction of a targeted DNA double-strand break at the desired integration location, and numerous significant numbers of carefully designed events were discovered in maize DNA where integration happened in extremely correct way with improved and optimised protocol with I-SceI gene for expression. This improved procedure worked for both *Agrobacterium* and particle bombardment DNA delivery methods, but the results indicated that targeted double-strand break-induced homologous recombination is an effective way to ensure precise changes in the maize genome and that targeted genome alteration of agronomic crops is possible (D'Halluin et al. 2008).

A transgene integrated with intrins was inserted at the exact locus using meganuclease in the model plant *Arabidopsis* (*A. thaliana*) to achieve an independent (not affected by transformation methods) and effective targeted insertion that established the development of premeditated endonucleases with site specificity. It was considered that such targeted insertion may boost the establishment of gene targeting (GT) techniques in a variety of species. Research in this emerging field of modifying gene is growing day by day, and a patent has been submitted in the United Kingdom for an engineering technique of I-CreI homing endonuclease variants capable of cleaving mutant I-CreI sites with variations in positions 8–10. An I-CreI homing endonuclease variation obtained by this strategy resulted in phytophthora-resistant potato with enhanced yield, as demonstrated by experiments in potato (Hogler and Timo 2012).

Meganuclease mutants are easily accessible and may be successfully used in plants for precise genetic alteration. Meganucleases are smaller (40 kD) than ZFNs and TALENs, which enables them to be used in vectors with smaller coding sequences specially that belongs to viruses (Iqbal et al. 2020). However, due to several restrictions, such as DNA binding and cleavage domains overlap (Stoddard 2011) that cause compromised catalytic activity of meganuclease, lack of the modular DNA-binding domain design and sometimes issue of sequence degeneracy for meganuclease, their use in genome editing/engineering is not as widespread as ZFNs or TALENs (Argast et al. 1998).

1.2.1 Zinc-Finger Nucleases

Plant phenotypes are the outcome of a complex array of biochemical, physiological and developmental processes culminating in physical appearance. All these activities are essentially governed by nucleotide base sequences found in nuclear, plastid and mitochondrial genomes, which supply both configurational and regulatory instructions to the live cell and, as a result, the growing organism. However, while the nucleotide sequences found in live creatures are similar, they differ from one another owing to changes within and recombinations among these sequences. The phenotypic variety observed across organisms is based on variations in their sequence and structure (Petolino 2015). Plant breeders can use naturally occurring and/or produced sequence changes and recombinations after analysing the sequence information. Plant breeders can use naturally occurring and/or produced sequence changes and recombinations after analysing the sequence information. They can adjust or alter the nucleotide sequence to suit their needs and change the phenotype. As a result, significant progress may be made in terms of improving the quality and performance of crops for agricultural and industrial purposes.

Sequences on DNA can be altered by using molecular scissors, and there are many present in living system. ZFNs (zinc-finger nucleases) are a type of DNA-binding protein that permits for customised genome editing by causing double-strand breaks in DNA at user-specified places. (Fig. 1.2). Individual ZFNs' DNA-binding domains generally include three to six zinc-finger repeats, each of which can identify between 9 and 18 bps (Ramirez et al. 2008). At present, most of the engineered ZFs arrays that are available are based on three individual zinc-finger domain that can recognise a nine base pair target location with high affinity (Christy and Nathan 1989). Other approaches that can build zinc-finger (ZF) arrays comprising six or more individual zinc fingers are combination of one-finger and two-finger modules (Shukla et al. 2009). A following research employed modular assembly to make zinc-finger nucleases with both three-finger and four-finger arrays, finding that the four-finger arrays had a substantially greater success rate (Kim et al. 2009).

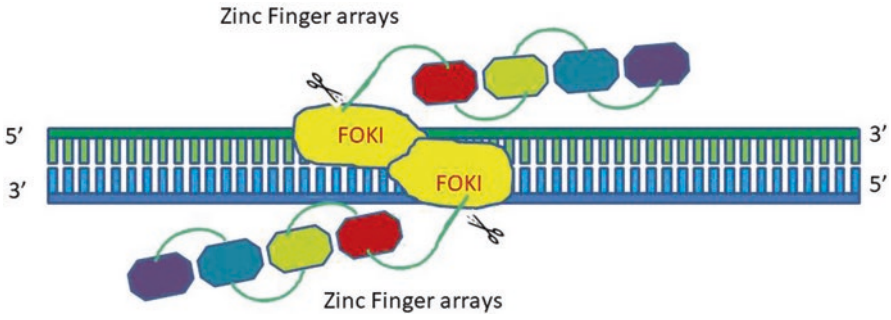

Fig. 1.2 Schematic representation to show mechanism of *zinc-finger nuclease (ZFN)* gene-editing system. *Zinc finger nuclease (ZFN) is composed of two monomers, and hexagon represent a zinc finger DNA-binding domain.* Each zinc finger typically recognises 3 bp

To construct ZF arrays capable of targeting specified sequences, a variety of selection approaches have been applied. Initially, phage display was used for identifying the proteins that bind a target DNA sequence from a huge pool of partly randomised ZF arrays, but recent research has focused on yeast one-hybrid systems, bacterial one-hybrid and two-hybrid systems and mammalian cells to select the constructed ZF arrays that are capable of targeting specified sequences (Chandrasegaran and Carroll 2016). The inventors of "oligomerised pool engineering (OPEN)", a promising new strategy for selecting innovative zinc-finger arrays, have named it after a bacterial two-hybrid system (Maeder et al. 2008). This technique combines pools of individually selected ZFs, each of which was preferred to bind a certain triplet, and then employs a second round of selection to generate three-finger arrays competent of binding a nine base pair sequence. This technique was developed by the Zinc Finger Consortium as an alternative to commercially available zinc-finger arrays.

Plant and animal genomes can both benefit from zinc-finger nucleases. In a study with *Arabidopsis*, researchers identified an effective technique for targeted mutagenesis of two genes (ADH1 and TT4) by controlling the production of zinc-finger nucleases that cause a double-strand breaks at specific target loci in DNA. The mutations produced were typically insertions of base pairs or deletions of base pairs, and the size of these varied from 1 bp to 142 bp. These mutations were found to be localised near the zinc-finger nuclease cleavage site and most probably resulted from non-homologous end-inaccuracy joining's in repairing chromosomal breaks. For about 70 percent of primary transgenics expressing the ADH1 ZFNs and around 33% of primary transgenics expressing the TT4, mutations created through use of ZFNs were passed down to the following generation. The findings revealed the applicability of ZFNs for obtaining the mutants in any target gene in *Arabidopsis* and it would have independent mutant phenotype (Zhang et al. 2010).

Zinc-finger nucleases enzymes was applied to create double-strand breaks at specific loci in acetohydroxyacid synthase (SuRA and SuRB) genes in tobacco. Some specific mutations in this gene are responsible for resistance to imidazolinone and sulfonylurea herbicides. Through this study, it was observed that utilisation of zinc-finger nuclease enzymes in tobacco was an efficient method for directed DNA sequence modifications (Townsend et al. 2009). The high rate of mutants with focused gene editing suggested that making precise sequence alterations in endogenous plant genes may be efficient. Curtin et al. (2011) employed ZFN genome engineering to target mutagenesis of nine endogenous genes and a transgene in soybean (*Glycine max*). Under an oestrogen-inducible promoter, cloning was done for specific zinc-finger nuclease targeting DICER-LIKE (DCL) genes and other genes that are involved in RNA silencing. The effectiveness of zinc-finger nuclease-induced mutagenesis at each marked locus was investigated using a hairy-root transformation technique. Transgenic roots demonstrated somatic mutations in genes DCL4a and DCL4b that were introduced through whole-plant transformation into soybean and generated independent mutation events to get mutants for seven out of nine targeted genes. The ZFN-induced mutation was efficiently heritable transmitted in the subsequent generation with the dcl4b mutation. The findings of

this study indicated that mutagenesis based on zinc finger nuclease can be an efficient method for making mutations in duplicate genes.

Custom-made ZFNs have been engineered to split DNA at specific sequences and are proven to be an effective tool in targeted gene manipulations. Also, they have the unusual property of blocking dominant mutations in heterozygous people. It causes breaks in both the strands of DNA (DSBs) in the mutant allele, which are restored by non-homologous end-joining in the absence of a homologous template (NHEJ). Durai et al. (2005) explored the gene targeting utilising the zinc-finger nuclease for plant and mammalian genome and found that there is great potential of ZFNs for "directed mutagenesis" and targeted "gene editing", that makes it more applicable for ZFN-based gene therapy for human therapeutics in future. It is possible to entirely erase whole vast portions of genomic sequence using numerous pairs of ZFNs in an experiment to inhibit the mutation (Paschon et al. 2019).

ZFNs are synthetic restriction enzymes that have been utilised in *Arabidopsis* to cause mutagenesis at particular sequence or homologous recombination at the repair location, and the result showed that no gene-targeted plants were produced at the end of the experiment. The study also demonstrated that in *Agrobacterium* T-DNA constructs, ZFNs improved creation of mutation at specific location and gene targeting by fully eliminating that occurrence (de Pater et al. 2009). ZFNs can also be utilised to redraft an allele's alignment or pattern by calling a machinery of recombination, i.e. homologous in nature to repair a double-strand splits or break (DSB) using the provided DNA fragment as a template. In an individual homozygous for the concerned allele, the technique of gene targeting using ZFN's efficacy would be reduced because the undamaged copy of allele can be used as a template for repair rather than the given fragment. ZFNs have also been used in genome/gene therapy, with the effectiveness of this method relying on the precise and proper insertion of genes under therapy into an appropriate and specific chromosomal site within the human genome without causing cell damage, cancer-causing alterations or an immune response. Vectors for this technique that are plasmid based can be created easily and quickly.

Off-target cleavage and immunogenicity are two possible issues with ZFNs. When zinc finger domains lack specificity and selectivity for their particular DNA location, off-target cleavage occurs, which can lead to genomic changes that aren't wanted. This causes chromosomal rearrangements, encourages random donor DNA integration and may even be lethal to the cells (Durai et al. 2005). When multiple foreign proteins are injected into the human body, an immune reaction to the therapeutic drug has been reported. As the protein must only be produced transiently, this raises the issue of immunogenicity (Durai et al. 2005).

Despite these two drawbacks mentioned above, ZFNs' capacity to accurately change the living organism's genomes offers a variety of effective applications in fundamental and applied research such as in the field of agriculture and human health. Improved ways of creating zinc-finger domains along with better supply of ZFNs from a commercial provider have made this technology available and assessable for increasing number of researchers, and it is now being utilised in conjunction with CRISPR to enhance plant agronomic features. Artificial zinc-finger

nucleases (AZFNs) were created based on the ones with the highest DNA-binding affinities for *Geminiviridae* DNA as an example of generating plants with begomovirus resistance. In vitro digestion and transient expression assays revealed that these AZFNs can effectively cleave the target sequence and suppress the reproduction of several begomoviruses (Chen et al. 2014), signifying that this strategy might be beneficial for the aforementioned goal.

1.2.2 Transcription Activator-Like Effector Nucleases (TALENs)

Transcription activator-like effector nucleases, in short TALENs, are another molecular scissor with a structure similar to ZFNs. The building block of TALENs is a highly conserved base sequence that are found to be expressed naturally in *Xanthomonas* proteobacteria as TALEs, i.e. transcription activator-like effectors. These are delivered into recipient cells of plants through a system of type III secretion, where they attach to DNA present in nucleus of cell and modify transcription, allowing harmful bacteria to colonise the cells more easily (Boch and Bonas 2010). TALEs mediate DNA binding by using arrays of highly preserved 33–35 amino acid repeats bordered by extra TALE-derived domains at the amino- and carboxy-terminal ends of the array. TALEs (DNA-binding proteins of 33–35 amino acids) (Fig. 1.3) are found in TALENs, derived from naturally existing plant pathogenic bacteria, and have ability to precisely detect one base pair of DNAs. Transcription activator-like effectors is connected together in the form of chain which may recognise and split a single location within the genome, similar to ZFNs. These nucleases are fusions of the cleavage domain *Fok*I and TALE protein-derived DNA-binding

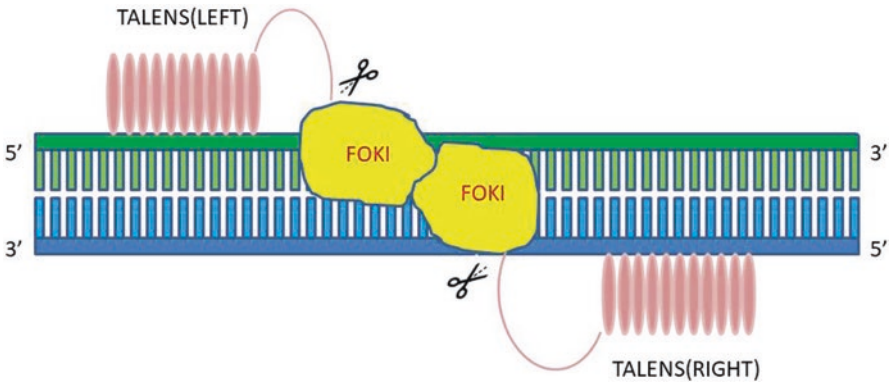

Fig. 1.3 Schematic representation to show mechanism of *transcription activator-like effector nuclease (TALEN)* gene-editing system. *TALEN comprises of two monomers, and light brown rectangles represent the DNA-binding domain.* The two TALEN target sites are typically separated by a 15–20-bp spacer sequence

domains. The structure of TALEs has depicted that it contains multiple amino acid repeat domains and each can distinguish a single base pair as ZFNs. TALENs induce double-strand splits in DNA that activate its damage response pathways and permit custom alterations like zinc-finger nuclease (Gaj et al. 2013). TALENs are comparable to ZFNs in that they can detect a single base rather than a triplet, which provides them more versatility than ZFNs (Gaj et al. 2016). Many effector domains, like as transcriptional activators and site-specific recombinases, have been created that may be joined to TALEN chains for targeted genetic alterations (Li et al. 2020).

One important difficulty with TALENs is their creation, which necessitates the assembling of many, virtually identical repeat sequences, which is a technical hurdle for a researcher (Cermak et al. 2011). Several revolutionary laboratory approaches, such as fast ligation-based automatable solid-phase high-throughput (FLASH) (Reyon et al. 2013), iterative capped assembly (ICA) (Briggs et al. 2012) and commercial DNA synthesis, have emerged as a result of this (Cermak et al. 2011). The ability to change any gene sequence quickly and effectively using TALENs assures a significant influence on research in the field of biosciences including health and agriculture, and it has the potential to boost yield potential as well as tolerance to biotic and abiotic stressors.

1.3 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9

The term "genome editing" is a collective form of technologies that provides biotechnologists and breeders the ability to modify an organism's DNA by adding or removing genetic material to it. Above we discussed different techniques that include different nucleases that allow the researcher to make a sequence-specific cut in genome and alter it. CRISPR and CRISPR/Cas9 are some other recent approaches that are easier, quicker, more efficient, less expensive and by far the most adaptable and simple to use, and its efficacy and accuracy have revolutionised the area of plant biology. It's a natural defence mechanism in bacteria against external DNA sources like bacteriophages and plasmids (Wiedenheft et al. 2009). When a virus infects a bacterial cell, a Cas (CRISPR-associated) protein extracts a piece of foreign DNA and inserts it into the CRISPR locus. The inserted foreign DNA, now referred to as a "spacer", is accommodated between two repeat sequences in a lengthy array of such repeat-spacer-repeat triplets, each from a distinct invader. The CRISPR array allows the bacteria to "remember" the viruses, even when the cell divides (Fig. 1.4), and thus the information is carried to the daughter cells (Horvath and Barrangou 2010; Sawyer 2013), and if the virus infects again, the bacteria employ Cas9 or a similar enzyme to cleave the virus's DNA apart, thereby rendering it inactive. Based on the structure and function of the Cas protein, the CRISPR/Cas systems may be classified into two classes (class I and class II), which can then be further divided into six types (type I–VI) (Makarova et al. 2015) among which type I, III and IV belongs to the previous class and the rest belongs to class II (Mohanraju et al. 2016).

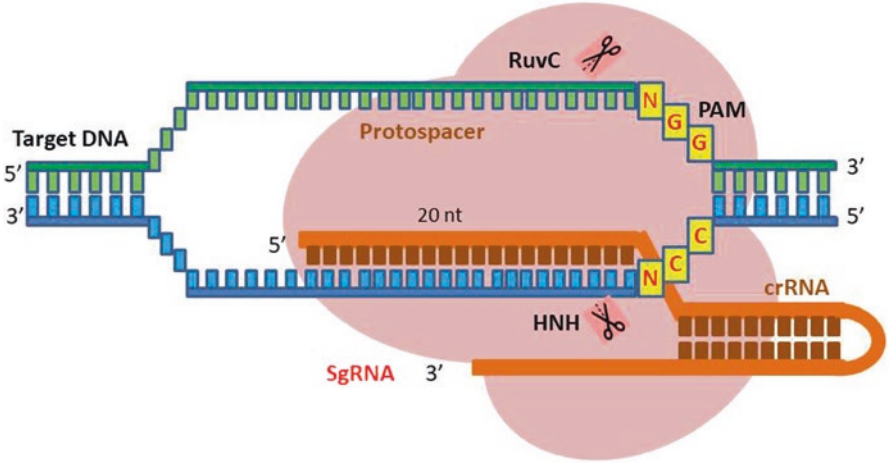

Fig. 1.4 Schematic representation to show mechanism of CRISPR/CAS9 gene-editing system. CRISPR/Cas9 system comprises a Cas9 protein (depicted in skin colour) with two nuclease domains (RuvC and HNH), and a single guide RNA (sgRNA). The sgRNA guides the Cas9 protein to the complementary sequences of the DNA target. The presence of a protospacer-adjacent motif (PAM) in yellow is a prerequisite for DNA cleavage by Cas9

Types I, II and V recognise the specific sequence in DNA and cleave it, whereas type VI has a feature to edit RNA and type III has editing attribute for both DNA and RNA (Terns 2018). Soyars et al. (2018) explored several types of Cas proteins and factors that are adjustable during optimisation of CRISPR/Cas9 systems for plants. There are several additional research and review publications that have covered the CRISPR/Cas9 approach (Wada et al. 2020; Zhu et al. 2020). Nucleases are employed in CRISPR to cleave DNA at particular sequences. Cas9 was the first nuclease found in this system that was tailed by Cpf1, discovered in *Francisella novicida*'*s* as CRISPR/Cpf1 system (Fonfara et al. 2016). CRISPR/C2c2, an RNA-guided CRISPR system with RNA as target rather than DNA, was identified later in *Leptotrichia shahii*, a bacterium. It can cleave/knock down single-stranded RNA targets (Abudayyeh et al. 2016).

Development of CRISPR/Cas9 system has permitted efficient and precise targeted mutagenesis. Because of its precision and effectiveness in altering the genome, CRISPR/Cas9 technology has exploded in popularity. CRISPR mutants were produced to examine the complete cleave gene PMR4 in tomato, which is responsible for powdery mildew pathogen susceptibility (S). For this, a CRISPR/Cas9 construct with four single-guided RNAs (sgRNAs) was applied that targeted PMR4 gene. This enhanced the likelihood of substantial deletions in mutants, as well as mutants with varying numbers of base pairs inversion, which were discovered following PCR-based transformant selection and sequencing. Visual assessment of symptoms and analysis of relative fungal biomass can be considered as the basis for grading these mutants that show a decreased sensitivity towards the pathogen. The efficacy and adaptability of this system as a valuable tool for studying and characterising

susceptibility genes by producing a number of mutations were established in the investigation (Santillán Martnez et al. 2020).

Equipped with novel edited gene delivery method, these newly discovered CRISPR/Cas systems in combination with other recent technologies for targeted gene editing thus, in the near future, will increase the use of the CRISPR toolset for plant genome editing. These tools will allow researchers to explore new approaches for specific and precise genome editing. It also guarantees that no transgenes will remain in genome-edited plants once the product is produced. There are many research and review articles available that elaborated and explained the methodology of CRISPR/Cas9 along with the delivery of genes in host genome in detail (Wada et al. 2020; Zhu et al. 2020). Delivery of gene or fragment of DNA is a tough task during CRISPR genome editing. Recently many delivery methods have been experimented for CRISPR/Cas9 genome-editing technique. Construct delivery in plant cells is largely accomplished by three methods: PEG-mediated *Agrobacterium*-mediated transformation, bombardment and biolistic transformation. Various delivery systems, their efficiency and accomplishments were explored in depth in a recent review paper (Sandhya et al. 2020). The paper found that genome editing's high efficiency is dependent on a number of variables. Using *Agrobacterium*-mediated transport of CRISPR/Cas9 components, 100% editing efficiency was reported for the banana plant (Naim et al. 2018). The effectiveness of various delivery strategies is determined by the tissue type and subsequent regeneration into entire plants. The characteristics of the plant species, tissue type, and culture method all influence regeneration problems. Naim et al. (2018) also emphasised the need to develop new methods for delivering CRISPR/Cas9 components, such as nanoparticle-mediated delivery (directly into the meristematic region) and pollen-mediated delivery, which would allow researchers to skip the time-consuming and labour-intensive tissue culture. Through the development of innovative delivery techniques, CRISPR/Cas technologies in agriculture will be boosted, and crops will be transformed. This technology will also overcome ethical and regulatory barriers, as it does not require any vector DNA for editing (Sandhya et al. 2020).

Above all, the CRISPR research community's open access policy might be one of the causes for the technology's recent rise in popularity. Through Addgene (a non-profit repository), the community makes plasmids available to the public, various web tools for gRNA sequences and predicting specificity, viz. http://cbi.hzau.edu.cn/cgi-bin/CRISPR; http://www.genome.arizona.edu/crispr/; http://www.rgenome.net/cas-offinder; and http://www.e-crisp.org/E-CRISP/index.html and also do hosts for forums for discussion groups, e.g. https://groups.google.com/forum/#!forum/crispr.

1.4 Gene-Editing Tools: Comprehensive Strengths and Limitations

In theory, all GenEd methods can cause identical variation in the nuclear genome, but each one differs in terms of mechanism of action, specificity, simplicity and, of course, cost effectiveness.

Following the initial reports suggesting the use of CRISPR/Cas9 technology in plants (Feng et al. 2013; Nekrasov et al. 2013; Jiang et al. 2013; Xie and Yang 2013), a large number of reports based on the CRISPR/Cas9 technology have found their way into PubMed, clearly demonstrating that CRISPR technology has outperformed all other Gen/Ed tools in the plant world. CRISPR/Cas9 has made ripples in the scientific world as a ground-breaking genome editing tool, even winning the Nobel Prize in Chemistry in 2020. Agronomic trait manipulation necessitates coordinated genetic regulation of several genes to manage the complicated metabolic network required for trait expression. As a result, CRISPR/Cas technology with multiplexing capabilities (several target sites may be edited at the same time) has leapfrogged the competition and shown to be extremely useful in both fundamental research and commercial applications. Several research papers have used Golden Gate-related cloning or the Gibson Assembly technique to integrate several sgRNAs into single Cas9/sgRNA expression vectors, with multiple sgRNAs driven by distinct promoters (Engler et al. 2008). A generic methodology for the synthesis of sgRNA from a polycistronic gene was developed by Xie et al. (2015). Improvement in the targeting and multiplexing efficiency of CRISPR/Cas 9 was achieved by Xie et al. (2015) by modulating the molecular intrinsic processing properties of t-RNA.

Ding et al. (2018) used this modified and enhanced tRNA-processing machinery in the CRISPR/Cpf1 system to achieve multiplex editing. Cpf1, unlike Cas9, is a binary nuclease that cleaves target DNA while also processing its own CRISPR RNA (Fonfara et al. 2016; Zetsche et al. 2017). Wang et al. (2017) took advantage of this property by engineering a sequence-specific nuclease CRISPR/Cas 9 (C-ERF922) and targeting multiple sites within the OsERF922 region, demonstrating that multiple sgRNAs can also be used to target a single gene in order to further improve editing rates in crops with minimal transformation or editing efficiency.

The CRISPR/Cas system has significant advantages over other sequence specific nucleases. A table that compares the features of various Gene Editing Tools (Table 1.1) are given and discussed below.

1.4.1 Simplicity (Ease of Designing)

CRISPR plasmid construction is simpler than ZFN and TALENS because target specificity is based on ribonucleotide complex generation rather than protein to DNA recognition. ZFN and TALEN both include DNA-binding domains that are connected to the FokI endonuclease, which needs dimerisation in order to cleave DNA. ZFN design necessitates rigorous protein engineering steps, and context-dependent specificity imposes limitations (Sander et al. 2011). Zinc-fingers construction step is simplified by procuring commercially engineered nucleases which are far superior to those designed individually (Ramirez et al. 2008). Sangamo Biosciences (Richmond, CA) has created a unique platform (CompoZr) for zinc-finger building in partnership with Sigma-Aldrich (St. Louis, Missouri), allowing scientists to bypass zinc-finger assembly and validation altogether. The

Table 1.1 Various gene-editing techniques: a comparative analysis

Attributes	Meganucleases	ZFN	TALENs	CRISPR/Cas9
Region of target loci	14–40 bp	9–18 bp	28–40 bp per TALENS pair	19–22 bp + PAM sequence
Specificity	High	High	High	Moderately high
Designing	Extremely difficult	Complex	Moderately difficult	Easy
DNA recognition mechanism	DNA and protein interaction	DNA and protein interaction	DNA and protein interaction	DNA and RNA interaction
DNA breakage and repair mechanisms	Double-stranded break with endonuclease	Double-stranded break by Fok 1	Double-stranded break by Fok 1	Cas 9-induced single- or double-stranded break
Off targeting	Low	Low to moderate	Low	High
Multiplexing	Difficult	Difficult	Difficult	Easily can multiplexes

development of TALENs has been facilitated by efficient DNA assembly and cloning methods such as Golden Gate (Engler et al. 2008), and unlike ZFN, its design has been improved by one-to-one recognition criteria between protein repeats and nucleotide sequences. Each ZNF recognises 3–6 nucleotide triplets on average, and since the cleavage domain Fok1 needs dimerisation to cleave DNA, every particular locus requires two ZNFs to target specific DNA fragment. TALENs are composed of highly repetitive sequences that can promote homologous recombination in vivo (Holkers et al. 2013), and they are also much easier to construct than ZNFs. Guide RNA-based (gRNA) cleavage, on the other hand, is based on a simple Watson–Crick base pairing with the target DNA sequence; therefore, no complex and difficult protein engineering is necessary for each target, and just 20 nt in the gRNA must be modified to recognise a different target. In addition, just 20 nucleotides in the gRNA sequence must be changed to confer a different target specificity, eliminating the need for cloning. Any number of gRNAs may be produced in vitro using two complementary annealed oligonucleotides (Cho et al. 2013). Vector systems for Cas9 expression are available in a variety of formats. SgRNA is available as a DNA expression vector, an RNA molecule, or a pre-loaded Cas9-RNA combination for delivery to cells. This allows for the creation of large gRNA libraries at a relatively low cost, allowing the CRISPR/Cas9 system to be used for high-throughput functional genomics applications and bringing GEN/Ed within reach of any lab interested in using CRISPR. Conventional TALENs and ZFN cannot cleave DNA containing 5-methylcytosine, but methylated cytosine is indistinguishable from thymidine in the major groove. Unlike ZFNs and TALENs, the CRISPR/Cas9 system in human cells can produce incisions in methylated DNA (Hsu et al. 2013), allowing for genomic modifications that other nucleases cannot (Ding et al. 2013). Although this element of the CRISPR/Cas9 system has not been fully researched in plants, it is reasonable to assume that it should be similarly efficient regardless of the kind of

genome targeted, given CRISPR's ability to cleave methylated DNA is an inherent characteristic of the system. In plants, the majority of CpG/CpNpG sites (≥70%), particularly CpG islands in promoters and proximal exons, have been found to be methylated (Vanyushin and Ashapkin 2011). CRISPR/Cas9 technology can therefore be more adaptable for genome editing in plants in general, but it's especially good for monocots with high genomic GC content (Miao et al. 2013).

1.4.2 Efficiency

Other targeted gene editing approaches are outperformed by the CRISPR/Cas system. RNAs encoding the Cas protein and gRNA can be infused directly into cell lines to provide modifications. When using classic homologous recombination procedures to create selected mutant lines, this avoids the time-consuming and labour-intensive transfection and selection steps. The relative efficacy of various nucleases (CRISPR associated) in plants is incomparable since the plant species studied by different scientists differs and each has employed a diverse set of CRISPR/Cas. Although CRISPR is more effective than current Gen/Ed methods, the regeneration aspect of engineered plants must be addressed since it significantly increases the tool's efficiency.

1.4.3 Multiplexing

The ease of multiplexing is CRISPR/key Cas9's practical advantage over ZFNs and TALENs. By injecting numerous gRNAs into several genes at the same time, mutations can be introduced in multiple genes at the same time (Li et al. 2013; Mao et al. 2013), which can be very effective for knocking off redundant genes or parallel pathways. By targeting two widely dispersed cleavage sites on the same chromosome, the same technique can be used to construct massive genomic deletions or inversions (Li et al. 2013; Upadhyay et al. 2013; Zhou et al. 2014). The monomeric Cas9 protein and any number of distinct sequence-specific gRNAs are all that's needed for multiplex editing with the CRISPR/Cas9 system. Multiplex editing with ZFNs or TALENs, on the other hand, necessitates separate dimeric proteins specialised for each target location.

All of the technologies – meganuclease, ZNFs, TALENs, and CRISPR/Cas – provide researchers with new ways to produce mutants more quickly than classic gene targeting approaches, but each come with their own set of restrictions and complications. Some of them are discussed below.

1.4.4 Off-Site Effects

One of the most significant drawbacks of these technologies is that mutations are frequently introduced at non-specific sites. These loci exhibit homology to the target locations that is similar but not identical. These can be difficult to spot, requiring a genome scan for mutations at places that are similar in sequence to the gRNA target sequence. CRISPR/Cas9 systems are more likely to elicit off-target actions than other systems (Zhang et al. 2014), because Cas9 can cut at other unintended sites in the genome in addition to the intended target region. Other systems have a high level of precision, but their construction or delivery are difficult. Actual Cas9 off-target activities are lower in *Arabidopsis*, maize, rice, tomato, and tobacco than in mammals (Nekrasov et al. 2013; Feng et al. 2014; Gao et al. 2015; Woo et al. 2015; Ishizaki 2016; Pan et al. 2016; Peterson et al. 2016; Tang et al. 2016). On target indel frequencies in *Arabidopsis* range from 33 to 92 percent of sequencing reads, but no off-target editing events were found elsewhere in the genome at expected or unexpected locations, corroborating findings from smaller scale studies (Peterson et al. 2016). During pathogen-related gene editing (Nekrasov et al. 2017) and targeted deletion of cis-regulatory regions (Rodríguez-Leal et al. 2017), no off-target mutations were observed in tomato. Backcrossing to a parental line can remove these so-called off-targets in some plant species. When targeting members of closely related gene families, the specificity of gene editing tools is particularly noticeable, especially when recent paralogues are co-located in the genome and unlikely to segregate. Another approach is to create a chimeric fusion between the FokI catalytic domain and a catalytically inactive Cas9 protein (dCas9). Guilinger et al. (2014) and Aouida et al. (2015) employed the inactive dCas9 as a targeting module to bring the FokI domain into close proximity and allow dimerisation, and the production of homodimers with the correct spacer sequence then allows the generation of DSBs. As it requires 40 bp of unique sequence and a unique distance between the two monomers, this greatly improves cutting specificity, limiting off-target actions (Yee 2016).

1.4.5 Mosaicism

As Cas9 nucleases may not always cut the DNA during the one cell stage of embryonic development, genetic mosaicism occurs when an individual species has more than two alleles with a mutant allele in only some of their cells. The CRISPR/Cas9 system may continually target and cleave genes at different phases of embryonic development, resulting in mosaicism of the introduced mutations, which is often documented in animal systems (Mizuno et al. 2014; Oliver et al. 2015; Luo et al. 2016). Small indel mutations in plants may have been missed by present detection methods, resulting in overall mosaicism rates being routinely overestimated or ignored.

1.4.6 Delivery

Despite the fact that CRISPR/Cas is a ground-breaking and unrivalled technology, there are still certain barriers to its widespread use in crop improvement and translational research. One of these obstacles is the efficient delivery of transformation vectors into the appropriate host cells, followed by successful plant regeneration. Transient transformation and steady transformation are two processes in the transformation of plants. Stable transformation is responsible for producing edited plants with heritable mutations, from which the nuclease incorporated transgene can be separated to produce transgene-free plants.

1.4.7 Multiple Alleles

Non-homologous end joining can heal the nuclease cleavage site, resulting in cohorts of mutants with different mutations from the same targeting constructs, necessitating genome sequencing to confirm the type and position of the individual mutation. It's also possible to create mutants with mosaics of numerous mutations, and breeding may be required to separate and isolate a cultivar with single mutations. Phenotyping bottlenecks are also created by the generation of mutants with many variations.

Despite these challenges, ZNFs, TALENs, and, in particular, the CRISPR/Cas systems are powerful new genome-editing tools. These methods are expected to be refined further, and they will be modified in novel ways to generate even more sophisticated plant models.

1.5 Plant Defence and Genome Editing

Interactions between plants and bacteria have piqued scientists' interest for ages. Microbes have been discovered to have either a protagonistic association with plants, in which they form a synergistic interaction with the plants that benefits both of them, or an antagonistic association with their hosts, in which they harm their hosts. Plants may undergo entire genome duplication events to counteract abiotic stress, and functional redundancy in multigene families may also be detected (Khan et al. 2018). One of the key goals of plant researchers is to have a full understanding of the molecular basis of abiotic stresses (such as drought, salinity, and heat) and associated tolerance mechanisms in order to engineer stress tolerance in plants. The antagonistic confrontations between plants and diseases, according to the Red Queen dynamic model, result in ever-changing co-evolutionary cycles (Han 2018). In the absence of an adaptive immune system, plants have evolved innate immune systems (including resistance proteins) to detect and respond to both biotic and