Chapter 13
Pearl Millet Blast Resistance: Current Status and Recent Advancements in Genomic Selection and Genome Editing Approaches

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Contents

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13.1 Introduction

Pearl millet is a hardy C_4 crop grown in the arid and semiarid tropics mainly in rainfed conditions where other cereals are likely to fail to produce economic yields due to drought and heat stresses. Adaptive evolution, a form of natural selection, shaped the crop to grow and yield satisfactorily with limited moisture supply or under periodic water deficits in the soil (Serba and Yadav 2016). *Pyricularia* leaf spot, also known as blast disease, is particularly important in pearl millet forage cultivars. It is an important disease in the southern United States and more recently it has emerged as a serious disease of dual-purpose (grain and fodder) pearl millet hybrids in India (Lukose et al. 2007; Anonymous 2009). In India, for the first time the disease was recorded from Kanpur, Uttar Pradesh (Mehta et al. 1953), and it was considered as a minor disease for a long time since its inception.

The pathogen infects several cereal crops, including rice, wheat, pearl millet, finger millet, and foxtail millet, and several grasses. The pathogen is highly variable, but highly specialized in its host range. Thus, *M. grisea* strains isolated from rice or any other hosts do not infect pearl millet and vice versa. The rice blast pathosystem has been extensively studied for several decades. In disease-conducive environments, the life span of many disease-resistant rice cultivars has been known to be ephemeral (Srinivasachary et al. 2002). Most of the deployed resistance genes in rice crop break down in a few years because of their race specificity, high rate of mutation, and rapid change in pathogenicity of the blast pathogen (Suh et al. 2009). Pathogenic variation in *M. grisea* populations adapted to rice, finger millet, foxtail millet, wheat, and several weed hosts has been reported by Takan et al. (2012). Various potential mechanisms, including sexual recombination, heterokaryosis, parasexual recombination, and aneuploidy, have been proposed to explain frequent race changes in *M. grisea* (Kang and Lee 2000). This implies that pathogenic variability might exist in the pearl millet-infecting strains of *M. grisea*. Therefore, for the management of disease through host plant resistance, it is important to know pathogen variation in populations of *M. grisea* infecting pearl millet and to identify potential resistance sources.

Disease resistance in plants occurs as a hypersensitive response (HR) at the site of infection by pathogen. This specific event is initiated in response to recognition of pathogen-associated molecular pattern (PAMP) and subsequent PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Boller and He 2009). Both PTI and ETI mechanisms are closely associated with reactive oxygen species (ROS) production and disease resistance that involves production of distinct biphasic ROS as one of its pivotal plant immune mechanisms. This unique oxidative burst is strongly dependent on the resistant cultivars because a monophasic ROS burst is a hallmark of the susceptible cultivars. However, the cause of the differential expression of ROS burst remains still unknown. A second phase of the innate immune system in plants, known as effector-triggered immunity (ETI), was discovered to be mediated by intracellular receptor molecules containing nucleotide-binding (NB) and leucine-rich repeat (LRR) domains that specifically recognize effector proteins

produced by the pathogen. The binding of receptors and effectors results in the activation of defense programs and often leads to localized cell death (Chen and Ronald 2011). In fact, ETI reactivation of defense responses is assumed to be mediated by the activity of cytoplasmic proteins and transcriptional reprogramming to make the first immune responses involving signaling from the cytoplasm to the nucleus (Wirthmueller et al. 2007).

Effectors are mainly involved in the colonization of pathogen or interrupt the activation of host cell defenses. On the contrary, rice plants have consequently showed immunity that is based on the sensitivity of effectors to host resistance proteins. *M. grisea* produces effector proteins to influence plant immunity, leading to the penetration of infection process. Avr proteins are a special group of effectors encoded by avirulence genes. These proteins can be distinguished by relative R proteins and this leads to the race-specific recognition (Wawra et al. 2013). Pathogen AVR effectors have been genetically proven to be essential components in plant immune responses (Flor 1971). However, the mechanism by which AVR effectors and R proteins are associated with these responses remains unclear. Initially, the ligand-receptor model (Gabriel and Rolfe 1990) was widely supported, but the lack of physical interactions between a number of R/AVR pairs has resulted in the generation of alternative guard and decoy hypothesis (van der Hoorn and Kamoun 2008). One of the interesting results have been reported recently for AVR-Pii and OsExo70-F3 interaction in which OsExo70-F3 physically interacts with AVR-Pii and is specifically involved in Pii-dependent resistance suggesting OsExo70-F3 as a helper in Pii/AVR-Pii interactions (Fujisaki et al. 2015). More recently, rice-resistant protein pair RGA4/RGA5 was shown to be required for recognition of *M. oryzae* effectors AVR-Pia and AVR1-CO39 revealing a mode of AVR protein recognition through direct binding to a novel, non-LRR interaction domain (Cesari et al. 2013, 2014). *M. oryzae* has evolved effector proteins such as Slp1 and AVR-Piz-t that can suppress rice immune responses, whereas the rice crop has developed an innate immune system that can recognize the presence of *M. oryzae* and initiate PTI and ETI defense responses (Liu et al. 2013; Singh et al. 2016). A model of AVR-Pii and Os-NADP-ME interactions near the BIC after *M. oryzae* infection was generated and it was also reported that the AVR-Pii-Os-NADP-ME2-3 pair provides key evidence on how AVR effectors reprogram host metabolism to establish compatibility and why the ROS issue has continued to be treated as a main defense mechanism in the innate immunity of most plants.

Insights into the function of secreted fungal effectors in the reprogramming of host defense and metabolism are gradually emerging. Among the plethora of secreted proteins encoded in the genomes of phytopathogenic fungi, only a few fungal effector targets have been identified to date, with less than a handful shown to impair host metabolic functions directly (Tanaka et al. 2014). Although eight AVR genes of *M. oryzae* have been isolated over the last several decades (Zhang et al. 2015), their functions as virulence factors have not been functionally addressed, except for evidences found in AVR-Pizt and AVR-Pii (Fujisaki et al. 2015; Park et al. 2012).

In recent years, more progress has been made in investigating the molecular mechanisms of innate immunity responses in rice and other cereal crops against *M. grisea*, as well as in identifying R genes, identification-triggered early signaling, signaling pathways in rice, and role of these signaling pathways in activating defense responses (Saitoh et al. 2011; Seo et al. 2011). However, a complete understanding of the molecular network regulating defense responses against pathogens in rice and millets is still obscured. For instance, despite the number of R genes present in rice, little information is available about the essential signaling needed to initiate effector-triggered resistance against *M. grisea*.

13.2 Plant Impedance and Genome Editing Tools

The traditional genetic breeding methods depend mainly on the existence of broad genetic variation in elite primary gene pool of particular crop species and its closely related crop species to introduce new resistant traits into local gene pool, which is purely naturally induced spontaneous mutation (Jung et al. 2018). The first artificially induced transgenesis was experimentally reported in bacteria by Avery et al. (1944). But for the first time gene modification or genome editing tools with respect to plants started during early 1980s. The work of Schilperoort et al. (1967) on *Agrobacterium tumefaciens* has become the key step in understanding the transfer of genes through the Ti plasmid (200 kb) of virulent strain and its integration into nuclear chromosomes (Zaenen et al. 1974; Van Larebeke et al. 1974; Chilton et al. 1980). Earlier to this a group of investigators at the Institute of Pasteur in Paris discovered a unique phenomenon of deviation from the Mendel's laws of genetic inheritance when they were studying the crossing in the yeast: they discovered that a particular mitochondrial allele, known as omega (ω), was unidirectionally inherited, which was later determined to be the gene for the large ribosomal RNA subunit which is further known to be type 1 intron; till then introns were regarded as junk DNA; after a few days, it became evident that this omega is encoded by separate gene producing an endonuclease which recognizes and introduces the double-strand break (DSB) over the LSU rRNA genes lacking the intron; these DNA nicks are repaired by DNA repair mechanism. This became the first basic foundation of homing meganucleases to use them in genetic engineering (Bolotin et al. 1971; Bos et al. 1978; Faye et al. 1979; Jacquier and Dujon 1985). But there was still a question as to how to introduce nick in the double-stranded DNA with unique locus of choice interest, which was addressed by Snyter and Brooks (1988) and his colleagues through the experimental characterization of best rare cutting endonuclease *Not1* enzyme with recognition site of 8 bp length and the I-Sce I endonuclease (omega of LSUrRNA) study pioneered the accuracy and precisions of editing of plant genomes (Puchta et al. 1993).

As per the records reported till now, for the first time plant genome editing was reported from *Agrobacterium* through chemical mutagenesis with the help of ethyl methane sulfonate (EMS) and ionizing radiations which created genome

modifications randomly. The second phase is associated with the discoveries of homing and meganuclease enzymes during the 1980s and 1990s as mentioned earlier, which are engineered to provide efficient tools for targeted editing in genome. During 2006–2012, a few crop plants were successfully and precisely modified using zinc-finger nucleases. A third phase (2009–2011) of improvements in genome editing led to a dramatic decrease in off-target events with the TALEN technology. In the year 2013, major breakthrough came with the development of CRISPR-Cas9 technology which has high efficiency and technical essence of use is quite impressive; this technology can be employed with the use of in-house kits or commercially available kits. The main requirements include careful selection of the location of the DNA double-strand breaks to be induced and then ordering of sequence of an oligonucleotide usually a primer (Francis 2016). Genome engineering is currently done by employing three different tools as shown in Fig. 13.1.

As it is known gene editing tools have the ability to implement DBS's double-strand breaks in the genome, which further requires repair of these breaks for a successful knock-in and knockout events. Subsequently, the damaged DNA is repaired mainly by two molecular repair mechanisms: homology-directed repair (HDR), where the broken DNA is repaired using a homologous DNA sequence as template, or nonhomologous end joining (NHEJ) where the broken ends are rejoined to each other at nonhomologous DNA sequence and NHEJ is mainly responsible for repair of one or two nucleotide breaks in the DNA leading to gene disruption whereas HDR is responsible for gene deletion, replacement, and gene insertion (as shown in Fig. 13.2). HDR works by precise copying of gene with the help and template at specific site, repairs the homologous DNA breaks, and is helpful for gene expression studies whereas NHEJ repair mechanism only repairs by insertion or

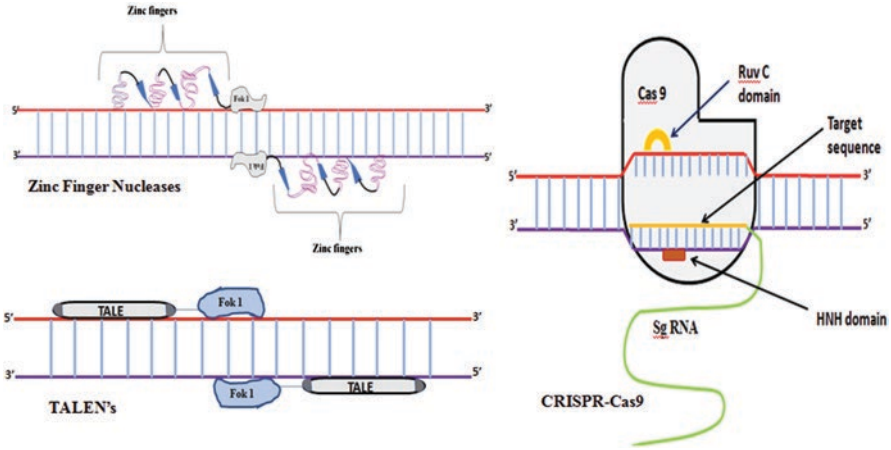

Fig. 13.1 Genome engineering via zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) and Cas 9

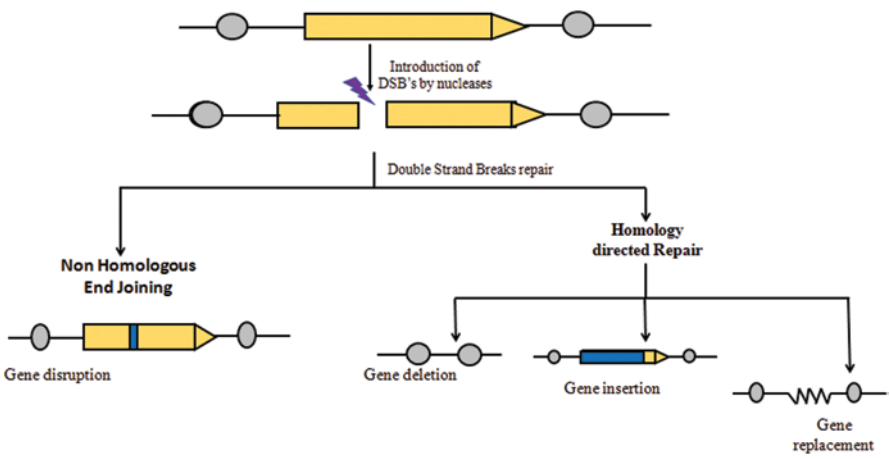

Fig. 13.2 DNA repair mechanism induced by double-stranded breaks leading to gene disruption via NHEJ pathway and gene deletion, insertion, and replacement by HDR mechanisms

deletion of one or two nucleotides where it is helpful in the SNP, knock-in, and knockout studies (Maresca et al. 2013; Lee et al. 2015).

13.3 Meganucleases or Homing Endonucleases (HEs)

HEs are small proteins (<300 amino acids) found in bacteria, archaea, and unicellular eukaryotes. A distinguishing characteristic of HEs is that they recognize relatively long sequences (14–40 bp) compared to other site-specific endonucleases such as restriction enzymes (4–8 bp). These lengthy recognition sites, and the name of the first such known enzyme, ω (also known as I-SceI), have given rise to the term "meganuclease" (Paques and Duchateau 2007).

The important feature that differentiates HEs from restriction endonucleases is their lack of absolute sequence specificity. Whereas restriction enzyme binding and/or cleavage depend on a perfect match to the recognition sequence, HEs are less discriminating, often tolerating multiple sequence changes within their recognition site (Colleaux et al. 1988). This is significant at the structural level making a great diversity between the number of contacts made by restriction endonucleases and HEs. Restriction endonucleases exploit most of the potential hydrogen bonds between the proteins and their target sites whereas HEs utilize only a fraction of the possible hydrogen bonds. The positions that are tolerated by HEs are often those at third positions of codons, which vary naturally between organisms. Such tolerance allows homing into new sites. Despite the imperfect fidelity, the lengthy recognition sites can make HEs highly specific, often cutting large genomes only once. This attribute makes the HEs amenable to genome editing, where spurious off-site cleavages are detrimental.

HEs have been historically categorized by small conserved amino acid motifs. At least five such families have been identified: LAGLIDADG, GIY-YIG, HNH, His-Cys Box, and PD − (D/E) × K, which are related to ED × HD enzymes and are considered by some as a separate family. At a structural level, the HNH and His-Cys Box share a common fold (designated ββα-metal) as same as the PD − (D/E) × K and ED × HD enzymes. The catalytic and DNA recognition strategies for each of the families vary and lend themselves to different degrees to engineering for a variety of applications among which LAGLIDADG homing endonucleases are well known (Belfort and Bonocora 2014). Another focus was directed to create hybrids which provide platform for increasing the specificity, employed for GIY-YIG and LAGLIDADG groups of enzymes. The idea of hybrids helped to produce catalytically nonactive with interest of recognition site enzymes coupling with catalytically active with undefined recognition site. The chimeric construct catalytically inactive LHE I-SceI fused to the restriction enzyme PvuII as the cleavage module where the two subunits are joined by linker (Fonfara et al. 2012). TALE-PvuII chimeric protein was also developed after the discovery of TALENS to increase the efficiency of inducing the breaks at a particular site (Yanik et al. 2013).

13.4 Zinc-Finger Endonucleases (Nucleases), ZFNs

ZFNs are artificially developed restriction enzymes that have been successfully used for a few years as a genome editing tool. As it is known that two most desired qualities of genome editing comprise (1) an endonuclease that must be able to recognize specific long target sequences and (2) adequate flexibility of re-targeting to desired sequences defined by use. The ZFN structure meets the above properties better than any other meganucleases. The ZFN carries the DNA-binding domain zinc-finger proteins (ZFPs) derived from eukaryotic transcription factors and the Fok I cleavage domain derived from *Flavobacterium okeanokoites* (Bitinaite et al. 1998; De Souza 2011). The zinc-finger nuclease is composed of 30 amino acids forming two antiparallel β-sheets opposite an alpha-helix. Each ZFN cleavage domain is linked to a range of three–six zinc fingers designed to specially recognize the target sequence flanking the cleavage site. The most commonly used zinc fingers have three DNA-binding domains, and nine base pairs. The zinc-finger proteins are used in pairs giving 18-base specificity of each bind to a single codon (i.e., three nucleotide codons). ZFN can become an optional tool for researchers, but due to the high cost and design complications, presently ZFNs have been successfully used for HDR-mediated gene KI and NHEJ-mediated KO approaches to many targets in prokaryotes as well as eukaryote gene editing experiments (Carroll 2011). Even though with so much advances in engineered systems, they also have some drawbacks like all possible nucleotide sequences of the genome cannot be targeted and its specificity to the sequence cannot be precisely defined as it may be influenced by other neighboring protein domains (Maeder et al. 2008).

Despite their complicated construction and off-targeting issues, ZFNs have already been used to make gene modifications in Arabidopsis (Osakabe et al. 2010; Petolino et al. 2010; Zhang et al. 2010; Even-Faitelson et al. 2011; Qi et al. 2013), tobacco (*Nicotiana tabacum*) (Wright et al. 2005; Townsend et al. 2009), and also in crops including maize (*Zea mays*), soybean (*Glycine max*), and canola (*Brassica napus*) (Shukla et al. 2009; Curtin et al. 2011; Gupta et al. 2012; Ainley et al. 2013). Resistance to bialaphos in maize (Shukla et al. 2009), resistance to herbicides in tobacco (Townsend et al. 2009), and ABA-insensitive phenotype in Arabidopsis (Osakabe et al. 2010). However, ZFNs lack specificity which results in the generation of undesired mutations, off targets, and some chromosomal abbreviations (Pattanayak et al. 2011; Radecke et al. 2010).

13.5 Transcription Activator-Like Effector Nucleases (TALENS)

After several years, Schornack et al. (2006) reported some proteins from *Xanthomonas* bacterial species that are capable of hijacking the plant immune response in rice. These proteins are 30–35-residue-long sequences with most positions occupied by conserved sequences and a series of tandem repeats, of which four conserved positions are with limited variability [two amino acids in positions 12 and 13 (called "diresidues") vary and specify the binding to A, C, G, and T as shown in Fig. 13.2] with DNA-binding domain and an effector domain. Proteins with similar properties were reported and were termed as transcription activator-like effector (TALE) (Moscou and Bogdanove 2009; Boch et al. 2009). The TALE proteins are engineered with a FokI nuclease domain which creates the DSB (Mussolino and Cathomen 2012). TALENs possess longer recognition sites which help in increasing the specificity and then ZFNs are highly efficient with less prone to mutations and cause very minimum deleterious effects (Puchta and Fauser 2014; Petersen and Niemann 2015). The plant pathogen TALE's N-terminal segment (NTS) harbors protein secretion signal peptides while their C-terminal segment (CTS) contains nuclear localization signal peptides and a transcription activator domain which play a very important role in cleavage efficiency by impairing the catalytic domain scaffold when fused with the FokI, so the scaffold optimization plays an important role during engineering the TALENs for developing efficient genome editing tool with precise on targets and DSB of our interest (White et al. 2009).

Just like ZFNs, TALEN construction also requires verification in vitro to have the knowledge of efficiency of site-specific cleavage instead of directly employing into the experiments because of the modular nature of the various segments (conserved sequences, NTS and CTS) in TALEN; the probability of rationally designing a TALEN that will be specific for a particular sequence appears to be greater than

that for ZFNs; however, it is not possible to rule out off-target cleavage in an experiment model without extensive testing and confirmation.

Rice bacterial blight is caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) and its pathogenesis is controlled by the interaction TALE of *Xanthomonas oryzae* and host target S genes. TALENs were developed with respect to S gene effector-binding element site (EBE) and induced the disruption of EBE which resulted in lack of interaction between *Xanthomonas* TALE protein and S genes resulting in subsequent establishment of blight resistance rice line (Li et al. 2012). The first TALEN-modified rice was made by mutating the OsSWEET14 promoter to generate heritable and effective disease-resistant lines. Powdery mildew pathogens cause yield loss up to 50% worldwide (Cao et al. 2011) and Wang et al. (2014) attempted to obtain a resistant variety of wheat by targeting the *mlo* (mildew locus O) gene coding for proteins repressing defense against the disease. A pair of TALENs was created that targeted a region in exon 2 of all three homolog genes, creating mainly small deletions. Homozygous mutation of all three homolog genes was necessary to have an effective and heritable powdery mildew resistance.

13.6 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and Associated Systems

Similar to eukaryotic mammalian immune system even bacteria and most of the archaea harbor a special type of immune response against various infections. Bacteria and archaea bacteria of known being are invaded by phages; bacteria fend this invasion of foreign bodies by employing clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins, which is similar to adaptive immunity of eukaryotic immune system; hence it is also referred to as adaptive immunity of bacteria and archaea bacteria (Jansen et al. 2002; Sorek et al. 2008). In eukaryotes the antigen and presentation are done by classical antigen-presenting cells, where a piece of fragment of that foreign body is being sliced and represented to recognize and defend the infection similarly in bacteria; as soon as it encounters the phage infection the bacterium with CRISPR acquires a piece of its DNA and tailors close to promoter sequence of CRISPR array. CRISPR array transcribes and generates m-RNA (referred to as crRNA) which serves as a guiding molecule for Cas9 proteins (helicase and nuclease proteins) to the target phage genome, cleaves the special nucleotide sequence (3–5 base pair) found upstream to protospacer adjacent motif (PAM) sequence in future invasion, and provides immunity to bacterial cell (Jinek et al. 2012). Even though CRISPR repeats were initially discovered in the 1980s in *E. coli* its function remained ununderstood (Ishino et al. 1987) but experimentally demonstrated resistance of *S. thermophilus* acquired against a bacteriophage by integrating a virus genomic fragment into its CRISPR locus (Barrangou et al. 2007).

Cas9 protein was characterized and known to have two catalytically active domains, namely N-terminal HNH domain with nuclease domain with histidine and asparagine residues in the middle region and RuvC domain with nuclease activity towards C-terminal end (Sapranauskas et al. 2011).

The CRISPR element consists of a complex of noncoding RNAs and Cas proteins (CRISPR associated), which have nuclease and helicase activity. In contrast to the chimeric TALEN proteins, recognition by the CRISPR/Cas system is carried out via the complementary interaction between noncoding RNA and target-site DNA, whereas TALEN tool is protein-directed gene editing. Simple construct of CRISPR plasmid contains elements necessary for CRISPR/Cas9 activity: U3 or U6 RNA polymerase III promoters, Cas9 nuclease/nickase, CRISPR mRNA, and tracrRNA. The crRNAs consist of approximately 40 bp sequences in association with tracrRNA that activates and guides Cas9 nuclease. Biolistic and agrobacterium-mediated transformation is being employed to transfer the sgRNA and Cas9 proteins into the desired cell when it is used for plant genome editing. The target sequence of choice of pathogen is inserted into this position by introducing DSB in the plasmid by employing site-specific endonucleases; once the cell encounters the invasion by respective pathogen this CRISPR-Cas9 protein gets activated and induces the cleavage in the target genome which is referred to as protospacers (Nemudryi et al. 2014; Barrangou et al. 2007). The advantages of CRISPR-Cas gene editing technology over other tools are that protein engineering is not required like ZFNs or TALENs, modification like DNA methylation can be done which cannot be done using ZFNs or TALENs, multiple targeting (multiplexing) can be done in single experiment, and even any number of gRNAs can be designed for a single target which makes it special (Ding et al. 2013; Doench et al. 2014; Mao et al. 2013).

One of the most powerful applications of CRISPR-Cas technology in plants will be to study the specific gene function involved in the metabolic or developmental pathways for knockout. For example, two key proteins in photosynthesis, highly homologous magnesium chelatase subunit I (CHLI) genes, CHLI1 and CHLI2, have been simultaneously targeted for elimination and have resulted in the production of albino plants as a useful tool for understanding the role of these enzymes in chlorophyll biosynthesis (Mao et al. 2013). Understanding the role of a particular gene in plant development like the auxin-binding protein (ABP1) is not required for either auxin signaling or Arabidopsis development (Gao et al. 2015). These two examples showed the precise elimination of one or more specific genes of character of interest to study the effects of its gene product role in the regulation of plant growth and development. Similarly multiplexing can be done because gRNA is small in size so that many genes can be targeted simultaneously. Golden gateway and Gibson assembly are the two important techniques which are employed to make multiplex targeting. Gibson assembly works based on primer overhangs whereas golden gateway of cloning works by employing restriction endonucleases which helps to target 5–8 genes. The other way of modifying plant growth and development and maximum yield of food grains for commercial benefit is to manipulate the specific gene timing and level of its expression by employing dead Cas9 (lacks the nuclease activity) coupling it with gene activator and gene repressor proteins, which

guides the polymerase to transcribe active and continuously or repress the expression continuously. The important issue after transformation is screening of positive clones with different techniques that have been developed, like annealing at critical temperature-polymerase chain reaction (ACT-PCR) (Hua et al. 2017), high-resolution melting analysis (HRMA) (Thomas et al. 2014), polyacrylamide gel electrophoresis (PAGE)-mediated genotyping (Zhu et al. 2014), T7 endonuclease I (T7EI) approach (Vouillot et al. 2015), and restriction enzyme site loss technique. After the successful editing of genome of Arabidopsis (Feng et al. 2013; Li et al. 2013) and rice (Miao et al. 2013; Shan et al. 2013) using CRISPR/Cas9 system has been reported, many independent applications of this genome editing tool in various crops have shown that CRISPR/Cas9 system is broadly acceptable and effective for crop improvement like generating herbicide resistance, disease resistance, enhancing of yield levels, and stress tolerance.

13.7 Development of Blast Resistance by Genome Editing

Potato crop is harvested and stored in cold rooms to avoid sprouting; due to storage under cold conditions the starch of potato degrades into glucose and fructose. These reduced sugars, when subjected to deep frying in oil, i.e., while making chips, French fries, etc., turn into a carcinogen called acrylamide. Chawla et al. (2012) used RNAi to overcome this problem and developed a new variety of potato called "innate potato"; similarly another group of scientists used a gene of enzyme called *vascular invertase* which plays a very important role in reducing the sucrose into glucose and fructose by using TALENs; it was successful in providing potatoes with very less starch and helps in brown-colored acrylamide formation (Clasen et al. 2016). Similarly, when apple cut was exposed to air it turned into brown color, because of reduction in polyphenols by polyphenol oxidase enzyme (PPO gene). RNAi was used to address this reduction by blocking the homologous *PPO* gene expression (Waltz et al. 2015). Herbicide-resistant crops were also developed by inducing the mutation in acetolactate synthase (ALS) gene which serves as a target for imidazolinone herbicides (Townsend et al. 2009) by employing the ZFNs. Citrus canker caused by *Xanthomonas citri* is a serious disease in many citrus cultivars, leading to huge economic losses around the world. Pathogen injects its effector (TALEPthA4) protein which has target of CsLOB1 gene EBE motifs resulting in active transcription of canker susceptibility products. It is known to possess two genes CsLOB1 and two genes where both are capable of functioning in the absence of other (Jia et al. 2016, 2017) induced mutation in both genes and generated canker-resistant citrus plants, which bind to EBE motifs and transcriptionally activate the downstream target canker susceptibility lateral organ boundary 1 (CsLOB1) gene in the host, leading to disease susceptibility using CRISPR-Cas system. Rice blast is the most devastating in all the rice-growing countries, with yield loss of 10–30% (Dean et al. 2012). Therefore, creating resistance to *M. oryzae* is one of the most effective approaches to mitigate the disease. The plant ethylene response factors

(ERF) are the main factors involved in multiple stress tolerance (Müller and Munné-Bosch 2015). The work of knockdown expression of rice ERF92 by employing RNAi enhances resistance to *M. oryzae* (Liu et al. 2012). CRISPR/Cas9 is used to mutate the OsERF922 gene through agrobacterium-mediated transformation of rice calli (Kuiku 131) where one sgRNA was designed for targeting the first exon of the gene, 21 mutant lines are obtained, and these mutant lines are evaluated for different agronomic parameters, but these mutant lines showed enhanced resistance to *M. oryzae* infection compared to the wild type. The lesion length in the mutant lines is about 66% smaller than that in the wild type (Wang et al. 2016). Ma et al. (2018) developed 273 ossec3a mutants that are more tolerant to *M. oryzae* infection by using CRISPR/Cas9. In the same way even pearl millet is highly affected by blast disease caused by *M. grisea* which can be managed by developing the resistant lines by employing genetic engineering tools. CRISPR-Cas9 tool can be used to target the genes responsible for the susceptibility to blast disease which are commonly termed as S genes and induce the DSBs in S gene by Cas system and direct it towards any of the DNA repair mechanism, resulting in the mutation of S gene which yields blast-resistant pearl millet crop lines (as shown in Fig. 13.3).

13.8 Conclusion and Future Prospects

Pearl millet blast severity has been increasing in recent years due to adoption of high-yielding varieties and use of chemical fungicides is not safe to environment. The use of recent technology CRISPR/Cas9 system which is simple, efficient, and highly specific produces fewer off-target events. It is a promising tool for genome modification in crop improvement program. Mutation in host susceptible gene/s by using CRISPR/Cas9 system has conferred broad-spectrum disease resistance against fungal diseases. This technology is successfully utilized in powdery mildew resistance locus O (*mlo*) which is one of the renowned S genes. It has been mutated via CRISPR/Cas9 and the mutants obtained were more resistant to powdery mildew fungus *Blumeria graminis* f. sp. *tritici* (Bgt) and *Oidium neolycopersici* in wheat and tomato, respectively. This technology is applied in rice blast which is one of the most damaging diseases in the world, which is caused by *Magnaporthe oryzae*. Targeted loss-of-function mutagenesis of negative regulator *Oryza sativa* ethylene response factor 922 (OsERF922) showed enhanced disease resistance against rice blast. Similarly, mutation in coding region of Pi21 gene via CRISPR/Cas9 has also improved the resistance in rice plants against blast fungus. This technology was utilized in rice blast resistance development with the help of the coding region of mitogen-activated protein kinase 5 (OsMAPK5). This technology has lot of potential in the development of blast disease resistance in pearl millet crop with suitable modifications in the genome.

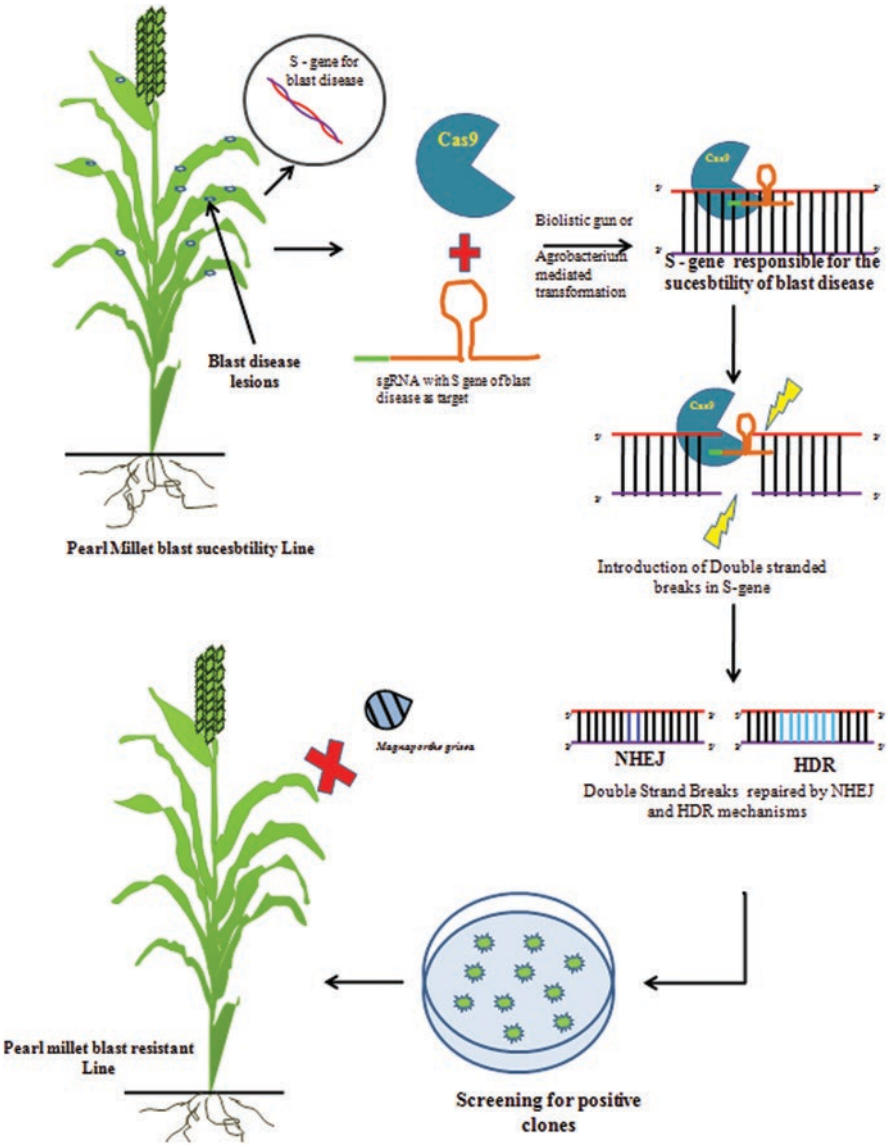

Fig. 13.3 Typical overview of generating blast disease-resistant pearl millet crop lines using CRISPR-Cas 9 system

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