Understanding the Plant-microbe Interactions in CRISPR/Cas9 Era: Indeed a Sprinting Start in Marathon

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Abstract: Plant-microbe interactions can be either beneficial or harmful depending on the nature of the interaction. Multifaceted benefits of plant-associated microbes in crops are well documented. Specifically, the management of plant diseases using beneficial microbes is considered to be eco-friendly and the best alternative for sustainable agriculture. Diseases caused by various phytopathogens are responsible for a significant reduction in crop yield and cause substantial economic losses globally. In an ecosystem, there is always an equally daunting challenge for the establishment of disease and development of resistance by pathogens and plants, respectively. In particular, comprehending the complete view of the complex biological systems of plant-pathogen interactions, co-evolution and plant growth promotions (PGP) at both genetic and molecular levels requires novel approaches to decipher the function of genes involved in their interaction. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 (CRISPR-associated protein 9) is a fast, emerging, precise, eco-friendly and efficient tool to address the challenges in agriculture and decipher plant-microbe interaction in crops. Nowadays, the CRISPR/Cas9 approach is receiving major attention in the field of functional genomics and crop improvement. Consequently, the present review updates the prevailing knowledge in the deployment of CRISPR/Cas9 techniques to understand plant-microbe interactions, genes edited for the development of fungal, bacterial and viral disease resistance, to elucidate the nodulation processes, plant growth promotion, and future implications in agriculture. Further, CRISPR/Cas9 would be a new tool for the management of plant diseases and increasing productivity for climate resilience farming.

Keywords: Beneficial microbes, phytopathogens, genome-editing, CRISPR/Cas9, durable disease resistance, plant-microbe interaction.

1. INTRODUCTION

Nature has multiple millions of microbes, which are continuously evolving and simultaneously interacting among themselves as well as with plants, animals, and the environment. In plants, microbes reside in the rhizosphere and additionally present as endophytes in roots and shoots. Based on the nature of the interaction, plant-microbe interactions can be broadly categorized into beneficial and harmful. The plant-beneficial (PB) microbes produce phyto-hormones, which support plant growth and afford protection against various plant pathogens [1]. As a direct mechanism, PB microbes enhance plant growth through biological nitrogen fixation, phosphorous uptake and production of phytohormones specifically, indole-3-acetic acid (IAA), gibberellic acid (GA) and cytokinins [2-4]. As an indirect mechanism, PB microbes suppress plant pathogenic microbes by producing different antibiotics, and promote induced systemic resistance in plants [5-8]. In contrast, many plant pathogenic (PP) microorganisms cause devastating diseases in various crops. Plant diseases extensively reduce crop yield and are considered as one of the major threats to food security worldwide [9]. Diseases caused by pathogens are generally controlled by the application of pesticides. Though pesticides play a significant role in sustainable food production and food security, their negative environmental impact and pesticide resistance are few major concerns in its usage [10]. The development of inherent genetic resistance to diseases is one of the sustainable approaches for ensuring food security. Several resistance genes (R genes) have been identified and successfully utilized in marker-assisted backcross breeding for the development of tolerant varieties in multiple crops [11]. However, efforts are persistent in the development of durable resistance to diseases. Recently, a genome editing...
technology named as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 (CRISPR-associated protein 9) is becoming highly prominent for understanding the biological function of genes in plants. Therefore, exploring the molecular components of plant-pathogen interactions using CRISPR-Cas9 based genome editing tools will be remarkably useful in developing durable, eco-friendly and sustainable disease management strategies.

2. PLANT-MICROBES INTERACTION IN CRISPR/Cas9 ERA

The interaction between plants and microbes can be either synergistic or antagonistic and elicit multiple responses. Pathogens infect plants and initiate the process of colonization, utilization of plant nutrition, which results in disease symptoms. In return, plants activate a defense response through antimicrobial and stress response pathways. The identification, isolation, and characterization of multiple resistance genes in plants were made possible adopting different techniques/methods inclusive of map-based cloning [12], whole-genome sequencing through next-generation sequencing (NGS) technologies [13], comparative genomics [14], RNA-seq analysis [15, 16], RNA-interference (RNAi) [17], proteomics [18, 19] and availability of mutant resources of genes used for functional characterization [20]. These technologies greatly assisted in translating the findings for application in the crop improvement programs [21]. For instance, reduction in the cost of sequencing has led to the sequencing of several plant genomes, including lupin (Lupinus angustifolius), a leguminous plant sequenced with the primary objective of understanding plant-microbe interactions [22].

3. CRISPR/Cas9 TECHNIQUE

CRISPR-Cas9 is a relatively new technique, which is being increasingly used nowadays for functional genomics [23]. Moreover, CRISPR/Cas9 is an emerging, fast, precise, eco-friendly and efficient tool to address the challenges in agriculture, especially deciphering plant-microbe interaction for resistance development in crops. Additionally, CRISPR-Cas9 is more robust and specific in gene knockdown when compared to RNAi, which induces partial gene silencing [24]. Historically, a repetitive sequence of 21-40bp was identified in E. coli, which was instrumental in the development of gene-editing technology. Later, CAS proteins (CRISPR-associated proteins) and repetitive sequences were predicted to be involved in prokaryotic immunity [25]. Specifically, gRNA (guideRNA) drives the CAS proteins to the complementary DNA sequence and induces DNA breaks. Further, first-generation CRISPR gene-editing technology has been successfully utilized to induce site-specific double-stranded breaks in the genome, exploiting two catalytic nuclease domains, namely HNH and RuvC, each cleaving a strand of DNA [26]. Site-specific cleavage by Cas9 is facilitated through single guide RNAs (sgRNAs) [27]. The second-generation CRISPR editing tool is capable of precisely converting a single base into another (Cytosine (C) to Thymine (T)) without causing double-strand DNA breaks [28, 29]. Moreover, with the improvement in ribosome engineering techniques, editing accuracy of Cas9 endonuclease has been enhanced by engineering hairpin secondary structures in guide RNA that brought about the concept of tuning CRISPR-Cas9, which increased specificity by several orders of magnitude [30].

4. CRISPR/Cas9 MEDIATED RESISTANCE TO PLANT DISEASES

Disease management using CRISPR/Cas9 is considered an emerging tool for plant disease management [31]. Besides, CRISPR/Cas9 has also been used for genetic manipulation of microorganisms for enhancing microbial benefits toward crops [32]. Recently, the CRISPR-Cas9 system was developed in order to decipher the mechanisms of Bacillus plant interactions [33]. Therefore, the present review provides an overview of the application of CRISPR/Cas9 in plant-microbe interactions, particularly for the development of disease resistance and improvement of growth promotion, biocontrol ability of beneficial microbes.

5. CRISPR/Cas9 AND RESISTANCE TO FUNGAL DISEASES

Plant diseases caused by fungal pathogens are considered to be the dominant factors responsible for the detrimental effect on plant yield. Nowadays, the emergence of virulent strains due to climatic changes is posing a far greater threat to crop production [34]. Besides, 30% of emerging diseases are caused by fungal pathogens, which can easily overcome R gene-mediated resistance due to high genetic diversity and evolutionary flexibility [35]. Therefore, gene-specific editing using the CRISPR/Cas9 approach will greatly assist in the development of durable resistance to fungal diseases. Rice blast caused by Magnaporthe oryzae is considered the most devastating disease worldwide and ranked one of the top fungal pathogens affecting food crops [36]. Previous studies reported that knockdown of ethylene-responsive factors (OsERF922) using RNAi approach enhanced disease resistance [37, 38]. In support of these findings, CRISPR/Cas9 based targeting of the OsERF922 gene in rice reduced blast lesion-symptom as compared to wild type in seedling and tillering stages. Besides, a significant difference in the agronomic traits was not observed between mutants and wild types. However, evaluation of the percent reduction in disease symptoms using multiple isolates within a geographical region will determine the efficacy of edited lines for commercial cultivation. In addition, ERF922 gene expression was up-regulated not only during infection of M. oryzae but also in abiotic stresses. Further, editing of the other three blast inducible ERF genes (OsBtERF1, OsBtERF3 and OsBtERF4) may provide additional insights into the role of ethylene response factors in regulating blast resistance in rice [39]. Similarly, the editing of a well-characterized Pi21 gene also enhanced resistance to blast fungus in rice [40]. Moreover, RNA-seq analysis between Pi21 and its loss-of-function lines showed pathogen-associated molecular patterns as one of the mechanisms conferring durable resistance [41]. Therefore, yeast two-hybrid assay of Pi21 protein with effector proteins will decipher the upstream signaling components in the Pi21-M. grisea interaction. Further, gene manipulation of exostalk subunit OsSEC3A and OsMPK5 via CRISPR/Cas9 enhanced the resistance against M. oryzae. However, both the mutants of sec3a and mpk5 had showed dwarf phenotype and susceptibility to abiotic stresses, respectively [42-44]. Specifically, localization studies on the protein translation
dynamics of SEC34: GFP into the plasma membrane (PM) during pathogen interaction and its differential loading into PM might provide us vital clues to decouple the enhanced resistance and plant development traits. Similarly, MPK5 protein interaction network and downstream regulators responsible for providing disease resistance require further investigation. Therefore, the above-mentioned examples of Sec3a and MPK5 suggest that knockdown might result in disease trade-off and different editing strategy is required for genes having multiple roles in plants.

Downy mildew and powdery mildew are the most devastating fungal diseases encountered in grape cultivation. A mildew locus O (MLO) gene up-regulated during Erysiphe necator infection induces susceptibility. RNAi based approach for silencing MLO S-genes viz., VvMLO7, VvMLO6 and VvMLO11 drastically reduced the severity (77%) of powdery mildew disease. Additionally, resistance to powdery mildew disease in other crops was provided by the silencing of MLO S genes [45, 46]. Three MLO alleles with TALEN-induced mutations were also conferred resistance to powdery mildew fungus Blumeria graminis f.sp. tritici in bread wheat [46, 47]. Furthermore, MLO-7 gene lines edited through CRISPR-Cas9 have been developed for enhancing resistance to downy mildew in grapes [48]. Mildew locus (MLO-S) genes form distinct clades and are evolutionarily conserved transmembrane domain proteins, which negatively regulate the penetration of powdery mildew fungus. Therefore, orthologous editing of MLO-S genes could be a prominent strategy in the development of mildew resistance in diverse crops, including fruits and vegetables [49, 50]. Besides, cell wall thickening was identified as one of the probable mechanisms contributing to resistance in the loss-of-function of the MLO1 gene in cucumber [51]. Recently, a medley of regulators were identified in regulating the cell wall thickening in plants [52]. Thus, enquiring the role of MLO signaling in regulating the medley of regulators, especially MYB and NAC genes involved in cell wall thickening, would be an attractive research hypothesis in exploring the mechanistic understanding of powdery mildew resistance. Similarly, CRISPR based knock-down of downy mildew resistance 6 (DMR6) also conferred resistance to downy mildew disease in grapevine. Thus, multiplex editing of MLO and DMR6 genes in grapes could provide durable resistance to downy mildew in grapes. Interestingly, editing of the tomato SLDMR6-1 gene also conferred resistance to different pathogens viz., Phytophthora capsici, Pseudomonas syringae, and Xanthomonas species [53]. Since dmr6 gene increased salicylic acid levels, thus targeting the orthologs in monocots and dicots might provide additional information on the development of broad-spectrum resistance and its associated pleiotropic effects. However, a slight reduction in plant height and its associated yield penalty due to the editing of the DMR6 gene needs to be better understood, considering its beneficial effects on plant immunity.

Ascomycetes fungus Leptosphaeria maculans causing ‘blackleg’ disease in oilseed crops such as Brassica napus is a major problem worldwide. Besides, only limited information is available regarding genes involved in conferring resistance against L. maculans. Gene knock-down through the CRISPR-Cas9 approach in L. maculans identified a gene cluster responsible for the synthesis of pathogenicity factor, abscisic acid (ABA), which increases pycnidiospore germination and appressorium formation [54, 55]. In addition, ABA has been reported to restrict the growth of some beneficial fungi like Aspergillus nidulans, suggesting that CRISPR-Cas9 can be used to unravel complex microbiome interactions [56]. Therefore, deregulating the components of ABA perception in plants during L. maculans infection might be considered a double-edged sword due to its effects on pathogen restriction and biocontrol agent promotion. Phytophthora spp is disease-causing agent of Theobroma cacao and reduces its yield drastically. A gene named Non-expressor of Pathogenesis-Related 1 (TcNPR1) acts as a major regulator of the defense system in cocoa [57]. Moreover, Arabidopsis NPR3 gene negatively regulates NPR1 activity [58]. Further, knock-down of TcNPR3 transcripts in cocoa leaf tissues demonstrated enhanced resistance to P. tropicalis infection. Thus, the CRISPR-Cas9 approach could effectively complement the knock-down of NPR3 transcripts for resistance development in cocoa. One of the important factors influencing the efficiency of CRISPR/Cas9 systems is the generation of homozygous mutations in the first generation, effective targets editing and elevated expression of Cas9 protein [59]. This is vital for plants having long generation time, particularly woody plants such as grape. In a report, Wang et al., (2018) [60] investigated the efficiency of CRISPR/Cas9 mediated targeted mutagenesis in the first generation of grape mutants. Results demonstrated that knockout of the VvWRKY2 gene increased disease resistance against Botrytis cinerea infection as compared to wild type plants. Further, there was no significant difference in phenotype between wild-type and biallelic edited plants and concluded that CRISPR/Cas9 can be efficiently utilized for the specific genome editing in the first generation plants. Moreover, efficient disease control found in edited lines having early termination of translation indicates efficient target site selection for obtaining better disease response. Most of the loss-of-function tools result in hemizygous/heterozygous mutants as compared to CRISPR-Cas9. Thus, homozygous mutants generated through CRISPR-Cas9 will be remarkably useful in woody crops for early ascertainment of phenotypic response. The list of genes edited through CRISPR/Cas9 related to the development of resistance is given in Table (1).

6. CRISPR/Cas9 AND RESISTANCE TO BACTERIAL DISEASES

Plant bacterial pathogens can spread rapidly and establish epidemics in a short term period. Especially, the management of bacterial pathogens is profoundly difficult because of their accelerated multiplication and high diversity. The characterized resistant (R) or susceptibility (S) genes are the major targets in marker-assisted breeding for the development of bacterial disease resistance [61]. Apart from traditional map-based cloning and transgenic methods, CRISPR/Cas9 tool has also been used to achieve resistance to bacterial diseases in various crops. Citrus canker is a severe disease in citrus caused by Xanthomonas citri subsp. citri (Xcc), resulting in huge economic losses worldwide [62]. Hu et al. (2014) [63] demonstrated that citrus Lateral Organ Boundary 1 (CsLOBI) gene is responsible for disease-susceptibility in bacterial canker disease. During Xcc
Table 1. List of target genes edited through CRISPR/Cas9 in different crops for understanding plant-microbe interaction and disease resistance.

| S. No. | Crop | Targeted Pathogen | Target Genes | Signaling Pathway/Processes | Physiological Function/Disease Response | References |
|--------|------|-------------------|--------------|-----------------------------|-----------------------------------------|------------|
| 1      | Citrus sinensis (Citrus) | Xanthomonas citri sub sp. citri (Xcc) and Xanthomonas axonopodis | LOB1 | TALE effectors binding | Susceptibility factor against Xcc | [64, 123] |
|        |      | Xanthomonas citri sub sp. citri (Xcc) | WRKY22 | Salicylic acid (SA) regulated defense | Induces pathogen-triggered immunity | [124] |
| 2      | Malus domestica (Apple) | Erwinia amylora | DIPM-1, DIPM-2, DIPM-4 | Leucine-rich repeat receptor kinase | Reduces host susceptibility | [48] |
| 3      | Oryza sativa (Rice) | Xanthomonas oryzae pv. oryzae | OsSWEET11,13,14 | Sucrose efflux transporter | Non-availability of sugar metabolites and broad-spectrum resistance | [73, 125] |
| 4      | Solanum lycopersicum (Tomato) | Pseudomonas syringae, Xanthomonas spp. | DMR6-1, DMR6-2 | Inactivation of salicylic acid to 2,3-dihydroxybenzoic acid | Broad-spectrum resistance | [53] |
|        |      | Pseudomonas syringae pv. Tomato | SIJAZ2 | Jasmonic acid-mediated regulation of stomata | Stomatal opening and disease susceptibility | [76] |

| S. No. | Crop | Targeted Disease | Target Genes | Signaling Pathway/Processes | Physiological Function/Disease Response | References |
|--------|------|------------------|--------------|-----------------------------|-----------------------------------------|------------|
| 1      | Gossypium hirsutum (Cotton) | Verticillium dahlia | GhMYB25 | Transcription regulation for fiber and trichome development | Enhanced resistance to Wilt | [126] |
| 2      | Oryza sativa (Rice) | Magnaporthe oryzae | OsSEC3A | Enhanced salicylic acid signaling | Disease resistance and plant development | [42] |
|        |      |                  | OsERF922 | Ethylene mediated signaling | Reduced susceptibility to blast disease | [38] |
|        |      |                  | Pi21 | Induces pathogen-associated molecular pattern response | Durable resistance | [40] |
|        |      | Magnaporthe grisea and Burkholderia glumae | OsMPK5 | MAP kinase pathway | Enhanced resistance | [43] |
| 3      | Solanum lycopersicum (Tomato) | Phytophthora capsici | SiDMR6-1, SiDMR6-2 | Inactivation of salicylic acid to 2,3-dihydroxybenzoic acid | Broad-spectrum resistance | [53] |
|        |      | Oidium neolycopersici | SiMlo1 | Negative regulation of vesicle-associated and actin-dependent defense pathways | Broad-spectrum and durable resistance | [127] |
|        |      | Fusarium oxysporum f.sp. lycopersici | Solyc08g075770 (CYCLOPS) | Inhibition of mycorrhizal colony | Enhanced defense response | [128] |

(Table 1) contd....
| S. No. | Crop | Targeted Disease | Target Genes | Signaling Pathway/Processes | Physiological Function/Disease Response | References |
|-------|------|------------------|--------------|----------------------------|----------------------------------------|------------|
| 4     | *Theobroma cacao* (Cacao) | *Phytophthora tropicalis* | NPR3 | Suppressor of defense response | Enhanced disease resistance | [129] |
| 5     | *Triticum aestivum* (Wheat) | *Blumeria graminis f.sp. tritici* | TaMLO | Negative regulation of vesicle-associated and actin-dependent defense pathways | Broad-spectrum and durable resistance | [47] |
| 6     | *Fusarium graminearum* | TaLpx-1 | Lipoxygenase activity | Enhanced resistance response | [130] |
| 6     | *Blumeria graminis f.sp. tritici* | TaEDR1 | MAP kinase pathway | Resistance response | [47] |
| 7     | *Vitis vinifera* (Grape) | *Botrytis cinerea* | WRKY52 | Transcription regulation of pathogenesis-related genes | Transcriptional reprogramming to regulate disease resistance | [60] |
| 7     | | *Erysiphe necator* | VvMlo7 | Negative regulation of vesicle-associated and actin-dependent defense pathways | Broad-spectrum and durable resistance | [52] |
| 8     | *Brassica napus* | *Sclerotinia* | WRKY11, WRK17 | Suppressor of jasmonic acid and salicylic acid signaling | Enhanced resistance | [131, 132] |

### Viral Diseases

| S. No. | Crop | Targeted Pathogen | Target Genes | Signaling Pathway/Processes | Physiological Function/Disease Response | References |
|-------|------|------------------|--------------|----------------------------|----------------------------------------|------------|
| 1     | *Nicotiana benthamiana* | Beet severe curly top virus (BSCTV) | A7, B7 and C3 sites | Replication | Interferes with replication of virus | [79] |
|       |       | TYLCV, BCTV, MeMV, CLCuKoV* | CP, Rep, IR | Virus maturation | Interferes with replication of virus | [79, 80] |
|       |       | Cotton Leaf Curl Multan virus (CLCuMuV) | IR | Transcriptional regulation | Inhibits bidirectional transcription | [133] |
| 2     | *Nicotiana benthamiana*, *Arabidopsis thaliana* | Bean yellow dwarf virus (BeYDV) | LIR, Rep/RepA | Replication | Interferes with replication of virus | [80] |
|       |       | Cucumber mosaic virus (CMV) and Tobacco mosaic virus (TMV) | Sequence target sites | Replication | Cleavage of RNA viruses | [85] |
| 3     | *Arabidopsis thaliana* | Turnip mosaic virus (TuMV) | eIF(iso)4E | Initiation factor | Disruption of viral translation | [84] |
|       |       | Cauliflower mosaic virus (CaMV) | CP | Virus assembly | Disables the assembly of virus | [134] |
| 4     | *Oryza sativa L. Japonica* | Rice tungro spherical virus (RTSV) | eIF4G | Initiation factor | Disruption of viral translation | [86] |
| 5     | *Tomata* | Tomato yellow leaf curl virus (TYLCV) | CP | Virus assembly | Disables the assembly of virus | [132] |
| 6     | *Hordeum vulgare* | Wheat dwarf virus (WDV) | MP/CP, Rep/RepA, LIR | Replication | Interferes with replication of virus | [135] |
| 7     | *Musa spp.* (banana) | Endogenous banana streak virus (eBSV) | ORF1, ORF2, ORF3 | Strand cleavage | Knockout of dsDNA | [136] |
| S. No. | Crop | Targeted Pathogen | Target Genes | Signaling Pathway/Processes | Physiological Function/Disease Response | References |
|-------|------|-------------------|--------------|-----------------------------|---------------------------------------|------------|
| 8.    | Manihot esculenta | African cassava mosaic virus (ACMV) | AC2, AC3 | Replication | Virus resistance | [137] |
|       |       | Cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV) | Novel cap-binding proteins (nCBP-1), and nCBP-2 | Translation and virus movement | Interferes with translation and movement of the virus | [138] |
| 9.    | Cucumis sativus | CVYV, ZYMV, PRSV** | eIF4E | Initiation factor | Broad virus resistance | [139] |
| 10.   | Solanum tuberosum | Potato virus Y (PVY) | Coilin gene | Host interaction | Impairment of host and virus interaction | [140] |

**Beneficial Microbes**

| S. No. | Crop | Targeted Trait | Target Genes | Signaling Pathway/Processes | Physiological Function | References |
|-------|------|----------------|--------------|-----------------------------|-----------------------|------------|
| 1.    | Lotus japonicas | Biological N fixation | LjLb1, LjLb2, LjLb3 | Oxygen binding | White color non-functional nodules | [103] |
| 2.    | Vigna unguicu- lata | Biological N fixation | RLKs | Receptor-like kinase signaling | Absence of nodules | [104] |
| 3.    | Glycine max | Biological N fixation | Rfkl | Strain-specific response | Symbiosis specificity | [105] |
| 4.    | Parasponia andersonii | Biological N fixation | PanHK4, PanEIN2, PanNSP1, PanNSP2 | Cytokinin, ethylene pathway | Conservation of nodulation in trees | [107] |
| 5.    | Medicago truncatula | Biological N fixation | MtGA2Ox10 | Gibberellin metabolism and signaling | Suppressed the infection thread formation | [109] |
|       |       |       | NFS2 | Biogenesis of Fe-S cluster | Strain specificity | [103] |

**Phytopathogens**

| S. No. | Pathogen | Targeted Disease | Target Genes | Signaling Pathway/Processes | Physiological Function/Phenotype | References |
|-------|----------|------------------|--------------|-----------------------------|-------------------------------|------------|
| 1.    | Alternaria alternate | Blight | pyrG (orotidine-5-phosphate decarboxylase gene) | Regulation of pyrimidine biosynthesis | Auxotrops for uracil and uridine | [110] |
|       |          |                  | pksA (polyketide synthase) | Melanin biosynthesis pathway | Disruption results in white colonies | [110] |
| 2.    | Colletotrichum sansevieriae | Anthracnose | SCD1 | Biosynthesis of dihydroxynaphthalene (DHN)-melanin | Lacks melanin biosynthesis resulting in white colonies | [113] |
| 3.    | Fusarium oxysporum | Wilt | BIK1 polyketide synthase | Synthesis of bikaverin | Lacks bikaverin synthesis | [111] |
|       |          |                  | URA5 | Uracil biosynthesis | Resistance to 5-fluoro-orotic acid and hygromycin | [111] |

(Table 1) contd....
infection, Xcc-derived transcription activator-like effector (TALE), i.e. PthA4 is translocated into plant cells and induced CsLOB1 expression resulting in canker development. Specifically, Xcc-derived effector protein PthA4, specifically binds to effector binding elements (EBE_{PthA4}) in the CsLOB1 promoter region (EBE_{PthA4}-CsLOBP) to activate its gene expression. Thus, promoter editing of the EBE_{PthA4} cis-regulatory element of CsLOB1 showed resistance to bacterial canker disease in Duncan grapefruit. Similarly, the coding region of CsLOB1 was targeted via CRISPR/Cas9 in Wanjincheng Orange [64] and provided enhanced resistance to Xcc signifying the importance of targeting LOB1 for the development of canker resistance in plants. LOB genes are essential for pattern formation in meristem cells [65], and few genes showed differential expression during biotic stress [66]. Moreover, CsLOB1 gene expression was correlated with hypertrophy and hyperplasia, and few downstream genes have also been identified to be direct targets of CsLOB1 [67]. The co-expression of LOB1 and its downstream genes in relation to pathogen colonization could provide mechanistic insights for the induction of hypertrophy and hyperplasia. Thus, LOB homologs could be potential targets for resistance development in crops.

Another best example is the control of bacterial leaf blight (BB) disease in rice caused by Xanthomonas oryzae pv. oryzae (Xoo). In rice, BB is one of the major pathogens and causes yield losses of up to 50% and sometimes even complete yield loss [68]. The Xoo pathogen through the type III secretion system translocates the transcription activator-like effector (TALE) proteins into host cells and induces the expression of susceptibility factor genes (S genes) in plants [69-72]. Xoo produces PthXo2 (effector protein) that induces OsSWEET13 (S gene) expression and results in bacterial blight disease. Mutagenesis in the OsSWEET13 coding region via the CRISPR/Cas9 system provided enhanced resistance to Xoo infection. Similarly, AvrXa7 is another TALE protein, which targets OsSWEET14 in rice. Gene editing in the OsSWEET14 promoter prevented the binding of AvrXa7 and consequently resulted in disease resistance. Recently, Oliva et al. (2019) [73] demonstrated that mutations in SWEET gene promoters of SWEET11, SWEET13 and SWEET14 provided a robust, broad-spectrum resistance to Xoo pathogen in rice. Thus, cis-TALE CRISPR (cis represents the promoter binding region of TALE effector) is an efficient disease control strategy in plants, which needs to be exploited in multiple crops. Further, differential disease response of multiple Xoo strains in indica and japonica varieties reinforces the requirement of evaluation of multiple pathotypes for disease screening. Additionally, SWEET11 and SWEET14 have also been found to be essential for reproductive development [74]. Thus, even though Oliva et al. (2019) [73] work showed no effect on percent spikelet fertility, three genes edited lines need to be evaluated under water-limiting or nutrient-limiting conditions for understandings its reproductive fitness. In tomato, Pseudomonas syringae pv. tomato (Pto) is the causal organism of bacterial speck disease. A refined strategy to manipulate hormonal crosstalk has been evolved in Pto strain DC3000 through the synthesis of coronatine (COR), which mimics the bioactive JA hormone. In plants, JA isoleucine (JA-Ile) stimulates stomatal opening, which facilitates bacterial invasion and promotes leaf colonization [75, 76]. Ortigosa et al. (2019) [77] showed SlJAZ2 is a major co-receptor of COR in stomatal guard cells of tomato. Further, the editing of the SlJAZ2 gene prevented stomatal reopening induced by COR and conferred resistance to Pto DC3000. Additionally, neither water use nor resistance to necrotrophic pathogen was affected. Moreover, salicylic acid regulated factors involved in imparting resistance in addition to stomatal closure needs to be understood independently for trade-off effects. This novel strategy provides insights into the trade-off between biotrophs and necrotoprophs in plants. Therefore, targeted knockout of susceptibility genes or/and negative regulators through genome editing provides a powerful strategy for disease resistance breeding.

7. CRISPR/Cas9 AND RESISTANCE TO PLANT VIRUSES

Plant viruses are emerging as a major challenge in agriculture and horticulture worldwide. Viruses are obligate parasites, which depend on host cells for their survival and replication. Chemical control is specifically unavailable for the management of viral pathogens. Therefore, the development of genetic resistance to viruses is a significant alternate strategy for the control of viral diseases. RNAi is successfully employed in the control of viral diseases. Recently, the
CRISPR/Cas9 system has been utilized to impart virus resistance by targeting either the viral genome or susceptibility genes of hosts [78]. Two families of ssDNA viruses (Geminiviridae and Nanoviridae) are known to infect plants. Geminiviridae is the largest known family of single-stranded DNA viruses, which infect both dicot and monocot plants, causing extensive crop losses globally [79]. Most of the CRISPR/Cas9 mediated viral resistance has been demonstrated against geminiviruses by targeting ssDNA of mono and bi-partite genome containing coat protein (CP), replication (Rep), intergenic region (IR) and nonanucleotide sequences [80] (Table 1). CRISPR/Cas9 system imparting resistance to geminiviruses has been demonstrated in Beet severe curly top virus (BSCTV) and Bean yellow dwarf virus (BeYDV) in Arabidopsis thaliana and Nicotiana benthamiana, respectively. The plants expressing viral gene-specific sgRNA-Cas9 exhibited lower viral titre or delayed accumulation of viruses and reduced symptom expression. Similarly, Ali et al. (2015) [81] reported the efficiency of editing various coding and non-coding regions such as viral coat protein (CP), replication protein (Rep) as well as the intergenic region (IR) in the control of Tomato yellow leaf curl virus (TYLCV) in N. benthamiana. Interestingly, none of the novel variants of viruses observed in N. benthamiana was carrying sgRNAs targeting IR sequences. Further, targeted editing in the non-coding IR region of three geminiviruses, namely Cotton leaf curl Kokhran virus (CLCuKov), Mellonit mosaic virus (MeMV) and Tomato yellow leaf curl virus (TYLCV) had imparted resistance to multiple begomoviruses and simultaneously conferred broad-spectrum resistance against geminivirus [82].

Majority plant viruses have an RNA genome, which is usually single-stranded (ss) or double-stranded (ds) in single or multiple fragments. Since the sgRNA-Cas9 system only recognizes DNA sequences, it becomes difficult to achieve resistance against RNA viruses. However, there are some Cas9 variants, which have the potential to target and cleave RNA sequences, which need to be explored. Therefore, the CRISPR/Cas9 system has been utilized to derive resistance to RNA viruses, mainly through targeting host genes responsible for susceptibility. Chandrasekaran et al., 2016 [83] demonstrated the utility of CRISPR/Cas9 system in developing disease resistance in cucumber plants against Zucchini yellow mosaic virus, Cucumber vein yellowing virus and Papaya ringspot virus by targeted editing of eukaryotic translation initiation factor 4E (eIF4E). The RNA viruses specifically attach to eIF4E through virus-encoded movement protein (VPg) and a mutation in the host eIF4E limits the viral establishment. Similarly, resistance against Turnip mosaic virus (TuMV) has been achieved in A. thaliana by targeting eIF(iso)4 locus, which plays a vital role in viral survival [84]. Zhang et al. (2018) [85] expressed CRISPR-Cas9 system from Franchesella novicida (FnCas9) in N. benthamiana and Arabidopsis plants to impart disease resistance against Cucumber mosaic virus (CMV) and Tobacco mosaic virus (TMV). Macovei et al. (2018) [86] developed tungro disease resistance (caused by Rice tungro bacilliform virus (RTBV) and Rice tungro spherical virus (RTSV)) in rice susceptible cultivar IR64 which was achieved by targeting translation initiation factor 4 gamma gene (eIF4G). Similarly, Aman et al. (2018) [87] exploited CRISPR/LshCas13a strategy to confer resistance against TuMV by targeting different viral genomic regions, namely helper component protease silencing suppressor (HC-Pro) and coat protein (CP) region. Further, a reduction in replication and spread of the virus was found to be efficient upon editing the HC-Pro region. Editing of host susceptibility factors to control RNA viruses requires comprehensive evaluation for understanding the fitness of edited plants in the field.

CRISPR based virus control is a novel strategy, which can knock out a host factor required by the virus for the development of resistance. Moreover, virus resistance would be durable than dominant R genes due to lower selective pressures on the virus to evolve counter defense strategies [88]. Besides, eukaryotic viruses have developed mechanisms to circumvent RNAi through the expression of RNAi suppressors [89]. Further, targeting essential cis-acting regions of the viral genome might help to prevent the escape of modified viruses [90]. Despite its appeal, CRISPR/Cas9 technology develops some off-target effects, and most of the CRISPR/Cas systems were based on the challenge results of agro-inoculations. Even though this method resembled natural inoculations, the differences in virion load between these two inoculation methods have not been systematically evaluated. Thus, it is better to perform inoculation on test plants with their respective vectors [91]. Finally, the virus has the ability to evolve constantly, and successful technology has to match the speed of the virus adaptability for providing better and quicker solutions.

8. CRISPR/Cas9 AND PLANT BENEFICIAL MICROBES

Plant beneficial microbes are often found associated with the plant rhizosphere. Few beneficial microbes are endophytes, which aid the host by improving efficient nutrient use of macronutrients (N, P), and micronutrients (Zn, Mn, Cu and Fe) [92]. Additionally, microbe-induced root architectural modifications also improve nutrient use efficiency. Microbes also participate in the acclimatization process of several abiotic, biotic stresses, osmoregulation, and carbon sequestration [93-97]. Accordingly, beneficial microbes are valuable candidates, which enhance crop productivity and participate in bi-directional interaction wherein plants provide carbon source and microbes assist the plant acclimatization to physiological stresses. CRISPR-Cas9 has been applied to understand soil microbiome-related processes, including nitrification and lignocellulose decomposition. Rice gene NRT1.1 B functions as nitrate transporter, which regulates the root microbiome in indica varieties [98]. Targeted allelic replacement of NRT1.1 in rice using CRISPR-Cas9 greatly enhanced nitrogen use efficiency in japonica rice, and the edited plants further served as biomarkers for understanding root microbiome. Thus, CRISPR technology is becoming a highly valuable tool for metagenomics assisted root microbiome studies. Additionally, CRISPR-Cas9 has been adopted for the alteration of plant cell wall components by specifically targeting OSH15 and OshAt10 genes for obtaining enhanced saccharification [99]. CRISPR elements are naturally present in microorganisms to provide anti-viral mechanism [100], and it was reported that polar soil has a higher abundance of CRISPR genes than tropical soil, which was correlated with greater disease pressure in the tropics.
[101]. Accordingly, horizontal gene transfers are greatly reduced in CRISPR-Cas9 edited Bacillus subtilis employing integrative plasmid wherein the risk of horizontal transfer could be lowered [102]. Hence, the presence of CRISPR repeats in plant beneficial bacteria may provide an evolutionary advantage for better adaptation.

9. CRISPR/Cas9 FOR MECHANISTIC INSIGHTS IN SYMBIONTIC NITROGEN FIXATION

Symbiotic nitrogen fixation is one of the major beneficial legume-Rhizobium interactions in this world. The CRISPR-Cas9 approach has been used to elucidate various genes involved in nitrogen fixation. Specifically, nodulation specific promoter region of leghemoglobin genes (LjLb1, LjLb2, LjLb3) was used for efficient expression of guideRNA in the nodules of Lotus japonica [103]. Further, the loss-of-function of cowpea (Vigna unguiculata) symbiotic receptor-like kinase (VaSYMRK) gene developed through CRISPR-Cas9 blocked the nodule formation [104]. In soybean, the dominant gene responsible for restriction of nodule formation by Sinorhizobium fredii was identified through the Rfg1 gene [105], and Rj4 gene [106] editing. Moreover, editing of four genes (PanHK4, PanEIN2, PanNSP1, and PanNSP2) related to hormonal regulation of nodulation in tropical tree, Parasponia andersonii, helped to identify conservation of nodulation process in comparison to that of legumes [107]. Recently, small fragments of tRNA (tRFs) synthesized in Rhizobia regulated 52 soybean genes were determined to be involved in the nodulation process. Further, CRISPR-Cas9 based editing of a few selected genes promoted nodulation in soybean [108]. In Medicago, CRISPR/Cas9 based editing of gibberlin oxidase (MtGA20ox10) suppressed the infection thread formation in the initial nodulation process [109]. In addition, MtNFS2 gene responsible for strain specificity was identified and validated through the CRISPR/Cas9 approach in Medicago [103]. Thus, genome editing by CRISPR/Cas9 facilitates not only mechanistic insights in nodule formation but also assists in the identification of target genes, especially for enhancement of nodulation in legumes.

10. CRISPR/Cas9 APPROACH IN MICROBES

The CRISPR/Cas9 system has already been reported in phytopathogens viz., Fusarium oxysporum, F. proliferatum, Alternaria alternata, Phytophthora spp, Sclerotinia sclerotiorum and Colletotrichum sansevieriae [110-114]. Specifically, F. proliferatum causes several diseases in plants and contributes to the production of diverse mycotoxins amongst which fusonins are the most toxic. In Fusarium, FUM1 encodes a polyketide synthase gene responsible for the synthesis of fusonins. CRISPR-Cas9 system was used to inactivate the FUM1 gene and the edited mutants did not produce fusonins [115]. Further, the CRISPR-Cas9 tool has also been used to understand the infection process of fungal pathogens through the development of CRISPR-Cas9 assisted endogenous gene tagging (EGT) techniques. The EGT has been used to examine the infection process of F. oxysporum, wherein CRISPR-Cas9 was utilized for tagging endogenous genes with fluorescent markers providing an efficient tool for subcellular localization studies of fungal proteins [116]. Additionally, biocontrol fungus consists of clustered genes responsible for the production of secondary metabolites. Fang and Chen (2018) [117] demonstrated that the silencing of ace1 gene in Trichoderma atroviride induces the expression of four polyketide biosynthetic genes, which enhance the biocontrol activity against Fusarium oxysporum and Rhizoctonia solani. Hence, it is possible to enhance biocontrol potential by activating gene clusters through the CRISPR-Cas9 approach. Further, this approach could discover novel aspects of secondary metabolites pathways and also assists in the development of improved strains for better eco-friendly disease management.

11. INSIGHTS GAINED FROM USING CRISPR/Cas9 APPROACH IN PLANT-MICROBE INTERACTIONS

The recently developed CRISPR/Cas9 technology has been so far extensively used in validating the previously identified genes/pathways involved in plant-microbe interactions. Additionally, the CRISPR/Cas9 approach has become one of the novel effective approaches for the control of viral diseases in crops. The differential disease response of novel alleles developed through genome editing in the resistance genes provides an indispensable tool in the hands of molecular breeders for crop improvement. Further, most of the genes studied so far either function at upstream or downstream components of plant-microbe interactions. However, none of the genes/pathways to our knowledge have been comprehensively understood from the initial pathogen interaction up to disease reaction in plants. In this regard, CRISPR/Cas9 based multiplex editing of target genes involved in a pathway has the potential to decipher the complete mechanistic understanding of plant-microbe interactions.

12. FUTURE THRUST

Currently, CRISPR/Cas9 is widely utilized for the development of durable disease resistance in plants. In most cases, the susceptibility gene(s) are edited for imparting durable disease resistance. However, nine different mechanisms were identified for resistance mediated through R genes in plants [118]. Therefore, targeting the genes involved in different pathways of resistance through the CRISPR/Cas9 approach could greatly assist in the development of durable resistance for multiple pathogens in crops. Besides durable resistance, editing of a few genes further resulted in broad-spectrum resistance. The applicability of broad-spectrum resistance in multiple crops will prove to be an interesting area of research in the near future. Additionally, the utilization of CRISPR/Cas9 techniques in disease management through the induction of gene activation will provide novel genetic regulators for the control of plant diseases [119]. Pathogens are recognized by plants extracellularly and/or intracellularly, which was recently highlighted by Van der Burgh and Joosten (2019) [120] as a ‘spatial-immunity model’. Therefore, CRISPR/Cas9 based targeting of host genes localized in different organelles (spatial) involved in regulating colonization will provide multilayered resistance against diseases. There is a recent report on CRISPR/Cas9 based enhancement of nodulation by small fragments of tRNA and the loss-of-function of few genes enhanced nodulation in legumes. Hence, nodulation processes could be better understood through high-throughput editing system using pooled gRNA.
feral crops. However, future farming techniques incorporating climate resilience [122] necessitates the cultivation of minor cereals, vegetables and pulses. In this scenario, diseases are the major limiting factor for the diversification of crops. Thus, utilization of the CRISPR-Cas9 approach for the development of durable disease resistance in minor crops ought to be given high priority, especially considering climate resilience, the profitability of cultivation, food, and nutritional security.

CONCLUSION

The applications of CRISPR/Cas9 in plant biology have exponentially increased in the last five years. The knowledge of plant-microbe interactions and the development of durable resistance in crops is a major research theme for scientists all over the world. The present review summarizes the utilization of genome editing (CRISPR/Cas9) tool targeting –81 genes in plants and phytopathogens for understanding bacterial, fungal and viral disease interactions. Further, genes involved in the nodulation process were also highlighted. However, the development of durable resistance in crops is a highly challenging task. The initial insights gained in the understanding of plant-microbe interaction through the CRISPR/Cas9 approach will greatly assist in developing sustainable disease management strategies in the future.

LIST OF ABBREVIATIONS

| Abbreviation | Definition |
|--------------|------------|
| LOB          | Lateral Organ Boundary |
| SWEET        | Sucrose transporter |
| Xa           | Xanthomonas |
| JAZ          | Jasmonate Zim domain |
| MYB          | Myeloblastosis |
| SEC          | Secretory |
| MPK          | Mitogen Activated Protein Kinase |
| MLO          | Mildew resistance gene |
| LIR          | Large Intergenic Region |
| Rep          | Replicase |
| ORF          | Open Reading Frame |
| Lb           | Leghemoglobin |
| NSP          | Nuclear Shuttle Protein |
| N3           | Bidirectional sucrose transporter |
| DMR          | Downey Mildew Resistance |
| JAZ          | Jasmonate Zim domain |
| MYB          | Myeloblastosis |
| WRKY         | Amino acid motifs |
| SEC          | Secretory |
| ERF          | Ethylene transcription factor |
| LPX          | Lipoxygenase |
| EDR          | Enhanced Disease Resistance |
| CP           | Coat Protein |
| IR           | Intergenic Region |
| eIF          | Eukaryotic Initiation Factor |
| RLKs         | Receptor-Like Kinases |
| HK4          | Histidine Kinase 4 |
| EIN          | Ethylene Insensitive |
| NSP          | Nuclear Shuttle Protein |
| pyrG         | Orotidine-5-phosphate decarboxylase |
| pksA         | Polyketide synthase gene |
| SCD1         | Scythaldehyde dehydratasegene |
| URA5         | Orotate phosphoribosyltransferase, conferring resistance to Hygromycin |
| FUM1         | Fumonisin biosynthesis |
| ORP1         | Oxysterol binding protein-related protein 1 |
| AVR          | Avirulence |
| pks13        | Polyketide synthase gene |
| Rfg1         | Thaumatin-like protein |
| GA20Ox       | Gibberelin Oxidases |

 AUTHORS’ CONTRIBUTIONS

PSR, PC, SS- Overall coordination, manuscript preparation and editing; KU, CB, AS, PP- Literature collection for bacterial and fungal diseases and graphical abstract preparation; BT, NK, SK, MKY- Literature collection for viral diseases and table preparation; PTKJ, AS- Literature collection on symbiotic N fixation and future prospects.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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