CRISPR/Cas9-mediated Immunity in Plants Against Pathogens

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Abstract

Global crop production is highly threatened due to pathogen invasion. The huge quantity of pesticides application, although harmful to the environment and human health, is carried out to prevent the crop losses worldwide, every year. Therefore, understanding the molecular mechanisms of pathogenicity and plant resistance against pathogens is important. The resistance against pathogens is regulated by three important phytohormones, viz. salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). Here we review the possible role of CRISPR technology to understand the plant pathogenicity by mutating genes responsible for pathogen invasion or up-regulating the phytohormones genes or resistant genes. Thus hormone biosynthesis genes, receptor and feeding genes of pathogens could be important targets for modifications using CRISPR/Cas9 following multiplexing tool box strategy in order to edit multiple genes simultaneously to produce super plants. Here we put forward our idea that the genes would be either mutated in case of plant receptor protein targets of pathogens or up-regulation of resistant genes or hormone biosynthesis genes will be better choice for resistance against pathogens.

Introduction

Immunity

Plants must evolve self-defence systems because they are immobile organisms unable to escape from biotic stress factors such as fungi, viruses and nematodes, and abiotic stress factors like a harsh environment condition. In response to pathogen attack, plants respond using two branched innate immune system (Jones and Dangl, 2006). The function of the first branch is more general to perceive and the response results against many groups of microbes consisting of non-pathogens. The second response is more specific to plant pathogen virulence. Thus, plant immune systems and pathogen elicitors make a complex molecular model across biological kingdoms. Pathogens attack plants to retrieve carbohydrate as a source of energy by

penetrating hyphae into plant cell, stylet or through stomata, hydathodes and wounds. First interaction of plant and pathogen establish at the plasma membrane interface to deliver virulence factors into plant cell in order to maintain pathogenicity. In first branch of plant immune system, AMP/pattern-triggered immunity (PTI) and transmembrane pattern recognition receptors (PRRs) respond to elicit microbial or pathogen-associated molecular patterns (MAMPS or PAMPs) (Jones and Dangl, 2006). The second branch of the immune system, effector-triggered immunity (ETI), majorly acts inside the cell using polymorphic NB-LRR protein encoded by R genes (Dangl and Jones, 2001; Marone *et al.*, 2013). The PTI system is a weak and vulnerable immune response system while ETI is associated with strong and robust responses that lead to reactive oxygen species and finally cell death due to hypersensitive response. Typical example of ETI system in which systematic pathway of immune response is triggered upon pathogen attacks (Fig. 5.1). Three plant hormones are associated with plant immune response system, viz. salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). CRISPR/Cas9 has been effectively employed against plant pathogens resistance in plants. Zhou *et al.* (2014) modified a promoter of *OsSWEET* genes for disease resistance, suppression of plant viruses in various plants (Ali *et al.*, 2015a,b; Baltes *et al.*, 2015; Ji *et al.*, 2015) and repression of *MLO* genes target for fungal pathogenicity (Wang *et al.*, 2014). Here we urge on the role of SA, JA and ET defence against pathogens and their regulation. Consequently, CRISPR/Cas9's role in the editing of SA, JA and ET genes mechanism is elaborated in order to explore the target genes which would be of better choice for contributing to plant resistance based on CRISPR/Cas9 system.

SA-, JA- and ET-mediated defence regulation against pathogens

SA is closely associated with immune responses against biotrophic pathogens (Vlot *et al.*, 2009). It has been reported that pathogens like *Ustilagomaydis* Cmu1, *Verticillium dahlia* VdISC1 and *Phytophthorasoja* PsIsc1 inhibited the formation of SA precursors (Djamei *et al.*, 2011; Liu *et al.*, 2014) or the down-regulation of SA signalling pathways (van Damme *et al.*, 2008). Therefore, the detailed binding or suppression of pathogen effectors to SA precursors is essential in order to delete the target binding sites of effectors without interruption of SA biosynthesis by CRISPR/Cas9-mediated genome editing.

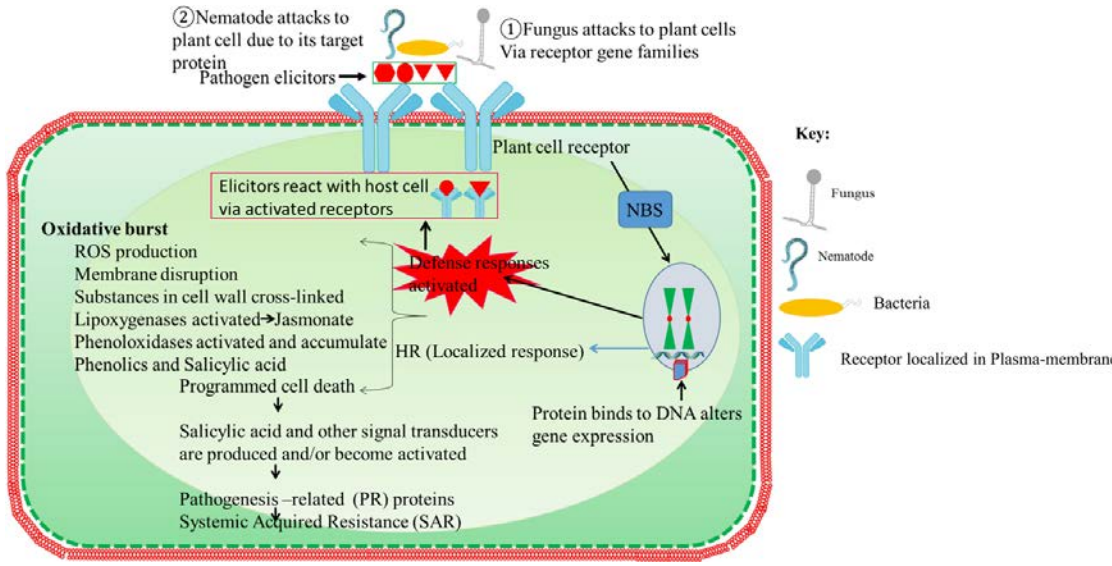

Figure 5.1 Model showing interaction of plant–pathogen at plasma membrane interface of plant cell through the receptors. Series of reactions occurred as a result of pathogen's attack on the plant cell also depicted.

Immunity triggered by JA and its regulation

Jasmonates are plant defence hormones which are lipid-derived molecules and enable plants to adapt under stressful situations due to pathogens and insect invasions. These hormones are produced in response to wounding of tissues and trigger local and long distance defence responses (Koo and Howe, 2009; Larrieu *et al.*, 2015). The defence mechanism triggered due to JA is termed as JA-triggered immunity (JATI). JATI confers broad-spectrum immunity in monocots and dicots. After tissue injury due to pathogens, rapid synthesis of JA and its receptor derivative jasmonoyl-l-isoleucine (JA-Ile) are triggered. Accumulation of JA-Ile in above- and below-ground tissues occurs due to nematode and other pathogens (Fragoso *et al.*, 2014; Grebner *et al.*, 2013; Koo *et al.*, 2009). JA also promotes the proteins and secondary metabolites that have roles in defence including terpenoids, alkaloids and pathogenesis-related proteins (Browse and Howe, 2008; De Geyter *et al.*, 2012; De Vleesschauwer *et al.*, 2013; Gonzales-Vigil *et al.*, 2011). It has been reported in *Arabidopsis* against *Alternaria brassicicola* and *Botrytis cinerea* (Thomma *et al.*, 1998). In addition to *Arabidopsis*, JA-related defence in maize and tomato has also been reported against *Pythium* species (Campos *et al.*, 2014; Staswick *et al.*, 1998; Vijayan *et al.*, 1998; Yan *et al.*, 2012). Constitutive overproductions of JATI regulate the negative effect on plant physiology and development. Since the JA is produced on the expense of limited metabolic resources (Baldwin, 1998; Yan *et al.*, 2007; Zhang and Turner, 2008), the negative feedback mechanism to stop the overproduction or CRISPR/Cas9-mediated pathogen-inducible up-regulation of would be beneficial to lose overburden of plant in terms of consuming metabolites in excess.

Role of ET in plant immunity

When plants are attacked by pathogens, they produce lot of ET (Erb *et al.*, 2012; Lai and Mengiste, 2013; Yang *et al.*, 2013). Few studies showed that the ethylene signalling pathway could contribute to resistance against pathogens (Botanga *et al.*, 2012; Lloyd *et al.*, 2011).

Detailed study of ethylene production due to bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Pst) in *Arabidopsis* was reported by Guan *et al.* (2015). The mutants of 1-aminocyclopropane-1-carboxylate synthase (ACS) defective in ethylene induction were highly susceptible to both Pst and Pst-avrRpt2, indicating a potential role of ethylene mediated resistance against bacterial pathogens (Guan *et al.*, 2015). High order mutants of ACS isoforms of *Arabidopsis* were highly susceptible to *Botrytis cinerea* infection compared with wild type, suggesting potential role of ACS genes in pathogen resistance (Tsuchisaka *et al.*, 2009). Two ACS isoforms ACS2 and ACS6 were studied under challenge of *Botrytis cinerea*-induced ethylene biosynthesis and the protein stability was maintained by two *Arabidopsis* pathogen-responsive mitogen-activated protein kinases (MAPKs) MPK3 and MPK6. Later, the reduced level of ethylene induction in the ACS2 and ACS6 mutants indicated that other ACS isoforms play their role in ethylene induction under *Botrytis cinerea* pathogenicity (Han *et al.*, 2010). Therefore, other isoforms of ACS such as ACS7, ACS8 and ACS11 were identified by genetic approach and these isoforms also contribute to pathogen-related ethylene induction. In addition to phosphorylation and protein stability of ACS2 and ACS6, MPK3 and MPK6 also regulate the expression of ACS2 and ACS6 genes by MPK3/MPK6 substrate and WRKY33 (a member of WRKY transcription factor family). Therefore, these findings compel that regulation of ACS genes at transcriptional and post-transcriptional level induce a high level of ethylene production under the challenge of pathogens (Li *et al.*, 2012a), suggesting that ethylene confers resistance against fungal and bacterial pathogens. The manipulation of above mentioned ACS genes by CRISPR/Cas9 following multiplexing can play key role in plant resistance.

CRISPR/Cas9 role in plant immunity

CRISPR/Cas9 defence mechanism against invasions has been exploited to use in biological disciplines to knock-out genes or enhance the expression of target genes. There are three types of CRISPR/Cas9; however, Type II gains more attention for its utility and versatility for genome editing (Bhaya *et al.*, 2011). In a typical example of CRISPR/Cas9 system, Cas9 enzyme has two

lobes REC and NUC which are activated by sub-genomic RNA (sgRNA) to develop Cas9–sgRNA complex. After complex formation, Cas9 searches for protospacer adjacent motif (PAM). Once PAM is determined, initial DNA destabilization and distortion occurs which leads to DNA cleavage in a systematic order (Belhaj *et al.*, 2015). Then Cas9, a sequence-specific nuclease, creates double-strand breaks (DSB) in the target region of DNA. These DSB are repaired by homology directed repair (HDR) or non-homologous end joining (NHEJ) that is a cell's endogenous repairing system. There are an increasing number of reports showing NHEJ repair in comparison to HDR to repair DSB in CRISPR/Cas9 genome editing. Based on the available literature, this trend has been drawn and presented in Fig. 5.2. With the advancement of transcriptomics of plants invaded by pathogens are available publicly such as NCBI gene expression omnibus (GEO) or at Array express. While utilizing KEGG pathways to understand the disease susceptible and resistant gene families for a particular pathogen under specific conditions, CRISPR/Cas has potential to address the functional characterization of gene families induced under pathogenicity in no time. On the other hand, due to the short life cycle of pathogens, they have evolved resistance against the plant native immune system by silencing host RNA and making it unsuitable for crop resistance (Pumplin and Voinnet, 2013). In order to overcome this bottleneck, a more robust strategy is to utilize gene targeting (GT), which permits disruption of endogenous or addition of *in vitro* manipulated copy of gene. Although this GT technology was a powerful tool to edit genes in plants for plant protection, it was limited due to its rare double-strand breaks (DSBs). However, this potential barrier has been overcome by the development of TALENS and CRISPR/Cas9 system by utilization of novel endonucleases (Puchta and Fauser, 2013).

Up-regulation of target genes by CRISPR (dCas9-VP64)

Transcriptional activation mediated by CRISPR was reported in humans at a genome-wide scale in a multiplex gene fashion (Konermann *et al.*, 2014). This was based on deactivated Cas9 (dCas9) mediated transcriptional system to enhance the target genes expression. The first tool box in plants was transcriptional activator VP64 fused with dCas9 that was reported to enhance the multiple endogenous genes expression in *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*) and tobacco (*Nicotiana benthamiana*) (Lowder *et al.*, 2015). In this study, it was suggested that the modification in promoter regions of endogenous genes with fused complex of dCas9-VP64 could enhance the gene expression in transient or stable transformation. The expression of the genes could enhance 2- to 7-fold in the case of AtPAP1 and 3- to 7.5-fold of miR319 as compared to control in *Arabidopsis*. The GUS reporter gene expression mediated by dCas9-VP64 enhanced remarkably compared to control. Thus this study strongly suggests the successful activation of transcriptional regulation of the endogenous genes by

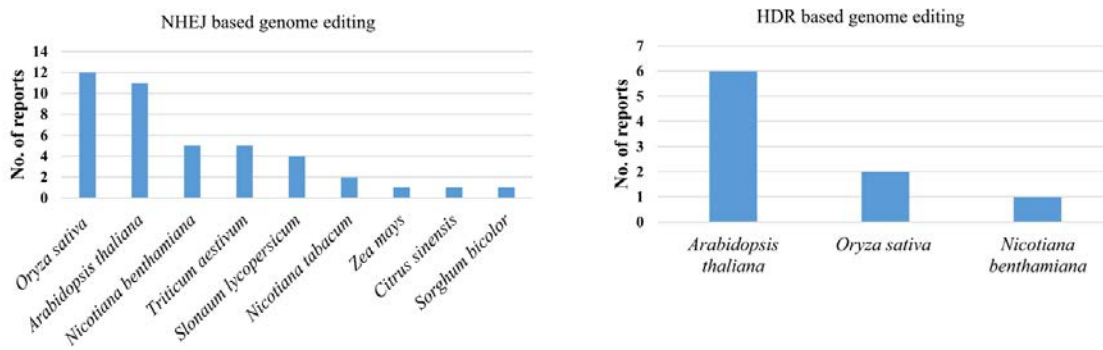

Figure 5.2 Frequency of genome editing based on NHEJ and HDR repair mechanism in plant species. The data are presented on the basis of number of available reports in literature regarding NHEJ and HDR repair in different plant species.

dCas9-VP64 system. Therefore, it will be feasible to adopt this up-regulation of transcriptional system in order to enhance the expression level of target genes related to pathogens resistance.

Multiplexing of genome editing

The editing of multiple genes is of great interest in plant immune systems since several gene family members could be contributing to pathogenicity responses. Multiplex gene editing in plants was reported by Li *et al.* (2013) in *Arabidopsis* and tobacco by editing *AtPDS3* or *NbPDS* target sgRNA by a single binary plasmid containing pcoCas9 in order to suppress carotenoid biosynthesis and chlorophyll oxidation with expected photobleached phenotype.

Li *et al.* (2012b) demonstrated TALEN based editing of *OsSWEET14* gene which is target of rice bacterial blight susceptibility. This gene encodes SWEET efflux transporter and manipulated by *X. oryzae* pv. *oryzae* while using TAL effectors AvrXa7 or PthXo3 to induce the gene expression and leading to stealing sugar from the plant cell in order to feed the pathogen for its growth. The successful editing of *OsSWEET14* by TALEN could produce disease resistant rice plants. This group also demonstrated the successful site-specific mutation in the promoter regions of disease susceptible genes *SWEET11* and *SWEET14* (Li *et al.*, 2012b) based on CRISPR/Cas9 system (Jiang *et al.*, 2013; Zhou *et al.*, 2014) where four SWEET transporters *OsSWEET1a*, *OsSWEET1b*, *OsSWEET11*, *OsSWEET13* were mutated successfully. The simultaneous editing of same gene family members was edited at higher efficiency (87–100%). In this study merely establishment of efficient multiplexing editing target for disease susceptible target genes. However, no pathogenicity test was performed to show the efficacy at plant pathogen interaction. The multiple sgRNA members of a gene family expressed by U3 and U6 small nuclear RNA promoters from rice: OsU3, OsU6b, OsU6c (Shan *et al.*, 2013a; Xie *et al.*, 2015; Zhou *et al.*, 2014), and OsU6a (Ma *et al.*, 2015). Therefore, multiplexing would facilitate in editing targets for several pathogens simultaneously by targeting multiple genes which could serve as target of pathogenicity.

Resistance against fungal pathogenicity

Botrytis cinerea (grey mould) is an important fruit rot fungus which reduces shelf life of fleshy fruits and berries and also causes leaf blight. Five resistance and two susceptible quantitative trait loci (QTLs) for grey mould were identified in wild tomato (Davis *et al.*, 2009). Three grey mould resistance locus (GML) and sensitive QTL shave been reported for tomato. Inactivation of sensitive gene or transcriptional activation of resistant gene would reduce the grey mould pathogenicity and ensure the crop safety thus increasing the yield potential and reduction of post-harvest losses. SWEET effluxers are target for pathogens/symbionts to acquire energy as a source of carbohydrate (Li *et al.*, 2012b; Sameeullah *et al.*, 2016). Modification in the promoter region of *OsSWEET* could enhance disease resistance (Zhou *et al.*, 2014). Powdery mildew devastates crop of cereals worldwide every year. The target of powdery mildew in plant cells is the MLO gene family which was first discovered in barley (Büschges *et al.*, 1997). Later on the homologues were identified in other plant species as well. Recently, CRISPR/Cas9 based edited MLO genes in wheat (*Triticum aestivum*) were reported to knock-out the target of powdery mildew in order to create resistant wheat (Wang *et al.*, 2014). The effector Avr4/6 of an oomycete pathogen *Phytophthora sojae*, causing root and stem rot of soybean, was effectively knocked out revealed the corresponding R gene loci RPS4 and RPS6 (Fang and Tyler, 2016). Ethylene response factor (ERF) gene *OsERF922* which is actively induced by multiple stress factors or plant hormones such as salt and rice blast causal agent pushovers of *Magnaporthe oryzae* and ABA. Enhanced resistance against *M. oryzae* was observed in RNAi based knockdown rice plants. On the other hand overexpression of the *OsERF922* gene made plants vulnerable and susceptible to *M. oryzae* (Liu *et al.*, 2012). Recently, Wang *et al.* (2016) demonstrated targeted mutagenesis in *OsERF922* by targeting multiple sites while using Cas9-multi-target-sgRNAs (C-ERF922S1S2 and C-ERF922S1S2S3) in order to obtain plants containing mutations at multiple sites. Edited rice plants showed a decreased level of pathogenicity compared to wild type at seedling and tillering stages. Moreover, there was no significant

difference among agronomic traits of mutant lines and wild type. Therefore, CRISPR/Cas9 presents a sustainable approach in plant protection against pathogens without disturbing genetic yield potential of commercial cultivars. This powerful tool can be applied to other high yielding elite cultivars of crop plants which has been obsolete due to invasion of pathogens. In this context there is an example of cotton cultivar NIAB-78 which had high yielding potential but was superseded due to cotton leaf curl virus disease (CLCuD).

Resistance against nematode infestation

Nematodes have feeding sites in plant cells to infect the plant cell to get energy. Inactivation of genes that are induced during nematode infestation would reduce the nematode infestation. Genes which are induced upon nematode infestation have been reported in many plants (Gheysen and Fenoll, 2002; Kyndt *et al.*, 2012). The power of CRISPR/Cas9 based genome editing would be a possible solution to repress the induced genes by following multiplexing tool box (Lowder *et al.*, 2015). Recently, novel target genes have been identified that could play role in establishment of parasitism between root knot nematode and the plant, especially plant nutrient transporters (Kyndt *et al.*, 2012). Inactivation of those genes by RNAi significantly reduced the degree of parasitism (Danchin *et al.*, 2013). Thus, CRISPR/Cas9 can be a promising strategy to inactivate multiple target genes simultaneously and observe the level of parasitism in host plants in no time.

Resistance against plant viruses

Plant viruses are the notorious pathogens of the plant kingdom. CRISPR/Cas9 has been effectively exploited to reduce the virulence of viruses in different crop plants against geminiviruses (Chaparro-Garcia *et al.*, 2015). sgRNA of tomato yellow leaf curl virus (TYLCV) were introduced into *Nicotiana benthamiana* that could effectively reduce the viral infection (Ali *et al.*, 2015b). Bean yellow dwarf virus (BeYDV) genome was edited by CRISPR/Cas9 and effectively reduced the copy number of the virus, thus reducing the infestation rate and conferred tolerance against virus (Baltes *et al.*, 2015). Ji *et al.* (2015) demonstrated induction of mutation and inhibition of beet severe curly top virus (BSCTV) by introducing sgRNA into wild tobacco. Their findings showed that overexpression in *Arabidopsis* and *N. benthamiana* conferred high resistance to virus infection.

Conclusion

Plant immunity is a rapidly growing subject to understand plant–pathogen interactions at a molecular level by genomic, transcriptomic, proteomic and metabolomic approaches. However, little is known so far about jasmonic acid and ethylene hormone impact in plant immunity. CRISPR/Cas9 tool is a hotcake tool for genome editing in crop plants for various traits of interest. CRISPR/Cas9 tool can knock-out or up-regulate the expression of target genes to confer tolerance and resistance against pathogens. In the latter case, manipulation of promoter of target genes is required. The multifaceted of CRISPR/Cas9 to manipulate plant resistant genes or susceptible genes to intervene resistance against multiple pathogens. A model is shown in (Fig. 5.3). The use of this highly precise tool can increase protection of crops. Additionally, post-harvest losses may also be reduced due to decrease in pathogens invasions especially in fleshy fruits. The application of this technology will not only boost crop yield but also ensure the acceptance of commodity to the consumer end.

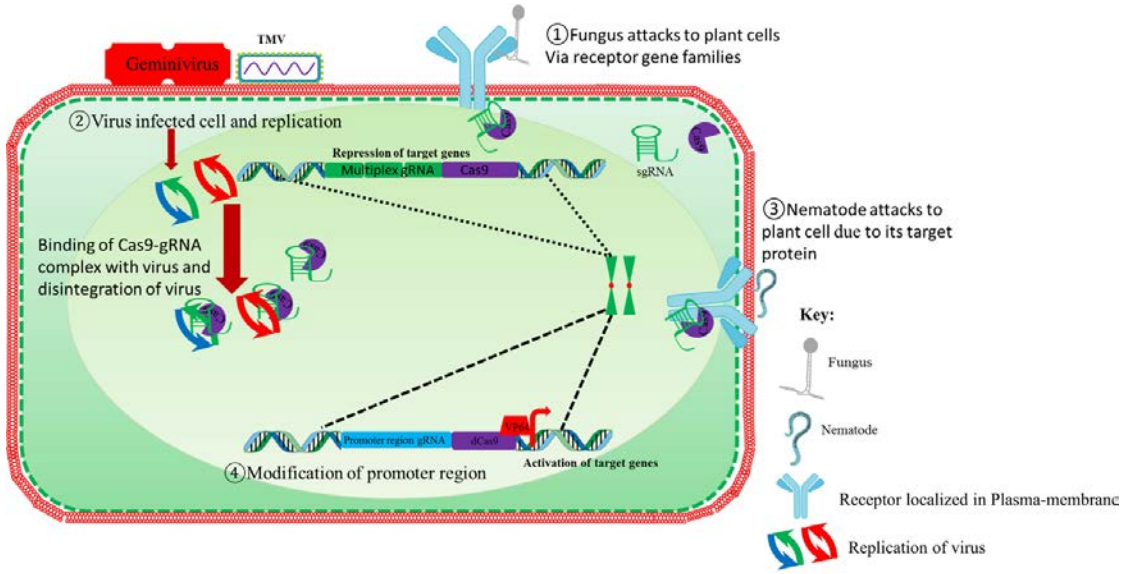

Figure 5.3 Model to show the repression or activation of sensitive or resistant genes in plant cell to confer resistance against the fungi, virus and nematode pathogens. Following multiplexing strategy of CRISPR/Cas9, genome editing would inactivate the targets of pathogens in a plant cell. Transcriptional activation of target gene by modification in promoter region mediated by dCas9 would enhance the expression level of resistant genes that will confer resistance against pathogens. (1) Shows the fungal interaction with plant receptors which are targets for fungal pathogenicity into plant cell. Inhibition of the target receptors by CRISPR/Cas9 localized at the plasma-membrane would induce plant resistance against the pathogens. (2) Attack of geminivirus or tobacco mosaic virus (TMV) into plant cell and its replication. Binding of target sgRNA to plant genome disintegrates the viral genome and thus induces the resistance against viruses in plant cell. (3) Nematode infection at plant cell interface induced plant nutrient transporters localized at the plasma membrane and thus facilitates transport of nutrients to nematode. Thus, targeting those nutrient transporters by CRISPR/Cas9 multiplexing tool to silence the target transporters would facilitate resistance against nematode infection. (4) Modification in promoter regions of target genes which on mutation could affect plant physiological development therefore, over-expression by modification promoter region mediated by CRISPR/Cas9 system contributes to plant resistance against pathogens. In this context promotion of genes like SA, JA and ET biosynthesis would be target to enhance expression in order to confer resistance against pathogens.

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