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CRISPR/cas9 gene editing tool for diseases resistant varieties

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Abstract
Plant pathogens, such as bacteria, fungi, and viruses infect plants, causing large financial losses and threatening sustainability of agriculture. The complexities of the plant immune system have been summarised, as well as the use of CRISPR-Cas9 to alter the various components of the plant immune system in order to obtain long-lasting resistance in plants against plant pathogens. CRISPR/Cas technology has proven to be one of the most effective genome editing method in all genome editing tools in modeling plants and crops against diseases. The recent breakthroughs in plant protection employing CRISPR/Cas9 technology in model plants and commodities in response to viral, fungal, and bacterial pathogens are the topic of this review. Here, we are giving an overview of history and different CRISPR/Cas9 systems, the derived methods for designing in plant pathology.

Keywords: CRISPR/cas9, gene editing tool, plant pathogens

Introduction
Crops are vulnerable to a wide range of infections, including fungus, bacteria, and viruses, all of which inflict significant economic losses. Disease control methods that relay on resistant cultivars and agrochemicals are typically extremely successful when it is used but agrochemicals have a negative impact on the environment. Plant breeding has shown to be the most effective method for producing disease-resistant new crop cultivars. Disease resistant cultivars in modern agriculture is mostly accomplished through cross breeding, mutation breeding, and transgenic breeding. Cross breeding takes 8-10 years to introduce desirable alleles, mutation breeding takes 8- 10 years to increase genetic diversity by introducing random mutations through the use of chemical mutagens or physical irradiation as well as transgenic breeding which takes 8-12 years to generates desirable features by transferring foreign genes into elite background varieties, has the potential to alleviate the barrier of reproductive isolation [1, 2]. Genome editing takes 4-6 years to improve a trait by precisely modifying the target genes or regulatory sequences or rearranging chromosomes. While in gene editing, precisely using clustered recurrently interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) and its variants, has become a important method in disease resistant cultivars and has the potential to revolutionize plant breeding. The discovery of mega-nucleases, sequence-specific engineered endonucleases, transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and type II clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) has laid the way for targeted gene editing in plant genomes [3]. Among them CRISPR/Cas technology have shown to be effective genome editing techniques in model plants and crops. On the one hand, CRISPR/Cas or techniques developed from this technology have made it possible to quickly and easily determine the function of various non-coding and coding DNA sequences in typical plants [4, 5].

Pathogen-defense mechanism in plants
Plants have evolved a multilayered defense system that includes both pathogen associated molecular patterns (PAMPs), effector-triggered immunity (ETI) and triggered immunity (PTI) to battle invading pathogens. PTI is activated quickly in general when PAMPs are recognized by pattern recognition receptors (PRRs). The invasion of non-adapted pathogens is usually prevented by this basal resistance response, which includes the production of reactive oxygen species (ROS), callose deposition and transcriptional reprogramming.

Pathogens produce effectors to disrupt PTI and change host cell physiology, resulting in effector-triggered vulnerability (ETS). In resistant plants, effectors or their by products can be detected by intracellular immune receptors, resulting in ETI, which is usually linked with localized plant cell death and pathogen arrest. Through the change of meta-effector interactions as well as ETI-eliciting effectors, several pathogens can overcome the host's ETI response [6]. As a result, the disease and the host engage in a never-ending arms competition. We still only have a limited understanding of the molecular mechanisms of host-pathogen interactions, after decades of research. As a result, more comprehensive studies of host-pathogen interactions, particularly the identification of important targets relevant to plant defense responses, would present a significant opportunity to design broad-spectrum and long-lasting resistance in a variety of crops. The newly developed CRISPR/Cas system, which allows for precise DNA alteration of the genome, has revolutionized plant study [7]. Because of its simplicity and efficiency, this technique has become a viable alternative to ZFNs and TALENs for genome engineering in a variety of eukaryotes. CRISPR-based technologies have been used in a various plant-pathogen interaction studies to date, including host reactions to fungus, bacteria, viruses, and other pathogens.

History of CRISPR/CAS 9

CRISPRs were discovered in E. coli in 1987 by Yoshizumi Ishino and his colleagues, who accidently cloned a strange pattern of repetitive sequences interspersed with spacer sequences while investigating a gene responsible for alkaline phosphatase conversion [8]. CRISPR systems were first assumed to be a unique DNA repair mechanism in thermophilic archaea and bacteria. In the early 2000s, Mojica and colleagues have found that the spacer sequences were similar to those observed in viruses, bacteriophages, and plasmids. They observed that viruses cannot infect bacteria that have homologous spacer sequences, implying that these sequences play a function in prokaryotic adaptive immune systems [9]. When a virus infects a prokaryote, the spacer sequences in CRISPR arrays are transcribed to form short CRISPR RNA (crRNA), which directs the Cas protein to break complementary DNA or RNA [10].

Methods for designing disease-resistant plants using CRISPR technology

1) Indels in coding sequences position to create disruption

The CRISPR-Cas9 technology is most commonly used for this purpose. It takes use of the cellular Non-homologous End Joining (NHEJ) DNA-repair machinery's error-prone tendency. As a result, one or more nucleotides are inserted or deleted (indel) at the sgRNA-guided location, causing a frameshift mutation and disrupting gene expression [11]. This method has been used to introduce desirable features into a variety of crops, including vital grains like rice and wheat [12]. This technology has been used to design disease resistance by altering a plant susceptibility (S) gene, which affects the plant-pathogen interaction, resulting in decreased pathogen fitness on the host plant [13]. By using of CRISPR-Cas9 to introduce indels affecting eukaryotic translation initiation factor 4E proteins (eIF4Es), which effectively produced resistance against several RNA viruses in Arabidopsis and cucumber, is a notable example of this use (*Cucumis sativus*).

2) Indels in promoter regions induces gene disruption

A similar strategy can be used to introduce indels in the promoter region of a plant gene rather than the coding area. CRISPR-mediated promoter editing can be used in two ways: to disrupt the promoter sequence in order to completely inhibit gene production, or to disrupt an effector-binding site in order to disrupt plant susceptibility by preventing a pathogen effector from binding to the promoter. The second technique was used to change the promoter of a rice sugar transporter gene OsSWEET14, breaking the link with a bacterial blight pathogen's effector and causing in blight resistance [14, 15].

3) Multiplex sgRNAs for gene deletion

Multiple sgRNAs can be applied in CRISPR systems to induce several DSBs at specified sites in the target genome. For example, two sgRNAs binding before and after the start and finish codons of the gene of interest will result in DSBs at the appropriate positions. These DSBs cause the loss of the DNA segment carrying the gene of interest before being repaired by the cellular repair NHEJ pathway. Because sgRNAs can be created at any genomic position that contains an acceptable PAM trinucleotide sequence, this approach can and has been used to delete both large and small amounts of DNA. chromosomal fragments and individual genes [16]. This is aided further by the creation of a rationally designed Cas9, SpCas9-NG, which can detect NG PAMs, a more relaxed stringency than the normal NGG PAMs [17]. Gene cluster is very useful in the context of engineered disease resistance, particularly in S gene clusters, where several S genes are distributed along the same chromosome [18]. The deletion of the chromosomal fragment will very certainly result in long-term resistance to the target pathogen.

4) Insertion of a gene by homology-directed repair

The aforementioned CRISPR approaches can be utilized to create disease resistance by modifying the S gene (s). However, all plant proteins, including the products of S genes, are essential and, for the most part, multifunctional; altering these proteins consequently has ramifications for plant health and/or productivity. There are alternate techniques to gene disruption, such as the previously stated cis-regulatory element and promoter editing, however it is often required to deploy resistance (R) genes against pathogens in circumstances where the plant-pathogen relationship is poorly understood and the S genes have not been thoroughly examined. In those kind of cases, the CRISPR toolkit can be used to insert the R gene. CRISPR-mediated gene insertion occurs via a different path that begins after Cas9 has established the sgRNA-directed DSB, This approach makes use of the cellular HDR machinery rather than the NHEJ mechanism. Cas9 and the sgRNAs are added to a delivery fragment that contains a R gene surrounded by sequence corresponding to the DSB ends. . This method has been used to insert one or more genes at specific genomic regions [19]. However, HDR efficiency in plants is really modest [20]. Although new ways to address this are being developed, this currently renders gene insertion in plants difficult to apply [21]. K. M. Veley *et al*. employed CRISPR-mediated homology-directed repair to tag genes in cassava [22].

Crispr/Cas9 techniques for biotic stress

Biotic stresses, such as fungal, viral, and bacterial infections, account for 20% to 40% of global agricultural productivity losses. [23]. Giving host plants pathogen resistance can decrease the incidence of disease on crop development and

productivity, tackling the problem of feeding the world's rising population. CRISPR/Cas9 technologies have mostly been used to combat viral infection, with efforts to increase fungal and bacterial disease resistance following.

Plant viral resistance mediated by CRISPR

Viruses are difficult to control, and their growth and spread are faster than any other infection due to the presence of insect vectors. In recent decades, significant attempts were undertaken to attack viruses using pathogen-derived resistance mechanisms. Nowadays, genetic engineering tools give a new weapon in the arsenal against plant viruses that utilize both DNA and RNA. The CRISPR/Cas9-mediated gene-editing technology has advanced quickly and has been utilized to investigate effective methods for creating viral resistance. Virus resistance can be achieved by either (23) targeting host components involved in virus replication or (24) targeting and destroying the viral genome directly, ultimately stopping virus replication. The majority of viral resistance experiments employing CRISPR-edited plants have focused on geminivirus (a group of single-stranded circular DNA viruses) genomes [25, 26, 27]. Geminiviridae is a major family of plant viruses that cause agricultural losses globally in numerous significant groups, including Cucurbitaceae, Euphorbiaceae, Solanaceae, Malvaceae, and Fabaceae. The first two studies focused on resistance to Beet Severe Curly Top Virus (BSCTV), geminiviruses and Bean Yellow Dwarf Virus (BeYDV), in model plants N. benthamiana and Arabidopsis [4, 5]. Ji and colleagues (2015) analyzed 43 potential sgRNA/Cas9 target sites in the BSCTV genome's coding and non-coding sections. Although all of the sgRNA/Cas9 constructions decreased viral accumulation in inoculated leaves to varied degrees, increased resistance to virus infection was found in *Nicotiana* and *Arabidopsis* plants with the higest levels of sgRNA and Cas9 expression. Parallel results were stated by Baltes *et al*. (2015), who found a reduction in targeted viral load of up to 87 percent in *N. benthamiana*, using, hairpins, Rep-binding sites, non anucleotide sequence of BeYDV and the 11 sgRNAs targeting Rep motifs.

RNA virus protection has seemed to be more difficult to implement, SpCas9 from *Streptococcus* pyogenes only recognizes dsDNA, therefore this is a problem. However, the identification of enzymes that can bind to and cut RNA, such as FnCas9 from *Francisella novicida* or LwaCas13 from *Leptotrichia wadei*, has resulted from the search for and characterization of similar nucleases. A first report showing resistance to RNA viruses in *N. benthamiana* and Arabidopsis plants using FnCas9 and RNA-targeting sgRNAs specific for cucumber mosaic virus (CMV) and tobacco mosaic virus (TMV) [27]. When compared to control plants, CMV and TMV accumulation was reduced by 40–80% in transgenic plants.

Table 1: Major uses of CRISPR/Cas9 technology in plants for viral resistance

Crop	Target gene	Pathogen	Results	Reference
Rice	elF4G	Rice tungro spherical virus (RTSV)	Resistance to viral infection	28
Nicotiana benthamiana and *Arabidopsis thalaiana*	CP, virus DNA-Rep and IR	Beet severe curly top virus (BSCTV)	Resistant to Gemini virus	29
Tobacco	IR and C1	Cotton leaf curl Multan virus (CLCuMuV)	Resistance to virus	30
Arabidopsis	Elf(iso)4E	Turnip mosaic virus (TuMV)	Potyvirus resistant plants	31
Tomato	CP and Rep	Tomato yellow leaf curl virus (TYLCV)	Transgenic plants achieved with reduced symptoms	32
Cucumber	elF4E	Cucumber vein yellowing virus (CVYV), Zuccbini mosaic virus (ZYMV), Papaya ring spot virus (PRSV)	Improved resistance to CVYV, ZYMZ, PRSV	33
Cassava	nCBP-1 and nCBP-2	Cassava brown streak virus (CBSV)	Reduced symptoms in field trials	34
N. benthamiana	Virus DNA, Rep A/Rep and LIR	Bean yellow dwarf virus (BeYDV)	Indels in virus DNA and resistance to Gemini virus	35
Banana	Virus sequences in plantain genome	Endogenous banana streak virus (eBSV)	Asymptomatic plants about 75%	36
Tomato	DCL2	Potato virus X and Tobacco mosaic virus (TMV)	Injected with targeted virus, mutants show viral symptoms	37,38
N. benthamiana	CP, Rep and IR	Cotton leaf curl Kokhran Virus (CLCuKoV), Merremia mosaic virus (MeMV), Tomato yellow leaf curl virus (TYLCV), Beet curly top virus (BCTV) and Tomato yellow leaf curl Sardinia virus (TYLCSV)	Symptomatic plant and no resistance	39
Arabidopsis, N. benthamiana	Virus RNA genome	Cucumber mosaic virus (CMV), Tobacco mosaic virus (TMV)	Tested crops are resistant	40

Plant fungal resistance mediated by CRISPR

Based on current knowledge of molecular pathways implicated in plant-pathogen interaction, several techniques have been developed to improve fungal resistance in plant species. Potential candidate genes and gene products implicated in plant resistance to fungus have been identified, and they are now viable targets for CRISPR/Cas9 editing. As past studies include MLO loci have been targeted by RNA-guided Cas9 endonuclease in three different plant species: tomato, grapevine and bread wheat [41, 42, 43]**.** MLO encodes a protein with seven transmembrane domains that are found in the plasma membrane and is found in all monocots and dicots [44]. MLO were previously identified as susceptibility (S) genes, with homozygous loss-of-function mutants greatly increasing resistance to powdery mildew in barley, Arabidopsis and tomato [45, 46, 47]. In Rice OsERF922, OsSEC3A was regulated with CRISPR-Cas9, resulting in total resistance to the blast disease *M. oryzae* without disrupting the plant's normal growth pattern [48]. Inhibitors of cell wall-degrading enzymes or genes involved in callose

deposition can be an effective target for GE to create fungus resistance. The Powdery Mildew Resistance 4 (PMR4) gene ortholog SlPMR4, which is involved in callose deposition (PRR gene), was genetically engineered using CRISPR-Cas9 to increase resistance to the powdery mildew disease Oidium neolycopersici [49]. CRISPR-mediated targeting of the Powdery mildew resistance 4 (Pmr4) S gene, whose resistance mechanism is unknown, has recently been shown to significantly reduce powdery mildew disease symptoms in tomato [50]. Zhang *et al*. found that CRISPR-mediated disruption of the gene encoding tomato mitogen-activated protein kinase 3 (SlMAPK3), which controls the generation of reactive oxygen species (ROS), resulted in resistance to B. cinerea in tomato plants [51]. The CRISPR-Cas9 technique was utilised to create a papaya plant mutant for a functional cysteine protease inhibitor (PpalEPIC8), which resulted in improved resistance to *P. palmivora* [52].

Table 2: Major uses of CRISPR/Cas9 technology in plants for fungal resistance

Crops	Target genes	pathogen	Results	Reference
Rice	Pi21/cds region	*Magnaporthe oryzae*	Increased disease resistance	53
Rice	OsMPK5	*Magnaporthe grisea*	Improved diseased resistance	54
Wheat	MLO-7	*Blumeria graminis f.sp tritici*	High tolerance to disease	55
Wheat	TaEDR 1	*Blumeria graminis f.sp. tritici*	Enhanced disease resistance	56
Soyabean	Avr 4/6	*Phytophthora sojae*	Enhanced disease resistance	57
Grapes	MLO-7	*Uncinula necator*	RNP based system to Enhanced resistance to disease	58
Rape seed	Hk	*Leptosphaeria maculans*	Enhanced disease resistance	59
Tomato	SlMlo1	*Oidium neolycopersici*	Enhanced disease resistance	60
Tomato	Multiple gRNA at Pmr4	*Oidium neolycopersici*	Mildew symptoms ae reduced significantly	61
Banana	RGA2, Ced9	*Fusarium oxysporum f.sp. cubense*	Observed disease reduction	62
Papaya	alEPIC8	*Phytophthora palmivora*	Enhanced disease resistance	63
Cocoa	NPR3	*Phytophthora tropicails*	Improved resistance against *Phytophthora tropicails*	64
Maize	bW2, bE1	*Ustilago maydis*	Tested the pathogen vilencce	65

Plant bacterial resistance mediated by CRISPR

In comparison to viral and fungal resistance, there are few reports on the use of CRISPR/Cas9 to treat bacterial infections in crops. Type III transcription-activator-like effectors (TALEs) are used by the proteobacteria *Xanthomonas oryzae* pv. *oryzae* to promote host gene expression, resulting in host susceptibility. OsSWEET13, a sucrose transporter gene, has been identified as a susceptibility gene for the PthXo2 effector protein of X. *oryzae* pv. *oryzae*. The OsSWEET13 allele was transferred from indica rice IR24 to japonica rice. Kitaake conferred disease susceptibility, but CRISPR/Cas9 alterations in the allele provided resistance to bacterial blight, on the other hand, mutations in the allele, are caused by resistance to bacterial blight was imparted by CRISPR/Cas9 [66].

Tomato bacterial speck disease is caused by *Pseudomonas syringae* pv. *tomato* (Pto). It generates coronatine (COR), which causes stomatal opening and bacterial invasion. This stomatal response to COR in Arabidopsis is reliant on the COR co-receptor AtJAZ2 (Jasmonate ZIM-domain-2). The shortened version of JAZ2 (JAZ21jas) that lacks the C-terminal Jas domain prevents stomatal opening by COR [67]. The tomato ortholog of AtJAZ2 (SlJAZ2) was targeted by CRISPR/Cas9 to develop the dominant JAZ2 repressor SlJAZ21jas, which blocked COR-induced stomatal opening and gave resistance to the biotrophic bacteria Pto [68].

Citrus canker is one of the most commercially important bacterial illnesses for which CRISPR gives a resistance option. This disease, caused by *Xanthomonas citri* subsp. *citri*, is one of the most damaging citrus diseases, causing yield losses in citrus-growing countries across the world. Two recent studies revealed the use of CRISPR/Cas9 to create citrus plants resistant to citrus bacterial canker (CBC). Jia et colleagues created canker resistant mutants in Duncan grapefruit by altering the PthA4 effector binding sites in the promoter of the Lateral Organ Boundaries 1 (CsLOB1) gene. Mutated lines demonstrated a reduction in typical canker symptoms 4 days after Xcc inoculation, but no other phenotypic changes were observed. Furthermore, PCR-sequencing revealed no probable off-target alterations in other LOB family genes [69]. Peng *et al*. (2017) established a relationship between CsLOB1 promoter activity and susceptibility to CBC disease in Wanjincheng orange (*Citrus sinensis*). The full deletion of the EBEPthA4 sequence from both CsLOB1 alleles resulted in increased resistance to CBC [70]. Furthermore, no changes in plant growth were seen following CsLOB1 promoter modification.

The enterobacterium *Erwinia amylovora* causes fire blight disease in apples and other Rosaceae plants. The pathogenicity effector (DspE) of E. amylovora interacts with four leucine-rich-repeat, receptor like serine/theonine kinases generated by the DspE-interacting proteins of Malus (DIPM) genes- DIPM 1, 2, 3, 4 [71]. Malnoy *et al*. used the CRISPR/Cas9 system to target DIPM 1, 2, and 4 genes in apple protoplasts in order to build resistance to fire blight disease [72]. Malnoy *et al*. (2016) report efficient direct delivery of CRISPR/Cas9 ribonucleoproteins (RNPs) (preassembled sgRNA/Cas9 complex) into plant protoplasts, which offers various advantages such as quick targeting efficiency, better ontarget and reduced off-target activity [72, 73].

Table 1: Major uses of CRISPR/Cas9 technology in plants for bacterial resistance

Crops	Target genes	Pathogen	Results	Reference
Rice	OsSWEETT11, OsSWEETT13 and OsSWEETT14	*Xanthomonas oryzae* pv. Oryzae	Enhanced broad spectrum disease resistance	74
Rice	OsSWEET13	*Xanthomonas oryzae* pv. Oryzae	No sign of resistance	75
Rice	OsMPK5	*Burkholderia glumae*	Resistance not detected	76
Apple	DIPM-1, DIPM-2, and DIPM-4	*Erwinia amylovora*	No sign of resistance	77
Pomelo	CsLOB1	*Xanthomonas citri* subsp. Citri	Increased resistance of disease	78
Wanjincheng orange	CsLOB1	*Xathomonas citri* subsp. Citri	Disease severity reduced (83.2-98.3%)	79
Tomato	SIDMR6-1	*Pswudomonas syringae*, *Phytoophthora capsica* and *Xanthomonas* spp.	Increased broad spectrum disease resistance	80
Tomato	Jaz2	*Pseudomonas syringae* pv. Tomato	Resistant to bacterial speck disease	81

Conclusion

CRISPR/Cas has been developed and tested in a wide range of host plants and pathogens in order to better understand the molecular mechanisms underlying plant-pathogen interactions and to improve host resistance to bacteria, fungi, and viruses. This system is useful for making gene loss-of-function and gain-of-function mutants, as well as understanding plant pathogen interactions and decreasing the harm caused by destructive pathogens in agricultural settings. New breeding techniques allow scientists to implant desired features more precisely and quickly than traditional breeding. CRISPR/Cas9-based genome editing is a game-changing technology. In the future, crop development using genome editing methods to boost yield, disease resistance, and other qualities will be a major focus. It has been used extensively in various plant systems for functional investigations, mitigating biotic and abiotic stressors, and improving other essential agricultural features in the last five years. Though various changes to this technology are needed to improve on-target efficiency, the majority of the work done so far is basic and needs to be improved.

Future prospects

Crop protection through genetic modification offers a promising option with no clear impact on human health or the environment in an era characterized by political and societal pressure to limit the use of pesticides. Many research have shown that the CRISPR-Cas9 system can be used to create disease-resistant transgenic plants. The viral load on these transgenic plants was very low, and they remained stable until the third generation. However, the use of such constantly expressing nuclease crops falls within the category of genetically modified organisms (GMOs), which are subject to GMO regulatory regulations, are not widely accepted in some countries, and have a significant risk of off-target mutation. As a result, transgene-free processes that can simultaneously employ many pathogen effectors and resistance genes, as well as grow foreign crops that are not subject to GMO restrictions, are attractive. CRISPR was recently exempted from the definition of a GMO under regulatory frameworks in the United States and Canada, allowing its cultivation and sale without a GMO label. CRISPR-edited crops that have already been developed and are seeking regulatory approval may also gain acceptance, allowing for the development of new crops with more desirable traits such as increased yield and medicinal capabilities (in the form of an edible vaccine). CRISPR-Cas9 technology is already speeding every field of study and has the potential to become a source of sustainable farming. In the years ahead, we expect its vast uses in plant and microbiological research will considerably increase our understanding of both fundamental biology and disease resistance. CRISPR/Cas-based techniques can be used to knock out single or several genes. Furthermore, CRISPR/Cas-based technologies may be utilised to generate elevated mutant libraries, allowing for faster gene function research on conferring resistance and pathogen pathogenesis. SNPs and quantitative trait loci (QTL), which are common sources of genetic variation among crop species individuals, are responsible for a variety of pleiotropic phenotypes, including crop resistance. Important SNP-typed QTLs and SNPs have been identified in association with a variety of resistance and immunity genes in numerous crops, including Pi-d2, bsr-k1, Xa4, CsSGR, and others. CRISPR/Cas9-mediated prime editors and base editors can be used in cash crops to rapidly accomplish precise genome editing of SNPs and SNP-typed QTLs, conferring multiple disease resistance. The base-editing-mediated gene evolution (BEMGE) approach was recently developed (82). To conclude that CRISPR/Cas system offer a fresh way to investigate the complicated field of plant-pathogen interactions. We expect CRISPR technologies to play a significant role in decoding the interaction between plant and pathogen and developing durable and broad-spectrum disease resistant plants in the future, given the constant changes in agricultural production activities and plant disease systems.

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