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Harnessing CRISPR/Cas9 system to engineer disease resistance in solanaceous crops: Current progress and future prospects

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Abstract: Crops belonging to the *Solanaceae* family, including potato, tomato, pepper, and tobacco possess considerable economic importance worldwide. However, their production is continuously under threat from plant pathogens. Farmers typically rely on resistant cultivars carrying one or several disease resistance (*R*) genes introduced through conventional breeding. Over time, a competitive host-pathogen coevolution can lead to major resistance breakdown. Genome editing is a significant research tool and avenue for the genetic improvement of crop species, as it enables the precise introduction of targeted genetic changes. This technology has been successfully used in various food crops, including those belonging to the *Solanaceae* family. The advent of the CRISPR/Cas9 genome editing system allows the rapid knockout of desirable genes. Plant pathogens often exploit host genes known as susceptibility (*S*) genes to facilitate their proliferation. Inactivation of these *S* genes may reduce the pathogen's ability to infect plants and confer durable and broad-spectrum resistance. This review provides an overview of the current application of CRISPR/Cas9 to disrupt the *S* genes for the development of disease-resistant solanaceous crops. The technological limitations and potential strategies for overcoming these challenges are discussed.

Keywords: genome editing; pepper; potato; susceptibility genes; tobacco; tomato

Crops which belong to *Solanaceae* family such as potato, tomato, pepper, and tobacco have substantial economic value due to their significant contribution to global food trade. Potato is the world's fourth staple crop after wheat, rice, and maize, with a global production of 376 million tons in 2021 (FAOSTAT 2021). In the same year, 189 million tons of tomatoes were produced worldwide, making it as the most popular vegetable crop (FAOSTAT 2021). However, the production of these crops is continuously threatened by plant pathogens such as bacteria, viruses, fungi, and oomycetes which can invade and cause disease in host plant cells. In the case of major outbreaks and epidemics, these patho-

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gens can cause substantial yield losses (Oerke, Dehne 2004). Therefore, developing new crop cultivars with higher resistance to diseases in a shorter period of time is vital to boost production.

The introduction of resistance (*R*) genes into elite cultivars through genetic crosses with wild relatives has been extensively practiced in traditional breeding for the development of resistant cultivars. However, the transfer of *R* genes is often accompanied by undesirable traits, such as deleterious traits on crop yield and quality (Niu et al. 2014; Wulff, Moscou 2014). To uncouple the undesirable traits, additional crossbreeding and selection are required, making this approach a lengthy process. In addition, the introduction of single or dual *R* genes into crops over large areas typically results in major resistance breakdown (McDonald, Linde 2002; Wulff, Moscou 2014; Phan et al. 2019).

Marker-assisted selection has improved the selection step in the crossbreeding program over the past decades. Since it is known that some genes encode for proteins and contain the instructions for specific traits, selection can be made at the DNA level instead of evaluating traits based on the appearance of the plant. By identifying a specific DNA fragment linked to an *R* gene, resistant progeny can be identified more efficiently and at an earlier stage. This approach has been widely adopted by the breeders owing to its significant reduction in selection costs and time. Nevertheless, another limitation of crossbreeding is that the crosses can only be made between individual plants of the same species (Wulff et al. 2011).

The emergence of genetically modified (GM) technology in the late 1970s has enabled the transfer of *R* genes from diverse plant species into a single plant as transgenes. Hence it has been suggested that GM technology is more precise, predictable and controllable as compared to crossbreeding. Despite its potential benefits, GM technology continues to face challenges related to public acceptance and political scrutiny. Novel techniques that generate DNA modifications with greater precision without incorporating foreign genes are required to overcome these issues.

In recent years, the advent of site-specific nucleases (SSN) based genome editing technologies has made precise DNA modifications in plants possible. The SSN can be employed to genetically engineer plants, which typically involves creating targeted DNA changes in order to modify the phenotypic traits. Since genes are composed of DNA, these changes can involve the substitution, insertion or deletion of a single or multiple DNA bases, leading to a change in gene function. Some genes code for proteins and contain instructions for specific traits, thereby leading to modification in physical characteristics.

Disease susceptibility genes (*S* genes) are the host genes that can either act as negative regulators of immunity or encode proteins that contribute to pathogen colonisation. The candidate *S* genes include effector targets in which the inactivation of these genes by mutation should diminish the pathogen's ability to infect plants and contribute to pathogen-specific resistance (Lapin, Van den Ackerveken 2013; Bozkurt et al. 2015; Koseoglou et al. 2022). Thus, it has the potential to offer an alternative to *R*-gene mediated resistance strategies for the generation of more durably resistant crop varieties. In contrast to *R* genes, where resistances are typically dominant, the majority of *S* genes are recessive (Jørgensen 1992; Duprat et al. 2002; Ruffel et al. 2002; Iyer, McCouch 2004; Gawehns et al. 2013; Koseoglou et al. 2022). The *Mildew Locus O* (*Mlo*) gene, in which a recessive mutant has conferred powdery mildew resistance in barley for the past 70 years is a classic example of an *S* gene's long durability in the field (Jørgensen 1992). In recent years, significant efforts have been made to target *S* genes in key crops (Wang et al. 2014; Zeng et al. 2020) using the most recent genome editing technology, CRISPR/Cas9.

In this review, we compiled the current progress in engineering disease resistance of solanaceous crops by targeting *S* genes via CRISPR/Cas9-mediated gene knockout. The limitations of the technology and prospective approaches to address these challenges are discussed.

GENOME EDITING TOOLS

The emergence of genome editing technologies has enabled precise DNA manipulation in plants through the utilisation of site-specific nucleases (SSN). Meganucleases (Smith et al. 2006; Paques, Duchateau 2007), zinc finger nucleases (ZFNs) (Maeder et al. 2008), transcription activator-like effector nucleases (TALENs) (Bogdanove, Voytas 2011; Miller et al. 2011), and type II clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) endonu-

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cleases (Jinek et al. 2012) are the four types of SSN that have been employed so far. Each of these SSN types varies in the manner in which the nuclease is directed to the target DNA sequence (Figure 1). The fundamental concept of genome editing involves the generation of DNA double-strand breaks (DSBs) at a specific genomic location, which can be repaired by the plant's endogenous repair mechanisms, namely non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms (Jinek et al. 2012) (Figure 1).

The first generation of genome editing techniques, namely meganucleases, ZFNs, and TALENs utilise a protein-dependent DNA cleavage system. Although these techniques have significant potential use, their applicability is hindered by tedious procedures required to achieve target specificity, as well as high cost and time-consuming. In contrast, the second generation of genome editing tools, such as CRISPR/Cas9 employs RNA-guided DNA or RNA cleavage system that is comparatively easier to design and execute, while also being cost-effective and time-efficient.

Meganucleases, which exhibit a high degree of specificity towards DNA sequences ranging from 18 to 30 bases, are derived from microorganisms such as bacteria and yeast (Daboussi et al. 2015). Such DNA fragments are uncommon in plants; thus the specificity of this method is entirely dependent on the recognition sequences of the meganucleases. Due to the limited number of meganucleases available in nature, precise cleavage of a specific target sequence is a challenging task. Scientists have been attempting for several years to alter the recognition sequences of pre-existing meganucleases to enhance their efficacy (Daboussi et al. 2015). A feasible approach to accomplish this goal is through the fusion of two nucleases, also referred to as dimers (Figure 1). Despite the increase of specificity to a certain extent, changes in the recognition sequence have led to a reduced activity of the nuclease.

ZFNs are proteins that are made up of two components – a zinc finger and a nuclease (Figure 1). The zinc finger protein exhibits precise binding to a specific DNA fragment, and when combined with the nuclease, it allows a targeted DNA cutting to occur (Urnov et al. 2010). To achieve greater accuracy, ZFN operates in tandem fashion, with one ZFN binding to each side of the double-stranded DNA. The efficiency of the cleavage process is de-

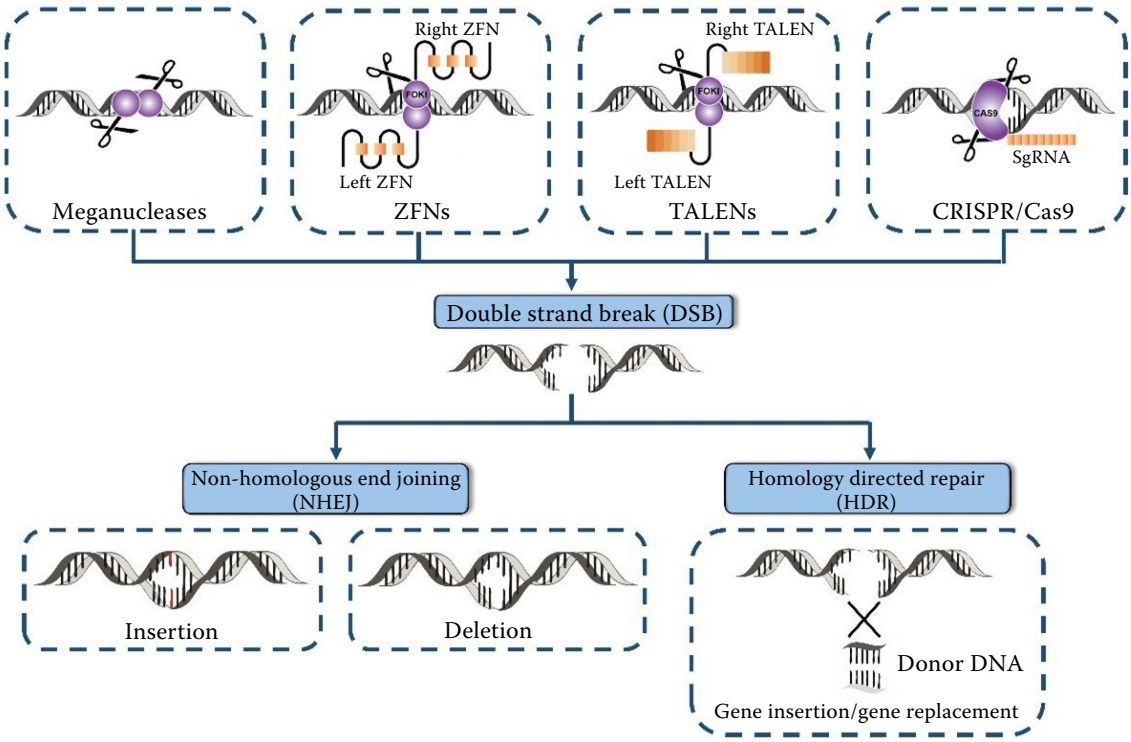

Figure 1. Schematic representation of various genome editing tools

ZFNs – zinc finger nucleases; TALENs – transcription activator-like effector nucleases; sgRNA – synthetic guide RNA; *FokI* – catalytic domain; Cas9 – enzyme; CRISPR/Cas9 – genome editing technology

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pendent upon the zinc finger domain, as the nuclease is restricted to cleave only at the designated site. In theory, it is possible to engineer the recognition sequence of the zinc finger domain to cleave any DNA sequences present in the plant. However, scientific evidence suggests otherwise as demonstrated by Urnov et al. (2005), owing to the laborious andexpensive process of generating such large modular proteins. Moreover, the adoption of this technology is limited due to the low cutting efficiency of the DNA targets.

TALENs are a type of proteins delivered to the plant host nuclei by the bacterial pathogen belonging to the *Xanthomonas* genus to modify plant gene expression (Christian, Voytas 2015). TAL effectors are composed of 13–28 copies of near-perfect repeats 34 amino acids and two hypervariable residues. In this modular structure, the hypervariable residues line up to generate a code that determines their DNA binding specificity (Boch et al. 2009; Moscou, Bogdanove 2009). The modular DNA-binding domain can be fused with the catalytic domain of the *FokI* endonuclease, enabling the design of synthetic TAL effectors that can bind to any target DNA sequence (Figure 1).

In contrast to ZFNs, the process of designing tailored TAL arrays is comparatively less complex, and the efficiency of the DNA targets binding is higher. However, the complexity and relatively high cost of constructing novel TAL arrays have impeded the wide utilisation of this technology.

CRISPR/Cas9 system represents a novel and precise method for genome editing that has emerged in recent years. Unlike ZFNs and TALENs, the CRISPR/Cas9 editing system offers a more simplified design at a lower cost. This system is derived from the natural immune system employed by bacteria as a defense mechanism against viral infections (Jinek et al. 2012). Upon the detection of viral DNA, the bacteria generate two types of short RNA, CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA) (Deltcheva et al. 2011). These two RNAs form a complex with Cas9 nuclease protein, which is an enzyme that is capable of cleaving DNA (Jinek et al. 2012). Upon reaching its target within the viral genome, a matched sequence, commonly referred to as guide RNA (gRNA), induces the Cas9 to cleave the target DNA, thereby causing the virus to become inactive (Sander, Joung 2014).

Researchers have discovered that this mechanism can be manipulated to cleave not only the viral DNA, but any DNA sequences with accuracy at specific chromosomal locations by modifying the gRNA to match the intended target (Figure 1). Upon entering the nucleus, the engineered CRISPR/Cas9 complex will selectively bind to a short nucleotide sequence known as a protospacer adjacent motif (PAM). The Cas9 enzyme is responsible for the denaturation of DNA strands and subsequent base pairing with the complementary RNA sequence. Following successful completion of the matches, the Cas9 enzyme will induce a DNA double-strand break (DSB) through cleavage of the DNA molecule. The cells will attempt to repair the cleavage either via non-homologous end joining (NHEJ) or homologous recombination (HR) mechanism (Jinek et al. 2012). In NHEJ, the ends of the break sites are typically repaired imperfectly, resulting in the introduction of small insertions or deletions (indels). On the other hand, HR mechanism can be employed when researchers aim to introduce or substitute a DNA fragment containing a desired sequence into a precise location. After a DSB, the DNA template exhibiting homology to the cleaved ends can undergo recombination, thereby substituting the original sequence with the new version.

Meganucleases are engineered restriction enzymes that generally recognize DNA target sequences and cleave as dimers. They do not have specific binding and cleavage domains. Zinc finger nucleases (ZFNs) act as dimer. Each monomer is composed of a DNA binding domain at N-terminus and the *FokI* nuclease domain at the C-terminus. Each DNA binding domain recognizes 9–18 nucleotides of target sequences. Transcription activator-like effector nucleases (TALENs) are dimeric enzymes similar to ZFNs. Each subunit consists of DNA binding domain (which contains 33–35 amino acid repeats specific for each nucleotide) and *FokI* nuclease domain. CRISPR/Cas9: Cas9 endonuclease bound to a synthetic guide RNA (sgRNA) complementary to 20 nucleotides of target sequence. Base pairing between the target sequence and sgRNA upstream of protospacer adjacent motif (PAM) allows DNA cleavage by the Cas9 endonuclease. All these systems induce double strand break (DSB), which then corrected by the cell repair mechanism either by non-homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ is typically resulted in insertion and deletion (InDel) while HDR led to gene insertion or gene replacement [adapted from Hamdan et al. (2022)].

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PLANT IMMUNE RESPONSE TO PATHOGEN

The plant immune system consists of two lines of defense to fight invading organisms (Figure 2). At the cell surface, pattern recognition receptors (PRR) (receptor kinases and receptor-like proteins) perceive evolutionarily conserved molecules among pathogens known as pathogen-associated molecular patterns (PAMPs) (Zipfel 2014) and initiate a defense response called PRR-triggered immunity (PTI) (Jones, Dangl 2006). To halt pathogen proliferation, the PTI typically results in intracellular immune signalling and elicitation of a set of biochemical and transcriptional responses including antimicrobial compounds and reactive oxygen species (ROS). In the cytoplasm, the second layer of defense known as effector-triggered immunity (ETI) is activated when more adapted pathogens subvert the PAMPs perception by translocating a group of virulence factors referred to as effectors (Jones, Dangl 2006). The effectors alter the host's physiological processes to facilitate the proliferation and survival of the pathogen.

On the plant side, disease resistance genes (*R* genes) mostly belong to the intracellular nucleotide-binding domain and leucine-rich repeat (NLR) gene family recognizes these effectors and initiates downstream signalling, which is often associated with localized plant cell death to stop pathogen growth (Krasileva et al. 2010). The recognition of *R* genes and their cognate effectors (or avirulence genes) is highly specific, following the gene-for-gene model (Cui et al. 2015) (Figure 2). Over evolutionary time, the more adapted pathogens may have accumulated sequence changes in a given effector that through adaptive selection have allowed them to circumvent ETI. In turn, novel alleles of *R* genes may arise via mutation or recombination, which are capable of recognising the newly evolved effectors. The repeated cycle of attack and defense over time has led to a competitive coevolutionary race between the host and pathogen.

This process may explain the 'boom and bust' cycle that occurs during the deployment of a single *R* gene across extensive agricultural lands. Over a period of a few growing seasons, the breakdown of resist-

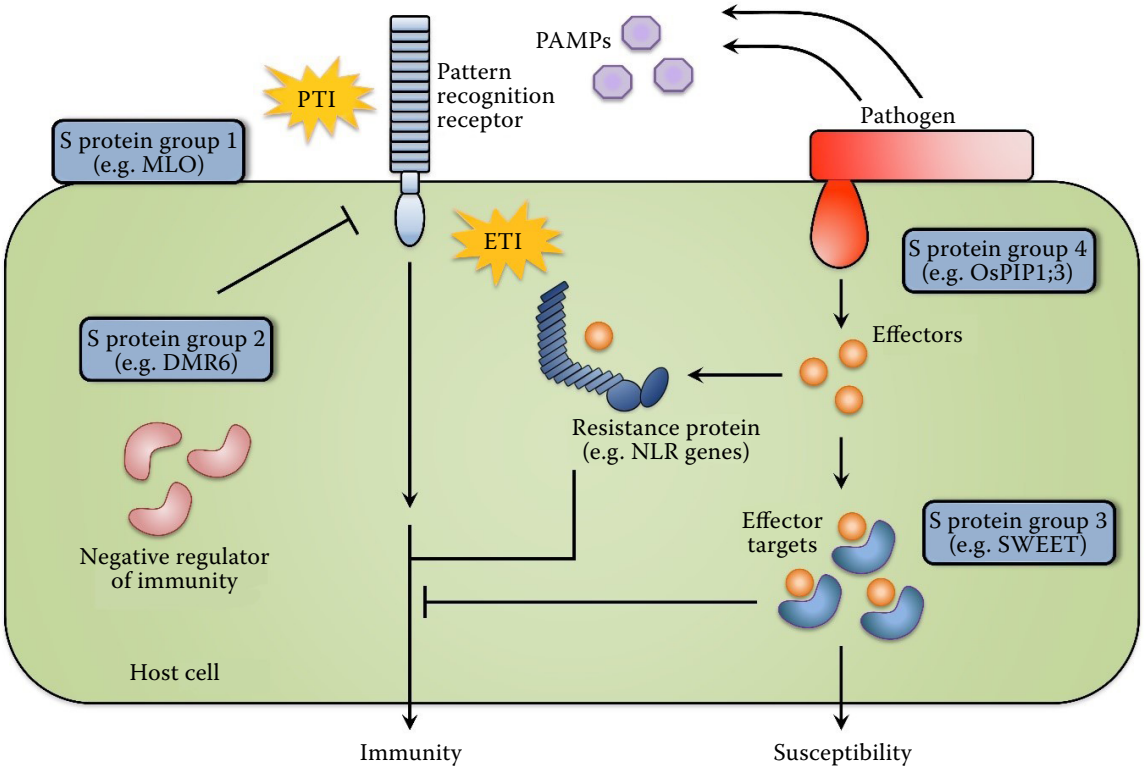

Figure 2. Principles of *S* genes in plant immunity and pathogen susceptibility

PTI – PAMP-triggered immunity; ETI – effector-triggered immunity; NLR genes – nucleotide-binding leucine-rich repeat genes; PAMPs – pathogen-associated molecular patterns; MLO, DMR6, SWEET, OsPIP1;3 – examples of S proteins for each group

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ance can be observed as a result of the natural selection of pathogen strains that exhibit enhanced virulence. Therefore, breeders must continually develop new resistant cultivars to keep pace with the evolution of the pathogen (Ali et al. 2022).

To achieve a more durable resistance, an alternative approach to *R*-gene mediated resistance is by deactivating the host genes that facilitate the pathogen colonisation, known as susceptibility (*S*) genes. This specific gene or a set of genes within the host's genome can be categorized into four groups (van Schie, Takken 2014; Koseoglou et al. 2022) (Figure 2). The first group includes genes that are required for pathogens to enter the host cells. A typical example is the *Mildew Resistance Locus O 1* (*MLO1*) gene that regulates the host's defense response against the fungal pathogen powdery mildew. The protein encoded by the *MLO1* gene plays a role in the formation of cell wall-associated papillae, which serves as a barrier against the penetration of fungal haustoria. However, the MLO1 protein recognizes the pathogen's effectors and initiates a signalling cascade that hinders the formation of papillae, thereby allowing the disease to progress further (Bai et al. 2008). The second group consists of genes that play an opposite role to the *R* genes, which negatively regulate the host immune response to the pathogen. For instance, *Downy Mildew Resistance 6* (*DMR6*) gene that regulates salicylic acid (SA) homeostasis during defense responses. Inactivation of *DMR6* induces elevated level of SA, resulted in enhanced resistance to the pathogen (van Damme et al. 2008; Zhang et al. 2017). Genes that are required for pathogen sustenance and growth, such as for nutrient acquisition and transport belong in the third group. A well-known example is *Sugars Will Eventually Be Exported Transporter* (*SWEET*) sugar transporter genes that are manipulated by effectors. The secretion of transcription-activator-like effectors (TALEs) by pathogen promotes the expression of *SWEET* genes, allowing the manipulation of nutrient uptake from the host (Chen et al. 2010; Chen et al. 2012). The fourth group of *S* genes is involved in translocation of effectors into host cells. An example is aquaporin *OsPIP1;3*, in which a loss of function of the gene disables the translocation of effector into cytosol during infection, leading to resistance (Li et al. 2019).

Pathogens secrete pathogen-associated molecular patterns (PAMPs) upon host colonisation which are recognized by plant pattern recognition receptors (PRRs) that activate PAMP-triggered immunity (PTI). To counteract plant immunity, pathogens secrete effectors to interfere with the PTI response. Plants can detect the presence of these effectors by resistance protein [majority belongs to nucleotide-binding leucine-rich repeat (NLR) gene families], leading to effector-triggered immunity (ETI) (middle). Pathogens exploit host proteins, specifically the S proteins, which are encoded by host susceptibility (*S*) genes, to enable their entry and proliferation, ultimately leading to susceptibility. The S proteins can be categorised into four groups according to Koseoglou et al. (2022) and van Schie and Takken (2014), and MLO, DMR6, SWEET, and OsPIP1;3 are the examples of S proteins for each group, respectively. Group 1 genes encode characteristics that facilitate pathogen entry, group 2 genes act as negative regulators of immunity, group 3 genes encode substrates required for the pathogen, and group 4 genes aid in the translocation of effectors into host cells. Inactivation of these S proteins has the potential to engineer enhanced disease resistance [adapted from Sun et al. (2016)].

STRATEGIES TO IDENTIFY SUSCEPTIBILITY GENE

Researchers can identify potential *S* genes using either forward or reverse genetic studies, with the former approach being the most commonly used strategy. This includes screening of mutagenised populations or screening of germplasm that has revealed many recessive alleles conferring resistance (Gawehns et al. 2013). However, the latter approach has also gained increasing interest in recent years. *S* genes are typically conserved across species. Therefore, the availability of pan-genomic, whole-genomic, and transcriptomic data owing to the substantial fall in sequencing costs has allowed the identification of orthologous *S* genes among closely related crop species (van Schie, Takken 2014) (Figure 3). Following the discovery of orthologous *S* genes, CRISPR-mediated functional analyses can be used to confirm their role in susceptibility. Such approach was successful to confirm the ortholog of *Arabidopsis S* gene, *Downy Mildew Resistance 6* (*DMR6*) in tomato (Thomazella et al. 2021) and potato (Kieu et al. 2021).

Pathogens typically employ overexpression of *S* genes as a strategy to invade the host plants.

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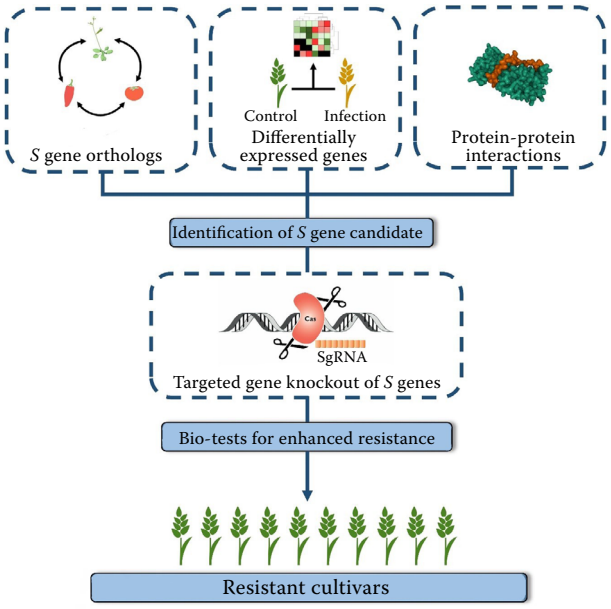

Figure 3. Workflow to identify potential *S* genes for engineering disease-resistant cultivars via CRISPR-mediated genome editing
sgRNA – synthetic guide RNA

For instance, in response to powdery mildew fungus *Oidium neolycopersici* challenge, *SlMLO* gene family expression increases, making tomato to become susceptible. *CaMlo2*, a gene involved in the susceptibility of pepper to powdery mildew is also overexpressed upon *Leveillula taurica* infection (Zheng et al. 2013). The identification of genes with differential expression can be facilitated by transcriptomics data generated through high-throughput techniques such as RNA sequencing (Figure 3). The genes that exhibit differential upregulation in infected plants as compared to non-infected ones, or in susceptible plants before and after infection may represent the *S* genes. Nine *S* gene homologs were identified using this approach by profiling genes specifically regulated in susceptible tobacco before and after *Phytophthora nicotianae* infection (Meng et al. 2021).

The effectors secreted by pathogens can promote susceptibility in their hosts. Understanding the different effectors carried by pathogens allows them to be used as molecular tools for the identification of *S* genes (Gibriel et al. 2016) (Figure 3). Several studies have shown that effectors and *S* gene proteins interact physically (Oh, Beer 2007; Hui et al. 2019; Zhang et al. 2019). Novel *S* genes can be discovered with the aid of protein-protein interaction assays, such as yeast two-hybrid assays, co-IP, or proximity labelling using the effector as a probe (Gibriel et al. 2016). Editing the *S* genes to prevent pathogen effectors from manipulating them will impair compatibility and render the plant more resistant.

Phylogenetic studies of known *S* genes across species can lead to the identification of *S* gene orthologs in other plant species. A typical pathogen strategy appears to be the overexpression of *S* genes. The generation of transcriptomics data can aid in the identification of classes of differentially expressed genes. Genes with differential expression in infected versus non-infected plants or in susceptible plants before and after infection may represent the candidate *S* genes. Multiple studies have revealed the physical interaction between pathogen effectors and proteins encoded by *S* genes. Protein–protein interaction analyses utilising pathogen effectors as molecular probes can be used to identify novel *S* genes. The candidate *S* genes identified from the abovementioned strategies can be knocked out using CRISPR for increased resistance. The editing of *S* genes can be confirmed via bio-tests for enhanced resistance, leading to the generation of resistant cultivars [adapted from Koseoglou et al. (2022)].

CURRENT PROGRESS OF TARGETED *S* GENE KNOCKOUT IN SOLANACEOUS CROPS

The engineering of disease resistance via CRISPR/Cas9-mediated gene knockout of *S* genes has been increasingly employed in solanaceous crops. A list of *S* genes mutated by this approach is summarized in Table 1. Powdery mildew-resistant tomato was developed by mutating *SlMlo1*, a gene that encodes a membrane-associated protein (Nekrasov et al. 2017; Pramanik et al. 2021). The *MLO* plant-specific gene family is also conserved in both monocots and dicots including other solanaceous crops such as pepper (Zheng et al. 2013) and tobacco (Fujimura et al. 2015). In addition to single gene mutation, CRISPR/Cas9 system has also been engineered to edit multiple genes or alleles at one time. Using this genome editing system, two homoeoalleles of *MLO* in allotetraploid tobacco have been simultaneously mutated, which led to enhanced resistance to powdery mildew (Xuebo et al. 2023). Another *S* gene that contributes to the development of powdery mildew disease is *Powdery Mildew Resistant 4* (*PMR4*) (Huibers et al. 2013). Disrupting the gene in tomato with

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CRISPR/Cas9 has resulted in reduced susceptibility not only to the powdery mildew (Santillán Martínez et al. 2020), but also to late blight disease (Li et al. 2022). *DMR6*, a gene that belongs to the superfamily of 2-oxoglutarate Fe(II)-dependent dioxygenases (2-ODDs) is another example of a conserved *S* gene (van Damme et al. 2008). Loss of function of its orthologs confer broad-spectrum resistance to bacterial speck, bacterial spot, blight, and powdery mildew in tomato (Thomazella et al. 2021), and increased late blight resistance in potato (Kieu et al. 2021). Tomato mutants carrying a dysfunctional *SlJAZ2* gene that lacks C-terminal Jas domain conferred bacterial speck resistance in tomato (Ortigosa et al. 2019). More recently, two putative negative regulatory genes associated with a major fungal disease of solanaceous crops, fusarium wilt were simultaneously edited using CRISPR/Cas9. Inactivation of *Xylem sap protein 10* (*XSP10*) and *Salicylic acid methyl transferase* (*SlSAMT*) genes resulted in a strong phenotypic tolerance to the disease in tomato (Debbarma et al. 2023).

CRISPR/Cas9-mediated gene knockout have also been extensively used to engineer viral resistance in solanaceous crops. One of the major targets is a group of genes belong to eukaryotic translation initiation factor (eIF) family. Loss of function studies using CRISPR/Cas9 on these genes in tomato have demonstrated that resistance is conferred against pepper mottle virus (PepMoV) (Yoon et al. 2020), potato virus Y (PVY) (Atarashi et al. 2020), cucumber mosaic virus (CMV) (Atarashi et al. 2020), and pepper veinal mottle virus (PVMV) (Kuroiwa et al. 2022). Deactivating the *eIF4E* gene has also led to enhanced resistance to PVY in other solanaceous crops such as potato (Noureen et al. 2022) and tobacco (Le et al. 2022). Other susceptibility factors that play a pivotal role in PVY infection are *Coilin* gene and *Ntab0942120* in potato and tobacco, respectively. CRISPR/Cas9-mediated site directed mutation in both genes have shown enhanced resistance to the disease (Makhotenko et al. 2019; Ruyi et al. 2021).

Another viral disease that severely affects tomato yield is tomato yellow leaf curl virus (TYLCV). The *SlPelo* gene is one of the factors of susceptibility as it plays a critical role in the infection cycle of TYLCV in tomato. CRISPR/Cas9-edited *SlPelo* mutants exhibited TYLCV resistance by restricting the viral DNA growth (Pramanik et al. 2021). The only *S* gene that has been targeted for CRISPR/Cas9-mediated gene knockout in pepper is *CaERF28*, which resulted in resistance to anthracnose (Mishra et al. 2021).

It is important to note that the deactivation of *S* genes, which play a pivotal role in developmental processes, may have a detrimental impact on the plant growth and productivity. A recent example is the *StDND1* gene, in which CRISPR/Cas9-mediated targeted mutagenesis led to increased resistance to late blight, but also resulted in undesired developmental changes in potato (Kieu et al. 2021). Therefore, dysfunctional *S* genes that lack pleiotropic effects are more desirable. In the same study, the mutation of the other two target genes, *StDMR6-1* and *StCHL1* conferred late blight resistance but did not show any significant differences in growth phenotypes as compared with the wild type, making them promising candidates for agricultural use (Kieu et al. 2021).

TRANSGENE-FREE GENOME EDITING TO SKIP REGULATORY PROCESS

The application of CRISPR/Cas9 system presents a great platform for accelerating crop improvement and commercialisation of new varieties. This system offers several advantages over previous genome editing technologies, including low cost, a simple design and high editing efficiency. In recent years, several methods have been developed to generate edited crop varieties that can be considered as 'transgene free' (Table 1). The absence or elimination of the CRISPR components in the improved crops provides opportunity to skip the lengthy regulatory process involved in their commercialisation.

A feasible approach for the removal of the Cas9-sgRNA construct relies on the segregation by selfing or backcrossing to the parental line as the CRISPR/Cas9-induced mutations are stable and heritable (Brooks et al. 2014). A notable example is demonstrated by the generation of transgene-free tomato plants that exhibit enhanced resistance to powdery mildew through the elimination of transgenes from segregating populations in subsequent generations. The absence of the T-DNA (transfer DNA) in the edited plants was confirmed by genome-wide sequencing analysis (Nekrasov et al. 2017). The same approach was employed to develop transgene-free pepper mottle virus resistant tomato (Yoon et al. 2020), PVY resistant tobacco (Ruyi et al. 2021), and anthracnose resistant chili pepper (Mishra et al. 2021) (Table 1).

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Table 1. List of disease-resistant solanaceous crops developed by CRISPR/Cas9-mediated gene knockout of susceptibility genes

Plant species	System	Target gene	Type of editing	Disease resistance (resistance to)	Reference
Tomato (*Solanum lycopersicum*)	transgene free	*SlMlo1*	deletion in coding region	powdery mildew (*Oidium neolycopersici*)	Nekrasov et al. (2017)
	transgene free	*eIF4E1*	deletion in coding region	pepper mottle virus (PepMoV)	Yoon et al. (2020)
	transgenic	*SlJAZ2*	deletion in coding region	bacterial speck (*Pseudomonas syringae* pv. *tomato)*	Ortigosa et al. (2019)
	transgenic	*DMR6*	deletion in coding region	bacterial speck (*Pseudomonas syringae* pv. *tomato*), *blight* (*Phytophthora capsici*), bacterial spot (*Xanthomonas* spp.), powdery mildew (*Pseudoidium neolycopersici*)	Thomazella et al. (2021)
	transgenic	*PMR4*	indels in coding region	powdery mildew (*Oidium neolycopersici*)	Santillán Martínez et al. (2020)
	transgenic	*SlMlo1*	indels in coding region	powdery mildew (*Oidium neolycopersici*)	Pramanik et al. (2021)
	transgenic	*XSP10, SlSAMT*	indels in coding region	fusarium wilt (*Fusarium oxysporum* f. sp. *lycopersici*)	Debbarma et al. (2023)
	transgenic	*PMR4*	indels in coding region	late blight (*Phytophthora infestans*)	Li et al. (2022)
	transgenic	*miR482b, miR482c*	indels	late blight (*Phytophthora infestans*)	Hong et al. (2021)
	transgenic	*eIF4E2*	indels in coding region	potato virus Y (PVY), cucumber mosaic virus (CMV)	Atarashi et al. (2020)
	transgenic	*eIF4E2*	indels in coding region	pepper veinal mottle virus (PVMV)	Kuroiwa et al. (2022)
	transgenic	*SlPelo*	insertion in coding region	tomato yellow leaf curl virus (TYLCV)	Pramanik et al. (2021)
Potato (*Solanum tuberosum*)	transgene free	*Coilin* gene	deletion in coding region	potato virus Y (PVY)	Makhoteno et al. (2019)
	transgenic	*StDND1,StCHL1, StDMR6-1*	deletion in coding region	late blight (*Phytophthora infestans*)	Kieu et al. (2021)
	transgenic	*StERF3*	indels in coding region	late blight (*Phytophthora infestans*)	Razzaq et al. (2022)
	transgenic	*eIF4E*	indels in coding region	potato virus Y (PVY)	Noureen et al. (2022)
Tobacco (*Nicotiana tabacum*)	transgenic	*NtMLO1, NtMLO2*	indels in coding region	powdery mildew (*Golovinomyces cichoracearum*)	Xuebo et al. (2023)
	transgene free	*Ntab0942120*	indels in coding region	potato virus Y (PVY)	Ruyi et al. (2021)
	transgenic	*eIF4E*	indels in coding region	potato virus Y (PVY)	Le et al. (2022)
Pepper (*Capsicum annuum*)	transgene free	*CaERF28*	indels in coding region	anthracnose (*Colletotrichum truncatum*)	Mishra et al. (2021)

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However, this strategy is less applicable for crops that reproduce via vegetative propagation such as potato, cassava, banana and other similar crops. Also, *Agrobacterium*-mediated transformation for plasmid DNA delivery may result in non-specific and unintended editing or known as off-target effects. Alternatively, Cas9 and gRNA can be delivered into plant cells in a form of ribonucleoprotein (RNP) complex using gene gun or through polyethylene glycol (PEG)-mediated protoplast transformation. In contrast to DNA, RNP undergoes rapid degradation after modifying the target sites without stably integrated into the genome. As a result, the edited crops are considered to be transgene-free (Liang et al. 2018a). To date, there has been only one study reported the utilisation of this method for editing *S* genes in solanaceous crops. The successful regeneration of apical meristem cells with editing activities has been demonstrated in the potato plants. The transgene-free PVY resistant potato exhibited enhanced resistance to the pathogen (Makhotenko et al. 2019). The limited study may be due to the low transformation efficiency for RNP delivery in certain crop species. This is also the case for transient expression of CRISPR components through particle bombardment of the callus (Zhang et al. 2016).

LIMITATIONS OF CRISPR/CAS9 SYSTEM

Although CRISPR/Cas9 exhibits remarkable potential for genome editing, it possesses some limitations. Several factors that could potentially constrain the efficiency and specificity of this technology include rational design of the sgRNA, PAM specificity, and possible off-target effects (Doudna, Charpentier 2014). The design of sgRNA is a crucial step in CRISPR/Cas9-mediated genome editing. Sequence and location of the sgRNA contribute to the efficiency of editing (Slaymaker et al. 2016). The selection of promoters for driving Cas9 (e.g. 35S and Ubiquitin promoters) and sgRNA (e.g. U3 or U6 promoters) is determined by the target plants, which may include monocots and dicots (Belhaj et al. 2015). Another factor that can directly affect the editing efficiency is the transformation method (e.g. transient or stable expression methods) (Scheben et al. 2017).

The precision of CRISPR/Cas9 may be constrained by the nature of the PAM sequence which requires the presence of an NGG nucleotide sequence during the process of selecting target sites. To allow for more target options, several studies have deployed PAM with multiple mismatches in the PAM distal region. This has led to a notable decrease in the affinity of Cas9 for the target sites (Fu et al. 2013; Hsu et al. 2013; Pattanayak et al. 2013).

The identification of potential off-target sites has emerged as a significant concern in the utilisation of genome editing technologies. This issue may arise due to the ability of Cas9 to tolerate mismatches in the sgRNA sequence, which can result in unintended editing at non-targeted sites. Although off-target effects have been detected in mammalian studies (Doudna, Charpentier 2014; Belhaj et al. 2015), they are typically low in plants as demonstrated by several studies in which whole-genome sequencing of the edited plants revealed a very low off-target cleavage frequency (Peterson et al. 2016; Nekrasov et al. 2017). Furthermore, it has been suggested that the unintended mutations could potentially be eliminated through backcrossing (Nekrasov et al. 2013; Belhaj et al. 2015) or minimized by employing RNP rather than DNA on vectors (Woo et al. 2015). The different Cas9 variants, such as eSpCas9 and HypaCas9 can reduce off-target activity as shown by Liang et al. (2018b). Other alternatives to the wild-type Cas9 such as base editors and prime editing are also prospective options for genome editing with higher efficiency and lower off-target effects (Kim et al. 2017; Lin et al. 2020). Finally, advances in computational biology have facilitated the development of various web tools for designing sgRNA and detecting potential off-target sites in plant genomes (Liu et al. 2017; Minkenberg et al. 2019).

CONCLUSION AND FUTURE PROSPECTS

In recent decades, the introduction of *R* genes into elite cultivars has emerged as the most predominant approach for developing resistance against diverse plant pathogens through breeding. However, the deployment of one or two *R* genes may jeopardize the resistant cultivars as most of pathogens have the adaptive potential to overcome resistance over time. In that scenario, the inactivation of *S* genes may present an attractive alternative for enhancing the resistance level of crops. Given the functional conservation of *S* genes across various plant species, the editing of these genes and their orthologs in economically significant solanaceous crops, such as po-

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tato, tomato, pepper, and tobacco could play a crucial role in the development of durable and broad-spectrum disease resistance. The advent of genome editing technologies, especially CRISPR/Cas9 represents a powerful tool for the development of crops that exhibit *S*-gene-mediated resistance response in a short period of time. Furthermore, it is feasible to generate transgene-free plants, providing a great potential for expedited commercialisation. In recent years, various studies have been conducted in solanaceous crops regarding the site-directed mutagenesis of *S* genes using CRISPR/Cas9 system. Despite the significant progress, the main challenges remain the off-target effects that negatively affect traits associated with crop yield. Therefore, it is expected that future research endeavours will prioritise enhancing the precision and efficiency of CRISPR/Cas9 technology in order to develop broad-spectrum and durable resistance in solanaceous crops with minor off-target effects.

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