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CRISPR Crops: Plant Genome Editing Toward Disease Resistance

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Abstract

Genome editing by sequence-specific nucleases (SSNs) has revolutionized biology by enabling targeted modifications of genomes. Although routine plant genome editing emerged only a few years ago, we are already witnessing the first applications to improve disease resistance. In particular, CRISPR-Cas9 has democratized the use of genome editing in plants thanks to the ease and robustness of this method. Here, we review the recent developments in plant genome editing and its application to enhancing disease resistance against plant pathogens. In the future, bioedited disease resistant crops will become a standard tool in plant breeding.

INTRODUCTION

Plants are continuously attacked by a multitude of pathogens and pests, including viruses, bacteria, oomycetes, fungi, insects, nematodes, and even parasitic plants. Collectively, these pathogens and pests cause substantial crop losses, threatening efforts to meet the food security needs of an ever-growing world population (54, 68, 71). During pathogen attack, a multilayer defense system is engaged to fight invading organisms. This system consists of a two-tier immune perception system that involves molecular receptors that detect potential pathogens at the cell periphery and inside the plant cell (59, 110). At the cell surface, pattern recognition receptors (PRRs) perceive conserved microbial patterns [also known as pathogen-associated molecular patterns (PAMPs)] and pathogen proteins that are secreted in the apoplast to initiate a defense response called PRR-triggered immunity (PTI) (213, 235). In the cytoplasm, intracellular nucleotide-binding domain and leucine-rich repeat–containing or NOD-like receptors (NLRs), encoded by disease resistance genes (*R* genes) recognize host-translocated pathogen effectors that generally alter plant processes to support pathogen growth (59, 110, 223, 235). This initiates an NLR-mediated response also known as NLR- or effector-triggered immunity (NTI/ETI), which leads to activation of the plant immune reaction that in turn stops pathogen growth (59, 235).

The *R*-gene type of genetic resistance has been extensively used in traditional plant breeding and allows the introduction of dominant or semidominant disease resistance genes into elite cultivars either by genetic crosses with wild relatives or via transfer of NLRs between plant species for engineering broad-spectrum resistance using genetic modification (54). Several single or stacked *R* genes have been introduced into crops with the aim of achieving durable resistance to invading microbes and pests (151, 183, 226). Although effective against a diversity of pathogens and pests, NLRs tend to have a narrow spectrum of pathogen recognition. Large monoculture systems in modern agriculture result in strong selection pressure on pathogen populations leading to effector diversification that allows them to adapt to new resistant varieties (59, 61, 226, 235). To address this problem, the research community has aimed at engineering NLR variants that recognize a broader range of pathogen effectors using a combination of in vitro evolution and rational design. Recent studies have shown that NLRs can be mutated to acquire new activities to improve disease resistance to different pathogens (79, 88, 120, 206). Another approach is to exploit noncanonical integrated domains found in some NLRs, which act as effector baits (78, 148, 188). In the future, as our mechanistic understanding of plant NLR function continues to advance, there will be renewed opportunities to develop synthetic immune receptors.

Susceptibility genes (*S* genes) have emerged as an alternative to *R*-gene-mediated disease resistance breeding, as they have the potential to be more durable in the field. *S* genes can either act as negative regulators of immunity or encode proteins involved in host plant development and, when manipulated by the pathogen, can suppress immune responses or promote pathogen growth (**Figure 1**). Effector targets are candidate *S* genes because removal or inactivation of these genes can impair the pathogen's ability to establish a susceptible state in the host and cause disease (32, 130, 173, 225). A typical example of an *S* gene is rice Pi21. Natural alleles of *pi21* carrying small deletions in a C-terminal proline-rich region confer recessive resistance against the blast fungus *Magnaporthe oryzae* (syn. *Pyricularia oryzae*) (74). Remarkably, the Pi21 protein contains a heavy metal–associated (HMA) domain with similarity to domains targeted by the *M. oryzae* effectors AVR-Pik, AVR-Pia, and AVR-Co39 (24, 40, 148, 169, 243). *S* genes can also function as negative regulators of immune response and their impairment can lead to recessive resistance. The archetypal example is *mlo*-based resistance, in which mutation in the barley mildew resistance locus (*MLO*) gene has been successfully used in European crop breeding for nearly four decades after its discovery in Ethiopian barley landrace in the 1940s (37, 111, 126). Plants homozygous

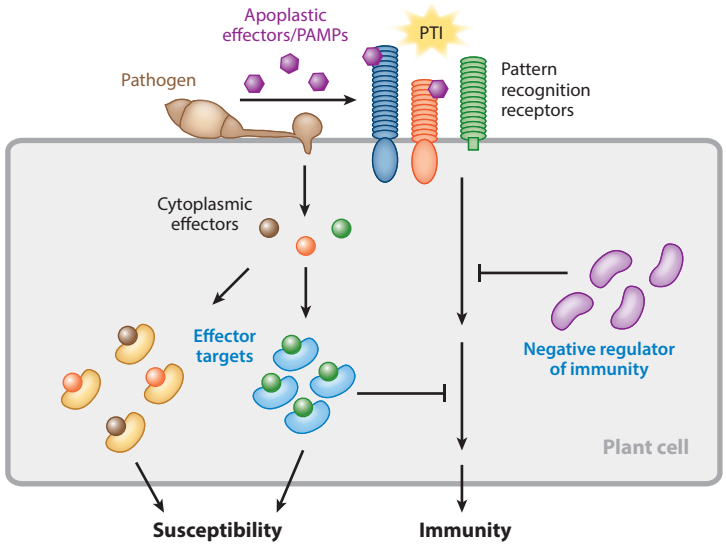


Figure 1

Functional categories of *S* genes in plant immunity and pathogen susceptibility. The graphic illustrates the different functional classes of *S* genes (*blue text*) that operate during plant-pathogen interactions. During infection, pathogen molecules can be recognized by plant pattern recognition receptors (PRRs) localized on the plant cell surface and triggering immune responses, such as PRR-triggered immunity (PTI). To counteract plant immunity, pathogens secrete effector proteins that interfere with host proteins by binding or modifying host targets. Host targets that are affected by pathogen effectors can either be involved in plant developmental or regulatory pathways (*left*) or act as negative regulators of immunity (*middle*). Both of these classes of effector targets can be *S* genes. Another category of *S* genes includes negative regulators of immunity that may operate independently of pathogen effector attack (*right*). Genome editing of each class of *S* genes has the potential to enhance resistance by removing or modifying important pathogen effector targets (*left*, *middle*) or by deletion or repression of negative regulators of immunity, sensitizing the plant immune response toward enhanced disease resistance.

for recessive *mlo* alleles possess broad-spectrum resistance to powdery mildew fungi not just in barley but also in other plants such as tomato and *Arabidopsis* (12, 53). Genetic screens, reverse genetics, and biochemical approaches were undertaken to understand the role of MLO, yet its exact biochemical function in immunity is not fully understood (3, 126).

Genetic mutants are critical for studying gene functions in plants and for genetic improvements of crops. Investigating gene functions through forward genetics screens and reverse genetics has emerged as a powerful approach to link genotype-phenotype causalities. In many agriculturally important crops and model plants, characterizing natural mutants has revealed important biological functions. However, conventional genetic screens using mutant libraries are labor intensive and time consuming. In recent years, modern genome-editing technologies have transformed plant research and breeding for resistant crops. The discovery of programmable nucleases has enabled precise DNA manipulation. These include zinc-finger nucleases (ZFNs) (145), TAL (transcription-activator-like) effector nucleases (TALENs) (28, 30, 49, 156), and CRISPR (clustered regularly interspaced palindromic repeat) RNA–guided Cas (CRISPR-associated protein) endonucleases, which can be programmed with different degrees of ease to target particular DNA sequences (51, 105, 146) (**Figure 2**). The development of efficient genome-editing techniques, especially CRISPR-Cas9, has opened a wide array of possible applications that can eventually be exploited in plant pathology and resistance breeding. The underlying principle of modern

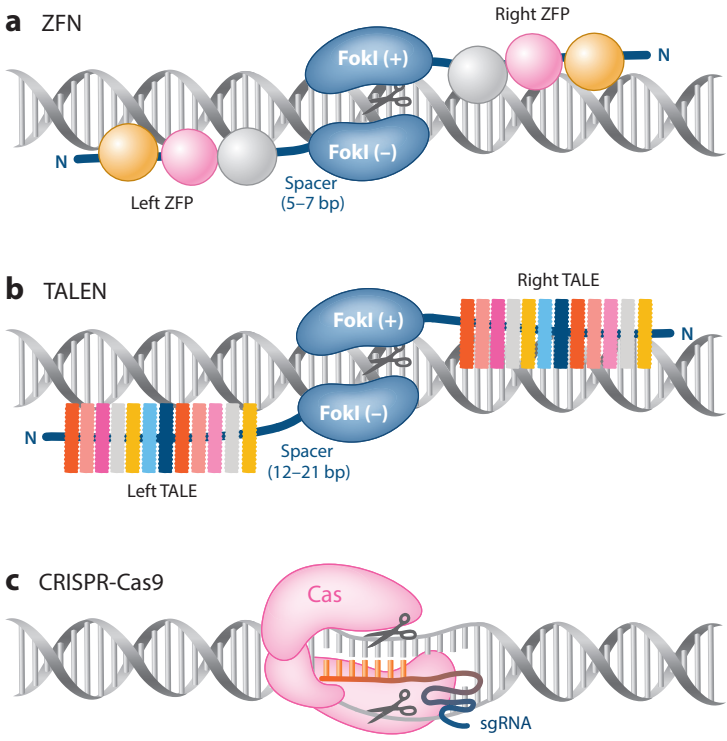


Figure 2

Schematic representation of three classes of programmable nucleases. (*a*) Illustration of zinc-finger nuclease (ZFN) pairs bound to DNA. Each ZFN is composed of a zinc-finger protein (ZFP) DNA-binding domain at the N terminus and the FokI nuclease domain at the C terminus. The linker between the DNA-binding and catalytic domains is represented by a blue line. Target sequences of ZFN pairs are typically 18–36 bp in length, excluding spacers. (*b*) Representation of a transcription-activator-like effector nuclease (TALEN) pair bound to DNA. Each TALEN consists of transcription-activator-like effector (TALE) DNA-binding domain at the N terminus and the FokI domain at the C terminus. Each TALE domain contains 33–35 amino acid repeats, with one repeat explicitly recognizing one single DNA base following a particular code. The amino acids at positions 12 and 13 confer DNA-binding specificity through the repeat variable di-residue (RVD). The target sequences of TALEN pairs are 30–40 bp in length, excluding spacers. (*c*) Depicted is a CRISPR-Cas9 nuclease bound to a synthetic guide RNA (sgRNA) complementary to a 20-bp target DNA sequence. Base pairing between a DNA sequence and sgRNA after protospacer adjacent motif (PAM) recognition allows DNA cleavage at the target site by Cas9 nuclease domains.

genome editing is the induction of DNA double-strand breaks (DSBs) by sequence-specific nucleases (SSNs) (e.g., Cas9, TALEN), which are subsequently repaired by plant endogenous repair mechanisms (179, 180, 228). DSBs are repaired by two major mechanisms, nonhomologous end-joining (NHEJ) and homology-directed repair (HDR). NHEJ occurs more frequently in plants but is error-prone, leading to small insertions or deletions (indels) at the target locus (122, 137, 180, 208). HDR is less frequent but more precise than NHEJ and can be used for precise gene replacement when a donor template containing homologous regions matching the target locus is provided (179).

Several reviews have summarized the exciting development of genome-editing technologies for plants (7, 20, 30, 139, 180, 191, 228, 242). Here, we provide an overview of the application of plant genome modifications to improve disease resistance and provide an outlook of future applications.

In addition, we discuss promises and limitations of this technology and future perspectives for genome-edited crops.

GENETIC MODIFICATIONS IN THE PRE-EDITING AGE

Genetic improvement of crop resistance against pathogens has been a continuous challenge for plant research and breeding. Sophisticated techniques have been utilized to create genetic changes in plant genomes, ranging from point mutations to integrations of chromosomal fragments from wild relatives into elite varieties. Natural mutations, classical wide crosses, hybridization, and random mutagenesis using physical (X or γ radiation), chemical [ethyl methanesulfonate (EMS)], and biological mutagenesis (e.g., transposon) have been routinely used by plant researchers and have led to a long list of plant varieties with improved disease resistance traits. However, recovering knockout lines by conventional forward genetics approaches (e.g., EMS, radiation) for a specific gene remains tedious and time consuming. In addition, these approaches are not targeted, with mutations randomly arising in the mutant population making it challenging to identify a desired genetic change.

With the advent of molecular techniques and the use of recombinant DNA technology, the toolbox has expanded. For example, delivery of foreign DNA via transfer DNA (T-DNA) from *Agrobacterium tumefaciens* into plant genomes has enabled the development of transgenic crops, colloquially known as genetically modified organisms (GMOs). Transgene insertion into the genome occurs at somewhat random positions, along with recombinant sequences from bacteria, viruses, and other species that drive expression of the introduced gene. This approach has been fodder for heavy criticism and regarded as problematic given that a mixture of DNA from different species could potentially have an unintended effect on targeted and untargeted organisms, and transgene insertion could interrupt the function of other important genes. To date, the most popular commercialized transgenic crops include the large-commodity crops maize and soybeans, which carry traits such as insecticide production [*Bacillus thuringiensis* (*Bt*-toxin)] and herbicide tolerance to glyphosate and glufosinate (101, 141). Although GM crop cultivation has increased over the past 30 years, only a few disease-resistant plant varieties have been commercially released, notably squash hybrids, and the Hawaiian GM papaya Rainbow and Sun-Up (54, 185, 216). The GM squash variety combines resistance against three different mosaic viruses by constitutive expression of three viral coat proteins. It was released to the US market in 1986 by the Asgrow Seed Company and continues to be sold by Seminis (54). Other examples of GM crops are the papaya Rainbow and Sun-Up with resistance to *Papaya ring spot virus* (PRSV). This product was approved and released in the US market in 1998 and resistance to PRSV remained durable for 15 years of commercial use (54, 101). However, many factors have limited the development and deployment of disease-resistant GM crops, including insufficient commercial benefits in comparison to development costs, intellectual property issues, public concern, and regulatory burdens (165). In most of Europe, cultivation of transgenic varieties is currently banned, except for Spain and Portugal, who continue to grow the GM corn MON810 on their territories.

Over the past 15 years, a new set of techniques (~20 techniques) collectively named new plant breeding techniques (NPBTs) have been developed. NPBTs have been proposed as alternatives to classical plant breeding and transgenic methods to improve crop varieties and create favorable genetic changes. These include cisgenesis (i.e., transfer of an unmodified genetic element from a crossable donor plant, often the same species), intragenesis (i.e., transfer of a hybrid genetic element combining different regulatory elements and open reading frames from a crossable donor species), RNA-dependent methylation, agro-infiltration, RNA interference (RNAi), reverse breeding, grafting on GM rootstock, oligo-directed mutagenesis (ODM), and SSNs (143). Whereas in

most cases these methods introduce heritable changes in the plant genome, some methods, such as virus-induced gene silencing, RNAi, or antisense RNA, alter gene function by repressing the corresponding mRNA. One possible drawback of mRNA targeting technologies is that transcript suppression typically produces a partial phenotype and therefore tends to be viewed as less robust.

To date, plants with novel traits produced by NPBTs are at advanced stages of development, and some of them have already been commercialized in the United States and Canada. In particular, a potato variety developed by Simplot with the trade name Innate® reached the US and Canadian markets in 2016 and 2017, respectively (101). This variety has been engineered for reduced black spots, lower levels of acrylamide and reducing sugar, and enhanced resistance to the late blight pathogen *Phytophthora infestans*. Innate® combines different approaches for genetic modification, namely the RNAi-based knockdown of two enzymes, *Ppo5* and *Asn1*, and cisgenetic introduction of the resistance gene *Rpi-vnt1* from the wild potato *Solanum venturii* to enhance resistance to late blight (**http://www.isaaa.org**). Other crops, such as nonbrowning Arctic® apple and herbicide-tolerant oilseed rape from CIBUS™, have been engineered using RNAi and ODM techniques, respectively, and have been commercialized in the United States and Canada (72, 101).

GENOME-EDITING REVOLUTION: FROM MEGANUCLEASES TO CRISPR-CAS NUCLEASES

The emergence of genome-editing technologies has revolutionized plant research, as it became possible to create genetic modification and modulate the function of DNA sequences in their endogenous genomic context in various organisms (16, 62, 75, 95, 112, 117, 187, 228). This era was made possible thanks to the discovery that SSNs can be reprogrammed to generate DSBs at a desired genomic location. To achieve effective genome editing and stimulate recombination at a locus, four major classes of customizable DNA-binding proteins have been utilized so far: meganucleases (170, 200), ZFNs (145), TALENs (30, 153), and several molecular endonucleases derived from CRISPR-Cas endonucleases (1, 2, 105, 106, 196, 250). Whereas first generation (ZFN-, TALEN-, and meganuclease-based) technologies are protein-dependent DNA cleavage systems, CRISPR-Cas endonucleases are programmable RNA-guided DNA or RNA cleavage systems. In this section, we review the different classes of SSNs.

Meganucleases

Meganucleases or engineered homing endonucleases are derived from naturally occurring enzymes that are encoded by mobile introns (170, 200, 228). These proteins are natural mediators of DNA targeting and can be engineered to recognize new DNA target sites. The yeast I-SceI meganucleases, encoded by an intron in the mitochondrial ribosomal RNA, are the best-characterized meganucleases for use in genome editing (181, 228). Meganucleases carry large DNA sequence recognition signatures ranging from 20 to 40 bp and typically act as a dimer of two identical subunits. Despite their small size (~165 amino acids for a meganuclease monomer), modulating their target specificity is challenging, and, as a consequence, they are now rarely used compared to other SSNs (181, 228).

Zinc-Finger Nucleases

Zinc-finger proteins were the first engineered endonucleases to recognize and cleave chromosomal DNA. They consist of artificial bipartite enzymes (~310 amino acids for a ZFN monomer) linked together via a linker peptide. A modular DNA-binding domain fused to a catalytic site of a FokI endonuclease allows DNA recognition and target cleavage (**Figure 2*a***) (220). The ZF binding

domain generally comprises four to six Cys2-His2 arrays derived from a human transcription factor and recognize three nucleotides in the DNA (75, 220, 228). Two ZFNs are required to bring FokI monomers into close proximity with the DNA and cleave the target sequence. Despite many successful demonstrations of ZFN-mediated genome editing in various organisms, the design and construction of such large modular proteins that perfectly match the triplet code are laborious and expensive. Although strategies to address these limitations have become available, the widespread adoption of programmable ZFNs remains limited because of the relatively high rate of failure for cleavage of the intended DNA sequence (181, 228).

Transcription-Activator-Like Effector Nucleases

TALENs are artificially engineered enzymes that originated from TAL effectors of the bacterial plant pathogen *Xanthomonas* spp. TAL effectors were initially developed as a better genome-editing tool than ZFNs (30, 49, 75, 112). Binding and specificity of TALENs are achieved by the protein's central domain, which comprises 13–28 copies of a tandemly repeated 34-amino-acid sequence, with one repeat specifically binding to a single DNA base. These repeats are almost identical, with the exception of two positions at amino acids 12 and 13 that define DNA-binding specificity following a particular code (**Figure 2*b***) (26, 28, 156, 192). This unique feature of the TAL effector led to the design of TALENs in which the modular DNA-binding domain of the TAL effector is fused to the catalytic domain of the FokI endonuclease. Custom TAL arrays are easier to engineer than ZFNs and bind their intended DNA targets at high efficiency (90%). However, construction of novel TALE arrays can be cumbersome and relatively costly, as a newly designed protein is required for each target sequence. In addition, the large size of TALENs, along with the necessity for a pair of proteins to recognize antiparallel DNA strands and induce a DSB, makes TALENs less suitable for multiplex gene editing (228).

CRISPR-Cas9 Nucleases

The application of *Streptococcus pyogenes* CRISPR-Cas9 nucleases to genome editing has emerged following fundamental discoveries on the class II bacterial adaptive immune system (15, 23, 124, 197, 201, 210). This led to the application of CRISPR-Cas9 as a genome-editing tool in human cells (51, 105, 106, 147), which was soon followed by applications in multiple organisms, including plants (133, 161, 194). Two simple components are required for CRISPR-Cas9 genome editing, the monomeric DNA endonuclease Cas9, and a customizable single guide RNA (sgRNA) sequence that binds to the DNA target by Watson-Crick base-pairing (**Figure 2*c***). Cas9 possesses a bi-lobed architecture with a large globular recognition (REC) lobe connected to a small nuclease (NUC) that accommodates two nuclease domains and a domain that binds the protospacer adjacent motif (PAM). The guide RNA is a customizable small noncoding RNA that consists of a fusion of two RNA moieties, namely the protospacer-containing CRISPR RNA (crRNA) with the *trans*-activating crRNA (tracrRNA). The Cas9-sgRNA complex probes the DNA double helix for the canonical G-rich PAM (5′-NGG-3′) sequence and for potential guide RNA complementarity in the PAM flanking sequence. Base pairing of matching nucleotides at the so-called seed region (8–12 bp) allows a step-by-step destabilization of the target DNA and formation of the guide RNA-DNA heteroduplex followed by DNA cleavage by the Cas9 nuclease (107, 167, 205).

The CRISPR-Cas9 system could not be initially used to target any sequence in the genome, as it requires a nuclease-specific PAM sequence (5′-NGG-3′). Although the canonical PAM sequences are frequently present in genomes, the PAM requirement for Cas9 activity limits the spectrum of potential genomic target sequences, especially in A-T-rich regions. Alternative PAM

sequences such as NAG and NGA can be recognized by the Cas9-sgRNA complex, potentially increasing the likelihood of off-target mutagenesis (253). Cas9 proteins from different bacteria have varying PAM sequence requirements and have yielded variants of the original *S. pyogenes* CRISPR-Cas9 (*Sp*Cas9), expanding the range of sequences that can be targeted (6). For example, Cas9 orthologs from *Staphylococcus aureus* (*Sa*Cas9), *Staphylococcus thermophilus* (*St*Cas9), and *Neisseria meningitidis* (*Nm*Cas9) have different PAM sequences from *Sp*Cas9. *Sa*Cas9 and *St*Cas9 require a 5′-NNGRRT-3′ PAM sequence, with R representing A or G, and a 5′-NNGGAA-3′ PAM sequence, respectively (167). *Nm*Cas9 also requires a different PAM: 5′-NNNNGATT-3′ or 5′-NNNNGCTT-3′ (94). In addition, unlike the *Sp*Cas9 and *St*Cas9 enzymes, *Nm*Cas9 binds a 24-nt protospacer sequence on its target DNA, conferring additional specificity over the previous 20-nt protospacer (94). Both *Sa*Cas9 and *St*Cas9 have recently been used in *Arabidopsis thaliana*, with comparable efficiency to the *S. pyogenes* Cas9 to enhance HDR (115, 203). Moreover, both *Sa*Cas9 and *St*Cas9 nucleases can be used simultaneously in one plant cell to target different sites in the genome, and their specific sgRNAs do not interfere with each other in plant cells (180, 203).

New CRISPR Nucleases: CRIPSR-Cas12a and Cas13a

Computational and functional analyses of bacterial genomes uncovered at least six new programmable class II system CRISPR-Cas nucleases. These include five DNA targeting nucleases (Cpf1, C2c1, C2c3, CasY, and CasX) and two RNA targeting nucleases (C2c2 and C2c6) belonging to type V and VI, respectively (36, 124, 154, 196, 197). The nomenclature of these enzymes was recently changed by Shmakov et al. (197) to Cas12a–e for subtype V and Cas13a–b for subtype VI, based on both sequence divergence and function (197). The enzymatic activity of Cas12a (formerly Cpf1) and Cas12b (formerly C2c1), Cas12d (formerly CasY) and Cas12c (formerly CasX), and Cas13a (formely C2c2) in genome editing was demonstrated in vitro and/or in vivo (1, 2, 36, 60, 140, 159, 196, 197, 204). Mechanistic and functional studies revealed important differences, such as the requirement for tracrRNA and crRNA, nuclease domains, and the nature of the DNA cleavage mechanism (159).

Cas12a (formerly Cpf1, CRISPR *Prevoltella* and *Francisella1*) employs similar mechanisms to those Cas9 uses to bind target regions in the genome but requires T-rich (-TTTN-) PAM sequences instead of Cas9's G-rich PAM. Reprogramming Cas12a for genome editing does not require a long chimeric guide RNA as it does for Cas9. Only a 42-nt crRNA guide region (versus ~100 bp for Cas9) is necessary to guide Cas12a and induce DSBs with 4- or 5-nt overhangs, leading to sticky ends as opposed to the blunt ends generated by Cas9 (249). Moreover, the insertion efficiency of a desired DNA fragment through the HDR mechanism into the Cas12a-cleaved site should be increased with the resulting cohesive DNA ends. In addition, Cas12a possesses not only DNA cleavage activity but also RNase III activity for pre-crRNA (i.e., unprocessed crRNA) processing. Both endoribonuclease and endodeoxyribonuclease activities of Cas12a can be exploited for multiplexing different targets via tandemly arrayed pre-crRNA expressing constructs (233). Recently, the activity of Cas12a was experimentally validated in plants using three Cas12a orthologs from *Francisella novicida* (*Fn*Cas12a), *Lachnospiraceae bacterium* ND2006 (*Lb*Cas12a), and *Acidaminococcus* sp. BV3L6 (*As*Cas12a) (19, 67, 118, 209, 238). Direct comparisons among these nucleases have shown that *Lb*Cas12a has higher efficiency than *As*Cas12a and *Fn*Cas12a (209, 233). Also, Cas12a seems to create lower rates of off-target edits relative to Cas9 nucleases (1, 19, 238, 246). Recently, Tang et al. (209) demonstrated that Cas12a has the potential for transcriptional repression in plants, making this system an attractive tool for regulating plant gene expression in addition to genome editing. The activity of Cas12a was found to be superior to Cas9, as it enables higher frequencies of precise gene insertions or replacements (8%) than other

editing nucleases and generates target gene insertion with a high rate of HDR. Also, the authors reported lower off-targets in plants compared to Cas9 and generation of biallelic mutations at nearly 100% efficiency in T0 plants. Moreover, by fusing nuclease deficient Cas12a to a repression domain, the authors could show that higher gene repression could be achieved than with nuclease-deficient Cas9 (dCas9)-based fusions (209). Cas13a (formerly C2c2) is the most recent addition to the CRISPR-Cas nuclease toolkit and enables precise editing at the RNA level (1, 2, 83, 196). Cas13a was identified in the bacterium *Leptotrichia shahii* and, unlike Cas9, is a dual ribonuclease that catalyzes crRNA maturation and RNA-guided single-stranded RNA (ssRNA) degradation interdependently using two separate catalytic sites in the two higher eukaryote- and prokaryote-binding (HEPN) domains. Similar to Cas12a, Cas13a is guided by a single crRNA sequence that is required for pre-crRNA processing, but Cas13a requires a protospacer flanking site (PFS) instead of PAM to induce single-strand breaks (SSBs). The cleavage preferentially occurs at uracil residues in ssRNA regions and depends on conserved catalytic residues in the two HEPN domains (2). To date, Cas13a was repurposed to achieve RNA-guided, PSF-dependent RNA cleavage in vitro (1). The Cas13a from *Leptotrichia wadei* (*Lwa*Cas13a) has been utilized in human and plant cells for targeted knockdown and, more recently, for RNA editing (1, 83). Highly efficient RNA cleavage was achieved using at least 22-nt crRNA with substantially reduced off targets compared to RNAi, making Cas13a-based gene silencing a promising alternative to RNAi (1). However, perhaps the most exciting development is the capacity to use Cas13 for RNA editing in vivo, which opens up a whole new set of applications for RNA engineering (83). The degree to which RNA engineering will emerge as an alternative application to genome editing in plant biology remains to be determined.

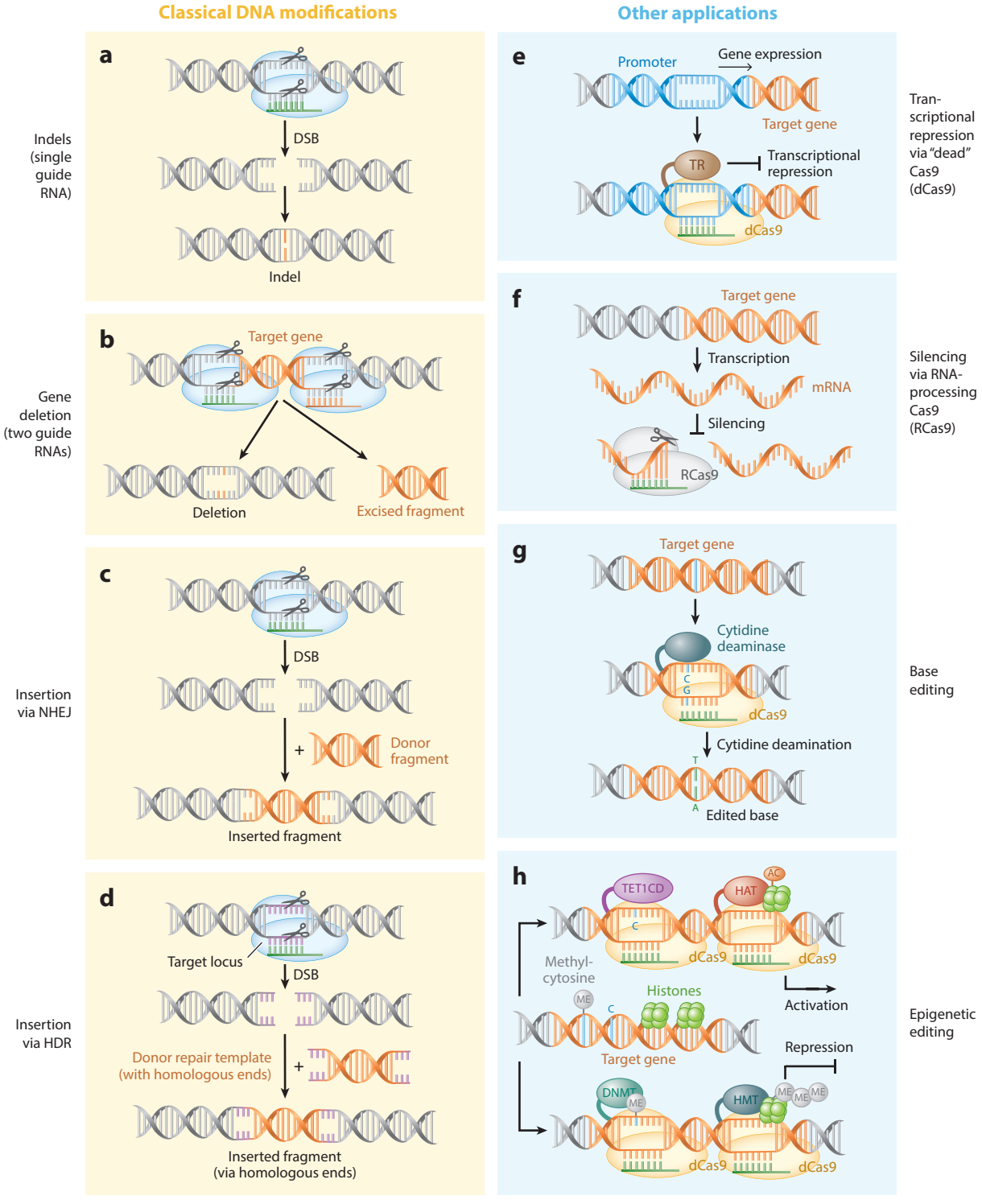
GENOME-EDITING APPLICATIONS: CUT-PASTE-REPLACE

Indels in the Protein Coding Sequence

The simplest and most widely used genome modification is the introduction of indels, which can be achieved by deploying SSNs to induce a site-specific DSB at the target locus, which is subsequently repaired by endogenous repair mechanisms. The most frequently occurring repair pathway is NHEJ, which, because of its error-prone nature, leads to small indels within the target region (**Figure 3*a***) (20, 122, 180, 181, 228). If the target region is within the protein coding sequence of a gene, these indels can cause frameshift mutations, leading to early stop codons and truncated proteins. In plant pathology and disease resistance breeding, this technique has been used to disrupt the function of *S* genes (173, 225), leading to enhanced resistance in various plant species, including *Arabidopsis*, tomato, citrus, rice, barley, and wheat (56, 134, 231).

Indels in Promoter Regions

The same approach can be used to introduce indels in specific promoter regions of *S* genes to modify their expression, particularly during pathogen infection, without altering potential developmental functions (8, 46, 184). A remarkable example for such promoter modifications is the rice sugar transporter *Os*SWEET14 (see section titled Examples of Genome-Edited *S* Genes). The promoter region of *Os*SWEET14 is targeted by bacterial TALEs, leading to overexpression of the gene and enhanced susceptibility. Small deletions within the TAL binding site abolish binding of the pathogen effector, which results in increased resistance without altering the developmental functions of the gene (25, 134). More studies investigating *S*-gene functions and their transcriptional regulation are needed to fully understand gene regulation dynamics during pathogen



(Caption appears on following page)

Figure 3 (*Figure appears on preceding page*)

Overview of genome-editing applications using Cas9 or modified nuclease variants. Sequence-specific nucleases induce double-strand breaks (DSBs) at the target locus that are subsequently repaired by the plant endogenous repair mechanisms nonhomologous end-joining (NHEJ) and homology-directed repair (HDR). In modern genome editing, these pathways can be exploited to induce desired mutations. (*a–d*) Classical Cas9-induced DNA modifications. (*a*) Indels. A single guide RNA-Cas9 complex induces a DSB at the target locus. The DSB is primarily repaired by NHEJ introducing indels at the target site, which can lead to frameshift mutations and thus disrupt the gene of interest. (*b*) Deletions. Two DSBs are induced at a genomic locus or chromosomal fragment leading to an excision of the targeted fragment. (*c*) Insertion via NHEJ. The NHEJ-mediated repair of a DSB can be exploited to insert a donor fragment into the target locus. Indels occur at the target site where the donor fragment gets inserted. If introns are targeted, this method can be used for exon or allele replacement without affecting the protein coding sequence. (*d*) Insertion via HDR. Codelivery of a donor fragment carrying homologous ends matching the target locus facilitates DSB repair by HDR. HDR can be exploited for gene replacement, seamless integration of donor repair templates into the target locus, or generation of in-frame fusion proteins. (*e–h*) Other Cas9 applications that extend the genome-editing toolkit. (*e*) Transcriptional repression. A nuclease dead Cas9 (dCas9) guide RNA complex can be targeted to promoter regions to repress gene expression. (*f*) Silencing via RCas9. An RNA processing variant of Cas9 (RCas9) binds and cuts to specific sequences of mRNAs leading to translational silencing. (*g*) Base editing. A Cas9-cytidine deaminase fusion protein can be targeted to specific regions leading to deamination of cytosine. The resulting uracil leads to a replacement of the G-C pair by A-T during replication in one of the daughter cells at low frequencies. (*h*) Epigenetic editing. dCas9 can be fused with additional protein domains to allow for epigenetic editing. Cytidine demethylase [e.g., ten-eleven translocation dioxygenase 1 (TET1CD) and histone acetyltransferase (HAT) fusion proteins] can be targeted to specific sites to enhance gene expression. In contrast, fusions of Cas9 with cytidine methylases [e.g., DNA methyl transferase 1 (DNMT)] or histone methylases (HMT) lead to transcriptional repression. Abbreviations: AC, acetyl group; ME, methyl group.

infection. However, the abovementioned example shows that simple genome modifications can be applied in plants to improve disease resistance.

Deletions of Genes or Chromosomal Fragments

Inducing two DSBs within the same chromosomal region can lead to the excision and deletion of the DNA between the DSBs (**Figure 3*b***). Two or more guide RNAs targeting a specific locus can be deployed to achieve a complete gene deletion or even deletion of chromosomal fragments containing gene clusters or recently duplicated genes (20, 254). In rice, CRISPR-Cas9-induced strand breaks have been utilized to delete chromosomal fragments of up to 245 kb in length, although in this case whole plants carrying the deletion were not regenerated (254). This approach can be applied in plant pathology to delete multiple members of a particular pathway or gene family located in proximity to each other on the same chromosome with putatively redundant functions during pathogen invasion. Modern genome editing via CRISPR-Cas9 also enables modification of numerous genes by deploying guide RNAs matching conserved regions within a gene family or by expression of multiple guide RNAs targeting different loci. Such a multiplexing strategy has been applied in tomato and rice to delete multiple genes from the same family or unrelated genes, respectively (33, 234). Recent advances in CRISPR-Cas9-mediated genome editing, such as the deployment of different Pol-III promoters to drive expression of multiple sgRNAs or expression of polycistronic sgRNA constructs, will further increase the efficiency and scope of applications for Cas9-mediated gene deletions and multiplex genome editing. In addition, multiplexing could be enhanced by combining multiple SSNs, such as various Cas9 orthologs (180, 203) or by using Cas9 in combination with Cas12a (209).

Gene Insertions and Allele Replacement

Modern genome editing offers great opportunities for gene insertions or gene/allele replacement by either the NHEJ or HDR pathway, making it a powerful tool to develop disease resistance in plants. Whereas NHEJ occurs spontaneously at the target site (**Figure 3*c***), HDR requires

codelivery of guide RNAs, Cas9, and a donor repair-template with sequence homology to each border of the target sites (**Figure 3*d***) (132). The donor template can be either single- or double-stranded DNA and can be delivered into the plant cell by particle bombardment or encoded on a plasmid along with the CRISPR-Cas9 machinery (50, 81, 232). Early proof-of-principle studies applying HDR in *A. thaliana*, *Nicotiana benthamiana*, and rice have shown that gene replacement by HDR can be achieved at varying frequencies, depending on the delivery method and the nature of the donor template (133, 194). More recently, the expression level of the donor template displayed a significant impact on editing efficiency by HDR. Accordingly, delivery of the repair template via viral replicons can significantly increase HDR efficacy (232).

Gene replacement via HDR is most attractive because it has the potential to seamlessly integrate new features at specific loci without inducing unwanted mutations. However, in plants, NHEJ occurs more frequently; thus, editing efficiency via HDR is relatively low. Another strategy to insert or replace a specific gene fragment (e.g., a certain domain or exon) uses intron-targeting in combination with NHEJ to avoid unwanted mutations in the protein coding sequence (132). This strategy deploys guide RNAs targeting introns to introduce DSBs in regions surrounding the fragment of interest in combination with a donor repair template. The repair template does not require homologous ends and is integrated into the target site during NHEJ. Using this strategy, exons can be replaced and desirable mutations can be inserted to, e.g., generate resistant alleles. Mutations that occur during error-prone repair are located in introns and most likely do not affect splicing; thus, the translated protein does not differ from the wild-type allele except for the introduced, desired mutations (132). Many studies investigating natural resistance have shown that allelic diversity of susceptibility or resistance genes has a major impact on plant-pathogen interaction (12, 82, 135). The coevolution of pathogen-derived effector proteins and their cognate immune receptors or targets leads to diversification and spawns natural, resistance-conferring alleles (17, 24, 39, 61, 100, 235, 241). However, traditional breeding of resistance alleles is slow, and, in some cases, these alleles are genetically linked to loci that influence plant development or crop quality (74, 150, 165, 225). The genome-editing strategies mentioned above have great potential to speed up resistance breeding and to overcome limitations caused by genetic linkage.

In-Frame Fusion Proteins

CRISPR-Cas9-mediated insertions can also help to study gene functions, protein localization, and spatio-temporal regulation of genes implicated in immunity and disease resistance. Wang et al. (232) demonstrated that it is feasible to generate in-frame GFP-fusion proteins at their endogenous locus using CRISPR-Cas9-induced HDR in rice, targeting the actin and glutathione S-transferase loci. Future applications of site-directed insertions will include the aforementioned replacement of susceptible alleles, promoter-reporter fusions to study transcriptional regulation of *S* genes or immune receptors, promoter replacement to modulate expression of *S* genes, and protein localization studies that have been difficult in nonmodel species so far. Applying modern genome editing for basic cell biology and mechanistic studies will help us to understand *S*-gene function and spatio-temporal regulation; thus, it will eventually contribute to improving resistance development.

Beyond Classical Genome Editing

In recent years, the development of new genome-editing applications skyrocketed, including modified Cas9 variants, such as deactivated or dead Cas9 (dCas9), RNA-processing Cas9 (RCas9), and Cas9-fusion proteins, offering a plethora of unprecedented opportunities in plant science (136,

190, 195, 256). Nuclease-dead dCas9 can be fused to transcriptional activator or repressor domains to generate synthetic transcriptional regulators that can either induce or repress expression of specific genes (**Figure 3*e***) (80, 136, 176). Similarly, RCas9, which binds and cuts RNA, enables Cas9-mediated post-transcriptional silencing in a sequence-specific manner (**Figure 3*f***) (163, 168). But the range of applications for RCas9 reaches beyond silencing. It is conceivable that future applications will include dead RCas9 fusions to fluorescent reporters to investigate spatiotemporal expression patterns during pathogen infection or even to investigate mRNA localization in living plant tissue (164). A dead RCas9 could also be combined with a library of guide RNAs to enrich for particular transcripts, e.g., a conserved *S*-gene family or immune receptors, which could enable targeted, sequence-specific transcriptional profiling. Similarly, deactivated, DNA-binding dCas9 could be used to enrich DNA sequences for targeted high-throughput sequencing approaches. Recently, dCas9 labeled with fluorescent probes has been used for in situ DNA labeling, and genetically encoded, fluorescent protein fusions enable live imaging of chromosomal dynamics in living tissue (45, 55). Combining different Cas9 variants for live cell imaging, e.g., imaging dynamic DNA rearrangements and RCas9-mediated mRNA profiling, has the potential to enhance our understanding of host-plant reprogramming during pathogen invasion.

Moreover, dCas9 can be fused to enzymatically active protein domains. One of the first described Cas9-fusion proteins was a Cas9-cytidine deaminase fusion, which enables targeted base editing, namely the conversion of cytosine to thymine (**Figure 3*g***) (123). Subsequently, the system has been further improved by combining so-called nickases (nCas9) and uracil glycosylase inhibitors into a single transcriptional unit with cytidine deaminases to increase base editing efficiency (142). Zong et al. (256) demonstrated the power of this system for applications in plant research by showing efficient base editing in rice, wheat, and maize. As our knowledge about *S*-gene or immune receptor alleles increases, it will become possible to edit specific bases in crop genomes to develop new, or mimic natural, resistance alleles in plant breeding.

So far, most technical advances are focused on the development and application of new or improved variants of the nucleases Cas9 and Cpf1. Both Cas9 and Cpf1 are guide RNA–mediated nucleases, but Cpf1 has unique features, including a T-rich PAM motif, nuclease activity generating sticky ends with 5′ overhangs, and the architecture of the guide RNAs used (118, 196, 197, 209, 246, 249). Whereas Cas9 requires guide RNAs with a 3′ scaffold, Cpf1 uses guide RNAs with a 5′ scaffold for sequence recognition. These features can be exploited to design fusion guide RNAs, which can function with both nucleases, allowing multiplexing and orthogonal genome manipulation, e.g., gene disruption and transcriptional activation of a second gene at the same time (129). In plants, one of the major limitations for precise genome editing is the low efficiency of HDR. The previously mentioned combination of fusion guide RNAs, Cas9, and Cpf1 could be exploited in plant genome editing to increase homologous recombination frequency by simultaneous silencing of components of the NHEJ machinery (e.g., Ku70) in combination with repair-template-mediated gene replacement. Transient silencing of Ku70 could significantly increase HDR frequency and could, therefore, pave the way for HDR as an efficient tool in plant genome editing, facilitating *cis*-genic allele replacement for resistance breeding.

The applicability of new Cas9 fusion proteins does not stop at modifications of the nucleotide sequence itself. More recently, Cas9 fusions were developed to enable epigenetic editing and manipulate gene expression patterns (180, 211) (**Figure 3*b***). Thus, transcriptional activation can be achieved by fusing Cas9 to the catalytic core of histone acetyltransferases, leading to either target-specific histone acetylation or methyl-cytosine demethylases (4, 48, 91). Conversely, Cas9 fusions to histone trimethylases or cytosine methylases can be used to repress transcription of target genes (212, 227). However, the opportunities for epigenetic modifications have not yet been fully exploited in plant resistance breeding.

Non-Genome-Editing Applications of CRISPR Nucleases

Since its first description, the CRISPR-Cas9 system has been modified and improved tremendously, giving rise to new applications that go far beyond genome editing. A number of recent studies demonstrated that the bacterial CRISPR-Cas9 immune system can be harnessed to enhance plant resistance against geminiviruses (5, 13, 43, 104, 247). Geminiviruses are single-stranded DNA viruses that replicate in the host plant nucleus and can cause severe damage to agronomically important crops, including tomato, sugar beet, and pepper. During viral replication, the single-stranded DNA is converted into a double-stranded intermediate, facilitating rolling-circle replication (87). Overexpression of Cas9 in combination with virus-specific guide RNAs in stable transformants strongly decreased viral replication and enhanced virus resistance (5, 13, 43, 104, 247). However, further studies are needed to evaluate off-target effects in plant genomes and possible selection for virus strains that evade Cas9-mediated degradation.

Among other applications of CRISPR nucleases, physical mapping using Cas9 nanoparticles in combination with atomic force microscopy has been demonstrated as a useful tool to complement genome-sequencing efforts and could develop into a powerful mapping tool in resistance breeding (152). Another recent study described a Cas13a (C2c2)-based nucleic acid detection system, named SHERLOCK, which allows attomolar DNA or RNA detection sensitivity with single-nucleotide specificity and can be applied for sensitive pathogen detection in the field (83). Cas13a exhibits RNA-targeting collateral ribonuclease activity that can be exploited to release fluorescent probes from artificial RNA substrates upon nucleic acid–sgRNA–Cas13a complex formation, which allows detection of specific sequence polymorphisms. This technique has the potential to be used for fast and large-scale pathogen surveillance, for example, as part of a response to major plant disease outbreaks (103).

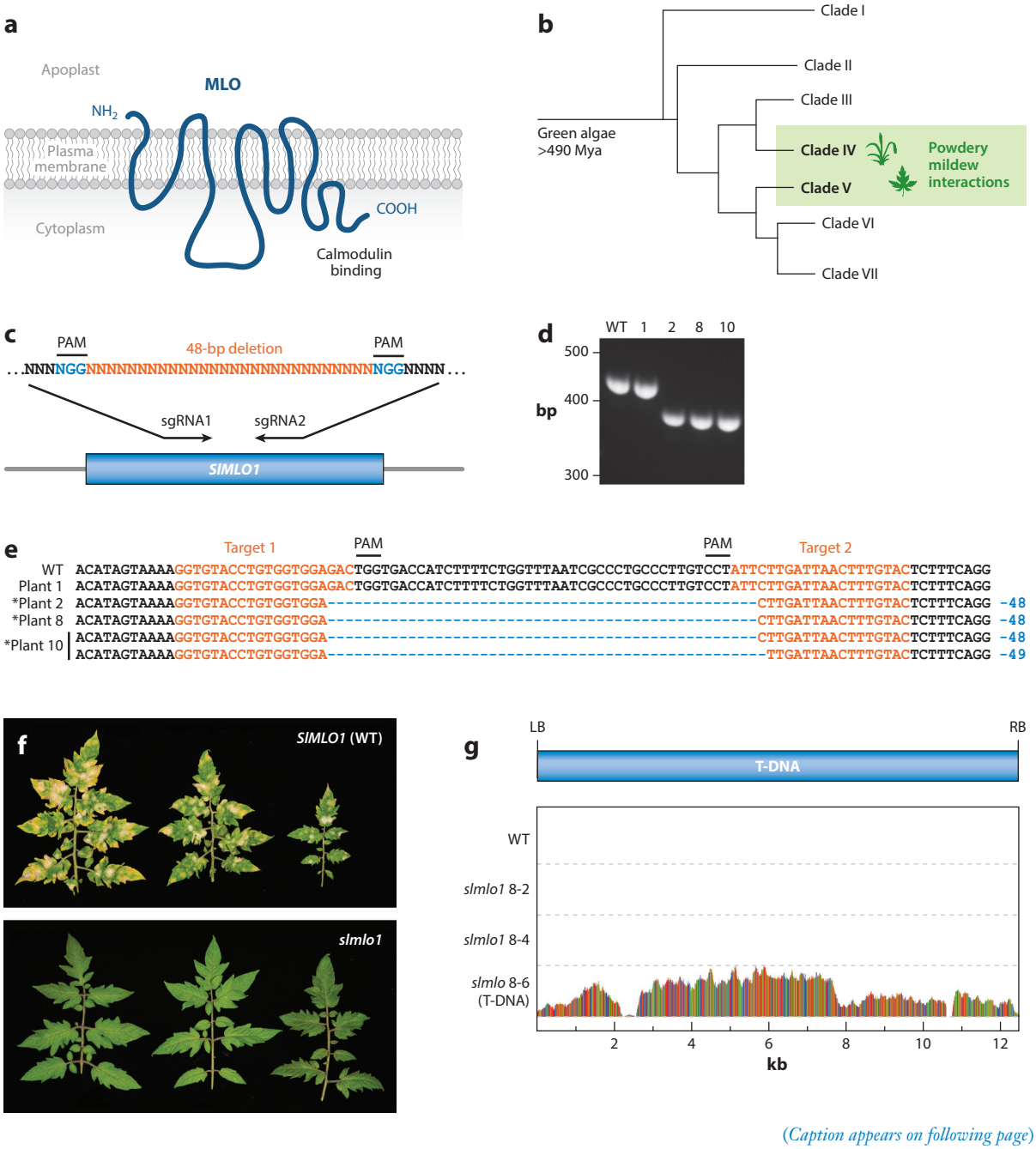
EXAMPLES OF GENOME-EDITED *S* GENES

Mildew Resistance Locus O

A classical *S* gene is the mildew resistance locus O (*MLO*). Wild-type alleles of *mlo* were first discovered in barley to confer susceptibility against all known isolates of the powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* (Bgh) (111). The gene was mapped and occurs in small- to medium-sized families in the genome of higher plants (**Figure 4**) (3, 57, 127). *MLO* encodes a plasma membrane–associated protein that binds calmodulin with its C-terminal cytoplasmic tail, a domain relatively well conserved among MLO family members (**Figure 4*a***) (119). The protein is structurally related to G-protein-coupled receptors in metazoans, and its function in susceptibility toward powdery mildew pathogens was confirmed in both monocotyledons (wheat and rice) and dicotyledons (*A. thaliana*, tomato, grapes, and pea) (12, 53, 98, 126). *Atmlo2* loss-of-function alleles also confers partial resistance to multiple powdery mildew species *Golovinomyces orontii* and *Golovinomyces chicoracerarum* in *A. thaliana* (53). Importantly, MLO-dependent susceptibility may not be confined to powdery mildew, as pepper *MLO2* was reported to contribute to susceptibility to the bacterial pathogen *Xanthomonas campestris* (116). Despite the exceptional efficacy and longevity of *mlo* resistance in the field, barley *mlo* alleles are associated with certain fitness penalties in the absence of pathogens, such as spontaneous callose deposition (papillae) and spontaneous cell death in leaf mesophyll cells. In addition, barley *mlo* alleles showed increased susceptibility to some pathogens, including the blast fungus *M. oryzae*, which could be a direct consequence of deregulated immune responses occurring in these plants (125).

Kusch et al. (127) performed a phylogenetic analysis comparing 341 MLO proteins from different plant species to uncover the evolutionary history and origin of the MLO family. The origin

of this gene family traces back 400–490 million years in unicellular, plastid-carrying eukaryotes (**Figure 4*b***) (127). During evolution, the family has diversified into seven phylogenetic clades with clade I being the most ancient. A massive expansion of the MLO family occurred in the eudicot lineage (e.g., 39 in soybean) but not in monocots (e.g., 7 in wheat). Each clade was attributed a putative function based on evolutionary origin and published information. MLO members of



(Caption appears on following page)

Figure 4 (*Figure appears on preceding page*)

Mildew resistance locus (*MLO*). (*a*) Topology of MLO protein with seven *trans*-membrane domain topologies (3). (*b*) The scheme shows a simplified phylogeny of the seven major clades of MLO as inferred by Acevedo-Garcia et al. (3) and Kusch et al. (127). MLO proteins involved in powdery mildew interaction appear to be restricted to clades IV for monocots (e.g., barley and wheat) and V for dicots (e.g., *Arabidopsis* and tomato). (*c*) Genome-editing strategy in tomato with sgRNA targeting *SlMLO1*. (*d*) Genotype of T0-edited transformants using polymerase chain reaction (PCR) band shift assay compared with wild-type (WT) tomato plant. (*e*) Sequencing reads from selected T0 transformants showing 48–49-bp chromosomal deletion in various transformants. Edited transformants are marked with an asterisk (*). (*f*) Leaves of edited tomato lines (named Tomelo) infected with *Oidium neolycopersici* showing full resistance toward this pathogen when compared to wild-type leaves. (*g*) Illumina sequencing data displaying coverage of T-DNA Illumina reads in three T1 segregants line from the left border (LB) to the right border (RB). No reads matching the T-DNA vector sequence were detected in two independent *slmlo* 8-2 and 8-4 mutant lines. These two tomato-edited lines are free of transgenic sequences. Panels *c*–*g* are modified with permission from Reference 162.

clade III could be linked to flower development, whereas members of clade IV and V are linked to susceptibility to pathogens (3, 127). Interestingly, MLO-like proteins in non-land-plant species contain Ca^{2+}-binding EF-hands domains. This is relevant because Ca^{2+}-binding proteins and MLO have previously been shown to physically interact and to be required for MLO function in barley, suggesting a role in Ca^{2+} signaling (119). Other types of MLO fusion proteins have also been found, such as glycosyl hydrolases, RNase H-like proteins, and zinc-finger domains (127). However, even though MLO susceptibility function was discovered decades ago, its precise mode of action is still unknown.

The conserved *mlo* function in disease resistance across monocot and dicot species prompted the application of TALEN and CRISPR-Cas mediated genome editing to knock out *MLO* in wheat (234) and tomato (162) (**Table 1**). Wang et al. (234) used TALENs and CRISPR-Cas9 to target a conserved region in the *MLO* exon 2. Allohexaploid winter wheat embryos were transformed using particle bombardment, and small deletions in the *MLO* locus were reported in all three genomes of primary transformants (T0), with a similar efficiency rate of 5.6–6% for both technologies. TALEN-induced mutations in *TaMLO* strongly enhanced resistance to powdery mildew *Blumeria graminis* f. sp. *hordei* in homozygous *mlo* plants, where no apparent macroscopic and microscopic fungal growth was observed on challenged leaves compared to wild-type plants (234). Similarly, Nekrasov et al. (162) edited *SlMlo1* for resistance against *Oidium neolycopersici* in tomato. CRISPR-Cas9-mediated genome editing using two guide RNAs targeting the *SlMlo1* locus resulted in deletions of 48 bp in edited T0 plants. Disease resistance assays in the Tomelo primary progeny (T1) revealed a resistance phenotype to the fungal pathogen *Oidium neolycopersici*, with no apparent pleotropic effects on plant morphology and fruit yield (**Figure 4*f***). In addition, whole-genome Illumina sequencing of three edited Tomelo segregant lines confirmed that two out of three lines analyzed were transgene free and no off-target mutations were detected, demonstrating the power and specificity of genome-editing technologies for rapid disease resistance development (**Figure 4*g***) (162).

OsSWEET14

The *OsSWEET14* gene (also called *Os11N3*) encodes a member of the MtN3/saliva/SWEET family of seven transmembrane domain–containing proteins involved in sugar transport. The first member of this family, MtN3 (*Medicago truncatula* nodulin 3), was identified in an expression screen in *M. truncatula* roots inoculated with *Rhizobium meliloti* (syn. *Sinorhizobium meliloti*) (77). Subsequently, a number of SWEET proteins from different species have been further characterized as being involved in diverse biological processes, such as pollen development and senescence, response to abiotic stress, and in plant-pathogen interactions (22, 41, 199). SWEET genes are phylogenetically conserved with many bacteria and animals encoding only one SWEET protein,

Table 1 Overview of genome-edited plants with improved resistance to pathogens

Target gene	Genome-editing method	Type of modification	Phenotype/disease resistance	Encoded protein	Plant species	References
Os11N (OsSWEET14)	TALEN	Deletion in promoter region	Resistance against *Xanthomonas oryzae* pv. *oryzae*	SWEET sugar transporter	*Oryza sativa*	134
OSERF922	CRISPR-Cas	Indels in ORF	Enhanced resistance against *Magnaporthe oryzae*	ERF family transcription factor	*Oryza sativa*	231
SlMlo1	CRISPR-Cas	Deletion in ORF	Resistance against *Oidium neolycopersici* (tomato powdery mildew)	Transmembrane protein	*Solanum lycopersicum*	162
TaMlo1	TALEN	Deletion in Exon2	Resistance against *Blumeria graminis* f. sp. *tritici* (powdery mildew)	Transmembrane protein	*Triticum aestivum*	234
TaEDR1	CRISPR-Cas	Deletion in Exon4	Resistance against *Blumeria graminis* f. sp. *tritici* (powdery mildew)	Raf-like mitogen-activated protein kinase kinase kinase (MAPKKK)	*T. aestivum*	252
SlDmr6	CRISPR-Cas	Deletion in Exon 3	Enhanced resistance against *Xanthomonas*, *Pseudomonas*, and *Phytophthora capsici*	2-oxoglutarate (2OG) Fe(II)-dependent oxygenases	*Solanum lycopersicum*	55
Cs-eIF4E (*Cucumis sativus*)	CRISPR-Cas	Deletion in Exon1	Resistance against *Cucumber vein yellowing virus*, *Zucchini yellow mosaic virus*, and *Papaya ring spot virus*; no resistance against CMV or CGMMV	Eukaryotic translation initiation factor	*C. sativus*	42
CsiLOB1 (*Citrus sinensis*)	CRISPR-Cas	Small deletions in promoter region	Enhanced resistance against *Xanthomonas citri* subsp. *citri* (citrus canker)	LOB domain protein, regulation of plant organ development	*C. sinensis*	174

Abbreviations: CGMMV, cucumber green mottle mosaic virus; CMV, cucumber mosaic virus; CRISPR-Cas, clustered regularly interspaced palindromic repeat–CRISPR-associated protein; ERF, ethylene response factor; indels, insertions/deletions; LOB, lateral organ boundary; ORF, open reading frame; SWEET, sugar will eventually be exported transporter; TALEN, transcription-activator-like effector nuclease.

whereas some plant species contain expanded gene families of more than 20 members (e.g., 10 in *Zea mays*, 17 in *A. thaliana* and *Brachypodium distachyon*, 21 in *Sorghum bicolor* and *Oryza sativa*, and 52 in *Glycine max*), suggesting more diverse functions and possibly redundancy, especially in land plants (171, 245). The phylogenies of SWEET genes from 13 species representing major plant groups suggest an expansion of SWEET genes in land plants, followed by further species-specific duplication and diversification (171). The importance of SWEET proteins in plant-pathogen interaction became apparent with the identification of specific SWEET genes from *O. sativa* and *A. thaliana* that are induced during infection of biotrophic and hemibiotrophic pathogens (8, 46, 239).

The rice genome encodes 21 members of the *Mt*N3/saliva/SWEET family. Two members, *Os*Sweet11 and *Os*Sweet14 (*Os*8N3 and *Os*11N3, respectively) have been shown to export sucrose and glucose into the apoplast and are associated with susceptibility to bacterial blight (22). Bacterial blight on rice is caused by the pathogen *Xanthomonas oryzae* pv. *oryzae* (Xoo), which spreads systemically through the vascular tissue and colonizes the plant apoplast. For successful colonization, Xoo relies on the secretion of transcription-activator-like effectors (TALEs) via the type III secretion system (T3SS). TALEs are secreted into the host cytoplasm and further translocated into the host nucleus, where they induce expression of specific target genes through combinatorial action of an N-terminal, sequence-specific DNA-binding domain and a C-terminal transcription-activator-like domain (**Figure 2*b***) (28, 29, 99). The TALEs bind to specific regions within the promoter, called effector binding elements (EBEs) of target genes to induce their expression. Accumulating evidence suggests that the interaction between TALEs and their respective target genes can be specific, i.e., one TALE induces one target, or that a number of TALEs functionally converge on the same target (25, 27) as a bet-hedging strategy of the pathogen.

Several Xoo TALEs have been shown to bind to specific EBEs of *Os*SWEET genes to induce their expression during infection. The Xoo TALEs PthXo1 and PthXo2 specifically induce expression of the rice genes *OsSWEET11* and *OsSWEET13*, respectively (239, 254). There is evidence that the induction of these *S* genes increases export of glucose and sucrose into the apoplast, facilitating colonization by bacteria (41). Similarly, the Xoo TALEs AvrXa7, PthXo3, TalC, and Tal5, which are present in different Xoo strains, functionally converge to bind EBEs of *OsSWEET14* and subsequently induce its expression. AvrXa7 and PthXo3 share a largely overlapping binding site, whereas Tal5 and TalC bind to unique EBEs in the *OsSWEET14* promoter (25). Intriguingly, there is a clear correlation between TALE-mediated expression of *OsSWEET14* and virulence in AvrXa7-, PthXo3-, and Tal5-carrying strains, suggesting a specific function of these TALEs in regulating *OsSWEET14* during infection. In contrast, modifications in the *OsSWEET14* binding site of TalC does not result in enhanced resistance, suggesting that TalC can induce other, not-yet-identified *S* genes and thus acts nonredundantly to promote host colonization (25, 244).

The identification of SWEET genes as susceptibility factors spawned the idea to genetically modify these genes to enhance resistance against bacterial blight (**Table 1**). However, their importance in pathogen-independent, developmental processes restricts the extent of possible modifications, as complete deletions of SWEET genes can lead to undesired, developmental phenotypes (41, 47). Li et al. (134) developed a method to precisely modify the EBEs of AvrXa7 and PthXo3 in the *OsSWEET14* promoter to prevent induction by the pathogen without altering its developmental function. The authors deployed designer TALEs (dTALES) and a fusion of the AvrXo7 DNA-binding domain with the nuclease FokI to generate small indels in the overlapping EBEs of AvrXa7 and PthXo3. T1 plants carrying either homozygous monoallelic or heterozygous biallelic mutations within the EBE are resistant to both AvrXa7- and PthXo3-dependent Xoo strains and morphologically indistinguishable from wild-type plants, indicating that pathogen-independent developmental functions of the gene are not altered (134). Importantly, approximately 13.5% of

the T1 plants carrying the desired mutations in the EBEs and showing resistance against Xoo did not contain the TALEN or hygromycin-resistance genes, proving that genetic segregation can be used to produce transgene-free plants (134).

Using a similar approach, Blanvillain-Baufum et al. (25) modified the EBEs of AvrXa7 and Tal5, which share a partly overlapping EBE, and the distinct TalC EBE of *SWEET14* in rice. Modifications in the AvrXa7/Tal5 EBE region result in resistance against Xoo strains that are dependent on either of the TALEs. Intriguingly, deletions in the TalC EBE result in a complete loss of *OsSWEET14* induction after inoculation with TalC-dependent strains as expected but do not confer resistance, indicating a virulence function of TalC on additional, genetically redundant susceptibility targets (25). These results demonstrate that a better understanding of susceptibility factors in agricultural crops is needed to design knowledge-based resistance strategies and efficiently deploy genome editing to enhance resistance.

Rice Ethylene Response Factor 922

OsERF922 encodes a transcription factor of the ethylene response factor (ERF) subfamily of the APETALA2/ethylene response factor (AP2/ERF) superfamily in rice. Members of this family are involved in various developmental and stress-response pathways, including abiotic and biotic stress responses, such as salinity and drought tolerance as well as response to pathogens (58, 138, 158). ERFs most likely function at nodes of different stress responses and might be involved in integrating and balancing both synergistic and antagonistic signaling pathways through cross talk with the phytohormones abscisic acid, jasmonic acid, and ethylene (155). Although it seems that ERFs are plant-specific transcriptional regulators, there is evidence suggesting horizontal acquisition of AP2/ERF domains from homing endonuclease genes of either viruses or bacteria followed by an expansion of the gene family in higher plants (160). The number of AP2/ERFs in monocots ranges from 53 members in barley to more than 180 in maize with the majority belonging to the ERF subclade (58). The rice genome harbors 164 AP2/ERF genes, of which 113 belong to the ERF subclade (160).

Functional analyses of AP2/ERF transcription factors proved that ERFs can act as both positive and negative regulators of immunity (21, 97, 251). Overexpression of *Arabidopsis* ERF1, tomato Pti4/5, tobacco OPBP1, wheat ERF1, and rice BIERF3 results in increased stress tolerance and enhanced resistance against various pathogens, indicating positive regulation of abiotic and biotic stress responses (21, 38, 84, 86, 255). In contrast, silencing of the potato ERF3 gene leads to enhanced resistance against *P. infestans* (214), and the rice OsERF922 acts as a negative regular of immunity during infection by *M. oryzae* (138). Therefore, the discovery of specific ERFs as negative regulators of plant immunity led to the idea to edit these genes to enhance resistance against pathogens (**Table 1**). Recently, Wang et al. (231) deployed CRISPR-Cas9-mediated genome editing with an sgRNA to introduce indels close to the translation initiation codon of *OsERF922* in the japonica rice variety Kuiku131. Six transgene-free, homozygous T2 mutant lines carrying different frameshift indels showed enhanced resistance against the *M. oryzae* isolate 06-47-6. Edited lines were further tested for effects on agronomic traits like plant height, flag leaf length, flag leaf width, number of panicles, panicle length, seed setting rate, and seed weight. None of the observed traits significantly differed from wild-type plants, indicating that modification of *OsERF922* can produce plants with enhanced resistance without negatively affecting plant development (231).

Downy Mildew Resistance 6

DMR6 (downy mildew resistance 6) belongs to the superfamily of 2-oxoglutarate (2OG) Fe(II)-dependent oxygenases. In plants, these oxygenases are often involved in hydroxylation or

desaturation steps in plant hormone synthetic pathways (90, 114). Van Damme et al. (221) originally identified *DMR6*, together with five other loci (*DMR1–6*), in a loss-of-susceptibility EMS mutant screen in *A. thaliana*. The identified *dmr3*, *dmr4*, *dmr5*, and *dmr6* mutants showed induced *PR* gene expression in the absence of pathogen infection, suggesting a function as negative regulators of immunity (221, 222). DMR6 is specifically upregulated during pathogen infection. The function of DMR6 as a negative regulator of plant defense response was further confirmed by Zeilmaker et al. (248) demonstrating partial functional redundancy of DMR6 with the phylogenetically related oxygenase DLO1 (DMR6-like oxygenase 1) during infection with *Pseudomonas syringae* DC3000 and *Hyaloperonospora arabidopsidis*. Both *dmr6* and *dlo1* single mutants show partial resistance and have increased salicylic acid levels, whereas overexpression mutants are more susceptible, suggesting a function in phytohormone regulation during pathogen attack (221, 222, 248).

In a translational approach, de Toledo Thomazella et al. (56) identified two DMR6 orthologs in tomato (*Solanum lycopersicum*), *Sl*DMR6–1 and *Sl*DMR6–2, of which only *Sl*DMR6–1 is upregulated during infection with *P. syringae* pv. *tomato* or *Phytophthora capsici* (**Table 1**) (113, 240). Using the CRISPR-Cas9 system, the authors introduced small indels in exons 2 and 3, generating mutant plants with truncated versions of *Sl*DMR6 that disrupt the active site of the enzyme. One homozygous T1 line, carrying a 7-bp deletion in exon 3 was further characterized in virulence assays against three bacteria (*Xanthomonas gardneri* Xg153, *Xanthomonas perforans* Xp4b, *P. syringae* DC3000) and the oomycete *P. capsici* LT1534. The obtained CRISPR-Cas9-induced mutation in *SlDMR6* enhances broad-spectrum, partial resistance against all tested pathogens, without altering plant development and morphology.

Eukaryotic Initiation Factor 4E

The eukaryotic initiation factor 4E (eIF4E) is part of the translation initiation complex, which is involved in mRNA translation in all eukaryotes (92). As a central part of the initiation complex, it binds to the 5′ 7-methylguanylate (5′-m7GpppN) cap structure of mRNAs and to eIF4G and, together with other factors, mediates the recruitment of the 40s ribosomal subunit to the mRNA 5′ end to ultimately initiate translation. Plant eIF4Es have also been identified as essential host susceptibility components for viral infection. Since then, natural resistance alleles have been identified and have since become the largest group of recessive virus resistance genes in both mono- and dicotyledonous species (93, 186).

In plants, eIF4E belongs to a multigene family, and recent evidence suggests that eIF4E is under strong positive selection. Intriguingly, most of the positively selected sites also seem to be involved in recessive immunity against viruses of the family *Potyviridae*, a group of single-stranded, positive-sense RNA viruses (10, 93, 157). The genomic RNA of these viruses is polyadenylated at the 3′ end, and the 5′ end is covalently linked to the viral genome-linked protein (VPg), which mimics the eukaryotic 5′ cap structure (215). VPg also binds to eIF4E to recruit the translation initiation complex and thus initiates viral replication (230). Natural resistance conferred by the eIF4E alleles is frequently overcome by the virus, mainly through nonsynonymous mutations within VPg (44, 76). This coevolutionary interplay led to a diversification of both eIF4E and VPg. Deployment of natural resistance alleles to confer enhanced virus resistance is often not durable because of high VPg mutation rates and genetic redundancy within the eIF4E family (17, 131).

Some eIF4E isoforms are linked to recessive resistance against viruses in various plant species, including *A. thaliana*, pepper, lettuce, tomato, cucumber, and melon (42, 63, 149, 166). Silencing or mutagenesis of eIF4E or the isoform eIF(iso)4E in tomato and melon enhanced resistance against viruses by disrupting the interaction with VPg (149, 184).

In a recent study, Chandrasekaran et al. (42) used CRISPR-Cas9 mediated gene disruption to enhance eIF4E-dependent, recessive resistance in cucumber (**Table 1**). The cucumber genome encodes for one copy of each eIF4E and eIF(iso)4E, which share 56% nucleotide identity. Targeting of eIF4E within nonhomologous regions of exons 1 and 3 with specific sgRNAs generated small deletions in either of the targeted sites. Nontransgenic, heterozygous T1 plants were crossed to produce homozygous T3 plant lines. The tested lines showed increased resistance against various types of viruses, including *Cucumber vein yellowing virus* (Ipomovirus) and potyviruses *Zucchini yellow mosaic virus* and *Papaya ring spot mosaic virus*-W, whereas heterozygous lines were highly susceptible, as is expected because of the recessive nature of eIF4E-mediated resistance (42).

PROMISES AND LIMITATIONS OF GENOME-EDITING TECHNOLOGIES

The emergence of precision genome-editing technologies has ushered in a revolution in fundamental and applied research by providing plant biologists with an impressive set of applications as described above and reviewed elsewhere (7, 16, 20, 62, 95, 112, 180, 229, 242). In particular, CRISPR-Cas nucleases have generated enormous excitement and huge business interest and investments across many biological fields, including the agribusiness and crop improvement sectors. From a business point of view, the technology allows speeding up trait improvement and commercialization of new varieties that respond to the need for more yield due to population growth, climate change, and the constant evolution of pathogens.

Advantages of CRISPR-Cas Technology Over ZFNs and TALENs

The original implementation of ZFN and TALEN site-directed nucleases for genome editing has proven to be cumbersome, as it requires sophisticated protein design, synthesis, and validation (112, 181, 228). Although these SSNs ultimately perform similar genome modifications, the CRISPR-Cas system offers a number of advantages in plant biology (7). The technology is cheap and relies on sgRNAs to confer target-site specificity (105). The guide RNA confers DNA target specificity and can be readily designed based on the genome of choice and in a multiplexed fashion in which several genomic loci can be perturbed or edited at the same time (159). Also, the system creates stable and heritable mutations, which can be easily segregated from the Cas-sgRNA construct to produce transgene-free plants in only a few generations (33, 69, 162). Moreover, the creation of transgene-free, edited varieties offers high potential for plant breeding. A recent example is highlighted by the rapid production of Tomelo in approximately nine months, resulting in a transgene-free tomato line that resists powdery mildew infections (**Figure 4**) (162). The 48-bp chromosomal deletion created by CRISPR-Cas9 in the genome is indistinguishable from naturally occurring mutations. Also, an unbiased approach using genome-wide sequencing analysis showed the absence of T-DNA and detectable off-target mutations in the genome of the edited plants (162). Finally, the translational potential of CRISPR-Cas-based technologies is particularly attractive, as the number of editing tools is continuously increasing (e.g., Cas9, Cpf1, and Cas13) (159, 246). The new sets of CRISPR-Cas systems, such as Cpf1 and Cas13a nucleases, underscore the promising applications in plants for genome editing, transcriptional regulation, and future pathogen surveillance of plant disease outbreaks (1, 2, 196, 249).

CRISPR-Cas Specificity and Efficiency

The CRISPR-Cas9 system undeniably possesses excellent potential for genome editing. However, some factors such as PAM specificity, rational design of the sgRNA, and off-target activity can

limit its efficiency and specificity (20, 62, 191, 242). One of the key factors constraining the Cas9 specificity is the nature of the PAM sequence located immediately downstream of the protospacer element. The stringent nature of the Cas9 PAM sequence (requirement of the nucleotide sequence NGG) limits the range of target sequences. Although multiple mismatches in the PAM distal region can be tolerated by Cas9, this was shown to significantly reduce its affinity to the target site (73, 96, 172).

A second factor that determines Cas9 specificity is the tolerance of mismatches in the PAM distal region. Although NGG-PAM sequences are frequently found in plant genomes, specific genomic regions may be difficult to target with these sequence constraints, especially in the context of highly AT-rich genomes (249). The target range limitations by the NGG-PAMs have been overcome by the discovery of alternative PAM sequences generated by introducing mutations into the PAM-interacting domains of wild-type *Sp*Cas9 (121). This PAM sequence includes motifs such as NGCG (Cas9 variant VRER, D1135V/G1218R/R1335E/T1337R), NGAG (Cas9 variant VQR, D1135V/R1335Q/T1337R), and NGAG (Cas9 variant EQR, D1135E/R1335Q/T1337R) (6). An extended protospacer region has also been discovered in *Nm*Cas9 (24 nts instead of 20 bp), which enhances Cas9 specificity and increases the number of genomic loci that are amenable to targeting by CRISPR-Cas9 (94). Moreover, CRISPR-Cpf1 has different PAM sequences that accommodate AT-rich regions and complement the popular *Sp*Cas9 system (249).

Editing efficiency of Cas9 depends on the sequence, location, and context of the target (198, 219). This varies among targeted plants (monocots and dicots) and depends on many factors, including codon optimization of Cas9 for the plant species and use of promotors to drive Cas9 (e.g., p35S, pUbiquitin) in the germline and/or the dividing tissues and sgRNA expression (U3 or U6 promotors) (20, 144). Moreover, the transformation method (i.e., transient or stable expression methods) directly affects Cas9 level in the cell and thus may affect editing efficiencies (191, 217, 242).

Off-site targeting has been a challenge for genome editing, especially in mammalian studies (20, 62, 219). It is defined by Cas9 tolerance to mismatches in the RNA guide sequence and is dependent on the number, position, and distribution of mismatches throughout the guide sequence beyond the initial seed sequence (62). Multiple methods have been developed to improve target recognition fidelity using sgRNA truncated by two or three nucleotides (73) and paired nickases (14, 20, 85, 95, 146, 182, 191). Here the RuvC domain of Cas9 is inactivated, converting the nuclease to a nickase, which induces a SSB. Two nicks are then induced in close proximity, ultimately producing a DSB and an efficient indel formation (95). The ratio of off-site targeting was monitored in various organisms, but it is acknowledged that off-target activity is commonly low in plants and that it is possible to eliminate undesired mutations by backcrossing (20, 31, 70, 161). Expectedly, off-site targeting is locus dependent and is affected by the complexity of the plant genome, rational design of the sgRNA, and delivery strategy (20, 31, 242). Tsai et al. (218) showed that target sequences with a GC content higher than 70% may increase the likelihood of off-target effects. Recently, off-target cleavage frequency was shown to be reduced when using preassembled complexes of purified Cas9 protein and guide RNA (ribonucleoproteins complexes or RNPs) instead of DNA on vectors (236). Woo et al. (236) reported that no off-target mutations were detected in the genome when transfecting RNP complexes into lettuce protoplasts. High-throughput sequencing detected no off-target mutations at 91 homologous sites that differed by one to five nucleotides from the target site. Finally, to design sgRNA and detect potential off-site targeting in plants, various bioinformatics tools, such as Cas-OFFinder (11), CasOT (237), and CRISPR MultiTargeter (178), were developed for multiple organisms. Whole-genome sequencing is unambiguously an unbiased approach to detect CRISPR-Cas9 off-targets, and so far the rates in plants have been very low (162, 175).

PERSPECTIVES FOR GENOME-EDITED CROPS

The pace and scale of genome editing have triggered a major revolution in plant biology and are poised to equally impact plant breeding. Genome-editing technologies can accelerate crop improvement, as they produce precise genetic modifications in a variety of species and can yield a desired trait in a relatively short time compared to traditional breeding (162, 189, 234). How genome-edited crops will be regulated is under evaluation, sometimes as part of a general evaluation of new breeding plant technologies (~20 techniques). Numerous reports have been produced by various advisory and regulatory bodies (18, 34, 35, 64–66, 193, 207) and scientific experts in Europe and elsewhere (9, 52, 89, 102, 108, 128, 189, 202, 224, 229). The existing legal framework in the European Union accommodates genome-edited crops within GMO legislation until clarification of their legal status. The United States Department of Agriculture (USDA) has recently changed regulation of genetically engineered plants in favor of new breeding technologies. Plants generated by genome editing are no longer regulated by the USDA if the resulting mutations are indistinguishable from mutations that could occur naturally or could be the result of traditional breeding technologies. However, this question is still under evaluation by the USDA regulatory partnerships, the US Food and Drug Administration (FDA), and the US Environmental Protection Agency (EPA). Overall, there is an urgent need to overhaul the regulations of GM products worldwide (128, 177). The present regulatory uncertainties have negatively impacted research and development of genome-edited crops and could interfere with international trade in commodity crops (52, 108, 109). Clearly, the current regulatory framework for genome-edited crops is untenable and contrasts sharply with the rapid dissemination of the technology. Nonetheless, it is evident that genome-edited crops are poised to become widely adopted and form a key tool in plant breeding.

SUMMARY POINTS

1. Genome-editing technologies and their applications to plant science have emerged rapidly to become an essential tool in plant biology.
2. The CRISPR-Cas9 system became the gold standard method for genome editing because of its simplicity, efficiency, and versatility.
3. A wide palette of natural and synthetic sequence-specific nucleases has further improved plant genome editing and given rise to new applications.
4. Genome editing has been applied to enhance plant resistance against various pathogens.
5. Susceptibility genes are prime targets for genome editing to improve plant disease resistance.
6. Modification of *S* genes offers exciting opportunities to complement classical *R* genes and help develop durable resistance.
7. The current legal framework for regulating genome-edited crops is in flux and is in urgent need of revamping.

DISCLOSURE STATEMENT

S.K. regularly consults for the plant biotechnology industry. T.L. and K.B. are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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