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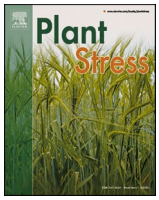

Review

Engineering biotic stress tolerance via CRISPR-Cas mediated genome editing in crop plants

Amjad Hussain [a,b], Mamoona Munir [c], Awais Khalid [d], Musrat Ali [e], Mohammed Amanullah [f], Qurban Ali [a,*], Hakim Manghwar [g,*]

[a] Department of Biology, College of Science, United Arab Emirates University, Al-Ain, Abu-Dhabi, UAE
[b] Hubei Hongshan Laboratory, National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China
[c] Department of Botany, Rawalpindi Women University, Rawalpindi, Pakistan
[d] Department of Physics, College of Science and Humanities in Al-Kharj, Prince Sattam bin Abdulaziz University, Al-Kharj 11942, Saudi Arabia
[e] Department of Plant Pathology, Faculty of Crop Protection, Sindh Agricultural University, Tandojam, Pakistan
[f] Department of Clinical Biochemistry, College of Medicine, King Khalid University, Abha, Saudi Arabia
[g] Lushan Botanical Garden Jiangxi Province and Chinese Academy of Sciences, Jiujiang, Jiangxi 332900, China

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ABSTRACT

Plants are incessantly challenged by a plethora of plant pests and pathogens, putting global agricultural productivity and food security at stake. Over several decades, various strategies have been developed in agriculture to overcome plant diseases and insect pests. With chemical control that remains effective but involves severe ecological and environmental concerns, conventional and transgenic breeding strategies have been primarily deployed to generate new varieties with novel genetic mutations. Though these strategies present a pivotal role in plant development, in part, they normally include extensive and labor-intensive processes. CRISPR-Cas technology, a genome editing tool, has opened new avenues to accelerate plant breeding by creating disease and pest resistance in a wide range of plants. CRISPR-Cas revolutionized agriculture by limiting yield losses due to biotic stress and minimizing reliance on pesticide usage. Here, we summarize the advances of CRISPR-Cas technology and the applications of this technology in disease and pest resistance development in crop plants. In addition, the review also discusses the advantages and concerns of CRISPR-Cas genome editing in crop plants.

1. Introduction

With an estimated 9.7 billion people on the planet by 2050, the need for agricultural production is anticipated to rise by 15 % over the next 10 years(Zhao et al., 2022). Global food security is significantly threatened by multiple factors, including the rapidly growing human population and climate change, the spread of various biotic stressors, such as bacteria, fungi, viruses, and insect pests (Manghwar and Hussain, 2022). According to estimates, pathogens and pests cause losses in crops that range from 19.5 to 41.1 % for maize, 24.6 to 40.9 % for rice, 10.1 to 28.1 % for wheat, 11.0 to 32.4 % for soybeans, and 8.1 to 20.0 % for potatoes (Savary et al., 2019). The most harmful plant pathogens are fungi and oomycetes, which are taxonomically distinct but have similar filamentous development and host-infecting structures (Manghwar et al., 2021; Ullah et al., 2018). For example, *Phytophthora infestans* is an oomycete pathogen that caused the Irish potato famine (Turner, 2005), and *Cochliobolus miyabeanus* is a fungal pathogen causing rice brown spot, which brought Bengal famine. These pathogens can rapidly devastate crops and create severe starvation (Chakrabarti, 2001). Combating diseases and pests depends primarily on protecting crops against the attack of pathogens and pests.

So far, numerous strategies have been applied in different agricultural systems for managing pests and diseases, including chemical control, which is considered a quick and effective way to get rid of infections. However, chemical control methods are being restricted because of their adverse effects on the environment and the rise of pathogen resistance (Yin and Qiu, 2019). Biological control is another method that is safe for the environment, but its poor efficacy, inconsistent application, and lack of cost-effectiveness limit its application (Ali et al., 2022; Gerbore et al., 2014). As an alternative, host resistance offers a desirable solution to the issues mentioned above. Over the past few decades, traditional plant breeding methods, including

* Corresponding authors.
E-mail addresses: rattarqurban@uaeu.ac.ae (Q. Ali), hakim@lsbg.cn (H. Manghwar).

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chemical/physical mutagenesis and conventional intergeneric crosses, have had a substantial global impact on guaranteeing food security (Tester and Langridge, 2010). Nonetheless, these techniques are non-specific and have a few limitations: (I) The creation of new cultivars using conventional breeding methods requires a sustained effort because genetic crosses and segregating progeny selection are typically labor- and time-intensive processes, (II) these techniques can only be deployed between plants that can only mate with each another, which prevents the introduction of new traits from other species (Hartung and Schiemann, 2014) and (III) it frequently adds other traits in addition to the intended resistance characters, such as those that have undesirable impacts on yield (Gao, 2018).

Nevertheless, the advent of genetic engineering involving biotechnology has brought new horizons in crop breeding. It has enabled us to introduce desired traits in crop plants by directly editing and inserting stable and heritable changes within the plant genome. It offers various benefits over conventional breeding approaches for instance, targeted trait modification, such as the development of resistance to insects and phytopathogens by inserting, deleting, or fine-tuning selected gene(s), is more straightforward and less laborious. Plants with desired traits can be achieved in fewer generations (Tyagi et al., 2021), Fig. 1. One of the major advantages of genetic engineering is that it has successfully bypassed the species barrier by offering the exchange of genetic material among different species (Das et al., 2022). Over the last twenty years, development in agricultural biotechnology has been dramatically enhanced due to increasing DNA knowledge. In addition, the complete genome sequencing of several plant species has revealed important insight into plant innate immunity, which provides an increasing number of targets for controlling pests and pathogens (Sun et al., 2024; Yin and Qiu, 2019). Particularly, negative regulators of plant disease resistance, considered as host susceptibility (*S*) genes, facilitate pathogen colonization and infection in host plants, have been the primary target for developing sustainable resistance in plants. Multiple *S* genes have been identified and successfully manipulated in various plants, including *MLO* in Barley, *IRG1* in *Medicago*, and *SWEET* in rice represent promising targets for genome editing (Li et al., 2014; van Schie et al., 2014). In addition, resistance development based on *R* (resistance) genes has been another target for plant genetic engineering. The largest group of *R* genes in plants encode nucleotide-binding site (NBS)–leucine-rich repeat (LRR) proteins. LRR domain recognizes pathogen-derived elicitors to provide resistance, and if modified, it could recognize elicitors from a broad spectrum of pathogens (Yue et al., 2012). To date, various *R* genes have been identified and introduced in plants, such as bacterial streak resistance was developed in rice by ex-pressing maize *Rxo1* gene (Zhou et al., 2010), bacterial spot resistance in tomato by utilizing peeper *Bs2 R* gene (Kunwar et al., 2018), resistance to stem rust in wheat (Brunner et al., 2012). Overexpression of the *Rpi-vnt1.1* gene rendered potato late blight resistance in potato (Dong and Ronald, 2019). Along with pathogens, transgenic technology has also shown promise in pest resistance and the success of this technology is no more evident than *Bt*-cotton. Most of the insect-resistant transgenic plants, including cotton, rice, and corn, have been developed by manipulating cry genes isolated from *Bacillus thuringiensis* (Chen et al., 2011; Li et al., 2020; Mao et al., 2011). Though transgenic technology has been successful in plant disease and pest management for several years, plants generated through transgenic method are considered genetically modified (GM), and the adoption of GM crops involves expensive and time-consuming regulatory approval, which has restricted the use of this technology to

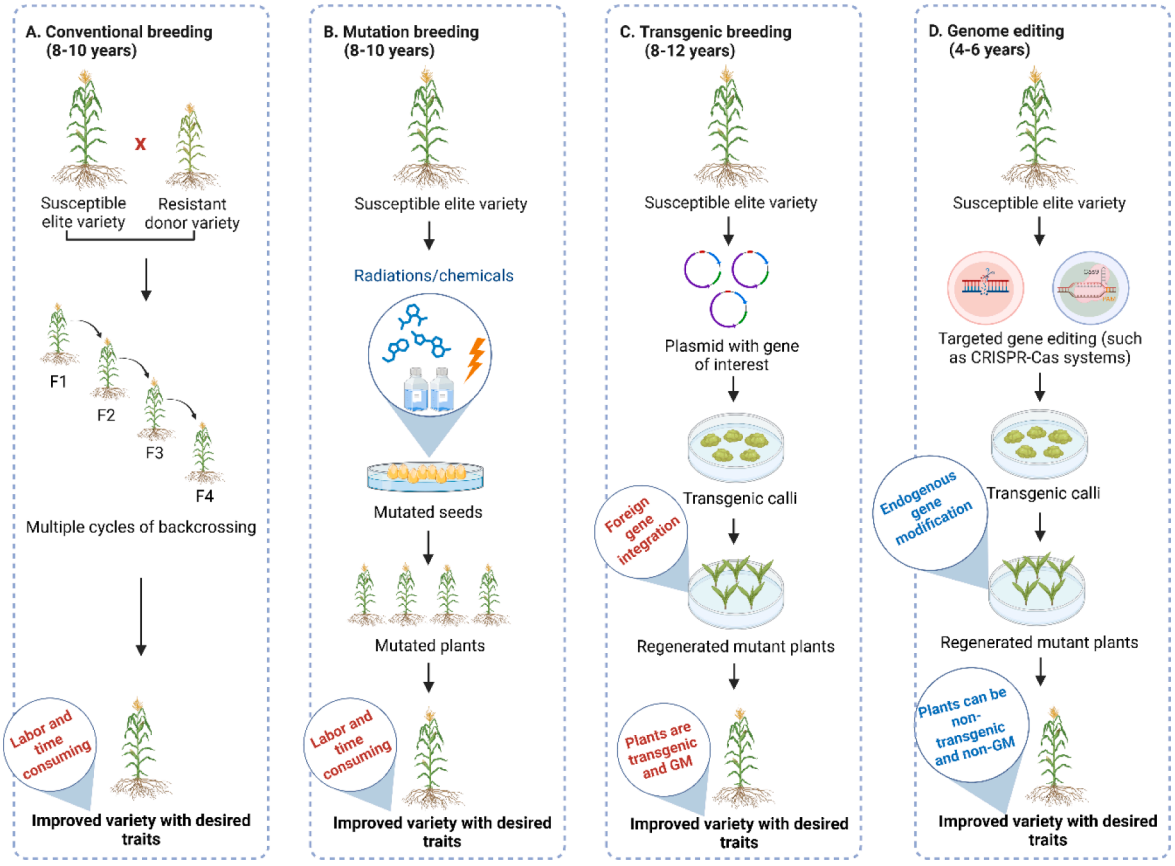

Fig. 1. Methods to create disease and insect pest-resistant plants. Though conventional, mutagenesis, and transgenic breeding techniques have long been deployed for disease and pest management, these systems require a lot of time and labor. Alternatively, CRISPR-Cas genome editing has brought a revolution in plant molecular breeding by enabling precise gene modification within the given plant genome, which can accelerate the creation of resistant crops that will ultimately lead to increased crop production in a sustainable manner that can alleviate the challenges levied by the growing world population and climate change.

a few crops (Prado et al., 2014). Moreover, most of the *S* and *R* genes utilized so far are monogenic, and the strong susceptibility and resistance can be encoded by multiple genes (van Schie et al., 2014). On the other hand, the resistance development in insect pests has raised another concern.

To overcome the problems of transgenic technology, genome editing technologies, particularly the clustered regulatory interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) system, is the most suitable option. CRISPR-Cas systems make targeted and precise genetic modification of crops more practical and hasten the transition toward precision breeding for improving desired traits (Wang et al., 2022). The CRISPR-Cas systems have developed into potent methods of altering genes during the past ten years and have significantly revolutionized the field of crop breeding. This technology has gained momentum due to its simplicity, versatility, cost-effectiveness, and high efficacy (Ahmad et al., 2021; Hussain et al., 2021). Current advances in genome editing technology have expanded the range of CRISPR-Cas toolkit. Base and prime editing, for instance, are the more accurate, effective, and promising methods that allow programmed targeted point alterations by nucleotide substitution (Naso and Petrova, 2019).

CRISPR-Cas9 also offers multiplex genome editing that targets multiple genes simultaneously to modify crops for better quality, greater yield, disease resistance, and insect resistance, which can be a useful and ecologically benign agricultural strategy. In particular, base editing technologies can produce non-genetically modified (non-GM) crops since they do not use foreign DNA. Many nations have exempted non-GM crops made with CRISPR-Cas systems from GMO rules (Zhang et al., 2020a). Therefore, the creation of non-GM disease and insect-resistant plants using genome editing is the most efficient alternative to conventional and transgenic technologies that can offer a financially feasible solution to help producers manage diseases and pests. In this work, we present the latest advances in CRISPR-Cas technology, with a focus on base editing, prime editing, and the CRISPRa and CRISPRi systems. We also discuss potential CRISPR-Cas applications for plant disease and pest resistance. The possible advantages and concerns of CRISPR-Cas genome editing in plants are also discussed.

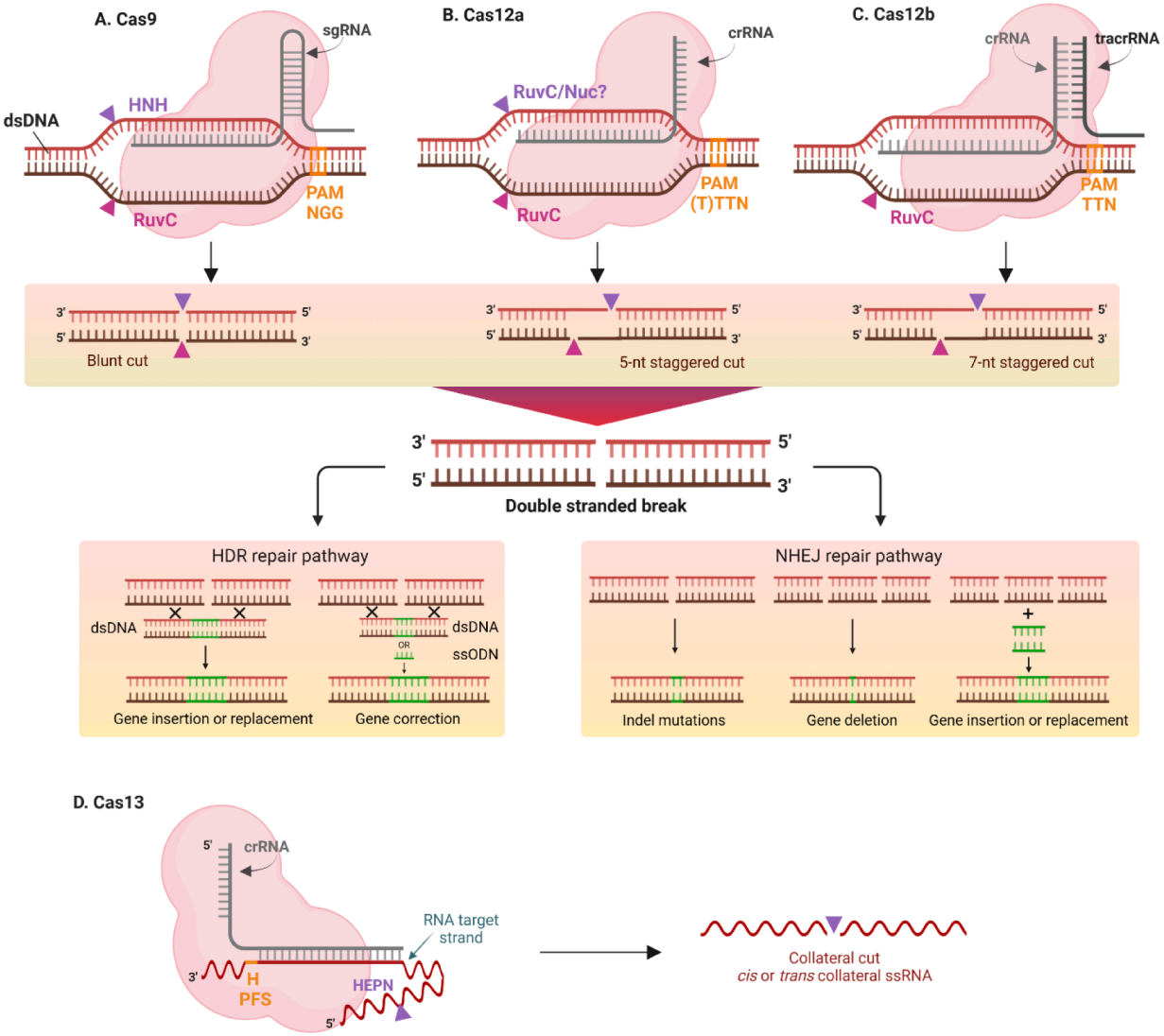

Fig. 2. Gene editing mechanism of different CRISPR-Cas systems. Guided by sgRNA, Cas9 induces DSBs 3 bp upstream of the PAM sequence (NGG) **(A)**. Unlike Cas9, Cas12a utilizes only crRNA and recognizes T-rich PAM sequence and causes 5–8 nucleotide 5′-overhangs by cleaving the target DNA **(B)**. Cas12b is a dual-RNA guided nuclease, recognizing a distal T-rich PAM that produces staggered DSBs **(C)**. The DSBs are subsequently fixed by HDR and NHEJ pathways. The HDR repair mechanism introduces precise DNA insertion as well as gene correction, while the NHEJ repair mechanism triggers loss-of-function mutation involving indels, gene deletion, and gene insertion. Cas13 is the newly evolved and distinctive CRISPR-Cas protein, which targets RNA instead of DNA. It involves two nucleotide-binding (HEPN) RNase domains that cleave target as well as non-target bystander RNA **(D)**.

2. Advances in CRISPR-Cas technology

For a variety of applications, including functional genomics and product delivery, the CRISPR-Cas-mediated genome-editing platforms have evolved into versatile tools for producing site-specific modifications in the genome at user-defined location(s) (Ahmad et al., 2020). Cas9 is a 160-kDa DNA-endonuclease and a Type II CRISPR Class 2 system. Due to the constantly changing requirements, many CRISPR-Cas systems have been created. These systems are divided into two classes with six types and 33 subtypes based on the effector proteins (Makarova et al., 2020). Since class 2 CRISPR-Cas systems use a single effector protein rather than the protein complex that class 1 systems use to induce double-stranded breaks (DSBs), they are more useful for gene editing. Class 2 CRISPR-Cas systems include Types II, V, and VI (Shmakov et al., 2017). The type II CRISPR-Cas system known as Cas9 has attracted almost every field of biological research. The Cas9 protein is a crRNA-dependent endonuclease, containing HNH and RuvC nuclease domains that respectively cut target and non-target strands of target DNA (Ishino et al., 2018). To guide Cas9 to the target sequence, a single-guide RNA (sgRNA) was created by joining crRNA and tracrRNA with a four-nucleotide tetra loop (Capdeville et al., 2021). Additionally, Cas9 requires a brief, orthologue-specific protospacer adjacent motif (PAM) right after the target sequence in order to connect to the foreign DNA (Manghwar et al., 2020). After binding to the target sequence (Fig. 2A), the two nuclease domains RuvC and HNH mediate DSB induction by cleaving the complementary and non-complementary strands, respectively, 3 bp upstream of the PAM sequence (Manghwar et al., 2019). Once the DSB is induced, the cell uses typically two processes to repair the DSB. One of these pathways, non-homologous end joining (NHEJ), frequently generates indels that result in loss-of-function mutations. The second method, known as homology-directed repair (HDR), corrects a mutation that already exists by introducing a template DNA sequence that helps to fix the break (Char et al., 2017).

A novel class II type V endonuclease called Cas12a (formerly Cpf1) demonstrates distinctive biochemical properties that make it an alluring tool for genome modification (Zetsche et al., 2015). Only a single crRNA is required to guide Cas12a endonuclease without the need of extra tracrRNA (Mahfouz, 2017). The diversity of protospacers is increased since just one RuvC domain is needed to detect the dsDNA targets employing T-rich PAM sequences, such as 5′-TTN/TTTN/TTTV-3′ (N = A/T/C/G; V = A/C/G) (Bandyopadhyay et al., 2020). Typically, it causes 5–8 nucleotide 5′-overhangs by cleaving the target DNA region 18–23 bases downstream from the PAM sequence (Das et al., 2022). Due to the longer cRNA and smaller Cas protein, the CRISPR-Cas12a technique is better suited for multiplexing as it can handle higher vector loads. It is gaining increasing attraction as a more potent replacement for CRISPR-Cas9 and a more flexible and powerful genome editing technology (Moon et al., 2018; Wang et al., 2017). Type V-B CRISPR effector Cas12b (previously C2c1) is another member of the Cas12 family (Fig. 2C). Unlike Cas12a, Cas12b is a dual-RNA guided nuclease (tracrRNA and crRNA, which are covalently linked and function as sgRNAs) (Liu et al., 2017). Similar to Cas12a, Cas12b recognizes a distal T-rich (5′-TTN-3′) PAM sequence and produces staggered DSBs (Strecker et al., 2019). DNA is cut between bases 14 and 17 in the target strand and 23 bases downstream of the PAM region in the non-target chain (Wu et al., 2017). As a result, Cas12b can produce DNA DSBs with 6–8 nt sticky ends. The application of Cas12b for gene editing has yielded positive results in plants (Ming et al., 2020).

The newest class 2, type VI CRISPR-Cas system, the CRISPR-Cas13 (Fig. 2D), typically targets RNA molecules rather than DNA molecules (Makarova et al., 2020; Shmakov et al., 2015). The most common Cas13 subtypes are Cas13a, Cas13b, Cas13c, and Cas13d (Abudayyeh et al., 2016; Cox et al., 2017; Konermann et al., 2018; Smargon et al., 2017). The Cas13 effector proteins contain two nucleotide-binding RNase domains (Gosavi et al., 2020). These two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains combine to form a single catalytic site that cleaves all bystander RNA randomly when activated by base pairing of its guide and a matched target RNA (Bot et al., 2022). To direct the Cas13-crRNA complex to the target site, the 5′- and/or 3′-protospacer-flanking site (PFS) is necessary. Cas13 proteins are used to locate, identify, and track various types of RNA molecules (Gosavi et al., 2020).

2.1. Base editing

The conventional CRISPR system uses the nuclease activity of Cas9 to create a DSB at a particular location. In gene knockout, this leads to numerous ambiguities (Chen et al., 2019). Base editors (BEs) are a new development in the CRISPR system. BEs use dCas9 (D10A) or nCas9 (D10A), the catalytically nuclease-deficient Cas proteins that enable exact base substitution in a programmable manner without DSBs or foreign DNA insertion, and resolve the problems associated with DSB (Molla and Yang, 2019). Different BE toolkits, such as the cytosine base editor (CBE), adenine base editor (ABE), and cytosine transversion base editor (CGBE), have been developed based on the various deaminases employed in the fusion.

The fusion of cytidine deaminase to nCas9 (D10a) and uracil glycosylase inhibitor (UGI) produces the CBE. U-G base pairing is the outcome of the CBE deaminating exocyclic amine to turn cytosine into uracil (Komor et al., 2016; Nishida et al., 2016). U is frequently recognized as an illegal base by uracil DNA glycosylase (UDG), which converts U back to the parent nucleotide C (Schormann et al., 2014). CBE initiates the mismatch repair system, which converts U-G to U, and employs UGI to prevent the conversion of U back to C. •A base pair is changed into a T-A base pair, which results in the replacement of C-G for T-A (Fig. 3A) (Eisen and Hanawalt, 1999; Jiang et al., 2020b). Numerous CBE variations have been created, the majority of which vary in the cytidine deaminase. Target-AID involves PmCDA—associated with activation-induced cytidine deaminase (AID) protein family fused to d/nCas9 (Molla and Yang, 2019), in contrast to BE1, BE2, BE3, and BE4, which use rAPOBEC1 (rate cytidine deaminase) (Nishida et al., 2016). It is well known that BE3 can introduce precise point mutations by deaminating cytosines from positions 3 to 9 in the protospacer inside editing window (Zong et al., 2017).

Unlike CBE, ABE was developed to target adenine bases. A-T to G-C substitution is produced through ABE by fusing Cas9n with DNA adenosine deaminase (Fig. 3B). The adenine in DNA is not known to be deaminated by any natural adenosine deaminases. In order to deaminate A in ssDNA, researchers created a deaminase variant (ecTadA*) based on *Escherichia coli* tRNA adenine deaminase (ecTadA) (Gaudelli et al., 2017). The ABE was created by fusing Cas9n with ecTadA-ecTadA* heterodimers. A mismatched DNA base pair with T is created when the sgRNA instructs ecTadA* to deaminate the A in the R-loop to inosine (I), which DNA polymerase interprets as G during DNA replication or repair. When Cas9n nicks the non-deaminated strand, it installs C as the broken strand is reformed, starting the DNA repair process. During DNA replication, the ABE succeeds in changing A-T to G-C base pairs (Li et al., 2021). The research continues to advance significantly, thanks to ongoing ABE tuning, which has dramatically increased the effectiveness, editing activity window, and breadth of the BEs (Huang et al., 2019; Zhang et al., 2021). The effectiveness of base editing has substantially boosted with the most recent ABE8e system. It evolved from an adenine base editor with the help of phages (Richter et al., 2020).

However, only base transitions can be induced by CBEs and ABEs. Thus, purine to pyrimidine and pyrimidine to purine base transversions have recently been catalyzed by CGBEs (Fig. 3C), which further expanded the base editing toolkit by incorporating C-G editing (Koblan et al., 2021; Zhao et al., 2021). A UNG (Uracil-N-glycosylase) fused to nCas9 (D10A) and either rAPOBEC1 (R33A) or rAPOBEC1 compose the CGBEs (Chen et al., 2021; Kurt et al., 2021). The enzyme cytidine deaminase turns C into U. UNG produces an apurinic/apyrimidinic (AP)

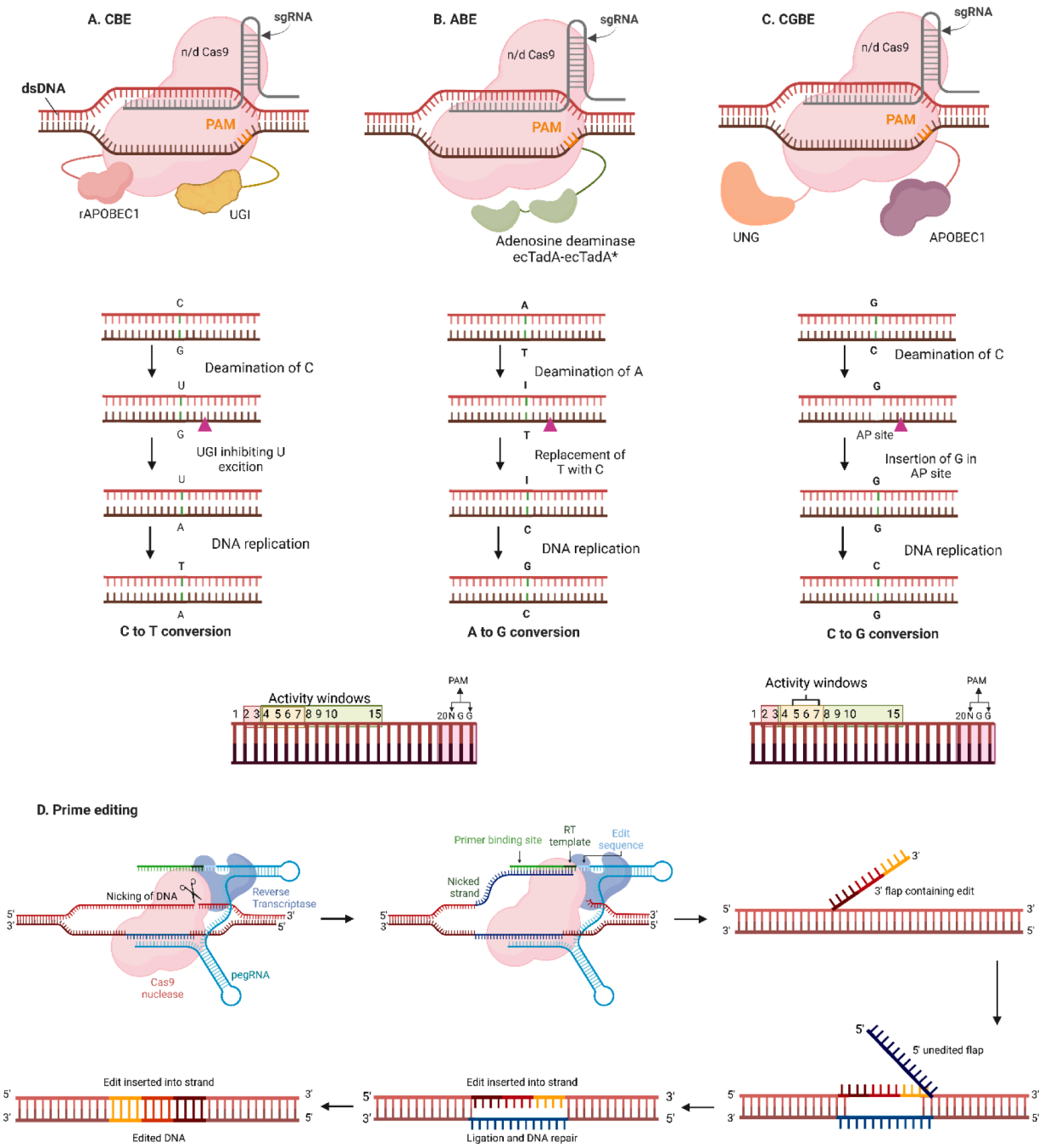

Fig. 3. Schematic representation of base editing and prime editing systems. CBE involves the fusion of nCas9 to cytidine deaminase rAPOBEC1 and UGI to carry out C-G to T-A base substitution at a target site **(A)**. The ABE was developed by fusing ecTadA-ecTadA* adenosine deaminase that deaminates adenine and provides A-T to G-C base substitution **(B)**. Another recently developed base editing system in plants is the CGBE system, comprising UNG fused to nCas9 with either rAPOBEC1 (R33A) or rAPOBEC1 and changes C to G at target loci **(C)**. Prime editing is the most advanced and versatile base editing system, which has greatly enhanced the ability of base editing by allowing the introduction of all mutation types, including insertions and deletions to transition and transversion base editing without causing a DNA DSB. In this system, a fusion protein made of nCas9 (H840A) and an improved Moloney murine leukemia virus reverse transcriptase (M-MLV RT) serves as the system's apoenzyme or Prime editor. The apoprotein involves an RT domain that uses the PE guide RNA (pegRNA) RT-template to reverse-transcribe a specific modification into DNA. Guided by the pegRNA, H840A binds to the target site through the spacer sequence in the pegRNA and creates a nick at the nontarget strand to create a single-strand break (SSB). It then binds to PBS to initiate reverse transcription followed by the formation of 3′ or 5′ flaps leading to DNA ligation and repair that result in DNA editing **(D)**.

site by removing U. Following error-prone polymerase activity, G is probably inserted at the AP site, resulting in base transversion editing (Molla et al., 2021). Another CGBE system was developed by combining X-ray repair cross-complementing protein 1 (XRCC1) with APOBEC1-nCas9 (Chen et al., 2021). The AP site is created in this version by a cellular UNG by eliminating the U generated by APOBEC1. A BER protein named XRCC1 then facilitates the preferred incorporation of a G at the AP site, leading to a C-to-G conversion. The CGBE has just recently been applied to plants after being initially developed in animal cells. Sretenovic et al. (Sretenovic et al., 2021), documented the simultaneous use of two different CGBEs in rice, tomato, and poplar plants. They found that rXRCC1-based CGBEs successfully edited C to G in

stably transgenic rice plants with mono-allelic editing efficiencies of up to 38 % in T0 lines. The study demonstrates how well CGBE systems work in plants, but more efforts are needed to improve the editing efficiency of CGBEs.

2.2. Prime editing (PE)

PE has raised genome editing to a new level since it enables the introduction of all mutation types—including insertions, deletions, and all putative 12 forms of base-to-base conversions—without creating a DNA DSB. All 12 types of point mutations can be introduced into target genes using the PE toolbox at locations up to 29 bp downstream and 3 bp upstream of a PAM sequence. Additionally, it allows deletions and insertions of up to 80 bp each (Anzalone et al., 2019). PE offers significant advances in genome editing compared to other systems, which only offer one base alteration at a time and require the challenging simultaneous delivery of a particular repair template. The system's apoenzyme or Prime editor is a fusion protein consisting of nCas9 (H840A) and an enhanced Moloney murine leukemia virus reverse transcriptase (M-MLV RT). The nCas9 variant has been modified to produce nicks rather than DSBs (Anzalone et al., 2019). Additionally, the apoprotein features an RT domain that reverse-transcribes a particular alteration (single or multiple nucleotide changes as well as brief indels) into DNA using the PE guide RNA (pegRNA) RT-template (Perroud et al., 2022). A featured pegRNA is created by combining an ordinary sgRNA with a spacer region for PE targeting, a primer binding site (PBS) for RT primer binding and RT initiation, and an RT template with edit(s) for targeted DNA modifications. Through the spacer sequence in the pegRNA, the H840A physically binds to the target genomic DNA site, creating a single-strand break (SSB) by nicking the non-target strand. The conjugated RTase effector then binds to the PBS in the pegRNA to initiate RT, which converts the pegRNA template sequence with the intended edit information to cDNA (Fig. 3D). The created cDNA is finally integrated into the target area through the endogenous mismatch repair process (Yang and Chen, 2020).

Following its initial use in mammalian cells, PE was then adopted for precise genome editing in plants. Success levels in rice, maize, wheat, potato, tomato, and legumes have ranged from moderate to high (Biswas et al., 2022; Butt et al., 2020; Jiang et al., 2020a; Lin et al., 2020; Lu et al., 2021). Although it has been demonstrated that utilizing various promoters, reverse transcriptase, and codon-optimized Cas9 nickase can boost the production of pegRNA, PE's editing efficiency in plants has only been moderate (Li et al., 2022; Xu et al., 2022). Later research considerably increased PE editing effectiveness by structuring the main editor and pegRNA. Xu et al. (Xu et al., 2022) enhanced the efficiency of plant prime editor 2 by using N-terminal reverse transcriptase-Cas9 nickase fusion and multiple nucleotide modifications in the reverse transcriptase template in rice and maize (PPE-2). Furthermore, by introducing T173I, A174V, and P177S (TAP-IVS) mutations in *EPSPS* and *OsACC1*, respectively, engineered PPEs have been used to create rice plants tolerant to glyphosate and aryloxyphenoxypropionate herbicides (Jiang et al., 2022; Xu et al., 2021). Achieving higher than 10 % desired PE in plants remained difficult, highlighting the need for further advancement. Although these studies have significantly increased the editing efficiency of PE, obtaining more than 10 % editing efficiency remained challenging in plants, calling for further advancement in this system. Additionally, PE in plants is still in its infancy, and the studies that have been reported so far have focused on systemic optimization. However, an optimized and highly effective PE system in plants can enhance crop productivity along with disease and pest resistance in plants.

2.3. CRISPRa/CRISPRi

In addition to gene editing, CRISPR-Cas systems have been used to control transcription and post-transcription regulation. It became possible by modifying the nuclease domains to create catalytically inactive Cas proteins (dCas), which can still bind to DNA with the aid of RNA but cannot induce DNA DSBs. The ability of the dCas proteins to fuse with effector proteins like transcriptional activators and repressors has enabled the development of CRISPR-mediated gene-specific activation and interference, i.e., CRISPRa and CRISPRi, respectively (McCarty et al., 2020). With the help of the dCas9 in CRISPRa, transcription activators are drawn to the region upstream of a target gene's promoter, which activates the gene. dCas-based CRISPRa proteins combined with activator domains present a promising alternative to the conventional gene overexpression technique in plants (Maeder et al., 2013; Pan et al., 2021a). It is theoretically possible for CRISPRa to precisely activate any target gene in the genome because it is RNA-guided. Furthermore, if numerous genes are implicated, CRISPRa is much more favorable than the traditional overexpression approach. dCas9-VP64 served as the foundation for the first-generation CRISPRa system in plants (Piatek et al., 2015). To promote the activation activity of CRISPRa system, different second-generation CRISPRa techniques have been developed, for example, dCas9-SunTag (Papikian et al., 2019), dCas9-TV (Xiong et al., 2021), and dCasEV2.1 (Selma et al., 2019). Because these methods were tested on different plant species or employing different gene and expression systems, it is unclear which method is the most efficient in plants. According to research by Lowder et al. (Lowder et al., 2018) in *Arabidopsis* and rice plants, CRISPR-Act2.0, a second-generation CRISPRa tool, provided more transcriptional activation than dCas9-VP64. Moreover, a third-generation CRISPRa system, called CRISPR-Act3.0, was recently created by Pan et al. (Pan et al., 2021b), and it has four to six times higher activation potency in rice and *Arabidopsis* compared to previous CRISPRa systems in plants.

CRISPRi is a relatively new programmable tool for targeted gene repression. Attaching to the promoter expanse in proximity to the transcription start site (TSS), and blocking RNA polymerase and transcription factor binding, the dCas proteins joined to transcriptional repression domains can hinder transcription start or elongation (Knott and Doudna, 2018). CRISPRi has recently been employed in plants, and a few studies have been reported yet. It has been demonstrated that the transcriptional repressors dCas9–3xSRDX (SUPERMAN Repression Domain X) and dCas9-SRDX reduced transcript levels to around 40 % of the control in *Arabidopsis* (Piatek et al., 2015) and *Nicotiana benthamiana* (Lowder et al., 2015). Two CRISPRi systems, 3SRDX and dCas9-SRDX, have recently been used in wheat and maize (Gentzel et al., 2020; Zhou et al., 2022). Nonetheless, the prevalent applications of CRISPRi for programmed targeted gene repression in plants are constrained due to the ineffectiveness of the available CRISPRi technologies. To maximize the potential of CRISPRi, the CRISPRa efficiency measures could be used (Fontana et al., 2020; Pan et al., 2021a). CRISPRi/a does not always affect gene expression levels to the desired extent. Future work is needed to increase the specificity of epigenetic modulation and the efficiency of activation and suppression systems in plants.

3. CRISPR-Cas applications for plant disease and pest resistance

Crop production is severely hampered by pests and diseases–caused by viruses, fungi, and bacteria, which diminish agricultural yield by 20–40 %. The use of modern genome editing tools in current agricultural settings can have a significant effect on crop resilience and productivity. By modifying either the host's *S* genes or the phytopathogenic agents' DNA to prevent their reproduction, we have been able to establish disease resistance in several crops through the use of several gene editing techniques (Karmakar et al., 2022). The endogenous plant genes, which induce infection and symptoms by pathogens in hosts during colonization are known as *S* genes. The absence of function of these genes may result in recessive resistance to plant diseases. Plants with *S* gene targeted resistance may offer a long-lasting defense. *S* gene-based resistance is caused by the silencing of a host component that is necessary for a pathogen to survive in the host (Tripathi et al., 2022). Precise plant

gene editing systems can facilitate obtaining these goals by the development of crops containing the traits of interest with greater acceleration and ease than conventional breeding approaches. Crop yields can be quickly increased by creating disease- and pest-resistant plants (Yin et al., 2017). Therefore, the use of these technologies to engineer plants to boost resistance to various diseases and pests has been driven by the distinctive qualities of modern gene editing techniques (such as CRISPR-Cas systems) outlined above. The applications of CRISPR-Cas systems in plant disease resistance are summarized in Table 1.

3.1. Resistance to fungal diseases

The majority of phytopathogens involve fungal species, posing a serious threat to agricultural output and crop protection costs worldwide. One of the most destructive fungal diseases that severely affects crop production is powdery mildew. Eliminating *S* genes or genes that encode negative regulators of disease resistance has been the primary objective of genome editing studies for fungal resistance (Karmakar et al., 2022). For instance, the well-known *S* genes in plants that protect them against various diseases include *MLO* (*MILDEW RESISTANCE LOCUS O*), *eIF4,* and *SR* (*Signal Response*). *MLO* is a powdery mildew fungus *S* gene that has undergone substantial research. More than 650 different types of powdery mildew fungus are affecting around 10,000 different plant species (Kusch and Panstruga, 2017; Moon et al., 2022b). Utilizing CRISPR-Cas9, all three *TaMLO* alleles were eliminated from wheat, resulting in the growth of plants that were more resistant to powdery mildew (Wang et al., 2014). Through *SlMlo1* gene deletion using CRISPR-Cas9 technology, powdery mildew-resistant tomato lines were developed (Nekrasov et al., 2017). Similar to this, CRISPR-Cas9 was used to silence the grapevine *VvMLO3* and *VvMLO7* genes, increasing the grapevine's resistance to powdery mildew (Malnoy et al., 2016; Wan et al., 2020). Additionally, a susceptible cucumber variety known as cv. Ilan was exploited to produce resistance to powdery mildew by CRISPR-Cas9-mediated targeted alteration of the *Csamlo8* gene (Shnaider et al., 2022). *Powdery mildew resistant 4* (*PMR4*), also known as *Callose synthase 12* (*CalS12*) gene, encodes callose synthase that produces callose in response to abiotic and biotic stress. It is also involved in salicylic acid (SA)-mediated defense pathway (Nishimura et al., 2003). *SlPMR4* was targeted using CRISPR-Cas9, which induced indel and inversion mutations in tomatoes. The study obtained reduced susceptibility to tomato powdery mildew in edited plants [104]. The *increased disease resistance1* (*EDR1*) is a known negative regulator of the defense response against powdery mildew in *Arabidopsis thaliana* and is highly conserved in all plant species (Frye et al., 2001). Interacting with MKK4/MKK5, negatively affects the kinase activity and protein levels of MPK3 and MPK6 to regulate plant innate immunity (Zhao et al., 2014). *TaEDR1* knock-down by RNAi or VIGS induced resistance to *Blumeria graminis* f. sp. *tritici* in hexaploidy wheat. The study then targeted three homologs of wheat *TaEDR1* using CRISPR-Cas9, where no off-target mutations were found, and the *TaEDR1*-edited plants showed increased resistance to the powdery mildew fungus without any mildew-induced cell death (Zhang et al., 2017). Microrchidia (MORCs) gene family in plants is associated with maintenance of genome stability and transcriptional gene silencing (Koch et al., 2017) and is involved in plant immunity (Kang et al., 2008). Barley mutants with single and double *HvMORC1* and *HvMORC6a* knockouts were produced using CRISPR-Cas9. The double knockout mutant lines (*hvmorc1/6a*) of barley were found to be more resistant as compared to single mutant lines employing bioassays with both biotrophic (*Bipolaris sorokiniana*) and necrotrophic (*Fusarium graminearum*) pathogens (Galli et al., 2022).

CRISPR-Cas9 has been effectively used to control a variety of fungal infections in plants, in addition to powdery mildew (Table 1). For instance, the second-worst disease to impact rice is sheath blight, which is caused by *Rhizoctonia solani* (Jung et al., 2022). Intriguingly, rice plants mutated with methyl esterase-like (*osmesl*) T-DNA insertion exhibited higher resistance to the sheath blight, rice blast, and bacterial blight brought on by *R. solani, Magnaporthe oryzae*, and *Xanthomonas oryzae* pv. *oryzae* (Xoo), respectively. In addition, RNAi and CRISPR knock out lines also showed resistance to all three pathogens. Based on qRT-PCR analysis, the study revealed that *osmesl* mutants had reduced expression of ROS (reactive oxygen species)-scavenging associated genes and ROS production, which led to resistance against these pathogens in rice (Hu et al., 2021). CRISPR-Cas9 was used to increase rice's resistance to the rice blast, a devastating fungal disease, by knocking out the *OsERF922* and *OsSEC3A* genes (Ma et al., 2018; Wang et al., 2016). The *WAT1* (*Walls are thin 1*) gene has been discovered as an *S* gene for *V. dahliae* in cotton and *Arabidopsis*. The *SlWAT1* gene disruption produced tomato mutants with growth retardation but resistance to a number of diseases, including *V. alboatrum, V. dahliae*, and *Fusarium oxysporum* f. sp. *lycopersici* (Hanika et al., 2021). A pentapeptide plant hormone called phytosulfokine controls signaling during plant growth and defense reactions. Higher resistance to *Fusarium oxysporum* f.sp. *niveum* was observed in edited watermelon plants as a result of the loss-of-function mutation of the phytosulfokine precursor encoding gene *Clpsk1* in watermelon. This mutation acts as a negative regulator in interacting with *Fusarium oxysporum* f.sp. *niveum* (Zhang et al., 2020b). *Brassica napus* was altered by targeting *BnCRT1a* to develop *Verticillium longisporum* (Vl43) resistance. *BnCRT1a*-mutated T2 plants exhibited reduced susceptibility to Vl43, possibly due to ethylene signaling pathway activation (Pröbsting et al., 2020). The Ta*NFXL1* gene was altered using the CRISPR-Cas9 technology to confer wheat with *Fusarium graminearum* resistance (Brauer et al., 2020). Using this method, *ZmFER1* in maize was modified to provide resistance to the *Fusarium* ear rot disease brought on by *F. verticillioides* (Liu et al., 2022). Anthracnose has been known as a major fungal disease caused by *Colletotrichum* spp. in chili (*Capsicum annuum*). Through targeted gene editing of *CaERF28* via Cas9, T-DNA and marker-free chili plants were achieved in T1 and T2 generations, resistant to *Colletotrichum truncatum* (Mishra et al., 2021). In addition, the applications of CRISPR-Cas systems have also been reported to increase potato resistance to *Phytophthora infestans*. Moon et al. (Moon et al., 2022a) created an RNP-mediated CRISPR-Cas9 method to target the *S* gene, *StSR4*, in potato (*Solanum tuberosum* L.) protoplasts and observed the higher expression of *StPR1, StEDS1*, and *StPAD4* in *StSR4*-edited plants. It enhanced *P. infestans* resistance but decreased the growth of plants. Moreover, Razzaq et al. (Razzaq et al., 2022) found that the gene knockout of *StERF3* resulted in the *P. infestans* resistance in potatoes.

3.2. Bacterial disease resistance

Many different types of plant pathogenic bacteria infect crops, leading to severe economic and crop yield losses. It is quite challenging to keep bacterial infestation under control because these phytopathogenic bacteria have a high rate of proliferation. To introduce desired genomic modifications into host crop plants' *S* genes and create resistance to several bacterial diseases, CRISPR-Cas9 offers an effective and affordable method (Table 1). *LATERAL ORGAN BOUNDARIES 1* (*CsLOB1)* is an *S* gene for citrus canker, which has an important role in promoting the growth of pathogens and the formation of erumpent pustule (Hu et al., 2014). Citrus canker resistance was successfully developed in grapefruit (*Citrus* x *pardisi*) by mutating the coding region of *CsLOB1*. By altering the promoter in *CsLOB1* with CRISPR-Cas9, it was possible to generate resistance to the *Xanthomonas citri* subsp. *citri* (*Xcc*) bacterium that causes citrus canker (Peng et al., 2017). Peng et al. (Peng et al., 2017) reported that the promoter of $CsLOB1^{-}$ and $CsLOB1^{G}$ alleles involves the effector binding element ($EBEP_{thA4}$) in *Citrus sinensis* Osbeck. The main effector of *Xcc*, the PthA4 recognizes $EBEP_{thA4}$ for the activation of *CsLOB1* expression for promoting citrus canker development. They designed five pCas9/CsLOB1 sgRNA constructs for altering the promoter region ($EBEP_{thA4}$) of *CsLOB1* and obtained 11.5–64.7 % mutation rate. $EBEP_{thA4}$-mutated plants showed increased citrus canker resistance as compared to the wild type. *Xanthomonas campestris* pv.

Table 1
Applications of CRISPR-Cas systems in plant disease resistance.

Resistance to Fungal Diseases					
CRISPR-Cas9	*Triticum aestivum* L.	*TaMLO-A1*	Indels	Resistance to *Blumeria graminis* f. sp. *tritici* causing powdery mildew	(Wang et al., 2014)
		TaEDR1	Frameshift mutations		(Zhang et al., 2017)
		TaNFXL1	Deletions	Resistance to *Fusarium graminearum*	(Brauer et al., 2020)
	Solanum lycopersicum	*SlMlo1*	Deletions	Resistance to Powdery mildew caused by *Oidium neolycopersici*	(Nekrasov et al., 2017)
	Vitis vinifera	*VvMLO3*	Indels	Resistance to Powdery mildew caused by *Erysiphe necator*	(Wan et al., 2020)
	Oryza sativa	*OsERF922*	Indels	Resistance to *Magnaporthe oryzae* causing of rice blast	(Wang et al., 2016)
		OsSEC3A	Indels	Resistance to *Magnaporthe oryzae,* dwarf stature, upregulation of SA synthesis- and pathogenesis-related genes	(Ma et al., 2018)
	Solanum tuberosum L.	*StSR4*	Indels	Conferred resistance to *P. infestans*, but reduced plant growth	(Moon et al., 2022a)
	Hordeum vulgare	*HvMORC1 and HvMORC6a*	Indels	Resistance to *Fusarium graminearum* and *Bipolaris sorokiniana*	(Galli et al., 2022)
	S. lycopersicum	*WAT1*	Deletions	Resistance to *V. albo-atrum, V. dahliae, and F. oxysporum f.* sp. *lycopersici*	(Hanika et al., 2021)
	O. sativa	*osmesl*	Knock out	Resistance to *Rhizoctonia solani, Magnaporthe oryzae*, and *Xanthomonas oryzae* pv. *oryzae*	(Hu et al., 2021)
	Cucumis sativus	*Csamlo8*	Indels	Resistance to Powdery mildew caused by *Podosphaera xanthii*	(Shnaider et al., 2022)
	Citrullus lanatus (Thunb.)	*Clpsk1*	Indels	Resistance to *Fusarium oxysporum* f.sp. *niveum*	(Zhang et al., 2020b)
	Brassica napus	*BnCRT1a*	Indels	Resistance to *Verticillium longisporum (Vl43)*	(Pröbsting et al., 2020)
	Zea mays	*ZmFER1*	Indels	Resistance to *Fusarium* ear rot disease caused by *Fusarium verticillioides*	(Liu et al., 2022)
	Capsicum annuum	*CaERF28*	Indels	Resistance to Anthracnose disease induced by *Colletotrichum truncatum*	(Mishra et al., 2021)
	S. tuberosum L.	*StERF3*	Indels	Resistance to *Phytophthora infestans*	(Razzaq et al., 2022)
Resistance to Bacterial Diseases					
CRISPR-Cas9	*Citrus sinensis* Osbeck	*CsLOB1*	Indels	Resistance to *Xanthomonas citri* subsp. *citri*	(Peng et al., 2017)
	Musa spp.	*DMR6*	Indels	Resistance to Banana Xanthomonas wilt caused by *X. campestris* pv. *musacearu*	(Tripathi et al., 2021)
	O. sativa	*OsSWEET11, OsSWEET13, and OsSWEET14*	Indels	Resistance to *Xanthomonas oryzae* pv. *oryzae* causing bacterial blight	(Xu et al., 2019)
		$EBE_{AvrXa23}$	Knock-in		(Wei et al., 2021)
		$EBE_{Tal2gXa23}$	Knock-in	Resistance to bacterial leaf streak, bacterial blight, and rice blast caused by *X. oryzae* pv. *oryzicola, Xoo*, and *Magnaporthe oryzae*, respectively	(Ji et al., 2022)
		Xa13, Pi21, and TMS5	Frameshift mutations	Rice blast and bacterial blight resistance	(Li et al., 2019)
		OsCUL3a	Deletions	Resistance to *Xanthomonas oryzae* pv. *oryzae*	(Gao et al., 2020)
	S. lycopersicum	*SlJAZ2*	Deletions	Resistance to *Pseudomonas syringae* pv. *tomato* DC3000 causing bacterial speck disease	(Ortigosa et al., 2019)
	Malus domestica	*MdDIPM4*	Indels	Fire blight disease resistance caused by *Erwinia amylovora*	(Pompili et al., 2020)
Resistance to Viral Diseases					
CRISPR-Cas9	*N. benthamiana*	*β-lactamase* and *ColE1* viral genes.	Indels	Resistance to bean yellow dwarf virus	(Baltes et al., 2015)
	S. lycopersicum	*TOM1*	Indels	Resistance to tomato brown rugose fruit virus	(Ishikawa et al., 2022)
		eIF4E1	Indels	Resistance to pepper mottle virus	(Yoon et al., 2020)
CBE	*Arabidopsis thaliana*	*eIF4E*	Single point mutation	Clover yellow vein virus resistance	(Bastet et al., 2019)
LshCas13a and FnCas9	*V. vinifera*	Hsp90h, Hsp70h, p5, CPm, and CP viral sequences	–	Resistance to Grapevine leafroll-associated virus 3 causing grapevine leafroll disease	(Jiao et al., 2022)
SpCas9	*S. lycopersicum*	*SlMlo1 and SlPelo*	Indels	Resistance to fungus *Oidium* sp. and *tomato yellow leaf* curl *virus*	(Pramanik et al., 2021)
CRISPR/Cas13a	*S. tuberosum* L.	Nib, CI, CP, and P3 conserved viral regions	–	Resistance to *Potato virus Y*	(Zhan et al., 2019)
	Ipomoea batatas	*RNase3*	–	Resistance to sweet potato virus disease caused by sweet potato feathery mottle virus and sweet potato chlorotic stunt virus	(Yu et al., 2022)
	N. benthamiana	*RNase3*	–	Resistance to cucumber mosaic virus and turnip mosaic virus	(Yu et al., 2022)

musacearu (*Xcm*), which is the cause of banana *Xanthomonas* wilt (*Musa* spp.)—one of the most devastating diseases in banana. It has been reported that all the cultivated varieties of banana are susceptible to *Xcm* (Tripathi et al., 2019). *DMR6* (*Downy mildew resistance 6*) is an *S* gene which encodes 2-oxoglutarate Fe(II)-dependent oxygenase that is expressed by pathogen infection (Low et al., 2020). Both the *DMR6* and its paralog *DLO1* (*DMR6-Like Oxygenase1*) are repressors of plant immunity (Zeilmaker et al., 2015). Recently, Tripathi et al. (Tripathi et al., 2021) developed enhanced resistance in a susceptible banana cultivar Sukali Ndiizi, via targeted mutagenesis in *MusaDMR6*. pMDC32-Cas9-MusaDMR construct was incorporated into the embryogenic cell suspension of Sukali Ndiizi. They obtained 30 transgenic events, which were then validated by Sanger sequencing that exhibited indel mutations with 100 % mutation frequency. Compared to the wild type, MusaDMR6-mutated banana plants showed broad spectrum resistance to Xcm without any effect on plant growth. By employing transcription activator-like effectors (TALEs), *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) promotes the expression of the *OsSWEET* family of putative sugar transporter genes, which increases the vulnerability of rice plants to rice bacterial blight (Xu et al., 2019). It is interesting to note that rice developed broad-spectrum resistance to a variety of harmful bacterial strains when CRISPR-Cas9 simultaneously edited many *S* genes in rice. Xu Z. et al. (Xu et al., 2019) generated alterations in the TALE binding sites of *OsSWEET11, OsSWEET13*, and *OsSWEET14* promoter regions to interfere with their function, which rendered *Xoo* incapable of recognizing TALE. As a result, the edited-MS14K rice lines exhibited higher resistance to multiple *Xoo* strains.

In addition, exploiting a different strategy of CRISPR-Cas9, a susceptible rice variety was turned into bacterial blight resistant variety (Wei et al., 2021). The promoter regions of the susceptible cultivar lack an effector binding element, $EBE_{AvrXa23}$, therefore an executor *R* gene cannot be expressed in response to *Xoo* attack. Wei Z et al. (Wei et al., 2021) employed CRISPR-Cas9 HDR system to successfully generate *Xoo* resistance in the susceptible rice variety Nipponbare by inserting $EBE_{AvrXa23}$ into the promoter region of the susceptible *xa23* allele. This specific change in *Xa23* gave rice broad-spectrum resistance to bacterial blight. This team developed rice plants that are resistant to a variety of diseases, such as rice blast, bacterial blight, and bacterial leaf streak, which are all caused by different strains of *Xanthomonas oryzae* pv. *oryzicola*, and *M. oryzae*. This resistance was generated by editing the *Xa23* gene via inserting *EBETal2g* in its promoter region (Ji et al., 2022). Additionally, the CRISPR-Cas9 technology was particularly used to modify the *Xa13, Pi21*, and *TMS5* genes in rice to produce resistance to bacterial blight and rice blast (Li et al., 2019). By altering *SlJAZ2*, tomato was made resistant to the bacterial speck disease (caused by *Pseudomonas syringae* pv. tomato (Pto) DC3000). *Pto* generates coronatine, which helps in the opening of stomata and colonization of bacteria in leaves. The edited plants produced truncated forms of JAZ2 lacking the C-terminal Jas domain, preventing the reopening of stomata in response to coronatine, decreasing the entry of bacteria via stomata, thereby rendering *Pto* DC 3000 resistance (Ortigosa et al., 2019). *MdDIPM4* gene was knocked out in two susceptible apple (*Malus domestica*) cultivars by Cas9, which dramatically reduced their vulnerability to *Erwinia amylovora* and thus conferred fire blight resistance (Pompili et al., 2020).

3.3. Resistance against viral diseases

In addition to fungi and bacteria, viruses account for a significant portion of developing plant diseases. This is mostly due to viruses' capacity to adapt to changing environmental conditions and their efficient spread made possible through vector transmission (Anderson et al., 2004; Robertson et al., 2022). The majority of economically essential crops are susceptible to viral infection, which results in severe viral diseases that significantly reduce global harvest yields and quality. Plant diseases are thought to be responsible for 15 % global agricultural yield loss, of which viruses account for one-third (Singh and Singh, 2018). In recent years, the CRISPR-Cas system has become one of the most reliable, accurate, and scalable DNA and RNA targeting platforms. It has been successfully used to develop plant tolerance to a variety of viruses (Table 1). The multiplex targeting capability of the CRISPR-Cas system at both the DNA and RNA level for creating targeted modifications in a transgene-free way offers a colossal bonus. Resistance to viral diseases in plants can be developed in plants using two major strategies, including directly targeting the virus via RNA silencing and secondly targeting endogenous *S* factors of the host plant (van Schie et al., 2014). When the transgenic *N. benthamiana* plants carrying CRISPR-Cas reagents were infected with *BeYDV*, they displayed lower viral load and symptoms, suggesting that CRISPR-Cas9 had successfully altered the *BeYDV* genome (Baltes et al., 2015). Studies have revealed that natural changes in eIF4E proteins in many plants can produce resistance to potyviruses (Hafrén et al., 2013). CRISPR-Cas9 presents the opportunity to introduce genetic resistance in plants that naturally lack eIF4E alleles. Bastet et al. (Bastet et al., 2019) introduced a viral-resistant *Pisum sativum eIF4E* allele into *A. thaliana* by inducing a single point mutation, N176K, using the CBE system, and the edited plants created were transgene-free and resistant to *Clover yellow vein virus*. Tomato brown rugose fruit virus (ToBRFV) is a recently emerged member of the *Tobamovirus* genus, which is rapidly spreading around the world. To control tobamovirus, studies have identified *TOBAMOVIRUS MULTIPLICATION1* (*TOM1*), which encodes a seven-pass transmembrane protein to interact with replication proteins of tobamovirus and is involved in tobamovirus multiplication (Ishikawa et al., 1991; Yamanaka et al., 2000). A combined loss-of-function mutation in TOM1 and its paralog TOM3 conferred a significant reduction in tobamovirus multiplication (Yamanaka et al., 2002). More recently, Ishikawa et al. (Ishikawa et al., 2022) introduced targeted mutation in four homologs of *SlTOM1* in tomatoes via CRISPR-Cas9. Intriguingly, the quadruple-mutated tomato conferred broad-spectrum resistance to different tobamoviruses, including ToBRFV, and did not show any effect on growth and fruit production.

Like Cas9 protein, Cas13 is an RNA-targeting CRISPR-Cas effector that has demonstrated remarkable promise for providing RNA phage defense. The class 2 type-VI CRISPR-Cas system Cas13 is an RNA-guided RNA-targeting defensive system against RNA and/or DNA bacteriophages in prokaryotes (Ali et al., 2018). Since 2016, several variants of the Cas13 protein belonging to multiple Cas13 subtypes (A-D) have been found (Abudayyeh et al., 2017; Konermann et al., 2018). Targeting RNA viruses in diverse plants has also been accomplished using multiple CRISPR-Cas13 variations (Table 1). To make potato plants resistant to the *Potato Virus* Y, CRISPR-Cas13a was utilized. Nib, CI, CP, and P3 were the four conserved viral areas that the study focused on. *Potato virus* Y accumulation was inhibited by the transient expression of Cas13a/sgRNA constructs in transgenic potato plants (Zhan et al., 2019). Yu Y et al. (Yu et al., 2022) exploited different Cas13 systems to silence multiple viral RNAs and create resistance in *N. benthamiana* and *Ipomoea batatas* plants. The research focused on RNase3, a component of the sweet potato chlorotic stunt virus' pathogenesis (SPCSV). Transgenic *Ipomoea batatas* plants that expressed RfxCas13d containing RNase3 exhibited greater resistance to SPCSV and sweet potato feathery mottle virus. Additionally, *N. benthamiana* plants that expressed the targeted-*RNase3-LwaCas13a* system exhibited resistance to the synergistic infection of TuMV-GFP and CMV-RNase3 (the cucumber mosaic virus) (turnip mosaic virus), respectively. One of the most serious diseases in grapevine is grapevine leafroll disease, which is brought on by Grapevine Leafroll-Associated Virus 3. Transient expression of the LshCas13a and FnCas9 systems led to the development of grapevine resistance to grapevine leafroll disease (Jiao et al., 2022). *Oidium* sp. and *tomato yellow leaf* curl *virus* (TYLCV) resistance were provided by SpCas9-mediated deletion of the *SlMlo1* and *SlPelo* genes, respectively (Pramanik et al., 2021). In tomato, target-specific mutation of *eIF4E1* resulted in increased resistance to the pepper mottle virus (Yoon et al.,

2020).

3.4. Applications of CRISPR-Cas technology in insect resistance in plants

Because they directly devour crops and propagate plant diseases, insects are the main biotic stressors that pose a serious threat to agricultural losses globally. According to estimates, insect pests destroy around one-fourth of the crop each year (Douglas, 2018). Sap-sucking and crop-chewing pests are the main insects responsible for large drops in agricultural productivity (Vanti et al., 2018). Recent advancements in the molecular interaction between insects and plants, as well as biotechnological techniques like genome editing, offer solutions to these problems. As mentioned above, the CRISPR-Cas technology in plants has been successfully employed to overcome a range of bacterial, fungal, and viral diseases. However, the practice of altering plants to control insect pests has lagged far behind. Here, we outline the possibilities that could be used to genetically modify plants to withstand insects.

For their growth, immunity, and behaviors that have been observed in rice, insects are dependent on essential chemical compounds contained in plants (Lu et al., 2018). According to research, most insects rely on essential plant compounds and volatiles for the growth and development of their immune systems (Tyagi et al., 2020). According to a study by Beale et al. (Beale et al., 2006), variations in volatile combinations attract insects away from host plants. As shown in transgenic plants, the emission of the sesquiterpene hydrocarbon (E)—farnesene by an aphid infestation prevents other host populations from eating on the plant and attracts the parasitic wasp *Diaeretiella rape*, which controls the aphid population (Beale et al., 2006). So, modifying volatile plant chemicals via genome editing might be an alternative pest management strategy. Through the use of CRISPR-Cas systems, plants can be modified to generate or not produce specific enzymes that can keep certain insect pests away from the plant or draw certain insect predators to the plant to eat the pest species that are attacking it (Rato et al., 2021). Important plant immunity genes can also be edited in order to generate plant species with insect resistance, and this approach is also a reliable way to improve host immunity to pests. Genome editing in plants has been found to boost insect pest resistance by removing the *S* genes from the plants. Lu et al. (Lu et al., 2018) transformed rice using CRISPR-Cas9 technology to make it resistant to the striped stem borer (*Chilo suppressalis*), and the brown plant hopper (*Nilaparvata lugens* Stl). They explained that tryptamine conversion to serotonin in plants results from tryptamine 5-hydroxylase encoding *CYP71A1* gene deletion, which inhibits plant hopper growth. Additionally, insects' capacity to identify and attack host plants is significantly influenced by the visual appearance of plants. It has been discovered that changes in plant pigmentation alter insect host preferences. This occurrence in transgenic tobacco, where an excess of anthocyanin pigmentation led the transgenic tobacco plant's leaves to appear red, was described by Malone et al. (Malone et al., 2009). The altered leaf color discouraged the herbivores *Spodoptera litura* (Cotton leafworm) and *Helicoverpa armigera* (Cotton bollworm), proving the importance of leaf color and appearance for insect host recognition.

Recently, Sun et al. (Sun et al., 2024) developed a high-throughput mutant library utilizing the CRISPR-Cas9 system to screen endogenous insect-resistant genes in cotton. To develop a mutant population, 969 sgRNAs were constructed targeting 502 endogenous genes related to insect resistance in cotton. The study identified the *GhMLP423* gene showing a broad-spectrum pest resistance by SAR (systemic acquired resistance) initiation of salicylic acid and *PR* genes via eliciting Ca^{2+}-mediated ROS signaling, inducing enhanced plant defense against insect pests. However, the knockout plants exhibited increased sensitivity and damage when fed by cotton bollworm and whitefly (*Bemisia tabaci*) compared to overexpression and wild-type plants. A similar strategy was used in another study (Wang et al., 2024a) of the same group, where a CRISPR-Cas9-mediated mutant library was constructed in cotton, targeting the *CDPK* (*Calcium-dependent protein kinases*) gene family to screen insect-resistant genes. In this study, 246 sgRNAs were used, and 89.49 % editing efficiency was obtained. From the mutant population, 14 *GhCPK* mutants, susceptible or resistant to insects, were identified. Through the molecular mechanism of insect resistance and the phenotypic analysis of the mutant library, *GhGPK33* and *GhCPK74* genes were characterized as candidate genes, which negatively regulate the synthesis of jasmonic acid (JA) produced by *S. litura* oral secretions. As a result, *GhGPK33* and *GhCPK74*-knockout plants showed increased resistance against *S. litura* (Wang et al., 2024a). In addition, PPI5 (peptidyl-prolyl trans-isomerase 5) is an *H. armigera* effector with peptidyl-prolyl isomerase activity, regulating the defense response through PCD (programmed cell death) via unfolded protein response in plants (Wang et al., 2024b). In cotton, PPI5 binds to *GhFKBP17–2* as a host target—restraining JA defense response and ER stress-mediated plant immunity by inhibiting *GhFKBP17–2* transcription and proline cis-trans isomerase enzymatic activity, rendering cotton plants more vulnerable to cotton bollworm invasion (Wang et al., 2024b). *GhFKBP17–1, GhFKBP17–2*, and *GhFKBP17–3* belong to plant immunophilins, which are known to be associated with higher plants' innate immunity (Aumüller et al., 2010; Wang et al., 2024b). Cotton mutants were created by knocking out the *GhFKBP17–1/3*, which showed higher susceptibility to cotton bollworm infestation (Wang et al., 2024b). *GhHAM* (*Hairy meristem*), a transcription factor related to the formation of pigment glands in cotton, was targeted by the CRISPR-Cas9 system. *GhHAM*-knockout mutants exhibited a significant reduction in gossypol production, resulting in increased susceptibility to aphids (*Aphis gossypii*) and cotton bollworm infestation (Long et al., 2024). Using these discoveries as a guide, plant metabolic pathways can be created that could be an effective method to establish insect resistance in plants. Finding new genes and effectors and elucidating their molecular mechanism could be highly useful in engineering resistance against insect pests utilizing CRISPR-Cas-based gene editing systems that could lay a foundation for modern pest resistance breeding in plants.

4. Advantages and concerns about CRISPR-Cas genome editing in plants

Creating resistance against diseases and insect pests has been challenging in crop plants, necessitating ongoing technological advancements. Natural resistance, which is primarily developed through conventional breeding methods, requires a lot of time and labor. Additionally, domestication frequently reduces the genetic variability of cultivated species and creates a bottleneck that prevents further improvement (Sikora et al., 2011). Even though natural diversity is a vital collection of desirable characteristics that must be enhanced, the quick advancement of biotechnological tools may be able to increase natural resistance to a new degree. By copying natural variability from one species to another, recent developments in the genome editing toolbox, such as the creation of durable CRISPR-Cas9 systems/variants, have made it possible for us to introduce precise modifications at specific target sites within the genome (Jacob et al., 2018). The CRISPR-Cas9-mediated base editing methods can successfully confer resistance because recent investigations have revealed that just one nucleotide alteration is required. Natural variation can successfully transfer resistance to another species if it does so in one species. In order to insert a single point mutation into the *eIF4E* gene and confer viral resistance in *A. thaliana* (Bastet et al., 2019). Therefore, by applying base editing techniques, we can successfully transfer distinctive resistance mutations from one species to another. New options for breeding plant resistance are opened up by such precise editing.

Base editing systems have proliferated as a result of the extraordinary advancements made in genome editing tools over the past ten years, and they are currently the best methods for enhancing disease and pest resistance in a variety of plant species. Notably, the CRISPR-Cas9-mediated base editing tools, including CBE, ABE, and CGBE, offer the chance to create plants through exact nucleotide base replacement

without involving the introduction of foreign DNA into the genome of a target species. Prime editing also offers the benefit of multiple base substitution, including base transition and transversion, as extensively discussed above. CGBE and prime editing are more recently developed systems and have been used as proof-of-concept in plants. However, to our knowledge, neither of these systems has been exploited for disease or pest resistance in pants to date. Therefore, the utility of these gene editing techniques for disease and insect pest resistance development could bring plant immunity and resistance to the next level due to their excellent promise for targeting multiple genes simultaneously that could build strong molecular immunity against multiple phytopathogens.

Broad-spectrum resistance has been achieved by simultaneously targeting many genes with the CRISPR-Cas system (Galli et al., 2022; Xu et al., 2019; Yu et al., 2022). Genome editing techniques are more precise than any other breeding methods currently in use because they allow us to change genomic sequences at target genes, regulatory sequences, and even target nucleotides. Crops created using CRISPR-Cas genome editing containing minor variations are considered to be non-GM. They are largely free of transgenes, making them identical to and almost indistinguishable from those created using conventional or transgenic breeding techniques. Therefore, researchers believe that genome-edited crops could be more widely accepted because they present little to no damage to the economy, environment, society, or human health (Zhang et al., 2020a).

Despite its clear advantages, CRISPR-Cas genome editing has several disadvantages. Many countries involve tight regulatory procedures regarding edited plants, where plants created by CRISPR-Cas systems are regarded as GM plants (Mao et al., 2019). When it comes to GM, the main obstacles to the development of gene-edited plants include the greater expenses associated with obtaining regulatory approval as well as problems with international trade (Jansing et al., 2019). The ability of robust technology to quickly generate and market genome-edited crops can, therefore, be limited as a result of these restrictions. Additionally, even though genome editing has increased accuracy, the technology still raises ethical, legal, and sociological issues in addition to having negative effects on plant yield and growth caused by some *R/S* genes. The technological issue also includes the potential for creating accidental genetic mutations in plants because of the unprecedented integration of artificial nucleases, which can lead to off-target modifications (Pineda et al., 2019). These technical issues must be resolved to advance technology for fair application in crop trait advances that are specifically targeted. Furthermore, the lack of understanding of technology's applications and guiding principles, which continues to be the principal barrier to this technology, is a contributing factor to the public's acceptance of genome-edited crops. Most significantly, by introducing *R* genes, removing *S* genes, and altering the relationship between the effector and the target, genome editing techniques have enabled plants to evolve diverse phytopathogen resistance. However, sometimes, these modifications have antagonistic effects on other crucial traits, like stunted plant growth that could lower the yield. Recent research has shown that gene deletion has negative impacts on plant growth in addition to conferring broad-spectrum disease resistance in several plants (Bastet et al., 2019; Hanika et al., 2021; Ke et al., 2020; Ma et al., 2018). Therefore, it must be considered that the target gene's modification will not have an adverse effect on other crucial traits. (Fig. 4).

5. Conclusion and perspectives

The entire agriculture sector at present is holding multiple pressures in terms of increasing crop yield and food production; for example, the attack of a plethora of plant pests and pathogens causes the direct loss of crop productivity. In addition, depleting environmental resources with respect to the constant decline in water and cultivable arable land resources contribute indirectly to decreasing crop yield. With chemical

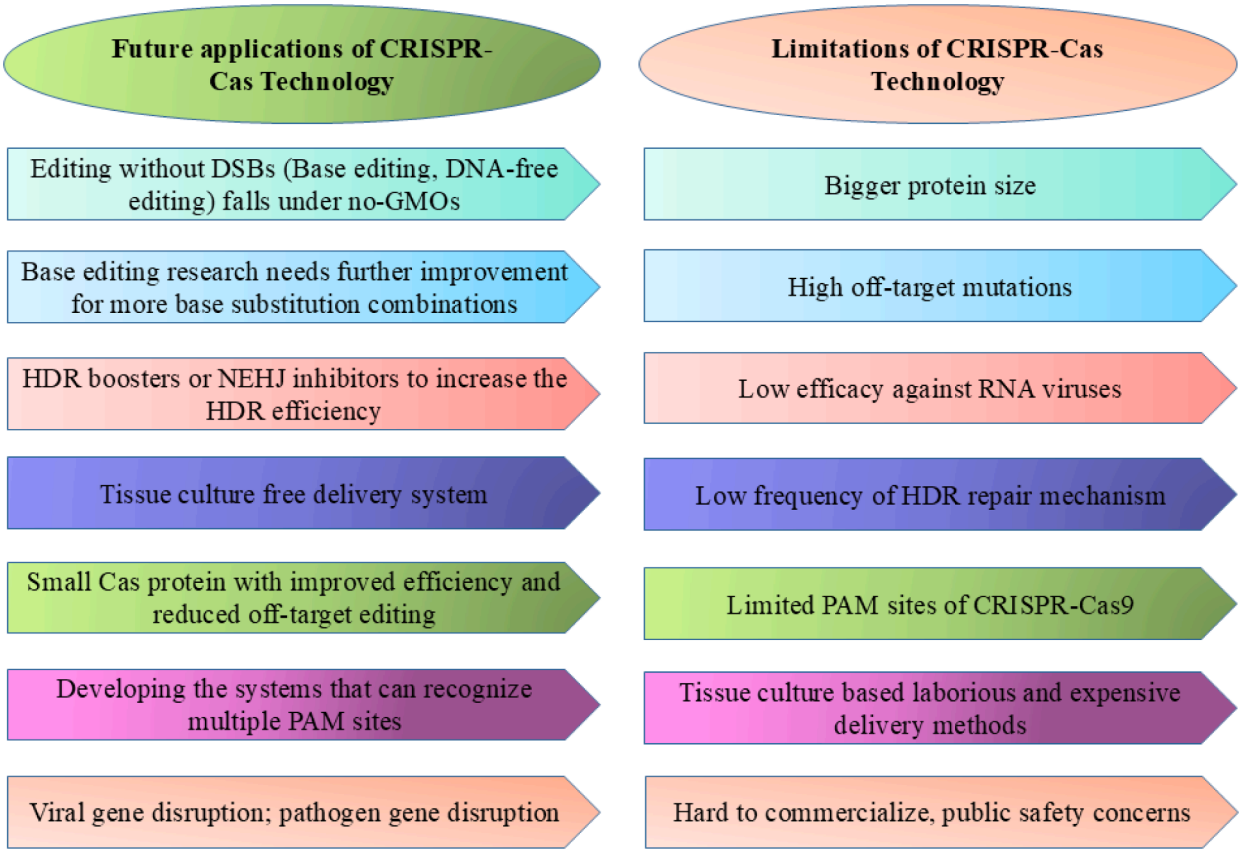

Fig. 4. Limitations and future applications of the current CRISPR-Cas systems in plants.

control, conventional and transgenic breeding techniques have been adopted to manage plant pests. Still, at present, these strategies cannot keep pace with the increasing demand for food supplies by expanding the human population in the near future. Currently, the advances in CRISPR-Cas techniques have emerged as powerful tools in biological research. This creates new opportunities for quickly and effectively targeting and altering genomic sequences, which will speed up gene functional studies as well as the breeding process by introducing useful alleles.

This review covered how several CRISPR-Cas9 applications can produce resilient, disease-and-insect pest-resistant plants with long-lasting resistance to increase their economic value and enhance food security and nutrition. The adoption of disease-resistant crops is currently one of the greatest ways to manage diseases. Using disease-resistant cultivars is the ideal technique to obtain optimum crop production without applying fungicides. There is no question regarding the role of *R* genes and their use in the development of resistance in plants; however, the loss of function of *S* genes or negative regulators through CRISPR-Cas systems plays a significant role in pathogenesis (Karmakar et al., 2022; Tripathi et al., 2022). Although gene editing has been shown to be effective in editing *S* genes against plant pathogenic bacteria and fungi, further research deploying these CRISPR-Cas9 systems is needed to promote plant protection against insect pests. We also discussed that the multiplex genome editing ability of CRISPR-Cas9 systems can present a stronger, durable, and broad-spectrum resistance to multiple diseases by simultaneous editing of multiple genes. For example, CRISPR-Cas9-mediated knockout of *OsSWEET14, OsSWEET13*, and *OsSWEET11* genes rendered rice plants resistant to multiple *Xoo* strains (Xu et al., 2019). Targeted mutations in the *Xa13, Pi21,* and *TMS5* genes resulted in rice that was resistant to both rice blast and bacterial blight (Li et al., 2019). Recently, Ishikawa et al. (Ishikawa et al., 2022) developed quadruple-mutant tomato plants showing durable and broad-spectrum tolerance against different viruses. To create crops resistant to insect pests and diseases, we suggest the identification and use of new *S* genes and multiplex genome editing using different CRISPR-Cas systems. CRISPR-Cas tools can provide an extra edge to accelerate the development of plant disease and pest resistance, which will ultimately lead to an increase in crop production in a sustainable way that can lessen the challenges posed by the growing global population and climate change.

CRediT authorship contribution statement

Amjad Hussain: Writing – original draft, Conceptualization. **Mamoona Munir:** Writing – review & editing, Software. **Awais Khalid:** Writing – review & editing, Software. **Musrat Ali:** Writing – original draft, Writing – review & editing. **Mohammed Amanullah:** Writing – review & editing. **Qurban Ali:** Writing – review & editing, Funding acquisition, Conceptualization. **Hakim Manghwar:** Conceptualization, Supervision, Writing – original draft, Software.

Declaration of competing interest

"The authors declare no conflict of interest."

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Data availability

No data was used for the research described in the article.

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