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AGRICULTURAL DEVELOPMENT BASED ON CRISPR-CAS9 AND RETRONs TECHNIQUES: A PERSPECTIVE APPLICATION ON TOMATO

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SUMMARY

Plant breeding has recently become a vital process in developing desired crop plants. Advances in genetic engineering occur more quickly than ever, with several crops generally created through traditional and modern techniques resulting in increased biomass and phytochemical compounds and adapted to the detrimental environment, such as biotic and abiotic stresses. More precisely, thousands of plant species gained enhancements suitable to various climatic and topographic conditions through genome editing; hence, people's dreams soon became a reality by implementing biotechnology to study many well-established fundamental grounds. Beliefs that biotechnology will progressively develop are happening in various aspects of modern sciences for crop development to be implemented based on genetic material. Besides the aforementioned benefits, this review manuscript will describe the progress of genome editing like CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9) with its modification and Retrons in crops and then determine its beneficial effects, which are more valuable through the application of these methods in crop development. The review further aims to assess the perspective application of CRISPR-Cas9 in the development strategies of virus-resistant tomatoes.

Keywords: Agriculture, tomato (*Solanum lycopersicum* L.), CRISPR-Cas9, Retron, pathogens, virus resistance

Key findings: The present review insights on describing the development of CRISPR-Cas9 and Retrons techniques and the benefits of its notable effects showed more valuable through the application on crop development. A prospective application on the tomato (*Solanum lycopersicum* L.) of CRISPR-Cas9 and Retrons technique in the development strategies for virus-resistance tomato also needs a concern.

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INTRODUCTION

Ensuring food security for nine billion people across the globe in 2050 remains an important topic of debate. Some propose that time and money should be invested in improving inputs, such as, fertilizers and soil and in developing new crops with desirable agronomic traits. Some justifications also emerged for making novel crops by exerting efforts for human food

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demand. For crop traits improvement, plant breeding activities have worked on crossbreeding and selection primarily based on homologous chromosome recombination derived from the parental genotypes to create varied genetic diversity. To enhance genetic variation and improve the populations for economic traits, conventional hybridization and mutation breeding applications have existed for a long time. The mutation with physical and chemical mutagens generates new variants with high economic value. In addition, molecular tools, such as, marker-assisted breeding and transgenic techniques can also produce more precise selection and shorten the breeding process (Ricroch, 2019). These tools still have certain limitations based on time, labor, knowledge of precision selection, and safety concerns due to the introduction of foreign genes from unrelated species through transgenic techniques.

The gene-editing technology has revolutionized genetic engineering, as well as, the field of plant breeding. Genome editing technology has improved selection accuracy for valuable traits being genetically stable. Gene-editing involves modifying the sequence of nucleotides at a predetermined target site through gene-editing systems, such as, zinc-finger proteins (ZNFs), Transcription Activator-Like Effector Nucleases (TALENs), and CRISPR/Cas-9. With these gene editing techniques, the traits of interest are targeted and edited at the desired DNA sequence position. This precise editing is through the design of a guide RNA (for CRISPR-based gene-editing systems) and a protein (Cas-9, Cpf1, Cas13a, and Cas14…) or target DNA recognition system based on TALENs and ZFNs protein combinations, which works as molecular scissors to cut the target DNA sequence at the desired locus. Right after the cleavage, the cell's natural repair mechanism helps rejoin the broken DNA fragment, which is probably imprecise to lead to new mutations (Indels or SNPs [single-nucleotide polymorphism], or minor nucleotide insertions). The repair mechanism involves non-homologous end-joining (NHEJ) and/or homologous recombination (HR) in which a homologous DNA sequence is found right at the site of the double-strand break (DSB), with the homologous donor sequence incorporated into the cleavage site.

Genome editing by site-directed nucleases (SDNs) such as TALEN or Cas9 is a versatile tool that generates variations in the recipient genome at specific target sites. Inducing changes in the DNA sequence of a gene to create mutations through gene-editing technology is not significantly different from the induced mutation through physical and chemical agents and spontaneous variations. The modification through a non-homologous end-joining mechanism generates small insertions and deletions that are indistinguishable from natural mutations (Ku and Ha*,* 2020; Schmidt *et al.,* 2020). Depending on the type of approach, one can distinguish between three types of alterations. SDN-1 introduces base-pair changes or small insertions/deletions without adding foreign DNA. The exact change cannot be predetermined and is quasi-random at the target site. SDN-2 uses a small DNA template to generate a specific change by homologous recombination. SDN-3 inserts higher DNA elements of foreign origin using a similar approach as SDN-2; the introduction of larger pieces of DNA is typically considered transgenic. Many countries have now adapted their biosafety legislation based on this classification of SDN-induced variants (Schmidt *et al.,* 2020). In general, considering gene-editing products as non-GMO will depend on the approach to produce a final product that is free from foreign DNA fragments. Also, it must have small nucleotide changes, such as, the insertion or deletion of a small piece of nucleotides without carrying foreign DNA.

In brief, gene-editing technology is an outstanding and versatile technology widely used in many fields, especially for the improvement of traits of crops and animals. Compared with transgenic methods, gene editing is a powerful tool by which one can change any DNA sequence in an organism's genome to achieve the desired goals, such as, treating rare genetic diseases and modifying plant and animal traits for improved yield, quality, and resistance to pathogens. This review will shed light on the following: a) describe the development of genome editings like CRISPR/Cas-9 and Retrons, introduced as a new plasmid-based gene editing method with the ability to generate millions of mutations at the same time that can attach "code" to mutant cells for easier screening by scientists in the United States aimed to overcome the limitations of CRISPR-Cas9, with the benefits of its treasured effects more valuable by applying to crop development, and b) determine the perspective of applying CRISPR-cas9 and Retrons in the developmental strategies for the improvement of virus resistant tomatoes.

Development of gene-editing tools

Zinc Finger Nucleases (ZFNs)

Zinc finger nucleases (ZFNs) are one of the first-generation gene-editing tools. The ZFNs are fusion proteins comprising a series of specialized DNA-binding domains of transcription factors containing the zinc finger along with an endonuclease domain of the bacterial *FokI* restriction enzyme. Each zinc finger domain recognizes a DNA sequence of 3–4 bp and can design tandem domains capable of binding to an extended nucleotide sequence (usually nine to 18 bp long). The ZFNs, engineered into a pair that recognizes two sequences adjacent to the target site, one on the forward strand and the other on the reverse strand, cut a target site in the genome. Once attached to both sides of the target site, the two *FokI* enzyme domains dimerize and cleave the DNA at the recognition site and produce a double-strand break (DSB) with a protruding end (5' overhang). Subsequently, repairing the DSB fragments used the NHEJ and HR mechanisms (Figure 1A) (Gupta and Musunuru, 2014).

ZFNs have been applied for gene-editing in plant and animal models (Carroll *et al.,* 2008; Doyon *et al.,* 2008; Mashimo *et al.,* 2010; Zhang *et al.,* 2010) and also in other plants, such as soybeans (Curtin *et al.,* 2011), canola (Gupta *et al.,* 2013), tomatoes (Hilioti *et al.,* 2016), and fruit trees, i.e., apples and figs (Peer *et al.,* 2015). However, the ZFNs have some limitations, such as, being complicated, time-consuming, and labor-intensive (Martinez-Lage *et al.,* 2017). Therefore, ZFNs are not widely used as CRISPR/Cas-9 and are less precise than Retrons.

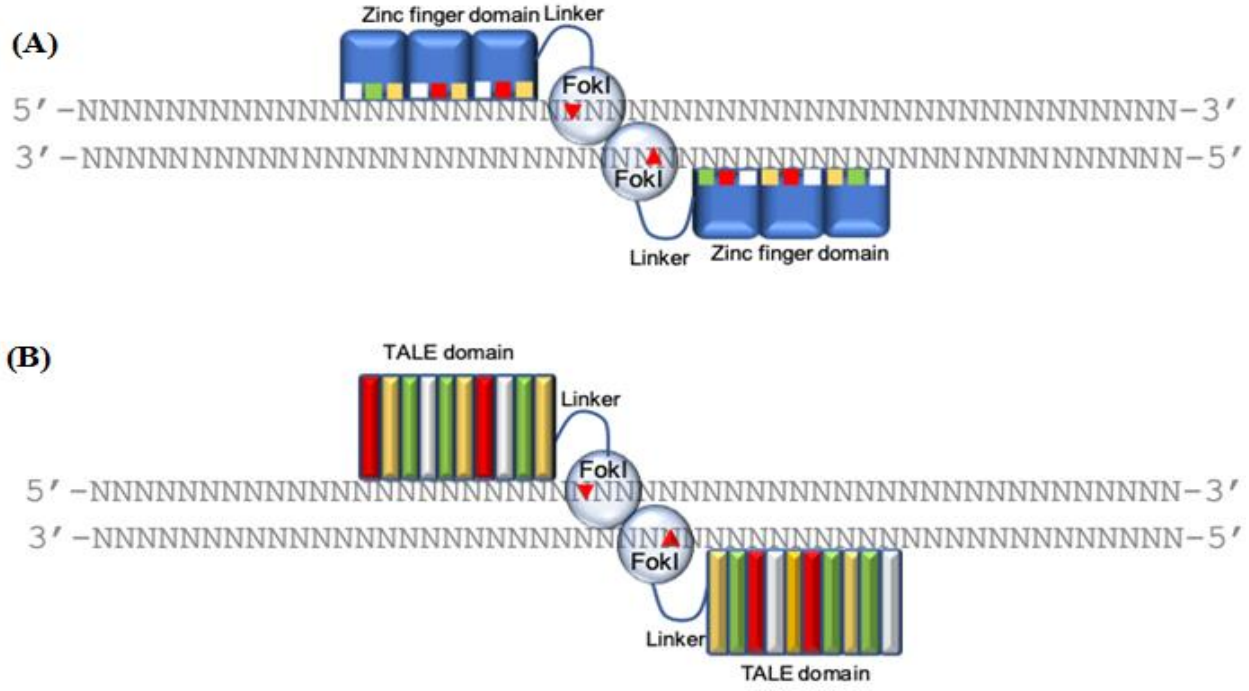

Figure 1. Model of the gene-editing system ZFNs and TALENs. (A) Variable-length ZFN DNA-binding domains bind to adjacent DNA sequences and localize their FokI nuclease domains such that they dimerize and induce DSBs between binding sites. (B) The heterodimer binding of TALENs, like ZFNs, is to bind variable-length regions to form DSBs between binding sites (Source: Martinez-Lage *et al.,* 2017).

Transcription Activator-Like Effector Nucleases (TALENs)

Like ZFNs, Transcription Activator-Like Effector Nucleases or TALENs are among the first-generation gene-editing tools. TALENs are fusion proteins comprising a DNA-binding domain associated with a *FokI* nuclease domain that induces DSB at the target site. The TALENs originated from the *Xanthomonas* bacteria. The TALENs comprising native DNA-binding modules, known as TALs, make the fundamental difference with ZFNs. TALs consist of a series of 34 amino acids conserved domains with a difference at positions 12 and 13, which are sites of DNA interaction known as repeat variable residues (RVDs). DNA binding is specific to each TALEN module defined by RVD and binds to DNA bases in a 1-RVD to 1bp ratio. Inversely, ZFNs recognize a DNA triplet. Same as ZFNs, it can bind TALEN's repeat motifs to each other for recognition of a target DNA sequence, as shown in Figure 1B (Martinez-Lage *et al.,* 2017).

Compared with ZFNs, TALENs have more advantages, such as, TALE repeat sequences that extend to any desired length, less difficulty in choosing a site, cleaner design, and time and labor-saving. However, TALENs are larger than ZFNs (the typical size for a cDNA encoding TALENs is about 3 kb, while a cDNA encoding ZFNs is only approximately 1 kb), which presents an obstacle to entry to cells of TALENs (Gupta and Musunuru, 2014; Martinez-Lage *et al.,* 2017). The TALENs gene editing system has been successfully applied to many crop plants, such as rice (Li *et al.,* 2012; Zhang *et al.,* 2016), tomato (Lor *et al.,* 2014), wheat (Wang *et al.,* 2014), and animals, as well as, in human cells (Hu *et al.,* 2013; Jinek *et al.,* 2013; Stroud *et al.,* 2013).

Clustered regularly interspaced short palindromic repeats (CRISPRs)

The clustered regularly interspaced short palindromic repeats or CRISPR-based genome editing system is a second-generation, gene-editing technology demonstrating superiority over the gene-editing systems of the previous generation (ZFNs and TALENs). CRISPR-Cas9 is the most widely used gene-editing tool today due to its simple design, fast design time, and ability to target any sequence at one time in the genome and multiple sites (Gupta and Musunuru, 2014).

CRISPR-Cas system

The CRISPR-Cas system is a bacterial immune system that defends against an invasion by phages and other mobile genetic factors, such as plasmids and transposons. Three main steps consist of the development of the CRISPR-Cas system in bacteria, i.e., a) CRISPR adaptation — addition of foreign gene fragments into the CRISPR sequence, called spacers, b) biological formation of crRNA — CRISPR sequences are transcribed into pre-crRNAs and processed into mature crRNAs, which integrate with Cas proteins to form an effector-crRNA complex, and c) CRISPR interference — these programmed effector complexes recognize and cleave the foreign gene fragments (Bandyopadhyay *et al.,* 2020). Some of the CRISPR/Cas-based genome editing systems widely used today follow (Bortesi and Fischer, 2015).

CRISPR/Cas9 gene-editing system

The CRISPR-Cas9 system consists of three components, i.e., CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA), and a Cas9 protein, one of the nucleases that plays a critical role during the process, a key component of CRISPR technology and available in different variations. Cas9 has a RuvC and His-Asn-His (HNH) DNA cleavage domain, which breaks double-stranded DNA (dsDNA) at 3bp before the PAM (protospacer adjacent motif) sequence on the target DNA sequence. The HNH domain cuts DNA strands complementary to guide RNA, while the RuvC domain cuts an opposite strand. Immediately after dsDNA cleavage, DNA repair occurs through NHEJ and HR mechanisms (Manghwar *et al.,* 2019). The CRISPR-Cas9 system design has a single guide RNA (sgRNA) that is a combination of crRNA and tracrRNA guiding the Cas9 protein to recognize specific PAM site and the 20-nt target site, afterward catalyzes to cleavage double-stranded DNA (Figure 2) (Martinez-Lage *et al.,* 2017).

Since the report on the CRISPR-Cas9 system came out in 2012 for the first time (Jinek *et al.,* 2012), the rapid adoption of said technique has ensued in a wide range of subjects (Jaganathan *et al.,* 2018; Jianguo *et al.,* 2019; Li *et al.,* 2020). Gene-editing techniques based on CRISPR-Cas rapidly evolved and constantly improved to extend the range of genome editing in various organisms.

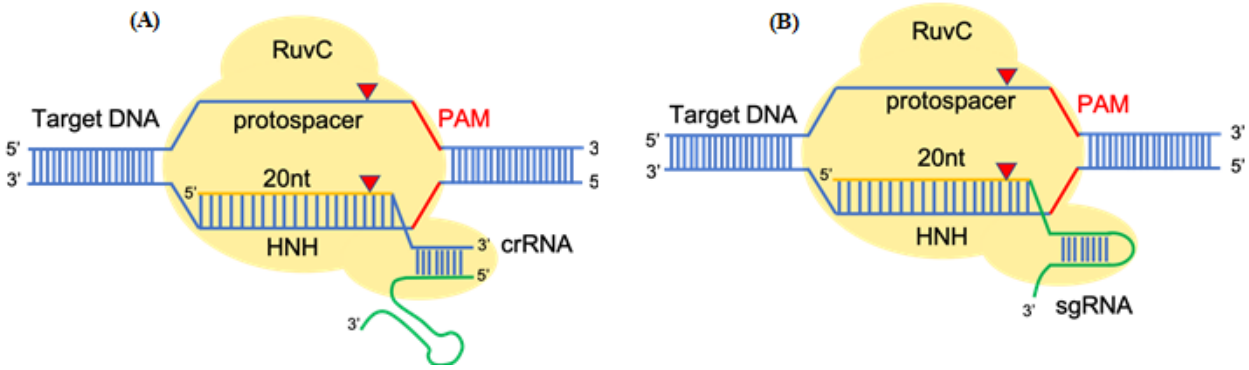

Figure 2. Model of the CRISPR/Cas9 gene-editing system. (A) In the native system, the Cas9 protein (light yellow) is guided by the structure formed by CRISPR RNA (crRNA, in orange), containing a 20-nt fragment that determines the specificity of the target and a tracrRNA in green. (B) Cas9 can be reprogrammed to cleave DNA by a single guide RNA molecule (sgRNA: 20nt guide sequence in orange and a scaffold in green), a chimera produced by fusing the 3′ ends of the crRNA with the 5′ ends of tracrRNA.

In addition, developing CRISPR-based versions has bloomed, such as CRISPR/Cpf1, base-editor techniques, and prime-editing.

CRISPR-Cpf1 system

The CRISPR-Cpf1 (CRISPR-associated endonuclease from *Prevotella* and *Francisella* 1) gene-editing system genetically consists of two components: a nuclease protein and a single crRNA of approximately the length of about 44 nucleotides that recognize the T-rich PAM sequence (5'-TTTN-3') at the 5' end of the target sequence, as illustrated in Figure 3 (Mishra and Zhao, 2018). Unlike Cas9, Cpf1 has only a single endonuclease domain that cleaves the target sequence 18-nt away from the PAM cutting the non-complementary strand and 23-nt for the complementary strand generating sticky ends (5 to 8 bp 5' overhang), in contrast to the blunt end in the case of Cas9. Hence, cleaving the target sequence far away from the PAM site can result in the subsequent round of Cpf1 cleavage (Bandyopadhyay *et al.,* 2020).

The Cpf1 is an alternative for Cas9 targeting T-rich sequences instead of G-like Cas9 (Zetsche *et al.,* 2015). Reports revealed the enzyme Cpf1 has shown to have a lower off-target ratio than the Cas9 nuclease. The advantages of Cpf1 make it a potential editing system to replace the CRISPR/Cas9 system (Mishra and Zhao, 2018). The wide use of the CRISPR/Cpf1 system continues in genome editing for desirable DNA sequences in bacteria and animal cells and plant trait improvement (Safari *et al.,* 2019; Bandyopadhyay *et al.*, 2020).

Base-editing system

Base editing also surfaced based on CRISPR-Cas. It converts one nucleotide of one type to another, namely, C to T or A to G or C to G and vice versa (Komor *et al.,* 2016; Gaudelli *et al.,* 2017; Chen *et al.,* 2021). The base-editing system consists of two components, i.e., the variant of the Cas9 proteins, namely, dCas9 (a catalytically inactive DNA-targeting Cas9 enzyme) and nCas9 (Cas9 with a nickase activity), and a deaminase system (adenine-base editor - ABE, cytosine-base editor - CBE, and C:G to G:C base editor - CGBE) for targeting the desired genomic location and triggering the conversion of nucleotides to make the desired edit.

Base-editor systems showed several advantages, such as being more efficient and generating far fewer off-targets than systems that induce double-strand breaks. In addition, targeting multiple sites at once will not cause chromosomal rearrangements, such as deletions and large inversions. The Base-editor use also generates nonsense mutations that avoid in-frame indels caused by double-strand breaks (Figure 4) (Chen *et al.,* 2019).

The application of base-editor techniques can overcome the barrier of strict

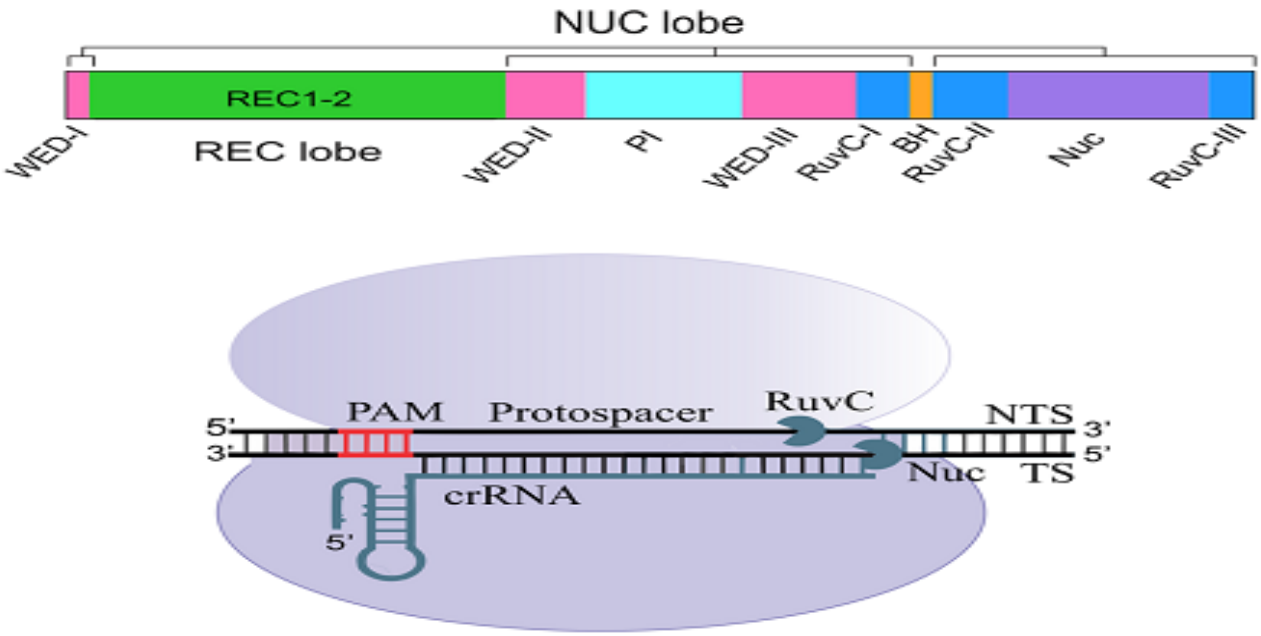

Figure 3. Illustration of the CRISPR/Cpf1 system and the organization of Cpf1 domains (Source: Safari *et al.,* 2019).

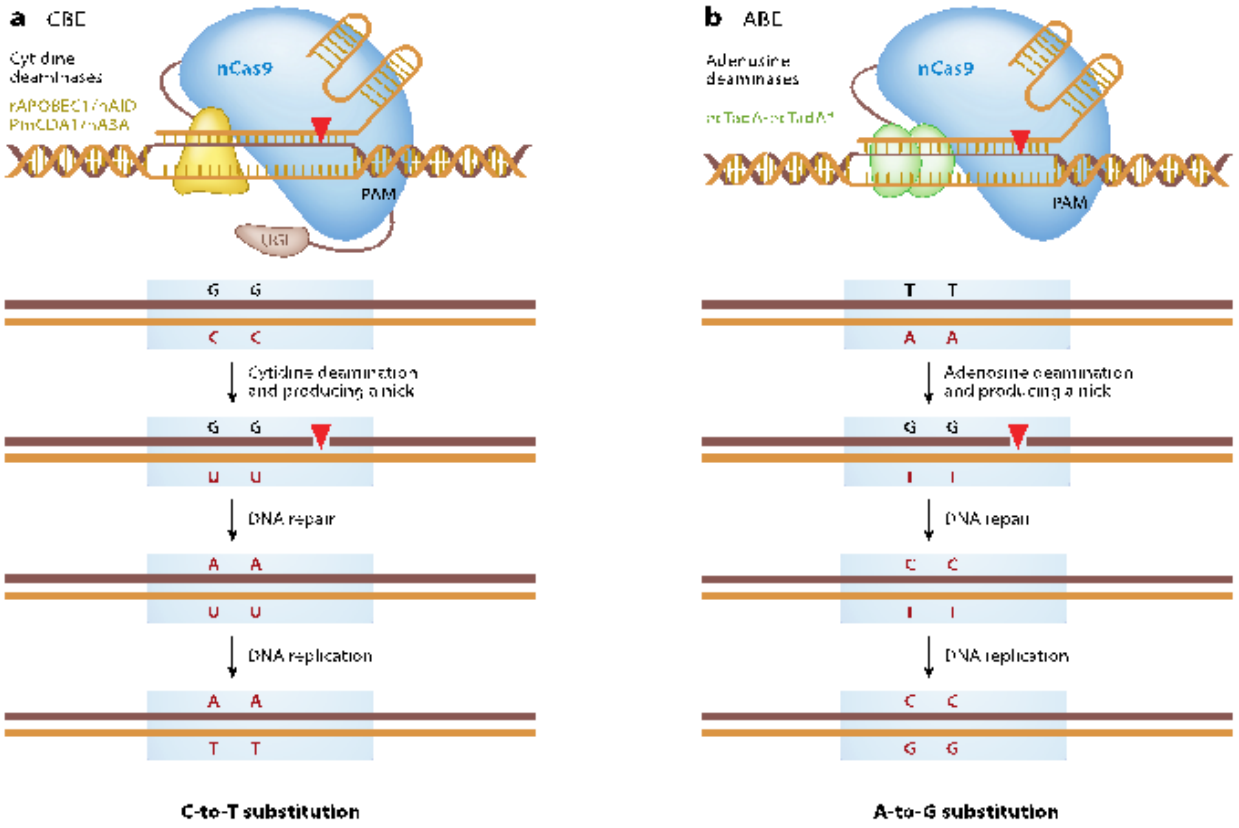

Figure 4. Base-editor system. (a) C-to-T system modulated by CBE (cytosine base editor). (b) A-to-G editing system modulated by ABE (cytosine base editor). (*Source*: Chen *et al.,* 2019).

regulations under GMO regulations because, base-editor replaces a single nucleotide at the target site, not causing double-strand breaks that result in mutations from insertion, deletion, and substitution of large chunks of DNA or frameshift mutations that cause loss of gene function (Kawall *et al*., 2020). The base-editor system is a promising and valuable tool for genetic research with many applications in agriculture and other fields.

Retrons system

Retrons' discovery happened in 1984 when a distinct DNA sequence resulted in the genome of many bacteria species. That sequence codes for an enzyme named reverse transcriptase (RT) and a unique single-stranded DNA/RNA hybrid called a multicopy single-stranded DNA (msDNA). Figure 5 summarizes the

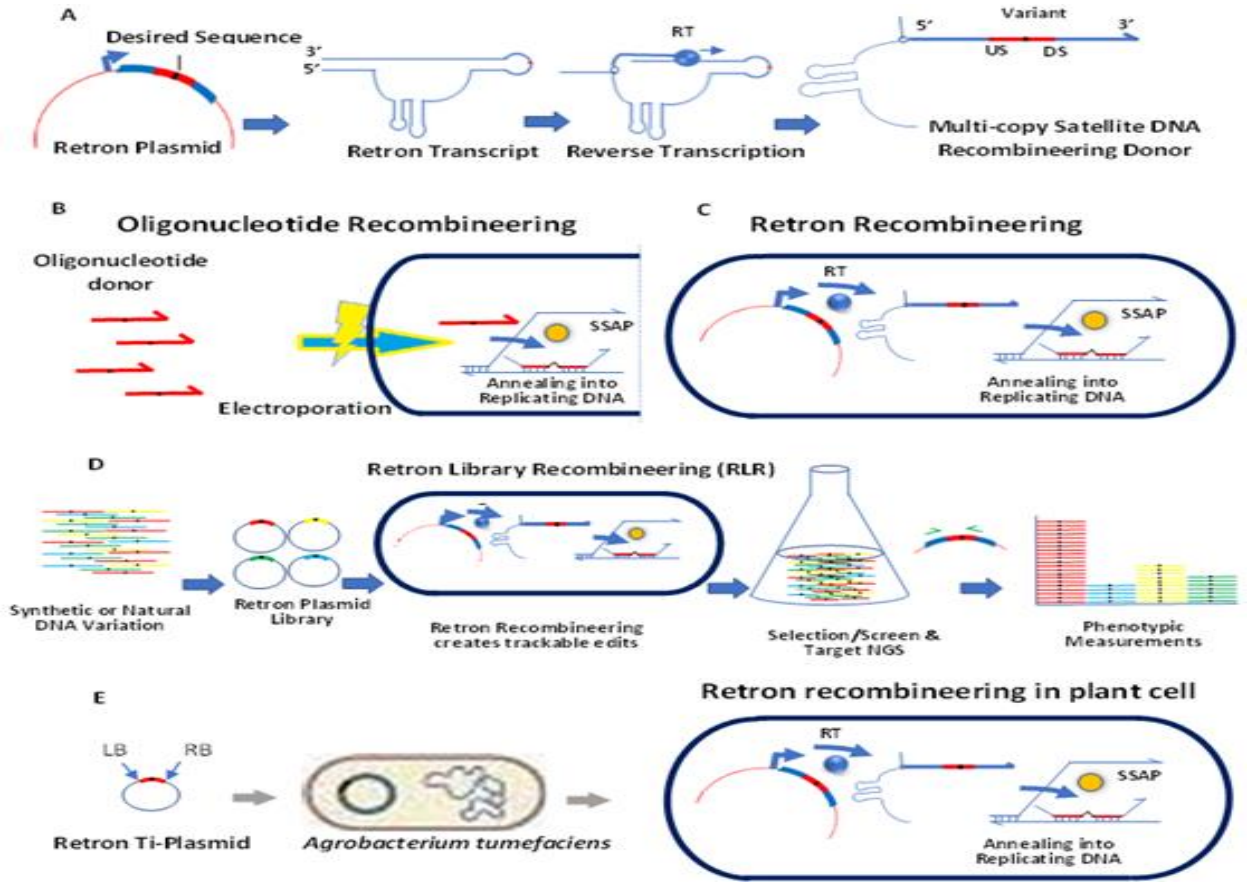

Figure 5. A summary of retron recombineering and modification to apply in the plant.
(*A*) The transcribing of retron *msr/msd* region, then retron reverse transcriptase (RT) catalyzes targeted reverse transcription leading to multicopy satellite DNA produced. When a homology upstream (US) and downstream (DS) in the red region contains a sequence alteration (black) is created, this DNA becomes a recombineering donor. Dashed lines describe RNA and solid lines describe DNA. (*B*) Oligonucleotide recombineering in that figure describes synthetic oligonucleotide donors entering bacteria anneal to start replicating DNA, thanks to the direction of a single-stranded annealing protein (SSAP) and as a result, introducing desired sequence alterations (black) into the genome. (*C*) A donor is a Retron recombineering uses RT-derived DNA further than transformed oligonucleotides, yet likely incorporates these into replicating DNA using a SSAP. (*D*) Synthesized libraries or natural DNA variants can be incorporated into retrons to perform recombineering which produces mutant cells bearing a retron plasmid, available for targeted amplicon sequencing using complementary primers (green) to measure mutant abundance in the multiplex. (*E*) Retron plasmid conferring the desired cassette for gene-editing is designed based on Ti-plasmid, then transferred the plasmid into *Agrobacterium tumefaciens.* This plasmid is transformed into a plantlet via an *Agrobacterium*-based mechanism to edit the target gene (Source: Schubert *et al.,* 2021).

detailed description of retron (Schubert *et al.,* 2021). The transcribing of retron *msr/msd* region, then retron reverse transcriptase (RT) catalyzes targeted reverse transcription leading to multicopy satellite DNA produced. When the creation of homology upstream (US) and downstream (DS) in the red region that contains a sequence alteration (black) occurs, this DNA becomes a recombineering donor. Dashed lines describe RNA and solid lines describe DNA. Oligonucleotide recombineering (*B*) in that figure defines synthetic oligonucleotide donors entering bacteria anneal to start replicating DNA, directed by a single-stranded annealing protein (SSAP); as a result, it introduces desired sequence alterations (black) into the genome (Meena and Bahadur, 2015; Simon *et al*., 2019; Schubert *et al.,* 2021).

Given the advances in biotechnology, CRISPR-Cas9 and its modification now give more capability in editing various crop traits. However, CRISPR-Cas9 can sometimes positively affect plant individuals, with its variation revealed more important. Using CRISPR-Cas9 as a newly developed technique showed advantages due to time-saving, exact results, with more choices. Concerning the former, CRISPR-Cas9 can perform faster than traditional techniques to save time. As far as precision is concerned, CRISPR-cas9 is comparatively exact with other gene-editing systems, i.e., ZFNs and TALENs. Finally, the gRNA constructing software can design multiple sequencing targets that are adequate, which provides the user with a broader range of options when they need them. However, drawbacks eclipsed these previously-mentioned benefits in terms of a lower level of exactness, which is considered some major limitations. A reason indicated, CRISPR-Cas9 often cuts unintended and/or off-target sites that cause the improper and unspecific design of the sgRNA. In particular, although CRISPR-Cas9 design can find and cleavage specific pieces of genetic material, the DNA editing to make desired mutations by striking the cell into taking a novel piece of DNA mends the break (Priyanka *et al*., 2015; Lopez *et al.,* 2022).

The Cas9 protein-mediated oligonucleotide insertion and large-scale leads to debate about GMO products (Gagnon *et al.,* 2014). In contrast, Retrons create genetic mutation without breaking DNA by using an alternate piece of DNA while genome replication happens in the cell. One of the chief benefits of that method is its use in several cells immediately to form a complex pool of the mutation. Seemingly, the drawbacks of the technological progress allowing genome editing through retrons recombineering do outweigh the benefits involved in exact genome editing. This technique opens doors of opportunities for successful genome editing not only for prokaryotes but also eukaryotes, such as, plants and human beings (Lopez *et al.,* 2022).

CRISPR-Cas9 use in crops development

CRISPR-Cas-based gene-editing technology developed rapidly, and between 2010 and 2022, immense reports related to its research and application were published (Bortesi and Fischer, 2015; Jaganathan *et al.,* 2018). So far, 62 crops have gained modification using CRISPR/Cas technique, with 587 publications (European Sustainable Agriculture Through Genome Editing, 2022). Tomato alone has 86 publications, with traits related to increased plant yield and growth, biotic and abiotic stress, industrial utilization, food/feed quality, color and flavor, and storage performance (European Sustainable Agriculture Through Genome Editing, 2022).

CRISPR-Cas can introduce mutation simultaneously at more than one site in the genome using multiple sgRNAs in a vector in any organism. CRISPR-Cas9 use has also ensued for a multiplex strategy, allowing the improvement of numerous traits into an elite background (Abdelrahman *et al.,* 2021). The multiplex strategy provides a powerful tool for targeting many members of polygenic families that are difficult to achieve with traditional breeding. Achieving this strategy comes in two ways: constructing multiple sgRNA-expressing backbones in separate vectors or collecting different sgRNAs in a single vector (Manghwar *et al.,* 2019).

Report of the multiplex strategy indicated as effective in a variety of crop plants. Cong *et al*. (2013) reported using multiplex gene editing was successful at multiple target sites in human and mouse cells. Ma *et al.* (2015) generated a multiplex gene-editing kit (toolbox) that combines multiple sgRNAs to target multiple genes in monocotyledonous and dicotyledonous plants. Similarly, Lowder *et al.* (2015) have created a gene-editing toolbox that targets multiple gene loci in the genome of several model plants, such as, *Arabidopsis*, tobacco, and rice. A study to optimize the multiplex efficiency in tomatoes used the *ELONGATION FACTOR-1α* (SlEF1α) promoter and simultaneously targeted two loci on the *SlNADK2A* gene (Hashimoto *et al.,* 2018). Li *et al.* (2018) successfully

domesticated wild tomato (*S. pimpinellifolium*) by editing its genes associated with morphology, flowers, fruit production, and ascorbic acid synthesis, resulting in developing tomato lines with increased vitamin C content, larger fruit size, disease resistance, and salt tolerance. Numerous studies applying a multiplex approach for simultaneously targeting multiple loci in the genome have come out (Rodriguez-Leal, 2017; Shen *et al.,* 2017; Li *et al.,* 2018; Zhou *et al.,* 2018; Hu *et al.,* 2019; Wang *et al.,* 2019; Fadhilah *et al*., 2022). These findings revealed that the multiplex strategy is effective for simultaneously modifying the many locations of the desired target gene.

Strategies for creating broad-spectrum viral resistance through genome editing technology

Plant viruses are infectious agents and cause serious diseases that lead to significant losses in yield and quality of crop plants. Climate change has rapidly accelerated the pandemic transmission of contagious viral illnesses and the emergence of new virus strains, leading to more difficulties in developing effective disease management measures in the long term (Varanda *et al.,* 2021).

Virus-plant interaction mechanism still needs complete understanding. Proposed numerous models have emerged for the pathogen-plant interaction that has served many years, including the guard model, which considers indirect interactions between plant R genes and pathogen AVR; the zigzag model, which proposes dynamic changes in plant-pathogen interactions; the "decoy" hypothesis, which explains the existence of functionally redundant R genes and non-functional effectors, and the iceberg model, which proposes crosstalk between intercrossing units (Zhang *et al.,* 2022). The zigzag model, known as the "central dogma" in plant pathology, presents as the most accepted and recognized model of interactions. Two distinct defense responses consist of the plant defense system in the zigzag model—the primary defense level, called PAMP/MAMP-triggered immunity (PTI), and the secondary defense level, called effector-triggered immunity (ETI). A prime defense mechanism presented by PTI is preventing invasion of the pathogen through cell wall thickening in response to specific structures or pathogen-associated proteins, so-called pathogen-associated molecular patterns (PAMPs), or microbe-associated molecular patterns (MAMPs). Plants show susceptibility only when a pathogen successfully establishes PTI response suppression and its pathogenic effector's facilitation. ETI, the second defense response level, is triggered when the products of R-gene directly or indirectly sense the presence of specific effectors called Avr factors (avirulence protein). Consequently, an effective ETI will keep the plants resistant; however, an insufficient ETI will lead to establishing the disease, i.e., the plant's susceptibility. Models of general resistance of most pathogens don't fit well with viral resistance because of the intracellular parasitic nature of the virus. For example, receptors of pattern recognition, serving as a major defense component by triggering the first layer of resistance when a receptor of plasma membrane perceives a fungal or bacterial MAMP or PAMP, can play no role in fighting plant viruses because viruses do not express extracellular PAMPs (Yadav and Chhibbar, 2018; Zhang *et al.,* 2022). There is always a never-ending battle between pathogens and their hosts, to which the pathogen responds by creating modifications in effectors and developing new effectors to defeat the ETI mechanism, as the plant also produces new R proteins, facilitating recognition of these new effectors. Therefore, new effectors and receptors continue to evolve in the interaction between plants and pathogens (Karibasappa *et al.,* 2021). Adaptive variability in plant-pathogen interactions is a major barrier in crop plant cultivar development with sustainable resistance to pathogens.

The realistic design of a plant's immune system could be one of the approaches that would allow it to cope with the rapid evolution of pathogens. Therefore, using the concept of effectors and receptors to improve the immunity of plants in combination with different types of resistance, acting at diverse stages during pathogen infection is an appropriate approach to creating effective and sustainable crop cultivars resistant to pathogens (Karibasappa *et al.,* 2021).

Recently, genome editing technology advanced as adopted in numerous fields, effectively applied in crop breeding to accelerate the various breeding processes. CRISPR-Cas9-based genome editing technique is a tool that enables the engineering of targeted genomic regions that induce variations and disruptions in the nucleotide sequence of genes that cause loss-of-function mutations. Utilizing the CRISPR-Cas9-based genome editing technology will help generate effective and sustainable virus-resistant crops by modifying host factors, such as, eukaryotic

initiation factors transcription (*eIF4E* and its isoform *eIF(iso)4E*), to prevent viral infection from entering host plants. Two strategies ably used to confer resistance in crops to viruses are a) conferring resistance by targeting the plant genome, where host factors regarding viral replication, and b) targeting viral genomes that destroy their genetic material to prevent their replication (Gosavi *et al.,* 2020). Therefore, approaching a strategy of targeting eukaryotic transcriptional initiation factors (*eIF4E*) to modify these receptors to block recognition of viral effectors for their infection into host cells, resulting in broad-spectrum virus resistance in tomatoes, needs action.

CRISPR-Cas9 gene editing to target host factors for virus resistance in plants

CRISPR-Cas9 is an effective tool to induce viral resistance traits in crop plants by engineering recessive resistance genes as transcriptional initiation factors to prevent viral entry into host cells. Recently, reports of success in conferring resistance in several plants based on gene-editing techniques targeting transcription promoters have surfaced. Chandrasekaran *et al.* (2016) knocked out the *eIF4E* gene via CRISPR/Cas9, showing complete resistance to zucchini yellow mosaic virus (ZYMV), papaya ringspot virus type-W (PRSV-W), and cucumber vein yellowing virus (CVYV). In rice, Macovei *et al.* (2018) targeted a novel rice allele (eIF4G) using CRISPR-Cas9 that demonstrated resistance to rice tungro spherical virus (RTSV).

The mutation caused in *eIF(iso)4E*, an isoform of *eIF4E*, induced by CRISPR-Cas9 in cassava and *A. thaliana,* confers complete resistance to cassava brown streak virus (CBSV) (Pyott *et al.,* 2016; Gomez *et al.,* 2019). Appearing most recently, the base-editing technique, based on CRISPR-Cas9n – cytidine, also revealed to induce antiviral, which can convert *Arabidopsis* susceptibility allele, *eIF4E1,* to resistant allele by introducing the N176K mutant to produce clover resistance to clover yellow vein virus (CYVV) (Bastet *et al.,* 2019). In tomatoes, Yoon *et al.* (2020) reported that mutation of the *eIF4E1* gene induced by CRISPR-Cas9 resulted in resistance to the PepMoV virus in tomato lines. Similarly, Atarashi *et al.* (2020) also disclosed the creation of mutated tomato lines with deletion (nine nucleotides) resistant to potyvirus using the CRISPR-Cas9 gene editing technique.

A prospective application of genome editing on tomato

Tomato (*Solanum lycopersicum*), originating from Latin America, is an essential and nutrient-rich crop. Tomato is one of the principal crops grown globally, with an annual global production of more than 160 million tons, accounting for more than 10% of total global vegetable production (Atarashi *et al.,* 2020). In Vietnam, tomatoes with high economic value are widely grown and consumed. In recent years, the area of tomato cultivation has varied around 23,000–25,000 ha, and about 40% in the South has an area of about 9,000 ha.

Tomatoes are vulnerable to more than 200 diseases caused by various pathogens, including viruses, bacteria, fungi, and nematodes (Atarashi *et al.,* 2020). In particular, viruses are one of the leading causes of severe losses to tomato yield and quality. However, chemical use is often ineffective and affects the environment, with chemical retention in food also affecting human health. Therefore, the generation of tomato varieties resistant to multiple viruses asserts vast importance in protecting tomato yield, reducing economic losses, and improving the lives of tomato producers, particularly in Vietnam.

Recently, a new technology emerged, revolutionizing genetic engineering—the genome editing technique. This technique allows manipulation of the desired region of DNA sequence in the genome to correct errors in the genetic code, insert genes, and replace, delete, and substitute the DNA sequences to improve the valuable traits in crops. Gene-editing technology is now more openly accessible in terms of GMOs, meaning that some products created from gene-editing technologies could not be considered GMOs (Urnov *et al.,* 2018; Schmidt *et al.,* 2020). Several countries, such as, the United States, Argentina, Brazil, Chile, Colombia, Japan, and Israel, have issued policies on genetically modified products that do not require regulations, i.e., GMOs if the final product does not contain foreign DNA sequences (Ku and Ha, 2020). For instance, in 2018, the US Department of Agriculture (USDA) decided not to impose regulation on new breeding technologies comprising genome editing. Most plants produced by SDN-1 or SDN-2 events are not subject to control by USDA once the

CRISPR gene has been crossed out (Schmidt *et al* ., 2020). In Canada, the regulatory trigger is a novelty, lacking existing history of safe use. For example, if natural variants used occurred in the context of breeding pathogen resistance, respective edited plants would not be subject to biosafety regulation (Schmidt *et al.,* 2020). Japan does not regulate plant varieties differently than conventionally bred varieties if they do not contain new DNA (SDN-1 and SDN-2) (Schmidt *et al.,* 2020). Indonesia and other countries in Southeast Asia are currently in the process of deciding whether to exempt crops produced by SDN-1 or 2 from GMO legislation. Although the GMO definition in the biosafety legislation of Bangladesh encompasses genome-edited products, the government is discussing whether or not to regulate genome-edited plants (Schmidt *et al.,* 2020). Thus, genome editing technology is a novel technology of massive potential that can substitute for traditional gene transfer methods.

Gene-editing technology has developed very quickly, now widely adopted around the globe in many fields, especially in agriculture. However, it is still relatively new in Vietnam and developing countries, with fewer applications. Therefore, applying gene-editing technology to induce mutation based on CRISPR-Cas9 to create broad-spectrum virus-resistant tomato lines is essential to meet the needs of new technology applications and generate and use new plant breeding methods for production in developing countries.

The eukaryotic translation initiation factor *eIF4E* - A key factor for viral infection

The *eIF4E*, also known as cap-binding protein, is a key factor in protein synthesis in eukaryotes (Figure 6). As part of a complex consisting of the eIF4G and eIF4A proteins, they bind to methylated guanine, which is post-transcribed to the 5' end of eukaryotic mRNA. It triggers the binding of the translation initiation complex, which eventually leads to ribosome binding and initiation of protein synthesis. The *eIF4E* organizes into small plant gene families that encode *eIF4E* and *eIF(iso)4E*, a form specific to terrestrial plants. The paralogs (homologous genes in the same organism, derived from a duplicated ancestor gene that may have a different DNA sequence and biological function) can encode each isoform , which increases gene family diversity, as is the case with *eIF4E1* and *eIF4E2* in tomatoes and peppers (Bastet *et al.,* 2019).

The *eIF4E* and its isoform *eIF(iso)4E* emerged to be vital factors for viral infection and function as recessive genes for resistance to different potyviruses in a variety of crops (Piron *et al.,* 2010; Jiang and Laliberte, 2011; Mazier *et al.,* 2011; Wang and Krishnaswamy, 2012; Gauffier *et al.,* 2016). Potyviruses showed to depend on eIF4E proteins to complete their infection cycle. Potyvirus genomic RNA contains a 5'-terminal protein, covalently associated with the genome, called VPg (Viral protein genome). The interaction between the VPg of several potyviruses and an eIF4E protein is primarily involved in the success of infection. Although the exact role of *eIF4E* in infecting the Potyvirus family is unclear, it is involved in the translation of the viral genome and the movement of viruses from cell to cell. The isoforms of *eIF4E* also displayed to be susceptibility factors to nepoviruses and poleroviruses, which led to the idea of discovering and engineering these genes to generate recessive resistance alleles for these viral groups (Bastet *et al.,* 2019).

Development of broad-spectrum potyvirus-resistant mutant tomato by CRISPR-Cas9

The eukaryotic translation initiation factor is a target for the study of broad-spectrum viral resistance in plants (Parrella *et al.,* 2002; Ruffel *et al.,* 2005; Mazier *et al.,* 2011; Van-Schie and Takken, 2014; Gauffier *et al.,* 2016; Lebaron *et al.,* 2016; Bastet *et al.,* 2019). The *eIF4Es* are determinants of plant susceptibility to RNA viruses, and viruses have utilized strategies to use different isoforms for infection (Piron *et al.,* 2010). Many plant species have multiple copies of the *eIF4E* or *eIF(iso)4E* gene that can be used specifically or interchangeably by potyviruses (Sanfaçon*,* 2015). Studies have shown that natural recessive resistance disrupts the interaction between the potyvirus and the eukaryotic transcription factors *eIF4E* or *eIF(iso)4E,* thereby preventing the virus from entering the host cells (Gauffier *et al.,* 2016).

Natural recessive resistance to potyvirus is generally associated with mutations of *eIF4E* or *eIF(iso)4E* that prevent their interaction with the viral VPg protein (Sanfaçon*,* 2015). In tomatoes, *eIF4E* has two homologs, *eIF4E* (*eIF4E1, eIF4E2*) and homolog *eIF(iso)4E,* with these factors associated with resistance to many positive-sense ssRNA viruses, especially potyviruses (Lebaron *et al.,* 2016). Natural resistance to potyvirus in lettuce, pepper, peas, and tomatoes is due to non-synonymous substitution mutations in the *eIF4E* coding sequence (Lebaron *et al.,* 2016). The molecular cloning of the recessive resistance gene *pot-1,*

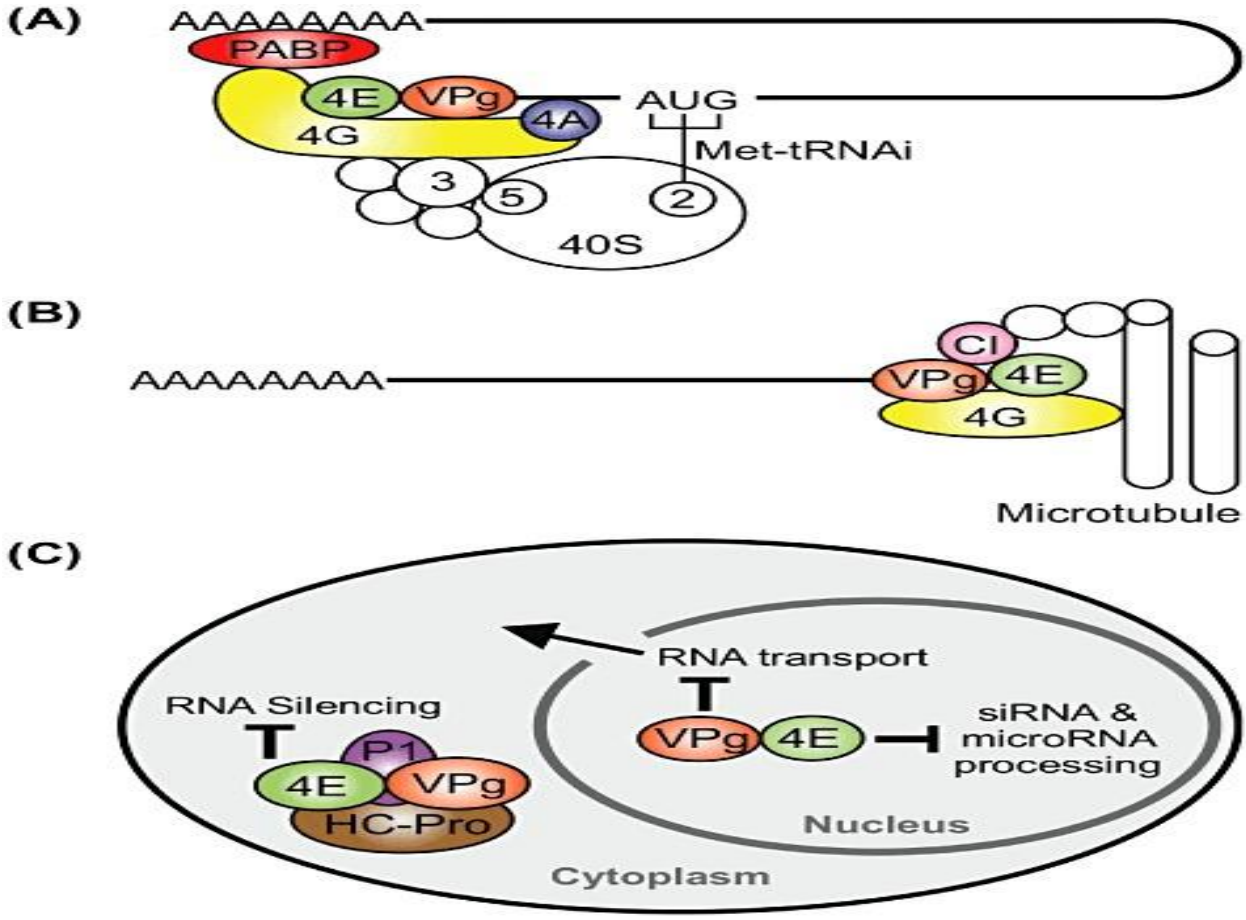

Figure 6. Proposed roles of eukaryotic translation initiation factor 4E (eIF4E) during potyvirus infection. (A) eIF4E binds to the genome-linked viral protein (VPg) and recruits the translation initiation apparatus for viral genome translation. (B) eIF4E, cylindrical inclusion (CI) protein and eIF4G may form a complex that binds to VPg to mediate intracellular trafficking of the viral genome for targeting plasmodesmata for cell-to-cell movement and, further, for systemic infection. (C) The VPg–eIF4E complex may be involved in the suppression of eIF4E-mediated transport of mRNA from the nucleus to the cytoplasm for translation and in the disturbance of siRNA and microRNA processing in the nucleus. eIF4E, P1, VPg, and HC-Pro (helper component–proteinase) may form a complex that functions as an RNA-silencing suppressor to safeguard virus translation/replication in the cytoplasm. 4A, 4E, 4G, 2, 3, and 5 represent eIF4A, eIF4E, eIF4G, eIF2, eIF3, and eIF5, respectively. P1, first protein; PABP, poly(A)-binding protein (Source: Wang and Krishnaswamy, 2012).

encoding the protein *eIF4E1,* demonstrated the role of *eIF4E* in resistance to two potyvirus strains, potato virus Y (PVY) and tobacco etch virus (TEV) in tomato, showing that the resistance allele and the susceptibility allele differ in the substitution of four amino acids (Parrella *et al.,* 2002; Ruffel *et al.,* 2005).

Several studies have genetically engineered the *eIF4Es* and *eIF(iso)4E* genes to induce mutation at the sites where the natural mutation is. Specifically, Piron *et al.* (2010), through the TILLING mutagenesis strategy, created a tomato line resistant to two strains of potato virus Y and pepper mottle virus; the resistance expressed through a loss of function mutation generating the truncated *eIF4E1* protein that led to inhibition of cap-binding activity. Mazier *et al.* (2011) used the RNAi technique to silence the *eIF4E* genes, resulting in lines that silenced both the *eIF4E1* and *eIF4E2* genes exhibiting broad-spectrum resistance to potyvirus. Similarly, Gauffier *et al.* (2016) also reported mutagenesis of the proteins encoding *eIF4E1* and *eIF4E2* by TILLING, resulting in loss-of-function of the *eIF4E1* gene without conferring broad-

spectrum resistance to the virus. But when combined with the loss of function on *eIF4E1* and *eIF4E2* conferred broad-spectrum resistance, it caused defects in plant growth. Their results showed that the *eIF4E1* or *eIF4E2* mutation did not confer resistance to the viral strains. It also suggests that most potyviruses interact with both *eIF4E1* and *eIF4E2*; in some cases, *eIF4E2* is a backup susceptibility factor when *eIF4E1* is mutated, using the virus *eIF4E2* for infection. Compared with the *eIF4E1* polymorphisms, the artificial mutants have a narrower resistance spectrum (Ruffel *et al.,* 2005; Piron *et al.,* 2010; Gauffier *et al.,* 2016). Thus, a dire need requires a strategy to generate mutation identical to natural resistance variants for broad-spectrum resistance to potyvirus without affecting plant growth and development.

A recent report by Moury *et al.* (2019) used the TILLING mutation technique to knock out the *eIF4E2* gene, leading to resistance to pepper veinal mottle virus (PVMV) strains. The said results also demonstrated the role of *eIF4E2* in resistance to potyvirus. In addition, the single mutation of *eIF4E2* alone is sufficient to confer partial or complete resistance to most of the PVMV isolates in tomatoes. Most recently, Yoon *et al.* (2020) reported using the gene-editing technique CRISPR-Cas9 to induce mutations in the *eIF4E1* gene, resulting in tomato lines resistant to pepper mottle virus (PMV). Another report performed by Atarashi *et al.* (2020) also mutated the *eIF4E1* gene using CRISPR/Cas9, resulting in a mutant tomato line that lost nine nucleotides showing resistance to the cucumber mosaic virus (CMV) strain. These two reports are the first to apply CRISPR-Cas9 gene-editing technology to generate mutation in the *eIF4E1* gene for broad-spectrum resistance to potyvirus. Aside from that, CRISPR/Cas9 application successfully modified *eIF4E* genes in other crops (Le *et al*., 2022; Pechar *et al.,* 2022).

Several studies have shown naturally resistant variants of *eIF4E1* with a few nucleotide substitutions for viral resistance with a broader spectrum than those generated by artificial mutation techniques (Ruffel *et al.,* 2005; Gauffier *et al.,* 2016). In addition, the results also elucidate an influential role in the recessive resistance of *eIF4E1* and *eIF4E2* to potyviruses and that *eIF4E2* is an alternative factor for the interaction of viral VPg in infection of the host plant in the absence of *eIF4E1* (Piron *et al.,* 2010; Moury *et al.,* 2019). Therefore, a method that produces mutations identical to natural resistance variation for stable resistance to potyvirus in crops, such as tomatoes, deems essential using gene-editing and genetic engineering TILLING technologies. Based on these studies, establishing a gene-editing system based on CRISPR-Cas9 to generate mutation in the target regions of genes *eIF4E1*, *eIF4E2*, and *eIF4(iso)4E* is of high consideration to apply in crop development strategies for viral-resistant tomato. Recently, the knockout of host eukaryotic initiation factors by CRISPR-cas9 has gained PVY-resistant tomatoes that promises potential tomato production (Kyoka *et al*., 2022; Surender *et al*., 2022).

CONCLUSIONS

Unquestionably, plant breeding, whether traditional or modern techniques, is vital for all communities because it can open doors of opportunities for successful crop development. However, modern techniques are a sound approach for application. Breeders having experience in applying CRISPR-cas9 or its modification forms and Retrons are more likely to edit desired traits, focusing on the effectiveness of crops like stress tolerance and nutrient-richness. In the present era, researchers seek to find efficient methods to breed germplasm before considering an application in production. Finally, the farmers also desire to expand minor and major crops with nutrition-rich, environmentally sustainable, and resilient traits derived from plant breeding. These creditabilities provide ample opportunity for securing a crop for worldwide use. The breeders should be encouraged for their efforts in studying crops and applying biotechnological engineering. This information will not only serve helpful but also shed hope for the success of breeders in preparing development strategies for future crops.

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