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REVIEW ARTICLE

Major CRISPR technologies for plant genome editing – A critical review of the molecular mechanisms

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Abstract

The CRISPR-Cas system, an adaptive immune mechanism in bacteria and archaea, is explored for gene editing in crops. This review critically explores the molecular mechanisms in major CRISPR types commonly used for plant genome editing. CRISPR-Cas9, Cas12a (Cpf1) and type I-D systems are the more recently studied systems frequently employed in plant genome editing. Cas9, a class II effector, has a dual-RNA structure to accurately target and cleave DNA, which makes it highly effective for gene knockouts. Cas12a, in contrast, offers unique features, including the ability to produce staggered DNA cuts and the absence of a requirement for tracrRNA, which broadens its editing capabilities by reducing off-target effects. The lesser-known type I-D system, a multi-complex Cas protein system, shows heritable editing in crops. Each system exhibits specific protospacer adjacent motif (PAM) requirements that influence target specificity. Moreover, base and prime editing expand CRISPR's potential for precise and multiplex genome editing. This review aims to provide a comprehensive understanding of the mechanisms, potential applications and limitations of these CRISPR-Cas systems to guide researchers in selecting the most suitable tool for precise gene editing in plants. Additionally, delivery methods and ethical considerations are discussed, emphasizing their role in optimizing crop improvement strategies.

Keywords

base editing; Cas9; Cas12a; guide RNA; prime editing; type I-D

Introduction

Most bacteria and archaea contain the CRISPR-Cas system, an adaptive immune system that protects them from invasion by phages, viruses and other foreign genetic elements (1, 2). The clustered regularly interspaced short palindromic repeats was identified in the late 1980s in *Escherichia coli* and early 1990s in *Mycobacterium tuberculosis*, but its adaptive character was described in 2005. Emmanuelle Charpentier and Jennifer Doudna created the groundbreaking CRISPR-Cas9 gene-editing technique in 2012. CRISPR repeat-spacer arrays, which transcribe CRISPR RNA (crRNA) and a group of CRISPR-associated (Cas) proteins that enable target DNA cutting, comprise the CRISPR-Cas system; the CRISPR-Cas genes function synergistically to initiate a molecular cascade (3). Prokaryotes acquire the spacer DNA from the invading genetic elements and integrate the foreign DNA into the spacer array, acting as an immune memory. Upon infection recurrence, CRISPR-Cas transcription and processing occur to recognize, bind and eliminate the foreign DNA (4).

The CRISPR-Cas system includes two major classes, six types and several subtypes. The class 1 system is a multi-Cas protein effector complex that comprises types I, III and IV. These effector complex in class 1 is a ribonucleoprotein (RNP) made up of several Cas proteins that bind to crRNA (CRISPR RNA) during interference (5). In contrast, class 2 is a single effector protein that initiates editing (6, 7). A short piece of DNA called the PAM sequence, "Protospacer Adjacent Motif," is next to the target DNA that CRISPR-Cas systems can read. It acts as a signal for Cas proteins to initiate target binding and cleavage. The specificity of the CRISPR-Cas system is determined by the PAM, which naturally varies in length and composition depending on the type of CRISPR system (8-12). type I systems locate PAMs at the 5′ end, while type II systems locate them at the 3′ end (Table 1).

Materials and Methods

This review was conducted by searching databases such as PubMed, Google Scholar, Elsevier and Web of Science for pertinent articles using keywords like "CRISPR in plants" and "plant genome editing." The studies were assessed according to the CRISPR used in plant genome editing, resulting edits, the molecular mechanism of editing and by CRISPR type (e.g., Cas9, Cas12a, base and prime editors). Comparative figures were created using the data visualization software Biorender to represent the molecular mechanism explicitly based on published research. A rigorous analysis of the findings highlighted the possibilities and limitations of each CRISPR type in plant genome editing.

Results

In plant genome editing, two predominantly used CRISPR-Cas systems are Cas9 and Cas12a, besides the newly found CRISPR type 1-D. This review focuses on the molecular mechanism of spacer acquisition and RNP-mediated DNA editing. It aims to improve the understanding of CRISPR-Cas-mediated DNA editing, the choice of the system for plant gene editing and the characteristics of the intended edits.

Type 1-D

Type 1-D is a less studied complex of several Cas proteins within the CRISPR-Cas system capable of targeting dsDNA and ssDNA. type I-D contains eight Cas proteins, Cas1, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas10 (Table 2). type 1-D differs from other type 1 Cas systems because it does not contain Cas8, an effector protein homologous to Cas10. Cas8 is typically a signature protein in other type I CRISPR-Cas systems (e.g., type I-E and I-F), where it plays a critical role in assembling and stabilizing the cascade complex. In type I-D, Cas8 is replaced by Cas10d for its structural and functional roles. Evolutionarily, there could be a common ancestor for the class I Cas system; both type I and type III possess Cas10, a homolog of Cas8, but type IV contains Cas8 and lacks Cas10 in the cascade (14).

Adaptation

Type ID Cas proteins perform various functions, such as acquiring spacers and facilitating DNA editing. The Cas1 (nuclease and integrase), Cas2 (endoribonuclease) and Cas4 (endonuclease) dimerize to form heterohexameric complexes. The complex consisting of Cas1:Cas4 and Cas1:Cas2 plays distinct roles in the CRISPR adaptation process. Cas1:Cas4

Table 1. Classification of the CRISPR system and the effectors involved in the cascade

Class	Type	Effector	PAM sequence	References
1	I-A	Cas1, Cas3,Cas4,Cas5,Cas6,Cas7 and Cas8a	No specific PAM	(4, 5)
	I-B	Cas1,Cas2,Cas3,Cas4,Cas5,Cas6,Cas7 and Cas8b		(4, 5)
	I-C	Cas1,Cas2,Cas3,Cas4,Cas5,Cas7 and Cas8c		(4)
	I-D	Cas1,Cas2,Cas3,Cas4,Cas5,Cas6,Cas7 and Cas10d		(5)
	I-E	Cas1, Cas2, Cas3,Cas5,Cas6,Cas7 and Cas8e		(9)
	I-F	Cas1,Cas2,Cas3,Cas5,Cas7 and Cas8f		(4)
	III	CASCADE		(5)
	IV	CASCADE		(4, 5)
2	II	SpCas9	NGG	(6, 7)
		SaCas9	NNGRRT	
		FnCas9	NGG	
		NmCas9	NNNNGATT	
	V	Cas12a	5′ AT-rich PAM	(7, 8)
		Cas12b		
		Cas12c		
	VI	Cas13a	3′PFS: non-G	(9-12)
		Cas13b	5′PFS: non-C; 3′PFS: NAN/NNA	
		Cas13c	-	
		Cas13d	-	

Table 2. Functions of the various Cas enzymes in type I-D system

Enzyme	Function	References
Cas1	Spacer acquisition	(13, 14)
Cas2	Endonuclease activity and conjugate with cas1 enzyme for spacer acquisition	
Cas3	Helicase and exonuclease activity and work as a conjugate with the other complex enzymes	
Cas4	Provides processed protospacers to the Cas1-Cas2 complex	
Cas5	Cas5 helps guide the cascade complex to the complementary sequence in the target DNA	
Cas6	It typically cleaves within the repeat regions of the pre-crRNA, resulting in the formation of mature crRNAs	
Cas7	It forms a backbone around which the crRNA is wrapped, creating a ribonucleoprotein complex	
Cas10d	Recognition of protospacer and functional nuclease	

identifies the PAM sequence strand, while Cas1:Cas2 binds to the non-PAM strand and subsequently recruits Cas4 endonuclease to cleave the DNA, leaving a 6-nucleotide overhang (Fig. 1). The length of the gRNA in type 1-D is more frequently 35 nt.

In type1-D CRISPR, Cas 6 endonuclease cleaves the primary spacer transcript into mature crRNA. Later, the crRNA is loaded onto a complex multi-subunit assembly that functions in surveillance and effector capacities, enabling the detection and cleavage of invading DNA sequences through RNA-DNA homology-directed mechanisms. The multi-subunit effector assembly consists of Cas4 (endo-nuclease), Cas1 (nuclease and integrase), Cas2 (endoribo-nuclease), Cas6 (endonuclease), among other Cas proteins (Cas3d, Cas5, Cas7, Cas10d and Cas11d), that help in binding and cleavage of the target DNA.

Type 1-D mediated editing

The protein Cas7d recruits the cascade complex to form a functional ribonuclease-effector complex for interference and to enhance the stability and efficiency of the editing. The 5' end of the crRNA is capped with Cas5 and Cas10d and the stem-loop at the 3' end is encased by Cas6d; the Cas5d bifurcates the double-strand DNA and forms an R loop that is stabilized by the positively charged amino acids at the C-terminal end of Cas10d and multicopy Cas11d encompassing the belly of the crRNA. The PAM recognition domain of Cas10d identifies the PAM; the glycine-rich loops of Cas10d insert into the minor groove of dsDNA, interacting with the guanine nucleotide at -3 and lysine with -3, -2,-1 of PAM. The Cas3" and Cas3' domains of Cas10d are responsible for cleavage and helicase activity, respectively. The

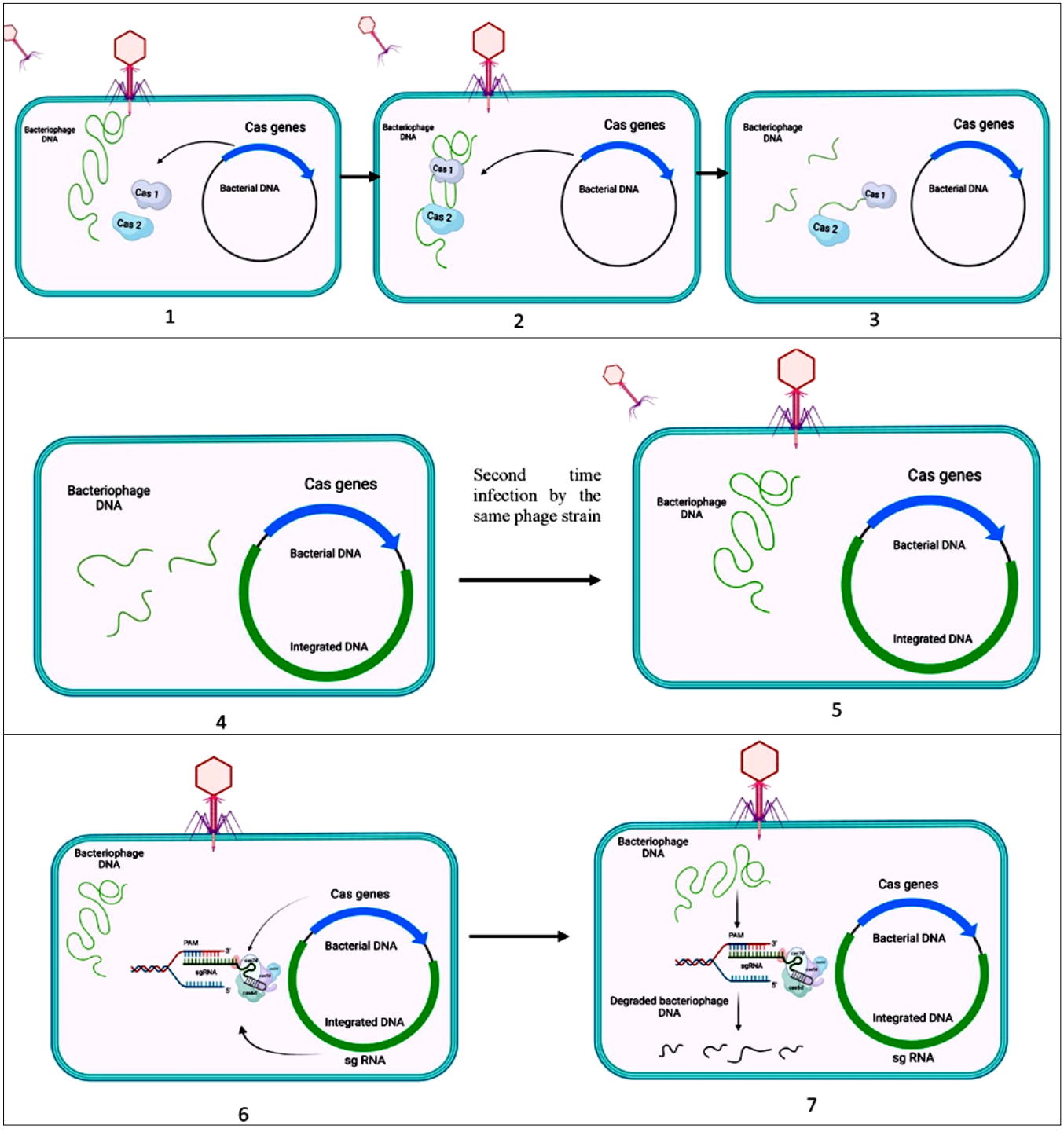

Fig. 1. The mechanism of CRISPR type I-D immune system in bacteria.

recruitment of Cas3' triggers Cas3" to nick bidirectionally the non-target and target strands on both sides of the PAM in an ATP-dependent reaction facilitated by the helicase activity (Fig. 2).

the target-specific RNA, PAM and tracr RNA. The two-component CRISPR system's simplicity, precision, reduced interference and flexibility make it a preferred choice for most gene editing applications.

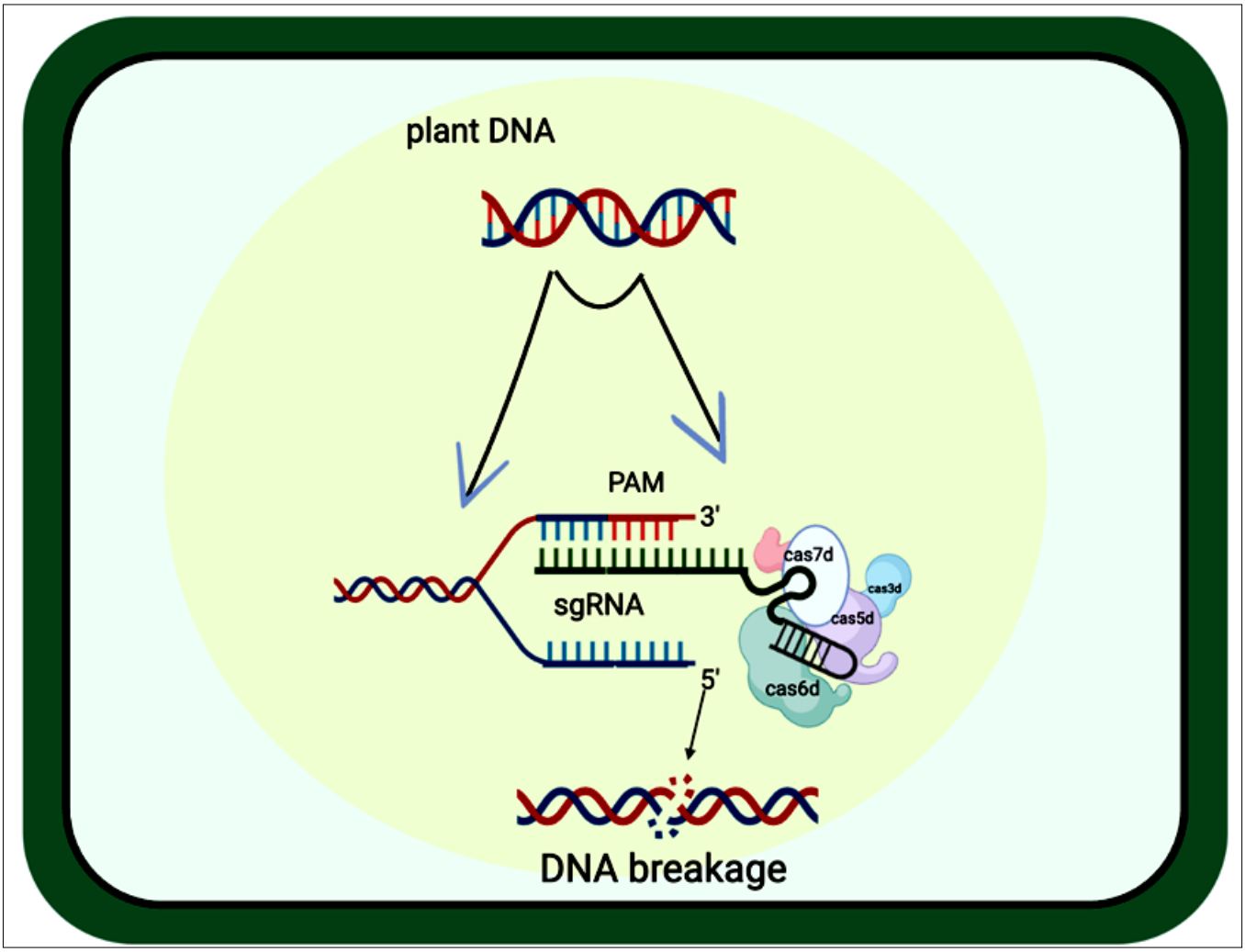

Fig. 2. Molecular mechanism of plant DNA editing using type I-D CRISPR system.

Properties of type 1-D

The type1-D-induced mutation in tomato slIAA9 (indole acetic acid 9) and NADK2 (nicotinamide adenine dinucleotide kinase 2) genes reveal that the mutations are bidirectional, heritable, long-range and short *indels,* without the effect of off-targets. The preferred PAM in tomato genes, sl1AA9 and NADK2 were GTT and GTC, respectively. However, knockout studies on type 1D Cas proteins in *Microcystis aeruginosa* show that the PAM sequence was '5-GTH-3' where H = A , C or T (13).

Pros and cons

Type 1-D involves a cascade complex that recognizes targets through multiple interactions, reducing off-target effects, but is limited by multiple Cas proteins.

CRISPR-Cas 9

The CRISPR-Cas9 mechanism, which was discovered in 1987 at Osaka University, utilizes the adaptive immune system of *Streptococcus pyogenes* to modify the genetic sequences in targeted organisms (15). The CRISPR Cas9 is a class II two-component system composed of Cas9 and a single guide RNA molecule (sgRNA). The sgRNA combines three components:

Adaptation

In this prokaryotic adaptive immune system, the conserved proteins Cas1 and Cas2 are involved in spacer acquisition. The Cas1 and Cas2 enzymes cleave the viral genome segments upstream of the PAM and integrate into the spacer sequence of the host. Although the molecular mechanism is unclear, Cas1 and Cas2 proteins complex into two distal Cas1 dimers connected to Cas2 dimers to form an integrase complex (16); the complex binds to the pre-spacer DNA and inserts it into the proximal leader region of the CRISPR array (Fig. 3). The pre-crRNA with the repeat and the tracrRNA with the anti-repeat form a repeat: anti-repeat duplex for the host endoribonuclease RNase III to cleave with a 2-nt overhang on the 3′ ends (17). The cleavage event divides the pre-crRNA into individual immature crRNAs consisting of a full-length spacer flanked by either half of the repeat. The immature crRNA is trimmed 10 nucleotides into the spacer by host ribonucleases that are yet to be described.

Cas9-mediated genome editing

The guide RNA is a two-component system consisting of the crRNA and tracrRNA that complexes with Cas9 to form the Cas9 ribonucleoprotein (RNP). The crRNA targets the double-

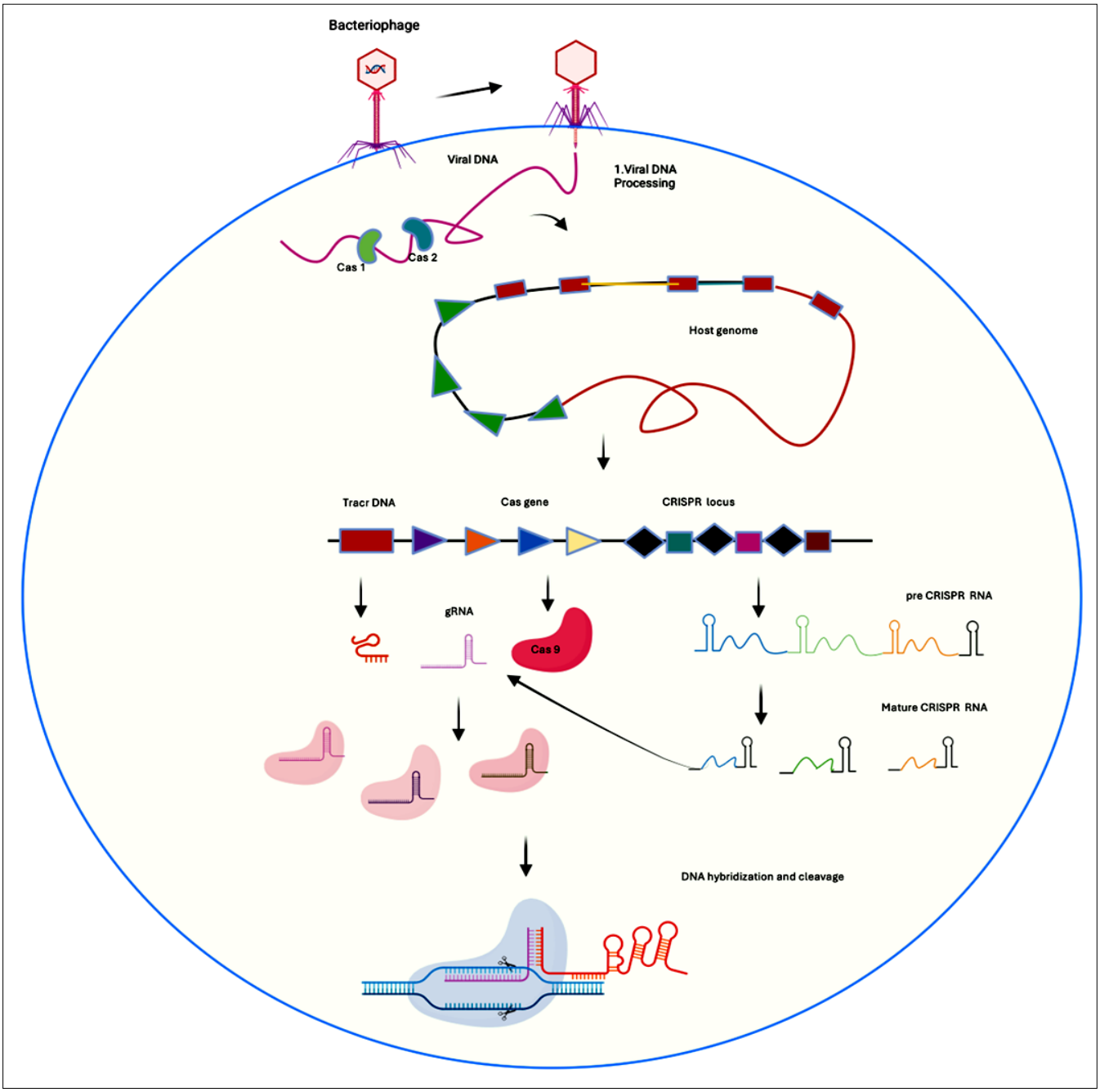

Fig. 3. The mechanism of CRISPR Cas9 system in bacteria.

stranded DNA to be cleaved and has a short homology region with the tracrRNA. The tracrRNA provides the required secondary stem-loop structure to associate with Cas9 protein. To cleave the target DNA, the guide RNA must possess 17-21 bases of RNA-DNA homology and a short protospacer adjacent motif (PAM) to bind the target DNA. The Cas9 is a single protein with 6 domains that include the recognition lobe, Rec 1, Rec2, arginine-rich bridge helix, PAM-interacting site, HNH and RuvC domains (Table 3), making it an efficient system to edit DNA. The binding of Cas9 to sgRNA triggers a conformational change, activating Cas9 to cleave the DNA (Fig. 4).

The sgRNA-activated Cas9 protein recognizes specifically the PAM in the target DNA; subsequently, crRNA mediates the complementary base pairing to the target DNA. The HNH and RuvC domains of Cas9 nuclease make double-stranded breaks to the complementary and non-complementary strands, respectively, at a site 3 base pair upstream to PAM (5'NGG-3') (20-22).

Properties of Cas9

The CRISPR-Cas9 system operates through Cas9 endonuclease, an RNA-guided nuclease, to target specific DNA sequences via complementary base pairing, inducing base deletion, insertion or replacement, disrupting gene function.

The apple PDs gene has mutants that target the third, sixth and seventh exons named ex3-20bp, ex6-20bp and ex7-20bp. The results show that ex7-20bp was the most effective target, showing that the sequence context of the target DNA is a key factor for its efficiency (23). Further, large-size deletions were attempted in tomato to target SlAGO7, a class of small RNAs known as trans-acting short interfering RNAs that regulate organ polarity through post-transcriptional silencing of the auxin response factor (ARF) by using two single guide RNAs that create large deletions of size ranging from 1 to 140 bp (24).

Table 3. Functions of the Cas9 and Cas12a protein domains

Enzyme	Domain	Function	References
Cas9	Rec 1	Responsible for binding of gRNA	(18)
	Rec 2	Not well studied, but part of a structural contribution	
	Arginine-rich bridge helix	Cleavage activity of the target DNA	
	PI	Interacting site to the PAM region of the target DNA	
	HNH	Nuclease domain is responsible for cleaving the DNA strand that is complementary to the gRNA	
	RuvC	Cleavage of the non-target strand of the DN	
Cas12a	Rec 1	Recognition and binding of crRNA	(19)
	REC 2	Stabilization of crRNA for the structural integrity of the binary complex	
	WED I	Stabilization and facilitating complex assembly	
	WED II	Involved in the stabilization of the crRNA within the complex	
	WED III	RNase site for processing crRNA to generate the active form of crRNA	
	RuvC	Nuclease lobe for endonuclease activity	
	PI	Interacting site to the PAM region of the target DNA	
	NUC lobe	Catalytic activity of Cas12a	
	BH	Connects rec and nuc lobe, facilitating communication	

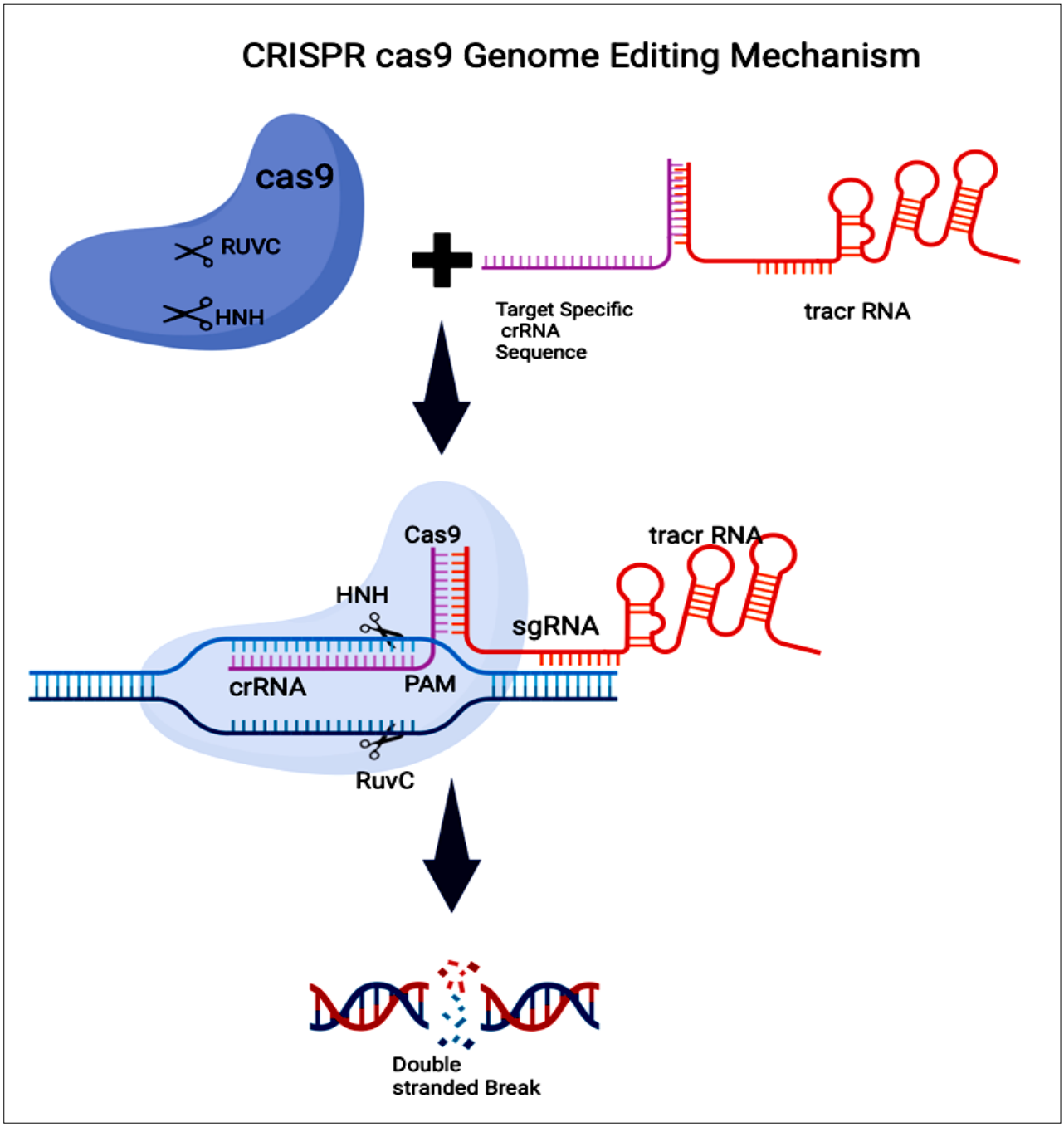

Fig. 4. Molecular mechanism of plant DNA editing using CRISPR Cas9 system.

Pros and cons

The Cas9-mediated plant DNA editing strategies are well-optimized for reliable editing in both prokaryotic and eukaryotic systems. This two-component system requires a single guide RNA (sgRNA) and Cas9, simplifying design and application. The large size of Cas9 is a limitation that complicates delivery via specific vectors. Moreover, natural SpCas9 requires NGG PAM, which may restrict target sites.

CRISPR-Cas12a

Cas12a, also referred to as Cpf1, is a component of the CRISPR-Cas system. It is classified as class 2 type V-a and functions by cleaving double-stranded DNA (dsDNA) by forming a ternary complex. The Cpf1 enzyme is classified as an RNA-directed endonuclease and requires only CrRNA but not tracrRNA (25). Several organisms include Cas12a in their immune systems; however, only a select handful can be utilized for genome editing.

Adaptation

In the Cas12a system, the Cas1 complexes with Cas2 to recognize, bind and process the pre-spacer DNA and to insert the pre-spacer sequence in the array (Fig. 5), while Cas4, an exonuclease, generates DNA ends of the pre-spacer for efficient integration into the spacer array (26, 27).

Cas12a-mediated editing

Cas12a is made up of the REC lobe (REC1, REC 2 and WED-I domains) and nuc lobe (ruvc, PAM interacting, WED-II, WED-III, Nuc and bridge helix domains) and does not require a tracrRNA or RNaseIII for processing of mature crRNA. The pre-crRNA is processed by the RNase site in the WED-III subdomain and stabilized by two mg^{2+} ions interacting with the WED, ruvc and rec2 domains (Table 3). The WED-II, III, REC 1 and PAM interacting domains are responsible for PAM recognition and binding; PI contains a lysine helix loop that inserts in the PAM duplex to unwind the double helix structure for ruvc and nuc-mediated catalytic cleavage.

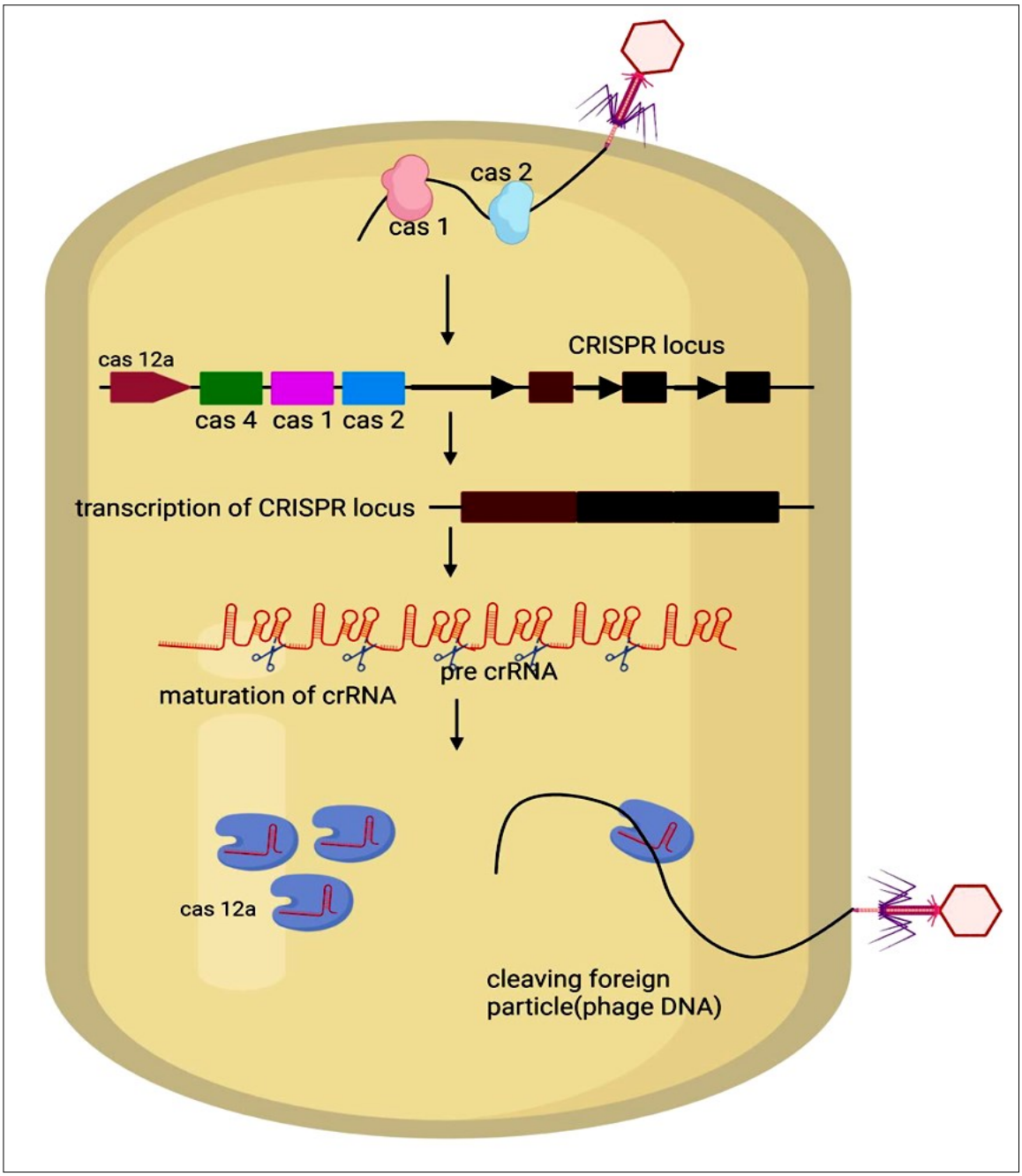

Fig. 5. The mechanism of CRISPR Cas12a system in bacterial cell.

The crRNA binds to the target DNA based on the conserved T-rich PAM site (TTTV, V=A/C/G) (Fig. 6) and produces large mutations (28-30). Studies reveal that the efficiency of the Cas12a system depends on the position of the second PAM site and the length of the PAM sites in delivering critical stability and increased fidelity, respectively.

Properties of Cas12a

The organisms commonly used for Cas12a include *Francisella novicida*, Acidaminococcus and Lachnospiraceae bacterium. In *Francisella novicida*, the fnCpf1 generates a 5' overhang on the non-target strand, the 18th base of the PAM, while on the targeted strand, at the 23rd base of the PAM producing staggered cut facilitating homology-directed repair (31-33). The efficiency of the Cas12a system relies on the effective design of the crRNA and temperature conditions; the efficiency of the process is influenced by crRNA intramolecular structure and reaction temperature. The Cas12a system works at different temperature ranges depending on the source of Cas12a.

More than 17 Cas12a orthologs have been described for genome editing; Ev1cas12a and Hs1cas12a show effective genome editing at lower temperatures across various plant species. In rice, Hs1cas12a and Ev1cas12a showed a 4-12 bp deletion, 12-23 bp away from the PAM site (Table 4). Similarly, in tomato protoplasts, the efficiency of genome editing with Ev1cas12a was higher at 25 c than at 32 c. The deletion size ranges from 3 to 13 bp, 12-23 bp away from the PAM site. The PAM sequence for both Ev1cas12a and Hs1cas12a is TTTV.

Pros and cons

The advantages of Cas12a are that it requires a short single crRNA (no tracrRNA), recognizes a T-rich PAM (e.g., TTTV) and is smaller than Cas9, facilitating easy design, expanding target sites and easy delivery, respectively. The T-rich PAM can restrict certain genomic target sites and is generally less

Table 4. Properties and functional characteristics of the CRISPR system

Enzyme	SgRNA length	Deletion length	Distance from PAM	Optimum temperature	References
TI-D	35 nt	Long-range and short indels	-	37 ^{0}C	(13)
Cas9	17-20 nt	Small and large deletions	3 nt upstream of PAM	22-26 ^{0}C	(32, 33)
Cas12a	42-44 nt	Small and large deletions	18-19 nt downstream of PAM	22-37 ^{0}C	(32, 33)

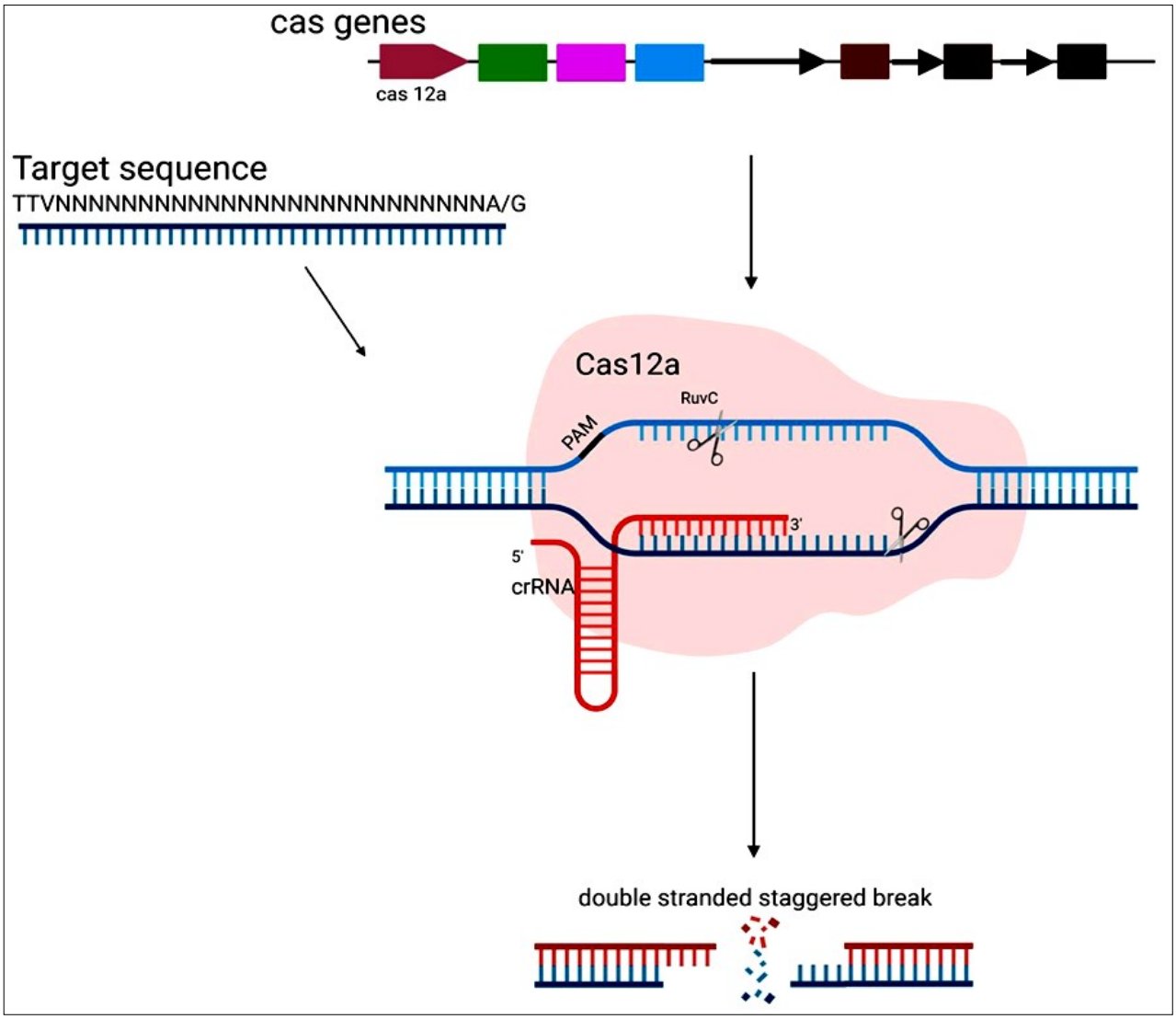

Fig. 6. Molecular mechanism of plant DNA editing using CRISPR Cas12a system.

efficient than Cas9 for some genomic loci.

Discussion

CRISPR for plant genome editing

CRISPR technology has revolutionized crop improvement by enabling precise and efficient genome editing. Gene editing has improved crop and product quality, shape, size, color, flavor and cooking quality. Gene-edited plants have shown resistance to plant viruses and tolerance to herbicides and they have demonstrated the potential to boost crop yield. Editing genes have also prolonged post-harvest shelf-life, biofortified crops with carotenoid and gamma-aminobutyric acid, modified the fatty acid composition in seed oils and eliminated antinutritional elements in crops (34, 35).

Technology for the future

Two recent technologies applied for plant genome editing are base and prime editing. Base editing allows for introducing specific nucleotide changes, such as transition and trans-versions, in the targeted DNA. On the other hand, prime editing can introduce insertions, deletions and all types of point mutations using the "search-and-replace" strategy.

Cytosine base editors (CBEs) and adenine base editors (ABEs) reduce the risk of off-target effects and improve precision by allowing the direct conversion of a cytosine (C) to a thymine (T) or adenine (A) to guanine (G) at a specific spot in the DNA without generating double-strand breaks (36). The ALS gene, coding for acetolactate synthase in *B. napus* has five genes (ALS 1, 2, 3, 4, 5). For example, base editing of the highly conserved genes Bn ALS 1 and Bn ALS 3 at position P197 converting C to T leads to high resistance to tribenuron-methyl herbicide (20).

Conclusion

The CRISPR-Cas system is pioneering new possibilities in agricultural biotechnology. When comparing the efficiencies and characteristics, Cas12a stands out due to its reliance on only crRNA for editing and its ability to create larger deletions. Understanding its mode of action, the characteristics of the edits and the nature of the mutations created enable the researchers to edit crops for various beneficial traits. As the agricultural sector continues to face increasing demands for higher production rates, the CRISPR system is poised to play a crucial role in crop production. However, there are a few bottlenecks to overcome: the policies to regulate gene-edited crops are ambiguous worldwide. It is important to establish a globally unified framework to regulate gene-edited crops. Further, technically, the CRISPR system offers limitations in target site selection due to the narrow PAM sequence and some produce off-target effects.

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Authors' contributions

RA conceptualized and designed the study, critically revised the manuscript and supervised the work. ACR, YN and SG contributed to the manuscript writing and prepared the representative diagrams. ACR drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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