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Emerging nucleases in crop genome editing: towards intellectual property independence and technical flexibility

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Abstract

As the global population grows and food security challenges increase, developing high-yield, stress-resistant crops have become crucial. Although CRISPR-Cas9 and CRISPR-Cas12a are the most widely utilized gene editing tools, their associated patent protections are notably stringent. This results in significant patent costs for commercializing breeding using these nucleases, highlighting the need for developing novel nucleases that possess autonomy of intellectual property (IP). In addition, novel nucleases exhibiting diverse recognition sites and hypercompact protein structures offer technical flexibility in gene editing. This review examines advancements in novel nuclease-based genome editing technologies and seeks to provide insights into the patent autonomy of plant genome editing, emphasizing the significant role of emerging compact nucleases in promoting sustainable agricultural practices and ensuring food security.

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Introduction

By 2050, the world population is projected to surge to 9.6 billion, resulting in an increased demand for staple crops of over 60%[1]. There is an urgent need to develop high-yield, stress-resistant, and adaptable crops to achieve sustainable agricultural development and meet the needs of the growing population. Using genetic resources for crop improvement is one of the key strategies to address this challenge. Traditional crop improvement primarily relies on spontaneous genetic variations that occur in nature or artificial mutations induced through physical or chemical mutagens. However, these approaches are limited by low efficiency and time-consuming processes.

Nuclease-mediated genome editing has been developing rapidly, representing a highly effective breeding technique[2,3]. Genome editing employs nucleases that generate double-strand breaks (DSBs) at specific genomic sites. These DSBs are then repaired by the host cell using two pathways: non-homologous end joining (NHEJ) or homologous recombination (HDR). This process allows for the introduction of mutations and enables precise modifications to the genome[4]. Pioneering genome editing technologies, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), has successfully facilitated the goal of genome editing in various crops[5–11]. However, the applications of ZFN and TALEN systems are restricted due to the time-consuming assembly process, high costs, and low editing efficiency[11,12]. The CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated proteins) system has emerged as a revolutionary genome editing tool. It offers significant advantages, including high efficiency, ease of design, low cost, and broad applicability, making it the predominant technology in gene editing. The Cas9 nuclease-based genome editing system has been effectively utilized in many plant species[13–20]. Currently, research on crop improvement predominantly relies on the Cas9 nuclease. However, restrictions on its use are imposed by intellectual property rights as well as technical limitations. There is an urgent need to explore novel nucleases that offer intellectual property independence[21,22]. Moreover, novel nucleases that exhibit diverse recognition sites, hypercompact protein structures, and evolutionary distinction from Cas9 are being identified, which offers technical flexibility in gene editing. Such development significantly expands the toolkit of genome editing in breeding applications. However, the efficiency of novel compact nucleases in plant genome editing still requires enhancement for practical breeding.

This review summarizes the development of nuclease-based gene editing technologies and explores the discovery and application of compact nucleases. With particular emphasis on plant genome editing, the work analyzes the unique opportunities and technical challenges confronting emerging compact nucleases in agricultural applications. Finally, the review proposes strategic implementation approaches and critical considerations for leveraging these novel compact editing systems to enhance editing efficiency and advance independent plant breeding initiatives.

Development of genome editing technologies

Programmable gene editing technologies have advanced through three distinct generations of nucleases. The first generation is represented by Zinc Finger Nucleases (ZFN), which are programmable chimeric nucleases consisting of a zinc finger protein (ZFP) DNA-binding domain linked to the Fok I nuclease cleavage domain at the carboxyl terminus[23]. The ZFP comprises a series of Cys2-His2 zinc finger motifs, each capable of recognizing approximately three bp of DNA, allowing for the artificial design of specific DNA sequences[24]. The Fok I enzyme, in contrast, requires the formation of a heterodimer to exert effective cutting action 6–8 bp downstream

of its recognition site[25,26]. Over the past two decades, ZFN technology has facilitated targeted modifications of endogenous genes in various crops[5–8,27]. The second generation of gene editing technology is the Transcription Activator-Like Effector Nuclease (TALEN) system. TALEN consists of Transcription Activator-Like (TAL) proteins that possess sequence-specific binding capabilities coupled with the Fok I nuclease, enabling targeted DNA cleavage. Each TAL monomer can recognize individual nucleotide target sites, thus offering enhanced specificity[28]. In plants, TALEN technology has been applied in genome editing of species such as tobacco[11], Arabidopsis[29,30], rice[10,31], and wheat[32,33]. ZFN and TALEN technologies rely on the customized design and synthesis of DNA sequence-specific protein modules. This process is laborious, time-consuming, and costly, limiting their broader applicability[34,35].

The third-generation technology, comprised of CRISPR-Cas gene editing, is characterized by its remarkable simplicity, robust programmability, and exceptional targeting efficiency, making it widely utilized in the genome editing of both plants and animals[36]. The most commonly applied systems within this category are CRISPR-Cas9 and CRISPR-LbCas12a (or Cpf1). The CRISPR-Cas system is an RNA-mediated adaptive immune system in prokaryotes that classifies various systems based on sequence similarities between Cas genes and modes of adaptation and interference, encompassing two significant classes and six types[37]. Class 1 systems include types I, III, and IV, primarily found in bacteria and archaea, typically forming multi-subunit protein-crRNA effector complexes[38]. Class 2 systems, comprising types II, VI, and V, rely on a single crRNA-guided protein nuclease to achieve localization and cleavage at target sites[39].

The engineered CRISPR-Cas9 system, derived from the Class 2 Type II CRISPR system, consists of two core components: the Cas9 endonuclease and a single-guide RNA (sgRNA). The sgRNA is a chimeric molecule formed by fusing crRNA (complementary to the target DNA) with tracrRNA (required for Cas9 activation), bypassing the need for RNase III-mediated processing in natural systems[40]. Cas9 contains two catalytic domains: the HNH domain cleaves the DNA strand complementary to the crRNA, while the RuvC-like domain cuts the non-complementary strand. This dual cleavage occurs three nucleotides upstream of the PAM sequence (typically 5'-NGG-3' on the non-target strand), generating blunt-ended double-strand breaks (DSBs)[36,41]. Various derivatives of Cas9, such as nCas9 and dCas9, further enhance CRISPR technology, enabling single-base editing, prime editing, transcriptional regulation, and epigenetic modifications[42–46]. PAM-relaxed variants like xCas9, Cas9-NG, and SpRY significantly expand the regions of CRISPR-Cas9 targets within the plant genome, enriching the plant gene editing toolbox[46–51]. On the other hand, exploring and utilizing SpCas9 ortholog (e.g., FrCas9, SaCas9, St1Cas9, NmCas9) further expands the options for nucleases in plant gene editing[52–56]. Notably, St1Cas9 exhibits reduced off-target effects and cytotoxicity, making it advantageous for plant genome editing[57].

The Cas12-type nuclease, classified as a Class II, Type V effector, can be subdivided into over twenty family proteins, ranging from Cas12a to Cas12n, each exhibiting distinct structural and functional characteristics[58]. CRISPR-Cas12a, a Class II Type V effector, differs fundamentally from Cas9 in both structure and function. It requires only a single crRNA for targeting and possesses intrinsic RNase activity to autonomously process precursor crRNA into mature forms, without the need for tracrRNA or auxiliary enzymes[59,60]. Cas12a recognizes T-rich PAM sequences (e.g., 5'-TTTN-3' for LbCas 12a) located upstream of the target site on the non-complementary strand. Its single RuvC domain cleaves both DNA strands sequentially, generating staggered double-strand breaks with 5' overhangs 18–23 bp downstream of the PAM[61–63]. This T-rich PAM preference enables efficient editing in AT-rich genomic regions (e.g., plant promoters)[64]. Cas12a system can process multiple crRNAs from a single transcript also facilitates multiplex genome editing, a critical feature for engineering plant polygenic traits[65–68]. Currently, several types of Cas12a nucleases have been discovered and applied in plants. LbCas12a and AsCas12a are the most commonly used due to their high editing efficiency and strong applicability[69].

IP landscape of CRISPR-Cas9 and CRISPR-Cas12 systems

Gene editing technology based on the CRISPR-Cas system has emerged as a pivotal advancement in genetic engineering. There has been significant interest from various research teams and enterprises, which has led to a heightened focus on the intellectual property (IP) landscape related to this technology. Cas9 is the first nuclease utilized within the CRISPR framework, demonstrating a broader patent scope than the overall CRISPR technology[70]. Given the complexity of CRISPR-Cas patents in different countries, here we focus on the patent landscape in China as a representative.

The foundational patent rights for CRISPR-Cas9 are held by two main groups: the CVC team (including the University of California, the University of Vienna, and the Emmanuelle Charpentier) and the Broad Institute team, which includes the Broad Institute, the Massachusetts Institute of Technology, and the President and Fellows of Harvard College. In 2013 (priority date in 2012), the CVC team filed a patent application for the CRISPR-Cas9 system, establishing a crucial foundation for the technology's subsequent development. The Broad Institute submitted corresponding patent applications in the same year, ultimately leading to a patent dispute with the University of California. Both parties claim rights to the CRISPR-Cas9 technology, leading to considerable overlap in the patents involved. After a decade of legal proceedings, the United States Patent and Trademark Office (USPTO) concluded in 2021 that specific patents held by the Broad Institute were valid and that the institute held rights to them. Nonetheless, disputes regarding the scope and legality of specific patents persist between the two entities[71–73].

The patent landscape for CRISPR-Cas9 system nucleases is quite complex, and the opportunities for operating freely within Cas9-based systems are limited. As a result, users of this technology must carefully assess patent risks and develop effective strategies to navigate these challenges. The patent issues surrounding Cas9 may pose significant restrictions on the commercial activities of emerging companies, particularly adversely affecting small enterprises that may find it challenging to afford the high costs of patent licensing. This situation could ultimately hinder technological development and application.Despite ongoing invalidation challenges to CRISPR-Cas9 related patents over the past years, Chinese patent authorities have consistently upheld the validity and broad claims of patent portfolios, particularly the enforceability of the divisional application CN201710585690.5 from the CVC team, encompassing rights related to sgRNA and its application, as shown in Table 1. Consequently, circumventing this patent while utilizing Cas9-sgRNA nucleases presents considerable challenges[74].

It is important to note that the gRNA patent held by the CVC team is expressly limited to its use in conjunction with the Cas9 protein. This patent does not cover gRNA interacting with other nucleases, such as Cas12[74–76]. Additionally, the scope of patent protection for crRNA associated with Cas12 proteins remains ambiguous. Given the diversity of Cas12 protein types, developing and applying novel Cas12-based systems for plant gene editing may represent an effective strategy for overcoming patent barriers. Currently, the earliest

Table 1. Published patents of CRISPR-Cas9 and CRISPR-Cas12 systems in China.

Assignee	Application number	Main claims	Expiration date
CVC team	CN201380038920.6	The genome editing system is composed of Cas9 and sgRNA.	2033-03-15
	CN201710585690.5	The genome editing system is composed of Cas9 and sgRNA.	2033-03-15
Broad Institute team	CN201380070567.X	The vector system for Cas9 and gRNA is utilized in eukaryotic cells.	2033-12-12

patent for LbCas12a (Cpf1) nucleases is held by the Broad Institute, primarily protecting the V-A type CRISPR system (Application No. CN201680035959.6) and a CRISPR system based on Cpf1 with a PAM of 5'T (Application No. CN201810911002.4)[77,78]. Additionally, several patents related to V-type nucleases, beyond the V-A type, have already been filed and granted in China, as detailed in Table 2.

Regarding the novel compact nucleases of the Cas12 class currently explored, most related patents ar encompass the design of new CRISPR/Cas systems, the development of novel enzymes, and specific gene-editing methods applicable to certain plants or animals. Chinese patent applicants primarily include universities, research institutions, biotechnology companies, and various startups. Many enterprises seek to expand their technological barriers and safeguard their commercial interests through patent applications. Furthermore, with the growing global interest in CRISPR technology, research institutions, and companies are increasingly engaging in international collaboration and exchange, aiming to enhance their global influence in terms of intellectual property rights.

Discovery of novel nucleases with genome editing abilities

With the widespread application of Cas9 technology and the increasing expansion of associated patent protections, significant challenges to the autonomy of intellectual property rights for gene editing have emerged. The currently utilized CRISPR-Cas9 nuclease system consists of over 1,300 amino acids, necessitating integrating regulatory elements such as promoters and termination signals during vector delivery. This size surpasses the cargo capacity of viral delivery of transgene in plants. Moreover, the strict recognition of the protospacer adjacent motif (PAM) sequence (NGG) by Cas9 limits its targeting ability in adenine-thymine (A-T) rich regions, such as promoters[79]. Thus, editing crop promoters targeted by nucleases like Cas12a can enhance regional coverage, create genotype-rich mutants, achieve variations in crop quantitative traits, and enable the screening of desirable crop characteristics[64,80]. Additionally, during the precise targeting of recipient genomes, the CRISPR-Cas system may encounter off-target effects, and the off-target efficiency depends not only on nucleases, sgRNAs, and tissue culture processes[81–83]. These challenges have significantly driven the exploration and application of novel compact nucleases. Consequently, more researchers are leveraging genomic prediction and bioinformatics tools to identify new compact nucleases with endonuclease activity and flexible intellectual property rights across diverse bacterial and archaeal species[84]. Building upon these findings, they aim to develop more comprehensive and adaptable programmable genome editing tools through synthetic biology approaches.

Based on phylogenetic relationships and classification, the novel nucleases identified were categorized into Class II-Type II and Class II-Type V analogs. Within the second class of type II, several effectors rich in arginine and lysine have been successfully identified, including the Cas9d and Cas9C2 series, which consist of approximately 600 to 1,050 amino acids[85]. These effectors differ from conventional Cas9 by encoding a zinc finger within the HNH structural domain and incorporating multiple zinc-binding motifs in their recognition domain. Cas9d has exhibited an editing efficiency of up to 90% in mammalian cells, presenting a promising alternative for efficient nuclease-mediated genome editing[85].

Additionally, the Type V Cas12 nuclease system encompasses a wide variety of variants characterized by their relatively small size. FnCas12a is particularly effective at low temperatures, allowing for efficient editing in cold conditions[69]. Additionally, Cas12a orthologs such as Lb5Cas12a, BsCas12a, Mb2Cas12a, TsCas12a, and MbCas12a nucleases have also been developed for plant gene editing[86]. With ongoing exploration, several novel compact nuclease systems have been discovered and applied, including CasX(Cas12e)[87,88], Cas14(Cas12f)[89], Cas12i[90], CasΦ(Cas12j)[91], and Casπ(Cas12l)[92]. It is noteworthy that in 2020, researchers identified the Casλ effector from a family of enzymes encoded by bacteriophages. This effector possesses a unique guide RNA (gRNA) capable of successfully inducing genome editing of endogenous genes in both human and plant cells[93].

Recent phylogenetic analyses have identified Fazor and TnpB from the OMEGA (specific mobile element-guided activity) system as ancestors of CRISPR-Cas12, while IsCB is recognized as the ancestor of CRISPR-Cas9. Notably, these nucleases possess an extremely compact size that facilitates the delivery of adeno-associated viruses (AAV), suggesting they may substitute CRISPR-Cas9 *in vivo* gene editing, thereby offering promising applications. TnpB is categorized as a programmable RNA-guided functional nuclease, cleaving DNA near the 5' end of the transposon-associated motif (TAM) through reRNA (right element RNA), enabling precise targeting and editing of the genomic DNA[94]. Researchers have identified various TnpB variants with distinct characteristics that allow genome editing across multiple cell types[95]. Subsequently, based on AlphaFoldDB, the researchers identified a eukaryotic TnpB-IS200/IS605-like protein named Fanzor, which exhibits RNA-induced editable nuclease functionality in eukaryotes[96]. In addition, a compact RNA-inducible nuclease, IscB (~400 amino acids, primarily composed of a RuvC structural domain and an HNH nuclease structural domain), has been identified from IS200/IS605 superfamily transposons. It is associated with evolutionarily conserved non-coding RNAs. Phylogenetic analyses indicate that all extant Cas9 enzymes are derived from a single common ancestor, IscB[97]. The compact IscB exhibits low editing activity in eukaryotic cells, thus limiting its further applications. An efficient enIscB system was developed for mammals, enhancing the toolkit for eukaryotic genome editing[98]. Those novel nucleases within the OMEGA system generally exhibit an extremely compact size, providing significant delivery and vector assembly advantages.

Application of compact nucleases in plant genome editing

Due to its unparalleled ability to precisely manipulate plant genomes, the CRISPR-Cas system has become a powerful tool in agriculture[99]. This technology facilitates the rational design of varietal traits, significantly accelerating the domestication process of various species and fundamentally transforming traditional breeding systems[2].

Table 2. IP landscape of representative nucleases in China.

	Assignee	Application number	Claims	Expiration date
Cas12b	Institute of Zoology, Chinese Academy of Sciences, and Beijing Institute of Stem Cell and Regenerative Medicine	CN202110591698.9	Cas12b variants	2040-12-07
chCas12b	Fudan University	CN202110606220.9	chCas12b (Cas12J-8)	2041-5-31
Cas12d/CasY	Longping Biotechnology (Hainan) Co., Ltd.	CN202310237362.1	CasD	2043-03-13
Cas12f/Cas14	Jilin Academy of Agricultural Sciences	CN202210204370.1	CasF2	2042-03-03
Cas12f (cas12i3)	China Agricultural University	CN201980014560.3	Cas12f	2039-10-29
		CN202110473640.4	Divisional Application, Under examination	/
		CN202110473632.X	Divisional Application	2039-10-29
	Bellagen Biotechnology Co., Ltd.	CN202210603607.3	Cas12i3 variants with S7R, Y124R mutations	2042-05-31
	Bellagen Biotechnology Biotechnology Co., Ltd.	CN202210314807.7	Cas12i3 variants with N417K, S638N mutations	2042-03-29
	Bellagen Biotechnology Co., Ltd.	CN202310088437.4	Cas12i3 variants with D233R, D267R, N369R, S433R mutations	2043-02-02
	Bellagen Biotechnology Co., Ltd.	CN202311039065.2	Fusion of Cas9-HNH with Cas12i3	2043-08-17
Cas12i	China Agricultural University	CN201980027152.1	Cas12i (Cas12i.1) protein and application	2039-04-19
	Institute of Zoology, Chinese Academy of Sciences, and Beijing Institute of Stem Cell and Regenerative Medicine	CN202011414384.3	Cas12i variants	2040-12-07
Cas12i12	HuiEdit Therapeutics Co., Ltd.	CN202111290670.8	Under examination	/
	Bellagen Biotechnology Co., Ltd.	CN202310096173.7	Cas12i12 variants with G179R, N269R, Q445R, V447R, E492R, T632R, A825R mutations, under examination	/
Cas12j/Casφ	China Agricultural University	CN201980014005.0	Cas12j.19	2039-11-15
		CN202110475316.6	Divisional Application of Cas12j.4	2039-11-15
		CN202110475336.3	Divisional Application, under examination	/
Cas12j.19	Bellagen Biotechnology Co., Ltd.	CN202310092231.9	Cas12j.19 variant with E100R mutation	2043-02-03
IscB	HuidaGene Therapeutics Co., Ltd.	CN202311617088.7	Novel IscB polypeptides and their applications	2043-11-30
TnpB	Beijing Coveland Biological Science Co., Ltd.	CN202311230195.4	Mini-TnpB after functional domain streamlining (less than 380 AA)	2043-09-22
	Huazhong Agricultural University	CN202310177144.3	TnpB identified from Archaea	2043-02-28
Gs12-7	Huazhong Agricultural University	CN202310086152.7	Nucleic acid endonucleases with high activity and temperature tolerance	2043-01-17
Gs12-16/Gs12-18	Huazhong Agricultural University	CN202310256014.9	Gs12-16 (PAM=NYYV), and Gs12-18 (PAM=YTYV) (V=A/C/G)(Y=C/T)	2043-03-16
Gs12-10	Huazhong Agricultural University	CN202310006257.7	Gs12-10 nuclease without PAM restrictions	2043-01-02
Gs12-3/Gs12-5	Huazhong Agricultural University	CN202310247616.8	Nucleases with a wider range of target sites	2043-03-10
CasD	Longping Biotechnology (Hainan) Co., Ltd.	CN202310237362.1	CasD nuclease with 5'-TNN PAM	2043-03-13
PaeCascade	Sun Yat-sen University	CN202010527709.2	Type I-F based CRISPR/Cas system	2040-06-11
Cascade-Cas3	China Pharmaceutical University	CN202211136496.6	Type I-B CRISPR-Cascade-Cas3	2042-09-19

With the ongoing exploration of the CRISPR-Cas system, novel compact nucleases have emerged in plant genome editing alongside the conventional Cas9 and Cas12a. These nucleases are characterized by their smaller size and greater flexibility, making them the focus of considerable interest (Table 3). Nucleases such as CasX, MAD7, and various Cas12 variants offer distinct advantages in targeting specific genomic sequences, thereby promoting precise modifications of plant genes.

The CRISPR-MAD7 is claimed to be a royalty-free nuclease derived from the *Eubacterium rectale* found in Madagascar. This nuclease shares about 76% nucleotide sequence similarity with its natural counterpart and consists of approximately 1,200 amino acids, while the amino acid homology with *Acidaminococcus* Cas12a (AsCpf1) is only 31%[86,100]. Nonetheless, it remains unclear whether CRISPR-MAD7, a Cas12a-type nuclease, is subject to existing LbCas12a or AsCas12a patents. MAD7 preferentially recognizes the YTTN protospacer adjacent motif (PAM), but its application in plants is limited. The CRISPR-MAD7 9 system has achieved editing efficiencies of 49.0% to 65.4% at *OsALS* and *OsEPSPS* sites in rice, though it has lower activity than other Cas12a nucleases[86,100]. In soybean root hairs, about 20% to 35% editing efficiency was achieved through co-expression of human FTO proteins[101].

The application of the nuclease from the sub-type V-I system (Cas12i) represents a significant development in using emerging compact nucleases for plant genome editing. Compared to SpCas9 and Cas12a, Cas12i effectors are smaller (ranging from 1,033 to 1,093 amino acids), and their mature crRNA is shorter (40-43 nucleotides), making Cas12i particularly suitable for multiplex genome editing and virus-based delivery systems[102]. Structure-guided rational design and protein engineering were employed to optimize the previously uncharacterized Cas12i3 nuclease, leading to the development of Cas-SF01. This variant exhibits a broader PAM range, enabling effective recognition of NTTN sequences. An upgrade to the Cas-SF01^{HiFi} system, achieved by incorporating the D876R modification, enhances its low off-target activity and high efficiency[90]. Approximately 77.2% editing efficiency was observed in stabilized transformation events at 12 targeting sites in monocotyledonous rice mediated by Cas-SF01, with an increased occurrence of homozygous, bi-allelic, and chimeric events in the E0 generation. Furthermore, the system demonstrated approximately 52.5% editing efficiency in dicot chili peppers and soybean root hairs, which is twice the editing efficiency of Cas9 (about 26.8%), underscoring its substantial potential in plant research and molecular breeding[90]. To enable the aggregation of multiple genetic traits in rice, the Cas12i3-based iMAGE serial system was constructed to edit multiple endogenous targets, achieving knockout efficiencies ranging from 38.4% to 47.3% at the *OsNramp5* gene locus[103]. Additionally, an ABE system based on Cas12i3 was developed, which resulted in base conversions ranging from 4.0% to 7.3% in rice callus[103]. In soybean, targeting the aroma candidate genes *GmBADH1* and *GmBADH2* using Cas12i3 enabled the successful

Table 3. Representative novel nucleases for genome editing in plant.

Nuclease	Origin	Editor type	PAM/TAM	Species	Ref.
MAD7 (ErCas12a)	*Eubacterium rectale*	Knockout/CBE	YTTN	Rice, wheat, soybean, arabidopsis	[86,100,101,113]
FnCas12a	*Francisella novicida*	Knockout	TTV	Rice, maize, arabidopsis, Populus, tobacco, wheat	[67,69,114–116]
Lb5Cas12a	*Lachnospiraceae bacterium* MA2020	Knockout	TTTV	Rice	[86]
BsCas12a	*Butyrivibrio* sp. NC3005	Knockout	VTTV	Rice	[86]
MbCas12a	*Moraxella bovoculi*	Knockout	TTTV	Rice	[86]
Mb2Cas12a	*Moraxella bovoculi* strain 57922	Knockout	VTTV	Rice and cotton	[86,117,118]
Mb2Cas12a-RVR	*Moraxella bovoculi* strain 57922	Knockout	TATV	Rice	[86]
Mb3Cas12a	*Moraxella bovoculi* AAX11 00205	Knockout	NTTV	Rice, maize, tomato	[80]
HkCas12a	*Helcococcus kunzii* ATCC 51366	Knockout	VTTV	Rice	[80]
PrCas12a	*Pseudobutyrivibrio ruminis* CF1b	Knockout	VTTV	Rice	[80]
TsCas12a	*Thiomicrospira* sp. XS5	Knockout	TTTV	Rice	[86]
AaCas12b	*Alicyclobacillus acidiphilus*	Knockout/CRISPRi/CRISPRa	VTTV	Rice	[119]
AacCas12b	*Alicyclobacillus acidoterrestris*	Knockout/CRISPRi/CRISPRa	TTV	Rice and cotton	[119,120]
BhCas12b	*Bacillus hisashii*	Knockout/CRISPRi/CRISPRa	VTTV	Rice	[119]
BthCas12b	*Bacillus thermoamylovorans*	Knockout/CRISPRi/CRISPRa	ATTN	Rice	[119]
BvCas12b	*Bacillus* sp. V3-13	Knockout	TTN	Arabidopsis	[121]
BhCas12b v4	*Bacillus hisashii* variant	Knockout	TTN	Arabidopsis	[121]
Cas12f	*Syntrophomonas palmitatica (Sp), Acidibacillus sulfuroxidans,* and uncultured archaeon (Un1)	Knockout	TTTR	Tobacco and rice	[108,122,123]
Cas12i3 (Cas-SF01)	*Lachnospiraceae bacterium*	Knockout	NNTN	Rice, pepper, soybean	[90,104,124]
CasΦ(Cas12j-2)	Biggiephage	Knockout/CRISPRa/Epigenome editor/CBE/ABE	NTTV	Arabidopsis, rice, tomato, tobacco, wheat, rye	[91,107,108,125]
Cas12j-SF05	*Caudoviricetes*	Knockout	TTN	Rice	[126]
Cas12j-8	Biggiephage	Knockout/CBE	TTN	Soybean and rice	[109]
TnpB-IsDra2	*Deinococcus radiodurans*	Knockout/ABE/CRISPRa	TTGAT	Rice and arabidopsis	[110–112,127]
TnpB-IsYmu1	*Youngiibacter multivorans*	Knockout	TTGAT	Rice	[111,127]
TnpB-IsAam1	*Anoxybacillus amylolyticus*	Knockout	TTTAA	Rice	[111,127]
TnpB-IsDge10	*Deinococcus geothermalis*	Knockout	TTAT	Rice	[111]
enIscB	Biggiephage	Knockout	NWRRNA	Rice	[111]

breeding of soybean varieties with enhanced aroma characteristics suitable for soybean milk production[104]. In addition to staple crops, the Cas12i3-based knockout of the *PmBR2-a* and *PmBR2-b* genes in broomcorn millet has successfully resulted in the production of plants exhibiting dwarf phenotypes, leading to denser planting and ultimately higher harvest indices[105,106]. Overall, Cas12i3 demonstrates remarkable performance in crop breeding, with Cas-SF01 achieving editing efficiencies at specific loci that meet or exceed those of Cas9. Furthermore, this nuclease reduces dependency on patent-constrained Cas12a systems, positioning it as a strategically valuable tool for modern crop breeding and commercialization.

CasΦ (Cas12j), a nuclease (about 700-800 amino acids) derived from the family of large bacteriophages, represents another compact nuclease that has been explored and applied in plants. This nuclease can recognize T-rich PAM sequences and demonstrates heightened sensitivity to chromatin environments[107]. CasΦ-2 (also known as Cas12j-2) has been employed to target the Arabidopsis *AtPDS* locus for heritable edits for plant genome editing. Additionally, several variants of vCasΦ and nCasΦ have been developed to enhance editing efficiency[107]. Despite recent advances, the overall efficiency remains unsatisfactory. Cas12j has been demonstrated to facilitate gene editing in several species, including tomato, tobacco, and poplar[91,108]. Through the rational engineering of crRNA and Cas12j-8 proteins, a gene knockout and cytosine base editor system based on en4Cas12j-8/cRNA-Rz was developed, showing promising editing activity in rice and soybean[109].

The TnpB nuclease from the OMEGA system, derived from the transposon family, is currently one of the most compact nucleases achieving genome editing in plants. Among these, the ISDra2 TnpB (about 400 amino acids, target adjacent motif (TAM: TTGAT) from *Deinococcus radiodurans* was the first to demonstrate targeted editing in rice protoplasts and regenerated callus, with editing efficiencies ranging from approximately 1.5% to 7.15%. Furthermore, employing a tandem tRNA-crRNA-HDV system achieved multiplex genome editing in rice, with a maximum editing efficiency of 14.84% ± 4.88% at the *OsHMBPP* locus[110]. Subsequent applications of the same strategy in *Arabidopsis thaliana* yielded an editing efficiency of around 1%[110]. As efforts to explore TnpB nucleases continue, additional variants such as ISAam1 (about 360 amino acids, TAM: TTTAA) and ISYmu1 (about 380 amino acids, TAM: TTGAT) have also been identified and utilized for editing in rice and *Arabidopsis*. However, the overall editing efficiency remains relatively low. Notably, the IsDge10 TnpB (about 390 amino acids, TAM: TTAT), isolated from *Deinococcus geothermalis*, demonstrated editing efficiencies ranging from 2.20% to 15.04% in rice protoplasts and from 4.2% to 25.0% in T0 regenerated seedlings. This TnpB also succeeded in editing seven target sites simultaneously in protoplasts using multiple target tandems, with efficiencies ranging from approximately 4.3% to 18.2%[111]. Moreover, IsDra2 has been utilized for genome editing in various medicinal plants, underscoring the promising applications of this novel compact nuclease in regulating secondary metabolite production in medicinal flora[112]. Currently, the efficiency of TnpB-like novel nucleases in genome editing across various plant species still presents significant opportunities for improvement. Additionally, TnpB's recognition of target TAM sequences shows considerable limitations. Therefore, mining novel TnpB nucleases with broader TAM recognition capabilities, or the engineering of existing nucleases to enhance genome editing efficiencies, is essential for advancing the utility of TnpB nucleases in plant biotechnology.

Prospects in the application of novel nucleases in plants

The discovery and efficient application of novel nucleases can significantly alleviate the constraints of intellectual property rights in plant breeding. Exploring novel nucleases and developing efficient editing systems are crucial for expanding a toolset of editing technologies with independent intellectual property rights. However, the successful application of novel nucleases derived from various sources, including microorganisms, in plants still faces challenges. One of the challenges includes the complex IP landscape surrounding CRISPR-Cas nucleases[128]. Furthermore, eukaryotic systems, such as plants, are inherently more complicated than prokaryotic systems, which presents additional challenges for applying novel nucleases in plants. These challenges include low editing efficiency, difficulties in delivery, and reduced activity of the nucleases.

Bioinformatics and associated tools are leveraged to discover new nucleases with independence of intellectual property. For example, by developing a large-scale mining approach for TnpB-related targeted gene-editing systems, researchers managed to identify 33 TnpB proteins that have targeted editing functions in prokaryotic systems. Among them, five were found to be active in eukaryotic systems. This eventually led to developing the most minor viable micro gene editor with independent IP to date[129]. Subsequently, the first miniaturized novel nuclease system, SisTnpB1, derived from the archaeon *Sulfolobus Islandicus* REY15A, was characterized using a mining approach, and an efficient gene editing method was developed[130]. Moreover, based on AlphaFold Protein Structure Database (AlphaFold DB), the researchers discovered and validated the characterization of Fazor proteins as nucleases in the eukaryotic system[96]. These examples demonstrate that employing relevant algorithms to identify novel nucleases with endonuclease activity can effectively circumvent the patent restrictions associated with CRISPR-Cas9 and Cas12 systems, leading to the establishment of various functional editors suitable for eukaryotic systems.

Engineering modifications can be utilized to boost the efficiency of genome editing when using new compact nucleases. Researchers have employed a variety of evolutionary approaches to improve the activity and specificity of these novel nucleases in host plants. One way to achieve this is through a process called directed evolution. This involves introducing random mutations into the nuclease genes and screening for enzyme variants performing exceptionally well under specific conditions. By doing so, researchers can obtain enzymes with enhanced activity. Several methods are used in directed evolution, such as extracellular mutagenesis, intracellular mutagenesis, and virus-assisted evolution. In 2011, the phage-assisted continuous evolution (PACE) system was reported, which has further advanced the field of nuclease engineering[131].

Additionally, using advanced technologies such as X-ray crystallography and cryo-electron microscopy enables researchers to analyze the three-dimensional structures of nucleases. These structural insights provide critical guidance for selecting mutation sites, allowing researchers to optimize enzyme structures effectively to enhance their functionality. The application of computational modeling will enable researchers to predict the impact of mutations on enzyme activity, which not only accelerates the optimization process but also provides a theoretical basis for designing more efficient nucleases. Researchers can further refine enzyme designs by integrating experimental data with computational models, improving their effectiveness in plant genome editing[126].

Different species exhibit unique codon preferences; implementing codon optimization can significantly influence the stability of heterologous mRNA in target species, thereby enhancing the expression levels of nucleases and ultimately improving gene editing efficiency[132,133]. In editing systems, optimizing nuclease expression levels relies not only on the design of coding regions but also on adjusting the combinations of non-coding region elements, such as selecting strong promoters for ubiquitous expression, varying the combinations of 5' and 3' untranslated regions (UTR), applying expression enhancers, and manipulating the number of introns within exogenous genes[134–137]. Furthermore, the secondary structure stability of sgRNA and cutting efficiency also significantly impact gene editing outcomes. Optimization strategies, such as incorporating HDV elements, tandem transfer RNA (tRNA), and improving secondary structures, have considerably enhanced editing effectiveness[138]. Additionally, the chromatin environment surrounding the target gene sites affects the accessibility of the nuclease complex, and regulating chromatin openness can significantly enhance the editing efficiency of endogenous genes in plants[101]. Optimizing expression systems can substantially improve the expression abundance of heterologous CRISPR-Cas systems within plant tissues, thereby facilitating increases in gene editing efficiency.

Enhancing the efficiency of plant genome editing primarily relies on effective delivery and transformation systems. While established methods such as *Agrobacterium*-mediated transformation, transformation of callus tissues with *Agrobacterium tumefaciens*, gene gun transformation, RNP transformation, and root-based cell transformation systems (CDB) are already in use, there is an urgent need to develop novel and efficient delivery strategies. These innovations are vital to maximizing the editing capabilities of the new nucleases[139,140].

In summary, with the rapid advancement of genome editing technologies, exploring novel compact nucleases offers unprecedented opportunities for plant genome editing. Optimizing and advancing new tools based on these compact nucleases would help avoid the fundamental IP issues linked to Cas9 and Cas12 and enable the autonomy of core technologies in gene editing systems to reduce the technical barriers.

Author contributions

The authors confirm their contributions to the paper: study conception and design: Guan Y, Li M; data collection: Lin W, Li C; draft manuscript preparation: Guan Y, Lin W, Li C. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in the published article.

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Conflict of interest

The authors declare that they have no conflict of interest.

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