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REVIEW

Research progress on nucleic acid detection and genome editing of CRISPR/Cas12 system

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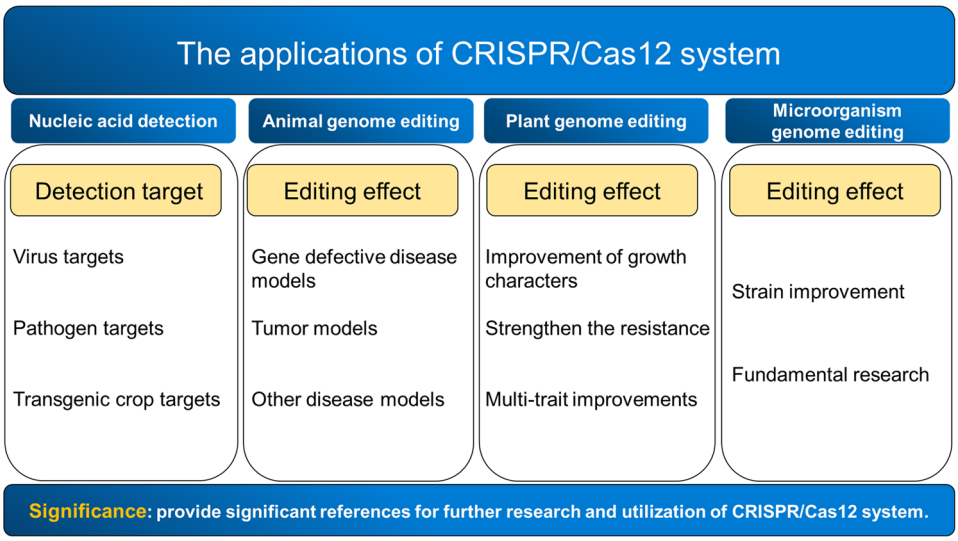
Abstract

Purpose This work characterizes the applications of CRISPR/Cas12 system, including nucleic acid detection, animal, plant and microbial genome editing.
Methods The literature on CRISPR/Cas12 system was collected and reviewed.
Results CRISPR/Cas system is an acquired immune system derived from bacteria and archaea, which has become the most popular technology around the world because of its outstanding contribution in genome editing. Type V CRISPR/Cas systems are distinguished by a single RNA-guided RuvC nuclease domain with single effector molecule. Cas12a, the first reported type V CRISPR/Cas system, targets double-stranded DNA (dsDNA) adjacent to PAM sequences and trans-cleaves single-stranded DNA (ssDNA). We present the applications of CRISPR/Cas12 system for nucleic acid detection and genome editing in animals, plants and microorganisms. Furthermore, this review also summarizes the applications of other Cas12 proteins, such as Cas12b, Cas12c, Cas12d, and so on, which further widen the application prospects of CRISPR/Cas12 system.
Conclusions Knowledge of the applications of CRISPR/Cas12 system is necessary for improving the understanding of the functional diversity of CRISPR/Cas12 system and also provides significant references for further research and utilization of CRISPR/Cas12 in other new fields.

Graphical abstract



Keywords CRISPR/Cas12 · Nucleic acid detection · Genome editing · Cas12 proteins

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Introduction

The CRISPR (Clustered regularly interspaced short palindromic repeat) system is a natural acquired immune system that prevents the invasion from viruses and phage through the combination with CRISPR associated (Cas) proteins [1]. According to the 2020 classification, CRISPR/Cas systems include 2 Classes, 6 types and 33 subtypes [2]. In Class 1 CRISPR/Cas systems (including type I, type III, and type IV), the effector module composes of multisubunit effector complexes (such as Cas3, Cas5-Cas8, Cas10 and Cas11). By contrast, the effector module of Class 2 CRISPR/Cas systems (including type II, type V, and type VI) is represented by single, large multidomain protein effectors (such as Cas9, Cas12 and Cas13). The CRISPR/Cas systems, consisting of Cas gene sequences, protospacer, direct repeat sequences and the spacer sequences, can specifically recognize foreign nucleic acid molecules by the aid of CRISPR RNAs (crRNAs), and then cleave them by Cas proteins. As the spacer sequences can be easily redesigned to introduce a double-strand break within a given sequence, the CRISPR-based technology has been widely employed in genome editing, gene transcription regulation, epigenetic engineering and many other fields [3]. Currently, three Class 2 effectors, Cas9, Cas12 and Cas13, have been extensively applied in many fields because of their high sensitivity, high specificity and easy operation. The genome editing systems represented by the CRISPR/Cas9 have been successfully applied in the fields of agriculture, medicine, biology, such as the establishment of new breeding methods, the development of new gene drugs, and the exploration of biological pathways. With the in-depth study, the other types of Cas proteins, such as Cas13a and Cas12a, have been successively developed and applied in genome engineering, nucleic acid detection and molecular diagnostics. Cas12a, also known as Cpf1, is a single crRNA-guided endonuclease with a single RuvC and Nuc domain, which is responsible for the cleavage of target DNAs adjacent to PAM sequences and generates DNA ends with a 5′ overhang [4]. Type V CRISPR/Cas systems are distinguished by a single RNA-guided RuvC nuclease domain with single effector molecule. Cas12a, the first reported type V CRISPR/Cas system, has been demonstrated to be a dual-nuclease with endoribonuclease and endonuclease activities that is specific to crRNA biogenesis and target DNA interference [5]. Meanwhile, CRISPR/Cas12a has both cis- and trans-cleavage activities on single-stranded DNA (ssDNA), which further expands its potential applications [6]. Subsequently, other subtypes of type V Cas proteins, such as Cas12b (C2c1) [7, 8], Cas12c (C2c3) [8], Cas12f (Cas14) [9] and so on, have been identified one after another. Recently, CRISPR/Cas12-based methods with high sensitivity and specificity have been employed for nucleic acid detection and CRISPR-based diagnostics (CRISPR-Dx) [3]. Compared with Cas13, Cas12-based methods are more popular because of its fewer steps for target nucleic acid amplification and trans-cleavage of ssDNA probes. Furthermore, genome editing using CRISPR/Cas12 system has been widely applied in of humans, animals and plants. Although Cas12-based CRISPR-Dx methods and their applications have already been reviewed [3], the latest detection methods and their applications in genome editing still need further illustrating. In this paper, the applications of CRISPR/Cas12 system, including the applications in nucleic acid detection and molecular diagnostics, genome editing of animals, plants and microorganisms, are summarized, which can provide significant references for further research and utilization of CRISPR/Cas12 in other new fields.

CRISPR/Cas12-based nucleic acid detection

Acting as the carrier of genetic information, nucleic acids have been becoming one of the most important research fields for the development of DNA and RNA detection technologies. Previous detection and imaging technologies are mainly based on traditional methods, such as in vitro amplification, protein hybridization and microarray. Although these methods can meet the requirements for detection and imaging of targets, they still have some defects due to time-consuming, high cost, low precision and sensitivity. The Cas12-based detection methods, such as DETECTR (DNA endonuclease targeted CRISPR trans-reporter) [10], HOLMES (one-hour low cost multipurpose highly efficient system) [11], HOLMESv2 (the improved version of HOLMES) [12], CDetection (Cas12b-mediated DNA detection) [13], and Cas12aVDet (Cas12a-based visual detection) [14], have been successfully carried out to detect various pathogens with quickness, high sensitivity and specificity [3]. More recently (2021–2022), the latest detection methods with time-saving, low-cost, high sensitivity and specificity, such as LAMP-Cas12a, G-CRISPR-Cas [15], LACD [16], CLAP [17] and vis-NEAA [18] and so on, have been performed to detect Hepatitis B virus (HBV), *Salmonella*, *Mycobacterium*, COVID-19 and other pathogens.

LAMP-Cas12a method (LAMP, loop-mediated isothermal amplification, combined with the CRISPR/Cas12a detection system) was developed to detect HBV, which innovatively solved the problems of point-of-care test and the nucleic acid extraction of samples. Based on LAMP-Cas12a, visualization of the assay results and a limit of detection (LOD) of 1 copy/μL within 13 min were achieved for HBV detection. Meanwhile, the sensitivity and specificity in the

evaluation of 73 clinical samples were 100%. The LAMP-Cas12a-based HBV assay provides rapid, accurate test results and low costs without specialized equipment, which has important practical implications for point-of-care HBV detection [19]. By contrast, G-CRISPR-Cas (G-quadruplex-probing CRISPR/Cas12 system), the label-free assay, was used to detect foodborne pathogen, *Salmonella enterica* (*S. enterica*). The introduction of G-quadruplex probe as Cas12a substrate enabled label-free analysis of foodborne pathogens. With the help of the amplification process induced by LAMP, G-CRISPR-Cas achieved highly sensitive detection for *S. enterica* as low as 20 CFU (Colony-forming unit). At the same time, the double recognition of LAMP primers and Cas12a-guided RNA ensured the specificity of pathogenic gene detection. Herein, G-CRISPR-Cas assay is useful for on-site detection of the infection or pollution of foodborne pathogens, which provides a guarantee for food safety [15]. Similar to G-CRISPR-Cas, vis-NEAA (CRISPR/Cas12a combined with nicking enzyme-assisted amplification) has been taken advantage of detecting *Salmonella* in food. CRISPR/Cas12a can specifically identify NEAA amplicons and convert the signal into fluorescent visual readouts, thereby having the advantages of rapidity and high efficiency of NEEA method, and achieving 100% fidelity at the same time. More importantly, it solved the problem of nonspecific amplification of NEAA method. With vis-NEAA assay, *Salmonella* with 80 CFU/mL in spiked eggs can be detected on-site within 20 min [18]. Taken together, both G-CRISPR-Cas and vis-NEAA can be used for the detection of foodborne pathogens, thus ensuring food safety. LACD (LAMP coupled with CRISPR/Cas12a-mediated diagnostic) was developed to detect *Mycobacterium tuberculosis* complex (MTC) in human tuberculosis (TB). Because the engineered LAMP primers contained the specific PAM site for CRISPR/Cas12a recognition, it can be utilized to detect any target sequence, even targets without PAM sites, which provides a new idea for the application of Cas12a and widens its application [16]. AuNP-based colorimetric assay coupled with Cas12 and RT-LAMP, also called Cas12a-assisted RT-LAMP/AuNP (CLAP), is a rapid, sensitive, and visual assay for SARS-CoV-2 detection. Under the optimal conditions, the detection of SARS-CoV-2 RNA can be reduced to 4 copies/mμL within 40 min by naked eye. In addition, the advantages of the superior specificity, easy operation, high-throughput detection make it suitable for large-scale population screening of SARS-CoV-2 in public places, which is conducive to controlling and alleviating the pandemic of COVID-19. Therefore, the CLAP method has important practical roles in screening suspicious population of COVID-19 [17]. To sum up, these latest methods make use of the low equipment requirements of LAMP and the high cleavage activity of Cas12a, which make them have broad application prospects because of rapidity, high sensitivity, specificity and accuracy, high throughput, and easy operation. It should be noted that among Cas12-based detection methods, RPA (recombinant enzyme polymerase amplification), RCA (rolling circle amplification), and PCR (including rapid PCR, rRT PCR, and asymmetric PCR, Table 1) can also be used to exponentially amplify target DNA molecules. With the help of RPA, CDetection and DETECTR can achieve attomolar (åM) sensitivity for DNA detection (Table 1). The characteristics and applications of main CRISPR/Cas12 assays developed in recent years were listed in Table 1.

CRISPR/Cas12 system involves in genome editing

As RNA-guided endonuclease, CRISPR/Cas9 and Cas12a have been widely used for genome editing in animals, plants, and cultured cells, based on their programmable ability to trigger DNA repair at the desired sites, thereby accelerating the pace of basic research and enabling clinical and agricultural breakthroughs [30, 31]. Meanwhile, Cas12b is also a powerful tool for genome engineering because of the small size and high specificity [32–34].

CRISPR/Cas12 system involves in genome editing of human and animals

As the second CRISPR/Cas system for mammalian genome editing, Cas12a recognizes the PAM sequence of 5′-TTN, which improves the recognition range of the genome compared with the PAM sequence of Cas9 recognizing 5′-NGG. The CRISPR/Cas12a and Cas9 systems complement each other and further accelerate the development of CRISPR genome editing tools. In 2017, LbCas12a-mediated genome editing was successfully used to induce pluripotent stem cells (IPSCs) from patients with Duchenne muscular dystrophy (DMD) and correct target genes of DMD in mouse embryos, making it an important contribution to DMD correction in gene therapy [35]. In the same year, by the aid of Cas12a's ability of processing its own crRNA, Zhang Feng's group accomplished simultaneously editing up to four genes in mammalian cells and three genes in mouse brains [36]. Furthermore, Liu et al. validated that the engineered variants of Cas12a with two different nuclear localization sequences (NLS) at the C terminus can improve the mutagenesis efficiency of LbCas12a and FnoCas12a in mammalians, which provide a scheme for broadening the application of Cas12a in vertebrates [37]. In addition, AsCas12a and LbCas12a were used for genome editing in mouse embryos, Drosophila and zebrafish by means of their high activity, but low

Table 1 The characteristics and applications of CRISPR/Cas12-based assays

Year	Methods	Detection targets	Reaction systems	Detection time	Sensitivity	Specificity	Multiple detections	Advantages	Applications	References
2018	DETECTR DNA Endonuclease targeted CRISPR *trans* reporter	Human papillomavirus	RPA + CRISPR/LbCas12a	1 h	åM	Single base	No	Simple, rapid, accurate, portable; high sensitivity and specificity	Molecular diagnostics; DNA detection	[10]
2018	HOLMES One hour low-cost multipurpose highly efficient system	DNA and RNA targets	RPA/LAMP /rapid PCR + CRISPR/ LbCas12a	1 h	10 åM	Single base	No	Simple, rapid, portable; high sensitivity and specificity, low cost	DNA and RNA detection; SNP identification	[11]
2018	Cas14-DETECTR	DNA and RNA targets	RPA + CRISPR/Cas14a	1 h	–	Single base	No	high-fidelity, high sensitivity, without PAM sequence	Molecular diagnostics; ssDNA pathogen detection	[9]
2019	HOLMESv2	DNA and RNA targets	RPA/LAMP/ asymmetric PCR + CRISPR/ Aac-Cas12b	1 h	10åM	Single base	No	Simple, inexpensive, accurate, portable; no cross contamination	Molecular diagnostics; DNA methylation quantitation e.g., SNP identification; DNA and RNA detection, DNA quantification; DNA methylation	[12]
2019	CDetection Cas12b-mediated DNA detection	DNA target	RPA + CRISPR/ AaCas12b	0.5 ~ 2 h	åM	Single base	No	Rapid, economical; high sensitivity, specificity, and accuracy	Molecular diagnostics and clinical research	[13]
2019	Cas12aVDet Cas12a-based visual detection	*Mycoplasma*	RPA + CRISPR/LbCas12a	~ 30 min	10åM	Single base	No	Convenient, rapid, visual, portable; 100% accuracy	Rapid nucleic acid detection	[14]
2020	iSCAN	COVID-19	RT-LAMP + CRISPR/ LbCas12a	< 1 h	10 copies/mμL	High specificity	No	Simple, rapid, portable; high sensitivity	Early detection of COVID-19	[20]

Table 1 (continued)

Year	Methods	Detection targets	Reaction systems	Detection time	Sensitivity	Specificity	Multiple detections	Advantages	Applications	References
2020	STOP SHERLOCK testing in one pot	COVID-19	LAMP + CRISPR/AaCas12b	~ 40 min	100 copies/mμL	100% specificity	No	Convenient, rapid, RNA extraction-free, low-cost, high sensitivity and specificity	Real-time detection of COVID-19	[21]
2020	SENA Specific enhancer for PCR-amplified nucleic acid	COVID-19	rRT-PCR + CRISPR/LbCas12a	< 2 h	1.2</=1.6</=2.1 copies/μL (95% confidence interval)	High specificity	No	Safe, simple, stable, quick, sensitive, specific; low-cost	Clinical diagnostics of COVID-19	[22]
2020	Cas12a-PB CRISPR/Cas12a based portable biosensor	GM soybean and maize powders	PCR/rapid PCR / LAMP + CRISPR/LbCas12a	~ 1 h	0.1% transgenic contents	High specificity	Yes	Simple, convenient, visual, portable; low-cost, high sensitivity and specificity	Multiple targets detection in clinic diagnostics, food safety, etc.	[23]
2021	LAMP-Cas12a	Hepatitis B virus (HBV)	LAMP + CRISPR/LbCas12a	< 20 min	1 copy/μL	Ultra-specificity	No	Rapid, accurate, visual; low-cost, high sensitivity and specificity	Point-of-care HBV detection	[19]
2021	LACD Loop-mediated isothermal amplification coupled with CRISPR-Cas12a-mediated diagnostic	*Mycobacterium tuberculosis* complex	LAMP + CRISPR/LbCas12a	~ 1 h	50 fg genome DNA (~ 10 copies)	5 ng of templates	No	Rapid, visual, ultrasensitive, and highly specific	Detection of a variety of target sequences	[16]
2021	G-CRISPR-Cas G-quadruplex-probing CRISPR-Cas12	*Salmonella enterica*	G-quadruplex probe + LAMP + CRISPR/LbCas12a	~ 20 min	20 CFU	High specificity	Yes	Rapid; label-free, high sensitivity, specificity, and accuracy	On-site diagnosis of the infection or contamination of foodborne pathogens	[15]
2021	PPCas12 Proximal DNA probe-based CRISPR-Cas12	*Salmonella enterica*	proximal DNA probe + CRISPR/LbCas12a	< 2 h	619 CFU	High specificity	No	Simple; low-cost, and without amplification steps	On-site monitoring of foodborne pathogens	[24]
2021	CLAP Cas12a-assisted RT-LAMP/AuNP	COVID-19	RT-LAMP/AuNP + CRISPR/LbCas12a	~ 40 min	4 copies/mμL	Superior specificity	Yes	Rapid; easy for operation, high-throughput, superior sensitivity, and specificity	On-site diagnosis of COVID-19	[17]

Table 1 (continued)

Year	Methods	Detection targets	Reaction systems	Detection time	Sensitivity	Specificity	Multiple detections	Advantages	Applications	References
2021	RPA-Cas12a-FS	DNA target	RPA + CRISPR/LbCas12a	45 min	10 copies/mμL	High specificity	No	Rapid; easy for operation, low-cost, high sensitivity and specificity	Detection of food-borne pathogenic bacteria, genetically modified crops, and meat adulteration	[25]
2021	Cas12c-DETECTOR	Psa, HPV COVID-19	RPA + CRISPR/Cas12c1	120 min	0.23pM	Single base	No	Rapid; high sensitivity and specificity	Plant pathogen detection, SNP identification, clinical diagnosis	[26]
2022	vis-NEAA vis-Nicking enzyme-assisted amplification	*Salmonella*	NEAA + CRISPR/LbCas12a	~20 min	80 CFU/mL	100% fidelity	No	Rapid, visual, and sequence-specific	Food quality monitoring, environmental analysis, and medical diagnostics	[18]
2022	isoCRISPR	Transgenic crops	RCA + CRISPR/LbCas12a	30 min	LOD: 45.0 pM	High specificity	No	Label-free; specific and rapid; isothermal operation; single-tube test	Detection of transgenic corn; on-site rapid detection of various food contamination	[27]
2022	CLEVER CRISPR-Cas integrated RT-LAMP easy, visual and extraction-free RNA	COVID-19	RT-LAMP + CRISPR/LbCas12a	~100 min	0.5 nM	100% specificity	No	Fast, direct, versatile; RNA extraction-free; superior sensitivity and specificity	Visually diagnostics of COVID-19	[28]
2022	DIRECT2 DNA-immunoglobulin reporter endonuclease cleavage test	Ribosomal intergenic spacer (IGS) of *Dickeya solani*	DNA-IgG probe + CRISPR/LbCas12a	0.5 ~ 1.5 h	0.5-1 nM	Low specificity	No	Rapid, first LFT-based platform	CRISPR-Cas12 activity detection	[29]

CFU: colony-forming units; *LAMP*: loop-mediated isothermal amplification; *LFT*: lateral flow test; *LOD*: limit of detection; *RCA*: rolling circle amplification; *RPA*: recombinase polymerase amplification; *AaCas12b*: *Alicyclobacillus acidiphilus* Cas12b; *AacCas12b*: *Alicyclobacillus acidoterrestris* Cas12b; *LbCas12a*: *Lachnospiraceae bacterium ND2006* Cas12a; –: no data

genome coverage and low targeting efficiency hinder their applications in biomedical fields [38].

CRISPR/Cas12b/C2c1 (type V-B) is a dual-RNA-guided DNA endonuclease, which contains RuvC-like endonuclease domain distantly related to Cas12a and relies on both crRNA and tracrRNA for DNA cleavage [7, 8]. The optimal cleavage activity of Cas12b from *Alicyclobacillus acidiphilus* (AaCas12b) was maintained in a wide temperature range (31 ~ 59 °C), together with a small size, high specificity, increased stability, and minimal off-target effects, making it suitable for mammalian genome editing and clinical applications, including single and multiplex genome editing, gene activation, and establishment of gene mutant mouse models, suggesting that CRISPR/Cas12b can be used as a versatile tool for mammalian genome engineering [33, 39]. Subsequently, Strecker et al. identified a promising candidate Cas12b for human genome editing from *Bacillus hisashii* (BhCas12b) and obtained the gain-of-function mutation of BhCas12b. The results showed that the mutant BhCas12b promoted in vitro genome editing in human cell lines and primary human T cells and displayed higher specificity. This work further confirmed that Cas12b can be used for genome editing of human cells [32]. More recently, Un1Cas12f1 (type V-I) was redesigned and optimized for genome editing in human cells. The results demonstrated that optimized Un1Cas12f1 system enabled efficient, specific genome editing in human cells with an efficiency comparable to SpCas9 and a specificity similar to AsCas12a [40]. These observations suggest that CRISPR/Cas12 can be used as a versatile tool for mammalian genome editing (Table 2).

CRISPR/Cas12 system involves in genome editing of plants

CRISPR/LbCas12a is a temperature-sensitive system in genome editing of plants, which has been successfully applied in rice, Arabidopsis, maize, soya bean, and other species [46]. The temperature tolerance and precise cleavage ability of Cas12a open up new prospects for creating GMO (genetically modified organism)-free crops with valuable traits. In rice, the average mutation frequency of target sites was 47.2%, indicating that FnCas12a can effectively induce gene-targeted mutations [47]. Subsequently, Zhong et al. used rice as a model system to investigate the PAM requirement of FnCas12a in plant genome editing, suggesting that FnCas12a preferred TTTV PAMs for efficient genome editing in rice [48]. AsCas12a was successfully used to induce heritable mutations with 77.8% and 92.8% frequencies at two target sites among rice T_0 lines [49]. In transgenic maize, LbCas12a-based genome editing achieved 100% high-frequency at a daytime temperature of 28 °C [49]. Nowadays, Cas12a has also been used for genome editing of woody plants. The CRISPR/AsCas12a has been verified to be the most effective at simultaneously knocking out the members of multigene families, which makes up for the deficiency of woody plant mutants and promotes the research of forest genetics [50]. Editing the coding sequences of *GhPGF* and *GhCLA1* genes by CRISPR/LbCas12a can accurately edit tetraploid cottons (*Gossypium hirsutum*). The results showed that non-transgenic and gossypol-free cottons were successfully created, which provided valuable germplasm resources for molecular breeding of cottons [46].

CRISPR/Cas12b has been proved to be effective in plant genome editing. AacCas12b and AaCas12b against *OsEPFL9*-sgRNA02 were transferred into rice callus by *Agrobacterium tumefaciens*-mediated transformation. The mutation rate of AacCas12b T_0 transgenic line was 36.4%, and that of AaCas12b was 54.2%, which can effectively generate stable rice mutants [34]. Moreover, the potential applications of the CRISPR/Cas12b in Arabidopsis were also explored. With BvCas12b and BhCas12b v4, a large number of deletions were produced at multiple sites in Arabidopsis, and stable mutants were successfully obtained without obvious mutations at potential off-target sites [51]. These results elucidated the potential utility of CRISPR/Cas12b system for genome editing in rice and Arabidopsis. The applications, characteristics and advantages of CRISPR/Cas12 system in plant genome editing were listed in Table 3. Notably, the CRISPR/Cas12 system mainly achieves plant-targeted mutagenesis through insertions and deletions, and its temperature dependence limits its utility in plant genome editing. In the future, it is necessary to further explore and resolve the temperature dependence of Cas12a, so as to facilitate its utilization in genome editing of other species.

CRISPR/Cas12 system involves in genome editing of microorganisms

As a versatile tool for genome editing, CRISPR/Cas12 can also be used for gene editing of microorganisms, including cyanobacteria, bacteria, and fungi. Cyanobacteria are photoautotrophic microorganisms and also an important model organism for physiological and ecological studies. CRISPR/Cas12a has been verified to be suitable for precise genome editing of cyanobacteria through homologous recombination [56, 57]. Subsequently, the Standard European Vector Architecture (SEVA)-based plasmids (containing CRISPR/Cas12a system) has been shown to be effective for genome editing of different genera of cyanobacteria [57]. Similarly, *Mycolicibacterium neoaurum* ATCC 25,795, a classical bacterium producing valuable steroidal drugs, has achieved efficient and accurate genetic manipulation through CRISPR/Cas12a system. Liu et al. confirmed that CRISPR/Cas12a system had great potential in precise genome editing of *M.*

Table 2 The applications, characteristics, and advantages of CRISPR/Cas12 system in genome editing of human and animals

Disease types	Editor	Target genes	Plasmids	Transformation methods	Editing strategies	Editing effect	Advantages	References
Duchenne muscular dystrophy (DMD)	AsCas12a LbCas12a	Exon 51 of the human *DMD* gene Exon 23 of the mouse *Dmd* gene	pLbCpf1-2 A-GFP pAsCpf1-2 A-GFP	Human: nucleofection Mouse: one-cell embryo injection	Reframing and exon skipping	Correct human *DMD* and mouse *Dmd* mutations in vitro and in vivo	High efficiency and specificity; low frequency of off-targeting effects; heritable	[35]
Human atherosclerosis	AsCas12a LbCas12a	*Apoe* and/or *Ldlr* genes	pcDNA3.1-hAsCpf1 pcDNA3.1-hLbCpf1	Microinjection	Knockout	Generate *Apoe*- and/or *Ldlr*-deficient rats	High efficiency and specificity; multiple gene targeting capacity; few or no off-targeting effects; heritable	[41]
Cryopyrin-associated periodic syndrome (CAPS)	AsCas12a	*NLRP3*	*NLRP3*-Cpf1-sgRNA	Electroporation	Point mutation	Produce *NLRP3* R259W homo-cloned pigs	No sign of off-targeting; precise NLRP3 point mutation; accurate	[42]
Hepatocyte-related disease	AsCas12a LbCas12a	*AAVS1* locus	pHM-CBh-LbCpf1 pHM-CBh-AsCpf1 pHM-U6-lbgRNA-AAVS1 pHM-U6-asgRNA-AAVS1	Co-transfection	InDels	Generate genome cleavages in PHHs	Larger capacity; efficiently targets human hepatocytes	[43]
Leukemia	AsCas12a	Exon 3 of *Lif* gene	AsCpf1/mRNA AsCpf1/RNP	Electroporation	Knockout	Generate *LIF* knock out mice	Easy, fast, and technically less demanding	[44]
Lung cancer	LbCas12a	*EGFR*	oAd/Cas12a/crEGFR	Liposome transfection	InDels	Precise genomic reprogramming	Efficient precise, and cancer-specific; no off-target; can be used as an alternative cancer therapy	[45]
Human T cell-related diseases	BhCas12b	Human CD4 + T cell containing TTTV PAM site	BhCas12b v4-sgRNA	Electroporation	InDels	Exhibit indel rates of 32 ~ 49% across three tested targets	Small size and high target specificity; low off-targeting activity; a promising tool for in vivo genome editing	[32]
RNF2 related cancer	AaCas12b	Human *RNF2* gene and mouse *Nrl* gene	AaCas12b RNPs	Microinjection	InDels	Produce indels with the frequency at up to 66.7%	Single and multiplex genome editing, gene activation, and generation of gene mutant mouse models; high specificity and minimal off-target effects	[33]

AaCas12b: *Alicyclobacillus acidiphilus* Cas12b; *FnCas12a*: *Francisella tularensis* Cas12a; *LbCas12a*: *Lachnospiraceae bacterium ND2006* Cas12a; *BhCas12b*: *Bacillus hisashii* Cas12b; *InDels*: insertions and deletions

Table 3 The applications, characteristics, and advantages of CRISPR/Cas12 system in plant genome editing

Species	Editor	Target genes	Plasmids	Transformation methods	Editing strategies	Editing efficiency or mutation rate	Advantages	References
Arabidopsis *Arabidopsis thaliana*	BhCas12b v4 BvCas12b	*AtPDS3* *AtFLS2* *AtGL2* *AtTT4*	BhCas12b-sgRNA BhCas12b v4-sgRNA	Agrobacterium-mediated transformation	InDels	0 ~ 4.3%	Multiplex genome editing; large deletions at multiple loci; no off-target mutations at the tested loci	[51]
	LbCas12a ttLbCas12a	*AtALS*	ttLbCas12a-sgRNA LbCas12a-sgRNA	Agrobacterium-mediated transformation	InDels	LbCas12a: 0.26% (22℃), 0.79% (28℃) ttLbCas12a: 0.63% (22℃), 1.34% (28℃)	ttLbCas12a increases gene targeting efficiencies and achieves high temperature plant gene targeting	[52]
	AsCas12a, LbCas12a	*AtGL2* *AtTT4*	AtGL2-crRNA1 AtTT4-crRNA1	Agrobacterium-mediated transformation	InDels	T2 plants *AtGL2*: ~ 35% *AtTT4*: ~ 15%	High-temperature regimes to achieve high editing efficiencies	[49]
Rice *Oryza sativa*	AaCas12b AacCas12b BhCas12b BthCas12b	*OsEPFL9 OsGS3*	OsEPFL9-sgRNA02 OsGS3-sgRNA02	Agrobacterium-mediated transformation	Deletions	T_0 lines OSEPFL9: 66.7% OsGS3: 70.85%	Multiplexed genome editing; no off-target mutations	[34]
	AsCas12a	*OsDEP1 OsROC5*	OsROC5-crRNA01 OsDEP1-crRNA02	Polyethylene glycol (PEG)-mediated transfection	Deletions	T_0 lines *OsDEP1*: 77.8%, *OsROC5*: 92.8%	High-frequency mutation	[49]
	FnCas12a	*OsDL* *OsALS*	crOsDL-1 ~ 2 crOsALS-1 ~ 2	Agrobacterium-mediated transformation	Deletions	The average mutant rate: 47.2%	Biallelic mutants obtained in the T_0 generation in rice	[47]
Maize *Zea mays*	LbCas12a	*ZmGL2*	LbCas12a-ZmGL2-crRNA1&2	Agrobacterium-mediated transformation	InDels	A842B-2-2-2: 51.4% A842B-5-1 :100%	High mutagenesis frequencies	[49]
Cotton *Gossypium hirrntnml*	AacCas12b	*GhCLA*	pRGEB32-AacCas12b-GhCLA	Agrobacterium-mediated transformation	Deletions (9 ~ 14 bp)	Mutation rate: ~ 20%	Efficient precise and faithfully inheritable; no off-target mutations	[53]
	LbCas12a	*GhPGF*	LbCas12a-GhPGF	Agrobacterium-mediated transformation	InDels	GhPGF-crRNA1: 91.5% (34 °C) GhPGF-crRNA2: 67.6% (34 °C)	Creation of nontransgenic, gossypol-free cotton; no off-target mutations	[46]

Table 3 (continued)

Species	Editor	Target genes	Plasmids	Transformation methods	Editing strategies	Editing efficiency or mutation rate	Advantages	References
Tobacco *Nicotiana tabacum*	LbCas12a	*NtPDS* *NtSTF1*	crNtPDS-1, 2 crNtSTF1-1 ~ 4	Agrobacterium-mediated transformation	InDels (majority)	The average mutant rate: 28.2%	Chimeric mutations	[47]
Citrus *Citrus reticulata*	LbCas12a	*CsPDS* *CsLOBP*	GFPp1380N-35 S-LbCas12a-crRNA-cspds GFPp1380N-Yao-LbCas12a-crRNA-lobp	Agrobacterium-mediated transformation	Short deletions	The average mutant rate: 28.3%	No potential off-targets	[54]
Poplar *Populus tomentosa*	AsCas12a LbCas12a FnCas12a	*PagPDS*	PagPDS-AsCas12a PagPDS-LbCas12a PagPDSFnCas12a	Agrobacterium-mediated transformation	Large-fragment deletions	AsCas12a: ~ 70% LbCas12a: ~ 33% FnCas12a: 6.7%	Simple and high efficiency; multigene simultaneous knockout mutants obtained	[55]

AaCas12b: Alicyclobacillus acidiphilus Cas12b; *AacCas12b: Alicyclobacillus acidoterrestris* Cas12b; *AsCas12a: Acidaminococcus* sp.BV3L6 Cas12a; *BhCas12b: Bacillus hisashii* Cas12b; *BhCas12b v4*: a variant of BhCas12b from *Bacillus hisashii*; *BthCas12b: Bacillus thermoamylovorans* Cas12b; *BvCas12b: Bacillus* sp. V3-13 Cas12b; *FnCas12a: Francisella novicida* U112 Cas12a; *FnoCas12a: Francisella tularensis novicida* Cas12a; *LbCas12a: Lachnospiraceae bacterium ND2006* Cas12a

neoaurum, such as integration of targeted genes into desired sites and targeted deletion of DNA sequences of different lengths [58]. Recently, CRISPR/Cas12a has been proved to be able to simultaneously edit *pyrG, pksP*, and *kusA* genes of *Aspergillus aculeatus* TBRC 277 (an industrially related cell factory), with an efficiency of up to 40% [59]. In conclusion, CRISPR/Cas12-mediated microbial genome editing mainly involves in strain improvement, such as the production of valuable steroidal pharmaceuticals [58] and bioproducts [59], as well as high-performance chassis [60], and fundamental research, for instance, the study of plant-fungal interactions [61, 62] and the pathogenesis of important opportunistic pathogens [63] (Table 4). For ease of understanding, based on existing reports, CRISPR/Cas12-mediated genome editing was represented in Fig. 1.

The other Cas12 proteins and their applications

As shown in Table 5, Cas proteins of type V can be divided into 11 subtypes, including subtype V-A (Cas12a), subtype V-B (Cas12b), subtype V-C (Cas12c), subtype V-D (Cas12d), subtype V-E (Cas12e), subtype V-F (Cas12f), subtype V-G (Cas12g), subtype V-H (Cas12h), subtype V-I (Cas12i), subtype V-K (Cas12k), and subtype V-U [2]. CRISPR/Cas12c (C2c3) and Cas12d (CasY) proteins represent compact CRISPR/Cas systems, which have limited sequence homology with the crRNAs of Cas12a and Cas9 [66]. The study demonstrated that Cas12d-catalyzed DNA cleavage required a short complementary untranslated RNA (scoutRNA) and crRNA, and the scoutRNA was an important cofactor for Cas12c-catalyzed pre-crRNA maturation. Cas12c can be applied for plant pathogen detection and single nucleotide polymorphism (SNP) identification. In addition, Cas12d can boost RNA-guided DNA interference in bacteria [66]. Cas12e (CasX) is an RNA-guided DNA endonuclease that modifies the genomes of humans, mouse, Drosophila, yeast, *Escherichia coli*, and other model organisms, thus having the potential as a universal genome editor [67]. The miniature Cas12f1 is an RNA-guided endonuclease, which has been demonstrated to be an efficient genome editing tool in bacteria and human cells [68]. Cas12g is an RNA-guided ribonuclease that cleaves single-stranded RNA (ssRNA) and ssDNA by targeting ssRNA substrate. CRISPR/Cas12g system provides a promising platform for RNA editing and nucleic acid detection by virtue of the small molecular weight and high thermal stability of Cas12g protein [69]. Cas12i, the subtype V-I CRISPR/Cas effector, is an endonuclease that predominantly recognizes and cleaves non-target strand of 28 bp double-stranded DNA (dsDNA) substrate. Compared with 20 bp dsDNA substrate recognized by Cas9,

Table 4 The applications, characteristics, and advantages of CRISPR/Cas12 system in microorganism genome editing

Species	Editor	Target genes	Plasmids	Transformation methods	Editing strategies	Editing efficiency or mutation rate	Advantages	References
Escherichia coli	FnCas12a	*ahpC* *tyrR*	pTF pTF-*lacZ* pSIM*cpf1*	Electroporation	Knock-outs knock-ins genome integration of large DNA fragment	Markerless deletion: > 80% genome integration: 50%	Simple; markerless deletion or genome integration; high efficiency	[64]
Cyanobacteria	FnCas12a	*nblA*	pSEVA351-Cpf1 pSEVA451-Cpf1 pSEVACpf1nblA	Natural transformation or conjugation	Deletions	40%	High efficiency; free cost; simplicity	[57]
	FnCas12a	*nblA* *psbA*	pSL2680	Conjugation	Point mutations knock-outs knock-ins direct gene replacement	~20%	Multiplex targeting; markerless editing; nontoxic engineering; low cost	[65]
Mycolicibacterium neoaurum	FnCas12a	*hsd4A* *KshA2*	p261-Cas12a pCM-cr_hsd4A pCM-cr_L2 pCM-cr_KshA2 pCM-cr_L1-L2 pCM-cr_T1-cr_T2 pCM-cr_T1-cr_T3 pCM-cr_T1-cr_T4 pCM-cr_T1-cr_T5 pCM-cr_T1-cr_T6	Electroporation	1 kb, 5 kb, 10 kb, 15 kb, 20 kb and 24 kb deletions knock-ins	Deletion efficiencies: 70%, 30%, 30%, 20%, 20% and 10% Integration efficiencies: single crossover: 100% double crossovers: 9%	Targeted deletion of DNA sequences and precise genomic integration	[58]
Magnaporthe oryzae	LbCas12a	*BUF1*	pFGL821 pFGL921	PEG-mediated transformation	Large-scale insertions and deletions	50%~100%	Variable DNA repair	[61, 62]
Enterococcus faecium	AsCas12a	*lacL* *acpH* *treA* *unaG*	pJC005.em.X*lacL* pJC005.em.X*acpH* pJC005.em.X*treA* pJC005.em.X*treA*::*unaG* etc.	Electro- transformation	InDels	> 80%	Rapid, efficient, and cost-effective	[63]
Shewanella oneidensis	AsCas12a BhCas12b	*ampC* *nagK* *gfp*	pAsCpf1-ampC pBhCas12b-ampC etc.	Conjugation	knock-outs knock-ins gene replacement	Insertion 94.4%, 83.9% Deletion: 41.67% Replacement: 25%	Ideally suitable to target T-rich DNA sequences	[60]
Aspergillus aculeatus	FnCas12a AsCas12a LbCas12a	*pyrG* *pksP* *kusA*	pCRISPR01-FnCpf1 pCRISPR01-AsCpf1 pCRISPR01-LbCpf1 pCRISPR01-FnCpf1-crRNA-kusA1-pksP2-pyrG3 etc.	Protoplast-mediated transformation	InDels	FnCas12a: 93% AsCas12a: 43% LbCas12a: 13% FnCas12a: three genes combined editing efficiency: 40%	Versatile, flexible, precise and highly efficient; multiplex gene-editing	[59]

AsCas12a: *Acidaminococcus* sp.BV3L6 Cas12a; *BhCas12b*: *Bacillus hisashii* Cas12b; *FnCas12a*: *Francisella novicida* U112 Cas12a; *LbCas12a*: *Lachnospiraceae bacterium ND2006* Cas12a

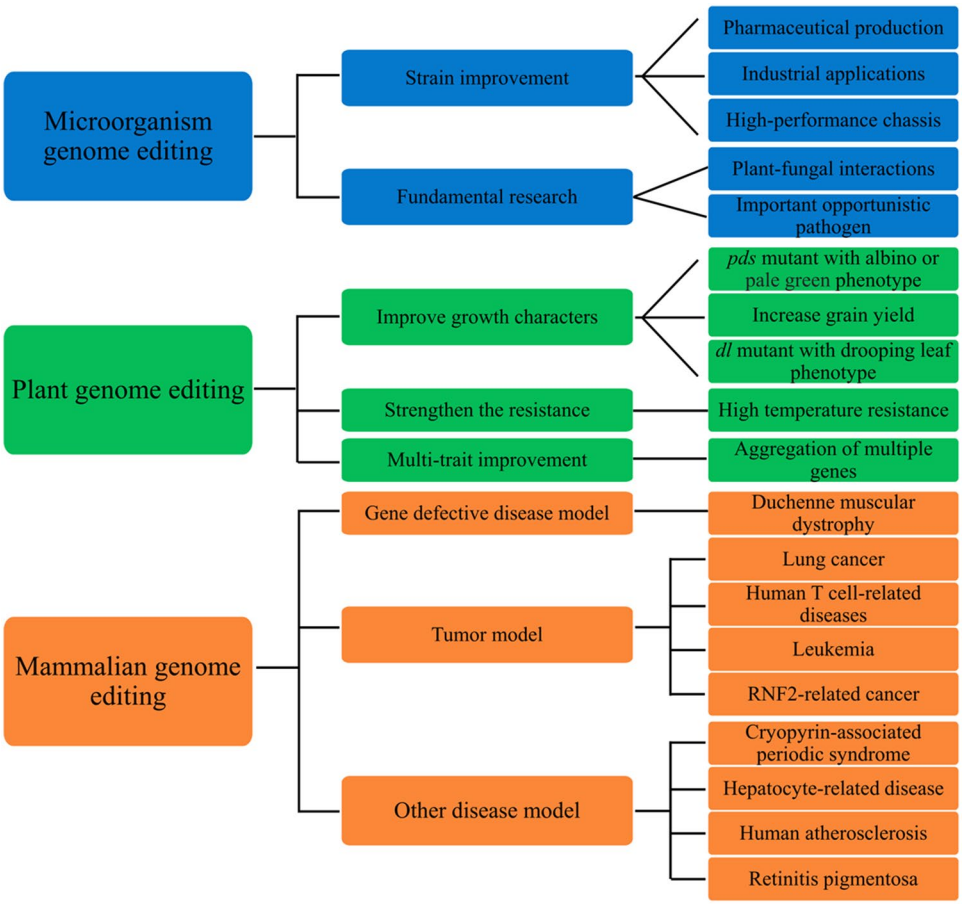

Fig. 1 CRISPR/Cas12-mediated genome editing of animals, plants and microorganisms

Cas12a, Cas12b, and Cas12e, Cas12i has the potential to be exploited into a high-fidelity genome editor [70]. Cas12k, encoded by cyanobacterial *Scytonema hofmannisystem* Tn7-like transposon, has no endonuclease activity and mediates guide RNA-dependent transposition [2, 71]. By combining the transposase with the CRISPR effector Cas12k, DNA fragments can be directly integrated into the target sites with 80% frequency, which laid the foundation for precise insertion of DNA [71]. Querques et al. demonstrated the feasibility of using CRISPR-associated transposons as a tool of programmable site-specific gene insertion [72]. The functions of Cas12 proteins from subtype V-H and V-U need to be further explored. The classifications and applications of CRISPR/Cas12 system were listed in Table 5.

It should be pointed out that in addition to the functions mentioned above, Cas12 also participates in gene regulation through CRISPR interference (CRISPRi) [64, 75]. Recent study has shown that CRISPRi can efficiently (> 90%) achieve transcriptional inhibition of target genes [64]. Taken together, these findings can improve our understanding of the functional diversity of CRISPR/Cas12 system. With further research, Cas12 will exhibit an increasingly extensive application prospect.

Conclusion

CRISPR/Cas technology, as the most commonly used genome editing tool, is known as the "magic scissors" for genome editing because of its simplicity, cheapness and high efficiency, which allows researchers to efficiently and precisely alter, edit or replace genes in plants, animals and even humans. Nowadays, the improved CRISPR/Cas technology is widely employed in the breeding of new varieties of animals and plants and biomedical fields, such as searching for genes related to signal pathways, screening drug targets, and gene therapy. Undoubtedly, CRISPR/Cas-mediated genome editing in vivo has certain off-target effects, which may lead to the loss of large DNA fragments at the distal end of the cleavage site, and even other more complex gene mutations [76, 77]. However, with the further development, modification, and improvement of CRISPR/Cas technology, the problems mentioned above will be solved gradually. Meanwhile, based on the

Table 5 The classifications, characteristics and functions of Cas12 proteins [2]

Subtype	Variant	tracrRNA[a]	Gene nomenclature	Cleavage activity	Amino acids	Functions	References
V-A		No	Cas12a[b] (Cpf1)[c]	dsDNA, ssDNA	1310	Gene editing; nucleic acid detection; molecular diagnosis	[8, 11, 47]
V-B	V-B1	Yes	Cas12b1 (C2c1)	dsDNA, ssDNA	~1100	Gene editing; nucleic acid detection	[12, 32, 39]
	V-B2	Yes	Cas12b2	dsDNA, ssDNA		Gene editing	
V-C		No	Cas12c (C2c3)	dsDNA	1253	Nucleic acid detection	[26]
V-D		No	Cas12d (CasY)	dsDNA	~1200	DNA interference	[73]
V-E		Yes	Cas12e (CasX)	dsDNA	~980	Genome editor	[67]
V-F	V-F1	Yes	Cas12f1 (Cas14a)	ssDNA	400~700	Gene editing High-fidelity SNP genotypes	[73]
	V-F2	No	Cas12f2 (Cas14b)			–	
	V-F3	No	Cas12f3 (Cas14c)			–	
V-G		Yes	Cas12g	RNA, ssDNA	768	Nucleic acid detection	[69]
V-H		No	Cas12h	dsDNA	871	–	
V-I		No	Cas12i	dsDNA	1055	Gene editing	[70]
V-K	V-U5	Yes	Cas12k (C2c5)	Transposases	–[d]	Help Tn-7 jump gene find specific sequence	[71, 72]
V-U	V-U1	No	C2c4	Transposon-encoded nucleases	–	–	[74]
	V-U2	No	C2c8				
	V-U3 (V-F1)	No	C2c10	Originate independently from different TnpB families			
	V-U4	No	C2c9				

dsDNA double-stranded DNA, *ssDNA* single-stranded DNA

[a]Transactivating CRISPR RNA (tracrRNA)

[b]Systematic name

[c]Legacy name

[d]–No data

CRISPR/Cas system, combined with isothermal amplification, electrochemical sensor, optical imaging and other technologies, the new biosensors with high specificity and sensitivity, as well as rapidity, simplicity and convenience have been established by researchers, which are extensively applied in the fields of disease-related detection and imaging, detection of various disease markers, thereby enabling high accuracy and reliability of disease diagnosis at the molecular level. Recently, Ivanov et al. developed a platform for detecting Cas12 trans-cleavage activity based on lateral flow test (LFT), and its assay result only depends on composite DNA-IgG probe [29]. In the future, the development of a CRISPR/Cas12-based probe detection platform holds great promise. Additionally, the novel RNA editing tool Cas7-11 can be fused together for genome editing, which may be used in tissue regeneration and antiviral drugs [78]. Cas12k has no nuclease activity and only mediates guide RNA-dependent transposition [2, 79]. The development of transposase component engineering may expand its application in eukaryotic cells in the future. A latest study reported a surprising finding that the designed CRISPR/Cas12 DNA device can degrade the native chromosome and convert bacterial cells into biosynthetic chassis for high-efficiency molecular biomanufacturing [80]. As a consequence, with the unremitting efforts of researchers from all over the world, the new functions and new types of Cas proteins will be further discovered and deciphered, which will further broaden their diverse biotechnological applications, so as to better utilize the CRISPR/Cas system to serve humans.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all the individual participants included in this study.

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