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Current advances in CRISPR-Cas-mediated gene editing and regulation in cyanobacteria

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Abstract

Photosynthetic cyanobacteria are important microbial models in basic research such as photosynthesis, biological rhythm, and the geochemical cycle of elements. Meanwhile, they attract significant attention to serve as "autotrophic cell factories", enabling the production of dozens of chemicals. In this case, genetic toolboxes especially gene editing and regulation tools with high efficiency are the basis of the development of related studies. Among them, clustered regularly interspaced palindromic repeats (CRISPR)-Cas related technologies have realized rapid and efficient gene editing, gene silence and activation in multiple organisms like *Escherichia coli*, budding yeast, plant and mammalian cells. To promote their understandings and applications in cyanobacteria, in this review, advances in CRISPR-Cas-mediated gene editing and regulations were critically discussed. Firstly, the elucidation of native CRISPR-Cas in cyanobacteria were concluded, which provided new tool candidates for further optimization. Secondly, basic principles and applications of CRISPR-Cas related gene editing and regulation tools used in cyanobacteria were respectively discussed. In the future, further studies on development of native CRISPR-Cas tools, continuous editing and dynamic regulation would significantly promote the synthetic biology researches in cyanobacteria.

Keywords Cyanobacteria, CRISPR-Cas, Gene editing, Gene regulation, Synthetic biology

Introduction

In the context of a low-carbon economy, cyanobacteria, as photosynthetic microorganisms, have garnered considerable attention for their ability to produce high-value-added chemicals from sustainable resources such as water, sunlight, CO_2, and mineral salts. Compared to land plants, cyanobacteria exhibit higher CO_2 fixation efficiency, attributed to the presence of a carbon concentration mechanism [1], which offers significant potential for achieving negative carbon production of chemicals and bioactive compounds. Leveraging synthetic biology approaches, cyanobacteria have successfully synthesized numerous high-value-added chemicals, including biofuels, bioplastics, bioactive compounds [2, 3], biologics [4, 5] and agents for environmental governance [6–8]. However, the efficient advancement of cyanobacteria-related research relies heavily on the availability of efficient gene manipulation tools.

The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas systems, widely recognized as adaptive immune systems in archaea and bacteria [9], are also prevalent in cyanobacteria. They have garnered significant attention due to their high efficiency, precision, and programmable characteristics for gene editing, and have found applications in various fields such as such

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as gene therapy [10], diagnosis [11], screening [12], and metabolic engineering [13]. Building on CRISPR-Cas9 and Cas12a, a plethora of genetic tools has been developed in cyanobacteria, including base editing [14], gene inhibition [15], gene activation [16], and genome large fragment knockout [17]. The exploration of endogenous CRISPR-Cas systems in cyanobacteria, coupled with the development of genetic tools based on these systems, has led to significant advancements. For instance, the Cas12k effector from the filamentous cyanobacterium *Scytonema hofmannii* was employed to generate homozygotes following the integration of target genes into the genome of *Anabaena* sp. PCC 7120 [18]. A wide variety of genetic tools based on the CRISPR-Cas systems have provided avenues for basic research and engineering in cyanobacteria.

To promote their understandings and applications in cyanobacteria, in this review, advances in CRISPR-Cas-mediated gene editing and regulations were discussed. Detailly, the elucidation of native CRISPR-Cas in cyanobacteria were first concluded, which provided new tool candidates for further optimization. Then, basic principles and applications of CRISPR-Cas related gene editing and regulation tools used in cyanobacteria were respectively discussed, which were the main focus of this article. Finally, future directions including development of native CRISPR-Cas tools, continuous editing and dynamic regulation were presented. The article here provides new insights for CRISPR-Cas related studies in cyanobacteria.

Native CRISPR-Cas systems in cyanobacteria

The adaptive immune process of the CRISPR-Cas system is mainly divided into three processes: foreign gene acquisition, crRNA biogenesis, and target interference [19] (Fig. 1). At the foreign DNA acquisition stage, a complex of specific Cas proteins binds to the invading target DNA to obtain fragments of 30–50 bases in length, known as the protospacers [19]. Adjacent to this

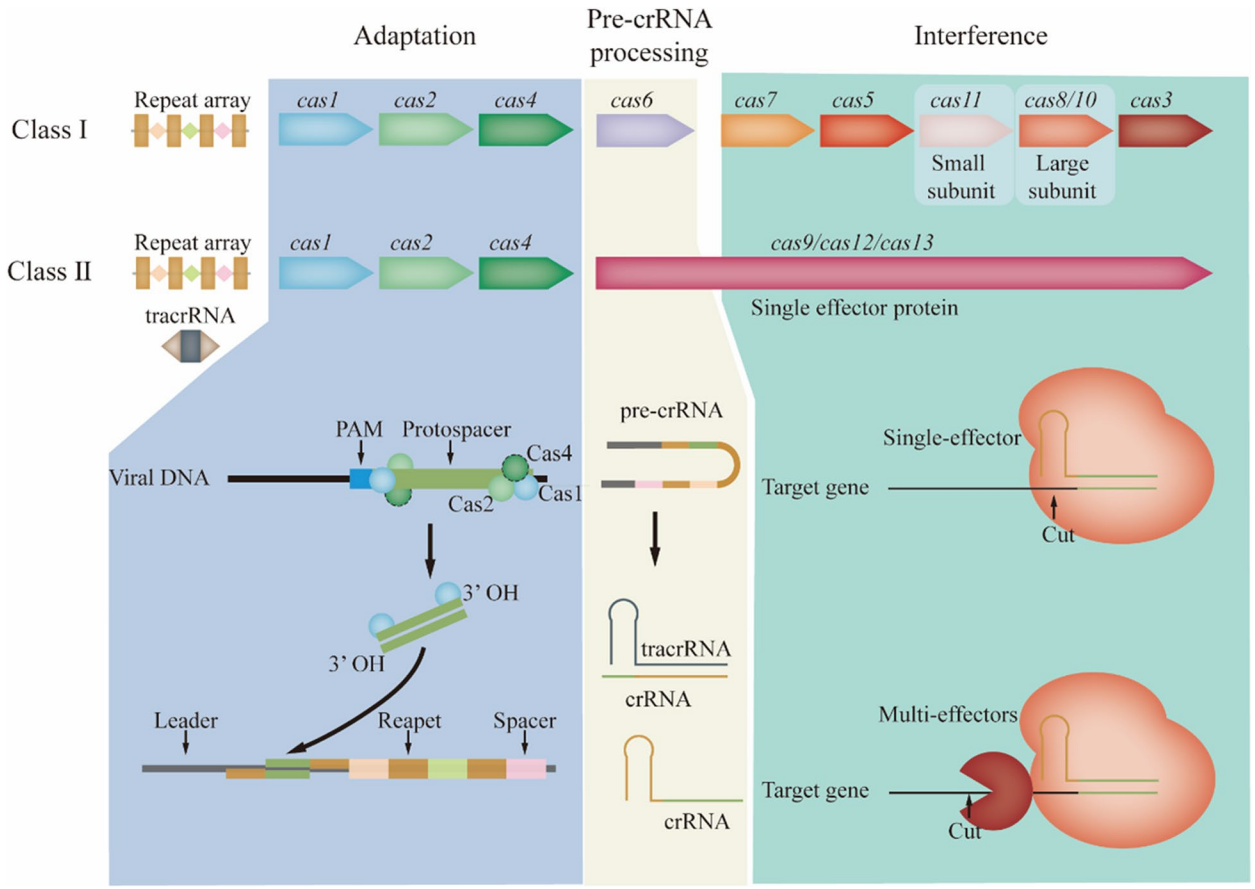

Fig. 1 Classification of CRISPR-Cas systems and the adaptive immune process. The CRISPR-Cas system is primarily categorized by function into three parts: spacer acquisition, pre-RNA processing, and interference. In the spacer acquisition process, the main functions are performed by Cas1 and Cas2, while Cas4 is absent in some systems (indicated by dashed lines). In Class I, Cas6 plays a role in pre-RNA processing and accompanies crRNA in the interference process. In Class I, multiple Cas proteins are recruited to accomplish interference, whereas in Class II, only one Cas protein is involved in pre-RNA processing and interference

sequence, there is a specific motif sequence recognized by the Cas proteins, called the protospacer adjacent motif (PAM) [20]. After the 5' end of the CRISPR repeat sequence is replicated, protospacers are inserted into the CRISPR array with the assistance of Cas1 and Cas2 proteins forming the spacer sequence [21]. At the crRNA biogenesis stage, single-stranded RNA transcribed by a CRISPR array, known as pre-CRISPR RNA (pre-crRNA), is processed into crRNA after undergoing cleavage by CRISPR-specific endoribonucleases [20]. These endoribonucleases vary across different CRISPR-Cas systems and may consist of multi-protein Cas complexes, single multi-domain Cas proteins, or non-Cas host RNAs [22]. At the target interference stage, crRNAs often remain bound to the processing complex and serve as guides to recognize invading DNA, which is subsequently degraded by Cas nucleases (or nuclease) [22].

The CRISPR-Cas system consists of a CRISPR array and Cas protein(s). The CRISPR array produces crRNA, which guides the effector composed of Cas protein(s) to cleave and degrade invading DNA or RNA. By analyzing the genetic composition and locus structure of the CRISPR-Cas systems, they have been divided into 2 classes, 6 types and 33 subtypes [22]. Class 1 systems (Type I, Type III, and Type IV) have effector modules composed of multiple Cas proteins, some of which form complexes mediate pre-crRNA maturation that, with contribution from additional Cas proteins, mediate interference [22–24] (Fig. 1). By contrast, class 2 systems (Type II, Type V, and Type VI) have a single multi-domain Cas protein that combines all the features for mature pre-crRNA and interference [22–24] (Fig. 1).

CRISPR-Cas systems are widely distributed among cyanobacteria, and Type I and Type III CRISPR-Cas systems are extremely abundant [25–27]. However, the CRISPR-Cas system was almost completely lost in marine cyanobacteria, despite being in an algae-rich environment, which is a primary driving force for the widespread distribution of CRISPR-Cas systems [25, 28, 29]. Additionally, the type I-F, I-B, and V-K systems, utilized by bacterial transposons of the Tn7 family to guide RNA-directed transposon insertion, have been identified in cyanobacteria [30, 31].

Class 1 CRISPR-Cas systems

Three CRISPR-Cas systems located in the pSYSA plasmid were identified in *Synechocystis* sp. PCC6803 (hereafter Syn6803), they belong to Type I-D, Type III, and Type-III-B, respectively [32–34]. The Type I-D and Type III systems contain Cas6 protein related to pre-crRNA processing, respectively, while the Type III-B system lacks this important protein, however, each of them functions independently [33]. *Synechocystis* sp. PCC6714, which was separated from the same environment as Syn6803, possesses a Type III-B system that is highly homologous to the one found in Syn6803 [32]. This may result from the horizontal transfer of genes. The Type III system mostly uses the Cas6 protein provided in trans by other CRISPR-Cas loci for pre-RNA processing but is not found in the Type III-B system of Syn6803, which uses endogenous RNase E for pre-crRNA processing [22, 33, 34].

There are abundant Type I-A, I-D, III-A, and III-B systems in *Microcystis aeruginosa*, and the direct repeat sequences (DRs) of the same type have similar secondary structures, and the existence of different and special secondary structures play an important role in the recognition of Cas proteins, and in addition, except for the DRs associated with Type III-A, all DRs have the same 3' sequence [26]. The foreign DNA acquisition stage of the CRISPR-Cas system may involve a conserved mechanism. The spacer sequences in the CRISPR array display homologous fragments with the sequences of bacteria (including cyanobacteria), plasmids, and viruses, implying that the CRISPR-Cas systems confer resistance against mobile genetic elements, plasmids, and viruses [27].

In *Anabaena (Nostoc)* sp. PCC 7120 (hereafter Ana7120), there are 3 different Class 1 systems (Type I-A, Type I-D, and Type III-D) and 1 Class 2 (Type V-K) system, containing 11 DRs, all capable of producing mature crRNAs [27]. Some of the DRs may belong to the residual CRISPR-Cas system and be able to play a partial role, although not experimentally verified. In the Type I-D system, the backbone protein Cas7 has been shown to have structural domains that bind to specific crRNAs [35]. This strongly suggests that the system is biologically functional with endogenous sources. The Cas6 protein of Type I-D cross-talks in recognizing DRs and can cleave DR sequences specifically recognized by Type I-A, a process that would limit the accumulation of crRNA in the Type I-D system [36]. Besides, the Type V-K effector protein All3613 may only be one subunit of the Cas12k protein [22, 27]. This type of CRISPR-Cas system is rare in cyanobacteria.

In a study of the CRISPR-Cas systems associated with transposition in cyanobacteria, an abundance of Tn7-like transposon genes was found in cyanobacteria, and a novel Type I-D system was identified [31]. In the classic Type I-D system, Cas1 and Cas2 proteins are involved in spacer acquisition, while Cas3 protein is responsible for unwinding double-stranded DNA, despite having separate functional domains [37]. However, in the novel Type I-D system, the Cas1, Cas2, and Cas3 proteins are missing, which is a conservative phenomenon in transposon-associated CRISPR-Cas systems [31]. The novel

Type I-D system, derived from *Myxacorys californica* WJT36-NPBG1, is biologically functional in *Escherichia coli* [31], whereas the Type I-D system derived from *M. aeruginosa* has been utilized for gene editing in mammalian cells [38].

Class 2 CRISPR-Cas systems

Class 2 CRISPR-Cas systems are relatively uncommon in cyanobacteria [25–27], with only Type V-K CRISPR-Cas systems discovered thus far [18, 27, 30]. Currently, there is a lack of direct evidence suggesting full activity in the Type V-K system in Ana7120, and its potential effector protein, All3613, is also likely to be only one of the subunits that make up the Cas12k core. The functionality of the Type V-K system derived from *Scytonema hofmannii* has been experimentally validated and applied in human cells as well as in Ana7120 [18, 30]. The Type V-K system consists of four components: the pseudonuclease Cas12k, the transposase TnsB, the AAA + ATPase TnsC, and the zinc finger protein TniQ [39]. The functioning process of this system involves the complexation of crRNA with Cas12K, guided by crRNA, recognizing and binding to the target DNA [30]. TnsC accumulates by interacting with Cas12k bound to the double-stranded DNA [30]. With the assistance of ATP, TnsC polymerizes to form a helical filament [30]. Subsequently, upon binding of TniQ to the helical filament, the generation of the filament is inhibited [30]. The filament serves as a platform for recruiting TnsB [30]. After recruiting TnsB, which carries the transposon, and completing the transposition process, the ATPase activity of TnsC is activated by interaction with TnsB [30]. This activation leads to the degradation of the helical filament [30]. Thus, the entire RNA-mediated transposition process is completed.

Off-target events may occur during the transposition process mediated by the Type V-K system, particularly after an increase in TnsC expression levels [39]. The occurrence of non-specific transposition events is primarily driven by TnsC, attributed to its preference for AT-rich regions in DNA binding. While currently suppressing non-specific transposition events by controlling the expression level of TnsC, this method does not fundamentally address the issue and still poses a high risk of off-target effects, thus limiting the universality of the system.

CRISPR-Cas mediated gene editing in cyanobacteria

Overview of CRISPR-Cas mediated gene editing tools

DNA fragment editing

Gene editing, which involves the insertion, deletion, or replacement of DNA fragments in the genome, is referred to here as DNA fragment editing. DNA fragment editing technology based on the CRISPR-Cas system relies on the recognition and cleavage of target sequences by the crRNA-effector complex, followed by repair by the organism's own DNA repair mechanisms [39]. To ensure the accuracy of repair outcomes, donor fragments containing homologous sequences are often provided (Fig. 2A).

CRISPR-Cas9 and CRISPR-Cas12a are the main CRISPR-Cas systems used for DNA fragment editing [39, 40]. While Cas9 exhibits higher toxicity in some species [41, 42], Cas12a demonstrates greater universality in cyanobacteria [43]. Cas12a possesses both endoribonuclease and endonuclease activities, enabling it to independently process pre-crRNA into mature crRNA [44]. In the CRISPR-Cas12a DNA fragment editing tool, after processing pre-crRNA into mature crRNA, Cas12a forms a complex with the crRNA to recognize and cleave the target sequence, resulting in double-strand breaks. The broken DNA can be repaired by either non-homologous end joining (NHEJ), resulting in random insertions or deletions, or homology-directed repair (HDR) mechanisms, which can generate precise editing outcomes when provided with a homologous repair template, within the cell [45, 46] (Fig. 2A). Another CRISPR-Cas3 tool developed based on Class I can degrade large fragments bidirectionally under the action of Cas3 helicase and exonuclease activities, without the need for a repair template [47] (Fig. 2A). This tool is able to cleave the recognition site bidirectionally, resulting in editing outcomes with different deletion lengths.

Base editing

Base editing entails modifying the bases of the genome. In classical base editing technology, a deaminase enzyme specific to the target base is fused with an effector protein. Under the guidance of crRNA, the fused effector complex targets the desired sequence and catalyzes the biochemical reaction to modify the corresponding base [48–52] (Fig. 2B).

The classical base editing tool is based on Class 2 CRISPR-Cas systems, where cytidine deaminase or adenine deaminase is fused to the C-terminal or N-terminal of the Cas protein. Cytidine deaminase converts C to U after deamination, then the copied DNA strand replaces U with T using the DNA containing U as a template, completing the C → T base editing process (CBE) [52]. Adenine deaminase converts A to inosine after deamination (A → I), then inosine is repaired to G, completing the A → G base editing process (ABE) [49]. Moreover, by fusing adenine deaminase and cytidine deaminase or mutating adenine deaminase, a dual-function base editor is developed based on CBE and ABE [53, 54]. Building upon CBE, uracil DNA glycosylase (UDG) is used to recognize

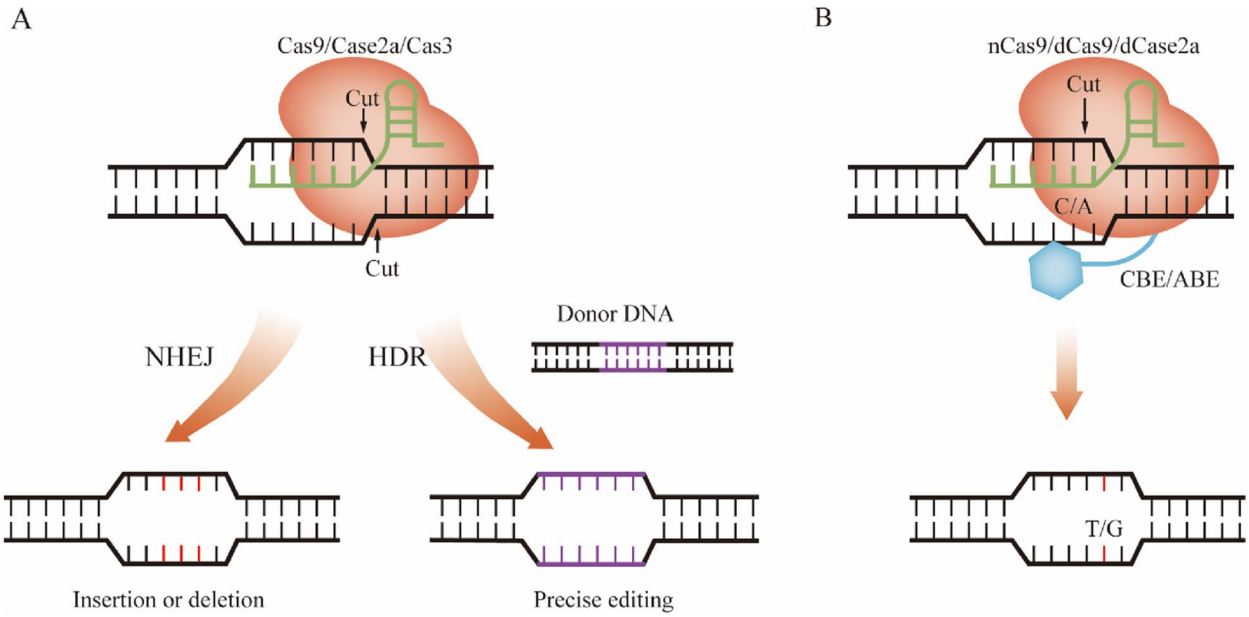

Fig. 2 Schematic diagram of the gene editing process. **A** Cas proteins with endonuclease activity cleave the target gene, resulting in a DNA double-strand break (DSB). DNA double-strand break repair mainly involves two repair mechanisms: Non-homologous DNA end joining (NHEJ) and Homology directed repair (HDR). In gene editing tools based on Cas3, Cas3 randomly cleaves both sides of the cleavage site, and the broken DNA is repaired through NHEJ. In tools based on Cas9 and Cas12a, Cas proteins with endonuclease activity cleave the target gene while providing a repair template, resulting in precise editing through HDR. **B** In base editors, Cas proteins that lack endonuclease activity or possess endonuclease activity but only generate nicks are fused with cytidine deaminase or adenine deaminase. The deaminase, when targeted to the site by the Cas protein, catalyzes the deamination of the target base, triggering a biochemical reaction at the target base, ultimately achieving the goal of base modification

and cleave the U base, generating a DNA strand without a base [55]. During the repair process, different species exhibit different preferences. For example, in *Escherichia coli*, primarily C→A results are produced, while in mammalian cells, primarily C→G results are observed, showing considerable randomness [55, 56]. Therefore, in CBE, a uracil glycosylase inhibitor is typically introduced to enhance the efficiency of CBE base editing outcomes [52]. In cyanobacteria, Type IV UDG is not inhibited by phage-derived UGI [57]. Although using CRISPRi to suppress UDG expression has improved the editing efficiency of CBE, it has also increased the complexity of the CBE system, making it not an ideal solution [57]. An ideal approach would be to find a UGI that can inhibit UDG activity in cyanobacteria. However, it is not necessary to be overly concerned about the impact of UDG activity on the CBE tool. Another study using the CBE tool in *Synechococcus elongatus* PCC 7942 (hereafter Syn7942) demonstrated that the CBE tool still exhibited very high editing efficiency without inhibiting UDG activity [14].

Due to the absence of an amino group in the T base, base editing tools based on deaminase cannot directly edit the T base. To expand the types of base editing, a base editing tool based on DNA glycosylase has been developed [58, 59]. DNA glycosylase creates an apurinic/apyrimidinic (AP) site at the target location, triggering the base excision repair mechanism, thereby achieving the goal of base editing [60]. While base editing tools based on DNA glycosylase have broadened the types of base editing, their use is limited due to the randomness of repair outcomes associated with the base excision repair mechanism. Currently, there have been no reports of the development of base editing tools based on glycosylase in cyanobacteria. This represents one of the future directions for the development of base editing tools in cyanobacteria.

Applications of CRISPR-Cas systems for gene editing in cyanobacteria

DNA fragment editing in cyanobacteria

DNA fragment editing tools based on CRISPR-Cas12a have been successfully developed and applied in cyanobacteria. In Ana7120, under the "two spacers" strategy, the editing efficiency reached 100%, with a maximum editing length of 118 kb [61]. Furthermore, by introducing the sucrose-sensitive counter-selection gene *sacB* onto the vector, rapid removal of edited plasmids was achieved [61]. Additionally, using this tool, the RBS sequence of the key gene *polA*, encoding DNA polymerase I, was replaced in Ana7120 [61], providing a powerful

tool for studying critical genes in cyanobacteria. Using DNA fragment editing tools developed based on CRISPR-Cas12a, successful deletions of predicted non-essential genes in Syn7942 have been achieved, along with the combination deletion of non-essential genes at different loci [17].

In *Synechococcus elongatus* UTEX 2973 (hereafter Syn2973), there have also been reports of using CRISPR-Cas3 for the deletion of non-essential genes [62]. Interestingly, Syn 2373, after deleting non-essential genes, exhibited advantages in growth and sucrose production, a phenomenon not observed in the study of non-essential gene deletion in Syn7942 [17, 62]. This could be due to the greater flexibility in deletion lengths generated by the CRISPR-Cas3 system. In the study of Syn2973's response to high light tolerance by truncating the light-harvesting antenna, strains after knocking out the gene encoding the rod-core linker gene *cpcG* based on CRISPR-Cas12a did not show an advantage [63]. However, targeting the gene encoding the rod-rod linker gene *cpcC2* using the CRISPR-Cas3 editing tool resulted in a mutant strain with the deletion of 3 rod-rod linkers (*cpcC1*, *cpcC2*, *cpcD*) and a phycocyanin gene *cpcB2A2*, exhibiting high-light tolerance characteristics [63]. In rational DNA fragment editing, systems based on CRISPR-Cas12a have an advantage, while in non-rational DNA fragment editing, systems based on CRISPR-Cas3 often yield some exciting results.

Base editing in cyanobacteria

CBE is used for gene silencing in Syn7942 by modifying the codons in the target gene coding region to stop codons, leading to premature termination of translation of the target gene [14]. Premature termination base editing of the *glgP* gene encoding glycogen phosphorylase and the *glgX* gene encoding glycogen debranching enzyme resulted in increased accumulation of glycogen [14]. Base editing tools have been less reported in cyanobacteria, and applications of other forms of base editing tools in cyanobacteria have not been widely observed yet.

CRISPR-Cas mediated gene regulation in cyanobacteria

Mechanisms of CRISPR-Cas mediated gene regulation

CRISPR interference (CRISPRi) primarily relies on catalytically inactive forms of dCas9 and dCas12a, guided by crRNA to bind to target genes, thereby interfering with the transcription process and reducing the expression levels of the target genes [64, 65] (Fig. 3A). By introducing multiple sgRNA/crRNA sequences, it is possible to achieve simultaneous suppression of multiple genes or achieve multiplexed suppression of the same gene, meeting the requirements for controlling gene expression [66] (Fig. 3B). In addition, CRISPRi can screen key genes on a genome-wide scale by synthesizing gRNA libraries [67].

Applications of CRISPR-Cas systems for gene regulation in cyanobacteria

CRISPRi-based gene regulation

To increase the production of succinic acid in Syn7942, the CRISPRi tool based on dCas9 was employed to individually inhibit three key genes: *glgC*, associated with sucrose accumulation, and *sdhA* and *sdhB*, associated with succinic acid decomposition metabolism [68]. All three interventions resulted in increased accumulation of succinic acid [68]. To further enhance the yield of succinic acid, in addition to overexpressing genes related to the succinic acid synthesis pathway, genes *glgC,* and

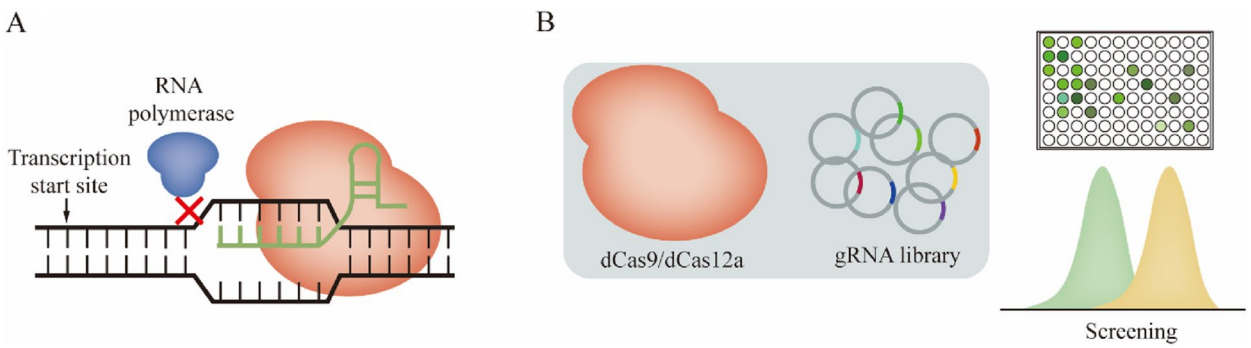

Fig. 3 CRISPRi working process and construction of CRISPRi library. **A** In the CRISPRi tool, guided by RNA, the complex of Cas without endonuclease activity binds to a site downstream of the transcription start site of the target gene. Through steric hindrance, it prevents the binding and movement of RNA polymerase, thereby interfering with the expression of the target gene at the transcriptional level. **B** Synthetic gRNA libraries are introduced into bacterial strains carrying Cas protein expression vectors, with each transformed strain containing one gRNA. Screening of the CRISPRi library is performed by coupling different conditions with growth or linking them with other signals, aiming to achieve rapid screening of the entire genome for gene selection

sdhB were simultaneously inhibited using the CRISPRi tool based on dCas9, resulted in a final yield increase of 82% [69]. In *Synechocystis* sp. PCC 6803 (Syn6803), it has also been demonstrated that the CRISPRi tool based on dCas9 is effective for simultaneously inhibiting multiple genes [15]. In Syn6803, the CRISPRi tool based on dCas9 was utilized to identify genes associated with extracellular polymeric substance (EPS) synthesis [70]. By individually inhibiting three candidate genes—*slr0977*, *slr2107*, and *sll0574*—it was determined that the *slr0977* gene is a key gene involved in EPS synthesis [70].

Compared to the CRISPR-Cas9 system, Cas12a has the ability to independently process pre-crRNA containing spacer-direct repeats into crRNA, giving it an advantage in multi-gene inhibition [44, 71]. In Syn7942, the CRISPRi system based on CRISPR-Cas12a, utilizing a single crRNA array, can achieve the inhibition of three genes [72]. Moreover, the strength of inhibition does not change with an increase in the number of genes targeted for inhibition [72]. Furthermore, increasing the yield of β-ionone has been achieved through the inhibition of the gene encoding aconitate hydratase, *acnB*, or the gene encoding the phycocyanin β-subunit, *cpcB2* [72]. It is worth noting that growth inhibition of the strain occurred during the inhibition of genes *acnB* or *cpcB2*, despite the use of a lactose operon to control the expression of dCas12a. However, the rigor of the induction system was not rigorously assessed in the relevant study. In Syn2973, utilizing a lactose operon to control dCas12a and the crRNA array, the expression of target genes was inhibited by 0–10% under non-induced conditions [73]. However, upon the addition of the inducer, the inhibition of target genes could reach over 90% [73]. The rigor of the induction system controlling the CRISPRi system remains an important issue that needs to be addressed. In Syn6803, controlling the expression of dCas12a with a riboswitch responsive to theophylline and controlled by a rhamnose inducible promoter, when inhibiting the gene *psbD* encoding the PSII reaction center protein D2, there was no effect on strain growth under non-induced conditions [74]. However, when only using the rhamnose-inducible promoter to control dCas12a, significant growth inhibition of the strain was observed under non-induced conditions [74]. A rigorous CRISPRi system provides a good solution for studying essential genes in cyanobacteria.

Screening library in cyanobacteria

Biochips enable the rapid synthesis of guide RNA libraries at a whole-genome scale, offering the possibility for the establishment of whole-genome scale CRISPRi libraries. The CRISPRi library can be coupled with growth under different conditions for screening, and it can also be combined with microfluidic techniques for screening. In cyanobacteria, construction of a whole-genome scale CRISPRi library has only been completed in Syn6803 so far [75]. Under L-lactate stress as the screening condition, after continuous cultivation of the CRISPRi library, a significant increase was identified in the *bcp2* mutant encoding the bacterioferritin comigratory protein [75]. Combining microfluidic techniques for screening the CRISPRi library, mutants with high L-lactate production were identified. The repression of gene *gltA* (citrate synthase) or *pcnB* (CCA-tRNA nucleotidyltransferase) resulted in significantly increased L-lactate yield [75].

Future direction

The development and utilization of endogenous CRISPR-Cas systems

Analysis of CRISPR-Cas systems in cyanobacteria with reference genomes has revealed their widespread distribution, indicating a rich resource of CRISPR-Cas systems within cyanobacteria [25–27]. Research on endogenous CRISPR-Cas systems in cyanobacteria such as Syn6803, Ana7120, and *M. aeruginosa* has reached a relatively mature stage [26, 31–34, 36, 76]. However, there are still numerous CRISPR-Cas resources waiting to be discovered. Further development of endogenous CRISPR-Cas systems in cyanobacteria into genetic manipulation tools still has a long way to go. It's worth noting that CRISPR-Cas systems, such as Type I-D from *M. aeruginosa* and Type V-K from *Scytonema hofmannii*, have been applied in genetic editing of other species [18, 38]. The evidence suggests that CRISPR-Cas systems sourced from cyanobacteria are versatile and have the potential to be developed into genetic manipulation tools applicable to various host cells.

Continuous editing of cyanobacterial genomes

Achieving continuous multi-step gene editing in host cells is a fundamental requirement for realizing artificial cell factories. Editing the cyanobacterial genome under untagged conditions involves removing the plasmid containing the editor after editing is completed. Currently, the approach involves introducing the *sacB* gene into the plasmid carrying the editor [61]. After the editing process is completed, the plasmid is removed by adding sucrose [61]. The characteristic of multiple copies in the cyanobacterial genome raises concerns about whether the genome can maintain the edited state stably after plasmid removal. Although tools have been developed to obtain homozygous mutants in cyanobacteria [18], the CRISPR-Cas systems used in these tools are relatively complex, which limits their flexibility. Therefore, it is necessary to develop a flexible gene editing tool that couples plasmid removal with homozygous screening.

Dynamic regulation

During the process of regulating genes in engineered strains to enhance product yield, premature inhibition often affects the biomass of the strains. Such phenomena have been observed during the use of CRISPRi to regulate genes in cyanobacteria [72]. To ensure dynamic regulation of target genes, strict induction system control over the expression of the CRISPRi system is necessary. In cyanobacteria, various control systems such as lactose operon, dehydrotetracycline inducible system, and theophylline-responsive RBS have been used to regulate the CRISPRi system. However, in related reports, instances of inhibitory effects on target genes under non-inducing conditions still exist [72, 74]. Therefore, the impact of the CRISPRi system on target genes under non-inducing conditions, as well as the degree of inhibition of target genes under induced conditions, is crucial for dynamic regulation. Currently, there hasn't been a systematic study on the dynamic range of dynamic regulation systems in cyanobacteria.

Conclusion

CRISPR-Cas systems have emerged as powerful tools for genetic manipulation in cyanobacteria. With their remarkable diversity, cyanobacteria present an extensive reservoir ripe for further exploration. As research into endogenous CRISPR-Cas systems in cyanobacteria advances, applications of these systems in genetic manipulation tools are beginning to surface. Utilizing Cas9 and Cas12a, tools have been developed enabling seamless deletion of large genomic segments, single-base modifications, and transcriptional level gene suppression in cyanobacteria, thereby propelling the realization of cyanobacterial cell factories. The development of CRISPRi libraries has expedited the screening of genes associated with specific phenotypes. Nevertheless, there are still areas for advancement in CRISPR-Cas-based genetic manipulation tools in cyanobacteria, including continuous gene editing, generation of homozygous mutants, removal of editing cassettes, ensuring the precision and induction levels of induction systems for dynamic regulation.

Abbreviations

Ana7120	Anabaena (Nostoc) sp. PCC 7120
ABE	A → G base editor
CBE	C → T base editor
CRISPR/Cas	Clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins
DRs	Direct repeats
HDR	homology-directed repair
PAM	Protospacer adjacent motif
NHEJ	Non-homologous end joining
pre-crRNA	pre-CRISPR RNA
Syn6803	*Synechocystis* Sp. PCC6803
Syn7942	*Synechococcus elongatus* PCC 7942
Syn2973	*Synechococcus elongatus* UTEX 2973
UDG	Uracil DNA glycosylase
AP	Apurinic/Apyrimidinic site

Authors' contributions

ZXD wrote the draft manuscript; LC and YW designed the manuscript; TS and WZ designed and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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