Applications and Prospects of CRISPR-Cas system in Cyanobacteria

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Abstract. Cyanobacteria are prokaryotic microorganisms with capacity to perform photosynthesis and provide valuable platform to produce high-value bioactive compounds in carbon-neutral pathway. However, due to the relative lack of high throughput genetic manipulation tools for cyanobacteria, their usage for complexity chemicals is lagging behind. The gene editing technology based on the CRISPR-Cas system has the advantage of simplicity and efficiency in recent years, making it a new tool for synthetic biology of cyanobacteria. In this review, we first introduced the CRISPR-Cas system and the types of CRISPR-Cas in cyanobacteria. In addition, we review the development of CRISPR-based systems in cyanobacteria, including the use of CRISPR systems for gene integration, deletion, and transcriptional regulation. Meanwhile, the CRISPR system can couple different functional proteins to achieve point mutations, such as base editing. Finally, we discuss the current limitations of CRISPR technology and its future application prospects in cyanobacteria to make an outlook. Finally, we discussed the current limitations of CRISPR technology and its future application prospects in cyanobacteria.

Key words: CRISPR-Cas system, cyanobacteria, synthetic biology, CRISPRi, Base editing, CAST.

1. Introduction

Cyanobacteria are prokaryotic microorganisms to perform anoxygenic photosynthesis and are widely found in marine and freshwater habitats[1,2]. Cyanobacteria can convert captured carbon into a variety of complex organic compounds, making it a valuable platform for the production of high-value chemicals[3,4]. As cell factories, cyanobacteria have unique advantages, including (1) intensive and large-scale use of bioreactor for rapid and high-density culture, (2) carbon neutral, which can absorb carbon dioxide directly from the atmosphere to resolve the problems such as global climate deterioration[5,6], (3) the efficiency of higher photosynthetic, which is 10 times higher than that of higher plants[7], (4) no organelles and various membranes compared with plant cells, which provides more ideas for synthesis pathway design, (5) the lower carbohydrate content and higher the protein content , which makes it ideal for the synthesis of target products using amino acids as substrates, (6) the strong ability to grow in harsh living conditions (such as extreme temperatures, salinity, and pH values)[2,7], (7) generation of relatively fast and inexpensive mutants (mainly in model species)[8],(8) no obvious pathological immune response to animal cells based on the theory of endosymbiosis[9]. Therefore, in the past few decades, cyanobacteria have been successfully designed to produce a variety of valuable chemicals, such as fatty acids [10], ethylene[11], ethanol[12], 1-butanol[13], sucrose[14], shinorine[15], terpenes [16] and so on.

However, cyanobacteria still have many obstacles as cell factories. On the one hand, compared with other microorganisms, the growth of cyanobacteria species is relatively slow. For example, under standard growth conditions, the doubling time of *Synechocystis sp.* PCC6803 is about 7 hours[17], compared with 20 minutes for *Escherichia coli* and 2 hours for *Saccharomyces cerevisiae*[18,19]. At present, a considerable number of genetically modified strains have the same growth rate as *S.cerevisiae*, such as *Synechoccoccus elongatus* UTEX 2973, which has shortened the division time of cyanobacteria to 2 hours[20]. On the other hand, lack of molecular tools limits the development of cyanobacteria engineering strains, so that the product yield of cyanobacteria is lower than that of *E. coli*, yeast, and other traditional hosts. Conversely, in the past few years, cyanobacteria-specific tools and components have increased at a staggering rate, including development based on CRISPR-Cas systems[21], which promoted the widespread adoption of synthetic biological paradigms. For example, The biological tools was designed by the combination of recombination standardized parts or modules (such as promoters, ribosome binding sites, coding sequences, and Terminators) and CRISPR-Cas system[22]. Among them, many of these tools were originally developed in *E. coli* or S*accharomyces*

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cerevisiae, which have been adapted and modified for cyanobacteria[23,24].

In recent years, CRISPR-Cas system has developed into a revolutionary method for genome editing[25,26]. RNA directs the Cas protein to the target site and induces double-strand break (DSB)[27]. DNA breaks can be repaired by non-homologous end joining (NHEJ) or homologous directed repair (HDR)[28]. Traditional gene editing tools, such as TALENs and ZFNs, have some disadvantages, such as complex module assembly, high cost, time-consuming, easy to miss, and difficult to operate [29], which greatly limit their application. In contrast, CRISPR-Cas system has the advantages of high efficiency and easy manipulation. It can induce double-strand breaks with reasonable specificity, locates flexibly in the genome, and complete a variety of genetic modifications in parallel, so it was rapidly developed as a versatile tool in genome editing[30].

Here, we introduced the composition and mechanism of the CRISPR-Cas system summarized the identification of natural CRISPR-Cas system of cyanobacteria. We had also introduced the research progress of genome editing and transcriptional regulation mediated by CRISPR technology in cyanobacteria. Finally, we discussed the current limitations of CRISPR technology and had made the prospective forecast for the development of CRISPR system in cyanobacteria.

2. Composition and mechanism of CRISPR-Cas system

CRISPR-Cas system is found in about 50% of bacteria and 90% of archaea [31,32], which are acquired immune systems for bacteria and archaea to resist infection by foreign phages, plasmids or other mobile elements[33,34]. CRISPR was first found in the flanking sequence of *E. coli* alkaline phosphatase *iap* gene[35]. Subsequently, a series of bioinformatics analysis revealed that the function of CRISPR-Cas system[36].

The CRISPR-Cas system consists of Cas nucleases and CRISPR array [37,38], and the CRISPR array consists of a leader, multiple short and conserved repeats, and various spacers[39,40]. The leader is located in upstream of CRISPR arrays, it could be the promoter sequence of CRISPR cluster and has species specificity[39]. The repeats length of different systems is 23~47 bp. In the same CRISPR array, these repeats are highly conservative and show similarities among phylogenetic related species[31]. Spacers are the trace left by the invasion of bacteriophages or plasmids, which give cells the ability to acquire immune defense against the corresponding bacteriophages or plasmids. In the same CRISPR array, the length of the spacers between the two repeats is similar, but the sequence is different[40]. Cas gene is located near the CRISPR array and mainly encodes Cas nuclease which performs to cleave nucleic acids, similar as DNA helicase [37,40]. Currently, according to the type and homology of Cas nuclease in CRISPR-Cas system, the known CRISPR-Cas system mainly divided into 2 classes with each consists of 3 types (For more information, see review[41,42]). Thereinto, the first class contains three major types: type I, III and IV, while the second class includes: type II, V and VI [43].

Take CRISPR-Cas9 which belongs to type II CRISPR system as an example, the mechanism consists three main stages: adaptation, expression and interference. In the adaptive stage, the CRISPR system recognizes and integrates the exogenous DNA. In the expression stage, the mature crRNA is synthesized. In the interference stage, the complex composed of crRNA, Cas9 and tracrRNA recognizes and cuts DNA[44,45]. As a new gene editing technology, CRISPR-Cas9 gene editing technology has the advantages of simplicity and rapidness. Cas9 enzyme has contains RuvC and HNH domains ribozyme regions, which are responsible for cutting two strands of the target DNA and inducing DNA repair pathways in vivo, including HDR and NHEJ, thus realizing gene integration and deletion[46,47]. So far, tracrRNA and crRNA have been simplified into a single-stranded guide RNA (sgRNA)[45]. The artificially designed sgRNA identify the target genome sequence, and guide the Cas9 to cut the target sequence effectively. In addition to CRISPR-Cas9, research of type V and VI are increasing, such as nucleic acid detection technology based on Cas12a[48] and Cas13 had been used for fast novel coronavirus detection[49].

3. CRISPR in cyanobacteria

Cas1 and Cas2 are widely used to identify CRISPR-Cas system[50,51]. Through the identification of Cas1/Cas2 gene in cyanobacteria, it was found that 86 of 126 cyanobacteria genomes contained CRISPR system[52]. At present, the CRISPR systems found in cyanobacteria are mainly type I, III and V.

Type I and III belong to the class I CRISPR-Cas systems, whose effect modules of class I composed of multiple Cas proteins. Type I CRISPR system is characterized by Cas3 which can recognize target and cleave single-strand DNA (ssDNA) [53] . Type I consists seven subtypes: I-A, I-B, I-C, I-D, I-E, I-F and I-G[43]. There are six subtypes of Type III, including III-A, III-B, III-C, III-D, III-E, and III-F. It is speculated that III-A, III-B and III-C can cut both DNA and RNA molecules, in compared to III-D and III-E can only cut DNA, and III-F can only cut RNA. Type V belongs to class 2 CIRPSR-Cas system [54,55]. Unlike class 1 system, class 2 CRISPR-Cas system mainly involve a single effector in the interference module, which is rarely observed in cyanobacteria[52,56].

Presently, I-A, I-B and I-D from type I and III-A, III-B and III-D from type III have been identified from cyanobacteria[43,57-61], and the type V system found in cyanobacteria are V-U2 and V-K, that is, C2c8 and C2c5 proteins [62]. For example, Syn6803 harbors type III-D subtype[43], while another strain from this species *Synechocystis sp.* PCC 6714 contains both I (I-A or I-B) and III (III-B) types[57]. The natural CRISPR system in multicellular filamentous cyanobacteria *Anabaena sp*. PCC7120 has been identified as at least three class 1 and one class 2.

Currently, there are few studies on cyanobacteria natural CRISPR, which only stay in the stage of bioinformatics analysis. Although CRISPR-Cas9 and CRISPR-Cas12a

are widely developed as genome engineering tools for eukaryotes and prokaryotes[63], such two systems are only found in less than 10% of the sequenced prokaryotic genomes[53], and exogenous CRISPR systems are difficult to use and toxic for most prokaryotes[64]. For this reason, the natural CRISPR-Cas system has been developed as an alternative strategy for archaea and bacterial prokaryotic genetic engineering. For instance, the development of the CRISPR I-F system of *Zymomonas mobilis* has effectively achieved a variety of genome engineering purposes and compared with the gene manipulation methods commonly used in the engineering strain, this CRISPR-based toolkit is simpler, more convenient and more time-saving[65]. The development method of this study supplies an important reference for the development and utilization of other microbial endogenous CRISPR-Cas systems, and we expect that the natural CRISPR system can also become a way to edit cyanobacteria.

4. Research progress of genome editing mediated by CRISPR technology in cyanobacteria

4.1 Research progress of CRISPR-Cas9 gene editing technology

The advent of CRISPR-Cas9 provides community new opportunity to explore an alternative method with potential to edit multiple genes simultaneously in cyanobacteria[66-68]. It was first reported in Syn2973 to realize markerless editing. This study provides a proof of concept for the introduction of CRISPR-Cas9 system into cyanobacteria and the successful removal of edited exconjugants[66]. Moreover, it developed a single plasmid CRISPR-Cas9 system pCRISPOmyces-2 to knock out a truncated thioesterase gene and obtain a Syn2973 mutant producing free fatty acid (FFA)[69]. Since then, more CRISPR-Cas9 applications have been conducted in cyanobacteria, the carbon flux was transferred from glycogen to succinic acid synthesis pathway in Syn7942, and the succinic acid titer was increased to 435.0 ±35.0 μg / L, which was 11 times higher than that of wild-type cells[67].

considering that the accumulation of Cas9 is toxic to cyanobacteria as previously mentioned strategies which could [66,67]strictly control or fine-tune the expression of Cas protein was established. The researchers developed a versatile theophylline-responsive riboswitch, a tightly regulated RNA device, to sustain the balance of Cas9 low enough to prevent its toxicity and high enough induce genome editing [70]. In addition, this circuit could be assembled into a shuttle vector to avoid labor and time consuming genetic integration [68] .

4.2 Research progress of Cas12a gene editing technology

Besides manipulation Cas gene expression level to eliminate toxicity caused on host cell, one could also identify some less toxic Cas protein with nuclease cleavage activities[71] . Cas12a is a less toxic protein that is more conducive to gene editing in cyanobacteria, its expression in cyanobacteria does not lead to toxic effect on Syn6803, Syn7942, *Nostoc* PCC 7120, *Chroococcidiopsis* B13, *Synechococcus elongatus* PCC 11801[111,113,115-117]. Cas12a is different from Cas9 in three perspectives: i) the PAM sequence recognized by endonuclease in Cas12a is guided by a single RNA and does not need auxiliary tracrRNA; ii) The PAM sequence recognized by Cas12a is rich in T (5′-TTN-3′), rather than G-riched Cas9 PAM; iii) Cas12a cut DNA by staggered DNA double-strand breaks [72,73] . In particular, due to simple composition of Cas12a sgRNA and its own ability to deal with crRNA, it is more favorable for multi-target editing compared with Cas9 system. Therefore, it can be considered that the CRISPR-Cas12a system developed in cyanobacteria genome editing is a more feasible and moderate method than Cas9.

So far gene editing by Cas12a (FnCas12a) from *Francisella novicida* has been implemented in various cyanobacterial species . The system was first introduced into three cyanobacteria strains Syn 2973, Syn 6803 and Ana7120[71] for a markerless gene replacement, it had been shown that Cas12a in general showed less toxicity to cell compared with Cas9. Interestingly, when pSL2680 plasmid plays load Cas12a, it cannot be cloned (90% of the plasmid preparation is single-stranded DNA), and they are highly unstable with low efficiency of gene editing. To overcome this, a study used cyanobacteria Ana7120 as a model strain, made modifications to the plasmid, improved and designed CRISPR-Cas12a-based tools and genetic strategies to make it more conducive to cyanobacteria-related research[71]. Through the employment of two editing plasmids containing the same homologous repair template but different spacer sequences, editing plasmids with different resistance markers, of course, gene fragments integration could be enhanced through introducing somecounter-selection marker, for example *sacB*[74]. In combination of *sacB* counter selection with Cas proteins, gene editing rate increased up to near 100%, meanwhile, the efficiency of multiple gene editing is feasible, and the process of plasmid stability is greatly simplified and accelerated. In addition, the study also found that CRISPR-Cas12 can manipulate essential genes and delete large segments of chromosomes by generating conditional mutants, which will greatly promote the reprogramming of genetic information[74]. Recently, a study constructed a CRISPR-Cas12a vector using Seva plasmid for genome editing of cyanobacteria. Compared with the editing plasmid used in previous studies, the developed pSEVA-Cpf1 vector has a broader host range, simplicity of modular structure, small size and low cost. These features make it a valuable genetic editing tool and had been successfully transformed into non-model extreme strain, *Chroococcidiopsis* B13. pSEVA-Cpf1, the genome of Syn6803 was edited successfully, and two copies of *nblA* were deleted. The development of pSEVA and its derivative vectors is a promising tool for transforming other biotechnology-related cyanobacteria, such as extreme bacteria, which could be further expanded to cover the genus cyanobacteria that cannot be edited by CRISPR[75].

In cyanobacterial metabolic engineering, the use of CRISPR-Cas12a greatly increased by introducing genes beneficial to increase yield and efficiency of limonene, succinate and ethylene production[76,77]. This is the first time to report a makerless editing gene editing in Syn11801 [115]. A stable recombinant strain was constructed by using CRISPR-Cas12a. Under the photoautotrophic condition, the specific production of ethylene and succinic acid was 338.26 and 1044.18 μg/g cell dry weight/hour, respectively. These results are significantly higher than the reported productivity, indicating that Syn11801 is a potential candidate for engineering strain.

Thus far, FnCas12a is the only reported Cas12a for genome editing of cyanobacteria. Nevertheless, PAM sequences recognized by other Cas12a subtypes have been found in different cyanobacterial genomes[78,79]. Studies have shown more Cas12a subtypes are available, such as AsCas12a from *Acidominococcus sp.* and LbCas12a from *Lachnospiraceae*. We can design alternative recognition PAM sequences to improve the flexibility of PAM recognition[78,79] and greatly improve the pool coverage in facing of the PAM site limitation in genome editing[78,80].

4.3 CRISPR-associated transposases

Cas9 mediated gene editing technology depends on host's DNA repair pathway, which remarkably limits its application. Recently, CRISPR-associated transposase (CASTs), as a gene integration tool independent of host HDR, has been deployed in a variety of bacteria, such as *Escherichia coli* and *Vibrio cholerae*[81,82], which is mediated by a combination between CRISPR and transposase elements.

It has been found that there are abundant CAST elements in cyanobacteria genome, including the best known guide RNA-directed transposons, the type V-K, I-B1, and I-B2 CRISPR- based systems, which can be used as one of the solutions for the development of natural CAST systems. Recently, the I-D type McCAST transposable system has been found in cyanobacteria, and a variety of new candidates belonging to I-D subtypes have been found, including the natural inactive Cas10 nucleases. Type I-D CRISPR-Cas transposable system is flexible in guiding RNA length requirements, and its transposon also has a naturally fused transposon protein for cut-and-paste[83]. Meanwhile, a Cas12k protein, C2c5, was identified from cyanobacteria *Cyanothess sp.* PCC 8801[43] and *Scytonema hofmanni*[82], which can cooperate with tracrRNA and CAST transposons[82]. Another study identified the freeze-fracture electron microscope structure of cyanobacteria *Scytonema hofmanni* Cas12k, which formed a complex with sgRNA and double-stranded target DNA, revealing the recognize and perform mechanism of Cas12k[84]. At present, the CASTs system used in cyanobacteria is V-K type, however, Cas12-K is actually only responsible for targeting and binding to targeted DNA in the absence of cleavage activity. While the transposase consists of three components, TniQ acts as a mediated protein to binds DNA and recruits TnsC, which then further recruits TnsB was act as ATP hydrolase, it is TnsB which conducts the cleavage[85]. The ends of the transposase include reverse repeat sequence named LE and RE [86] .

Recently, a study has established a high throughput gene assemble strategy through Golden-gate methods, with the advantage to freely assemble multiple components in a modular assembly program. In this process, Arévalo has successfully used CAST to integrate two targeting two different sites on the genome, the results showed that CASTs could mediate gene integration at different sites on the genome, which verified that CAST is effective in Ana7120. It successfully transferred the comparatively large plasmids containing the CAST and the engineered transposon. In four out of six cases analyzed over two distinct target loci, the insertion site was exactly 63 bp after the PAM., which confirmed that sgRNA that target the leading, but not the reverse complement strand were effective with the PAM sequence included in the sg RNA. Subsequently, different insertions were obtained by changing the target locus and donor genes to determine the insertion mechanism and unlock the final miss effect. It was only the transposon defined by the sequence ranging from left and right elements was inserted at the target loci, therefore, RNA-guided transposition resulted from cut and paste[85]. This work lays a foundation for genome editing through RNA-guided transposons in filamentous cyanobacteria and provides a new tool.

4.4 Base Editing

Base editing is a new CRISPR-based single-nucleotide resolution genome editing tool, which combines the catalytically dead Cas9 mutant (dCas9) with base deaminase. Therefore, this tool can be used to catalyze the deamination of bases at specific sites on the DNA chain to elicit a mismatched base pair pairing, and resulting in base substitution from cytosine to thymine (C-to-T) or adenine to guanine (A-to-G) in the genome[87-89]. With the advancement of base editing toolkit, single nucleotide substitution has been extended to more combinations, including C-to-G and C-to-A[90,91]. Compared with gene editing, base editing has the advantages of less toxicity, no donor DNA, no DSB, non-homologous terminal repair and homologous recombination pathway independent of host, and does not need high transformation efficiency to screen viable cells, so it has become an ideal tool for accurate gene editing[92-94]. So far, base editing has been successfully adapted to a variety of bacteria, such as *Streptomyces griseus*, *Agrobacterium rhizogenes*, *Pseudomonas* aeruginosa and *Clostridium Yongda*[94-97]. And recently, it has been preliminarily studied in cyanobacteria because of its advantages.

The base editing technology in cyanobacteria was firstly reported in Syn7942 in 2023 [98]. The researchers designed a modular assembly program to generate the base editing construct, PSY. The construct includes the dCas9 of Streptomyces pyogenes and the activation-induced cytidine deaminase (PmCDA1) from sea lamprey (*Petromyzon marinus*). The nucleotide substitution of C-to-T was successfully demonstrated on the targeted locus. By introducing the early termination codon into the related genes to regulate the glycogen metabolic pathway in cyanobacteria, the engineering strain with the potential

for improvement was constructed, so that the inactivation of *glgC* which is targeted to block the glycogen synthesis and the inactivation of *glgP* and *glgX* which are the targeted genes of the glycogen degradation pathways successfully increased the glycogen production, which was 95.72% higher than that of the wild type. Accurate and efficient genome editing of two target genes was achieved at single-nucleotide resolution, and multi-base editing was demonstrated in the first model of cyanobacteria *Synechococcus elongatus*. This study provides an example of cyanobacteria base editing to promote metabolic engineering and synthetic biology of cyanobacteria[98].

In another study, researchers developed a dCas12a-mediated base editing in Syn7942. To overcome this limitation, phage-derived the glycosylase inhibitor (UGI) could not inhibit type IV DNA glycosylase (UDG) in cyanobacteria. In order to overcome this limitation, the researchers successfully applied orthogonal dCas12a interference to inhibit the expression of UDG gene in cyanobacteria, thus allowing base editing at the desired target and producing the correct phenotype of the desired mutant in cyanobacteria. This study proposes for the first time the development of cyanobacteria CBE suitable for oligopoly *Streptomyces longissimus* Syn7942 as a model strain of synthetic biology and oligoploidy, and reveals that the control of uracil DNA glycosylase (UDG) in cyanobacteria is the key to the successful development of CBE, which will open up new opportunities for base editing using type IV UDGs in archaea and green algae[99].

5. Transcriptional regulation mediated by CRISPR system

The CRISPR system regulates the transcription of downstream genes by using inactivated dCas proteins to bind to sgRNA, and activates or knocks down genes through binding to targeted protein or gene expression regulated range to interference gene translation, which are named CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi), respectively. Both CRISPRa and CRISPRi regulalte cyanobacterial gene expression at the transcriptional level. CRISPRa relies on modified guide RNAs (gRNAs) that are extended with hairpin sequences. The function of gRNAs is to recruit RNA-binding proteins (RBP) to fuse with transcription factors ω, one of the subunit of RNA polymerase (RNAP)[100]. When the dCas9-gRNA complex binds to the target gene, modified gRNAs recruits transcription factor to drive transcription. CRISPRi technology uses catalytic inactivation of dCas9, dCas12a and other Cas proteins to combine with sgRNA to form a ribonucleoprotein complex to reach the designated site of the gene, preventing the binding of RNAP instead of cutting DNA, to achieve gene knock down[101,102]. As multiple gRNAs can be generated in the same cell, therefore, CRISPRi and CRISPRa could be simultaneously applied to inhibit and activate target genes[100,103]. With this intent, one could easily perform the "push-pull strategy" to enhance orthogonal introduced metabolic pathway meanwhile inhibit native competitive flow, therefore could at least theoretically redirect carbon and energy flux toward desired bioproducts. Considering the toxicity of Cas protein, and the damage of gene knock-in or knock-out on host viability, the application of CRISPR in cyanobacteria is greatly limited. On the contrary, CRISPRi has become a popular tool for cyanobacteria research and development because of its simple mechanism, low toxicity, reversibility and ability to regulate multiple genes at the same time[101].

dCas9 is the most commonly used Cas protein in CRISPRi. Through the transformation of metabolic engineering to adjust the carbon flux of some metabolic pathways, inhibit the carbon sequestration of high-throughput metabolic pathways, and introduce carbon into the target product metabolic pathways, many studies are related to this[104-107]. It was the first time to applied in Syn6803. Inactive dCas9 was implemented for target gene down-regulation, with an inhibition rate of 50-95% on the genes of 4 putative aldehyde reductases and dehydrogenases, thus increasing the yield of fatty acids and alkanes[104]. The study also carried out the photoproduction of sesquiterpene valenene through the metabolic engineering of Syn6803, increased the flux to valence and down-regulated the formation of carotenoids to CRISPRi-dCas9 through unlabeled deletion, while almost maintained the both the level of chlorophyll and performance at similar level to wild type, and successfully created the mutant resulted in a valencene production of 19 mg/g DCW [105]. Recently, the inhibition of effective gene targets by CRIPSRi-dCas9 in Syn7942 resulted in an 82% increase in succinic acid production. The yield of the strain reached 4.8g/L within 28 days, and then the succinic acid could accumulate continuously through the re-inoculation strategy, and the final titer reached 8.9g/L, which was by far the highest titer of autotrophic succinic acid production[106]. Additionally, in this study, an inducible promoter was used to control the expression of dCas9, through which hope to separate cell proliferation and bioproducts generation, and minimize Cas protein toxicity on cell factory. Based on these, lactic acid production reached 1g/L, it is a 100% increase in cumulative effect[107].

Gene down-regulation can also be used to study the growth mechanism of cyanobacteria cells[108,109]. In this study, we used CRISPRi-dCas9 to knockdown the gene of a detoxified H2O2 peroxiredoxins called 2-Cys-Prx (annotated as Alr4641) to analyze the physiological role in the oxidative stress response Syn7120. Gene knockdown reduced the content of Alr4641 by more than 85%. It was found that 2-Cys-Prx encoded by Alr4641 seems to be the main peroxiredoxins (Prxs) responsible for maintaining redox balance in different photosynthetic systems from chloroplasts to cyanobacteria[108]. Another study manipulated DNA supercoils in Syn6803 through CRISPRi-based rotase knockdown in combined with topoisomerase I overexpression. It was found that increased DNA relaxation (topAOX strain) or decreased DNA supercoiling activity (gyrkd strain) inhibited cell division and widely affected physiology[109].

CRISPRi also successfully realizes the expression of multiple genes and can regulate multiple genes in parallel rather than alone. The concept was first implemented in Syn6803 using dCas9 to study the key genes that inhibit

four competitive pathways at the same time[104]. In comparison with dCas9, the multiplexing of dCas12a is not limited by the requirement of expressing multiple single-guide RNA (sgRNAs). It directly recognizes the sequence derived repeatedly in the pre-crRNA transcript to produce mature crRNA, so it can control more gene loci[110]. dCas12a is used in Syn7942 to suppress 3 target genes by crRNA array, and up to 3 genes are edited on each array [111]. The multiple properties of CRISPRi promote the metabolic engineering of cyanobacteria, such as inhibiting six genes simultaneously in Syn6803 to redirect fatty acid flux to fatty alcohol production[112].

Following the development of CRISPRi in cyanobacteria, sgRNA libraries have recently been developed in Syn6803 and Syn7942[113,114]. This library can be used to target gene inhibition and collect sgRNA genes to be transformed into the target strain, to explore the interaction between genotype and phenotype. The designed platform cultivated an industry-related strain with high lactic acid production by inhibiting the necessary genes related to lactic acid synthesis (i.e. GLTA and pcnB) based on CRISPRi[113]. The CRISPRi library was also used to increase the yield of 2,3-butanediol. The study integrated the 2,3-BDO pathway genes and a CRISPRi library into the Syn7942 using the orthogonal CRISPR system to overexpress pathway genes and attenuate genes that inhibit 2,3-BDO formation. A strain that increased the yield of 2,3-BDO nearly 2 times to 1583.8mg/L was screened rapidly[114].

In addition to dCas9, dead Cas12a (dCas12a) has recently been developed and applied in cyanobacteria[111,115,116]. Compared with dCas9, dCas12a is less toxic and has a more effective inhibition mechanism[102,117]. A new suppression system has been developed for a series of commonly used "L" promoters inhibited by TetR and induced by dehydrated tetracycline (ATC)[118]. A Cas12a-based CRISPRi platform regulated by promoter *lac* was constructed to inhibit the expression of genes related to necessary pathways, such as CPC (photosynthetic pigment), *glgC* (glycogen accumulation) and *btpA* (PSI content). The elaborate regulated system can effectively knock down the highly binding genes in cyanobacteria, which will be helpful to study the photosynthetic process that cannot be obtained by gene knockout mutants[116].

A rhamnose-inducible promoter system contained a theophylline responsive riboswitch was constructed to drive CRISPRi-ddCpf1(DNase-dead Cpf1 nuclease), effectively and reversibly inhibit the PSII reaction center gene psbD (D2 protein) to regulate the activity of II and the growth of Syn6803 in the photosystem[115]. Likewise, the study developed a dCas12a-mediated CRISPRi in Syn7942, successfully achieved multiple genes suppression between 53-94% through a single crRNA. The metabolic engineering was carried out to increase the yield of photosynthetic squalene by inhibiting metabolic essential but competitive genes and promoting the construction of biological solar cell factories [111].

6. Conclusion

In this review, we summarize the phylogenetic and current application development of CRISPR in cyanobacteria, but there are still many limitations, such as Cas protein toxicity and miss effect, which can be solved by introducing innovative technologies and looking for new Cas enzymes. On the one hand, we can introduce prime-editing technology, which allows the insertion of small DNA fragments without DNA double-strand breaks or the need for donor DNA through the combination of Cas9 nickase (nCas9) and reverse transcriptase[119], so it may be less lethal than Cas9 or Cas12a. nCas9 is a Cas9 variant of a high on-target effect and nondetectable off-target effect, which contains a mutation in the RuvC domain, requires two sgRNA to target Cas9 to a specific genomic region and mediate DSB[120,121]. Some studies have further applied this method to bacteria, making it possible to introduce deletions, insertions and nucleotide substi tution in *Escherichia coli* through primer editing[122]. On the other hand, Cas enzymes with high fidelity and low miss ability can be introduced into cyanobacteria, such as CasX[123]. Recently, the structural engineering design of CasX has improved its efficiency to the same level as the commonly used Cas9 from *Streptococcus pyogenes* (spCas9)[124], and it is found that CasX has lower fault tolerance than Cas9 and Cas12a[125]. Additionally, until now the application of CRISPR system in cyanobacteria is mostly limited to several model strains. In order to extend this technique to other strains, the characterization of thermostable Cas9 variants may provide a platform for genome engineering of cyanobacteria species adapted to extreme conditions [126], which has been applied in thermophilic strains *Thermoanaerobacter kivui* and *Methanothermobacter* thermautotrophicus[127,128]. We look forward to introducing these methods into cyanobacteria.

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