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CRISPRi-assisted metabolic engineering of cyanobacteria for photosynthetic hyaluronic acid from CO_2

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Abstract

Background Hyaluronic acid (HA) is widely used in pharmaceuticals, medicine, and cosmetics. Sustainable production has shifted to microbial fermentation using engineered GRAS strains. Diverse carbon sources and CO_2 conversion via engineered microorganisms enhance HA production. Herein we applied advances in CRISPR technologies and tools to optimize metabolic pathway by redirecting carbon portioning in cyanobacterium *Synechoccous elongatus* PCC 7942, demonstrating enhanced HA production.

Results *S. elongatus* PCC 7942 lacking hyaluronan synthase (HAS) required pathway engineering for HA production. By expressing heterologous Class I HAS, a modular gene expression system was employed, incorporating *hasB* and *hasC* for the HA-GlcA module and *glmU*, *glmM*, and *glmS* for the GlcNAc module. This approach resulted in construction of four engineered cyanobacterial strains. Optimizing metabolic pathway involving the HA-GlcA and GlcNAc modules led to SeHA220 (wild-type with HA-GlcA and GlcNAc modules) producing 2.4 ± 0.85 mg/L HA at 21 d, a 27.5-fold increase compared to the control. Targeting F6P and G6P metabolic nodes via CRISPR interference to repress *zwf* and *pfk* genes further improved production, with SeHA226 (SeHA220 with a gene repression module) achieving 5.0 ± 0.3 mg/L HA from CO_2 at 15 d. Notably, SeHA226 produced photosynthetic HA with a molecular weight (Mw) of 4.2 MDa, comparable to native producers, emphasizing the importance of precursor balance and growth conditions.

Conclusions This study engineered cyanobacteria for efficient HA biosynthesis using modular gene expression and CRISPR-interference systems. Optimizing heterologous metabolic pathway was key to achieving high-molecular-weight photosynthetic HA production from CO_2. The findings provide insights into tunable HA production, with future efforts aimed at scaling up photosynthetic HA production for larger-scale applications.

Keywords Cyanobacteria, CRISPR-dCas12a, Synthetic biology, Hyaluronic acid

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Background

Hyaluronic acid (HA), a linear and unbranched biopolymer of a repeating unit of *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) catalyzed by hyaluronan synthase (HAS), has been widely used for pharmaceutical, medical, and cosmetologically industry with specific requirements [1–3]. Microbial fermentation using native HA producers, such as *Streptococcus zooepidemicus* and *S. dysgalactiae subsp. equisimilis*, has emerged as a sustainable alternative to traditional animal-based extraction methods for HA production [4, 5]. To mitigate the potential risks associated with using pathogenic strains in fermentation, metabolic engineering of GRAS (Generally Recognized As Safe) strains has been extensively explored for heterologous HA production. *Escherichia coli* [6, 7], *Corynebacterium glutamicum* [8, 9], *Bacillus subtilis* [10], and *Komagataella pastoris* [11] have been metabolically engineered by introducing heterologous metabolic pathway and HAS enzyme [12]. Subsequently, diverse carbon sources such as cheese whey [13], potato peel waste hydrolysate [14], sugar cane molasses [15], has been explored to economically enhance HA production. In addition, growing attention has been directed toward addressing climate change by converting CO_2 into valuable biochemicals using engineered microorganisms [16]. *Synechococcus* sp. PCC 7002 has been engineered to photosynthetically produce HA production from CO_2 [17].

Fine-tuning gene expression within heterologous pathways in engineered hosts significantly enhances the production of target compounds. Synthetic protein scaffolds have facilitated modular control of heterologous pathways [18]. Additionally, addressable phase-separated RNA has enabled spatial engineering in *E. coli* for precise metabolic control [19]. Recent advancements in programmable gene regulation tools, such as the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (CRISPR-Cas) system, have been applied to metabolic engineering. These tools enable the programmable repression and activation of gene expression at both the transcriptional level [20, 21] and the translational level [22, 23]. In cyanobacteria, a dCas12a-mediated CRISPR interference (CRISPRi) system has been developed to repress single or multiple genes on the chromosome, demonstrating its application in enhancing squalene production [24, 25].

Here, we report the metabolic engineering of *Synechococcus elongatus* PCC 7942 to enable photosynthetic production of HA from CO_2. A modular gene expression system was employed to overexpress heterologous genes, effectively enhancing precursor availability for HA biosynthesis. Furthermore, a CRISPRi system was utilized to redirect carbon flux from biomass synthesis towards HA production. Analysis of the molecular weight of the photosynthetically produced HA revealed a size comparable to that of HA derived from native producers. This study provides valuable engineering insights for expanding the applications of photosynthetically driven biopolymers.

Methods

Strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table 1 and Table S1. *E. coli* strain DH5α was used for gene cloning and was grown in Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) at 37°C on a rotary shaker at 200 rpm. When appropriate, the medium was supplemented with antibiotics at the following concentrations: 50 μg/mL kanamycin, 100 μg/mL spectinomycin, and 30 μg/mL chloramphenicol. The plasmids used in this study were derived from SyneBrick expression vectors, which are standard tools for chromosomal integration at neutral sites I (NSI), II (NSII), or III (NSIII). pSe1Bb1s-GFP and pSe2Bb1k-GFP, pSe3Bb1c-dCas12a using the BglBrick standard cloning method as a synthetic platform for gene expression in *S. elongatus* PCC 7942 [26]. For gene induction, 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the culture medium at an optical density at 730 nm (OD_{730}) of 1 after inoculation. Cell growth was monitored by OD_{730} with an Eppendorf BioSpectrometer® (Eppendorf AG, Hamburg, Germany).

To construct a HA-producing cyanobacterial strain, the *hasA* gene, originating from *S. zooepidemicus*, *hasB* from *S. pyogenes* [27], *hasC* from *S. pyogenes* [28], and the *glmU*, *glmM*, and *glmS* genes originated from *E. coli* were codon-optimized (co) using Gene Designer 2.0 (DNA2.0, Menlo Park, CA) for effective heterologous gene expression in *S. elongatus* PCC 7942. These optimized genes were synthesized (Genscript, Piscataway, NJ) and used for plasmid construction. The *hasA*(co), *hasB*(co), and *has*C(co) genes were cloned into pSe1Bb1s-GFP to generate plasmids like pSe1Bb1s-HasA or pSe1Bb1s-HasA-HasB-HasC for the endogenous production of UDP-glucuronic acid and HAS (Table S1, Table S2). Similarly, the *glmU*(co), *glmM*(co), and *glmS*(co) genes were cloned to construct pSe2Bb1k-glmU-glmM or pSe2Bb1k-glmU-glmM-glmS for the endogenous production module of UDP-*N*-acetylglucosamine (UDP-GlcNAc). The heterologous genes were expressed under an IPTG-inducible P_{Trc} promoter.

For CRISPR-dCas12a-mediated gene repression, a crRNA containing the target protospacer sequence was cloned into the pSe3Bb1c-dCas12a(co) vector, resulting in the construct pSe3Bb1c-dCas12a(co)-crRNA-X, where "X" represents the target gene. Briefly, target protospacer (PS) sequence for gene repression was selected 23-nt after the PAM (5'-TTV) and near the translational start site (5'-ATG) of target gene (Table S3). Then, the PS sequence and direct repeat (DR) (19-nt) were cloned

Table 1 Bacterial strains and plasmids used in this study

Strain	Relevant characteristics	References
E. coli DH5α	F−(80d ***lac***Z M15) (*lac*ZYA-argF) U169 *hsd*R17(r− m+) *rec*A1 *end*A1 *rel*A1 *deo*R96	[39]
S. elongatus PCC 7942	*Synechoccous elongatus* PCC 7942, Wild type	PCC
SeHA100	*S. elongatus* PCC 7942 NSI::Bb1s-HasA, the *hasA*(co) originated from *Streptomyces zooepidemicus*	This study
SeHA200	*S. elongatus* PCC 7942 NSI::Bb1s- HasA-HasB-HasC, the *hasA*(co), the *hasB*(co) originated from *S. pyogenes*, the *hasC*(co) originated from *S. pyogenes*	This study
SeHA210	SeHA200 NSII::Bb1k-glmU-glmM, the *glmU*(co), *glmM*(co) originated from *E. coli*	This study
SeHA220	SeHA200 NSII::Bb1k-glmU-glmM-glmS, the *glmS*(co) originated from *E. coli*	This study
Se3R-none	*S. elongatus* NSIII::Bb1c-dCas12a(co)	[24]
SeHA211	SeHA210 NSIII::Bb1c-dCas12a(co)	This study
SeHA212	SeHA210 NSIII::Bb1c-dCas12a(co)-nagB, native *nagB* gene repression by dCas12a-crRNA(nagB)	This study
SeHA213	SeHA210 NSIII::Bb1c-dCas12a(co)-glgC, native *glgC* gene repression by dCas12a-crRNA(glgC)	This study
SeHA214	SeHA210 NSIII::Bb1c-dCas12a(co)-zwf, native *zwf* gene repression by dCas12a-crRNA(zwf)	This study
SeHA215	SeHA210 NSIII::Bb1c-dCas12a(co)-pfk, native *pfk* gene repression by dCas12a-crRNA(pfk)	This study
SeHA221	SeHA220 NSIII::Bb1c-dCas12a(co)	This study
SeHA222	SeHA220 NSIII::Bb1c-dCas12a(co)-nagB, native *nagB* gene repression by dCas12a-crRNA(nagB)	This study
SeHA223	SeHA220 NSIII::Bb1c-dCas12a(co)-glgC, native *glgC* gene repression by dCas12a-crRNA(glgC)	This study
SeHA224	SeHA220 NSIII::Bb1c-dCas12a(co)-zwf, native *zwf* gene repression by dCas12a-crRNA(zwf)	This study
SeHA225	SeHA220 NSIII::Bb1c-dCas12a(co)-pfk, native *pfk* gene repression by dCas12a-crRNA(pfk)	This study
SeHA226	SeHA220 NSIII::Bb1c-dCas12a(co)-zwf-pfk, native *zwf* and *pfk* gene repressions by dCas12a-crRNA(zwf)-crRNA(pfk)	This study

Note: (co) represents that the gene sequence is codon-optimized to the PCC 7942. NSI, NSII, and NSIII are neutral site I, II, and III, respectively. The plasmids used for constructing strains are listed in Supplementary Table S1 and S2. The protospacer sequences for the crRNA were listed in Supplementary Table S3. The [dCas12a-crRNA] describes Bbc-lacI^q-P_{trc}-dCas12a-P_{J23119}-crgRNA-[protospacer]

into pSe3Bb1c-dCas12a to form pSe3Bb1c-dCas12a-crRNA[DR(S)-PS], which was used for transformation of *S. elongatus* strains. The dCas12a and crRNA were expressed under an IPTG-inducible P_{Trc} promoter and a P_{J23119} constitutive promoter, respectively.

Transformation of *S. elongatus* PCC 7942

Transformation of *S. elongatus* PCC 7942 was performed as described previously [24, 29, 30] The SyneBrick vectors constructed in this study were transferred for chromosomal integration. To obtain fully segregated mutants, recombinant colonies were transferred to fresh selective plates, accounting for the oligoploid nature of cyanobacteria. Successful chromosomal integration into NSI, NSII, or NSIII was confirmed using PCR (Fig. 1). For sequence verification, the primers Se1-fw (5'- GCA TGG ATC TGA CCA ACA TG-3') and Se1-rv (5'- CAA GGC AGC TTG GAA GGG CG-3') for NSI, Se2-fw (5'- CAT GGA TGG GTG TGC AAT GA-3') and Se2-rv (5'- TTG GAT GCT CTT GAA TTG CC-3') for NSII were used. Se3-fw (5'- GTA GCG AAG CAG TGC ACA CC-3') and Se3-rv (5'- TAT AAA CGC AGA AAG GCC CA-3') for NSIII were used. The recombinant strains used in this study were listed in Table 1.

Growth conditions for hyaluronic acid (HA) production from engineered cyanobacteria

Recombinant *S. elongatus* PCC 7942 used for HA production were cultivated at 30 °C under continuous fluorescent light (100 μmol photons/m^2/s) measured using a LightScout Quantum meter (3415FXSE; Spectrum, Aurora, IL). The cultivation was conducted in 100 mL BG-11 medium (UTEX) supplemented with 10 mM MOPS (pH 7.5) inside a Duran bottle equipped with a three-port cap at pH 7.5, with 5% (v/v) CO_2 gas (monitored by online gas analyzer) and 95% (v/v) filtered air supplied at a constant flow rate of 10 cc/min into the medium [30, 31]. Selection pressure was maintained using final concentration of 10 μg/L spectinomycin, 10 μg/L kanamycin and 3 μg/L chloramphenicol. For HA production, 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added into the culture medium 24 h after inoculation for induction.

Quantification of photosynthetic hyaluronic acid (HA) and HA molecular determination

HA was quantified using the HA analysis kit (Sandwich HA ELISA; Catalog #: K-4800, Echelon Bioscience, Utah, U.S.A.). The Sandwich HA ELISA enables to detect HA over 130 kDa. After cultivating the cyanobacteria, 1 mL of the culture samples were harvested and were centrifuged at 10,000 × *g* for 3 min for separation of cell pellet and supernatant. For HA in the medium, the supernatant samples were collected. For HA in the cell, the cell pellets were resuspended with 1 mL distilled water and disrupted with 0.2 g of glass beads (150–212 μm) using a bead beater. After centrifugation at 15,000 × *g* for 3 min, supernatant samples were collected for HA analysis. The HA present in the sample was analyzed according to

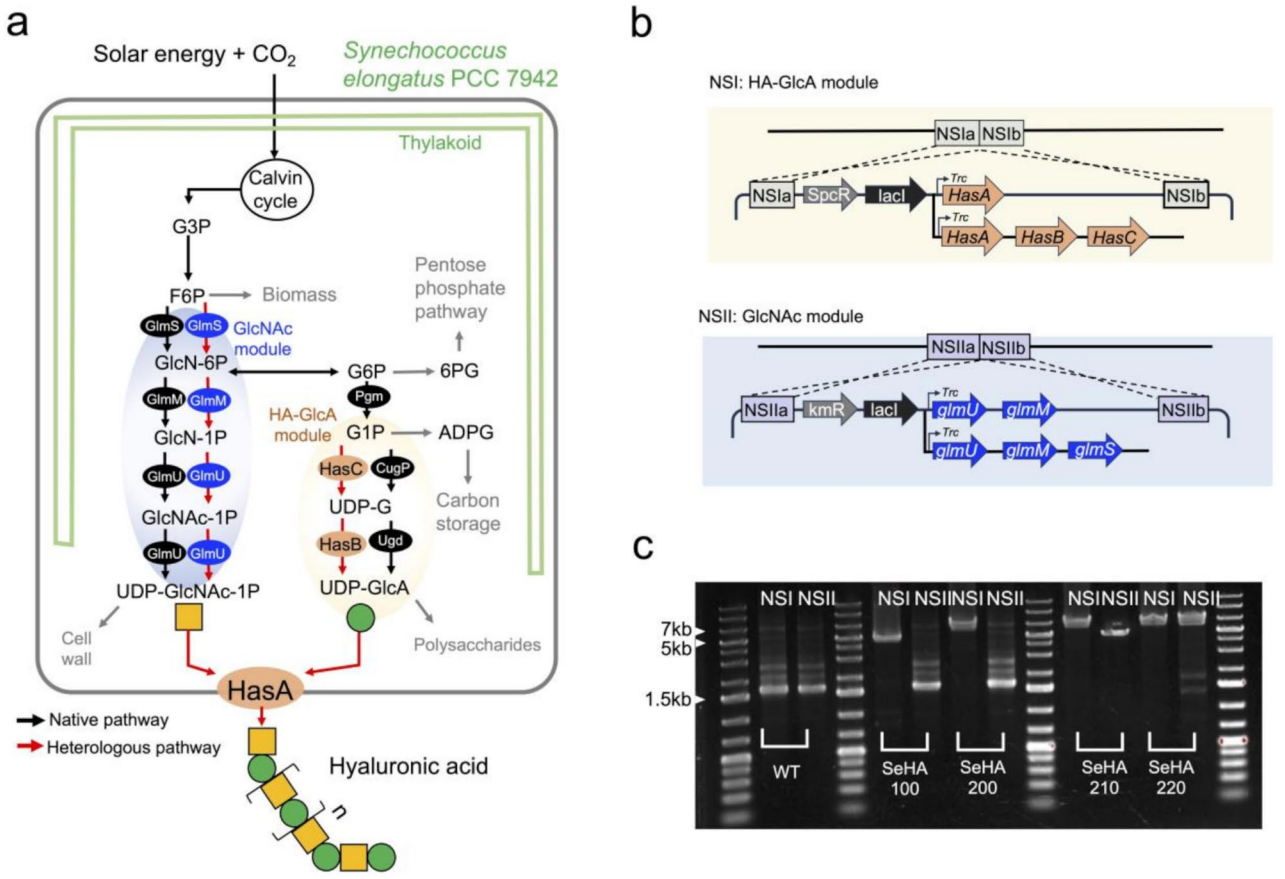

Fig. 1 Development of photosynthetic hyaluronic acid (HA) production using engineered ***S. elongatus*strain.** (**a**) A schematic pathway for HA production in recombinant *S. elongatus* PCC 7942 strain by introducing a heterologous HA-producing pathway. Two metabolic pathway modules (HA-GlcA module, GlcNAc module) were used for heterologous HA-producing pathway. (**b**) Schematic diagrams of the module construction of *S. elongatus* PCC 7942 strains for production of HA. The heterologous *hasA*, *hasB*, and *hasC* genes were introduced into neutral site I (NSI) for HA-GlcA module. For GlcNAc module, the heterologous *glmU*, *glmM*, and *glmS* genes were introduced to neutral site II (NSII) of *S. elongatus* genomic DNA. (**c**) A gel image showed colony-PCR results verifying recombinant *S. elongatus* strains using a pair of Se1-fw/rv and Se2-fw/rv for the NSI and NSII integrations, respectively. The DNA sequences were also verified. The target size of each PCR product for wild-type or mutant cyanobacteria: wild-type (1.6 kb), SeHA100 (5.0 kb), SeHA200 (7.5 kb), SeHA210 (7.5 kb), and SeHA220 (7.5 kb) at NSI and wild-type (1.6 kb), SeHA100 (1.6 kb), SeHA200 (1.6 kb), SeHA210 (5.0 kb) and SeHA220 (7.8 kb) at NSII. The genotypes of the recombinant strains are described in Table 1. Abbreviation: G3P, glyceraldehyde 3-phosphate; F6P, fructose 6-phosphate; GlcN-6P, glucosamine 6-phosphate; GlcN-1P, glucosamine 1-phosphate; GlcNAc-1P, *N*-acetylglucosamine 1-phosphate; UDP-GlcNAc-1P, UDP-*N*-acetylglucosamine 1-phosphate; G6P, glucose 6-phosphate; G1P, glucose 1-phosphate; 6PG, 6-phosphogluconolactone; UDP-G, UDP-glucose; UDP-GlcA, UDP-glucuronic acid

the manufacturer's instructions (Sandwich HA ELISA). Briefly, 25 µL sample was used to perform a quantitative immunoassay in the HA detection plate. The provided HA standard was used along with the samples and measured in technically-duplicate at 450 nm using Infinite® M Nano + microplate reader (Tecan AG, Männedorf Switzerland).

For determination of HA molecular weight, the cyanobacterial strain was cultured in 500 mL of BG-11 medium. The supernatant containing HA was collected after centrifugation at 3600 × *g* for 10 min. Then, the sample was lyophilized using a freeze-dryer (IlShinBioBase, Seoul, Korea). The lyophilized sample was resuspended in 5 mL of phosphate buffered saline (pH 7.0), and 20 mL isopropyl alcohol was added to the sample at a final concentration of 80%. The mixture sample was incubated overnight at 4 °C. After centrifugation at 3600 × *g*, the resulting pellet was dried under nitrogen gas. For gel filtration chromatography analysis, the pellet was resuspended with 51 mL 0.1 M $NaNO_3$. Gel filtration chromatography in combination with a refractive index detector (GPC-RI, EcoSEC HLC-8420, Tosoh), equipped with TSKgel® GMPWXL (13 µm) and TSKgel® G2500PWXL (7 µm) HPLC Columns, was used. A 50 µL sample was injected into 0.1 M $NaNO_3$ mobile phase at 40 °C with a flow rate of 1.0 mL/min. Standard markers were used for calculation of HA molecular weight. Polyethylene glycol and polyethylene oxide standards: 1.25 MDa, 690 kDa, 120 kDa, 73 kDa, 21 kDa, 10 kDa, 3.8 kDa, 1.5 kDa, 610 Da, 106 Da.

Results and discussions

Designing modularized pathways for photosynthetic production of hyaluronic acid

S. elongatus PCC 7942 does not produce HA by its natural metabolism due to lack of capability of having activity of a HAS that catalyzes the polymerization of UDP-GlcNAc and UDP-d-glucuronic acid (UDP-GlcA) via a glycosidic linkage formation [32]. Thus, pathway engineering in *S. elongatus* PCC 7942 must be needed for heterologous HA production (Fig. 1). Microbial HA production has been successfully demonstrated by either native producer, *S. zooepidemicus* [15] or engineered producers (*E. coli* [6], *C. glutamicum* [8, 9], *B. subtilis* [10], and *Synechococcus* sp. PCC 7002 [17]) from various carbon sources (sugarcane molasses, glucose, lactic acid, sucrose, CO_2). both producers employed metabolic pathways to synthesize the precursors UDP-glucuronic acid and UDP-*N*-acetylglucosamine. Thus, we modularized the biosynthesis pathways into two modules (HA-GlcA and GlcNAc) for optimization (Fig. 1).

First, to increase the intracellular pools of UDP-GlcA substrate, we overexpressed the heterologous genes encoding key enzyme HasA (HAS), HasB (UDP-glucose dehydrogenase), and HasC (UTP-glucose-1-phosphate uridylyltransferase) enzyme by using chromosomal integration vectors (pSe1B1s [26]) targeting neutral site I (NSI) of *S. elongatus* PCC 7942. Two version of the HA-GlcA module was designed (HasA; HasA-HasB-HasC) to investigate whether the native UDP-GlcA pathway can contribute the HA production with sole HAS expression, constructing the strains SeHA100 and SeHA200 (Table 1).

For the GlcNAc modules, over-expressions of *glmU* (encoding for *N*-acetylglucosamine uridyltransferase), *glmM* (encoding for phosphoglucosamine mutase), *glmS* (encoding for glutamine-fructose-6-phosphate aminotransferase) were tested to confirm if they are critical engineering targets as shown previously [6, 8, 10, 17] to SeHA200, resulting in the strains SeHA210 and SeHA220. The difference between SeHA210 and SeHA220 lies in the absence or presence of overexpression of GlmS. As a result, four recombinant *S. elongatus* strains were generated for the HA-GlcA and GlcNAc modules (Table 1).

Metabolic pathway engineering of photosynthetic HA production

As the first step toward HA production, strain SeHA100, which expresses HasA, was constructed and cultivated in 100 mL BG-11 medium with 5% (v/v) CO_2 bubbling under constant light. Heterologous module expression was induced with 1 mM IPTG when OD_{730} reached at 1. This strain achieved OD_{730} of 8.1 ± 0.2 at 20 d. However, no HA production was detected using the HA ELISA assay (limit of detection: 10 μg/L) (Fig. 2). To investigate whether the UDP-glucuronic acid pool limits HA production, the HA-GlcA module was introduced. The resulting strain, SeHA200, reached a final OD_{730} of 8.9 ± 0.2 at 20 d and showed 0.08 ± 0.02 mg/L total HA production at 20 d, with 0.01 ± 0.005 mg/L detected in the cell pellets and 0.07 ± 0.02 mg/L in the supernatant. Although Class I HAS, a single domain integral membrane protein, catalyze the substrates in cytosol to secret into medium, some portion of HA could be attached to inner membrane or peptidoglycan layer [9, 17], resulting in ELISA detection (> 130 kDa HA) in cell pellets. In previous studies, metabolic engineering of *E. coli* [6], *C. glutamicum* [9], and *B. subtills* [10] also utilized the strategies of overexpression of HasA, HasB, and HasC in various combinations and demonstrated that the HA-GlcA module improved HA production. Interestingly, engineered *E. coli* with co-expression of HasA and GlmU did not show improved HA production compared with *E. coli* strain with HA-GlcA module only [6]. This study proposes that prioritizing the exploration of the GlcNAc module over the HA-GlcA module may be more effective in enhancing HA production.

To enhance the substrate pools of UDP-*N*-acetylglucosamine 1-phosphate (UDP-GlcNAc-1P) and UDP-glucuronic acid (UDP-GlcA), a GlcNAc module was integrated into SeHA200, resulting in strain SeHA210. After cultivation, SeHA210 achieved a final OD_{730} of 6.3 ± 0.01 at 21 d, slightly lower than the OD_{730} of the parental strain SeHA200. SeHA210 produced a total of 1.5 ± 0.5 mg/L HA at 21 d, comprising 0.8 ± 0.3 mg/L in cell pellets and 0.7 ± 0.2 mg/L in the supernatant, representing a 17.2-fold increase in total HA production compared to SeHA200. Subsequently, the *glmS* gene was co-overexpressed in the GlcNAc module to enhance the flux from glucose 6-phosphate (G6P) to glucosamine 6-phosphate (GlcN-6P) via glutamine-fructose-6-phosphate aminotransferase, resulting in the strain SeHA220 derived from SeHA210. SeHA220 achieved a final OD_{730} of 4.67 ± 0.4 at 21 d and produced 2.2 ± 0.85 mg/L total HA at 21 d, with 0.2 ± 0.02 mg/L in cell pellets and 2.0 ± 0.83 mg/L in the supernatant. This represents a 27.5-fold increase in total HA production compared to SeHA200 and a 1.6-fold increase compared to SeHA210. Interestingly, the proportion of secreted HA in SeHA220 increased compared to SeHA200 and SeHA210. This observation may be attributed to balanced gene expression, leading to higher pools of UDP-GlcNA [17]. The previous study reported that 50% of the total HA production in cyanobacteria was attached to the cell surface. Understanding the secretion mechanism through the cell wall after HA biosynthesis in the cytoplasmic membrane remains crucial. Additionally, the overexpression of GlmS in the GlcNAc module serves as a key enzymatic

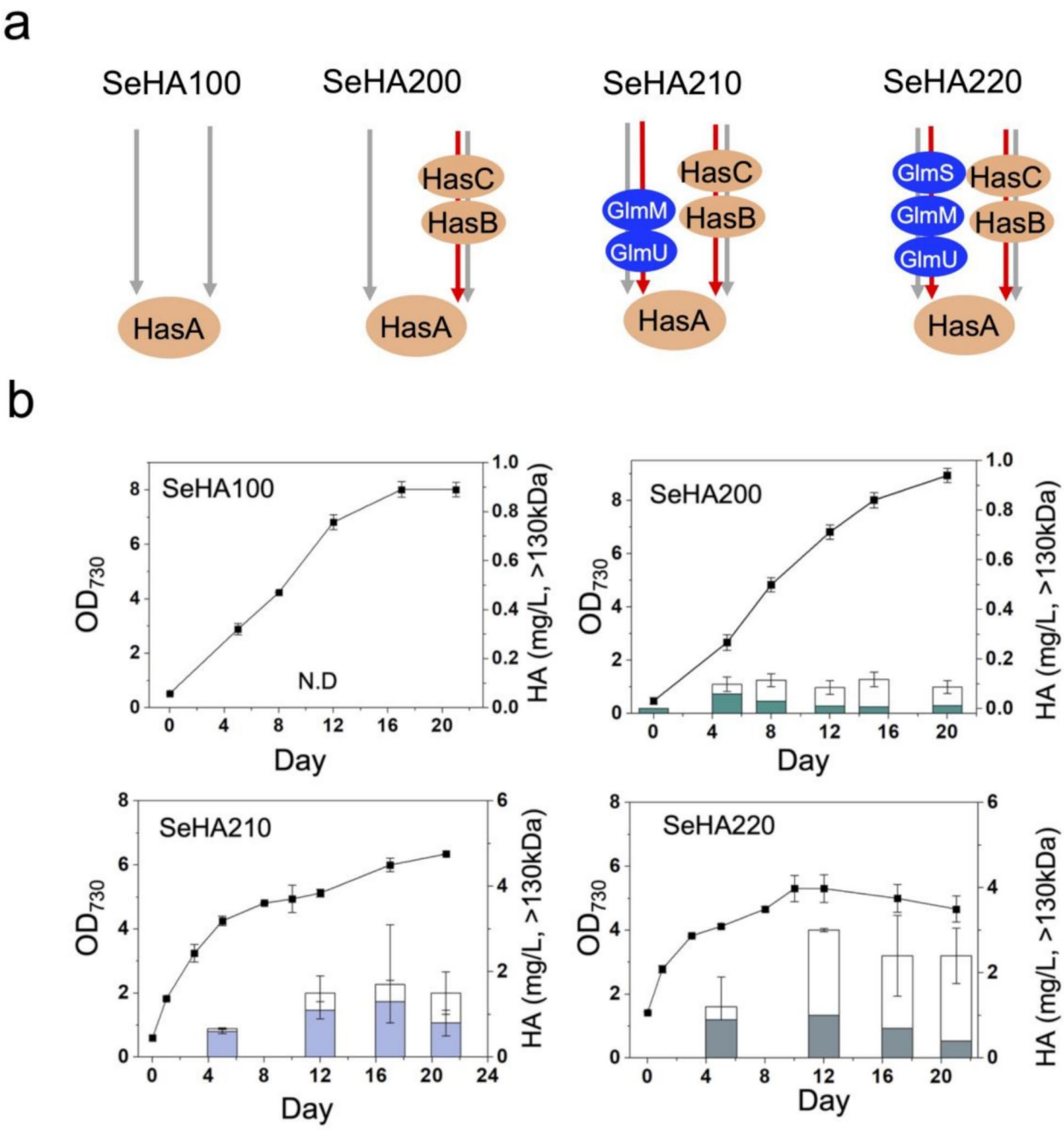

Fig. 2 Photosynthetic HA production from engineered ***S. elongatus*strains.** (**a**) Strategies of introducing various modules into cyanobacteria and their corresponding strains. (**b**) Time-course analysis of cell growth and HA production for various strains. Cell growth was measured at OD_{730}. HA production was analyzed using enzymatic assay. Open bar; supernatant, filled bar; cell extract. Data represent mean values of at least triplicated cultivations, and error bars represent standard deviations

step, redirecting carbon flux from fructose 6-phosphate toward the UDP-GlcNAc pathway rather than glycolysis. Thus, optimizing the substrate pool of UDP-GlcNAc-1P and UDP-GlcA is crucial for enhancing HA production.

Similarly, engineered *C. glutamicum* and *B. subtilis* also utilized two-modular metabolic engineering by expressing both modules, which included HasA and HasB, as well as GlmS or GlmUMS. Consistent with the findings of this study, where GlmS was identified as a critical factor for HA production within the GlcNAc module, the overexpression of GlmS significantly improved HA production in both *C. glutamicum* and *B. subtilis* [9, 10]. In addition, overexpression of GlmU-GlmS contributed improved photosynthetic HA production along with

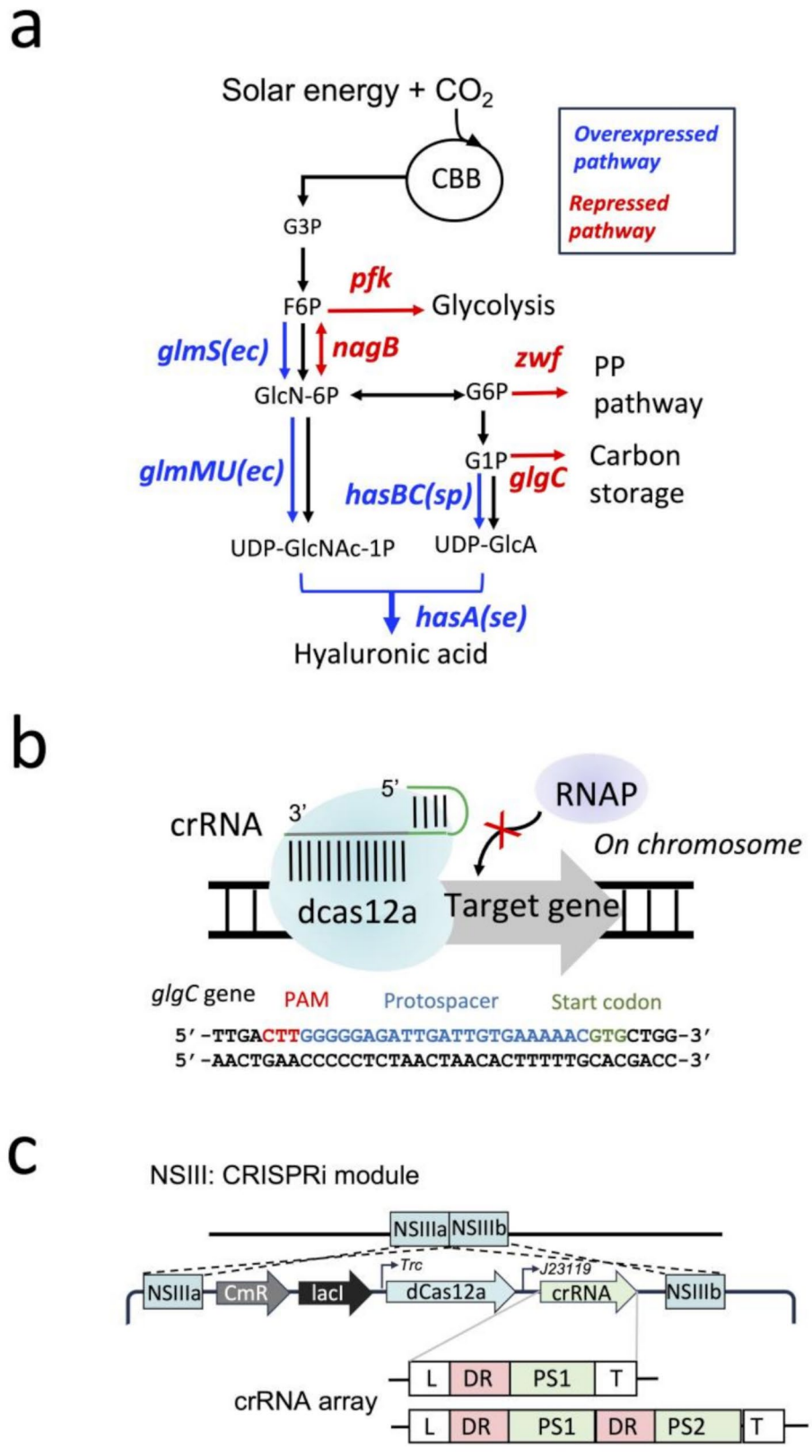

Fig. 3 (See legend on next page.)

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Fig. 3 Design of CRISPRi-assisted metabolic engineering of ***S. elongatus*strains for photosynthetic HA production** (**a**) A scheme of the metabolic pathway for HA production in engineered *S. elongatus* strain. Heterologous gene was introduced for overexpression of metabolic enzymes (GlmS, GlmM, GlmU, HasA, HasB, and HasC; shown in blue). The CRISPRi (or dCas12a-mediated system) was used to repress the target gene. The target gene (*nagB*, *zwf*, *glgC*, and *pfk*) was selected for gene repression (shown in red). (**b**) A programmable dCas12a-mediated gene repression for *S. elongatus* PCC 7942. A protospacer was selected to interfere the activity of RNA polymerase (RNAP). The protospacer sequences for the targets were shown in Table S3. (**c**) A scheme of the CRISPRi module with target crRNAs. A CRISPRi module, comprising inducible dCas12a and crRNA expression, was integrated into neutral site III (NSIII). DR; direct repeat, PS; protospacer. (Table 1)

HasA expression in *Synechococcus* sp. PCC 7002 [17]. Overall, we demonstrated that the introduction of the heterologous HA-GlcA and GlcNAc modules into the cyanobacterium *S. elongatus* PCC 7942 enabled photosynthetic HA production directly from CO_2.

Improvement in photosynthetic HA production using the CRISPR-dCas12a

To enhance HA production from CO_2 in cyanobacteria, we aimed to increase the pools of UDP-GlcNAc-1P and UDP-GlcA by boosting the levels of fructose 6-phosphate (F6P) and G6P/glucose 1-phosphate (G1P) (Fig. 3a). Previously, we developed a CRISPR interference system for cyanobacteria to repress gene expression on chromosomes [24, 25]. This programmable system utilizes a dCas12a and crRNA complex, which interferes with RNA polymerase activity to inhibit transcription initiation (Fig. 3b). In addition, dCas12a-mediated CRISPRi enables the regulation of essential gene expression in cyanobacteria, which cannot be deleted but are critical for metabolic reconstitution.

In cyanobacteria, the GlcNAc module utilizes the substrates of F6P and GlcN-6P, which are also essential for cell growth. The *pfkA* gene encodes 6-phosphfructokinase that catalyze the phosphorylation of F6P to fructose 1,6-bisphosphate (FBP), while *nagB* gene encodes glucosamine 6-phosphate deaminase that catalyze the reversible isomerization-deamination of glucosamine 6-phosphate (GlcN-6P) to form fructose 6-phosphate (F6P). To conserve F6P and GlcN-6P for the GlcNAc module, *pfkA* and *nagB* were individually repressed (Fig. 3a). In parallel, HA-GlcA module utilizes the substrates of G6P and G1P, which are also required for cell growth and carbon storage. The *zwf* gene encodes G6P dehydrogenase that catalyze the oxidation of G6P to 6-phosphogluconolactone and *glgC* encodes G1P adenyltransferase that catalyze the reaction of G1P to convert to ADP-glucose for carbon storage. Thus, we selected four different gene repression targets: *pfkA* and *nagB* for GlcNAc module, *zwf* and *glgC* for HA-GlcA module.

To initiate gene repression, we selected the HA production host SeHA210. Subsequently, a dCas12a expression system cassette containing four distinct crRNAs was introduced into the strain (Fig. 3c), resulting in strain SeHA211 with no crRNA, SeHA212 with crRNA-nagB, SeHA213 with crRNA-glgC, SeHA214 with crRNA-zwf, and SeHA215 with crRNA-pfk. To induce gene, 1 mM IPTG was added when OD_{730} reached at 1 for both inducible dCas12a expression system and the heterologous module expression. As a result, SeHA210 exhibited an OD_{730} of 5.2 ± 0.1 at 15 d and SeHA211 exhibited an OD_{730} of 5.7 ± 0.1 at 15 d. We did see no growth inhibitions between SeHA210 (as a control strain) and SeHA211 (none-target strain) (Fig. 4a). For HA production, SeHA212 and SeHA213 did not show any improvement compared to SeHA210 (Fig. 4b). However, SeHA214 produced 3.7 ± 0.4 mg/L total HA, with 2.0 ± 0.6 mg/L in cell pellets and 1.7 ± 0.6 mg/L in the supernatant, while SeHA215 produced 3.5 ± 0.3 mg/L total HA, with 1.8 ± 0.3 mg/L in cell pellets and 1.7 ± 0.3 mg/L in the supernatant. SeHA214 and SeHA215 demonstrated statistically significant increases in HA production, with 1.57-fold and 1.50-fold improvements, respectively, compared to SeHA210 (*Student t*-test, p-value < 0.05). The growth of SeHA214 (OD_{730} of 3.9 ± 0.3 at 15 d) and SeHA215 (OD_{730} of 4.9 ± 0.1 at 15 d) exhibited slightly lower than SeHA210 (OD_{730} of 5.2 ± 0.1 at 15 d). This is due to the fact that F6P and G6P serves key metabolite nodes in both GlcNAc and HA-GlcA module, competing with metabolic pathway essential for cell growth. Thus, we concluded that the repression of either *zwf* or *pfk* were effective for improving HA production.

In parallel, we utilized the second HA production host SeHA220, in which GlmS was also overexpressed to strongly pull the carbon flux from F6P to GlcN-6P within the GlcNAc module. Similar to the results observed with SeHA210, SeHA221 did not exhibit significantly changes in either cell growth (OD_{730} of 4.6 ± 0.1 at 15 d) or HA production (3.6 ± 0.07 mg/L total), compared to SeHA220 (OD_{730} of 4.6 ± 0.4 at 15 d; 3.4 ± 0.05 mg/L total HA). The dCas12a-mediated CRISPRi without a target did not affect cell growth and HA production. The *nagB* and *glgC* repression in SeHA222 and SeHA223, respectively, did not improve HA production over SeHA220. However, SeHA224 produced 4.26 ± 0.3 mg/L total HA at 15 d, with 3.3 ± 0.2 mg/L in cell pellets and 0.9 ± 0.2 mg/L in the supernatant, while SeHA225 produced 4.3 ± 0.1 mg/L total HA at 15 d, with 3.8 ± 0.1 mg/L in cell pellets and 0.5 ± 0.1 mg/L in the supernatant. SeHA224 and SeHA225 demonstrated statistically significant increases in HA production, with 1.25-fold and 1.26-fold improvements, respectively, compared to SeHA220 (*Student t*-test, p-value < 0.05). On the other hand, the growth

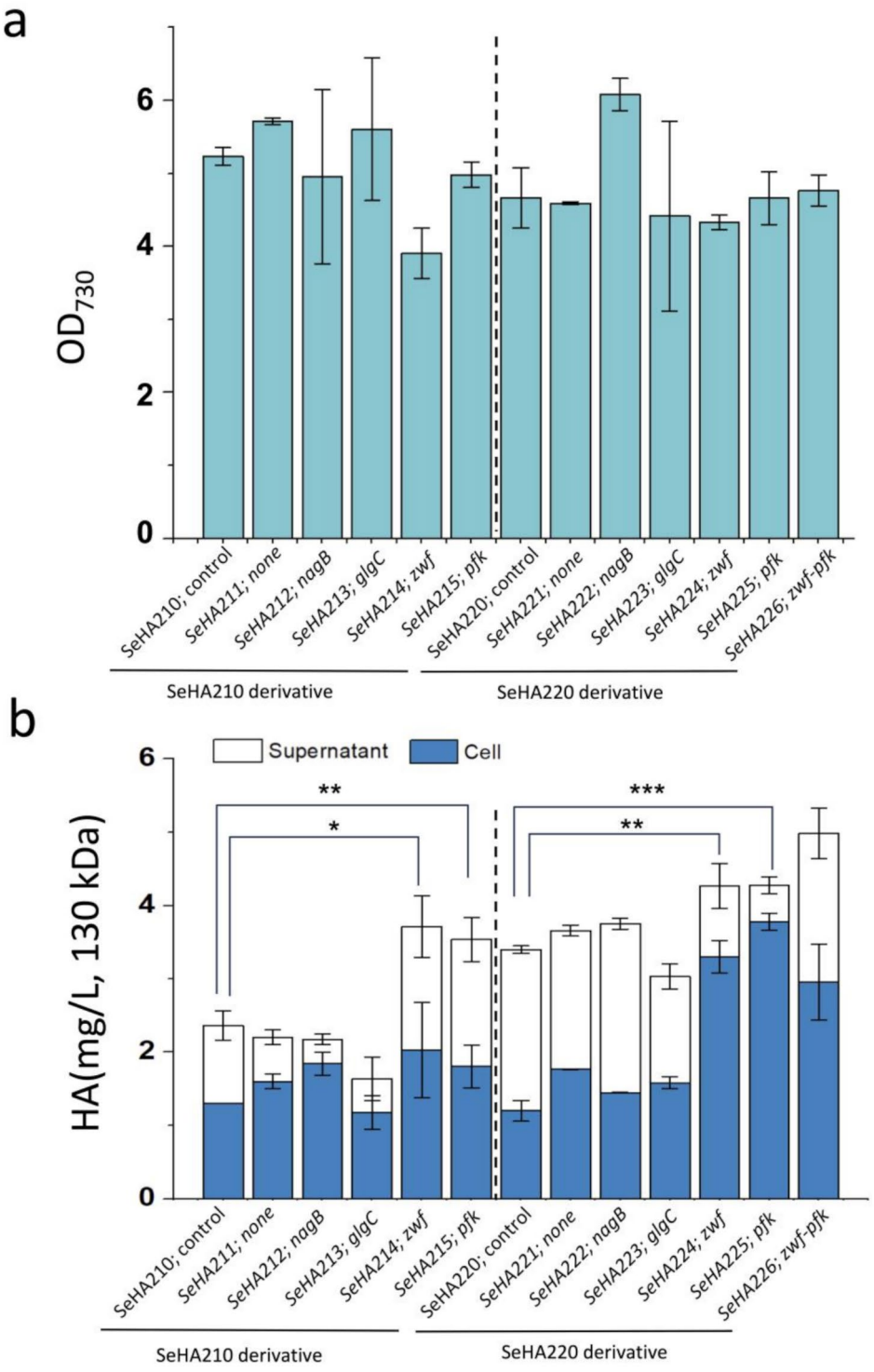

Fig. 4 CRISPRi-assisted metabolic engineering of ***S. elongatus*strains for photosynthetic HA production** (**a**) Cell growth of various strains used for CRISPRi-assisted metabolic engineering (Table 1). Cell growth was measured at OD_{730} after 15 d cultivation. Data represent mean values of at least triplicated cultivations, and error bars represent standard deviations. (**b**) Photosynthetic HA production from engineered strains. Open bar; supernatant, filled bar; cell. Data represent mean values of at least triplicated cultivations, and error bars represent standard deviations. A *Student's t*-test was performed for statistical analysis (**p*-value < 0.05; ***p*-value < 0.005, ****p*-value < 0.0005). Abbreviation: G3P, glyceraldehyde 3-phosphate; F6P, fructose 6-phosphate; GlcN-6P, glucosamine 6-phosphate; UDP-GlcNAc-1P, UDP-*N*-acetylglucosamine 1-phosphate; G6P, glucose 6-phosphate; G1P, glucose 1-phosphate; 6PG; UDP-GlcA, UDP-glucuronic acid

of SeHA224 (OD_{730} of 4.33 ± 0.1 at 15 d) and SeHA225 (OD_{730} of 4.6 ± 0.3 at 15 d) exhibited slightly lower than SeHA220 (OD_{730} of 4.6 ± 0.4 at 15 d). Notably, the *zwf* and *pfk* gene repressions also improve HA production over SeHA220. The partitioning of carbon to biomass may be altered to achieve less biomass and more intermediates for HA production.

Furthermore, we double-repressed *zwf* and *pfk* genes as SeHA226, in which HA was produced from CO_2 at 5.0 ± 0.3 mg/L total HA at 15 d, with 3.0 ± 0.5 mg/L in cell pellets and 2.0 ± 0.5 mg/L in the supernatant. SeHA226 slightly improved the HA production compared to either SeHA224 or SeHA225. Consistently, downregulation of the *zwf* and *pfk* genes and upregulation of the *glmUM* genes in *B. subtilis* showed the improved HA production [10]. In this study, we demonstrated that F6P and G6P are important metabolite nodes for improving HA production. In addition, the CRISPRi systems are effective for regulating gene expressions of *zwf* and *pfk* in cyanobacteria. Yet, scaling up photosynthetic HA production in cyanobacteria presents several challenges, including ensuring a cost-effective CO_2 supply, host engineering for toxic materials in flue gas, developing photobioreactors with high photon efficiency, efficiently harvesting biomass, and isolating the product during downstream processing [33, 34].

Molecular weight analysis of photosynthetic HA in engineered cyanobacteria

To investigate the molecular weight of photosynthetic HA in engineered *S. elongatus* PCC 7942, strain SeHA226 (recorded in the highest HA production in this study) was cultivated with 5% (v/v) CO_2 under constant light condition for 15 d and the secreted HA in the medium was harvested and analyzed to determine the molecular weight using GPC. As a result, the weight-average molecular weight (Mw) and number-average molecular weight (Mn) of the photosynthetic HA secreted by strain SeHA226 were calculated to be 4.2 MDa and 0.8 MDa, respectively (Fig. 5). No HA larger than 25.7 MDa or smaller than 62 kDa was detected in this strain.

Interestingly, engineered strains including *E. coli* and *C. glutamicum* have produced rather smaller molecular weight (less than 2.5 MDa) of HA than native producer (*S. zooepidmicus*; a range of 1 MDa to 4 MDa) [35]. However, engineered cyanobacteria in this study showed

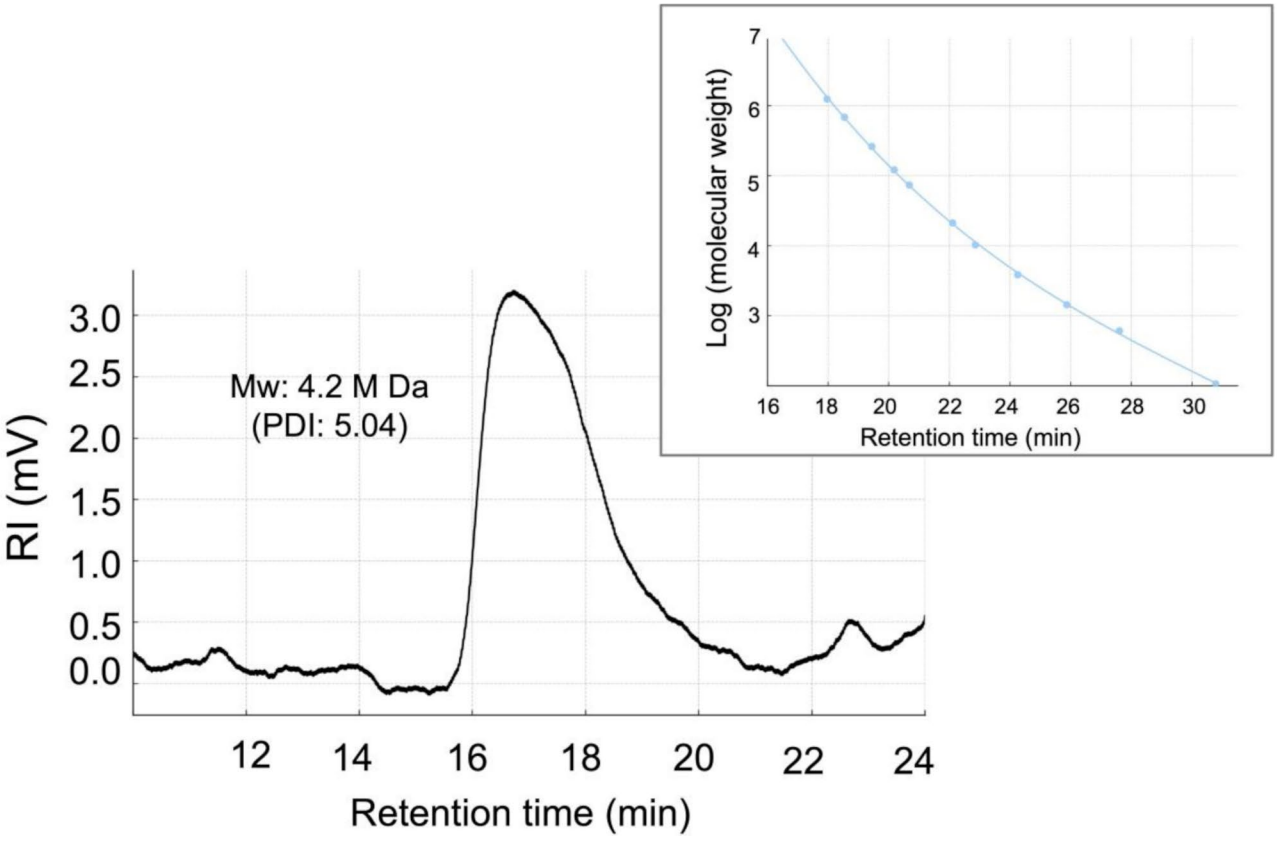

Fig. 5 The molecular weight of photosynthetic HA produced by engineered cyanobacteria. Weight average molecular weight (Mw) and number average molecular weight (Mn) of photosynthetic HA was determined by gel filtration chromatography-a refractive index detector (GPC-RI) at 1 mL/min. Polydispersity (PDI) was calculated as a ratio of Mw/Mn. Inset plot: A calibration curve for determination of molecular weight. Polyethylene glycol and polyethylene oxide were used as molecular standards

Table 2 Heterologous HA productions in various engineered strains

Strain	Source of the hasA gene	Carbon source	Titer*	Mw	Reference
E. coli	Class I (*Streptococcus pyogenes*)	16 g/L glucose	190 mg/L, f	0.35 MDa	[6]
B. subtilis	Class I (*Str. equisimilis*)	20 g/L sucrose	2.26 g/L, b	0.35 ~ 1.9 MDa	[10]
C. glutamicum	Class I (*Str. equisimilis*)	625 g/L glucose	28.7 g/L, b	0.21 ~ 0.9 MDa	[8]
C. glutamicum	Class I (*Str. pyogens*)	400 g/L glucose	74.1 g/L, b	0.053 MDa	[9]
E. coli	Class II (*Pasteurella multocida*)	50 g/L glucose	3.8 g/L, b	1.5 MDa	[7]
Komagataella pastoris	Class I (*Xenopus laevis)*	20 g/L glucose	0.17 g/L, b	2.5 MDa	[11]
Synechococcus sp. PCC 7002	Class I (*Str. equisimilis*)	1% (v/v) CO_2 and air	112 mg/L, a	2 ~ 2.2 MDa	[17]
Syn. elgonatus. PCC 7942	Class I (*Str. zooepidemicus*)	5% (v/v) CO_2 and air	5 mg/L, a	4.2 MDa	This study

*The value for titer listed in Table 2 was obtained from the literature. a, air-lift bioreactor; b, stirred-bioreactor; f, flask

comparable molecular weight to the native producer (Table 2). One of the key factors determining the molecular weight of HA is the balance of precursors (UDP-GlcNAc-1P and UDP-GlcA), which are utilized by HAS for HA chain elongation [12, 32]. An equal mole ratio of the precursors UDP-GlcNAc-1P and UDP-GlcA had led high molecular weight HA production in engineered *Lactococcus lactis* [36]. Additionally, modifications to the glycolytic and HA synthetic pathways under nitrogen-limiting conditions resulted in slower growth rates, which were inversely correlated with the production of higher molecular weight HA [37]. Recent studies have shown that Class I HAS polymerizes HA-UDP at the reducing end of the UDP-sugar substrate [38]. Further investigation into the mechanisms controlling HA polymerization will be necessary to enable the design of HA with specific molecular weights in engineered bacteria.

Conclusions

In this study, metabolic engineering of cyanobacteria was conducted to enable the efficient biosynthesis of HA using a heterologous modular gene expression system. Additionally, a CRISPR-interference gene repression system was implemented to optimize metabolic pathways, further enhancing HA production in conjunction with the modular gene expression. As a result, strain SeHA226, which redirected the pools of UDP-GlcNAc-1P and UDP-GlcA within photosynthetic carbon partitioning, produced 5.0 ± 0.3 mg/L of HA directly from CO_2 at 15 d, with a weight-average molecular weight of 4.2 MDa. Optimizing heterologous metabolic pathway was key to achieving high-molecular-weight photosynthetic HA production from CO_2. Future efforts will focus on scaling up the photo-bioprocess to demonstrate the feasibility of photosynthetic HA production at larger scales.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13036-025-00494-z.

Supplementary Material 1

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Author contributions
J.S., H.M.W.: Conception and design of the work; J.S.: data acquisition and analysis, J.S., H.J.L., H.M.W.: interpretation of data, have drafted the work or revised it.

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Data availability
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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References

1. Fallacara A, Baldini E, Manfredini S, Vertuani S. Hyaluronic Acid in the Third Millennium. Polymers. 2018;10:701.
2. Stecco C, Stern R, Porzionato A, Macchi V, Masiero S, Stecco A, De Caro R. Hyaluronan within fascia in the etiology of myofascial pain. Surg Radiol Anat. 2011;33:891–6.
3. Averbeck M, Gebhardt CA, Voigt S, Beilharz S, Anderegg U, Termeer CC, Sleeman JP, Simon JC. Differential regulation of hyaluronan metabolism in the epidermal and dermal compartments of human skin by UVB irradiation. J Invest Dermatol. 2007;127:687–97.
4. Zhang Y, Luo K, Zhao Q, Qi Z, Nielsen LK, Liu H. Genetic and biochemical characterization of genes involved in hyaluronic acid synthesis in *Streptococcus zooepidemicus*. Appl Microbiol Biotechnol. 2016;100:3611–20.
5. Pourzardosht N, Rasaee MJ. Improved yield of high molecular weight hyaluronic acid production in a stable strain of *Streptococcus zooepidemicus* via the elimination of the Hyaluronidase-Encoding gene. Mol Biotechnol. 2017;59:192–9.
6. Yu H, Stephanopoulos G. Metabolic engineering of *Escherichia coli* for biosynthesis of hyaluronic acid. Metab Eng. 2008;10:24–32.
7. Mao Z, Shin H-D, Chen R. A Recombinant *E. coli* bioprocess for hyaluronan synthesis. Appl Microbiol Biotechnol. 2009;84:63–9.

8. Cheng F, Yu H, Stephanopoulos G. Engineering *Corynebacterium glutamicum* for high-titer biosynthesis of hyaluronic acid. Metab Eng. 2019;55:276–89.
9. Wang Y, Hu L, Huang H, Wang H, Zhang T, Chen J, Du G, Kang Z. Eliminating the capsule-like layer to promote glucose uptake for hyaluronan production by engineered *Corynebacterium glutamicum*. Nat Commun. 2020;11:3120.
10. Westbrook AW, Ren X, Oh J, Moo-Young M, Chou CP. Metabolic engineering to enhance heterologous production of hyaluronic acid in *Bacillus subtilis*. Metab Eng. 2018;47:401–13.
11. Jeong E, Shim WY, Kim JH. Metabolic engineering of *Pichia pastoris* for production of hyaluronic acid with high molecular weight. J Biotechnol. 2014;185:28–36.
12. de Oliveira JD, Carvalho LS, Gomes AMV, Queiroz LR, Magalhães BS, Parachin NS. Genetic basis for hyper production of hyaluronic acid in natural and engineered microorganisms. Microb Cell Fact. 2016;15:119.
13. Amado IR, Vázquez JA, Pastrana L, Teixeira JA. Cheese Whey: A cost-effective alternative for hyaluronic acid production by *Streptococcus zooepidemicus*. Food Chem. 2016;198:54–61.
14. Mousavi S, Esfandiar R, Najafpour-Darzi G. Hyaluronic acid production by *Streptococcus zooepidemicus* MW26985 using potato Peel waste hydrolyzate. Bioproc Biosyst Eng. 2024;47:1003–15.
15. Pan NC, Pereira HCB, da Silva MdLC, Vasconcelos AFD, Celligoi MAPC. Improvement production of hyaluronic acid by *Streptococcus zooepidemicus* in sugarcane molasses. Appl Biochem Biotechnol. 2016;182:276–93.
16. Woo HM. Solar-to-chemical and solar-to-fuel production from CO 2 by metabolically engineered microorganisms. Curr Opin Biotechnol. 2017;45:1–7.
17. Zhang L, Toscano Selão T, Nixon PJ, Norling B. Photosynthetic conversion of CO2 to hyaluronic acid by engineered strains of the Cyanobacterium *Synechococcus* Sp. PCC 7002. Algal Res. 2019;44:101702.
18. Dueber JE, Wu GC, Malmirchegini GR, Moon TS, Petzold CJ, Ullal AV, Prather KLJ, Keasling JD. Synthetic protein scaffolds provide modular control over metabolic flux. Nat Biotechnol. 2009;27:753–9.
19. Guo H, Ryan JC, Song X, Mallet A, Zhang M, Pabst V, Decrulle AL, Ejsmont P, Wintermute EH, Lindner AB. Spatial engineering of E. coli with addressable phase-separated RNAs. Cell. 2022;185:3823–e38373823.
20. Qi Lei S, Larson Matthew H, Gilbert Luke A, Doudna Jennifer A, Weissman Jonathan S, Arkin Adam P, Lim Wendell A. Repurposing CRISPR as an RNA-Guided platform for Sequence-Specific control of gene expression. Cell. 2013;152:1173–83.
21. Fontana J, Sparkman-Yager D, Faulkner I, Cardiff R, Kiattisewee C, Walls A, Primo TG, Kinnunen PC, Garcia Martin H, Zalatan JG, Carothers JM. Guide RNA structure design enables combinatorial CRISPRa programs for biosynthetic profiling. Nat Commun. 2024;15:6341.
22. Ko SC, Woo HM. CRISPR–dCas13a system for programmable small RNAs and polycistronic mRNA repression in bacteria. Nucleic Acids Res. 2024;52:492–506.
23. Kim G, Kim HJ, Kim K, Kim HJ, Yang J, Seo SW. Tunable translation-level CRISPR interference by dCas13 and engineered gRNA in bacteria. Nat Commun. 2024;15:5319.
24. Choi SY, Woo HM. CRISPRi-dCas12a: A dCas12a-Mediated CRISPR interference for repression of multiple genes and metabolic engineering in cyanobacteria. ACS Synth Biol. 2020;9:2351–61.
25. Lee M, Heo YB, Woo HM. Cytosine base editing in cyanobacteria by repressing archaic type IV uracil-DNA glycosylase. Plant J. 2023;113:610–25.
26. Kim WJ, Lee S-M, Um Y, Sim SJ, Woo HM. Development of SyneBrick vectors as a synthetic biology platform for gene expression in *Synechococcus elongatus* PCC 7942. Front Plant Sci. 2017;8:293.
27. Blank LM, Hugenholtz P, Nielsen LK. Evolution of the hyaluronic acid synthesis (has) Operon in *Streptococcus zooepidemicus* and other pathogenic Streptococci. J Mol Evol. 2008;67:13–22.
28. Hurst JR, Kasper KJ, Sule AN, McCormick JK. Streptococcal pharyngitis and rheumatic heart disease: the superantigen hypothesis revisited. Infect Genet Evol. 2018;61:160–75.
29. Golden SS, Brusslan J, Haselkorn R. Genetic engineering of the cyanobacterial chromosome. Methods Enzymol. 1986;153:215–31.
30. Chwa JW, Kim WJ, Sim SJ, Um Y, Woo HM. Engineering of a modular and synthetic phosphoketolase pathway for photosynthetic production of acetone from CO2 in *Synechococcus elongatus* PCC 7942 under light and aerobic condition. Plant Biotechnol J. 2016;14:1768–76.
31. Choi SY, Lee HJ, Choi J, Kim J, Sim SJ, Um Y, Kim Y, Lee TS, Keasling JD, Woo HM. Photosynthetic conversion of CO2 to Farnesyl diphosphate-derived phytochemicals (amorpha-4,11-diene and squalene) by engineered cyanobacteria. Biotechnol Biofuels. 2016;9:202.
32. Agarwal G, Prasad KVK, Bhaduri SB, Jayaraman A. Biosynthesis of hyaluronic acid polymer: dissecting the role of sub structural elements of hyaluronan synthase. Sci Rep. 2019;9:12510.
33. Choi SY, Sim SJ, Ko SC, Son J, Lee JS, Lee HJ, Chang WS, Woo HM. Scalable cultivation of engineered cyanobacteria for squalene production from industrial flue gas in a closed photobioreactor. J Agric Food Chem. 2020;68:10050–5.
34. Choi HI, Hwang S-W, Kim J, Park B, Jin E, Choi I-G, Sim SJ. Augmented CO2 tolerance by expressing a single H+-pump enables microalgal valorization of industrial flue gas. Nat Commun. 2021;12:6049.
35. Shikina EV, Kovalevsky RA, Shirkovskaya AI, Toukach PV. Prospective bacterial and fungal sources of hyaluronic acid: A review. Comput Struct Biotechnol J. 2022;20:6214–36.
36. Hmar RV, Prasad SB, Jayaraman G, Ramachandran KB. Chromosomal integration of hyaluronic acid synthesis (has) genes enhances the molecular weight of hyaluronan produced in *Lactococcus lactis*. Biotechnol J. 2014;9:1554–64.
37. Jagannath S, Ramachandran KB. Influence of competing metabolic processes on the molecular weight of hyaluronic acid synthesized by *Streptococcus zooepidemicus*. Biochem Eng J. 2010;48:148–58.
38. Tlapak-Simmons VL, Baron CA, Gotschall R, Haque D, Canfield WM, Weigel PH. Hyaluronan biosynthesis by class I *Streptococcal hyaluronan* synthases occurs at the reducing end. J Biol Chem. 2005;280:13012–8.
39. Hanahan D. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol. 1983;166:557–80.

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