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Anchoring the ethylene-forming enzyme to photosystem-related proteins to improve ethylene production in engineered *Synechocystis* PCC 6803

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ABSTRACT

Ethylene production in engineered cyanobacteria offers a sustainable alternative to the traditional production from fossil resources. Protein fusions and anchoring strategies are promising routes to augment the stability of recombinant proteins. Here we attempted to anchor the ethylene-forming enzyme (Efe) to several photosystem-related proteins (including D1, PetF, and PsaE) in order to improve the ethylene producing capability in engineered cells of *Synechocystis* PCC 6803. Generated strains harboring fused Efe with D1 or PetF showed respectively 1.6- and 1.5-fold increase in ethylene production compared to a control strain harboring only Efe. Additionally, strains co-expressing a non-fused Efe with PsaE or PetF showed respectively 3.5- and 3.1-fold increase in ethylene production compared to the control strain. This work demonstrates the feasibility to incorporate (anchor or co-express) target enzyme(s) with specific photosystem components for facilitating highly efficient production of bulk chemicals in engineered cyanobacteria.

1. Introduction

Ethylene is a most widely used petrochemical in several industries including plastics, textiles and solvents. The traditional production of ethylene from fossil resources can result in a large emission of CO_2 and extensive amounts of toxic compounds (Ghanta et al., 2013; Zhao et al., 2018). A realistic need is to develop environmentally friendly and sustainable ways to produce this chemical. Ethylene can be naturally biosynthesized in higher plants and a wide range of microorganisms (Eckert et al., 2014; Ju et al., 2015). Ethylene production in engineered cyanobacteria by introducing prokaryotic ethylene biosynthetic genes and using CO_2 as the feedstock offers a sustainable alternative to the conventional ethylene production (Kallio et al., 2021; Ungerer et al., 2012).

Plant pathogens, like *Pseudomonas syringae* and *Pencillium digitatum*, synthesize ethylene directly from 2-oxoglutarate (2-OG) in a single-step reaction catalyzed by the ethylene forming enzyme (Efe) (Eckert et al., 2014; Sakai et al., 1997). Currently, the *efe* gene originating from *P. syringae* has been widely introduced into cyanobacteria to produce ethylene (Carbonell et al., 2019; Durall et al., 2020; Ungerer et al., 2012; Veetil et al., 2017; Xiong et al., 2015). Since 2-OG is an important tricarboxylic acid (TCA) cycle intermediate in organisms, a good example in engineering *efe*-expressing cyanobacteria is overexpression of phosphoenolpyruvate carboxylase or phosphoenolpyruvate synthase resulting in enhanced carbon supply to the TCA cycle and thus increased ethylene production (Durall et al., 2020). However, a critical problem is the unstable expression of Efe that may result in low ethylene productivity in cyanobacteria, particularly in *Synechococcus elongatus* PCC 7942 (Carbonell et al., 2019; Sakai et al., 1997; Takahama et al., 2003;). Therefore, the *efe* gene has been modified and the resulted codon-optimized *efe* (*Sy-efe*) can be stably expressed in cyanobacteria without any cytotoxicity (Ungerer et al., 2012). These studies imply that stabilizing the Efe activity should be another important direction when engineering cyanobacteria for efficient ethylene production.

Protein immobilization by using the support matrix is a promising technology that can enhance the enzyme stability and efficiency (Sharma and Singh, 2020). Anchoring the target protein to a specific protein serves as a distinct type of immobilization approach in host cells. A well-known example is the fluorescent proteins that have been used to track protein localizations or monitor physiological processes (Enterina et al., 2015). Recently, it was reported that targeting the pathway expression to subcellular compartments through enzymes fusion yielded larger productivity of astaxanthin than expression of individual enzymes

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in the yeast *Yarrowia lipolytica* (Ma et al., 2021). Additionally, it was demonstrated that fusion of a heterologous protein to a glycosylphosphatidylinositol anchoring domain enabled efficient utilization of the cell wall space and facilitated ethanol production in the yeast *Saccharomyces cerevisiae* (Inokuma et al., 2020). In cyanobacteria, the outer membrane porin SomA and the major pilus subunit PilA1 have been adopted as anchoring motifs for surface display of functional proteins (Cengic et al., 2018; Chungjatupornchai et al., 2011; Fedeson and Ducat, 2017). Moreover, a plant cytochrome P450 was successfully fused to the photosystem I (PSI) subunit PsaM of *Synechococcus* PCC 7002, with the purpose of utilizing the electrons released from PSI to drive the enzymatic reaction (Lassen et al., 2014).

Due to the above discussed advantages of protein-fusion anchoring technology, we aimed to develop an anchoring strategy to positively modulate the stability of Efe in *Synechocystis* PCC 6803 (hereinafter *Synechocystis*). As we know, cyanobacterial cells have double outer cell membranes and folded inner thylakoid membranes that play important roles in photosynthesis (Liberton et al., 2006; Nevo et al., 2007). Photosynthetic light capture, electron transport and ATP synthesis, via PSI and photosystem II (PSII), take place on the abundant internal thylakoid membranes (Frain et al., 2016). PSI is thought to be much more stable than PSII, as PSI-catalyzed reactions do not occur at extremely oxidizing redox potentials (Powles, 1984). PSI proteins (half-life time of 30–75 h) are longer-lived than PSII proteins (half-life time of $<1-11h$) (Yao et al., 2012). In addition, a key component of PSII, D1 protein, is stable in darkness but undergoes rapid turnover in the light (Mulo et al., 2012; Tyystjärvi et al., 2001). Therefore, in this study, we aimed to anchor Efe to several PSI-related proteins and the D1 protein to achieve higher ethylene production in engineered strains of *Synechocystis*.

2. Material and methods

2.1. Protein anchoring design

Cyanobacterial PSI-related proteins considered include PsaA, PsaB, PsaC, PsaD, PsaE and PetF. Among them, PsaA and PsaB are two integral membrane proteins of PSI, while PsaC, PsaD and PsaE are three stromal subunits that are attached to the PsaA/PsaB heterodimer (Fischer et al., 1998; Jeanjean et al., 2008; Jolley et al., 2006; Klukas et al., 1999). Ferredoxin I (PetF) is a small soluble electron carrier protein that can mediate the electron flux from PSI to the downstream electron acceptors, which is assumed to loosely bind PsaD and PsaE (Barth et al., 1998; Jeanjean et al., 2008; Sawyer and Winkler, 2017). In addition, D1 (PsbA) is an integral membrane protein of PSII. Overall, the native PsaA (Slr1834), PsaC (Ssl0563), PsaD (Slr0737), PsaE (Ssr2831), PetF (Ssl0020), and D1 (Slr1311) of *Synechocystis* were chosen as the targets of Efe fusion. Their N-terminal peptides were used as the anchoring sites in this study.

2.2. Plasmid construction

The starting plasmid for construction was pEERM-0168, which contains 1000 bp upstream sequence and 1000 bp downstream sequence of *slr0168*, a neutral integration locus in the genome of *Synechocystis* (Liu et al., 2019). An illustration of the fused (or anchoring) and non-fused constructions of each target gene is shown in Fig. 1. The non-fused construction served as a counterpart of the anchoring construction for comparative evaluation. The *efe* gene (*Sy-efe*) was amplified from the plasmid pJU158 (Xiong et al., 2015). A strong, light inducible P*psbA2* promoter (a 315 bp sequence starting from "GCGTGCAAGG" to "ACAAATACAT") (Englund et al., 2016) was used to drive the gene transcription. A strong ribosomal binding site RBS* (TAGTGGAGGT) flanked by a *Xba*I site was inserted between the promoter and the start codon (Englund et al., 2016). Protein fusion with suitable linkers offers the advantages in construction of stable, bioactive fusion proteins (Chen et al., 2013). Thus, for the gene fusion construction (Fig. 1A), the stop codon-removed *efe* gene and the target gene were linked by a 36-bp linker sequence (GGAGGAAGTGGTGGATCTGGAGGTAGTGGTGG-TAGT). For the non-fusion construction (Fig. 1B), the *efe* and target genes were driven together by P*psbA2* promoter but were independently translated. In addition, another control plasmid was constructed in pEERM-0168, in which only *efe* was expressed under the control of the P*psbA2* promoter. The primers used in this study are shown in Supplementary Tables S1 and S2. Competent *Escherichia coli* T7 Express strain was employed for constructing the plasmids and the resulted constructs were verified by sequencing.

2.3. Generation of recombinant Synechocystis strains

The host strain (WT + 2xPEPc) used in this study was a previously engineered *Synechocystis* PCC 6803 strain (WT-P) containing three copies of the native *pepc* gene, which encodes phosphoenolpyruvate carboxylase (Durall et al., 2016). The plasmids constructed were transformed into WT-P via natural transformation as described (Liu et al., 2019). Transformed cells were selected on BG11 plates containing 25 μg

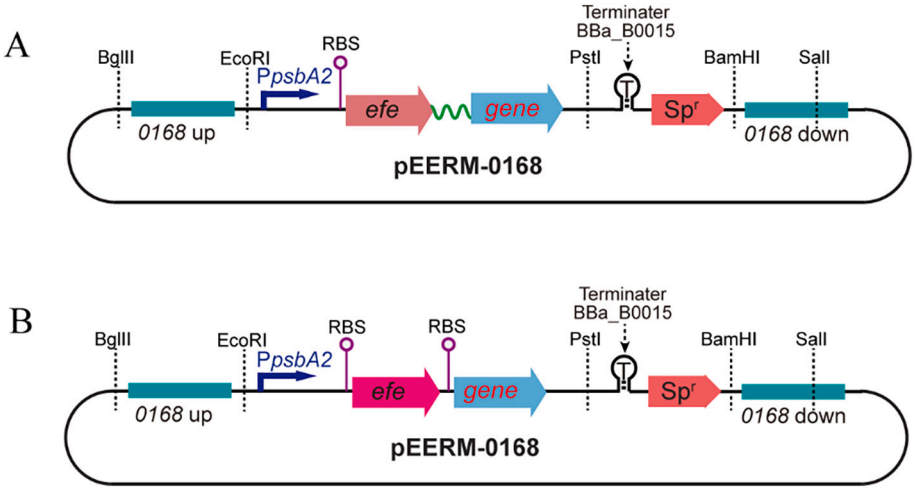

Fig. 1. Schematic illustration of the fused (A) and non-fused (B) genes constructions. In panel A, the stop codon-removed *efe* gene was fused with the target gene by a 36-bp linker. In panel B, *efe* gene and the target gene were independently translated. Abbreviations: *0168* up, *slr0168* upstream; *0168* down, *slr0168* downstream; RBS*, the strong ribosomal binding site; Sp^r, spectinomycin resistance cassette.

mL^{-1} kanamycin (Km) and 25 μg mL^{-1} spectinomycin (Sp). Positive transformants were confirmed by PCR amplification with the gene-specific primers (Supplementary Table S1), inoculated into 6-wells plates and further cultivated until fully segregated. The segregation of each strain was examined by PCR using *slr0168*-specific primers (Supplementary Table S1) and extracted genomic DNA.

2.4. Cultivation and ethylene measurement

The *Synechocystis* strains were cultivated in BG11 medium containing 25 μg mL^{-1} Km and 25 μg mL^{-1} Sp in 250 ml conical flasks at 30 °C in a shaker (120 rpm). Growth was compared by cultivation for 4 days with an initial OD_{730} of 0.15. The ethylene production of the recombinant strains was measured as described previously (Durall et al., 2020) with slight modifications. In brief, the *Synechocystis* cells of OD_{730} at 0.4–0.6 were harvested at 5000 rpm for 10 min and the pellet resuspended in 2 mL of fresh BG11 containing 10 mM Tris-HCl (pH 8.0) and 5 mM $NaHCO_3$. The culture of each strain was then placed into a 9 mL glass vial and completely sealed. The vials were horizontally placed in the shaker (120 rpm) under 20 μE m^{-2} s^{-1}. After 24 h, 100 μL of the gas phase was collected from the vial with a gas syringe and produced ethylene detected by a Clarus 580 Perkin Elmer FID gas chromatograph (GC) equipped with a packed column (1.8 m × 2 mm i.d., Cat No. N9305013-ZW5531, Perkin Elmer). The carrier gas was N_2 at a flow rate of 20 mL min^{-1} and the GC program was 200 °C for 1 min. The area of the ethylene peak at 0.4 min was converted into mg mL^{-1} using the equation obtained from the standard curve established with pure ethylene (purity ≥99.95 %, Sigma-Aldrich).

2.5. Statistical analysis

The experiments were all performed in triplicate and the results are expressed as the mean ± SD. Significant difference (P value <0.05) was assessed by the student's *t*-test.

3. Results and discussion

3.1. Generation of recombinant Synechocystis strains

Plasmids were constructed using the PSI-related proteins PsaA, PsaC, PsaD, PsaE and PetF. The genes were sequentially linked by fusion PCR method with high-fidelity DNA polymerase before the fused genes were ligated into pEERM-0168 and transformed into competent *E. coli* cells for plasmid amplifications. However, only very few transformants related to *psaA*, *psaC*, and *psaD* were obtained, and sequencing of the extracted plasmids showed that they all had nonsense mutations in the respective open reading frames. Replacement of the P*psbA2* promoter with the P*trc2* promoter (Khalil and Collins, 2010) resulted in similar results. In addition, the correct plasmid harboring non-fused *efe* and *D1* was also not obtained. Membrane proteins are toxic to cells or even cause cell death when overproduced in host, possibly by jamming the inner membranes (Kaur et al., 2018; Terpe, 2006). The finally obtained correct plasmids and their corresponding recombinant *Synechocystis* strains are shown in Table 1. The fully segregated recombinants were verified by PCR using genomic DNA (without any PCR bands being corresponding to *slr0168*) and then subjected to growth and ethylene production assessments.

3.2. Growth and ethylene production

The growth and ethylene production of the six engineered *Synechocystis* strains (Table 1) were assessed (Figs. 2 and 3). As shown in Fig. 2, strains ED (WT-P + Efe ~ D1), EE0 (WT-P + Efe ~ PsaE) and EE1 (WT-P + Efe + PsaE) seemed to grow more slowly than the control strain E0 (WT-P + Efe). On day 4, the biomass levels (in terms of OD_{730}) of EE0 and EE1 strains were significantly lower (P value <0.05) than that of E0

Table 1
The plasmid constructs and generated *Synechocystis* PCC 6803 strains.

Strain	Plasmid construct[a] (#Efe anchoring)	Resistant cassette[b]
WT-P + Efe (E0)	P*psbA2*-RBS*-*efe*-T	Km^r + Sp^r
WT-P + Efe ~ D1 (ED)	P*psbA2*-RBS*-*efe* ~ linker~*D1*-T #	Km^r + Sp^r
WT-P + Efe ~ PsaE (EE0)	P*psbA2*-RBS*-*efe* ~ linker~*psaE*-T #	Km^r + Sp^r
WT-P + Efe + PsaE (EE1)	P*psbA2*-RBS*-*efe*-RBS*-*psaE*-T	Km^r + Sp^r
WT-P + Efe ~ PetF (EF0)	P*psbA2*-RBS*-*efe* ~ linker~*petF*-T #	Km^r + Sp^r
WT-P + Efe + PetF (EF1)	P*psbA2*-RBS*-*efe*-RBS*-*petF*-T	Km^r + Sp^r

[a] RBS*, the strong ribosomal binding site; T, terminator BBa_B0015.
[b] Km^r, kanamycin resistance cassette; Sp^r, spectinomycin resistance cassette.

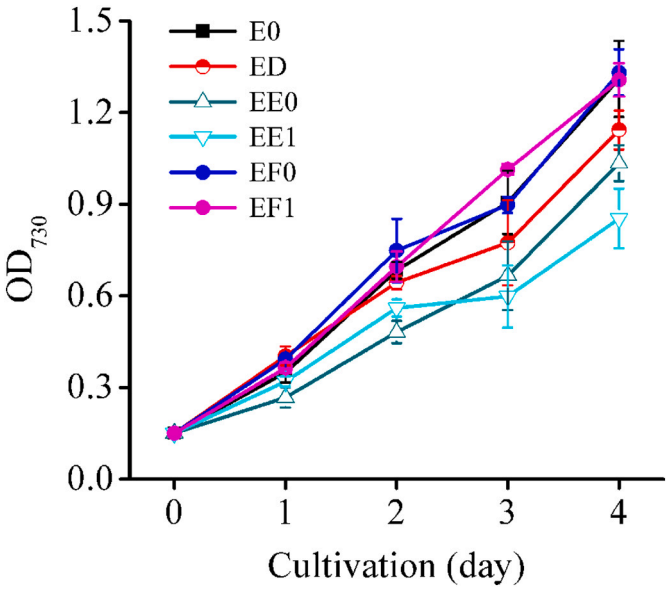

Fig. 2. Growth curves of the engineered *Synechocystis* strains E0, ED, EE0, EE1, EF0 and EF1 (see Table 1). Data shown as mean ± SD (n = 3).

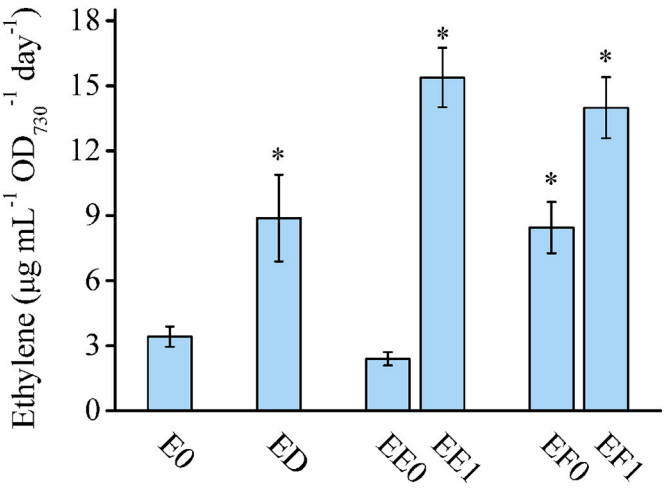

Fig. 3. The ethylene producing capability of the engineered *Synechocystis* strains E0, ED, EE0, EE1, EF0 and EF1 (see Table 1). Data shown as mean ± SD (n = 3). * Significant difference ($p < 0.05$, *t*-test).

strain, being decreased by 21.0 % and 34.8 %, respectively; while the biomass level of ED strain was slightly but not significantly lower than that of E0 strain. In contrast, both EF0 (WT-P + Efe ~ PetF) and EF1 (WT-P + Efe + PetF) strains showed growth curves very similar to that of E0 strain. As mentioned above, D1 is an integral membrane protein of PSII, and PsaE is a stromal extrinsic PSI subunit, while PetF is a small soluble protein which loosely binds to PsaE (Barth et al., 1998; Jeanjean et al., 2008; Mulo et al., 2012). Overexpression of D1 or PsaE may lead to a disruption of cellular membrane homeostasis, and thus inhibit cell growth. Only PetF-related constructions did not result in the growth inhibition of engineered strains. These results imply that overproduction of photosynthetic membrane proteins or their tightly attached proteins may impair cell growth to a certain extent.

Further, the ethylene producing capability was compared among the *Synechocystis* strains (Fig. 3). As compared to E0 strain, ED and EF0 strains showed significantly elevated ethylene production, being increased by 1.6 and 1.5 folds, respectively. However, a slightly decreased ethylene production was observed in EE0 strain. The obtained results demonstrate that anchoring Efe to photosystem-related proteins can serve as an effective strategy for enhancing ethylene production. However, it was confined to a limited number of proteins such as D1 and PetF.

Additionally, EE1 and EF1 strains, which harbor non-fused but co-expressed gene constructs, were generated (Table 1). Unexpectedly, both strains showed much higher ethylene production than E0 strain, being increased by 3.5 and 3.1 folds, respectively (Fig. 3). Thus, both strains were more effective in ethylene production than their counterpart strains EE0 and EF0. Interestingly, EE1 and EF1 did not show any increased growth compared to strain E0 as well as their respective counterpart strains EE0 and EF0 (Fig. 2). This new finding implies that the non-fused pattern for two-gene co-expression could also favor efficient ethylene production.

3.3. An explanation for the potential mechanisms

The thylakoid membranes in cyanobacteria are arranged inside the cell as concentric cylinders, thereby creating a separate and isolated cytosolic compartment in the interior of the cell (Liberton et al., 2006). The thylakoid membrane-intrinsic proteins and their attached proteins are supposed to be relatively abundant and stable. Although D1 is easy to be damaged in the light, a rapid turnover guarantees a steady-state level of this protein in the cells (Mulo et al., 2009). In this study, the N-terminus of D1 (in the stromal side) (Knopf and Adam, 2018) was fused with Efe by a flexible linker and the formed fusion protein was ectopically expressed. Theoretically, this construction would increase the abundance of D1 but not affect its normal function in the engineered strain. A previous study reported that overexpression of a maize *psbA* (*D1*) increased D1 abundance and enhanced drought tolerance in tobacco (Huo et al., 2015). As expected, the engineered ED strain showed only slightly inhibitory growth but highly increased ethylene production (Figs. 2 and 3). Similarly, the anchoring way with the electron carrier protein PetF was also proved to be successful. It has been reported that overexpression of *petF* could enhance tolerance to heat stress and increase starch and oil yields in *Chlamydomonas reinhardtii* (Huang et al., 2015; Lin et al., 2013). Relevant studies also showed that "fusion constructs as protein overexpression vectors" can increase the stability of eukaryotic plant- and animal-origin recombinant proteins in cyanobacteria (Zhang et al., 2021). Thus, the increased ethylene production in the ED and EF0 strains may be largely attributed to the increased abundance and functional stability of Efe. However, for the unimproved ethylene production of EE0 strain, disturbance on the normal function of PsaE (PSI subunit) caused by protein fusion may be a main reason.

The finding that EE1 and EF1 strains showed even higher ethylene producing capability was beyond our expectation (Fig. 3). In prokaryotes, RNA polymerase and ribosomes often bind concurrently to the same RNA transcript, leading to the functional coupling of transcription and translation (Fan et al., 2017; Irastortza-Olaziregi and Amster-Choder, 2021). The non-fused two-gene co-expression in EE1 and EF1 strains may facilitate the direct interaction between RNA polymerase and ribosomes, thus contributing to the coupling of transcription with translation thereby improving the gene expression efficiency.

4. Conclusions

Protein fusion and anchoring strategy were employed to elevate the ethylene producing capability in cyanobacteria. The fusion constructions of Efe with D1 or PetF protein resulted in above 1.5-fold increase in ethylene production in the engineered strains, as compared to the control strain, which provides the feasibility and valuable information of anchoring the target enzyme to certain photosystem components for establishing a stable biocatalytic system. In addition, an unexpected finding is that the co-expression of Efe with PsaE or PetF protein resulted in above 3.1-fold increase in ethylene production, providing new inspiration for future engineering design.

CRediT authorship contribution statement

Xiang Gao: Conceptualization, Methodology, Validation, Investigation, Visualization, Formal analysis, Writing – original draft, Funding acquisition. **Xufeng Liu:** Methodology, Visualization, Writing – original draft. **Xin Jing:** Visualization, Formal analysis. **Peter Lindblad:** Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2022.101178.

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