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Enhancing photosynthetic production of ethylene in genetically engineered *Synechocystis* sp. PCC 6803[†]

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Ethylene is widely used in the petrochemical industry and has traditionally been produced *via* the steam cracking of petroleum-based feedstock. The exploration of sustainable and carbon-neutral methods of producing ethylene from the renewable feedstock seems promising. The direct photosynthetic production of ethylene after the recycling of carbon dioxide shows great potential. In this study, continuous and stable ethylene production was achieved in *Synechocystis* sp. PCC 6803 by introducing a codon-optimized ethylene-forming enzyme (EFE) from *Pseudomonas syringae* pv. *sesami* and using 2-oxoglutarate (2-OG) as the substrate. Based on diverse promoter screening, *PcpCB* was proved to be a highly efficient promoter for ethylene production in cyanobacteria. The genes encoding 2-OG decarboxylase (OGDC) and succinic semialdehyde dehydrogenase (SSADH) in the tricarboxylic acid (TCA) cycle in *Synechocystis* sp. PCC 6803 were identified, and the TCA cycle was genetically modified by blocking these two enzymes with the simultaneous overexpression of EFE. Meanwhile, a gene encoding 2-OG permease (KgtP) from *E. coli* was introduced into the *phaAB* loci to increase the 2-OG supply. A peak volumetric production rate of 9.7 mL L⁻¹ h⁻¹ for ethylene was eventually achieved in the *Synechocystis* recombinant (XX110), with the genetic modification of the TCA cycle and heterologous expression of 2-oxoglutarate permease by the modified semi-continuous cultivation.

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Introduction

Ethylene is the raw material for a number of downstream chemical products, such as polyethylene (PE), polyethylene terephthalate (PET), polyvinyl chloride (PVC) and polystyrene (PS), as well as fibers widely used in the chemical industry and consumer markets. It is also a gaseous plant hormone frequently used in agriculture to promote crop growth and control the ripening of fruit.¹ Although the global ethylene market has exceeded 143 million tons per year, the demand for ethylene is expected to continue rising.² The traditional approach for ethylene production mainly relies on the steam cracking of unrenewable petroleum derivatives, and this process causes serious environmental pollution and the greenhouse effect.³ Bioethanol-based ethylene production has already been excessively explored. However, food competition

(from food-based biomass) and cost-effectiveness (from lignocellulose-based biomass) are hurdles to be overcome, and the related production processes still require a full commercial demonstration.⁴ Hence, it is more and more urgent to explore innovative and sustainable ways to produce this fundamental raw material.

Thus far, three biological pathways for ethylene synthesis have been identified. (i) High plants produce ethylene *via* a three-step reaction (the ACC pathway), in which methionine is first converted to *S*-adenosyl-*L*-methionine, then to *L*-aminocyclopropane-*L*-carboxylic acid (ACC), and finally to ethylene, as progressively catalyzed by methionine adenosyltransferase, ACC synthase and ACC oxidase.^{5,6} (ii) In most prokaryotes, ethylene is formed from methionine *via* 2-keto-4-methyl-thiobutyric acid (KMBA) catalyzed by an NADH:Fe(III)EDTA oxidoreductase in a two-step process (the KMBA pathway).^{7,8} (iii) In many plant pathogens, such as *Pseudomonas syringae*, ethylene is directly synthesized by a single enzyme (ethylene forming enzyme, EFE) from an important TCA cycle intermediate, 2-oxoglutarate (2-OG)^{9,10} (Fig. 1A). The 2-OG dependent ethylene-producing pathway has been tentatively explored in bacteria,¹¹ fungi^{12,13} and yeast^{14,15} using genetic and metabolic engineering strategies. However, ethylene productivity was still at low levels in these microbial model systems.

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Fig. 1 Biochemical reaction catalyzed by ethylene-forming enzyme (EFE) (A) and the schematic diagram for ethylene production in *Synechocystis* sp. PCC 6803 (B).

Unlike heterotrophic microorganisms, cyanobacteria are a type of photoautotroph that can perform oxygen-evolving photosynthesis.¹⁶ It is this ability of sequestering and recycling CO₂ into organic matter that qualifies cyanobacteria as potential candidates for the direct photosynthetic production of bio-fuels and biochemicals.¹⁷ Early studies on the cyanobacterial production of ethylene were performed in *Synechococcus elongatus* PCC 7942. The *efe* gene from *Pseudomonas syringae* was introduced into host cells by two strategies: using a shuttle vector^{18,19} or being incorporated into the chromosome by homologous recombination.²⁰ In both cases, the exogenous *efe* was under the control of the *psbA1* promoter, and the amount of carbon incorporated into ethylene could be up to 5.84% of the total carbon fixed, according to Sakai *et al.*¹⁹ However, all *Synechococcus* recombinants in these studies were genetically unstable, and the ethylene-producing phenotype was lost over a few generations. Recently, stable *efe* expression and continuous ethylene production have been achieved in *Synechocystis*

sp. PCC 6803 by the modification of the *efe* sequence.³ The regulated expression of *efe* was also investigated in *Synechocystis* sp. PCC 6803, and a well-regulated Lac promoter variant was identified.²¹ By increasing the *efe* copy number and re-suspending ethylene-producing recombinants in 5 × BG11 in a semi-continuous cultivation, a peak production rate of 5650 μL L⁻¹ h⁻¹ was finally achieved when *efe* was under the control of the *psbA* promoter from pea plant chloroplasts.³ The metabolic fate of photosynthetically fixed carbon in recombinant ethylene-producing cyanobacteria mainly includes ethylene production, biomass accumulation and storage compounds (e.g. glycogen and polyhydroxybutyrate, PHB), *etc.* It has been reported that less than 5% of the fixed carbon is directed to the TCA cycle in *Synechocystis* sp. PCC 6803 under photoautotrophic growth conditions.^{3,22} As one of the most iconic pathways in the central metabolism, the TCA cycle has a dual role in ATP synthesis and precursor metabolite (e.g. oxaloacetate, succinate and 2-OG) supply.²³ The introduction of two copies of *efe* redirected ~5.5% of the total fixed carbon toward ethylene production (surpassing the carbon partition rate into the TCA cycle), indicating that the amount of EFE enzyme was one of the rate-limiting factors for ethylene production.³ Multiple copies of *efe* might meet the need for a higher partition rate of fixed carbon toward ethylene production. On the other hand, EFE catalyzes the conversion of 2-OG to succinate, along with the production of ethylene; therefore, the exogenous EFE pathway provides an alternative to the 2-OG decarboxylase (OGDC) and succinic semialdehyde dehydrogenase (SSADH) of the cyanobacterial TCA cycle.^{3,24} Based on such recognition, inactivating the genes encoding the identified OGDC and SSADH by inserting the *efe* gene should reduce the metabolic flux toward the native TCA cycle and actively redirect metabolic flux toward the ethylene-producing pathway (Fig. 1B).

In addition to the EFE enzyme, the intracellular pool of 2-OG is an important factor affecting ethylene production. 2-OG is not only the substrate of ethylene biosynthesis but also serves as a sensitive signal of the carbon and nitrogen status of the host cells. It is also the precursor of a wide variety of compounds in cyanobacteria, including heme, chlorophyll and phycobiliproteins.²⁴ Perturbations in the 2-OG pool are likely to manifest themselves as changes in carbon flux and carbon partitioning within the cell.³ Cyanobacteria bearing KgtP (2-oxoglutarate permease) from *E. coli* were previously reported to be able to assimilate extracellular 2-OG actively,²⁵ so the introduction of *kgtP* was thought to make a positive contribution to ethylene production by controlling 2-OG substrate provision and/or metabolic regulation.

After a promising recombinant ethylene-producing strain was constructed, we developed an efficient and cost-effective method for cell cultivation and gas product collection. The initial cultivation of these ethylene producers was mainly performed in shake flasks, and the collection and the production analysis of ethylene took place in sealed bottles with a volume of approximately 10 mL.^{3,18–21} Here, we not only evaluated the ethylene-producing capacity of the *efe* transformants using traditional cultivating and collecting methods, but we also

preliminarily developed a gaseous/aqueous two-phase photobioreactor for the successive cultivation of cyanobacterial cells and continuous collection of ethylene products.

In this study, *Synechocystis* sp. PCC 6803 was chosen as the starting strain for ethylene production. Enhanced ethylene production ($9.7 \text{ mL L}^{-1} \text{ h}^{-1}$) was achieved by codon optimization of the *efe* gene, promoter screening, genetic modification of the TCA cycle, heterologous expression of 2-oxoglutarate permease, and optimization of the cultivation process. Approximately 51 mL of the ethylene gas product could be collected using the 1 L gaseous/aqueous two-phase photobioreactor during 16-day continuous cell cultivation. Herein, the *Synechocystis* recombinants constructed and the bioreactor developed show great potential for the direct photosynthetic production of ethylene from carbon dioxide.

Materials and methods

Chemicals and reagents

Taq DNA polymerase and restriction enzymes were obtained from Fermentas (Canada) or Takara (Japan). The kits used for molecular cloning were from Omega (USA) or Takara (Japan). The antibodies and chemicals used for western blotting were from Tiangen (China). Pure ethylene gas standard was purchased from Heli (Qingdao, China). The 2-oxoglutarate (α -ketoglutarate) assay kit was ordered from Sigma-Aldrich (USA). Unless otherwise specified, all other chemical reagents were purchased from Sigma-Aldrich (USA).

Plasmid construction

All plasmids and primers used in this study are listed in Table 1, and the details are described as follows. The clones of PCR products were confirmed by sequencing.

(i) **Plasmids used to inactivate *sll1981* and *slr0370*.** The DNA fragment generated by PCR using primers *sll1981*-1 and *sll1981*-2 was cloned into the pMD18-T sample (Takara), resulting in pTZ14. Plasmid pTZ14 was digested completely with *Bal*I (402 bp was cut off) and ligated with the kanamycin resistance cassette (Km^r) excised from pRL446²⁶ with *Pvu*II, resulting in pTZ16. The DNA fragment generated by PCR using primers *slr0370*-1 and *slr0370*-2 was cloned into the pMD18-T sample, resulting in pTZ15. Plasmid pTZ15 was cut with *Apa*I, blunted with T4 DNA polymerase, and ligated with the blunted chloramphenicol/erythromycin resistance cassette C.CE2 excised from pRL271²⁷ with *Sal*I and *Bgl*II, resulting in pTZ17.

(ii) **Plasmids used to express *sll1981* and *slr0370* in vitro.** The DNA fragment generated by PCR using primers *sll1981*exp-1 and *sll1981*exp-2 was cut with *Bam*HI and *Xho*I, and inserted into the same sites of pET32a (Novagen), resulting in pZL105. The DNA fragment generated by PCR using primers *slr0370*exp-1 and *slr0370*exp-2 was cut with *Bam*HI and *Xho*I and inserted into the same sites of pET32a (Novagen), resulting in pZL106.

(iii) **Promoter-probing vector used to screen high-efficient promoters for ethylene synthesis and a series of plasmids**

constructed based on this vector (Fig. 2A). The DNA fragment generated by PCR using primers *efe*-5 and *efe*-6 was cloned into pMD18-T, resulting in pXX16. The cloned DNA fragments were excised from pXX16 with *Nde*I and *Xho*I and inserted into the same sites of pXT37b,²⁸ resulting in the promoter-probing vector pTZ10b. The promoter regions fell into the following genes (operons): *psbA2*, *cpcB*, *rbcL* and *psbD* of *Synechocystis* sp. PCC 6803, *psbA1*, *psbA2* and *groESL* of *Synechococcus elongatus* PCC 7942, *glnA* of *Anabaena* sp. PCC 7120, and *cI* of λ -phages were amplified from their own genomic DNA by PCR using the following primers: 03psbA2-1/2, cpcB-1/2, rbcL-1/2, psbD-1/2, 42psbA1-1/2, 42psbA2-1/2, groESL-1/2, glnA-1/2 and cI-1/2. They were cut with *Nde*I and *Kpn*I and inserted into the same sites of pTZ10b, resulting in pXX29, pXX47, pXX48, pXX50, pXX32, pXX33, pXX54, XX53, and pXX49, respectively.

(iv) **Construction of BioBrick "T" vector pFL-XS for functional block assembling.** The chloramphenicol resistance (Cm^r) cassette was amplified from pRL271²⁷ with primers X-F1 (containing *Xcm*I cassette) and S-R1 (containing *Xcm*I cassette and *Spe*I site) before being cloned into pMD18-T, and then the *Nde*I site on the plasmid was eliminated by site-directed mutagenesis, resulting in pFL-XS.

(v) **Plasmids used to express *efe* at *sll1981* and *slr0370* gene loci (Fig. S5†).** The Cm^r cassette was amplified from pRL271²⁷ with primers Cm -F and Cm -R and cloned into pMD18-T; then, the *Nde*I site was eliminated by site-directed mutagenesis, resulting in pXT170n. Plasmid pXT170n was cut with *Bgl*II and *Nco*I and blunted with T4 DNA polymerase. The 3 kb fragment was purified using a gel extraction kit and ligated with Km^r excised from pRL446²⁶ with *Pvu*II, resulting in pXT170k. The $\text{P}_{\text{cpcB}}\text{-efe}$ expression cassette was amplified from pXX47 by PCR using primers cpcB-1 and *efe*-6 and cloned into pFL-XS, resulting in pXX55. The Km^r cassette was then excised from pXT170k with *Eco*RI and *Spe*I and inserted into the *Eco*RI and *Xba*I sites of pXX55, resulting in pXX60. The Cm^r cassette was then excised from pXT170n with *Kpn*I and *Spe*I and inserted into the *Kpn*I and *Xba*I sites of pXX55, resulting in pXX61. The $\text{Km}^r\text{-P}_{\text{cpcB}}\text{-efe}$ expression cassette was excised from pXX60 with *Xba*I and *Spe*I, blunted with T4 DNA polymerase, and inserted into *Bal*I completely digested pTZ14, resulting in pXX64. The $\text{Cm}^r\text{-P}_{\text{cpcB}}\text{-efe}$ expression cassette was excised from pXX61 with *Xba*I and *Spe*I, blunted with T4 DNA polymerase, and inserted into *Apa*I digested and blunted pTZ15, resulting in pXX65.

(vi) **Plasmids used to express *kgtP* at the *phaAB* operon (Fig. S5†).** *E. coli kgtP* gene was amplified from *E. coli* K12 using the primers *kgtP*-1 and *kgtP*-2 and was then cloned into pFL-XS, resulting in pXX56. The gentamicin resistance cassette (Gm^r) was amplified from pLY216 (unpublished data) with primers Gm -1 and Gm -2 and cloned into pFL-XS, resulting in pXX58. P_{cpcB} of *Synechocystis* sp. PCC 6803 was amplified with primers cpcB-1 and cpcB-2 and cloned into pFL-XS, resulting in pZZ008. The Gm^r cassette was excised from pXX58 with *Eco*RI and *Spe*I and inserted into the *Eco*RI and *Xba*I sites of pZZ008, resulting in pXX62. The $\text{Gm}^r\text{-P}_{\text{cpcB}}$ cassette was excised from pXX62 with *Eco*RI and *Spe*I and inserted into the *Eco*RI

Table 1 Plasmids, strains and primers used in this study

Plasmids, strains and primers	Derivation and/or relevant characteristics ^a	Reference or source
Plasmids		
pXT37b	Ap ^r Sp ^r , pUC9' derivative containing upstream and downstream fragments of <i>slr0168</i> , omega, <i>PpetE</i> and <i>LacZ</i>	28
pMD18-T	Ap ^r , cloning vector	Takara
pMD18-T simple vector	Ap ^r , cloning vector	Takara
pET32a	Ap ^r Km ^r , overexpression vector	Novagen
pFL-XS	Ap ^r Cm ^r , a Biobrick "T" vector used for functional block assembling	This study
pTZ14	Ap ^r ; pMD18-T simple vector derivative containing upstream and downstream fragments of <i>sll1981</i>	This study
pTZ15	Ap ^r ; pMD18-T simple vector derivative containing upstream and downstream fragments of <i>slr0370</i>	This study
pTZ16	Ap ^r Km ^r , pTZ14 derivative used to inactive <i>sll1981</i>	This study
pTZ17	Ap ^r Cm ^r (Em ^r), pTZ15 derivative used to inactive <i>slr0370</i>	This study
pZL105	pET32a derivative used to express <i>sll1981</i> <i>in vitro</i>	This study
pZL106	pET32a derivative used to express <i>slr0370</i> <i>in vitro</i>	This study
pXX16	Ap ^r , pMD18-T derivative containing synthetic <i>efe</i> gene	This study
pTZ10b	Ap ^r Sp ^r , pXT37b derivative used as a promoter-probing vector	This study
pXX29	Ap ^r Sp ^r , pTZ10b derivative used to detect promoter strength of P _{6803psbA2}	This study
pXX32	Ap ^r Sp ^r , pTZ10b derivative used to detect promoter strength of P _{7942psbA1}	This study
pXX33	Ap ^r Sp ^r , pTZ10b derivative used to detect promoter strength of P _{7942psbA2}	This study
pXX47	Ap ^r Sp ^r , pTZ10b derivative used to detect promoter strength of P _{cpcB}	This study
pXX48	Ap ^r Sp ^r , pTZ10b derivative used to detect promoter strength of P _{rbc}	This study
pXX49	Ap ^r Sp ^r , pTZ10b derivative used to detect promoter strength of P _{ci}	This study
pXX50	Ap ^r Sp ^r , pTZ10b derivative used to detect promoter strength of P _{psbD}	This study
pXX53	Ap ^r Sp ^r , pTZ10b derivative used to detect promoter strength of P _{glnA}	This study
pXX54	Ap ^r Sp ^r , pTZ10b derivative used to detect promoter strength of P _{groESL}	This study
pXX55	Ap ^r , pFL-XS derivative containing P _{cpcB-efe} expression cassette	This study
pXT170n	Ap ^r Cm ^r , pMD18-T derivative (<i>NdeI</i> eliminated) containing Cm ^r cassette with <i>BglIII</i> and <i>SpeI</i> added	This study
pXT170k	Ap ^r Km ^r , pXT170n derivative containing the Km ^r cassette	This study
pXT170s	Ap ^r Sp ^r , pXT170n derivative containing the Sp ^r cassette omega	This study
pXX60	Ap ^r Km ^r , pFL-XS derivative containing Km ^r -P _{cpcB-efe} expression cassette	This study
pXX61	Ap ^r Cm ^r , pFL-XS derivative containing Cm ^r -P _{cpcB-efe} expression cassette	This study
pXX64	Ap ^r Km ^r , pTZ14 derivative used to express <i>efe</i> at <i>sll1981</i> gene locus	This study
pXX65	Ap ^r Cm ^r , pTZ15 derivative used to express <i>efe</i> at <i>slr0370</i> gene locus	This study
pXX56	Ap ^r , pFL-XS derivative containing <i>E.coli kgtP</i> gene	This study
pXX58	Ap ^r , pFL-XS derivative containing the Gm ^r cassette aacC1	This study
pZZ008	Ap ^r , pFL-XS derivative containing P _{cpcB} of <i>Synechocystis</i> sp. PCC 6803	This study
pXX62	Ap ^r Gm ^r , pFL-XS derivative containing Gm ^r -P _{cpcB} expression cassette	This study
pXX69	Ap ^r Gm ^r , pFL-XS derivative containing Gm ^r -P _{cpcB-kgtP} expression cassette	This study
pKC104	Ap ^r , pMD18-T derivative containing upstream and downstream fragments of <i>phaAB</i> operon	29
pXX70	Ap ^r Gm ^r , pKC104 derivative used to express <i>kgtP</i> at <i>phaAB</i> operon	This study
pRL446	Ap ^r Km ^r , plasmid containing the Km ^r cassette	26
pRL271	Ap ^r Cm ^r (Em ^r), plasmid containing the Cm ^r /Em ^r cassette	27
pLY216	Ap ^r Gm ^r , pLY216 containing the Gm ^r cassette	Unpublished data
<i>Synechocystis</i> strains		
PCC 6803	<i>Synechocystis</i> sp. PCC6803 wild type	Prof. X. Xu
XX55	<i>slr0168::Sp^r-P_{7942psbA1-efe}</i>	This study
XX56	<i>slr0168::Sp^r-P_{7942psbA2-efe}</i>	This study
XX57	<i>slr0168::Sp^r-P_{6803psbA2-efe}</i>	This study
XX76	<i>slr0168::Sp^r-P_{cpcB-efe}</i>	This study
XX77	<i>slr0168::Sp^r-P_{rbc-efe}</i>	This study
XX78	<i>slr0168::Sp^r-P_{ci-efe}</i>	This study
XX79	<i>slr0168::Sp^r-P_{psbD-efe}</i>	This study
XX80	<i>slr0168::Sp^r-P_{glnA-efe}</i>	This study
XX81	<i>slr0168::Sp^r-P_{groESL-efe}</i>	This study
XX102	<i>sll1981::Km^r</i>	This study
XX103	<i>slr0370::Cm^r/Em^r</i>	This study
XX104	<i>sll1981::Km^r/slr0370::Cm^r/Em^r</i>	This study
XX105	<i>sll1981::Km^r-P_{cpcB-efe}</i>	This study
XX106	<i>slr0370::Cm^r-P_{cpcB-efe}</i>	This study
XX107	<i>slr0168::Sp^r-P_{cpcB-efe/sll1981::Km^r-P_{cpcB-efe}}</i>	This study
XX109	<i>slr0168::Sp^r-P_{cpcB-efe/sll1981::Km^r-P_{cpcB-efe/slr0370::Cm^r-P_{cpcB-efe}}}</i>	This study
XX110	<i>slr0168::Sp^r-P_{cpcB-efe/sll1981::Km^r-P_{cpcB-efe/slr0370::Cm^r-P_{cpcB-efe/phaAB::Gm^r-P_{cpcB-kgtP}}}}</i>	This study
Primers (5'→3') ^b		
sll1981-1	GGGCTTCGTTAGGTTGTGTGGC	
sll1981-2	CCGCATGGCCGTTTCCAACCTCC	
slr0370-1	GCCGAGGAATACTTAGCCGATG	
slr0370-2	CTGCCCTATGAACCGAATATGG	
sll1981exp-1	AAATTTGGATCCATGGGGCAAATGAACACCGCAGACC	<i>BamHI</i>

Table 1 (Contd.)

Plasmids, strains and primers	Derivation and/or relevant characteristics ^a	Reference or source
sl1981exp-2	AATAATCTCGAGTTATTCCCAAATTTACAGGCC	<i>XhoI</i>
slr0370exp-1	AAGGATCCATGGCGATCGCCACTATTAAATCCG	<i>BamHI</i>
slr0370exp-2	TTCTCGAGCCCTTAAACTGGAAGTTCCC	<i>XhoI</i>
03psbA2-1	CGGTACCGCGTGCAAGGCCAGTG	<i>KpnI</i>
03psbA2-2	GGCATATGTTGGTTATAATTCCTTAGTTCAGATTGGAAGTACTGACT	<i>NdeI</i>
42psbA1-1	CCGGTACCTGGATTTAGCGTCTTC	<i>KpnI</i>
42psbA1-2	GGCATATGATCGATCTTGAGGTTG	<i>NdeI</i>
42psbA2-1	CGGTACCGAAAAGTCTGAAAGTTC	<i>KpnI</i>
42psbA2-2	GCATATGTTTGCGATGAGTCCTTAGGAGACTTAATAGCCTCG	<i>NdeI</i>
cpcB-1	TGGTACCTTCGTTATAAAATAAAT	<i>KpnI</i>
cpcB-2	TCATATGATTAATCTCCTACTTGAC	<i>NdeI</i>
rbcL-1	GGGTACCCCTTTACCCGCTTACTGCGTCC	<i>KpnI</i>
rbcL-2	GCATATGGGTGAGTCCCTCCATAAACATTGAAT	<i>NdeI</i>
psbD-1	CGGTACCCACCTTCAACAGTCTCCACG	<i>KpnI</i>
psbD-2	CCATATGTGCAAATCCTCTTGCGTAGCTAGC	<i>NdeI</i>
ci-F	TGGTACCTCGAGTAACACCGT	<i>KpnI</i>
ci-R	TCATATGTTTTCCTCCAGCA	<i>NdeI</i>
groESL-1	GGGTACCTGCTTCGCGAGCGGACAACTGT	<i>KpnI</i>
groESL-2	GGGCATATGAGTTTTTGAGTAAACGTCG	<i>NdeI</i>
glnA-1	GGGTACCGCTTGTACTGCATCGCGCATTCC	<i>KpnI</i>
glnA-2	GCGCATATGTGTACTCCTTCTCTGCC	<i>NdeI</i>
efe-1	ATGACTAACTTACAAACCTTCG	
efe-5	GCATATGACTAACTTACAAACCTTCG	<i>NdeI</i>
efe-6	GCTCGAGTTAGTGGTGGTGGTGGTGGGAACCGGTGGCGCGCGTA	<i>XhoI</i>
kgp-1	ATGGCTGAAAGTACTGTAACGG	
kgp-2	TTAAAGACGCATCCCTTCCCT	
Gm-F	TCCTTGTTGATAAGGGGACACT	
Gm-R	GGCCGGAAGCCGATCTCGGCT	
slr0168-1	ACCTCTCCACGCTGAATTAG	
slr0168-2	TTCCAGGCCACATTGTTGTC	
phaAB-1	GGGGACCATCCTGACTACACGG	
phaAB-2	TGTTGATGGTGGGTATCGTGGT	
Cm-F	TCAGATCTCGAATAAATACCTGTGACGGAAGATCATC	<i>BglII</i>
Cm-R	TTACTAGTTCTGCCATTTCATCCGCTTATTATCAC	<i>SpeI</i>
X-F1	TTCCCAAGATTATCGTGGCGAATAAATACCTGTGACGG	<i>XcmI</i>
S-R1	TTCACTAGTCCAAGATTATCGTGGTCTGCCATTTCATCCGCTTAT	<i>XcmI</i>

^a Ap, ampicillin; Km, kanamycin; Sp, spectinomycin; Cm, chloramphenicol; Em, erythromycin; Gm, gentamicin. ^b Restriction enzyme sites added in the primers were underlined and listed.

and *XbaI* sites of pXX56, resulting in pXX69. The Gm^r-P_{cpcB}-*kgp* expression cassette was excised from pXX69 with *EcoRI* and *SpeI* and blunted with T4 DNA polymerase. Next, this cassette was inserted into *NcoI*, digested, and blunted with pKC104,²⁹ resulting in pXX70.

Generation of cyanobacterial transformants

The transformation of wild-type and mutants of *Synechocystis* sp. PCC 6803 was performed as in the established procedures, with minor changes.³⁰ The cells of the mid-exponential phase were collected by centrifugation, washed three times with fresh liquid BG11 medium and resuspended to a cell concentration of approximately 1×10^9 cells mL⁻¹. Next, the cell suspension was mixed with plasmid DNA (final concentration of 10 µg mL⁻¹). After incubation for approximately 4 h at 30 °C, 30 µmol photons m⁻² s⁻¹, the mixture was spread onto nitrocellulose filters, which were rested on BG11 agar plates without antibiotics for 24 h. The filters were then moved to selective BG11 agar plates containing antibiotics at an appropriate concentration. After 1–2 weeks incubation, the resulting transformants were streaked on plates and cultured in liquid

medium under selective pressure from antibiotics. Plasmids pXX32, pXX33, pXX29, pXX47, pXX48, pXX49, pXX50, pXX53, pXX54, pTZ16, pTZ17, pXX64 and pXX65 were transformed into *Synechocystis* sp. PCC 6803 to generate the mutant strains XX55, XX56, XX57, XX76, XX77, XX78, XX79, XX80, XX81, XX102, XX103, XX105 and XX106, respectively. The plasmid pTZ16 was transformed into the *Synechocystis* mutant strain XX103 to generate the double mutant strains XX104. The plasmid pXX64 was transformed into the *Synechocystis* mutant strain XX76 to generate the XX107 mutant strain. The plasmid pXX65 was transformed into the recombinant *Synechocystis* strain XX107 to generate XX109. The plasmid pXX70 was transformed into the recombinant *Synechocystis* strain XX109 to generate XX110. The *Synechocystis* mutant strains used are listed in Table 1, and their genotypes were confirmed by PCR (Fig. 4A) using the primers listed in Table 1.

Cultivation of cyanobacterial strains

(i) **Liquid shaking cultivation in a 50 mL flask.** For inoculum purposes, *Synechocystis* strains were cultivated in 30 mL

Fig. 2 Promoter screening for improving ethylene production. The ethylene forming enzyme gene in genetically engineered *Synechocystis* strains was under the control of *PcpbC* (XX76), *Prbc* (XX77), *PpsbD* (XX79) and *PpsbA2* (XX57) from *Synechocystis* sp. PCC 6803; *PpsbA1* (XX55), *PpsbA2* (XX56) and *PgroESL* (XX81) from *Synechococcus elongatus* PCC 7942; *PglbA* (XX80) from *Anabaena* sp. PCC 7120; and *Pcl* (XX78) from λ -phage. Ethylene productivity was measured daily for 8 days. (A) The schematic structure of the promoter-probing vector pTZ10b shows how the expression cassettes were integrated into the *str0168* neutral site of *Synechocystis* sp. PCC 6803. (B) Comparison of the highest specific ethylene production rate. (C) Comparison of the highest volumetric ethylene production rate. Data presented are the means and standard deviation of two independent experiments, each with three replicates.

BG11 medium at 30 °C, 30 μmol photons m⁻² s⁻¹, with rigorous shaking at 220 rpm at the initial OD₇₃₀ ~ 0.05.

(ii) Cultivation on plates. BG11 agar plates, used to maintain the *Synechocystis* strains, were supplemented with 1.4% agar, 8 mM TES (pH 8.2), and 0.3% Na₂S₂O₃.

(iii) Aerated cultivation in 250 mL flasks. Cyanobacterial seed cultures of the exponential phase were diluted to OD₇₃₀ ~ 0.05 in 250 mL flasks containing 200 mL BG11 medium and then were bubbled continuously with 5% CO₂-air (v/v). For semi-continuous cultivation, stationary phase cultures were collected

by centrifugation and resuspended at the same cell concentration in fresh 5 × BG11 but with 1 × nitrate (hereafter referred to as 5 × BG11*), allowing it to resume log phase growth at a light intensity of 200 μmol photons m⁻² s⁻¹. The aerated cultivation included a culture process (for 6 days) and a collecting-resuspending process (repeated every 3 days for a total of 21 days).

Mutants were grown by adding the corresponding antibiotics of appropriate concentrations for all of the cultivation manners mentioned above (kanamycin, 30 μg mL⁻¹; spectinomycin 10 μg mL⁻¹; chloramphenicol, 10 μg mL⁻¹; gentamycin, 10 μg mL⁻¹. When two or more antibiotics were used, the concentrations of antibiotics were reduced by half).

Chlorophyll a determination and photosynthetic activity analysis

Wild type and recombinant *Synechocystis* strains were grown autotrophically to a mid-exponential growth phase (OD₇₃₀ ~ 2.5). Cells were then collected by centrifugation and resuspended in fresh BG11 medium to OD₇₃₀ ~ 1. Oxygen evolution rates were measured with a Clark-type oxygen electrode at 30 °C with a saturating actinic light of 300 μmol photons m⁻² s⁻¹. Chlorophyll a concentrations in the cultures were determined spectrophotometrically in 90% methanol extracts of the cells, according to Meeks and Castenholz.³¹

Protein expression and purification in *E. coli*

E. coli BL21 (DE3) (Takara) was transformed with plasmids pZL105 and pZL106, respectively. One liter of LB medium was inoculated with 10 mL of the overnight cultured transformed *E. coli* BL21 (DE3) from a single colony and grown at 37 °C. Cell cultures were induced by adding 0.1 mM isopropyl- β -thiogalactopyranoside (IPTG) at OD₆₀₀ ~ 0.6, and the cultures were incubated at 16 °C overnight while shaking at 180 rpm. Thereafter, cells were harvested by centrifugation, resuspended in binding buffer (20 mM Tris-HCl, 50 mM NaCl and 5 mM imidazole, pH 7.5), and lysed by sonication. The cell lysate was pelleted by centrifugation at 10 000g and 4 °C for 30 min. The supernatant was immediately added to the Ni-NTA resin (Novagen) and pre-equilibrated with the binding buffer, which was gently agitated at 4 °C for 1 h. The resin was transferred into a 5 mL column that was washed sequentially with 5 column volumes of the binding buffer, five column volumes of the washing buffer containing 20 mM, 60 mM, and 100 mM imidazole to remove nonspecifically bound proteins, and then 25 mL of the washing buffer containing 250 mM imidazole to elute the target protein. The eluted proteins were examined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified proteins were subsequently desalinated with 20 mM Tris-HCl (pH 7.5) and concentrated in 30% PEG20000 before quantitation using the Bradford method and measurements of enzymatic activity.

Sample preparation for the crude enzyme analysis of cyanobacterial cells

Cyanobacteria cultures (30 mL) of the mid-exponential phase (OD₇₃₀ ~ 2.0) were centrifuged. The harvested cells were

quickly frozen with liquid nitrogen and resuspended in 1 mL ice-cold Tris buffer (50 mM Tris-HCl, pH 8.0) with 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were disrupted using glass beads (0.25 to 0.30 mm; Sigma-Aldrich) by vigorous vortex mixing of the tube for 10 min in cycles of mixing for 30 s and cooling on an ice-water bath for 30 s. The homogenate was subsequently centrifuged to remove the cell debris and unbroken cells at 12 000g, 4 °C, for 30 min. The supernatant was then collected for western blotting analysis or enzymatic assay.

OGDC and SSADH activity assay *in vitro*

OGDC- and SSADH-coupled reactions were conducted according to Zhang and Bryant,²⁴ with minor modifications. The reaction mixture (0.2 mL) contained 4 mM 2-OG, 100 mM K-phosphate, pH 7.0, 0.3 mM TPP, 2 mM DTT, 1 mM MgCl₂, 2 mM NADP⁺, 50 µg purified Sll1981 (OGDC) and 50 µg purified Slr0370 (SSADH). The production of NADPH was monitored at 340 nm immediately after the reaction mixture was incubated to room temperature using a Beckman-coulter DU-800 spectrophotometer. Control experiments were performed by removing the 2-OG substrate or one of the enzymes. We also used the crude enzyme of wild type, *sll1981* mutant (XX102), *slr0370* mutant (XX103) and the double mutant (XX104) to complete the functional complementation enzymatic assay.

Western blotting analysis

Total soluble proteins extracted from the samples were separated by 12% SDS-PAGE according to the standard procedure and electroblotted onto PVDF membranes, which were sealed in 5% nonfat milk-TBST (0.05% Tween-20 in TBS) at room temperature for 30 min. First, the membranes were incubated with the anti-His₆-tag monoclonal antibodies for 1 h and washed three times with TBST (15 min each). Second, the membranes were incubated with an alkaline phosphatase-linked secondary antibody for 1 h and washed three times with TBST (15 min each). Finally, the membranes were colored with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

Addition and concentration determination of 2-oxoglutarate

One milliliter of cyanobacterial culture of OD₇₃₀ ~ 3.0 was transferred to a sterile 10 mL penicillin bottle. After adding 150 nmol 2-OG (the final 2-OG concentration in the culture was 150 µM), the bottle was sealed and incubated for 1 h under the standard culturing conditions. Then, the culture was centrifuged at 10 000g, and the changes of 2-OG in the supernatant was determined using a 2-OG assay kit (Sigma-Aldrich, USA).

In this kit, the 2-OG converting enzyme (glutamic-pyruvic transaminase) catalyzes the reaction of 2-OG with pyruvate and glutamate. The 2-OG development enzyme mix (Pyruvate oxidase and horseradish peroxidase) then catalyzes a nearly colorless specialized fluorescent probe (fluorescent peroxidase substrate) into a red-fluorescent oxidation product (resorufin),

which has fluorescence excitation and an emission maxima of approximately 571 nm and 585 nm and an absorbance maxima of 570 nm. The colorimetric detection (OD₅₇₀) approach was employed in this study. The procedures were as follows: (i) preparation of the standard. Six concentrations of 0 (blank), 2, 4, 6, 8, and 10 nmol per well standards were prepared using 2-OG assay buffer for a total volume of 50 µl in a 96-well flat-bottom plate. (ii) Preparation of samples. The pipetted supernatant medium samples needed to be tested on the 96-well plate (50 µl per well). (iii) Preparation of the reaction mixture. Add 50 µl of the appropriate reaction mix (containing 46 µl 2-OG assay buffer, 2 µl 2-OG converting enzyme, 2 µl 2-OG development enzyme and 2 µl fluorescent peroxidase substrate) to each of the blank, standard, and test wells. Mix well by pipetting, and incubate the reaction for 1 h at 37 °C, protecting the plate from light during the incubation. (iv) A standard curve for 2-OG quantification was constructed based on the 2-OG standard by measuring the OD₅₇₀. Changes of the 2-OG in the sample were calculated based on this standard curve. Prior to extracellular 2-OG determination, the ethylene production was analyzed using a gas chromatograph (GC) equipped with a flame ionization detector (FID), as mentioned below.

Ethylene production assay

During the experiment, one milliliter of the cultured *Synechocystis* cells was transferred to a sterile 10 mL penicillin bottle, sealed with a sterile rubber stopper, and then incubated for 1 h under the same conditions as those for cultivating the cells. At the end of the incubation period, 200 µl gas samples were extracted from the headspace with a gas-tight syringe (500 µl measurement range) for the following analysis. Qualitative analysis of the gas product was performed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890A system equipped with a HP-INNOWax column (30 m × 250 µm × 0.25 µm) through the comparison of retention times and mass spectra with the pure ethylene standard (Fig. S2B†). The quantitation analysis of ethylene production was performed on a GC-FID with the help of an ethylene calibration curve (Fig. S2A†). The injection and oven temperature was 100 °C, and the detector temperature was 200 °C. Nitrogen (constant flow 1 mL min⁻¹) was used as the carrier gas.

Results and discussion

Stable production of ethylene in *Synechocystis* sp. PCC 6803 by introducing a codon-optimized *efe* gene

The ethylene forming enzyme (EFE) is a member of the superfamily of Fe²⁺/ascorbate oxidases. Two reactions, the L-arginine-dependent ethylene formation reaction and the 2-OG-dependent dioxygenase reaction, are simultaneously catalyzed by EFE.³² The EFE-catalyzing ethylene synthetic pathway was first reported for *Penicillium digitatum* and has been extensively studied in *Pseudomonas syringae*.^{18,33} Although it had been reported that there is a functional ethylene-binding domain in

the *slr1212* coding sequence in *Synechocystis* sp. PCC 6803,³⁴ ethylene was proved to have no influence on the growth of *Synechocystis*, either at high levels (95%) or low levels (1%).^{3,21}

Although the *efe* of *Pseudomonas syringae* pv. *phaseolicola* was frequently used for ethylene biosynthesis in genetically engineered cyanobacteria and other microorganisms in previous studies,^{3,14,15,18–21} *Pseudomonas syringae* *sesami* was proved to be more productive (approximately 4×10^{-7} nL cell⁻¹ h⁻¹) than other strains, according to the ethylene production rate screening assay of different *Pseudomonas syringae* pathovars described by Weingart *et al.*³⁵. Hence, the *efe* of *Pseudomonas syringae* *sesami* was used for ethylene production in this study. Actually, there are two single-base pair substitutions present, leading to one amino acid sequence change between the *efe* copies of these two ethylene-producing strains.³⁵ The *efe* of *Pseudomonas syringae* *sesami* was synthesized by Sangon (Shanghai, China) with two major modifications. It was codon-optimized according to *Synechocystis* sp. PCC 6803, and three hot spots of spontaneous mutations (CGATG) were avoided by silent mutations (Fig. S1†). We also introduced a His₆-Tag to EFE using the primer *efe*-6 (Table 1) in order to track the expression of the EFE protein in recombinant *Synechocystis* strains.

The promoter of *psbA2*, which encodes a D1 protein homolog in the reaction center of the photosynthetic photosystem II of *Synechocystis* sp. PCC 6803, is an efficient promoter widely used for protein expression in cyanobacteria.^{36,37} In this study, we modified the sequence of *PpsbA2* (using primers 03psbA2-2 in Table 1) by deleting the AT box (9–18 bp upstream from the ATG start codon) to enhance the mRNA stability.^{36,38} The synthetic *efe* gene was driven by the modified *PpsbA2* and integrated into the *Synechocystis* genome at a neutral site (*slr0168*) by homologous recombination. Compared with the wild-type, the resulting recombinant strain (XX57) exhibited a similar growth rate (Fig. S3A†). In order to calculate the ethylene yield of the transformants, a serial dilution of vaporized pure ethylene standards was prepared, and an ethylene calibration curve was constructed based on the amount of ethylene relative to the peak area from GC analysis (Fig. S2A†). We further confirmed that the gas product generated by *efe* transformants was ethylene by GC-MS analysis (Fig. S2B†). Our results indicated that the XX57 strain could reach a peak specific ethylene production rate of 518 $\mu\text{L L}^{-1} \text{h}^{-1}$ OD730⁻¹ (ethylene production rate of 121 $\mu\text{L L}^{-1} \text{h}^{-1}$) under standard culture conditions (Fig. S3B†) while maintaining a stable ethylene synthetic capacity in the following subculture process (data not shown).

Promoter screening for higher ethylene productivity

While photoautotrophic cyanobacteria are well-recognized as promising microbial cell factories for the production of biofuels and biochemicals, the development of biosynthetic parts for this organism lags behind what has been developed for their heterotrophic companions.³⁷ In previous studies, promoters or their variants developed in *E. coli* and yeast often did not function as expected in cyanobacteria.³⁹ In terms of ethyl-

ene biosynthesis in cyanobacteria, native metal-inducible promoters (*PpetE*, *PcoaT*, *PsmtA*, *etc.*) have been tested using a shuttle vector but failed to produce a high ethylene yield.²¹ The *E. coli*-originated Trc promoter and Lac promoter variant (*P_{A1lacO-1}*) were also investigated, and it was found that the control of the Lac-derived promoter became progressively more relaxed as the cyanobacterial culture grew denser.²¹ Thus, it is important to select an appropriate promoter for effective EFE expression to achieve higher ethylene productivity.

Eight identified strong promoters, including *PcpcB* (promoter of the *cpcBAC* operon, which encodes the protein phyco-cyanin⁴⁰), *Prbc* (promoter of the *rbc* operon, which encodes ribulose-1,5-bisphosphate carboxylase/oxygenase⁴¹) and *PpsbD* (promoter of the *psbD* gene, which encodes the photosystem II D2 protein⁴²) from *Synechocystis* sp. PCC 6803; *PpsbA1* (promoter of *psbA1*, which is one of the three genes encoding the photosystem II D1 protein⁴³), *PpsbA2* (promoter of *psbA2*, which is one of the three genes encoding the photosystem II D1 protein) and *PgroESL* (promoter of the *groESL* operon, which encodes the heat shock protein⁴⁴) from *Synechococcus elongatus* PCC 7942; *PglnA* (promoter of the *glnA* operon, which encodes glutamine synthetase⁴⁵) from *Anabaena* sp. PCC 7120; and *PcI* (promoter of the CI repressor protein) from λ -phage were incorporated into the promoter-probing vector pTZ10b and integrated into the genome of *Synechocystis* sp. PCC 6803 at the *slr0168* neutral site (Fig. 2A). To evaluate the strength of the aforementioned promoters, both the ethylene production rate and specific ethylene production rate of the resulting *Synechocystis* recombinants were measured. Our results indicated that the specific ethylene production rate and volumetric ethylene production rate varied greatly based on different promoters. *PcpcB* and *Prbc* were stronger than the frequently used *PpsbA* in cyanobacterial ethylene biosynthesis. This was especially true for *PcpcB* (XX76), which reached a peak specific ethylene production rate of 774 $\mu\text{L L}^{-1} \text{h}^{-1}$ OD730⁻¹ and a volumetric ethylene production rate of 221 $\mu\text{L L}^{-1} \text{h}^{-1}$ during a 8-day cultivation (Fig. 2B,C). Guerrero *et al.* described that the method of *efe* introduction into the host cells (chromosomal integration *versus* plasmid-based) appeared to have no influence on the ethylene's evolutionary stability.²¹ Our findings were consistent with those reported by Sakai and coworkers, who also emphasized the importance of promoters.¹⁹ According to the results of promoter screening, *PcpcB* was used for ethylene production in the following study.

Improving ethylene production by genetic modification of the TCA cycle

The recent identification of 2-OG decarboxylase (OGDC) and succinic semialdehyde dehydrogenase (SSADH) in *Synechococcus* sp. PCC 7002, which perform the function of the missing 2-OG dehydrogenase (OGDH), closed the long-lasting misinterpreted cyanobacterial TCA cycle.^{24,46} Zhang and Bryant showed that both the deletion and overexpression of either SynPCC7002_A2770 (encoding OGDC) or SynPCC7002_A2771 (encoding SSADH) in *Synechococcus* sp. PCC 7002 affected the

cell growth rate (particularly the overexpression of SynPCC7002_A2770). Although the reasons resulting in these phenotypes remain unclear, interpretations based on the intracellular levels of TCA intermediates have been proposed.^{46,47} As the ethylene-producing pathway employed in this study can catalyze the conversion of 2-OG and arginine into ethylene and succinate, it can actually provide an alternative to OGDC and SSADH of the cyanobacterial TCA cycle. Hence, substituting EFE for the OGDC-SSADH pathway might be a feasible strategy to redirect the metabolic flux toward the alternative EFE pathway and to improve the ethylene productivity.

(i) Identification of OGDC and SSADH homologs in *Synechocystis* sp. PCC 6803. OGDC and SSADH are highly conserved throughout the vast majority of cyanobacteria with known genome sequences. Amino acid sequence identities $\geq 60\%$ with OGDC and SSADH from *Synechococcus* sp. PCC 7002 were partially listed in Table S1.† Gene analogs encoding these two enzymes can be on co-located genes (like in *Synechococcus* sp. PCC 7002) or not. In *Synechocystis* sp. PCC 6803, *sll1981* and *slr0370*, which are not neighboring genes, are supposed to encode OGDC and SSADH by sequence alignment. Up to date, there are no experimental observations on the functional role of these two proteins in the TCA cycle except for a hypothetic metabolic flux analysis.²³ First, we verified that Sll1981 and Slr0370 functioned as OGDC and SSADH by *in vitro* enzymatic assays. Their coding genes were separately expressed in *E. coli*, and the resulting soluble fusion proteins were purified (Fig. S4†). When the purified Sll1981 and Slr0370 were incubated with 2-OG and other necessary cofactors, NADPH was synthesized in the reaction mixture. If 2-OG was removed, no NADPH was produced. If the reaction mixture lacked either of these enzymes, NADPH would not be produced (Fig. 3A). Subsequently, *sll1981* and *slr0370* were interrupted individually and in combination (Fig. 4A), and the cell

crude extract was used to further verify the enzyme activity of these two enzymes by the functional complementation assay *in vitro*. When the purified Sll1981 was incubated with the crude extract of *slr0370*-interrupted mutant (XX103) or *sll1981/slr0370* double-interrupted mutant (XX104) (Table 1), NADPH was synthesized at the background level. However, when Sll1981 was combined with the crude extract of *sll1981* mutant (XX102), an OGDC and SSADH coupled reaction could be well conducted, and NADPH was produced at a higher level (Fig. 3B).

Previously, Sll1981 of *Synechocystis* sp. PCC 6803 was putatively assigned as a bifunctional enzyme with conserved domains for both acetolactate synthase (ALS) activities⁴⁸ and L-myo-inositol 1-phosphate synthase (MIPS).⁴⁹ Nevertheless, our enzymatic analysis showed that Sll1981 and Slr0370 functioned as OGDC and SSADH, respectively. Meanwhile, our results were in line with the metabolic flux analysis observed by Knoop and coworkers.²³

(ii) Introduction of EFE eliminates the metabolic burden caused by the inactivation of OGDC and SSADH. As shown in Fig. 4B, the inactivation of *sll1981* or *slr0370* resulted in a slight reduction of cell growth. The growth rate of the *slr0370* mutant (XX103) without SSADH activity was more affected than that of the *sll1981* mutant (XX102). If these two genes were interrupted together (XX104), the cell growth rate lost approximately 20% compared with the wild-type (Fig. 4B). To further understand the possible reasons causing growth inhibition in the *Synechocystis* mutants, oxygen evolution abilities were measured. Not surprisingly, photosynthesis in the mutants was more severely affected. More than half of the oxygen evolution was lost in the double mutant strain (XX104) (Fig. 4C). Zhang and Bryant proposed two possible reasons leading to these growth defects. One is the lower intracellular level of 2-OG, the other one is the accumulation of toxic succi-

Fig. 3 Enzymatic assays for the OGDC and SSADH activity of *Synechocystis* sp. PCC 6803 by monitoring the absorbance increase at 340 nm for NADPH production. (A) Enzymatic assay using purified Sll1981 and Slr0370. The reaction mixture was set as indicated in the materials and methods section. (Sll1981+Slr0370)+2-OG: Sll1981 and Slr0370 coupled reaction; (Sll1981+Slr0370)-2-OG: coupled reaction lacking of 2-OG; Sll1981+2-OG: coupled reaction without Slr0370 protein; Slr0370+2-OG: coupled reaction without Sll1981 protein. (B) Functional complementation assay using the purified protein and the whole-cell crude extracts of mutants. The reaction mixtures were set as indicated, and 50 μ g of the whole-cell extracts was used. The Sll1981+XX102: coupled reaction was catalyzed by the purified Sll1981 and the whole-cell crude extracts of the *sll1981* mutant (XX102); the Sll1981+XX103: coupled reaction was catalyzed by the purified Sll1981 and the whole-cell crude extracts of *slr0370* mutant (XX103); and the Sll1981+XX104: coupled reaction was catalyzed by the purified Sll1981 and the whole-cell crude extracts of the *sll1981/slr0370* double mutant (XX104).

Fig. 4 (A) Genotype analysis of *Synechocystis* sp. PCC 6803 recombinants. Lane 1: 1 kb DNA Ladder; lane 2: genomic DNA of wild-type was amplified by primers *sll1981*-1 and *sll1981*-2 (control); lane 3: genomic DNA of XX102 was amplified by primers *sll1981*-1 and *sll1981*-2; lane 4: genomic DNA of wild-type was amplified by primers *slr0370*-1 and *slr0370*-2 (control); lane 5: genomic DNA of XX103 was amplified by primers *slr0370*-1 and *slr0370*-2; lane 6: genomic DNA of XX104 was amplified by primers *sll1981*-1 and *sll1981*-2; lane 7: genomic DNA of XX104 was amplified by primers *slr0370*-1 and *slr0370*-2; lane 8: genomic DNA of wild-type was amplified by primers *slr0168*-1 and *slr0168*-2 (control); lane 9: genomic DNA of XX76 was amplified by primers *slr0168*-1 and *slr0168*-2; lane 10: genomic DNA of XX105 was amplified by primers *sll1981*-1 and *sll1981*-2; lane 11: genomic DNA of XX106 was amplified by primers *slr0370*-1 and *slr0370*-2; lanes 12, 13: genomic DNA of XX107 was amplified by primers *sll1981*-1/*sll1981*-2 and *slr0370*-1/*slr0370*-2, respectively; lanes 14–16: genomic DNA of XX109 was amplified by primers *slr0168*-1/*slr0168*-2, *sll1981*-1/*sll1981*-2 and *slr0370*-1/*slr0370*-2, respectively; lane 17: genomic DNA of wild-type was amplified by primers *phaAB*-1 and *phaAB*-2 (control); lanes 18–21: genomic DNA of XX110 was amplified by primers *phaAB*-1/*phaAB*-2, *slr0168*-1/*slr0168*-2, *sll1981*-1/*sll1981*-2 and *slr0370*-1/*slr0370*-2. (B) The cell growth ability of *Synechocystis* sp. PCC 6803 wild type and mutants. All strains were initially inoculated at OD₇₃₀ ~ 0.05 and cultured for 7 days under standard conditions. The data presented are the ratios of the final OD₇₃₀ to that of the *Synechocystis* sp. PCC 6803 wild type. (C) Photosynthesis activities of *Synechocystis* sp. PCC 6803 wild type and mutants. Oxygen evolution rates were measured as indicated in the materials and methods section. Data presented are the mean and standard deviation of two independent experiments, each with three replicates.

nic semialdehyde (SSA). 2-OG is the major precursor of several essential components of the photosynthetic reaction center (chlorophyll, phycocyanobilin *etc.*). The reduction of the photosynthetic activity might be caused by failing to synthesize an

appropriate light-harvesting complex owing to the disruption of *sll1981* and/or *slr0370*. Another possibility was increased oxidative stress on the photosynthetic machinery triggered by interruption of the essential TCA cycle.

To determine the effects of introducing EFE on the OGDC and SSADH pathways, the *efe* gene was inserted into the *sll1981* and *slr0370* sites, respectively. Interestingly, we found that the introduction of the ethylene-producing pathway eliminated the metabolic stress caused by the lack of OGDC and SSADH. As shown in Fig. 4B and C, *sll1981::Km^r-P_{cpcB}-efe* (XX105) and *slr0370::Cm^r-P_{cpcB}-efe* (XX106) completely recovered their cell growth and oxygen evolution ability. Meanwhile, the fact that the physiological phenotypes of these *efe* transformants showed no significant differences from those of *Synechocystis* sp. PCC 6803 wild-type further verified the stable expression of the codon-optimized *efe* gene (Fig. 4B, C).

(iii) Ethylene production was enhanced in *Synechocystis* recombinants with multiple *efe* copies and a modified TCA cycle. *Synechocystis* recombinants, which harbor two *efe* copies at the *slr0168* neutral site and *sll1981* locus (XX107) or three *efe* copies at the *slr0168* neutral site, *sll1981* locus and *slr0370* locus (XX109), were constructed (Table 1, Fig. S5†). The EFE protein expression level in the recombinant strains of single, double and triple *efe* copies were detected by western blotting using the anti His₆-tag antibody. The expression of *efe* was predicted to increase, along with the increase in *efe* copy number (Fig. 5A, B).

The XX107 recombinant strain, which harbors two *efe* copies at the sites of *slr0168* and *sll1981*, reached an ethylene productivity of 439 μL L⁻¹ h⁻¹ in the aerated cultivation (5% CO₂) in 250 mL flasks. The strain with triple copies of *efe* (XX109) achieved a maximum ethylene production rate of 542 μL L⁻¹ h⁻¹, accompanied by the highest EFE expression level among all of the recombinants (Fig. 5A, B). All of these *efe* transformants showed similar growth abilities, according to the growth curve shown in Fig. 5D. Comparing the ethylene productivity of XX105 with that of XX76 indicated that the disruption of *sll1981* led some metabolic flux to the ethylene-producing branch and then to higher ethylene productivity. Regarding XX106 and XX76 only inactivation of *slr0370* seemed to have a tiny effect on the ethylene productivity. The improvement of the ethylene productivity might be explained as an integrated effect of increasing *efe* copy numbers and blocking the TCA cycle shunts.

Improving ethylene production by heterologous expression of 2-OG permease

The supply of 2-OG, the substrate of the EFE enzyme, is of great importance for ethylene production; 2-OG is also the carbon skeleton for the assimilation of nitrogen within cyanobacteria. 2-OG permease (KgtP) from *E. coli*, which showed 28% amino acid sequence identity to the glucose permease (GlcP) of *Synechocystis* sp. PCC 6803, has been reported to be able to actively incorporate 2-OG in *Synechococcus elongatus* PCC 7942 but to be detrimental to the growth of the *kgtP*-bearing strain.²⁵ Since the inactivation of the *phaAB* operon

Fig. 5 Ethylene production by *Synechocystis* sp. PCC 6803 mutants with expression of multiple *efe* copies at different gene loci. (A) Western blotting detection of EFE in recombinant ethylene-producing strains. *Synechocystis* sp. PCC 6803 wild-type was used as a control. (B) SDS-PAGE electrophoretogram. The proteins were loaded at equal amounts. (C) Comparison of ethylene production from *Synechocystis* recombinants harboring one copy of *efe* driven by P_{cpbB} at *slr0168* locus (XX76), *slr1981* locus (XX105) and *slr0370* (XX106); two copies of *efe* driven by P_{cpbB} at both *slr0168* and *slr1981* loci (XX107); or three copies of *efe* driven by P_{cpbB} at these three gene loci (XX109). *Synechocystis* sp. PCC 6803 wild-type was used as a control. (D) Growth curves of *Synechocystis* sp. PCC 6803 mutants. *Synechocystis* sp. PCC 6803 wild-type was used as a control. Data presented are means and standard deviation of two independent experiments, each with three replicates.

had no effect on the cell growth reported by Taroncher-Oldenburg *et al.*⁵⁰ the *E. coli kgtP* driven by P_{cpbB} was integrated into the *phaAB* sites on the genome of *Synechocystis* strain XX109 in this study, resulting in strain XX110 (Fig. 4A, S5†). The XX110

strain grew normally in $1 \times \text{BG11}$ medium when it was cultivated in 50 mL shaking flasks without aeration and on the plate (data not shown). However, its growth was severely impacted when it was cultured in $1 \times \text{BG11}$ medium bubbled with 5% CO_2 (Fig. 6A). Furthermore, XX110 lost the growing ability completely when it was cultivated under the aeration conditions with the addition of 1000 μM 2-OG (Fig. 6A). Possibilities leading to the negative effect of 2-OG feeding on the cell growth of the *kgtP*-bearing cyanobacteria mutant include the following: imbalance of metabolites within the cells, bioenergetic shortage caused by 2-OG transport and affecting other membrane transport functions, and the change of intracellular pH caused by the influx of a dicarboxylate, *etc.*²⁵ Our finding that bubbling with 5% CO_2 imposed greater stress on the growth of the *kgtP*-bearing mutant provoked us to focus on changing the culture conditions for a stable growth of XX110 under 2-OG feeding conditions. When the culture medium was changed to $5 \times \text{BG11}^*$, the recombinant *Synechocystis* strain XX110 exhibited a stable growth phenotype, similar to XX109 under aerating conditions, regardless of whether 2-OG was added or not (Fig. 6A). Hence, these optimal culture conditions were used for the following ethylene production assays.

For the sake of determining whether the addition of 2-OG promotes ethylene production, a series of concentrations of 2-OG were added to the $5 \times \text{BG11}^*$ culture medium. As shown in Fig. 6B, the ethylene productivity of XX110 increased more than that of XX109 when both strains were fed with less than 1 mM 2-OG. It is reasonable to suggest that XX110 can assimilate 2-OG actively from the medium; however, the uptake of 2-OG in XX109 is dependent on diffusion. Surprisingly, when the concentration of 2-OG rose beyond 1 mM, the ethylene productivity of XX109 decreased sharply, whereas XX110 encountered a similar problem when the concentration of 2-OG exceeded 2 mM (Fig. 6B). This phenomenon was most likely caused by the acid stress on the cell because the pH value in the medium started to decrease at 1 mM 2-OG. As a result, 1 mM was used as the optimal concentration of exogenous added 2-OG for ethylene production.

The amount of 2-OG flowing to the ethylene-forming pathway was calculated using the 2-OG concentration assay kit. To avoid the effect of low-affinity uptake mentioned by Vazquez-Bermudez and coworkers at high 2-OG concentrations,²⁵ a total amount of 150 nmol 2-OG was added to 1 mL culture sealed in a penicillin bottle (2-OG concentration in the culture was 150 μM). As shown in Fig. 6C, the extracellular 2-OG in the culture of XX110 ($\text{OD}_{730} \sim 3.0$) dropped from 150 nmol to 80 nmol, while no obvious decrease was found in the culture of XX109 ($\text{OD}_{730} \sim 3.0$). In the meantime, there was an increase in ethylene production from 548 $\mu\text{L L}^{-1} \text{h}^{-1}$ to 769 $\mu\text{L L}^{-1} \text{h}^{-1}$ in XX110 culture. Based on the reaction catalyzed by EFE (Fig. 1A),⁵¹ the increased 221 nL (221 $\mu\text{L L}^{-1} \text{h}^{-1} \times 1 \text{ mL} \times 1 \text{ h}$) ethylene needs approximately 9.886 nmol of 2-OG substrate, accounting for 14% of the uptaken 2-OG (70 nmol), which means that 14% of the uptaken 2-OG flows into the EFE pathway in 1 hour at a feeding concentration of 150 μM . As mentioned above, 2-OG stands at the crossroad

Fig. 6 Effect of introducing the *kgtP* gene from *E. coli* on the growth and ethylene production in *Synechocystis* recombinants harboring triple copies of *efe*. (A) Growth curve of XX109 and XX110 in 1 × BG11 and 5 × BG11* medium, with or without 2-OG added. (B) Ethylene production and pH value (after 1 h incubation) of the culture of XX109 and XX110 at different concentrations of 2-OG. One milliliter normally cultivated cyanobacterial culture (OD₇₃₀ ~ 3.0) was split into sterile 10 mL penicillin bottles. Then a gradient concentration of 2-OG (from 0 to 5000 μM) were added to the corresponding bottles. The bottle was sealed and incubated for 1 h under standard culture conditions. Ethylene productivity was measured as mentioned in the materials and methods section. The pH values of the culture after the 1 h incubation were measured using a pH electrode. (C) Analysis of the incorporation of 2-OG within the ethylene-forming pathway in *Synechocystis* recombinants XX109 and XX110 fed with 2-OG. The amount of 2-OG in the culture medium was shown by grey columns. Left columns represent before 1 hour incubation, and right columns represent after 1 hour after incubation. The increasing ethylene production was shown by lines above the grey columns. Data presented are the means and standard deviation of two independent experiments, each with three replicates.

between the carbon and nitrogen metabolism. The incorporated 2-OG in XX110 might function not only as a substrate for ethylene synthesis but also as a regulator affecting C/N meta-

bolic flux. Transporting 2-OG into the cells, in combination with increasing the concentration of nutrients, afforded the *kgtP*-bearing strain XX110 a stable and improved ethylene-producing capacity. The phenotypes of cell growth and oxygen evolution of XX110 in 5 × BG11* indicated a coordinated metabolic status in this recombinant strain (Fig. 4B, C). Ultimately, 2-OG feeding led to a higher ethylene volumetric production rate of 1038 μL L⁻¹ h⁻¹ (Fig. 6B).

Improving ethylene production by modifying semi-continuous cultivation

It was reported that fresh BG11 medium resuspension could recover the ethylene synthetic capacity of the recombinant ethylene-producing strains.³ Although the components of such a medium leading to this recovery were not clear yet, it was known that neither nitrogen-addition nor nitrogen-depletion contributed to the ethylene yield.³ At the same time, it was found that the intracellular concentration of 2-OG increased under nitrogen starvation conditions.^{52,53} Because ethylene production and nitrogen assimilation are competitive pathways, the condition of relatively limited nitrogen might facilitate cyanobacterial self-regulation of an intracellular pool of 2-OG, glutamine, amino acids and γ-aminobutyric acid (GABA), enabling higher ethylene productivity. So, in the semi-continuous cultivation here, 5 × BG11* was used to resuspend the previously cultured recombinant strain XX110 in 250 mL flasks containing 200 mL culture. The initial inoculation concentration was OD₇₃₀ ~ 0.1, and a 6-day culture process followed by a 21-day collection–resuspension repeating process was employed. The resuspension of the previous culture (without dilution) in fresh 5 × BG11* was repeated every 3 days. Ethylene production was measured every day over the course of the experiment. During incubation in the sealed penicillin bottle, 1 mM 2-OG was added to XX110 culture; the sample without 2-OG was used as the control. As shown in Fig. 7, the ethylene production rate varied according to the resuspension time point and as to whether 2-OG was added. High-level ethylene production resumed gradually after resuspension in fresh 5 × BG11* and declined after reaching a peak yield at the end of each resuspension cycle (Fig. 7A). Obviously the ethylene productivity of XX110 fed with 2-OG was higher than that without 2-OG. At the end of the third resuspension cycle, the ethylene productivity had reached 6120 μL L⁻¹ h⁻¹, which surpassed the highest cyanobacterial ethylene production (5650 μL L⁻¹ h⁻¹) that had ever been reported.³ The peak ethylene production rates of 9739 μL L⁻¹ h⁻¹ (2-OG feeding) and 7514 μL L⁻¹ h⁻¹ (no 2-OG feeding) were achieved finally in the semi-continuous culture of XX110 (Fig. 7A). Compared with the collection–resuspension process, the rise in volumetric ethylene production rate was not so high in the early culture process (the first 6 days); this might be the result of the primary carbon flux toward biomass accumulation. Hence, it is reasonable to suggest that the specific ethylene production rate went through a rapid decline when normalizing a high OD₇₃₀ (Fig. 7B). Even so, the specific ethylene production rate achieved in this study exceeded all others ever

Fig. 7 Cultivation and resuspension of XX110 recombinants in fresh 5 × BG11* medium, with 2-OG added or not. (A) Total ethylene production rate; (B) specific ethylene production rate; (C) growth curve of XX110 in 5 × BG11* medium in the semi-continuous cultivation. Data presented are the means and standard deviation of two independent experiments, each with three replicates.

reported, and the highest rate of $858 \mu\text{L L}^{-1} \text{h}^{-1} \text{OD}_{730}^{-1}$ was reached in the culture process (Fig. 7B). Regular medium replenishment could recover the cell growth which it otherwise should have ceased, and the tendency toward cell growth was generally in agreement with what was reported by Ungerer and coworkers³ (Fig. 7C). 2-OG feeding was proven to have no obvious promoting effect on the ethylene production of the XX109 (Fig. S6†), further verifying the viewpoint that extracellular 2-OG cannot be effectively assimilated by the *Synechocystis* sp. PCC 6803 wild-type.

Finally, a modified gaseous/aqueous two-phase photobioreactor⁵⁴ was used for ethylene continuous collection

(Fig. S7†). As shown in Fig. S8A,† the three dilutions administered in the experiment changed the cell density from $\text{OD}_{730} \sim 4.0$ (prior to the dilution) to about $\text{OD}_{730} \sim 2.5$ (after the dilution) for both XX109 and XX110 cultures. A total of more than 31 119 μL ethylene in XX109 and 50 633 μL ethylene in XX110 with 2-OG feeding was finally collected during the fed-bath culture process (Fig. S8B†). These results once again reinforced the advantage of introducing KgtP and 2-OG feeding for ethylene biosynthesis. Although a preliminary gaseous/aqueous two-phase photobioreactor for cyanobacterial ethylene production was developed and showed potential in this study, it remains to be optimized for ethylene production with higher efficiency and feasible scalability.

Conclusions

In this study, a series of genetically engineered *Synechocystis* strains were constructed for the photosynthetic production of ethylene. First, stable ethylene production was achieved by introducing a codon-optimized *efe* gene from *Pseudomonas syringae* pv. *sesami*. Second, *PcpC*B was identified as an appropriate promoter for ethylene biosynthesis with a high efficiency in *Synechocystis* sp. PCC 6803. Third, the EFE protein expression level and the ethylene production were significantly enhanced with the increasing *efe* copy number at the gene loci of *ogdc* and *ssadh* of the TCA cycle, and the metabolic stress caused by the inactivation of *ogdc* and/or *ssadh* was well released by the introduction of the EFE enzyme as an alternative. Fourth, feeding 2-OG was efficiently assimilated by the recombinants with an exogenous 2-OG permease (KgtP) at the *phaAB* operon, and at least 14% of the incorporated 2-OG was used for ethylene biosynthesis. The recombinant *Synechocystis* XX110 strain displayed robust growth and high-efficiency ethylene productivity under the modified cultivation conditions, and a peak ethylene production rate of $9739 \mu\text{L L}^{-1} \text{h}^{-1}$ ($295 \text{ mg L}^{-1} \text{day}^{-1}$) was achieved. Finally, a modified gaseous/aqueous two-phase photobioreactor with an efficient ethylene collecting section was developed. By the fed-bath cultivation of the ethylene producer XX110 with a period of 16 days in the 1 L photobioreactor, a total of more than 50 mL ethylene product was collected. The achieved ethylene productivity in the study shows the promising application potential for direct photosynthetic production of ethylene.

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