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SEQUENCE OPTIMIZATION OF EFE GENE FROM *P. SYRINGAE* IS NOT REQUIRED FOR STABLE ETHYLENE PRODUCTION IN RECOMBINANT *SYNECHOCYSTIS* SP. PCC 6803

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Abstract

Ethylene (C_2H_4) is a simple alkene of high commercial value due to multitude of large-scale uses in plastic industry, and as a potential fuel for spark-ignition piston engines. Currently ethylene is derived entirely from non-renewable sources, but it can also be produced directly from atmospheric CO_2 via microbial biosynthesis in photosynthetic cyanobacterial hosts by the expression of ethylene forming enzyme (efe) from *Pseudomonas syringae*. One of the obstacles encountered in expression system design and optimization has been the instability of the *efe* gene in *Synechocystis* sp. PCC 6803 and *Synechoccocus elongatus* PCC 7942, which has previously been overcome by sequence optimization and altered expression strategies. This study focuses on understanding ethylene expression system stability in *Synechocystis* sp. PCC 6603, and demonstrates that gene optimization and removal of sequence elements implicated with gene inactivation are not essential for sustained production. The results suggest that the observed loss of production efficiency is more likely associated with other factors such as the chromosomal integration site or homologous sequence elements within autonomously replicating expression plasmids, which are central considerations in the development of any future production platforms.

Introduction

Ethylene (C_2H_4) is a volatile alkene, which is used in massive quantities by the chemical industry worldwide. Due to its chemical properties it serves as a precursor for vast array of materials including commonly used plastics such as polystyrene, polyvinyl chloride and polyethylene [1]. Ethylene is also highly combustible, and technically viable alternative fuel for spark-ignition engines, with properties comparable to gasoline in performance and emissions [2]. The current market and potential for further expansion, together with multitude of potential uses makes ethylene a significant commercial target.

Ethylene is currently produced by the petrochemical industry at a multimillion ton annual scale from longer chain hydrocarbons by steam cracking. Consequently, the production and use are faced with increasing economic and environmental global issues common to all petroleum-derived products. Alternative renewable systems for sustainable ethylene production are thus of increasing biotechnological interest, and especially the potential of photosynthetic microbial systems has been investigated in several contexts [3].

Besides being a natural hormone in higher plants [4], ethylene is also produced by a prokaryotic plant pathogen *Pseudomonas syringae*, and serves as a chemical mediator for virulence [5]. The bacterial biosynthesis of ethylene proceeds via a common key metabolic intermediate of the citric acid cycle, 2-oxoglutarate, which is converted to ethylene by ethylene-forming enzyme (efe) (EC 1.13.12.19)[6]. Based on this capacity, the *efe* gene from *P. syringae* has been used for the assembly of heterologous ethylene pathways in photosynthetic cyanobacterial hosts including *Synechococcus elongatus* PCC 7942 (S7942) [7] [8] [9] [10] [11] [12] [13] and *Synechocystis* sp. PCC 6803 (S6803) [14] [15] [16] [17] [18] [19]. These platforms have allowed the microbial biosynthesis of ethylene directly from water and CO_2 using sunlight as energy, but the production levels remain below commercial viability, and the systems need further evaluation and stringent optimization.

Several different cyanobacterial ethylene production systems have been described in literature, yet there are still unanswered questions regarding the most optimal over-expression strategies for efe. The aim of this study was to investigate the performance and factors influencing the genetic stability of the original non-optimized *P. syringae efe* sequence in a plasmid-based expression system in S6803. The corresponding *efe* sequence has previously been reported to be unstable in a chromosomal integration construct at *psbA* site [12] as well as in autonomously replicating plasmid harboring *psbA* promoter/terminator [8] in S7942, observed as a loss of ethylene production over several generations [8] [12]. Synthetic codon optimized *efe*

gene, however, has been shown to enable stable ethylene production when expressed in S6803 [14] [15] [16] [17] [18]. Based on the earlier findings, the requisite for genetic optimization and the role of sequence elements associated with insertional mutations in gene inactivation thus still remain unclear. As the initiative for this study, understanding these interactions is of fundamental interest and relevant for the rational design of optimal autotrophic expression systems in cyanobacterial hosts.

Materials and methods

Expression constructs and *Synechocystis* sp. PCC 6803 transformants

All DNA constructs were generated using standard recombinant molecular biology techniques, commercial enzymes (New England Biolabs, Ipswich, MA, USA) and DNA kits (Qiagen, GmbH, Hilden, Germany). *E. coli* DH5α was used for plasmid propagation. Primers were obtained from MWG-Biotech AG (Eurofins Genomics, Ebersberg, Bavaria, Germany). All the plasmids and strains used in the study are listed in Table 1, and primers in Table 2.

The original *P. syringae efe* gene (o-*efe*) from pYX212-EFE [20] (Chalmers University of Technology, Sweden) was amplified by PCR using the primers 1 and 2. The fragment was subcloned (KpnI-PstI) into pDF-trc [15] under the control of trc promoter, and the resulting construct pDF-trc-o-*efe* was confirmed by sequencing using the primers 1-3. Previously generated plasmids pDF-trc-sy-EFE carrying the optimized gene (sy-*efe*) and pDF-trc-EFEh with additional sequence encoding a C-terminal Histidine tag [15] (sy-*efe*h) were used as controls.

The expression constructs carrying the genes o-*efe* , sy-*efe*h and sy-*efe* were transformed into *Synechocystis* sp. PCC 6803 [21] thus generating the expression strains S6803-o-*efe* S6803-sy-*efe*h and S6803-sy-*efe*, respectively. The strains were stored at -80 °C with 5% methanol as cryoprotectant.

Culture conditions

The S6803 transformants were grown in BG11 liquid medium (pH 8.0) with 20 mM TES-KOH, supplemented with 1 mM IPTG required for full induction at low cell densities [15], and 50 µg/ml of spectinomycin and 20 µg/ml of streptomycin to sustain selection pressure for the expression constructs. The main cultures were carried out in 100 ml volume in 250 ml Erlenmayer flasks under continuous light (50 µmol photons $m^{-2}s^{-1}$) at 30 °C in 3 % CO_2 and in an orbital shaker at 120 rpm in a Sanyo Chamber (SanyoElectric Co. Ltd).

Table 1. Strains and plasmids used in this study

Plasmids /Strains	Description	Ref.
Plasmids		
pYX212-EFE	Plasmid harboring the original *efe* sequence from *P. syringae.*	[20]
pDF-trc-o-*efe*	pDF-trc plasmid with *efe* from pYX212-EFE	This study
pDF-trc-sy-*efeh*	pDF-trc carrying sequence optimized *efe* with N-terminal His-Tag sequence	[15]
pDF-trc-sy-*efe*	pDF-trc harboring sequence optimized *efe*	[15]
Strains		
S6803:o-*efe*	S6803 with pDF-trc-o-*efe* plasmid	This study
S.6803:sy-*efeh*	S6803 with pDF-trc-sy-*efeh*h plasmid	[15]
S.6803:sy-*efe*	S6803 with pDF-trc-sy-*efe* plasmid	This study
DH5α	The *E. coli* strain used for engineering work	

Measuring ethylene production *in vivo*

Ethylene production of the three strains (with two biological replicates each) was monitored in a step-wise experiment of five repeated consecutive cultivation batches. Each cultivation batch was started at $OD_{750nm} \approx 0.1$ and grown until the optical density reached 1, followed by the inoculation of the successive batch. Throughout the experiment, ethylene production was measured once a day. The analysis was done by transferring 1 ml of the cell cultures into sealed 10 ml serum bottles, followed by 2-4 h incubation under the specified conditions, and GC-MS analysis of the headspace (25-250 µl samples) as described earlier [15]. The integrated MS peak areas were used to calculate the relative ethylene production as nl ethylene / ml culture / h, and normalized to cell density to allow comparison.

Table 2. PCR Primers (5'→3' direction) with complementary region in capital and restriction sites underlined

ID	Name	Description	Ref.
1	o-*efe* _KpnI_Fw	gctcGGTACCATGACCAACCTAC AGACTTTCGAGTTGC Cloning (KpnI overhang)/ sequencing	This study
2	o-*efe* _PstI_Rev	tgcCTGCAGAAGCTTCAGGATCC TGAGCCTGTCG Cloning (PstI overhang)/ sequencing	This study
3	pLac1_fw	GCTGCAACTCTCTCAGGGCCAG Sequencing	
4	FD_RT_o-*efe*	GAGCACGATGAACCTTGGAC RTqPCR of o-*efe*	This study
5	RV_RT_o-*efe*	GCTTAACCTTGTGCGGAGTG RTqPCR of o-*efe*	This study
6	FD_RT_sy-*efe*	TTGGACAGTTTTCCCTGGTG RTqPCR of sy-efe	This study
7	RV_RT_sy-*efe*	TCGTGGAAATAAGCGCAAG RTqPCR of sy-*efe*	This study
8	FD_rnpB	GTGAGGACAGTGCCACAGAA RTqPCR of *rrnpB* reference gene	[22]
9	RV_rnpB	CCTTTGCACCCTTACCCTTT RTqPCR of *rnpB* reference gene	[22]

RNA isolation and cDNA synthesis

For mRNA analysis, three parallel 10 ml culture samples were collected at OD_{750nm} = 1. The cells were centrifuged at 5000 g for 6 min at 4 °C, supernatant was removed and pellets were stored at -80 °C. Total RNA was extracted by TRIsure™ (Bioline, London, UK) according to the manufactures instructions and quantified spectrophotometrically (NanoDrop, Thermo scientific, Wilmington, DE, USA). Samples were treated with Turbo DNase (Ambion, Life technologies, Carlsbad, CA, USA). One µg of RNA was used for cDNA synthesis by QuantiTect® Reverse Transcription kit (Qiagen, GmbH, Hilden, Germany), using random primers according to the manufactures instructions. After reverse transcription, the cDNA obtained was diluted 5-fold with water and used as template for RTqPCR.

Real-Time quantitative PCR

The relative *efe* transcript levels of the expression strains were measured from at least three independent biological replicates by RTqPCR (iQ5 RT-PCR, Bio-Rad Laboratories, Inc, Hercules, CA, USA) using rnpB [22] as a reference gene. The amplification reactions were carried out using specified primers (Table 2) with 2 µl of the isolated cDNA as template in 25 µl total volume (iQ™ SYBR® Green supermix, Bio-Rad Laboratories, Inc, Hercules, CA, USA). LineRegPCR software was used for estimating the efficiency of each reaction [23]. The relative levels were normalized to the reference gene *rnpB* and calculated as described earlier [24].

Statistical analyses

One-way ANOVA (analysis of variance) and post hoc comparisons using the Tukey HSD test were conducted to define the significance differences between means. $P < 0.05$ was considered statistically significant.

Results

Plasmid construction and strain generation

A plasmid construct for the expression of original non-optimized *efe* from *P. syringae* was assembled by subcloning the amplified gene into a pDF-trc plasmid compatible with S6803 [15]. The sequence was confirmed by gene sequencing after which the construct was transformed into S6803. The transformants were selected based on spectinomycin and streptomycin resistance, and the presence of the *efe* gene was confirmed by colony PCR.

Monitoring ethylene production

Using the constructed S6830 expression strain, function of the native *efe* gene (o-*efe*) from *P. syringae* was compared against the codon-optimized gene with and without a sequence coding for an N-terminal His-tag (sy-*efe*h and sy-*efe*, respectively) [15]. Monitoring of ethylene accumulation in the headspace of closed culture vials by GC-MS showed that the productivity was in a similar range for all the three strains, and continued stable throughout the five repeated four-day cultivation batches without any signs of expression system instability (Figure 1A; bars). At the same time, the growth rate of the strain expressing o-*efe* was comparable to the controls (Figure 1A; lines), and no adverse phenotypic effects could be observed. Further comparison of the total ethylene amount over the 20-day cultivation period suggested that the overall production was slightly higher (31%) for the o-*efe* construct than for the optimized controls (Figure 1B), and altogether remained in the range of 150-250 µl ethylene per liter culture per hour normalized by the optical density.

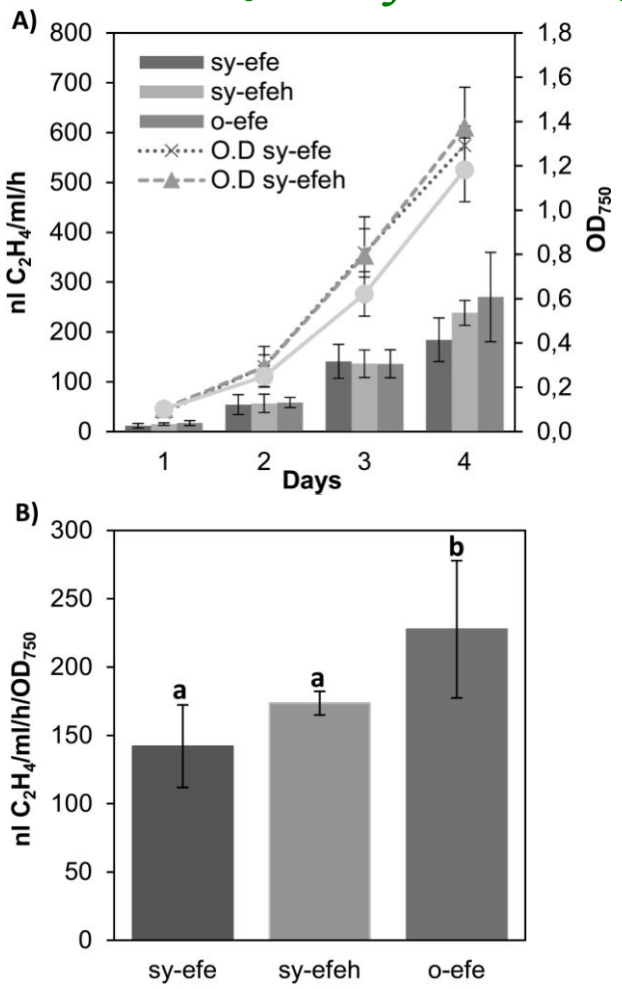

Figure 1. Comparison of ethylene production and growth rate of three *Synechocystis* sp. PCC 6803 mutants expressing different variants of the *efe* gene. O-*efe* represents the original unoptimized gene sequence from *P. syringae*, while the sy-*efe*h and sy-*efe* are the corresponding codon optimized sequences with and without an N-terminal His-tag, respectively. A) Ethylene production measured by GC-MS from the headspace of closed culture vials at the end of each day over a four-day batch culture. This was repeated five times in a row by diluting the culture batch to OD_{750nm} = 0.1 after each batch (OD_{750nm} ≈ 1). B) The total ethylene production of the three expression strains over the entire batch cultivation. The mean and standard deviation have been calculated in each case from five consecutive (two parallel) batch cultivation (n = 10). ANOVA df 2,23, p = 2.07E-05; Tukey HSD test shown as letters above the columns where a and b are significantly different, p = 0.05.

Transcriptional analysis of *efe*

RT qPCR analysis was further carried out to compare the *efe* transcript levels of the three strains. Despite small variation and slightly lower mRNA levels of the o-*efe* in reference to the controls observed in several independent repetitions, the differences between the three strains were marginal (Figure 2).

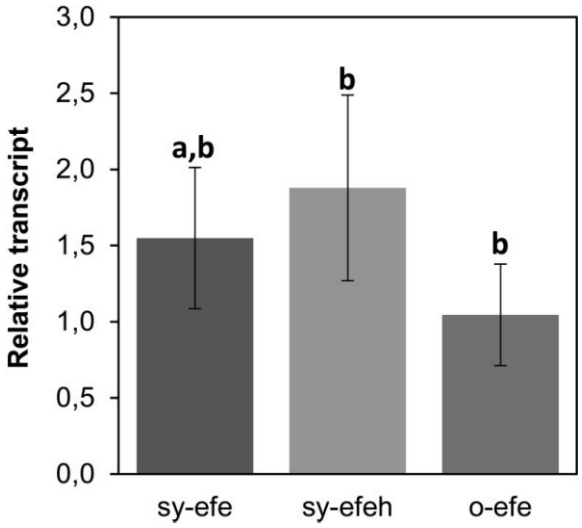

Figure 2. Relative mRNA transcript levels of *efe* variants isolated from the *Synechocystis* sp. PCC 6803 expression strains. The relative transcript was compared to o-*efe* in the same amplification reaction and normalized with the reference gene (rnpB). Bars are SE from three independent biological replicates. ANOVA df 2,24, p = 0.005; Tukey HSD test shown as letters above the columns where a and b are significantly different, p = 0.05.

Discussion

Genetic stability is a primary requirement for any microbial expression system used for biotechnological applications. This study focused on the stability of cyanobacterial ethylene production strains which are based on heterologous expression of ethylene forming enzyme (efe) from *P. syringae*. In previous studies, ethylene productivity of engineered S7942 was shown to be lost in a few generations due to the disruption of the *efe* gene integrated at the *psbA* locus [12]. Subsequently, this problem was overcome by simultaneous sequence optimization of the *efe* gene and transfer to an alternative expression system in S6803, which has thereafter been shown to maintain stable production in different studies [14] [15] [16] [17] [18]. This suggested that the encountered instability issues would not be primarily due to ethylene toxicity or metabolic stress resulting from the ethylene precursor 2-oxoglutarate depletion, but some other factors in the original expression system [12]. This study aimed to clarify the role of the *efe* gene sequence and the alleged need for sequence

optimization – information which may be important for the rational design of future expression systems in S6803.

The results demonstrated that ethylene production was similar for all the compared S6803 strains, irrespective of *efe* gene sequence optimization, and corresponded to the values reported previously [15]. This showed that the primary sequence of the *efe* gene and the sequence elements previously associated with mutational inactivation [12] [14], did not compromise ethylene production of S6803. Altogether, using the original unoptimized gene systematically resulted in slightly higher ethylene levels in comparison to the controls, although the reason is not completely clear. The results also verified that optimizing the *efe* codon usage does not have a positive impact on the outcome, indicating that translational efficiency at current production levels is not a limiting factor in S6803.

Similar effects were also seen at transcript level in RT qPCR comparison. Several repeated analytical series did not reveal dramatic differences in the mRNA levels between the three parallel ethylene producing strains. This demonstrated that the use of the original unoptimized *efe* sequence did not compromise transcriptional efficiency or mRNA stability in S6803 to any clear extent.

Taken together, this study shows that there is no need for codon optimization or removal of specific sequence elements in the *P. syringae efe* gene to allow sustained ethylene production in S6803. Thus, it is clear that the limitations in performance in regards to stability stem from other factors than the ethylene production per se or the primary sequence of the *efe* gene. Comparison of the different expression strategies presented in literature suggests that these factors may include, for example, the choice integration site in the host chromosome [12] or the presence of homologous sequence elements in extrachromosomal expression vectors [8], which promote generic rearrangement and instability via unwanted homologous recombination.

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