Metabolic Engineering Communications 4 (2017) 29–36

Contents lists available at ScienceDirect

Metabolic Engineering Communications

journal homepage: www.elsevier.com/locate/mec

Synechocystis PCC 6803 overexpressing RuBisCO grow faster with increased photosynthesis

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ARTICLE INFO

Keywords:
Synechocystis PCC 6803
RuBisCO
Increased growth
FLAG tag
Photosynthesis
Strep-tag II

ABSTRACT

The ribulose-1,5-bisphosphate (RuBP) oxygenation reaction catalyzed by Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is competing with carboxylation, being negative for both energy and carbon balances in photoautotrophic organisms. This makes RuBisCO one of the bottlenecks for oxygenic photosynthesis and carbon fixation. In this study, RuBisCO was overexpressed in the unicellular cyanobacterium *Synechocystis* PCC 6803. Relative RuBisCO levels in the engineered strains FL50 and FL52 increased 2.1 times and 1.4 times, respectively, and both strains showed increased growth, photosynthesis and *in vitro* RuBisCO activity. The oxygen evolution rate increased by 54% and 42% on per chlorophyll basis, while the *in vitro* RuBisCO activity increased by 52% and 8.6%, respectively. The overexpressed RuBisCO were tagged with a FLAG tag, in strain FL50 on the N terminus of the large subunit while in strain FL52 on the C terminus of the small subunit. The presence of a FLAG tag enhanced transcription of the genes encoding RuBisCO, and, with high possibility, also enhanced the initiation of translation or stability of the enzyme. However, when using a streptavidin-binding tag II (strep-tag II), we did not observe a similar effect. Tagged RuBisCO offers an opportunity for further studying RuBisCO expression and stability. Increased levels of RuBisCO can further improve photosynthesis and growth in the cyanobacterium *Synechocystis* PCC 6803 under certain growth conditions.

1. Background

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39) has attracted intensive research interest as an abundant, low efficiency but critical enzyme (Spreitzer and Salvucci, 2002) since it was firstly purified from spinach in 1947 (Wildman and Bonner, 1947). Presently, food supply becomes an outstanding social problem as world population increases rapidly. Optimizing RuBisCO performance is one of the main strategies to increase crop yield to meet the high food requirement (Durall and Lindblad, 2015; Parry et al., 2013).

RuBisCO evolved about 3.8 billion years ago. Until now, three types of RuBisCO (Form I, II, and III) and one RuBisCO like protein (RLP, also called form IV) which lacks the carboxylation ability have been demonstrated (Tabita et al., 2008). Form I hexadecameric RuBisCO exists in most plants, algae, cyanobacteria and proteobacteria. It has eight large subunits forming the catalytic core (4L2) and four small subunits capping on the top and the bottom. L2 is anti-paralleled and the catalytic site is formed by N terminus of one large subunit and C terminus of the neighbor large subunit, altogether 8 catalytic sites in one holoenzyme (Tabita et al., 2008). Significant efforts have aimed to increase RuBisCO performance. Unfortunately, only limited successes have been achieved so far due to the turnoff between CO_2 affinity and catalytic ratio (Carmo-Silva et al., 2015; Durall and Lindblad, 2015). RuBisCO is an abundant protein in leaves consisting of almost half of the soluble proteins. This makes it difficult to further increase RuBisCO amount in the cells. The complex regulatory system of RuBisCO does not only make homologous expression difficult, but also make heterologous expression challenging (Parry et al., 2013). Overexpressing *Synechococcus elongatus* PCC 6301 *rbcLS* in *Synechococcus elongatus* PCC 7942 resulted in cells with higher *in vitro* RuBisCO activity and twofold isobutyraldehyde production whereas oxygen evolution remained the same as the strains without additional *rbcLS* expression (Atsumi et al., 2009). This is a successful example of heterologous expression of RuBisCO even though the *Synechococcus elongatus* PCC 6301 genome is almost identical to the *Synechococcus elongatus* PCC 7942 genome, except for a 188.6 kb inversion (Sugita et al., 2007). In addition, positive effects on the free fatty acid production in *Synechococcus* sp. PCC 7002 have been reported by heterologous expression of RuBisCO from *Synechococcus elongates* PCC 7942 (Ruffing, 2014). In addition, an increased RuBisCO activity in engi-

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http://dx.doi.org/10.1016/j.meteno.2017.02.002
Received 3 October 2016; Received in revised form 28 November 2016; Accepted 17 February 2017
Available online 20 February 2017

neered cells of *Synechococcus elongates* PCC 7942 containing heterologous *rbcLS* has been observed (Iwaki et al., 2006). These positive examples of heterologous overexpression of RuBisCO may benefit from avoiding post-transcriptional regulations of the introduced, foreign RuBisCO by the native machinery (Ruffing, 2014).

Cyanobacterial and algae RuBisCO are reported to have higher efficiency compared to plant RuBisCO and are regarded as alternatives to increase crop yield (Whitney et al., 2011). Unfortunately, there is no breakthrough report on expressing cyanobacterial or algae RuBisCO in any plant for now. One of the reasons is the differences and necessary of folding and assembling chaperones. RuBisCO has complex folding and assembling processes with chaperones involved (Hauser et al., 2015). In addition, RuBisCO activation and activity maintenance require an activase that belongs to the AAA+ family. Successful expression of cyanobacterial *Synechococcus elongatus* RuBisCO in tobacco was reported only recently (Lin et al., 2014). *Synechococcus elongatus* RuBisCO were assembled in tobacco chloroplasts both with and without the assembling chaperone RbcX, or the carboxysome protein CcmM35, and replaced the tobacco RuBisCO (Occhialini et al., 2016). Cyanobacterial RuBisCO functioned in tobacco, but the transformant line could grow autotrophically only under elevated CO_2 concentration (3% v/v CO_2/air) (Lin et al., 2014; Occhialini et al., 2016). Even though being an important step this transformant line is far away from an ideal high yield plant.

Fusion tag (protein tag) is widely used for protein purification (Kosobokova et al., 2016). Fusion tag can be a larger protein (like Glutathione S_Transferase, GST) or short peptides containing several amino acids (like FLAG tag, Streptavidin_binding tag). The short peptide tag may have positive effects on protein expression, protein solubility, protein efficiency or even folding (Kosobokova et al., 2016). There are reports that the presence of a His tag increased *slr1192* and aldehyde reductase gene (from *Synechocystis* PCC 6803) expression in *Escherichia colicoli* and trans-2-enoyl-CoA reductase gene (from *Treponema denticola*) efficiency in *Synechococcus elongates* PCC 7942 (Lan and Liao, 2011; Akhtar et al., 2013). Even though the mechanism is not elucidated, it is possible that the tag may confer some stability at (post)transcriptional and/or (post)translational level. Other short protein tags like FLAG tag and Streptavidin-binding tag II are also well studied even though there are no reports about their positive effects on protein expression (Kosobokova et al., 2016). A FLAG tag consists of eight amino acids with the sequence DYKDDDDK, only 1 kDa (Einhauer and Jungbauer, 2001). A Streptavidin-binding tag II (strep-tag II) is also an 8 amino acid tag with the sequence WSHPQFEK (Schmidt and Skerra, 2007). It was developed from the original strep tag (WSHPQFEK) to conquer the constraint that a strep tag can only be used on the C terminus of the partner protein (Korndörfer and Skerra, 2002).

In this study, we engineered *Synechocystis* PCC 6803 (*Synechocystis* hereafter) strains with higher level of RuBisCO protein and characterized these strains. RuBisCO overexpression was only observed when the gene was tagged with a FLAG tag. RuBisCO overexpressed strains had higher *in vitro* RuBisCO activity, growth and oxygen evolution rate under the experimental conditions. This work indicates that improving RuBisCO can further enhance photosynthesis, growth and potentially improve yield.

2. Materials and methods

2.1. Strains and culturing conditions

Escherichia coli DH 5α (*E. coli* hereafter) strain was cultured with LB medium (liquid or agar petri dish) under 37 °C. *Synechocystis* PCC 6803 (*Synechocystis*) wild type and engineered strains were grown with BG11 medium (liquid or agar petri dish) in 30 °C room. 50 μg/ml kanamycin was supplied for *E. coli* and 25 μg/ml for *Synechocystis* when screening stress was required.

Synechocystis strains were grown in tubes (20 cm length, 2.6 cm inner-diameter, 3 cm outer-diameter) bubbled with air under 100 μmol photons m^{-2} s^{-1} light intensity with 0.01% antifoam (polypropylene glycol 1025, VWR) from day 1. For strains having kanamycin cassette on vectors, 25 μg/ml kanamycin was added. For strains having kanamycin cassette on chromosome, BG11 was used.

2.2. Plasmids and engineered strains

The RuBisCO gene operon (*rbc*, *slr0009-slr0011-slr0012*) and P*psbA2* were amplified from wild type *Synechocystis* genome with corresponding primers (Supplementary Table 1) using Phusion polymerase (Thermo Fisher Science). *rbc* was expressed either on pPMQAK1 (Huang et al., 2010) or on the chromosome.

Synechocystis strain carrying pPMQAK1 (cutting off *ccdB* gene, WT +Km^r-vector) is control to engineered strains introducing another *rbc* gene on pPMQAK1. P*psbA2* was flanked with *Eco*RI and *Xba*I digesting sites. RuBisCO gene was flanked with *Xba*I and *Pst*I digesting sites. In total, five versions of RuBisCO genes were designed and constructed, encoding large subunit N terminus tagged with FLAG tag (FLAG- *slr0009-slr0011-slr0012*), encoding small subunit C terminus tagged with FLAG tag (*slr0009-slr0011-slr0012*-FLAG), encoding large subunit C terminus tagged with FLAG tag (*slr0009*-FLAG-*slr0011-slr0012*), encoding large subunit N terminus tagged with strep-tag II (strep- *slr0009-slr0011-slr0012*), and encoding small subunit C terminus tagged with strep-tag II (*slr0009-slr0011-slr0012*-strep). To insert FLAG tag on C terminus of large subunit, overlap extension PCR was used. pPMQAK1 was digested with *Eco*RI and *Pst*I and larger fragment was recovered. These three fragments were ligated using Quick ligase (New England Bio-labs). The resulting plasmids (pFL50, pFL52, pFL50C, pFL50strep, pFL52strep) were conjugated into wild type *Synechocystis* cells, resulting into engineered strains FL50, FL52, FL50C, FL50strep and FL52strep respectively. Conjugation was performed as described previously (Liang and Lindblad, 2016).

Synechocystis strain carrying a kanamycin cassette on the *slr0168* site (WT+Km^r-genome) was used as a control to the engineered strains with genetic constructs on the chromosome. P*psbA2* together with the operon encoding RuBisCO (FLAG-*slr0009-slr0011-slr0012* or *slr0009-slr0011-slr0012*-FLAG) were amplified from pFL50 and pFL52 and inserted onto pEERM3 Km (Englund et al., 2015) using *Eco*RI and *Pst*I sites. The resulting plasmids were conjugated into wild type *Synechocystis*, resulting in the engineered strains FL50G and FL52G. Complete segregation was confirmed with PCR. Kanamycin cassette and P*psbA2* (with ribosome binding site and an ATG-FLAG tag) were flanked with upstream sequences of *slr0009* and the initial part of *slr0009* (without start code) before introduced into *Synechocystis* wild type cells. Flanking sequence was about 800 bp. The resulting strain, named FL75 (Fig. 1A), was confirmed to be completely segregated using PCR (Fig. 1B). FLAG-TAA and kanamycin cassette flanked with downstream sequences of *slr0012* and the final part of *slr0012* (without stop codon) were introduced into *Synechocystis* wild type cells, resulting in strain FL76 (Fig. 1A). Flanking sequence was about 800 bp and full segregation was confirmed using PCR (Fig. 1C). All digestion enzymes were fast digestion enzymes from Thermo Fisher Science. *Synechocystis* strains used in this study are summarized in Table 1.

2.3. Optical density, chlorophyll a content and oxygen evolution measurement

Synechocystis optical density was measured at 750 nm (OD_{750}) using a spectrophotometer (Cary® 50 UV–visible Spectrophotometer, Varian). Chlorophyll *a* was extracted with 90% methanol. Chlorophyll *a* content and oxygen evolution measurement as detailed previously (Liang and Lindblad, 2016). Three independent experiments were conducted, each experiment with biological replicates and technical