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A phycocyanin · phellandrene synthase fusion enhances recombinant protein expression and β-phellandrene (monoterpene) hydrocarbons production in *Synechocystis* (cyanobacteria)

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ABSTRACT

Cyanobacteria can be exploited as photosynthetic platforms for heterologous generation of terpene hydrocarbons with industrial applications. Transformation of *Synechocystis* and heterologous expression of the β-phellandrene synthase (*PHLS*) gene alone is necessary and sufficient to confer to *Synechocystis* the ability to divert intermediate terpenoid metabolites and to generate the monoterpene β-phellandrene during photosynthesis. However, terpene synthases, including the PHLS, have a slow K_{cat} (low V_{max}) necessitating high levels of enzyme concentration to enable meaningful rates and yield of product formation. Here, a novel approach was applied to increase the PHLS protein expression alleviating limitations in the rate and yield of β-phellandrene product generation. Different *PHLS* fusion constructs were generated with the *Synechocystis* endogenous *cpcB* sequence, encoding for the abundant in cyanobacteria phycocyanin β-subunit, expressed under the native *cpc* operon promoter. In one of these constructs, the CpcB · PHLS fusion protein accumulated to levels approaching 20% of the total cellular protein, i.e., substantially higher than expressing the PHLS protein alone under the same endogenous *cpc* promoter. The CpcB · PHLS fusion protein retained the activity of the PHLS enzyme and catalyzed β-phellandrene synthesis, yielding an average of 3.2 mg product g^{-1} dry cell weight (dcw) versus the 0.03 mg g^{-1} dcw measured with low-expressing constructs, i.e., a 100-fold yield improvement. In conclusion, the terpene synthase fusion-protein approach is promising, as, in this case, it substantially increased the amount of the PHLS in cyanobacteria, and commensurately improved rates and yield of β-phellandrene hydrocarbons production in these photosynthetic microorganisms.

1. Introduction

Cyanobacteria are good microbial platforms for the generation of commodity product useful for industrial and domestic consumption (Ducat et al., 2011; Oliver and Atsumi, 2014; Savakis and Hellingwerf, 2015). Compounds synthesized in the cyanobacterial cell and spontaneously separated from the biomass and the extracellular aqueous medium are particularly attractive because product segregation and harvesting are simplified. This is a parameter that weighs heavily on the economics of a microbial production system, as a spontaneous product separation from the biomass alleviates negative effects associated with product accumulation inside the cells. The latter include potential inhibitory or toxic effects of the product molecule on cellular metabolism, and considerably higher costs associated with product extraction from the cell interior, harvesting, and downstream processing (Melis, 2012; Wijffels et al., 2013).

β-Phellandrene ($C_{10}H_{16}$) is a monoterpene with commercial value as a key ingredient in synthetic chemistry, medical, cosmetic and cleaning products, and potentially as a fuel (Bentley et al., 2013). It is a component of plant essential oils, naturally synthesized in plant trichomes from geranyl-diphosphate (GPP) by a nuclear-encoded and plastid localized β-phellandrene synthase (PHLS) enzyme. Heterologous production of β-phellandrene was achieved by genetic engineering of the cyanobacterium *Synechocystis*, showing spontaneous and quantitative separation of the molecule from the biomass and the extracellular aqueous phase. β-Phellandrene efficiently diffused through the plasma membrane and cell wall and, because of its hydrophobicity, accumulated as a floater molecule on the surface of the culture, from where it was

Abbreviations: β-PHL, β-phellandrene; APC, allophycocyanin; Car, carotenoid; Chl, chlorophyll; dcw, dry cell weight; GPP, geranyl-diphosphate; PBS, phycobilisome; Phc, phycocyanin; PHLS, β-phellandrene synthase; PS, photosystem; wt, wild type

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harvested by siphoning (Bentley et al., 2013; Formighieri and Melis, 2014a).

Heterologous expression of the *PHLS* gene *via* genomic DNA chromosome-based transformation is necessary and sufficient for the constitutive photoautotrophic generation of β-phellandrene in *Synechocystis* transformants. More specifically, the codon optimized *PHLS* gene from *Lavandula angustifolia* (lavender) (Demissie et al., 2011) was expressed under the control of the strong endogenous *psbA2* (Bentley et al., 2013) or *cpc* promoter (Formighieri and Melis, 2014a) *via* homologous recombination and replacement of the respective *psbA2* gene or *cpc* operon. In both cases, β-phellandrene was constitutively generated, but the rate and yield of production were low, attributed to the low levels of transgenic protein accumulation. This consideration is important, as high levels of terpene synthase expression are required for enhanced yields in product generation, considering the slow K_{cat} of these enzymes (Demissie et al., 2011; Zurbriggen et al., 2012).

The *cpc* operon (locus 724094–727466 in the *Synechocystis* genome, ⟨http://genome.microbedb.jp/cyanobase⟩) encodes five proteins, i.e., the phycocyanin (Phc) β- and α- subunits (*CpcB* and *CpcA* genes), and their linker polypeptides (encoded by the *CpcC2*, *CpcC1* and *CpcD* genes), all of which assemble to form the peripheral rods of the phycobilisome (PBS) light-harvesting antenna complex. Phycocyanin (Phc) is one of the most abundant proteins in cyanobacteria, suggesting strong expression elements in the promoter and 5′UTR of the *cpcB* gene (Zhou et al., 2014), including aspects of the function of the *cpc* operon transcription and translation processes that, theoretically, could be used to efficiently drive expression of transgenes. Removal/replacement of the *cpc* operon resulted in a Phc-less mutant with a truncated PBS antenna size (Kirst et al., 2014).

Expression of the *PHLS* gene under the control of the *cpc* operon promoter improved accumulation of the PHLS protein over that under the *psbA2* promoter (Bentley et al., 2013) to a point where the transgenic protein was, for the first time, visible in the Coomassie-stained SDS-PAGE of *Synechocystis* total protein extracts (Formighieri and Melis, 2014a). Correspondingly, the yield of β-phellandrene hydrocarbons also increased from about 0.01 to about 0.2 mg of β-phellandrene g^{-1} dry cell weight (Formighieri and Melis, 2014a). It was concluded that limitations in rate and yield of β-phellandrene hydrocarbons production are in part due to the limited concentration of the transgenic enzyme in the transformant cells. However, in spite of this improvement upon use of the *cpc* promoter, levels of PHLS accumulation were nowhere near those of the native phycocyanin β- or α-subunits, and the yield in β-phellandrene was still low, corresponding to a 0.02% β-phellandrene:biomass (w:w) carbon partitioning ratio. This yield accounted for only a small fraction of the carbon flux through the cell's own terpenoid biosynthetic pathway, which was estimated to be 4–5% of all photosynthetically fixed carbon (Lindberg et al., 2010).

It became clear, therefore, that a strong promoter is necessary (Camsund and Lindblad, 2014) but not sufficient to yield high levels of transgenic terpene synthase and product yield. In order to further enhance the level of transgenic enzyme accumulation, as a pre-requisite for greater rates and yield of monoterpene production, we made a fusion of the entire *cpcB* gene with the *PHLS* transgene, expressed under the control of the *cpc* promoter. As a result, we report on the successful generation of a productive *Synechocystis* strain expressing the CpcB · PHLS fusion protein to about 20% of the total cell protein, a feature that allowed us to obtain substantially greater yields of β-phellandrene hydrocarbons from the photosynthesis of the cyanobacteria.

2. Materials and methods

2.1. Synechocystis strains, recombinant constructs, and culturing conditions

Synechocystis sp. PCC 6803 (*Synechocystis*) was used as the recipient strain and referred to as the wild type (wt) in this study (Williams, 1988). The recombinant plasmid for expression of the codon optimized β-phellandrene synthase (PHLS)-encoding gene in the *cpc* genomic locus (Formighieri and Melis, 2014a) was modified in order to fuse the *PHLS* to the C-terminus of the complete *CpcB*-encoding sequence. The upstream 500 bp of the *cpc* operon and the *cpcB* sequence were amplified by PCR from the *Synechocystis* genome, using cpc_us-XhoI as forward primer and cpcB-NdeI as reverse primer (Supplementary material, Table 1S). The resulting PCR product was cloned upstream of *PHLS via* XhoI and NdeI digestion, removing the native stop codon of *cpcB*. Homologous recombination was designed to occur between the 500 bp of the upstream and downstream sequences of the *cpc* operon, leading to replacement of the *cpc* operon by the recombinant CpcB · PHLS construct. Resulting *Synechocystis* transformants in this case are referred to as Δcpc+cpcB · PHLS (Fig. 1b). Homologous recombination was alternatively performed between the upstream sequence of the *cpc* operon and the CpcA-encoding sequence. The latter was amplified by PCR from the *Synechocystis* genome, using cpcA-BamHI and cpcA-SacI as forward and reverse primers, respectively (Supplementary material, Table 1S). The CpcA-encoding DNA was then cloned downstream of the recombinant CpcB · PHLS construct *via* BamHI and SacI digestion, thus replacing the 500 bp of the downstream sequence of the *cpc* operon previously employed. Transformation of *Synechocystis* and homologous recombination allowed substitution of the native *cpcB* sequence by the *cpcB · PHLS* fusion construct while maintaining the other *cpc* genes in the downstream portion of the operon (Fig. 1c, cpcB · PHLS+cpc transformant). In addition, homologous recombination was designed to occur between the upstream sequence of the *cpc*

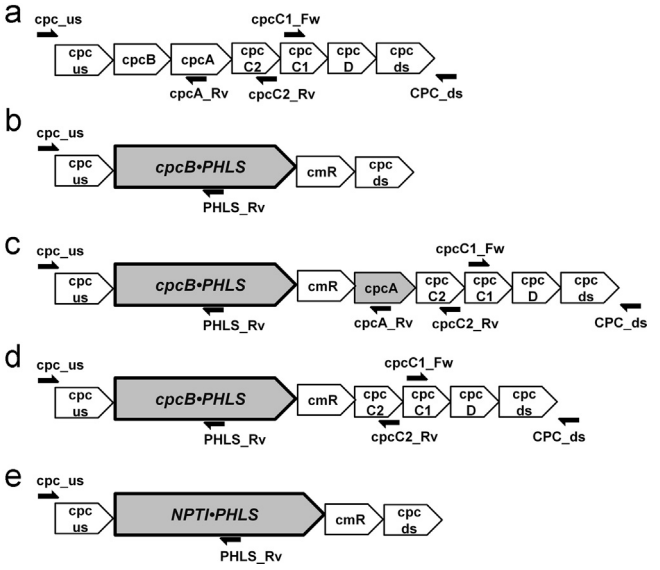

Fig. 1. Schematic overview of DNA constructs designed to transform the genome of *Synechocystis*, as used in this work. (a) The *cpc* operon, as it occurs in wild type cyanobacteria. (b–d) Constructs Δcpc+cpcB · PHLS, cpcB · PHLS+cpc, and cpcB · PHLS+cpc(-cpcA) express a fusion *CpcB · PHLS* and chloramphenicol resistance (*cmR*) genes in the absence of other *cpc* operon genes (b), in the presence of the remainder *cpc* operon genes (c), or in the presence of the remainder *cpc* operon genes, minus the *cpcA* gene (d). (e) Construct Δcpc+NPTI · PHLS was designed to replace the coding sequence of the endogenous *cpc* operon in *Synechocystis* with a kanamycin-resistance phellandrene-synthase fusion (NPTI · PHLS) encoding sequence.