Metabolic Engineering 12 (2010) 70–79

Contents lists available at ScienceDirect

Metabolic Engineering

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

Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism

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ARTICLE INFO

Article history:
Received 7 July 2009
Received in revised form
18 September 2009
Accepted 2 October 2009

Keywords:
Biofuel
Cyanobacteria
Isoprene
Photosynthesis
Renewable energy
Synechocystis

ABSTRACT

The concept of "photosynthetic biofuels" envisions application of a single organism, acting both as photo-catalyst and producer of ready-made fuel. This concept was applied upon genetic engineering of the cyanobacterium *Synechocystis*, conferring the ability to generate volatile isoprene hydrocarbons from CO_2 and H_2O. Heterologous expression of the *Pueraria montana* (kudzu) isoprene synthase (*IspS*) gene in *Synechocystis* enabled photosynthetic isoprene generation in these cyanobacteria. Codon-use optimization of the kudzu *IspS* gene improved expression of the isoprene synthase in *Synechocystis*. Use of the photosynthesis *psbA2* promoter, to drive the expression of the *IspS* gene, resulted in a light-intensity-dependent isoprene synthase expression. Results showed that oxygenic photosynthesis can be re-directed to generate useful small volatile hydrocarbons, while consuming CO_2, without a prior requirement for the harvesting, dewatering and processing of the respective biomass.

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1. Introduction

A variety of herbaceous, deciduous and conifer plants possess the genetic and enzymatic capability for synthesis and release of isoprene (C_5H_8) into the surrounding environment (Sharkey et al., 2008; Sharkey and Yeh, 2001). This short-chain volatile hydrocarbon is derived from the early Calvin-cycle products of photosynthesis, and is produced at substantial rates under certain environmental stress conditions. Heat stress of the organism is particularly important for the induction of this process in plants (Sasaki et al., 2007; Sharkey et al., 2001; Singsaas et al., 1997). The process of heat stress-induction and emission of short-chain volatile hydrocarbons by plants has been discussed in the literature as undesirable pollution of the atmosphere (for a review, see (Sharkey et al., 2008)). However, there has been no description of a system for the generation, harvesting and sequestration of isoprene for use as a renewable biofuel, or feedstock in the synthetic chemistry industry. Herbaceous, deciduous and conifer plants would be unsuitable for large-scale generation of isoprene, due to the volatility of the product, the difficulty of harvesting it from an extended and difficult-to-contain canopy, and the inherently low solar energy conversion efficiency of these plant species (Melis, 2009). In this endeavor, microbial hosts offer a distinct advantage (Stephanopoulos, 2007; Fischer et al. 2008).

There is an urgent need to develop renewable biofuels that will help meet global demands for energy and synthetic chemistry feedstock, but without contributing to climate change or other environmental degradation (Melis et al., 2007; Dismukes et al., 2008). The current work addresses this need by providing novel methods for the generation of volatile isoprene hydrocarbons in photosynthetic microorganisms. Isoprene, derived entirely via photosynthesis, i.e., from sunlight, carbon dioxide (CO_2) and water (H_2O), could serve as a renewable biofuel or feedstock in the synthetic chemistry industry. Photosynthetic microorganisms, such as cyanobacteria or unicellular microalgae, can grow to high densities within fully enclosed photo-bioreactors (Chisti, 2007; Angermayr et al., 2009; Beer et al., 2009). Such a system would enable oxygenic photosynthesis to convert solar energy and store it in the form of isoprene, while permitting collection and sequestration of this volatile product.

There are two biosynthetic pathways leading to the formation of isoprenoids; the mevalonic acid pathway, which operates in the cytosol of eukaryotes and archaea; and the methyl-erythritol-4-phosphate (MEP) pathway, which is of prokaryotic bacterial origin and present in plant plastids (Lichtenthaler, 2000). Formation of isoprene in plants is due to the presence of an isoprene synthase (*IspS*) gene (Miller et al., 2001); a nuclear gene encoding a chloroplast-localized protein that catalyzes the conversion of

Abbreviations: DXP, deoxyxylulose-5-phosphate; HMBPP, hydroxy-2-methyl-2-butenyl-4-diphosphate; G3P, glyceraldehyde-3-phosphate; Isp, isoprene; IspS, isoprene synthase; MEP, methyl-erythitol phosphate; PCR, polymerase chain reaction; PS, photosystem

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doi:10.1016/j.ymben.2009.10.001

dimethylallyl diphosphate (DMAPP) to isoprene (Silver and Fall, 1991). Plant isoprene synthases, encoded by *IspS*, have been cloned and characterized from poplar (*Populus alba*, *Populus tremuloides* and *Populus nigra*) (Fortunati et al., 2008; Miller et al., 2001; Sasaki et al., 2005) and kudzu vine (*Pueraria montana*) (Sharkey et al., 2005). Isoprene is a small hydrophobic and volatile molecule that can easily go through cellular membranes and the stomata of leaves and, thereby, be emitted from the leaves into the atmosphere. Isoprene production and release can function as a protective mechanism for the plant, via which the thermotolerance increases (Behnke et al., 2007; Sasaki et al., 2007; Sharkey et al., 2001; Singsaas et al., 1997).

Cyanobacteria are not endowed with an isoprene synthase by which to specifically catalyze the last committed step in isoprene biosynthesis. However, they do express the MEP pathway and utilize the corresponding enzymes for the biosynthesis of a great variety of needed terpenoid-type molecules (carotenoids, tocopherols, phytol, sterols, hormones, among many others; Lichtenthaler, 1999; Ershov et al., 2002; Poliquin et al., 2004; Okada and Hase, 2005). The MEP isoprenoid biosynthetic pathway uses pyruvate and glyceraldehyde-3-phosphate (G3P) as substrates, which are combined to form deoxyxylulose-5-phosphate (DXP), as first described for *Escherichia coli* (Rohmer et al., 1996). DXP is then converted into methyl-erythitol phosphate (MEP), which is subsequently modified to form hydroxy-2-methyl-2-butenyl-4-diphosphate (HMBPP). HMBPP is the substrate required for the formation of IPP and DMAPP as the final step in this biosynthetic pathway (Fig. 1). Cyanobacteria also contain an IPP isomerase that catalyzes the inter-conversion of IPP and DMAPP (Barkley et al., 2004; Poliquin et al., 2004). Genetic inactivation of the IPP isomerase gene results in impairment of isoprenoid biosynthesis from photosynthetic substrates (Poliquin et al., 2004).

Previous studies with *Synechocystis* sp. PCC 6803 have shown that, under photosynthetic growth conditions, substrates for the MEP pathway may not strictly be pyruvate and G3P, as outlined above. Rather, substrates originating from the pentose phosphate cycle may enter the pathway at steps later than MEP (Ershov et al., 2002; Poliquin et al., 2004) (Fig. 1), providing a more direct link between primary products of photosynthesis and the isoprenoid biosynthetic pathway. Furthermore, it has been shown in the cyanobacterium *Thermosynechococcus elongatus* BP-1 (Okada and Hase, 2005), and also under *in vitro* experimental conditions using plant enzymes (Seemann et al., 2006), that the reaction catalyzed by GcpE (Fig. 1), an Fe–S cluster-containing enzyme responsible for the formation of HMBPP, is dependent on reduced ferredoxin for its activity (Fig. 1), providing yet another direct link between photosynthesis and the isoprenoid biosynthesis pathway.

In the present work, genetic engineering of the cyanobacterium *Synechocystis* sp. PCC6803 was employed to generate transformant strains endowed with the property of photosynthetic isoprene production. This modification resulted in the generation of strains that can be used in an enclosed mass culture system, e.g. a photo-bioreactor, to provide a renewable hydrocarbon suitable as biofuel or feedstock in chemical synthesis. The work further discusses guiding principles for the selection of organisms and design of processes for the renewable generation of biofuels.

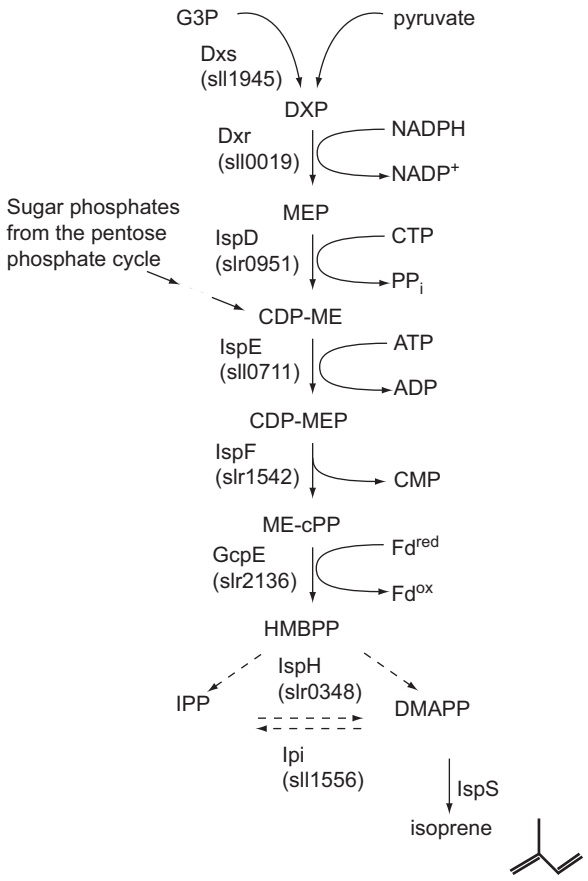

Fig. 1. The MEP pathway for isoprene biosynthesis. Abbreviations used: G3P=glyceraldehyde 3-phosphate; DXP=deoxyxylulose 5-phosphate; MEP=methylerythritol 4-phosphate; CDP-ME=diphosphocytidylyl methylerythritol; CDP-MEP=CDP-ME 2-phosphate; ME-cPP=methylerythritol 2,4-cyclodiphosphate; HMBPP=hydroxymethylbutenyl diphosphate; IPP=isopentenyl diphosphate; DMAPP=dimethylallyl diphosphate. Enzymes: Dxs=DXP synthase; Dxr=DXP reductoisomerase; IspD=CDP-ME synthase; IspE=CDP-ME kinase; IspF=ME-cPP synthase; GcpE (IspG) HMBPP synthase; Fd=ferredoxin; IspH=HMBPP reductase; Ipi=IPP isomerase; IspS=isoprene synthase. Where applicable, corresponding ORF names in the *Synechocystis* genome database (http://genome.kazusa.or.jp/cyanobase/ (Kaneko and Tabata, 1997)) are given in parentheses. In addition to reactants G3P and pyruvate, the MEP pathway consumes reducing equivalents and cellular energy in the form of NADPH, reduced ferredoxin, CTP and ATP, ultimately derived from photosynthesis (see also Ershov et al., 2002; Sharkey et al., 2008).

Materials and methods

2.1. Strains and growth conditions

E. coli strain XL1-Blue (Stratagene, La Jolla, CA, USA), was used for routine subcloning and plasmid propagation. *E. coli* strain Rosetta (Novagen (EMD), San Diego, CA, USA), was used for overexpression of recombinant protein for antibody generation. All *E. coli* strains were grown in LB media with addition of appropriate antibiotics at 37 °C, according to standard protocols. To promote accumulation of the recombinant protein in a soluble rather than insoluble (inclusion body) form, overexpression of *IspS* in *E. coli* was performed at 15 °C. *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*) was grown routinely in BG11 medium (Stanier et al., 1971) at 25 °C, and at a light intensity of approximately 40 μmol photons m^{-2} s^{-1}. For selection and growth of transformed strains, 5 μg/ml kanamycin (Invitrogen, Carlsbad, CA, USA) was added to liquid BG11 medium. For maintenance of strains on agar plates, the BG11 medium was supplemented with 1.5% (w/v) agar, 0.3% (w/v) sodium thiosulfate, and buffered with 10 mM TES-NaOH at pH 8.0.

2.2. Chlorophyll determination

For chlorophyll (Chl) measurements, pigments were extracted in 90% methanol, cell debris removed by centrifugation at 20,000 g for 5 min, and the absorbance of the supernatant was measured using a Shimadzu UV160U spectrophotometer. Chl *a*

concentrations were calculated as described previously (Meeks and Castenholz, 1971).

2.3. Codon optimization and gene synthesis

The nucleotide sequence of the *P. montana* (kudzu) isoprene synthase (*IspS*) gene (Sharkey et al., 2005), GenBank accession no AY316691, without its predicted chloroplast transit peptide (hereafter referred to as *kIspS*), was optimized to the preferred codon usage of *Synechocystis* using the GeneDesigner software (DNA 2.0, Menlo Park, CA, USA) and a *Synechocystis* codon usage table from the Kazusa Codon Usage Database (Nakamura et al., 2000). The native kudzu (*kIspS*) and *Synechocystis* codon-optimized kudzu *IspS* (*SkIspS*) genes were augmented by suitable restriction sites. These newly designed DNA sequences were synthesized by DNA 2.0 (Menlo Park, CA, USA). Nucleotide and corresponding amino acid sequences of the synthesized genes are provided in Fig. 1S.

2.4. Construction of plasmids for transformation of Synechocystis with the isoprene synthase gene.

For heterologous expression of the isoprene synthase gene in *Synechocystis*, plasmid constructs were generated, which allowed replacement of the *psbA*2 gene of *Synechocystis* with either the *kIspS* or *SkIspS* genes via double homologous recombination. Two regions of the *Synechocystis* genomic DNA containing 500 bp of sequence located immediately upstream and downstream, respectively, of the *psbA*2-gene were amplified by PCR. The primers used were A2us_Eco_F, 5′-GAGAGAGAATTCAGCGTTCCAGTGGAT-3′, and A2us_NdeI_Bam_R, 5′-GTTGGATCCGTCGTTGTCATATGGT-TATAA-3′, for amplification of the upstream region; and A2ds_Bam_F, 5′-GAGAGAGAGGATCCTTGGTGTAATGCC-3′, and A2ds_SacI_R, 5′-GAGAGAGAGAGCTCGATCGCCTTGGCAAAACAA-3′, for amplification of the downstream region. The upstream fragment was cloned in the *Eco*RI and *Bam*HI sites of pBluescript KS+ (Stratagene, La Jolla, CA, USA). The downstream fragment was subsequently cloned in the *Bam*HI and *Sac*I sites of the resulting vector to form pBA2A2. Thereafter, the *kIspS* or *SkIspS* synthetic genes were introduced into the *Nde*I and *Bam*HI restriction sites of pBA2A2, forming plasmids pBA2kIA2 and pBA2SkIA2, respectively. The *Nde*I site allowed cloning of the *IspS* genes in frame with the translation start site of the replaced *psbA*2. The final step in the generation of the plasmid constructs was cloning of an antibiotic resistance cassette carrying *npt*, which is conferring resistance to kanamycin, into the single *Bam*HI site of pBA2kIA2 and pBA2SkIA2, to form plasmids pBA2kIKmA2 and pBA2SkIKmA2. These two vectors were used to transform *Synechocystis*, as described below.

2.5. Transformation of Synechocystis

Synechocystis cultures were grown in liquid BG11 medium for 2–3 days until the cell density reached about 3×10^7 cells/ml (OD_{730}=0.3). Cells were harvested by centrifugation and resuspended in fresh BG11 to a density of 10^9 cells/ml. One microgram of plasmid DNA was added to 100 μl of cell suspension in a microcentrifuge tube and mixed. The mixture was incubated at 25 °C under low light for 4–6 h before spreading on nitrocellulose filters (HAWG, Millipore, Billerica, MA, USA), which were placed on top of BG11 agar plates. After incubation for 24 h on BG11 agar, the filters were moved to selective media on BG11 agar plates containing 5 μg/ml kanamycin. Single colonies were isolated after about 2 weeks incubation, and grown in liquid culture for analysis.

2.6. Generation of IspS polyclonal antibodies

The native kudzu isoprene synthase cDNA sequence (*kIspS*) (Sharkey et al., 2005) (GenBank accession no. AY316691) was cloned in an expression vector carrying a His-tag, which was subsequently used to transform *E. coli* (Rosetta, Novagen (EMD), San Diego, CA, USA). After expression, the recombinant protein was purified on a Superflow Ni-NTA agarose column (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The purified His-tag-kIspS protein was used as antigen for the generation of specific polyclonal antibodies (Covance, Princeton, NJ, USA).

2.7. Reverse transcription (RT-) PCR

Cultures of wild type and transformant cell lines carrying the *kIspS* or *SkIspS* constructs were harvested in the exponential growth phase, and RNA extracted for analysis. For RT-PCR experiments, total RNA was extracted from 10 ml of cell culture, using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RT-PCR was performed using 0.25 μg of total RNA as starting material for cDNA generation by SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), followed by amplification by PCR. The following gene-specific primers were used:

RT_kIspS_F, 5′-TCTTGGCTTTGAGGGAGAAA-3′, and RT_kIspS_R, 5′-CCACCACCTTGACAGGTCTT-3′, for amplification of *kIspS*; RT_SkIspS_F, 5′-CGGTCCTTAACGGACTTTCA-3′, and RT_SkIspS_R, 5′-ATCGCCGTATTGGTAAGTGC-3′, for amplification of *SkIspS*; RT_rbcL_F, 5′-GTATCACCATGGGCTTCGTT-3′, and RT_rbcL_R, 5′-CACAAGCTTCCAAAGCAACA-3′, for amplification of *rbcL*, which was used as a positive control of transcription.

2.8. Southern blot analysis

Genomic DNA from *Synechocystis* strains was extracted as previously described (Tamagnini et al., 1997). For Southern blots, 1 μg of each DNA sample was digested with a combination of two restriction enzymes; *Xba*I and *Sac*I, or *Xba*I and *Nde*I. The digested DNA samples were separated on agarose gel and blotted onto a nylon membrane (Amersham Hybond N+, GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. Detection of DNA fragments was performed using the Amersham Gene Images AlkPhos Direct Labeling and Detection System (GE Healthcare, Little Chalfont, UK). The probe used for detection was generated by PCR, using primers A2us_probe_R, 5′-GAGTTTTG-TAAAGCTTTGTAACAGGA-3′, and A2us_Eco_F, 5′-GAGAGAGAATT-CAGCGTTCCAGTGGAT-3′, with *Synechocystis* genomic DNA as template for the reaction, and labeled according to the manufacturer's instructions.

2.9. SDS-PAGE and Western blot analysis

For Western blot analysis, *Synechocystis* crude extracts were prepared by sonication of cells in Tris–EDTA–NaCl (TEN) buffer (50 mM Tris–HCl pH 8, 5 mM EDTA, 100 mM NaCl) with addition of a mix of protease inhibitors (P2714, Sigma-Aldrich, St Louis, MO, USA). After sonication, insoluble material was removed by centrifugation, and the protein concentration of the supernatant determined using the Bio-Rad DC assay (Bio-Rad Laboratories, Hercules, CA, USA). The soluble protein extracts were separated on 8–12% SDS-PAGE according to Laemmli, (1970), blotted onto PVDF membranes (Millipore, Billerica, MA, USA), according to standard procedure, and probed sequentially with primary specific polyclonal antibodies and horseradish peroxidase conjugated second-

ary antibodies (Bio-Rad Laboratories, Hercules, CA, USA). Cross-reactions between protein bands and antibodies were visualized using the Supersignal ECL detection kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA) following the manufacturer's instructions. The antiserum against the AtpA protein was described previously (Park and Rodermel, 2004).

2.10. Isoprene production assays

To assay isoprene synthase activity, gas from the headspace of sealed cultures was sampled and analyzed by gas chromatography using a Shimadzu 8A GC (Shimadzu, Columbia, MD, USA) equipped with a flame ionization detector (FID) and a column selected to detect short-chain hydrocarbons. Amounts of isoprene produced were estimated by comparison with a pure isoprene standard (Acros Organics, Fair Lawn, NJ, USA).

3. Results

3.1. IspS protein overexpression

The isoprene synthase *IspS* gene from the vine *P. montana* (kudzu) was used for expression of the enzyme in *E. coli* and *Synechocystis*. The native cDNA sequence (GenBank accession no. AY316691) was first employed (Sharkey et al., 2005). The nucleotide sequence encoding the 45 amino acid chloroplast transit peptide was removed, resulting in a cDNA sequence encoding the mature IspS protein only. This modified kudzu native *IspS* gene, from here on termed *kIspS*, was cloned in an expression vector also including a 6xHis-tag epitope. The tagged recombinant protein was expressed in *E. coli*, and could readily be visualized on Coomassie stained SDS-PAGE of whole-cell extracts. Fig. 2 shows such an SDS-PAGE analysis, where protein samples were prepared from whole-cell *E. coli* protein extracts of un-induced (lane 1; not expressing His-kIspS) and induced (lane 2; expressing the His-kIspS) *E. coli* cells. The over-expressed His-kIspS protein is shown as a dominant 65 kD band (569 amino acids) in the induced cell extract only (Fig. 2, lane 2).

3.2. IspS gene codon-use optimization

Among photosynthetic microorganisms, the unicellular cyanobacteria *Synechocystis* sp. are most amenable to genetic transformation. Because the native kudzu cDNA sequence has a codon usage different from that preferred by *Synechocystis*, a *de novo* codon-optimized version of the gene was designed and synthesized. In this optimized version of the gene, termed by us *SkIspS*, the codon usage was adapted to eliminate codons rarely used in *Synechocystis*, and to adjust the AT/GC ratio to that of the host. Rare codons were defined using a codon usage table derived from the sequenced genome of *Synechocystis* (Nakamura et al., 2000). Fig. 3 presents a graphic illustration of the difference in codon usage between the original *kIspS* and the codon-optimized *SkIspS* gene, plotting, for each codon in the two gene sequences, the actual frequency of use of that particular codon in the genome of *Synechocystis*. In the native kudzu *IspS* sequence (Fig. 3, *kIspS*), a substantial number of codons are present that are used with a frequency of less than 12% by *Synechocystis*. In the codon-optimized gene (Fig. 3, *SkIspS*), such low-frequency codons were not allowed. To test the effectiveness of codon optimization in the expression of the *IspS* gene, both the native kudzu (*kIspS*) and the codon-optimized *SkIspS* sequence were employed for expression in *Synechocystis*.

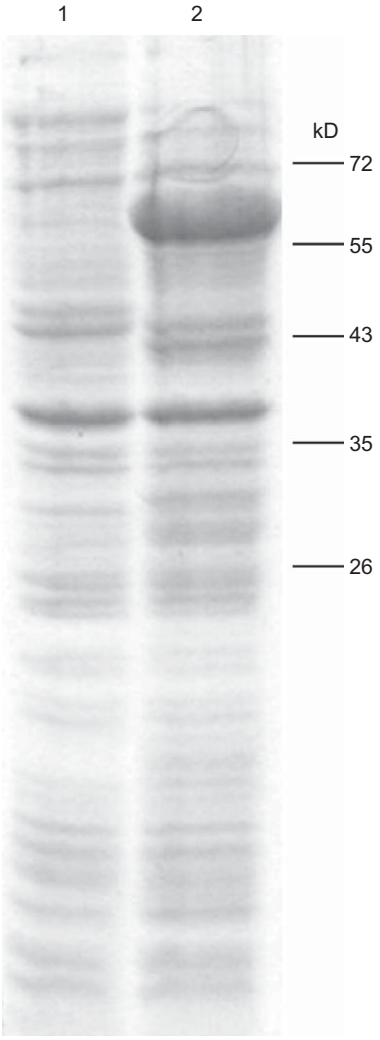

Fig. 2. Coomassie-stained SDS-PAGE profile of proteins from whole cell *E. coli* extracts. Total protein was isolated from un-induced (lane 1) and induced (lane 2) cell lines. The IspS protein in the induced sample (lane 2) is visible as a pronounced 65 kD polypeptide.

3.3. IspS plasmid constructs and Synechocystis transformations

The two versions of the kudzu isoprene synthase gene, *kIspS* and *SkIspS*, each were cloned in a plasmid construct, where the respective gene, together with an antibiotic resistance cassette, was placed in-between the sequences immediately flanking the *psbA2* gene in *Synechocystis* (Fig. 4). The resulting plasmids, containing *kIspS* or *SkIspS*, respectively, were used for double homologous recombination into the *Synechocystis* genomic DNA, yielding transformants, in which the *psbA*2 gene was replaced by the *kIspS* or *SkIspS* constructs, respectively (Fig. 5A). The correct insertion of the new genes into the *Synechocystis* chromosome, and segregation of the transformed DNA copy from all wild type copies, was confirmed by Southern hybridizations (Fig. 5B and C) and PCR analysis (Fig. 6, DNA control lanes). After sub-culturing of the transformant strains in selective media, no wild type copies of the *Synechocystis* DNA could be detected by Southern hybridization to a specific probe, corresponding to the *psbA*2 upstream region. For example, digestion of the wild type *Synechocystis* genomic DNA with *Sac*I (S) and *Xba*I (X) (Fig. 5A, top map, Fig. 5B, left panel, lane 1), gave rise to a hybridization signal from a band of different size compared to that obtained from digested genomic DNA of three different transformant lines

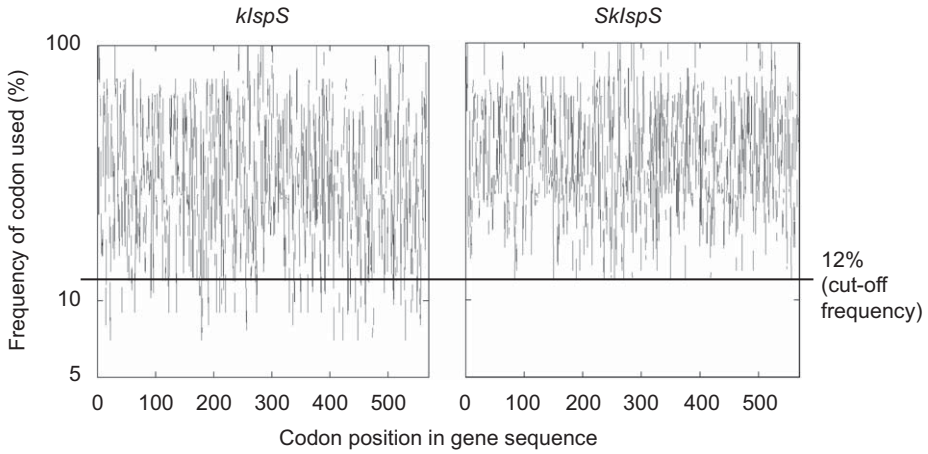

Fig. 3. Codon use optimization for the *IspS* gene. Shown is the frequency of codon usage in *Synechocystis* for each codon of the *kIspS* and *SkIspS* genes. In the optimized sequence, codons used with a frequency lower than 12% were not allowed. The 12% cut-off frequency used in the gene optimization is shown as a horizontal line. In order to better emphasize the difference between the two sequences with respect to usage of rare codons, the *Y*-axis was plotted as the log of the frequency of codons used.

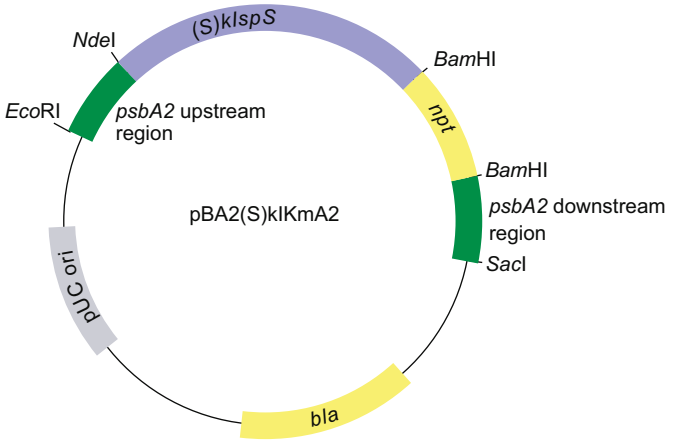

Fig. 4. Plasmid constructs for the transformation of *Synechocystis*. The two versions of the isoprene synthase gene, *kIspS* and *SkIspS* (indicated in the figure as (*S*)*kIspS*), were each cloned in a pBluescript-based plasmid, also containing an antibiotic resistance cassette and the flanking sequences of *psbA2*. The resulting plasmids were used for insertion into the *Synechocystis* genome via double homologous recombination. Restriction sites used for cloning are indicated (see Section 2). *npt*=neomycin phosphotransferase gene, conferring kanamycin resistance; *bla*=β-lactamase gene, conferring ampicillin resistance.

carrying the *kIspS* gene (Fig. 5A, middle map, Fig. 5B, left panel, lanes 2–4). This was due to the presence of an extra *Sac*I (S) restriction site in these transformants only. Similarly, digestion of the wild type *Synechocystis* chromosome with *Xba*I (X) and *Nde*I (N) (Fig. 5A) gave rise to a hybridization signal corresponding to a band of larger size (Fig. 5B, right panel, lane 1) compared to the *kIspS*-transformants (Fig. 5B, right panel, lanes 2–4). Similar results were obtained for the *Synechocystis* transformants carrying the *SkIspS* construct (Fig. 5A, lower map, and Fig. 5C).

Fitness of the *kIspS* and *SkIspS* transformants, relative to that of the wild type, was tested by comparative measurements of the rate of growth, under conditions of limiting illumination at 25 °C. Under such growth conditions, no significant growth differences could be detected between transformant strains and the wild type (results not shown), suggesting that expression of the *IspS* gene, and replacement of the *psbA2* gene, did not adversely affect cell physiology and growth.

3.4. Expression of the IspS trangenes

RT-PCR was employed to test whether the *IspS* genes, as cloned downstream of and in frame with the *psbA2* promoter, were transcribed in *Synechocystis*. The RT-PCR analysis showed that both versions of the gene (*kIspS* and *SkIspS*) were indeed expressed. This is shown in Fig. 6A (lane+RT) for *kIspS*, and Fig. 6B (lane+RT) for *SkIspS*, where *IspS* amplification products were visualized on agarose gel, demonstrating the presence of *kIspS* and *SkIspS* mRNA in these different transformants. In control samples where no RT enzyme was added, no amplification products could be seen from the PCR reaction. Conversely, when genomic DNA of the transformed strains was used as a template in the PCR reaction (Fig. 6, DNA), a band of the same size as in the RT-PCR analysis was evident, both in the *kIspS* and *SkIspS* transformants, but not in the *Synechocystis* parent strain (Fig. 6, lanes DNA wt). Several independent transformant lines were examined by RT-PCR, and all tested strains showed similar expression (data not shown).

Western blot analysis and immunodetection of the isoprene synthase enzyme, using specific polyclonal antibodies raised against the *E. coli*-expressed recombinant protein, confirmed the presence of the IspS protein in *Synechocystis* (Fig. 7A). The IspS protein was localized in the soluble fraction of *Synechocystis* cell extracts, consistent with the notion of a soluble protein (Sharkey et al., 2005). Fig. 7A, wt, shows absence of cross-reaction between the IspS polyclonal antibodies and any protein of the *Synechocystis* wild type soluble extract. Fig. 7A, lanes 1–3, show a specific cross-reaction between the IspS polyclonal antibodies and a protein band at about 65 kD from soluble extracts of three independent lines of *Synechocystis* transformants carrying the *kIspS* construct. A much stronger cross-reaction was observed in extracts of three independent clone lines of *Synechocystis* transformants carrying *SkIspS* construct (Fig. 7A, lanes 4–6). These results clearly show presence of the recombinant IspS protein in the *Synechocystis* transformants. It was consistently observed that the level of the IspS protein present in the codon-optimized *SkIspS* transformants was substantially greater (by a factor of about 10, as estimated by quantitative Western blot analysis) than that in the non-optimized *kIspS* transformant lines (Fig. 7A), suggesting a substantial positive effect on the expression, as a result of the codon optimization of the *IspS* gene. This outcome is testimony to

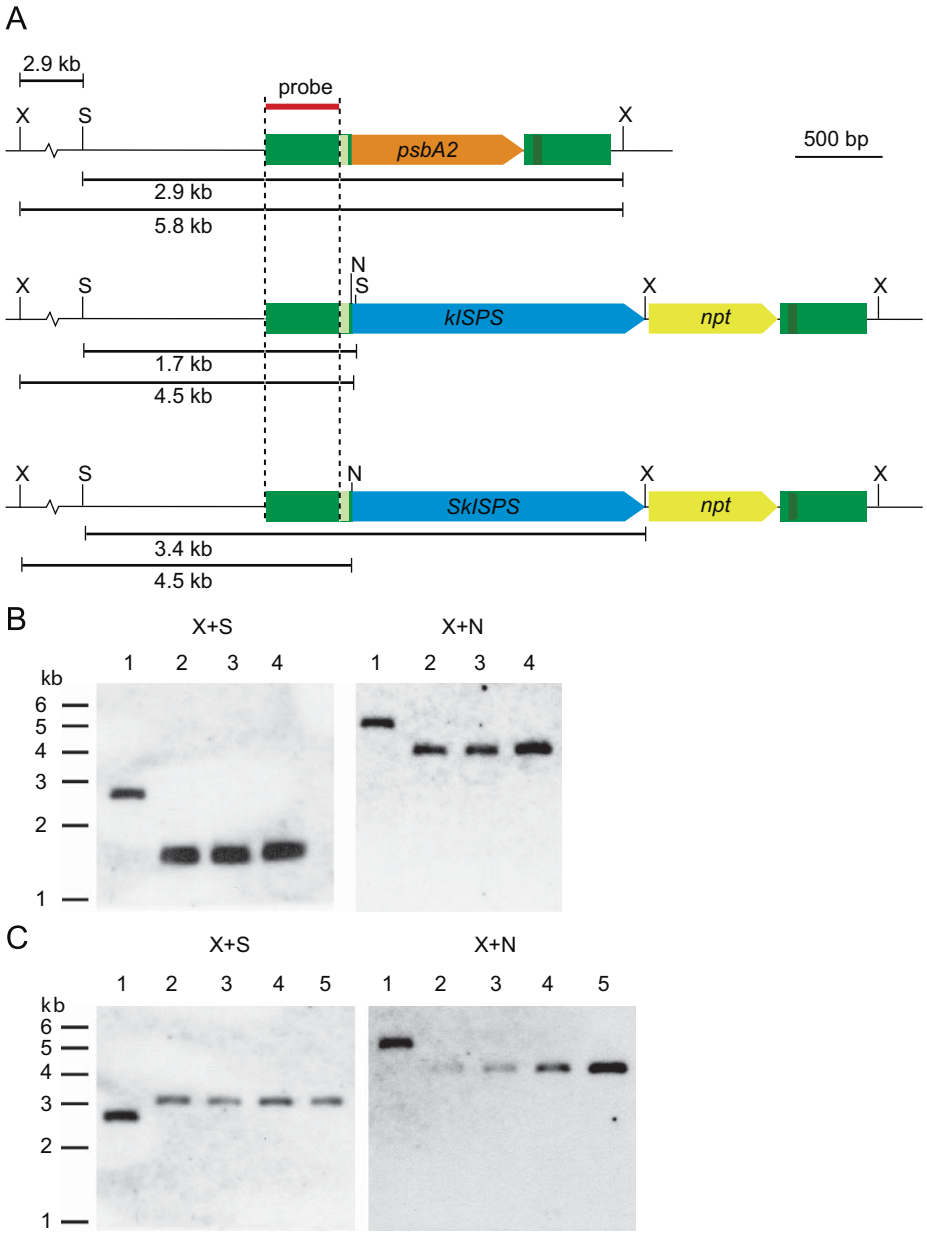

Fig. 5. Genetic maps and Southern blots of wild type and *IspS* transformants. (A) Map of the *Synechocystis psbA2* locus, with the inserted *kIspS* and *SkIspS* genes, compared to the wild type. Restriction sites for XbaI (X), NdeI (N), and SacI (S) are indicated. Bars under each map show expected sizes of restriction fragments detected in Southern blots using the *psbA2* upstream region probe ("probe"). (B) Southern blot analysis of *Synechocystis* wild type and *kIspS* transformants. Left panel: restriction by XbaI and SacI. Right panel: Restriction by XbaI and NdeI. Lane 1: wild type; lane 2: kI-6; lane 3: kI-14; lane 4: kI-17. (C) Southern blot analysis of *Synechocystis* wild type and *SkISpS* transformants. Left panel: restriction by XbaI and SacI. Right panel: Restriction by XbaI and NdeI. Lane 1: wild type; lane 2: SkI-3.1; lane 3: SkI-2.1; lane 4: SkI-1.2; lane 5: SkI-1.1.

the advantage that is conferred by rational design of codon usage in heterologous gene expression.

To test the efficacy of the *psbA2* promoter on the expression of the *IspS* gene in *Synechocystis*, experiments were conducted, where cultures of *Synechocystis* transformants, expressing *IspS* gene were subjected to a shift from low light (10 μmol photons $m^{-2} s^{-1}$) to high light (500 μmol photons $m^{-2} s^{-1}$) growth conditions. It is known that high light conditions enhance expression of the *psbA2* gene (Mohamed and Jansson, 1989). Fig. 7B (top panel) shows that expression of IspS in *Synechocystis* was induced as a function of time (0–6 h) under high light, with levels of the IspS protein increasing steadily between 0 and 6 h of high light incubation. Contrary to this induction, expression levels of the control protein AtpA were stable and unaffected by the shift in light intensity (Fig. 7B, middle panel). These results demonstrate that the *psbA2* promoter is able to regulate the expression of the heterologously expressed *IspS* gene in response to shifting irradiance conditions. Accordingly, use of the *psbA2* promoter under high light conditions can be used as a tool for the overexpression of the *IspS* gene.

3.5. Isoprene production by the IspS transformant Synechocystis

The above results clearly show that *Synechocystis*, as wells as *E. coli* strains, are amenable to heterologous transformation by the *IspS* gene, and that they express and accumulate the respective protein in their cytosol. To determine whether the expressed IspS protein is metabolically competent, gas samples were obtained from the headspace of sealed cultures of wild type and

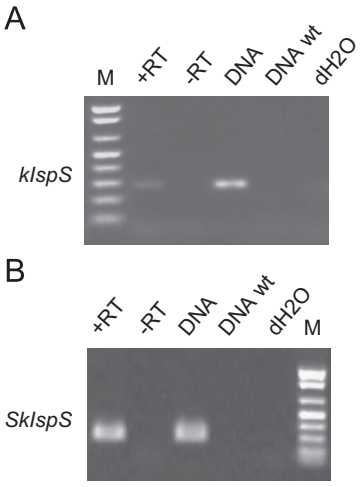

Fig. 6. RT-PCR analysis of *kIspS* and *SkIspS* transformants. Shown is the transcription of *kIspS* and *SkIspS* genes in transformed strains of *Synechocystis*. (A) Transcription of *kIspS*. Lanes are M: molecular size markers; +RT: RT-PCR using RNA from *kIspS* strain 6 as template for the reverse transcription; −RT: as for +RT but without addition of RT enzyme in the reaction; DNA: positive control for the PCR using genomic DNA from *kIspS* strain 6 as template; DNA wt: negative control for the PCR using genomic DNA from wild type *Synechocystis* as template; dH_2O: negative control for the PCR using water as template. (B) Transcription of *SkIspS*. Lanes are +RT: RT-PCR using RNA from *SkIspS* strain 1.2 as template for the reverse transcription; −RT: as for +RT but without addition of RT enzyme in the reaction; DNA: positive control for the PCR using genomic DNA from *SkIspS* strain 1.2 as template; DNA wt: negative control for the PCR using genomic DNA from wild type *Synechocystis* as template; dH_2O: negative control for the PCR using water as template; M: molecular size markers.

IspS transformants and analyzed by gas chromatography. In all such transformants, but not in their wild type counterparts, an isoprene-specific peak was clearly evident, showing that the expressed enzyme is indeed active in the *IspS* transformant strains. Fig. 8 shows an example of a comparative GC analysis of headspace gases from photosynthetically grown *Synechocystis* wild type (left panel) and an *IspS* transformant (middle panel), showing the isoprene peak at about 5 min after sample injection in the GC in the transformant but not in the wild type. The identity of isoprene in the corresponding GC peak was established by comparison with an isoprene standard (Fig. 8, right panel). Preliminary measurements showed accumulation of ~50 μg isoprene per g dry cell weight per day in the sealed *Synechocystis* culture headspace.

4. Discussion

"Photosynthetic biofuels", as defined in this laboratory, are produced in a system where the same organism serves both as photo-catalyst and producer of ready-made fuel. A number of guiding principles have been applied in the endeavor of

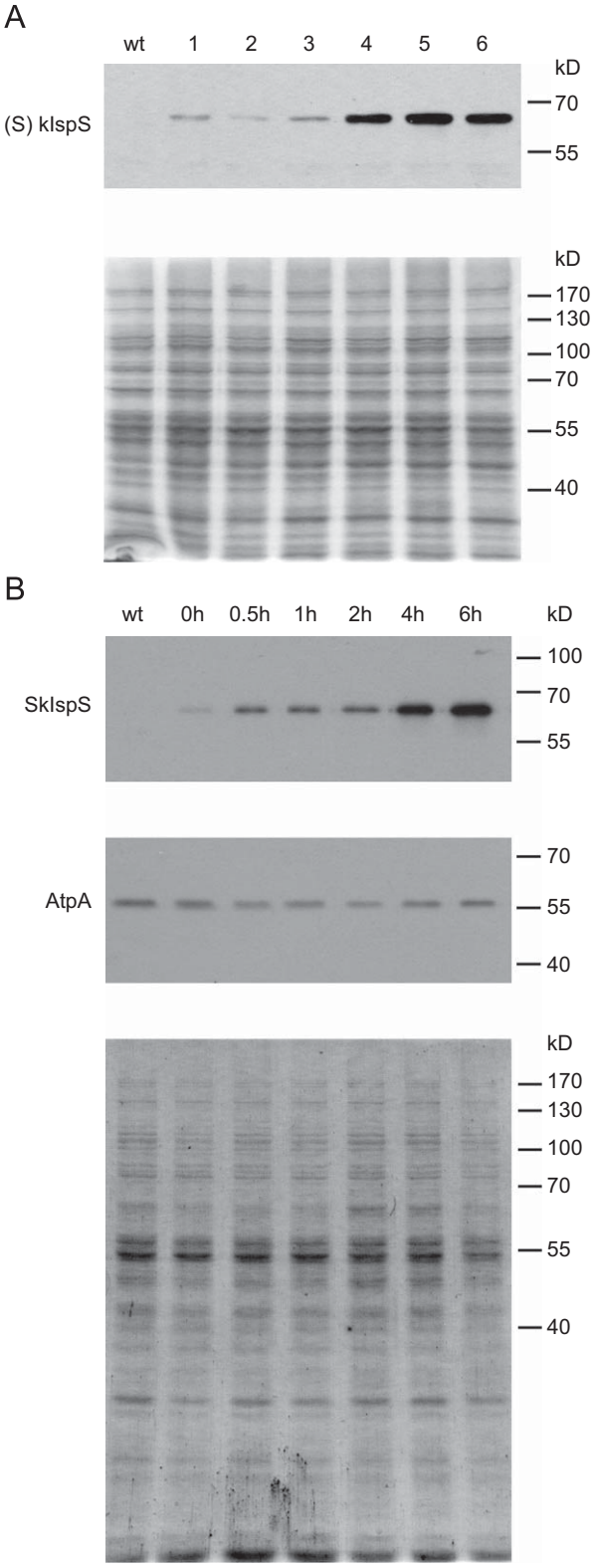

Fig. 7. Western blot analysis of *Synechocystis* wild type and *IspS* transformants. (A) Top panel: Western blot analysis with IspS specific polyclonal antibodies. Lane 1: wt *Synechocystis*; lanes 2–4: three different lines of *kIspS*-expressing *Synechocystis* transformants; lanes 5–7: three different lines of *SkIspS*-expressing *Synechocystis* transformants. Lower panel: Coomassie-stained SDS-PAGE profile of proteins corresponding to the Western blot above, shown as a control of protein loading. 20 μg protein were loaded in each lane. (B) Western blot analysis showing induction of expression of SkIspS in response to high-light treatment of *Synechocystis*. Soluble extracts from samples of *Synechocystis* were collected between 0 h and 6 h after a shift from low-light to high-light conditions. Top panel: Western blot analysis with IspS specific polyclonal antibodies. Lane 1: wt *Synechocystis*; lanes 0.5–6 h, *SkIspS*-expressing *Synechocystis* transformant samples taken at $t=0$ to $t=6$ h after a shift of the culture from low (10 μmol photons m^{-2} s^{-1}) to high (500 μmol photons m^{-2} s^{-1}) light. Middle panel: protein levels of the α subunit of ATP synthase (AtpA) (same samples as above), probed with polyclonal antibodies against this AtpA protein, and used as a control to show stable expression independent of the change in the light regime. Lower panel: Coomassie-stained SDS-PAGE profile of proteins corresponding to the Western blots above, shown as a control of protein loading. 20 μg protein were loaded in each lane.

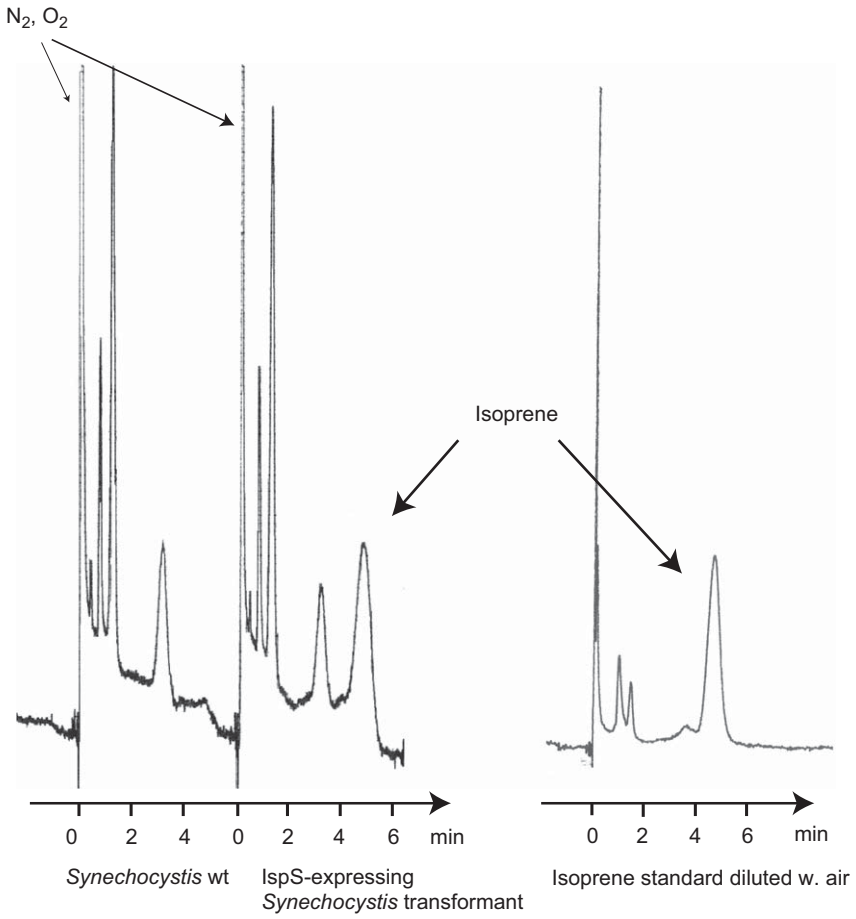

Fig. 8. Isoprene production measurements. An example of gas samples from the GC measurements is shown. Left panel: GC profile of gases from the headspace of a *Synechocystis* wild type culture. Middle panel: GC profile of gases from the headspace of an *IspS*-expressing *Synechocystis* transformant culture. The isoprene peaks are shown with an elution time of 4–5 min. Right panel: GC profile of an isoprene standard in air.

photosynthetic biofuels, as they pertain to the selection of organisms and, independently, to the selection of potential biofuels. Criteria for the selection of organisms include, foremost, the solar-to-biofuel energy conversion efficiency, which must be as high as possible. This important criterion is better satisfied with photosynthetic microorganisms than with crop plants (Melis, 2009). Criteria for the selection of potential biofuels include (i) the relative energy content and potential utility of the molecule. Pure hydrocarbons are preferred over sugars or alcohols because of the greater relative energy stored in hydrocarbon molecules (Schakel et al. 1997; Berg et al., 2002); and (ii) the question of product separation from the biomass, which enters prominently in the economics of the process and is a most important aspect in commercial application. Volatile isoprene hydrocarbons are suitable in this respect, as they are not miscible in water, spontaneously separating from the biomass and, thus, alleviating the requirement of time-consuming, expensive, and technologically complex biomass dewatering (Danquah et al. 2009; Saveyn et al., 2009) that otherwise would be needed for product separation.

In the pursuit of renewable biofuels, photosynthesis, cyanobacteria and isoprene meet the above-enumerated criteria for "process", "organism" and "product", respectively. Evidence was provided in this work to show that isoprene can be produced photosynthetically by cyanobacteria, genetically modified to express a plant isoprene synthase. In this study, the kudzu *IspS* gene was employed via heterologous expression in *Synechocystis*. Expression of the *IspS* gene in this cyanobacterium was improved upon codon-use optimization of its DNA sequence (Fig. 3). This resulted in a much stronger expression of the encoded protein, compared to expression of the non-optimized gene driven by the same promoter (Fig. 7). These results clearly show that codon optimization conferred an advantage in *IspS* gene expression.

Generation of high-value plant bio-products upon genetic and metabolic engineering of microorganisms has been successfully applied (Vannelli et al., 2007; Anthony et al. 2009). Moreover, the terpenoid biosynthetic pathway in plants, microalgae and cyanobacteria has been the subject of many investigations, as essential cellular compounds are derived from it (Chappell, 1995; McGarvey and Croteau, 1995; Lichtenthaler, 1999, 2000). Understanding terpenoid metabolism to improve carotenoid production in plants was the subject of an authoritative analysis by Sandmann et al. (2006). Along these lines, the present work demonstrated, for the first time, "proof of concept" in photosynthetic isoprene hydrocarbons production by cyanobacteria.

Further assessment of the potential of cyanobacteria and microalgae as hosts for photosynthetic isoprene synthesis is offered upon examination of the challenges encountered and metabolic engineering strategies that would be required to improve titer (Stephanopoulos, 2007; Fischer et al., 2008). In the context of this work, photosynthetic carbon partitioning among the sugar, terpenoid and fatty acid biosynthetic pathways must be

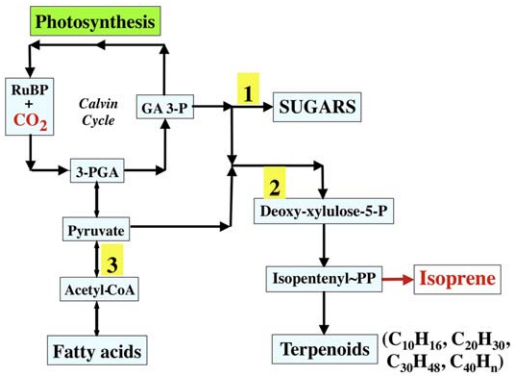

Fig. 9. Photosynthetic carbon flux and partitioning among the sugar (1), terpenoid (2) and fatty acid (3) biosynthetic pathways. Carbon allocation is primarily directed toward sugar biosynthesis (80–85%), with fatty acid biosynthesis (~10%) and terpenoid biosynthesis (3–5%) lagging far behind that of sugar.

considered. Fig. 9 shows the metabolic flux relationship between these three biosynthetic pathways. In preliminary measurements from this lab (unpublished), it was determined that photosynthetic carbon allocation among these three pathways is primarily directed toward sugar biosynthesis (80–85%), while fatty acid biosynthesis (~10%) and terpenoid biosynthesis (3–5%) are lagging far behind that of sugar. It is evident from these considerations that, to improve titer of isoprene production, metabolic engineering strategies would be needed to alter this carbon partitioning in the cell and to re-direct photosynthate away from sugar and more toward the terpenoid biosynthetic pathway.

In plasmid constructs employed for the expression of the isoprene synthase in *Synechocystis*, we used the *psbA*2 gene locus for insertion of the transgenes. Upon transformation of *Synechocystis* with those constructs, the coding sequence of the *psbA*2 gene was replaced by the *IspS* gene, and the *psbA*2 promoter was used to drive expression of *(S)kIspS. psbA*2 is one of three homologous genes in cyanobacteria, the other two being *psbA1* and *psbA*3, that encode the 32 kD/D1 reaction center protein of photosystem-II. The promoter region and regulation of expression of the *psbA*2 gene has been characterized (Eriksson et al., 2000; Mohamed et al., 1993; Mohamed and Jansson, 1989). It has also been shown that a knock-out mutant of either *psbA*2 or *psbA*3 is able to grow photo-autotrophically, provided that the other *psbA* genes are still active, while *psbA*1 on its own was not able to compensate for the loss of both *psbA*2 and *psbA*3 (Mohamed and Jansson, 1989). Inactivation of *psbA*2 resulted in a strong up-regulation of *psbA*3 (Mohamed et al., 1993). This is in agreement with results in this study, where replacement of *psbA*2 by the *IspS* gene did not significantly alter normal photosynthesis and growth of the transformants. On the contrary, it conferred the advantage of up-regulation of the *IspS* gene expression concordant with the level of irradiance (Fig. 7B).

Isoprene is an energy rich 5-carbon volatile hydrocarbon molecule, useful industrially as the basic unit of synthetic rubber polymer. Currently, isoprene used by the rubber industry is produced mainly from petroleum, as a byproduct of the refining process. However, this work has shown that isoprene can be produced by photosynthetic cyanobacteria, through heterologous expression of the gene encoding for the isoprene synthase (*IspS*), in a reaction of the MEP pathway, driven by the process of cellular photosynthesis. Since the carbon atoms used to generate isoprene in such a system originate from CO_2, this would make cyanobacterial isoprene production a carbon-neutral source of synthetic chemistry feedstock and fuel. Isoprene would also be suitable as a building block for the production of longer chain hydrocarbons, to be used as renewable and carbon-neutral biofuels.

Acknowledgments

The work was conducted with financial support by Ecoprene, Inc. under UCB-Ecoprene R&D agreement # 20071085.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2009.10.001.

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A *kIspS*:

NdeI NcoI

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XbaI BamHI

Deduced amino acid sequence of kIspS:

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LIDDVQRLGLTYKFEKDIIKALENIVLLDENKKNKSDLHATALSFRLLRQHGFEVSQDVFERFKDKEGGFSGEL
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YEPKEPHHQLLLELAKLDFNMVQTLHQKELQDLSRWWTEMGLASKLDFVRDRLMEVYFWALGMAPDPQFGECRK
AVTKMFGLVTIIDDVYDVYGTLDELQLFTDAVERWDVNAINTLPDYMKLCFLALYNTVNDTSYSILKEKGHNNL
SYLTKSWRELCKAFLQEAKWSNNKIIPAFSKYLENASVSSSGVALLAPSYFSVCQQQEDISDHALRSLTDFHGL
VRSSCVIFRLCNDLATSAAELERGETTNSIISYMHENDGTSEEQAREELRKLIDAEWKKMNRERVSDSTLLPKA
FMEIAVNMARVSHCTYQYGDGLGRPDYATENRIKLLLIDPFPINQLMYV*

B *SkIspS*:

NdeI

CATATGCCCTGGCGTGTAATCTGTGCAACTTCTTCCCAATTTACTCAAATTACCGAGCACAATTCCCGGCGTAG
TGCCAACTATCAACCCAATCTGTGGAACTTTGAGTTCTTACAGAGCCTGGAAAATGATTTAAAGGTCGAGAAAT
TGGAGGAGAAGGCCACTAAATTGGAAGAGGAAGTGCGGTGTATGATTAATCGTGTAGACACCCAACCATTGAGT
CTGTTAGAATTGATCGATGATGTGCAACGTCTCGGCCTGACATACAAATTCGAAAAAGATATCATTAAGGCCCT
AGAAAACATTGTCTTATTGGATGAAAACAAGAAAAATAAGTCTGACTTGCATGCCACCGCTTTAAGTTTCCGCT
TGTTGCGGCAGCACGGCTTTGAAGTGTCCCAAGATGTTTTTGAACGGTTCAAAGACAAGGAGGGCGGCTTTTCC
GGCGAACTCAAAGGGGATGTTCAGGGCCTATTGTCTTTGTATGAAGCTAGTTACTTGGGATTTGAAGGCGAGAA
TCTGTTAGAAGAAGCTCGCACTTTTTCCATTACACATTTAAAGAACAACCTAAAGGAAGGGATTAACACAAAAG
TGGCTGAGCAGGTGTCTCATGCTCTGGAGTTGCCGTATCATCAACGCTTACACCGGCTCGAAGCCCGCTGGTTT

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TTGGATAAATATGAACCGAAAGAACCGCATCATCAATTACTGCTCGAACTGGCGAAGCTGGACTTTAATATGGT
CCAAACACTACATCAGAAAGAACTCCAGGACCTAAGTCGGTGGTGGACTGAAATGGGTCTGGCATCCAAGCTAG
ATTTTGTGCGCGACCGTTTGATGGAGGTGTACTTCTGGGCACTAGGCATGGCTCCCGACCCGCAGTTTGGTGAG
TGTCGTAAGGCAGTGACCAAGATGTTTGGTTTAGTAACGATCATCGACGACGTTTACGATGTCTATGGCACCCT
AGACGAATTACAACTCTTTACAGATGCCGTCGAACGTTGGGATGTTAATGCCATCAATACCTTACCTGATTACA
TGAAATTGTGCTTCCTCGCCTTGTATAATACCGTTAATGACACCAGCTATTCTATTCTGAAGGAAAAAGGCCAC
AATAACTTAAGCTACCTAACCAAAAGTTGGCGGGAATTGTGTAAGGCTTTCTTACAGGAAGCCAAATGGTCCAA
CAACAAAATTATCCCCGCATTTTCTAAATACCTGGAAAATGCCTCCGTGTCCTCTTCCGGGGTGGCTTTGCTAG
CACCCAGCTACTTTTCTGTTTGTCAGCAACAGGAGGACATCAGTGACCATGCCTTGCGGTCCTTAACGGACTTT
CATGGCTTAGTGCGGAGTAGCTGCGTCATTTTTCGTTTATGTAACGATTTGGCTACAAGTGCTGCGGAATTGGA
ACGTGGGGAAACAACCAACAGCATTATCAGTTATATGCACGAAAACGATGGCACCAGTGAAGAGCAGGCACGGG
AAGAACTGCGCAAATTAATCGACGCTGAATGGAAGAAGATGAATCGCGAACGTGTGTCTGATAGTACCTTATTA
CCTAAAGCCTTCATGGAAATTGCGGTGAATATGGCCCGCGTCAGTCATTGCACTTACCAATACGGCGATGGATT
AGGTCGGCCCGATTACGCAACGGAAAATCGGATCAAATTGCTATTGATTGATCCGTTCCCAATTAATCAATTAA
TGTACGTGTAATctagaggatcc
            |   |
            XbaI BamHI

Deduced amino acid sequence of SkIspS:
MPWRVICATSSQFTQITEHNSRRSANYQPNLWNFEFLQSLENDLKVEKLEEKATKLEEEVRCMINRVDTQPLSL
LELIDDVQRLGLTYKFEKDIIKALENIVLLDENKKNKSDLHATALSFRLLRQHGFEVSQDVFERFKDKEGGFSG
ELKGDVQGLLSLYEASYLGFEGENLLEEARTFSITHLKNNLKEGINTKVAEQVSHALELPYHQRLHRLEARWFL
DKYEPKEPHHQLLLELAKLDFNMVQTLHQKELQDLSRWWTEMGLASKLDFVRDRLMEVYFWALGMAPDPQFGEC
RKAVTKMFGLVTIIDDVYDVYGTLDELQLFTDAVERWDVNAINTLPDYMKLCFLALYNTVNDTSYSILKEKGHN
NLSYLTKSWRELCKAFLQEAKWSNNKIIPAFSKYLENASVSSSGVALLAPSYFSVCQQQEDISDHALRSLTDFH
GLVRSSCVIFRLCNDLATSAAELERGETTNSIISYMHENDGTSEEQAREELRKLIDAEWKKMNRERVSDSTLLP
KAFMEIAVNMARVSHCTYQYGDGLGRPDYATENRIKLLLIDPFPINQLMYV*
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Fig. 1S. *IspS* nucleotide and amino acid sequences employed in this work. (A) *kIspS* is the native *Pueraria montana* (kudzu) cDNA sequence without the native chloroplast transit peptide. (B) *SkIspS* is the *Synechocystis* codon optimized version of the *Pueraria montana* (kudzu) cDNA *minus* a chloroplast transit peptide. Introduced restriction sites are underlined: NdeI site at the start of the sequence to facilitate in-frame cloning of the gene; XbaI and a BamHI sites after the stop codon. The *kIspS* sequence also contains an NcoI site near the 5' end. Start and stop codons are also indicated.