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博士論文(要約)

Basic research for photosynthetic production of isoprenoids in cyanobacteria

(シアノバクテリアを用いたイソプレノイドの光合成的生産に関する基礎研究)

2014 年 12 月博士（理学）申請

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Abstract

Cyanobacteria are oxygenic photosynthetic prokaryotes. I tried to make a model cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis*) the photosynthetic factory of isoprenoids using metabolic engineering. This thesis consists of two chapters; Chapter I, isoprenoid production using *Synechocystis*; Chapter II, enzymatic analysis of Slr0739, geranylgeranyl pyrophosphate (GGPP) synthase of *Synechocystis*.

In Chapter I, I constructed *Synechocystis,* which expresses plant derived limonene synthase as a model for photosynthetic production of isoprenoids. Cyanobacteria synthesize their own isoprenoids such as carotenoids and phytol using MEP pathway (also called non-mevalonate pathway). Limonene synthase produces limonene by one-step enzymatic reaction from geranyl pyrophosphate (GPP), which is the end product of MEP pathway. I have constructed a strain expressing the limonene synthase under the control of the *trc* promoter which is a constitutive expression promoter commonly used in *Synechocystis*. When the extract of this strain was analyzed by gas chromatography-mass spectrometry (GC-MS), the peak eluting at the same retention time as authentic limonene was detected. I confirmed that this strain synthesize limonene in the cells.

To quantify the volatile limonene, I designed gas-stripping method for collecting the product limonene. Gas stripping is a method removing the volatile products from the culture medium by aeration, and recovering the product in the exhaust gas by cold trap. When the strain expressing limonene

synthase was cultured using this system for 168 hours, limonene was exclusively recovered in the cold trap. The limonene content of the cell pellet was 0.4% and supernatant of the culture medium was practically free of limonene, confirming the efficiency of the gas-stripping and the cold trap. As a result, continuous limonene production and recovery was achieved over 100 hours at a rate of 41 $\mu g \cdot L\ culture^{-1} \cdot day^{-1}$.

To improve the supply of substrate for limonene synthase, I introduced three critical genes of the MEP pathway into the recombinant strain that overexpresses the limonene synthase: *dxs* gene for deoxyxylulose-1-phosphate synthase, *crtE* gene for GPP synthase, and *ipi* gene for isopentenyl pyrophosphate (IPP) isomerase. These genes were cloned from *Synechocystis* and introduced into a neutral chromosomal site as extra copies under the strong promoter *trc*. This MEP pathway-enhanced strain produced limonene at a rate 1.4 times higher than the parent strain. I also introduced a whole set of yeast genes for mevalonate pathway into the limonene synthase-expressing strain. This mevalonate pathway-enhanced strain produced limonene at a rate 1.7 times higher than the parent strain.

To expand my strategy, I constructed another recombinant strain that overexpresses amorpha-4, 11-diene synthase gene derived from a plant *Artemisia annua*. This strain produced amorpha-4, 11-diene at a rate >10-fold higher than the rate of limonene production. This finding suggests that *Synechocystis* is suitable for production of various isoprenoids by photosynthesis.

In Chapter II, I performed enzymatic analysis of intrinsic GGPP synthase of *Synechocystis* (Slr0739) to dissect its multistep enzymatic reactions. Generally, GGPP synthase catalyzes three sequential steps: addition of IPP to dimethylallyl pyrophosphate (DMAPP) to produce GPP, addition of IPP to GPP to produce farnesyl pyrophosphate (FPP), and addition of IPP to FPP to produce GGPP. At first, I established a quantitation method of these products GPP, FPP, and GGPP using acid hydrolysis and GC-MS. The Slr0739 protein was expressed in and isolated from *E. coli*. I examined the long-term accumulation profiles of GPP, FPP and GGPP, when the enzyme was incubated with DMAPP and IPP. As a result, the accumulation of intermediates GPP and FPP was transient and relatively low, while the final product GGPP was continuously accumulated. The accumulation profile indicated transient accumulation in the order of GPP -> FPP -> GGPP. Next, I analyzed the short-term reactions of GPP production from DMAPP and IPP and GGPP production from FPP and IPP. I estimated K_m and k_{cat} values for DMAPP and FPP. I could not obtain the k_m and K_{cat} for GPP. I performed simulation analysis of these multistep reactions using the experimental K_m and k_{cat} values and found that the k_{cat} and K_m for GPP are also comparable to those for FPP. Moreover, the whole reaction in experiments proceeded faster than the reaction in the simulation. It is suggested that produced intermediary products (GPP and FPP) more efficiently rebind to the enzyme than in the free solution. These findings also explain why the production rate of amorpha-4, 11-diene derived from FPP was higher than that of limonene derived from GPP as shown in Chapter I.

In this study, I achieved photosynthetic production of limonene and amorpha-4, 11-diene in cyanobacteria for the first time. I also succeeded in improving the production of limonene by increased expression of three enzymes for the MEP pathway or by introduction of an alternative mevalonate pathway for supply of the precursors. I studied the multistep enzymatic reactions of the *Synechocystis* GGPP synthase (Slr0739) by experiments and simulation and found that channeling of the intermediary products (GPP and FPP) efficiently supports the production of the final product GGPP. Taken together, it is suggested that the level and supply of the substrate for the isoprenoid synthases must be optimized for the isoprenoid species. In order to further improve the photosynthetic production of various isoprenoid compounds, it is essential to remodel the natural metabolic pathways including photosynthetic carbon fixation reactions. I believe that my work here provides the first and firm basis for the photosynthetic biomass production utilizing cyanobacteria. Further extensive genetic engineering of cyanobacteria should be done to meet with the social demand and also would open the new possibility of the biotechnology of photosynthesis.

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Abbreviations

ACC	acetyl-CoA carboxylase
DMAPP	dimethylallyl pyrophosphate
Dxs	deoxyxylulose-1-phosphate synthase
FPP	farnesyl pyrophosphate
GGPP	geranylgeranyl pyrophosphate
GPP	geranyl pyrophosphate
Ipi	isopentenyl pyrophosphate isomerase
IPP	isopentenyl pyrophosphate
MEP	2-methylerithritol-4-phosphate
PCR	polymerase chain reaction
PEP	phosphoenol pyruvate
PEPC	phosphoenol pyruvate carboxylase
PPi	inorganic pyrophosphate
RubisCO	ribulose-1,5-bisphosphate carboxylase/ oxygenase

General Introduction

Photosynthesis is the biological process that converts light energy into chemical energy, which is the foundation to support most of living organisms into on the Earth. Human has been developing a way to convert the solar energy biomass through agriculture from its early days. These days, the photoelectric conversion efficiency of solar power generation into a direct electrical energy has been improved gradually, and now reached approximately 40% (Green et al., 2012). However, artificial photosynthesis to produce organic compounds from carbon dioxide is still under investigation, and has never reached the application level (Mikkelsen et al., 2010; Tachibana et al., 2012).

On the other hand, the recent progress of molecular biological techniques have opened a door to the applied stage by introducing foreign enzymes or metabolic pathways to achieve production of useful compounds such as drugs in microorganisms or culture cells (Lee et al., 2012). The technique can also be applied for photosynthetic organisms (plants, green algae, cyanobacteria and photosynthetic bacteria), aiming for real "mass" production of fuels, industrial resources and foods/feeds. Currently, many targets of photosynthetic biomass production are fuels such as alcohols, hydrogen and fatty acids (Ho et al., 2014; Yu et al., 2013).

Isoprenoids are major secondary metabolites, which consist of isoprene units (C_5). Precursor of isoprenoids, dimethylallyl pyrophosphate (DMAPP) (C_5) and isopentenyl pyrophosphate (IPP) (C_5), are synthesized by two distinct pathways (Fig. GI-2) (Kuzuyama, 2002). Mevalonate pathway is

mainly used in eukaryotic cells. Non-mevalonate pathway or MEP pathway is distributed in prokaryotic cells and plastids of eukaryotes. Many molecules of IPP are successively condensed into initial substrates (mostly DMAPP) by extension enzymes called prenyltransferase to produce various prenyl pyrophosphate (C_{5n}) (Fig. GI-3). At the last step, the linear isoprenoid backbones are changed into a variety of ring structures via carbocation rearrangement by the action of terpene synthases (Degenhardt et al., 2009).

Various isoprenoids play critical roles for basic cellular activities; diverse species of ubiquinones for respiratory electron transport, plastoquinone for photosynthetic electron transport, chlorophylls and carotenoids for photosynthetic light capture and for dissipation of excess light energy, and steroids (eukaryotes) or hopanoids (bacteria) for biomembranes (Lambreva et al., 2014; Nowicka and Kruk, 2010; Sáenza et al., 2012). Isoprenoid side chain of ubiquinone ranges from C_{30} to C_{45}, depending on bacteria and eukaryotic mitochondria. The side chain of plastoquinone is usually C_{45}. These isoprenoid side chains of ubiquinone and plastoquinone are very important for their hydrophobic behavior in the quinone cycle of the electrogenic electron transport (Nowicka and Kruk, 2010). Phytol chain (C_{20}) also confers hydrophobic nature to rather hydrophilic chlorophyllides except chlorophyll *c* (Wettstein et al., 1995). Carotenoids are chromogenic compounds, whose conjugated double bonds are generated by successive desaturation of phytoene (C_{40}) or dehydrosqualene (C_{30}), whereas many other isoprenoids are colorless (Lu and Li, 2008). Steroids and hopanoids are cyclized derivatives of squalene and play critical roles for membrane fluidity,

steroid hormones, and bile acids mostly in eukaryotes (Sáenza et al., 2012). There are many other related isoprenoid compound as specific secondary metabolites, which are found in certain groups of higher plants. They are limonene in *Schizonepeta tenuifolia*, artemisinin in *Artemisia annua,* patchoulol in *Pogostemon cablin*, paclitaxel in *Taxus brevifolia*, polyisoprene in *Hevea brasiliensis,* etc. (Knudsmark Jessing et al., 2014; Park et al., 2006).

Many useful isoprenoid compounds have been isolated from plant leaves, roots, bark and essential oils, and consumed as perfumes, pharmaceuticals, dyes, natural rubber, fuel and raw materials for production of related compounds in the long history of human. Many of them are replaced with petroleum-derived synthetic compound, whereas certain pharmaceuticals such as baccatin III which is precursor of paclitaxel are still prepared from plant materials due to low cost compared with the chemical synthesis (Fu et al., 2009).

Various isoprenoids have been produced by metabolic engineering using microbes such as *Escherichia coli* and yeast. For example, production of isoprene (hemiterpene), geraniol (monoterpene), patchoulol (sesquiterpene) and taxadiene (diterpene) was reported (Albertsen et al., 2011; Ding et al., 2014; Liu et al., 2013; Zhou et al., 2014). However, production using these species must consume organic carbon sources and emit carbon dioxide. Therefore, it is urgent to establish a production method using a photosynthetic organism.

In my thesis, I attempted to establish a basic framework for the photosynthetic production of unnatural isoprenoids in recombinant cyanobacteria. Personally, I have interested in photosynthetic metabolism, chemical background of metabolic process, and metabolic engineering for years. In my master thesis, I developed protocol for gas chromatography-mass spectrometry of amino acids and studied the effect of nutrient starvation on amino acid pools in a model cyanobacterium *Synechocystis* sp. PCC 6803 and its nutrient starvation mutant (*ΔnblA1/A2*) (Kiyota et al., 2014). Through this work, I learned various analytical techniques and metabolic background of cyanobacterial photosynthesis and metabolic pathways. Then, I started my Ph.D. work on metabolic engineering aiming for photosynthetic production. I chose *Synechocystis*, because it is a simple photosynthetic organism and highly tractable to genetic engineering. Metabolic analyses at transcription and metabolite levels have been performed many in *Synechocystis* (Hihara et al., 2001; Osanai et al., 2005; Yang et al., 2002; Yoshikawa et al., 2013). There are many reports on photosynthetic production using *Synechocystis*, but is biased to compounds oriented fuel such as alcohols or fatty acids. If photosynthetic production of isoprenoids succeeds, useful compounds other than fuel can be supplied by photosynthesis.

In Chapter, I introduced a gene for limonene synthase into *Synechocystis* sp. PCC 6803 as a model photosynthetic production of volatile isoprenoid, and constructed gas stripping method to recover the product and evaluate production rate. This is the first example as monoterpene production

using cyanobacteria. Although production was successful, production rate of limonene was significantly lower than intrinsic isoprenoids, phytol chain of chlorophyll *a* and carotenoids. The substrate of limonene synthase, geranyl pyrophosphate (GPP), is also the substrate for intrinsic prenyltransferases. It suggests that introduced limonene synthase could not snatch the substrate from prenyltransferase and could not effectively utilize the substrate. Therefore, the characteristics of an enzyme supplying prenyl chain were thought to be important for efficient isoprenoid production in cyanobacteria.

In Chapter II, I analyzed Slr0739, putative geranylgeranyl pyrophosphate synthase (CrtE) in *Synechocystis*. Geranylgeranyl pyrophosphate synthase is a kind of prenyltransferase which synthesize geranylgeranyl pyrophosphate (GGPP) via GPP and farnesyl pyrophosphate (FPP) by condensing IPP into DMAPP. Cyanobacterial prenyltransferases have been only analyzed for their substrate specificity but not for detailed kinetics. To perform enzymatic analysis of Slr0739, I established product quantification method using GC-MS. I obtained kinetic parameters (K_m (FPP), K_m (DMAPP), k_{cat} (FPP) and k_{cat} (DMAPP)) of Slr0739. Although I could not get K_m (GPP) and k_{cat} (GPP) by unknown reason, simulation analysis revealed that K_m (GPP) and k_{cat} (GPP) are similar to K_m (FPP) and k_{cat} (FPP).

In this study, I showed the key points of efficient photosynthetic production of isoprenoid through the photosynthetic production of limonene and analysis of key enzyme, CrtE.

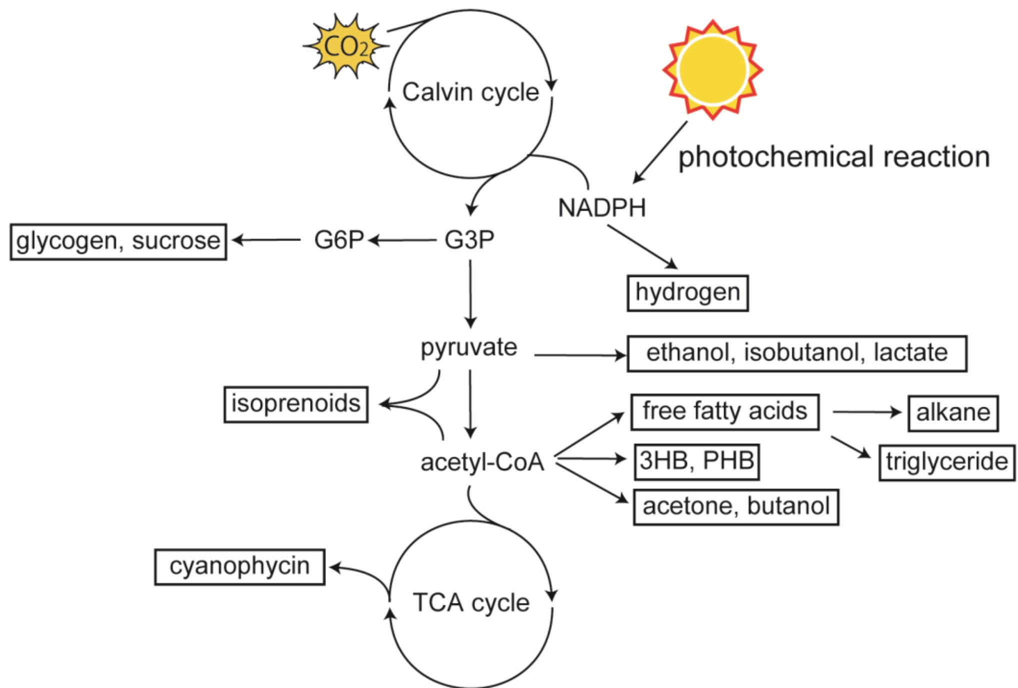
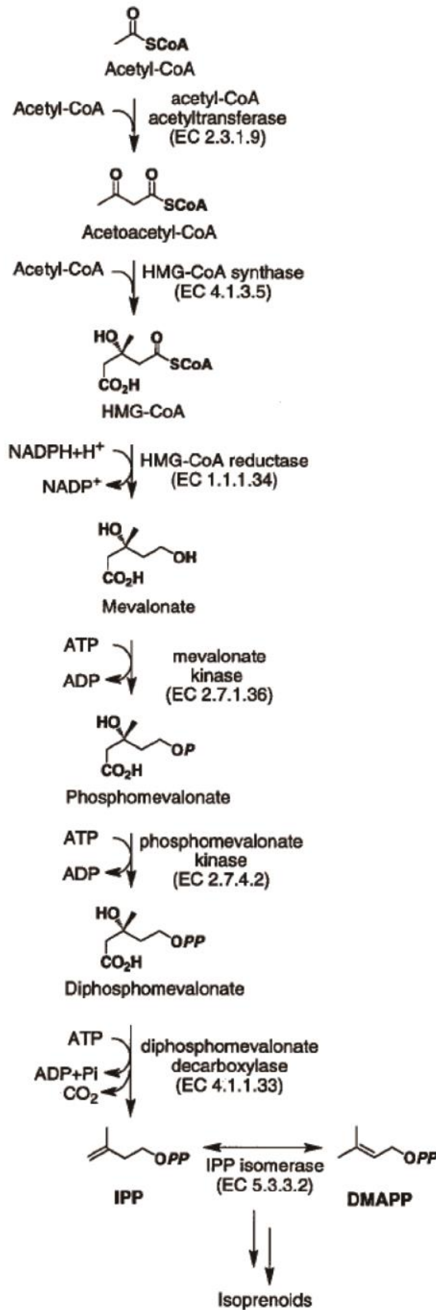


Fig. GI-1 Biomass production in a cyanobacterial cell. Compounds that have been reported as the targets of metabolic engineering are boxed.

Mevalonate pathway



Non-mevalonate pathway

(2-Methylerythritol-4-phosphate pathway)

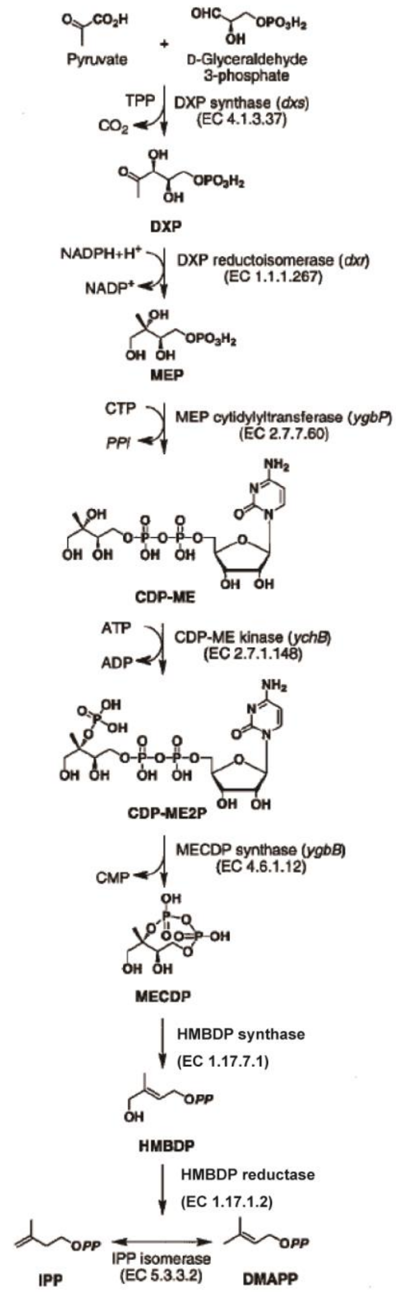


Fig. GI-2 Two types of biosynthetic pathways, mevalonate pathway and non-mevalonate pathway (MEP pathway), for supply of DMAPP and IPP. Modified from (Kuzuyama, 2002).

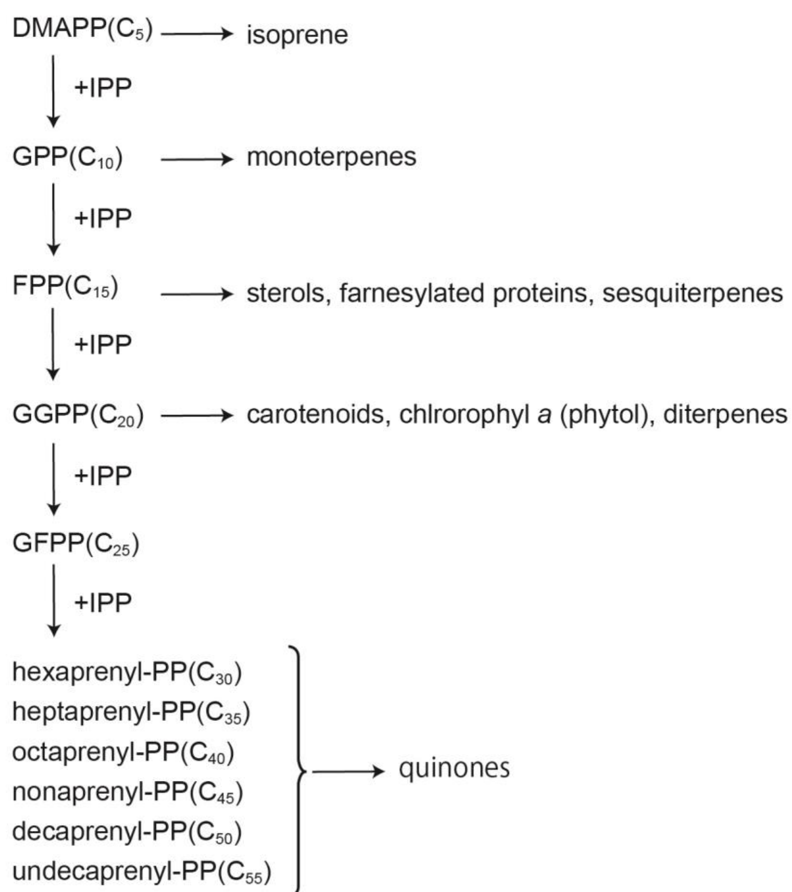


Fig. GI-3 Elongation of various lengths of prenyl chains. Isoprenoid compounds derived from these prenyl pyrophosphate species are shown.

Chapter I

Photosynthetic production of isoprenoids in *Synechocystis*

I.1 Introduction

Isoprenoids that are composed of isoprene units (C_5) are major secondary metabolites in many organisms. They are major components of essential oils (Fornari et al., 2012), steroids (Ghayee and Auchus, 2007) and carotenoids (Takaichi, 2011) and are also useful for various commercial applications. These include the production of perfumes, medicines, materials, and biofuels (Bohlmann and Keeling, 2008). These products can be manufactured in part from petroleum or other materials via chemical synthesis, and many of them are extracted and purified from plant materials such as barks, leaves, roots and essential oils of certain plants. However, isoprenoid production in plants is limited to their slow growth. To overcome this, isoprenoid production has been studied using genetically engineered microorganisms such as *Escherichia coli* and yeast. Isoprenoids that have commercial use or other benefits can be produced using these organisms (Curran and Alper, 2012). For example, carvone (Carter et al., 2003), amorpha-4,11-diene (Martin et al., 2003), bisabolane (Peralta-Yahya et al., 2011), astaxanthin (Miura et al., 1998) and sterols (Wriessnegger and Pichler, 2013). Production levels in these heterotrophic organisms reached several grams per liter. However, although metabolic engineering of these organisms achieved such high productivity in synthesizing exogenous isoprenoids, these microbes require an organic carbon source and emit carbon dioxide during their growth and production. As carbon dioxide is one of the major greenhouse gases, it has become imperative to develop methods to photosynthetically produce isoprenoids directly from carbon dioxide using phototrophic microbes.

For the photosynthetic production of isoprenoids, we took advantage of the model cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis*), which is highly tractable to genetic engineering. It is becoming more widely known that metabolic engineering enables cyanobacteria to photosynthetically produce various important biofuels such as isopropyl alcohol (Kusakabe et al., 2013), isobutyl aldehyde (Atsumi et al., 2009), fatty acids (Liu et al., 2011), alkanes (Wang et al., 2013), ethylene (Takahama et al., 2003) and hydrogen (Masukawa et al., 2012). However, there have been few studies of the potential for photosynthetic production of functional isoprenoids as commercially useful compounds and also as potential biofuels. Cyanobacteria naturally accumulate functional isoprenoids such as carotenoids, the phytol of chlorophyll and quinones as essential cofactors for photosynthesis (Nowicka and Kruk, 2010). Some cyanobacteria also produce odorous monoterpenes (Li et al., 2012), sesquiterpenes (Agger et al., 2008) and hopanoids (Sáenza et al., 2012). Thus, the cyanobacteria may provide a potential platform for the large-scale photosynthetic production of various isoprenoids. There is a pioneer work to produce a hemiterpene isoprene (C_5H_8) in *Synechocystis* by plant enzyme isoprene synthase (Bentley and Melis, 2012; Lindberg et al., 2010).

Here, I chose to test the production of the popular volatile monoterpene limonene ($C_{10}H_{16}$) in *Synechocystis* as this compound can be used in various applications. Limonene is a water-insoluble liquid under atmospheric pressure and may be released from cells without specific treatments. There are two naturally occurring enantiomers, (*R*)-limonene and

(*S*)-limonene, which are produced by stereo specific enzymes in plants. (*R*)-limonene has a lemon-like fragrance, whereas (*S*)-limonene has a petroleum-like odor. These limonene enantiomers have been used not only in perfumes but also as a solvent for polystyrene. Limonene can be produced by a single enzyme, limonene synthase (*lms*) (EC 4.2.3.20) from GPP, which is a natural intermediate for the biosynthesis of carotenoids and phytol (Fig. I-1).

In this Chapter, I constructed recombinant *Synechocystis* strains that would heterologously produce this monoterpene using a plant-derived *lms*. These recombinant strains stably express the enzyme and successfully produce limonene. I evaluated the production and accumulation of limonene in my cyanobacterial system using a gas-stripping method. I studied effects of environmental conditions (nitrogen depletion and high light) on the limonene production. I also constructed an overexpression mutant of three enzymes for the intrinsic MEP pathway and introduced the mevalonate pathway enzymes derived from yeast into the limonene producing *Synechocystis.* The mevalonate pathway converts three molecules of acetyl-CoA to one molecule of DMAPP or IPP. This is the first study to describe the construction of cyanobacteria that heterologously produce a plant monoterpene and that show increased production of this compound through genetic engineering.

I.2 Materials and Methods

I.2.1 Strains and plasmid construction

A glucose tolerant strain of the unicellular cyanobacterium

Synechocystis sp. PCC 6803 was used as a platform in this study. The *lms* gene from the medicinal herb *Schizonepeta tenuifolia* (GenBank accession number AF282875) (Maruyama et al., 2001) was used for expression in *Synechocystis*. The initial experiments were done using the native DNA sequence of this plant *lms* gene after truncation of the transit region for targeting to chloroplasts. However, the other experiments in this study were done using a synthetic DNA (Fig. I-S1), in which the codons were optimized for *Synechocystis* (GenScript, Piscataway, USA). The optimized *lms* gene was cloned into an expression vector (pT31CTH-TePixJ) that incorporated a 6xHis-tag epitope, a *trc* promoter and a chloramphenicol resistant cassette (Ishizuka et al., 2006). The *lms* insert in the resulting plasmid (pT31CTH-lms) was confirmed by nucleotide sequencing. The resultant plasmid DNA was introduced by double homologous recombination into the *Synechocystis* chromosome, yielding transformants in which part of a silent *slr2031* gene was replaced (Satoh et al., 2001).

Codon optimized amorpha-4, 11-diene synthase gene (*ads*) from *Artemisia annua* (GenBank accession number AAF61439) (Mercke et al., 2000) (Fig. I-S2) or geraniol synthase gene (*gs*) from *Perilla setoyensis* (GenBank accession number FJ644545) (Masumoto et al., 2010) (Fig. I-S3) was cloned into an expression vector (pSISCT) that carries a *trc* promoter and a chloramphenicol resistant cassette to allow strong expression in *Synechocystis* cells. The resultant plasmid DNA was introduced by double homologous recombination into a neutral site between *slr1340* and *sll1255* of the *Synechocystis* chromosome.

The genes encoding three enzymes of the 2-methylerythritol-4-phosphate pathway (MEP pathway) in *Synechocystis*, *dxs* (*sll1945* for deoxyxylulose-1-phosphate synthase), *crtE* (*slr0739* for GGPP synthase) and *ipi* (*sll1556* for IPP isomerase) were cloned into pT7blue (Merck, Darmstadt, Germany) and then combined as a single operon (Fig. I-2A and Fig. I-S4) in another expression vector, pPCRScript-slr0846-1/9, by replacing the *slr0846* gene insert (Midorikawa et al., 2012). The resulting plasmid, pS46KT-MEP, was used to integrate these three genes with the kanamycin-resistant cassette by double homologous recombination into the chromosome at an intergenic neutral site between *sll0821* and *slr0846*. In the recombinant chromosome, an extra copy of *dxs-crtE-ipi* is expressed from the *trc* promoter at this intergenic neutral region in addition to the intrinsic copies. Since cyanobacterial cells possess multiple copies of identical chromosomes (Watanabe et al., 2012), complete segregation of the integrated genes was confirmed by PCR. The genes for mevalonate pathway were cloned from pBAD33MevT (acetoacetyl-CoA synthase, hydroxymethylglutaryl-CoA synthase and hydroxymethylglutaryl-CoA reductase) and pMBIS (mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, IPP isomerase, and FPP synthase) into pPCRScript-slr0846-1/9 as two contiguous operons: the former three genes were driven by the *trc* promoter and the latter five genes were driven by the lac promoter. These genes were derived from yeast and optimized for *E. coli* codon usage (Martin et al., 2003). pBAD33MevT and pMBIS were purchased from Addgene, non-profit global plasmid repository (Fig. I-S5).

I.2.2 Western blotting

Cells were collected by centrifugation. Protein extraction buffer (100 mM NaCl, 10% (w/v) glycerol, 20 mM HEPES-NaOH (pH 7.5)) including 0.5 μM phenylmethylsulfonylfluoride and zirconia beads were then added to the cell pellet, followed by vortexing twice for 30 s with a 1 min interval. The resulting homogenate was centrifuged and the supernatant was subjected to SDS-PAGE and western blotting as described previously (Yoshihara et al., 2004). I used peroxidase conjugated HisProbe (Pierce, Rockford, USA) to detect the LMS protein.

I.2.3 Limonene extraction from cells

Limonene was extracted from cells using chloroform and the organic layer was collected and subjected to gas chromatography-mass spectrometry (GC-MS) analysis.

I.2.4 GC-MS analysis

GC-MS was performed using QP2010 Plus GC-MS (Shimadzu, Kyoto, Japan) equipped with a ZB-AAA column (Phenomenex, Torrance, USA). The analytical conditions were as follows: He (1.55 $mL{\cdot}min^{-1}$) as a carrier gas, ionization voltage 70 kV, split ratio of 15:1, injector temperature of 150°C, and an oven program of 50°C for 1 min increasing at 20°C min^{-1} to 150°C in 5 min and a held at 150°C for 2 min. Analyses were carried out in the selected ion monitoring mode (m/z = 68, 93, 121, 136). The limonene peak was identified

as the retention time in the selected ion chromatogram. In the quantitative analysis, we used α-pinene as an internal standard.

I.2.5 Culture conditions and gas-stripping method

Cyanobacterial cells were grown under normal conditions at 30°C in BG11 medium supplemented with 20 mM HEPES-KOH (pH 7.8) (Rippka, 1988) with bubbling of 1% (v/v) CO_2 in air and continuous illumination with white fluorescent lamps at 50 μmol photons·s^{-1}·m^{-2}. Recombinant cells were maintained in the presence of antibiotics (20 μg·mL^{-1} chloramphenicol and/or 20 μg·mL^{-1} kanamycin) but cultivated in the absence of antibiotics for limonene production. To start the experiments, growing cells were collected by centrifugation and resuspended in a fresh BG11 medium or BG11-N medium, in which 17 mM $NaNO_3$ was replaced by 17 mM of NaCl, at a density of 2×10^8 cells/ml. The cultures were bubbled with 1% (v/v) CO_2 in air under continuous illumination with white fluorescent lamps at 100 μmol photons·s^{-1}·m^{-2}. I used a polytetrafluoroethylene tube to connect the culture flask and the cold trap. Limonene was trapped using a Dimroth condenser at –15°C and recovered into the octane phase (cold trap)

I.2.6 Quantification of chlorophyll a and carotenoids

Chlorophyll *a* and carotenoids were extracted from cells using methanol and the extract was subjected to photometric determination. Concentration of chlorophyll *a* was obtained from the absorbance of 665 nm. I subtracted spectrum of authentic chlorophyll *a* from the spectrum. The

concentration of carotenoids was obtained from absorbance of the subtracted spectrum at 450 nm. I used 140000 as molar absorbance coefficient of carotenoids.

I.3 Results

I.3.1 Construction of LMSox cyanobacterial strain

I introduced the plant *lms* gene into a silent locus on the *Synechocystis* chromosome (Fig. I-2A) and confirmed complete segregation by PCR analysis (Fig. I-2B). I used the segregated strain as the LMSox strain. The *lms* gene is expressed from a strong *trc* promoter and expression of the LMS protein was confirmed by western blotting with proteins from the LMSox strain (Fig. I-2C).

I.3.2 Limonene detection

First, I detected limonene accumulation in LMSox cells. The cells were collected by centrifugation and limonene was extracted using chloroform. Figure I-3 shows GC-MS chromatograms of three mass fragments, which could be derived from limonene (m/z = 68, 121, 136). The limonene peak at 1.58 min was unambiguously detected in each chromatogram in LMSox but not in wild type cells. In contrast, the other peaks were not commonly detected, implying an origin other than limonene.

I.3.3 Gas-stripping method

For efficient photosynthesis and carbon assimilation of *Synechocystis*

cells, CO_2 must be supplied continuously. However, the volatile limonene product must be collected from the exhaust. To achieve both, we chose the gas-stripping system (Fig. I-4A) in which the bubbling snatches limonene from the medium and releases it into the octane phase through the cold trap. The octane phase was analyzed by GC-MS. Limonene was detected as a single peak at 1.58 min in the GC chromatogram of LMSox cells, but was not detected in wild type cells (Fig. I-4B). The mass spectrum of this peak gave fragments of 136, 121, 93 and 68, which matched the fragments of authentic limonene (Fig. I-4C). The complete recovery of limonene in this trap was confirmed by its absence in the second trap. The recovery of limonene was 99.4% in the octane phase, 0.6% in the cell and 0% in the culture medium, indicative of exclusive recovery in the trap (Fig. I-4D).

I.3.4 Limonene production

The photosynthetic growth of LMSox cells was found to be almost identical to wild type under various growth conditions. As shown in Figure I-5, the growth of both cell types measured from inoculation at a low cell density showed no difference in the log phase. It is important to note that the limonene production and/or accumulation did not repress the growth of *Synechocystis*. Time course analysis of the limonene accumulation in the trap is shown in Figure I-6. The accumulation of limonene increased continuously for 168 hours, at a nearly constant rate of 41 $\mu g \cdot L\ culture^{-1} \cdot day^{-1}$.

I.3.5 Overexpression of the MEP pathway

Synechocystis possesses an active MEP pathway to biosynthesize its own isoprenoids, such as carotenoids and phytol. I introduced an additional copy of three genes (*dxs*, *crtE* and *ipi*) under the control of a strong *trc* promoter at a neutral site of the *Synechocystis* chromosome to increase the supply of GPP, which is the direct substrate of Lms (Fig. I-1). Dxs and CrtE catalyze reactions with a large free energy difference and Ipi enhances the interconversion between IPP and DMAPP, which combines to produce GPP (Ajikumar et al., 2010; Kim and Keasling, 2001). I generated a double recombinant cyanobacterial strain, LMSox-MEPox. I confirmed its complete segregation by PCR (Fig. I-2D). In this LMSox-MEPox strain, the rate of limonene production (56 $\mu g \cdot L$ $culture^{-1} \cdot day^{-1}$) was improved by 1.4-fold compared with the parent LMSox strain (Fig. I-6). Again, this improvement was achieved without growth inhibition (Fig. I-5).

I.3.6 Long-term production

I investigated the sustainability of the limonene production in my system without changes of the culture medium for up to one month (Fig. I-7). The LMSox-MEPox strain produced limonene linearly for 300 hours after which this productivity gradually attenuated and was measured at 19 $\mu g \cdot L$ $culture^{-1} \cdot day^{-1}$ at 712 hours. The overall production achieved was 1 $mg \cdot L$ $culture^{-1}$. Thus, limonene can be produced sustainably in cyanobacteria during the stationary phase of growth.

I.3.7 Effect of nitrogen deprivation

Nitrogen is a key macronutrient for microalgae. Expecting that the carbon flow into limonene could be enhanced in the absence of nitrogen assimilation, I compared the limonene production under nitrogen starvation with normal condition. However, production of limonene was much lower than that under the normal growth condition (Fig. I-8)

I.3.8 Correlation between light intensity and MEP pathway

Effects of light intensity on growth and the limonene production was investigated (Fig. I-9). Both growth rate and limonene production increased with light intensity. However, ratio of the limonene production rate to the growth rate, which represents carbon partitioning into limonene, reduced with the light intensity. Moreover, cellular content of chlorophyll *a* and carotenoids also reduced with light intensity. These results suggest that the carbon partitioning into the total isoprenoids may limit the production of limonene.

I.3.9, I.3.10

これらの節については、5 年以内に雑誌等で刊行予定のため、非公開。

I.4 Discussion

I successfully constructed a system to produce limonene at a rate of 41 μg·L culture^{-1}·day^{-1} (LMSox) and 56 μg·L culture^{-1}·day^{-1} (LMSox-MEPox)

using genetically engineered cyanobacterial cells of the *Synechocystis* strain that exogenously express a plant limonene synthase and three intrinsic enzymes to promote supply of the substrate for this enzyme. Although limonene is growth inhibitory for microorganisms (Aggarwal et al., 2002; Chambon et al., 1990), I achieved its sustainable production in cyanobacteria without any defect in growth, pigmentation, or other properties, likely because the produced limonene was efficiently removed from the culture system by gas-stripping.

I employed the gas-stripping system (Atsumi et al., 2009) to collect volatile limonene and to sustain its production in cyanobacteria without medium exchange. My system supported the sustained production of this compound for up to 300 hours. Even at 700 hours, the *Synechocystis* cells produced limonene at nearly one-third of the initial rate. In this system, the produced limonene does not stay in the cell or in the culture medium and is exclusively recovered in the trap due to air bubbling to supply CO_2 (Fig. I-4D). This likely underlies why a sustained production could be achieved for weeks. Further, our method would also help to avoid possible product-induced feedback inhibition, which is often observed in many metabolic pathways.

Bently and Melis (2012) have reported the photosynthetic production of a hemiterpene (isoprene) in a recombinant *Synechocystis* system. In that report, the culture system was semi-closed, and needed to be exchanged with fresh medium every 48 hours. In my thesis, I demonstrated the sustained production of limonene using *Synechocystis* cells at high cell density (late log to stationary phase). This will be an important consideration in the future

when constructing an efficient bioreactor for the photosynthetic production of limonene and other related isoprenoids.

Regulation of the intrinsic MEP pathway or bottlenecks in this pathway has not been studied in cyanobacteria to date, although the genes in this pathway have been identified (Cunningham et al., 2000; Harker and Bramley, 1999; Yin and Proteau, 2003). It is generally known that various protective carotenoids accumulate while the chlorophyll content decreases under high light conditions (Kilian et al., 2007). In many cyanobacteria, myxoxanthophylls and zeaxanthin often accumulate under high light conditions (Montero et al., 2012). In terms of gene regulation, only two genes (*crtB* for phytoene synthase and *crtP* for phytoene desaturase) are reported to be induced by high light in cyanobacteria (Fernández-González et al., 1998). Carotenoids and phytol of chlorophylls are derived separately from a common precursor, GGPP, which is the end product of the MEP pathway. In *E. coli*, several enzyme steps have been implicated as rate-limiting in the MEP pathway in gene engineering trials, which lead to the overproduction of carotenoids and other isoprenoids (Ajikumar et al., 2010; Kim and Keasling, 2001). I chose these putative rate-limiting enzymes (Dxs, CrtE and Ipi) to enhance the MEP pathway in *Synechocystis* and, indeed, obtained marked improvement of the limonene production (1.4-fold) in my cyanobacterial system. However, I did not see any effects on the accumulation of chlorophylls or carotenoids, likely because their biosynthesis is regulated after GGPP as mentioned above. Further fine-tuning of our operon design and screening of other genes are now in progress.

Native isoprenoids in cyanobacteria comprise mostly carotenoids and phytol of chlorophyll *a*, in addition to minor cofactors (plastoquinone and phylloquinone). We estimated the accumulation of these compounds during the early phase of the limonene production as follows: carotenoids ca. 1000 $\mu g \cdot L\ culture^{-1} \cdot day^{-1}$ and phytol ca. 600 $\mu g \cdot L\ culture^{-1} \cdot day^{-1}$. This indicated that the flow to limonene is approximately 2.5 % (LMSox) or 3.4% (LMSox-MEPox) of the total isoprenoid biosynthesis. During this period, the cyanobacterial cells grow rather linearly instead of exponentially with an approximate rate of 94 mg dry cell weight$\cdot L\ culture^{-1} \cdot day^{-1}$. Assuming that the carbon content of the dry cell weight is approximately 50%, the carbon flow to limonene would be 0.1% (LMSox-MEPox) of the total fixed carbon. It should be noted, however, that the content of carotenoids and chlorophyll in our overexpression mutants were comparable to wild type. This may suggest that the Lms activity is limiting the flow to limonene.

Despite that the limonene molecule does not require any nitrogen atom, the nitrogen starvation did not enhance the limonene production. Just after transfer of cells to the nitrogen free medium, the limonene production was somehow suppressed compared with the production under the normal BG11 conditions, and the production was almost stopped after three days of nitrogen starvation. This may be due to degradation of introduced enzyme or carbon fixation as adaptive responses to the nitrogen starvation. In my Master Thesis (Kiyota et al., 2014), I studied specific and non-specific responses of *Synechocystis* cells during the nitrogen starvation. However, little is known about stability of heterologously expressed proteins and/or

maintenance carbon fixation and carbon flow in these cells. These issues may be dramatically changed when the severe nitrogen starvation is introduced. Mild nitrogen starvation such as lower concentration of nitrate in the culture medium or partial suppression of nitrate reductase by engineering might help for improvement of the biomass production in cyanobacteria in future.

Under high light conditions, limonene production and chlorophyll *a* and carotenoids accumulation per cell basis, tended to decline during the late phase of growth. It was reported that the expression of phytoene synthase and phytoene desaturase is induced by intense light conditions in *Synechocystis* cells (Fernández-González et al., 1998). I assumed that the decrease in carotenoids and chlorophyll *a* content was due to decline of the flux of the MEP pathway. Under high light, the flux of the MEP pathway may be down-regulated or not much up-regulated compared with the increased cell growth. If this is due to the intrinsic metabolic regulation for the high light acclimation, the limonene production may be enhanced by introducing foreign enzymes for the MEP pathway.

The limonene production in the strain co-expressing the mevalonate pathway enzymes was increased slightly, but not as much as in a similar *E. coli* strain (Martin et al., 2003). This may be due to insufficient supply of their substrates or bottleneck in the pathway. The codon optimization of their genes and evaluation of each enzyme activity must be taken into consideration in future.

Production of monoterpenes by introduction of amorpha-4, 11-diene synthase and geraniol synthase seemed quite high compared with the

production of limonene. Quantification of their products requires authentic compounds but the product amorpha-4, 11-diene is not commercially available and the major products in the geraniol synthase-expressing cell was not geraniol. Anyway, MS peaks of ads-expressing cells are more than ten-fold higher than the peaks of LMSox cells (Fig. I-11). This may mean that further optimization of the precursor supply including carbon partitioning should be studied in the ads expressions. It also should be mentioned that the gas-stripping method works fine for recovery of other volatile monoterpenes or sesquiterpenes.

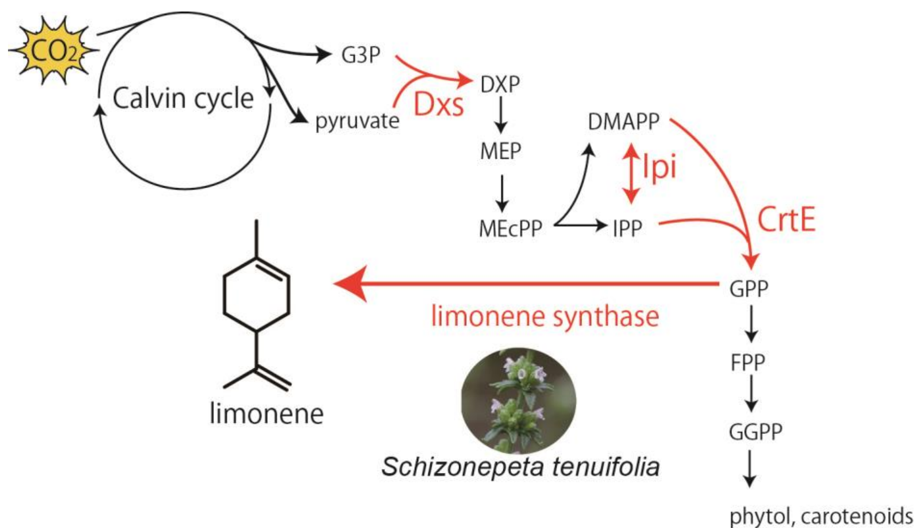


Fig. I-1. Biosynthesis of limonene from GPP by limonene synthase derived from *Schizonepeta tenuifolia.* GPP is a metabolic intermediate of the MEP pathway, which is supplied from CO_2 fixation via the Calvin cycle. Three engineered enzymes of the MEP pathway are highlighted in red.

G3P, glyceraldehyde-3-phosphate; DXP, 1-deoxyxylulose-5-phosphate; MEP, 2-methyl erythritol-4-phosphate; MEcPP, 2-methyl erythritol-2,4-cyclodiphosphate; DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

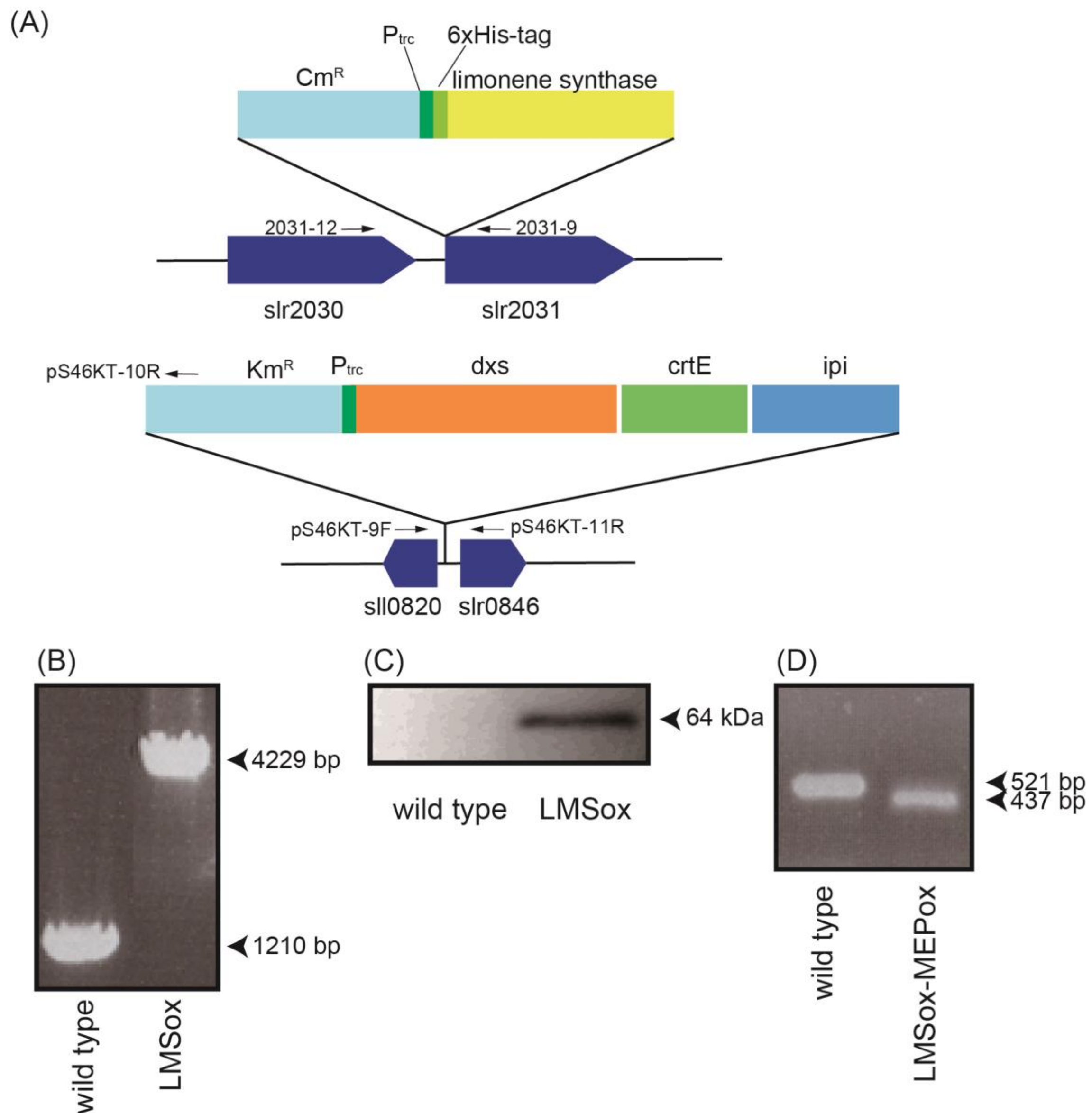


Fig. I-2. Integration and expression of engineered genes in the *Synechocystis* genome. A, Schematic representation of gene integration into the cyanobacterial chromosome by homologous recombination. P_{trc}, trc promoter; Cm^R, chloramphenicol resistance cassette; Km^R, kanamycin resistance cassette. Primers for PCR analysis are highlighted by arrows. B, PCR analysis of the integration of the gene for limonene synthase using the primers 2031-9 and 2031-12. C, Western blotting of His-tagged limonene synthase. D, PCR analysis of the integration of the *dxs-crtE-ipi* genes for the MEP pathway using the primers pS46KT-9F, pS56KT-10R and pS46KT-11R.

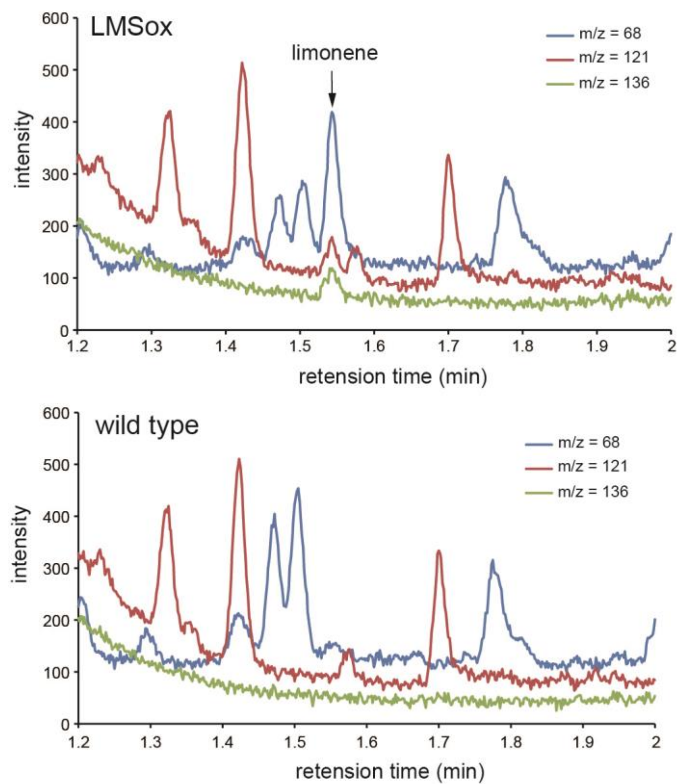


Fig. I-3. GC-MS analysis of extracts from LMSox and wild type cyanobacterial cells. Each line indicates a selected ion chromatogram.

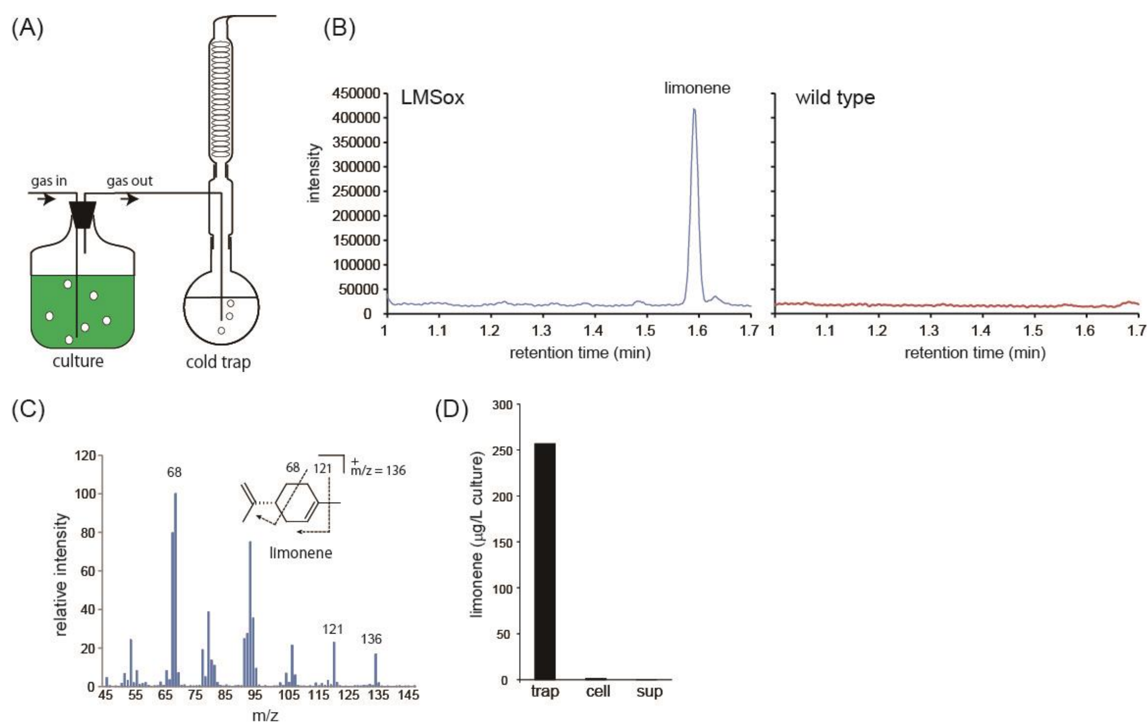


Fig. I-4. Extraction of limonene produced in cyanobacterial cultures using the gas-stripping method. A, Scheme for the gas-stripping system. B, GC-MS total ion chromatogram of the octane phase in the cold trap. C, mass spectrum of the limonene peak in panel B. D, distribution of the accumulated limonene in the octane phase of the trap (trap), cells collected from the culture (cell) and supernatant of the culture after centrifugation (sup).

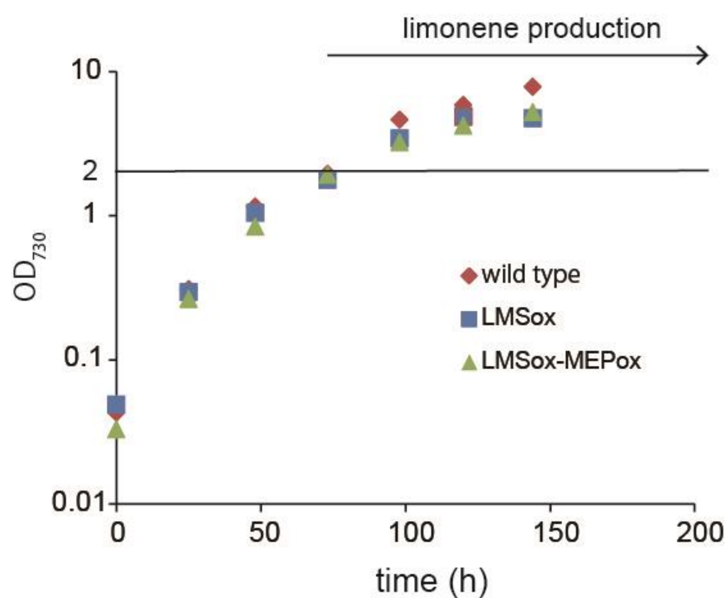


Fig. I-5. Growth of LMSox, LMSox-MEPox, and wild type cyanobacterial cells with bubbling under light at 50 μmol photons·m^{-2}·s^{-1}. The growth phase for the limonene production is denoted with an arrow.

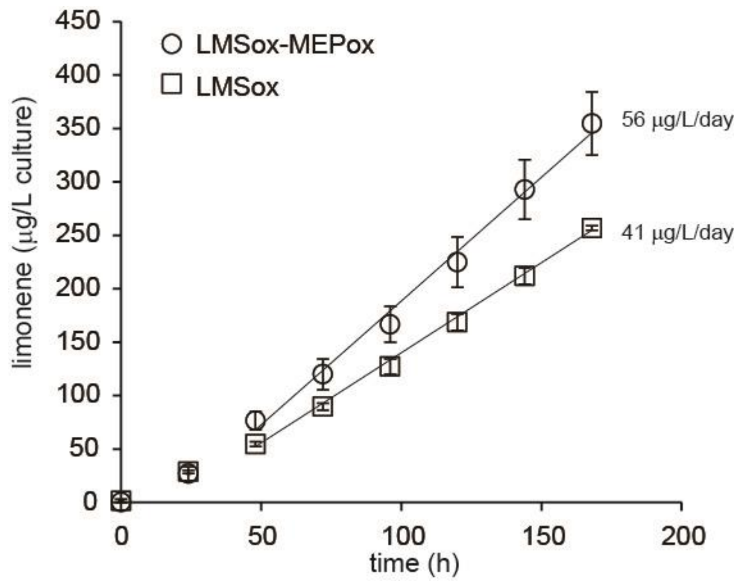


Fig. I-6. Limonene production in LMSox and LMSox-MEPox cyanobacterial cells. The standard error for three independent experiments is shown.

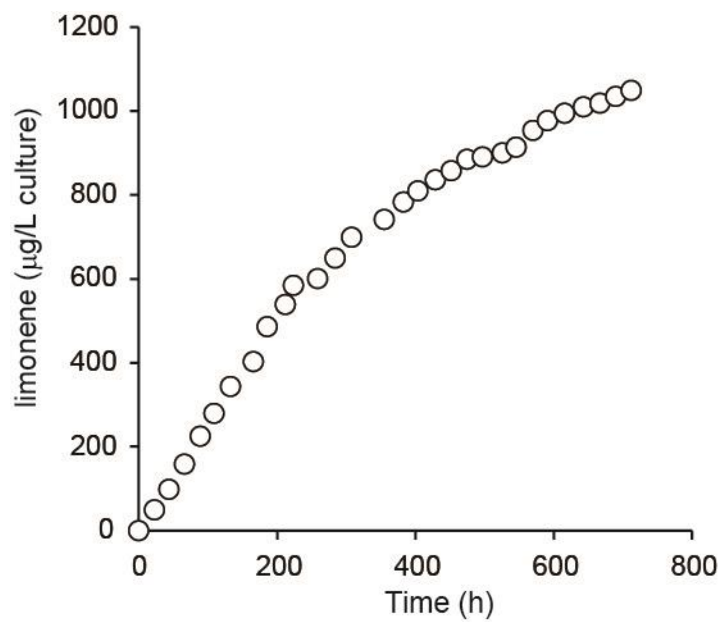


Fig. I-7. Augmented production of limonene in LMSox-MEPox cells.

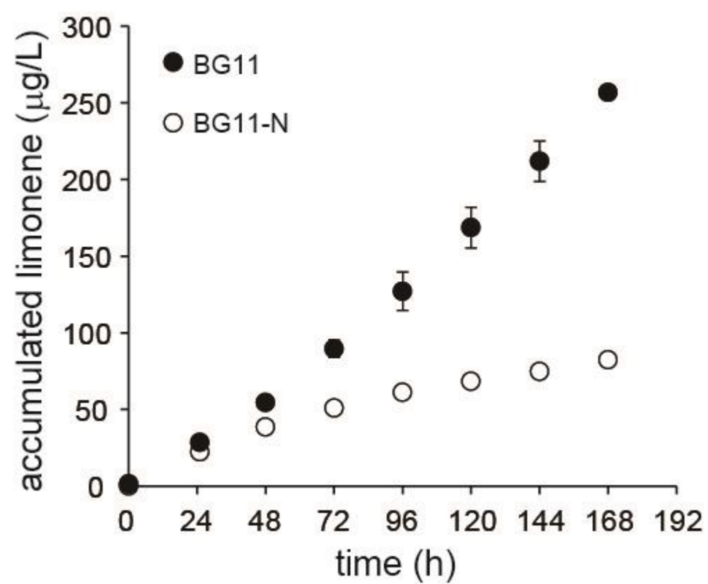
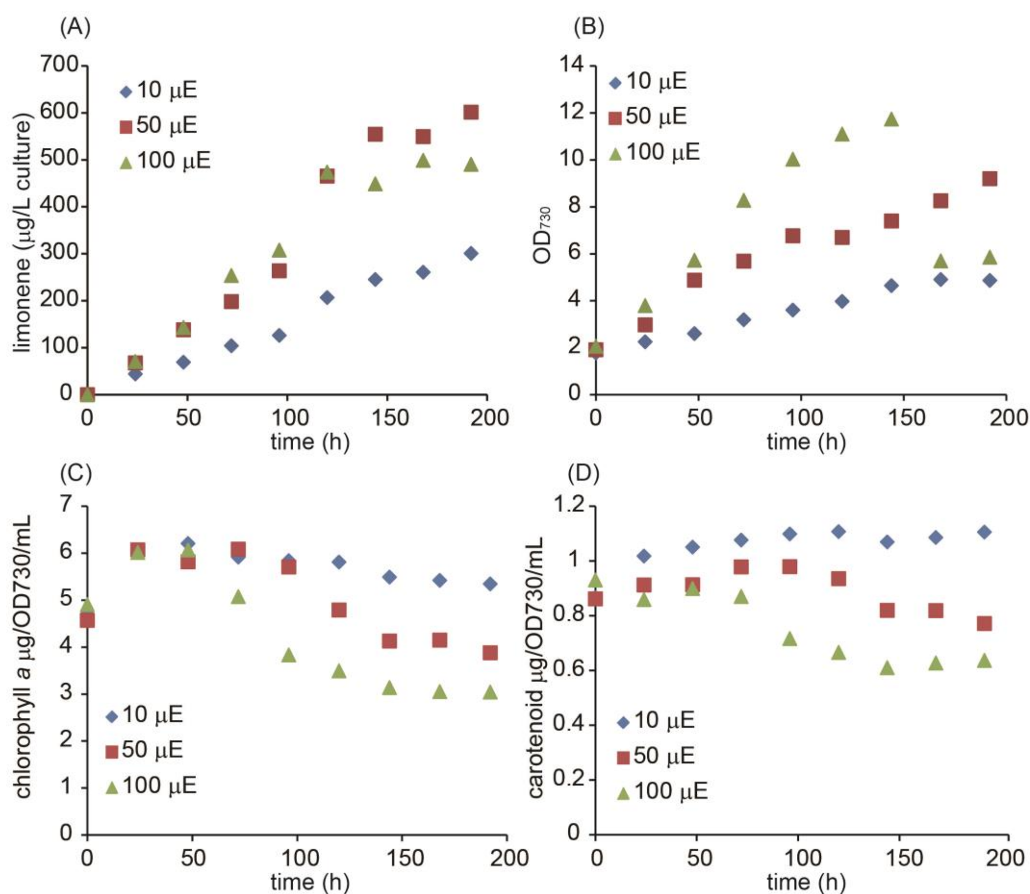


Fig. I-8. Limonene production in LMSox cyanobacterial cells under BG11-N (BG11 without nitrate) or BG11 condition.



(E)

	limonene production/h	OD/h	limonene production/OD
10 μE	1.30	0.0191	68.2
50 μE	2.74	0.0518	53.0
100 μE	3.38	0.0851	39.7

Fig. I-9. Light intensity dependence of limonene production (A), growth (B), amount of chlorophyll *a* (C) and carotenoids (D) in LMSox. Limonene production rate and growth rate obtained by first 92 h in panel A and B (E).

Fig. I-10, 11, 12

これらの図表については、5 年以内に雑誌等で刊行予定のため、非公開。

第２章

本章については、5 年以内に雑誌等で刊行予定のため、非公開。

General discussion

この部分の内容については、5 年以内に論文として刊行予定の内容を含むため、非公開。

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TATCATTACGGCGACGGTCACGGGACTCAGCACGCTAAAATTCACCAGCAGATTACCG
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Supplementary Fig. I-S1 Transit-peptide truncated and codon optimized sequence of limonene synthase

Fig. I-S2, S3

非公開とした本文 I.3.10、図表 Fig. I-11, 12 に関連する内容であるため、非公開。

CAATATTCTGAAATGAGCTGTTGACAATTAATCATCCGGCTCGTATAATGTGTGGAAT
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Fig. I-S4. Combined sequence of trc promoter, *dxs, crtE* and *ipi*

Purple, trc promoter; blue, *dxs*; light green, up-stream region of *crtE* gene; dark green, *crtE*; orange, up-stream region of *ipi* gene; red, *ipi*.

Fig. I-S5

上で非公開とした本文 I.3.9、図表 Fig. I-10 に関連する内容であるため、非公開。

Acknowledgements

My deepest appreciation goes to Prof. Masahiko Ikeuchi for advising my research. His colleagues gave constructive suggestion. Special thanks also go to Dr. Takafumi Midorikawa for useful advice about Chapter II. Dr. Masami Y. Hirai and her colleagues gave me constructive comments about GC-MS and metabolites analysis. Dr. Michiho Ito gave me genes for terpene synthase (limonene, geraniol, and linalool) used in this thesis. Dr. Daisuke Umeno gave insightful comment about biosynthesis pathways and metabolic engineering for isoprenoids. I also would like to thank Dr. Tetsuhiro S. Hatakeyama for valuable discussion about sequential reaction of prenyltransferase. Dr. Masaki Ihara and his colleagues gave me helpful comment about enzymes for MEP pathway. I would also like to express my gratitude to my family for their moral support and warm encouragements.

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