博士論文

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)

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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 μg·L culture^{-1}·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.

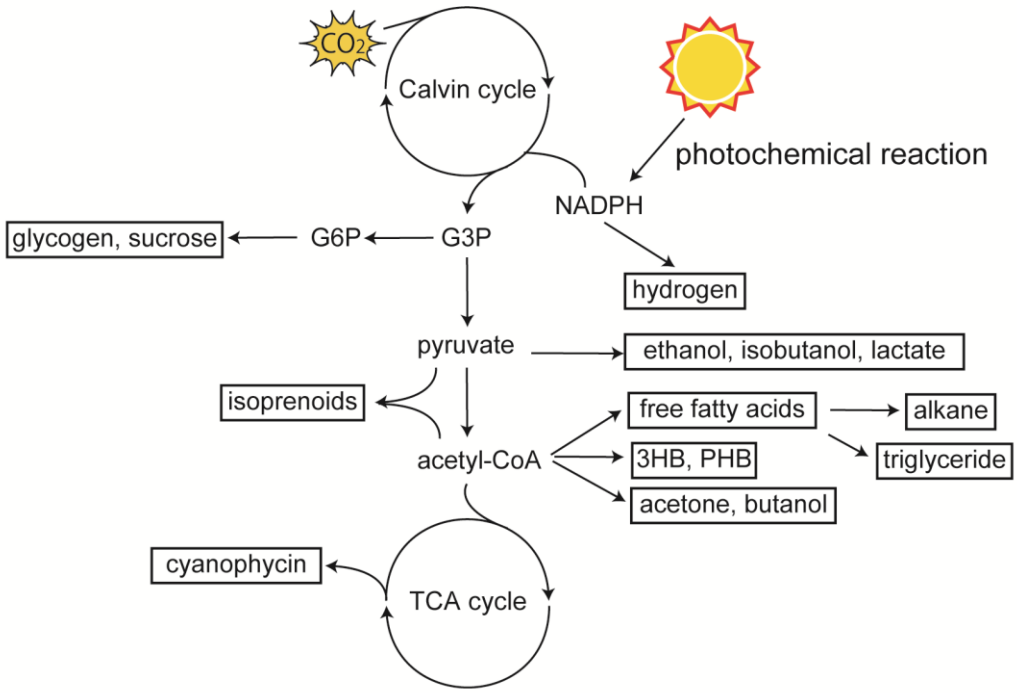


Fig. GI-1 Biomass production in a cyanobacterial cell. Compounds that have been reported as the targets of metabolic engineering are boxed.

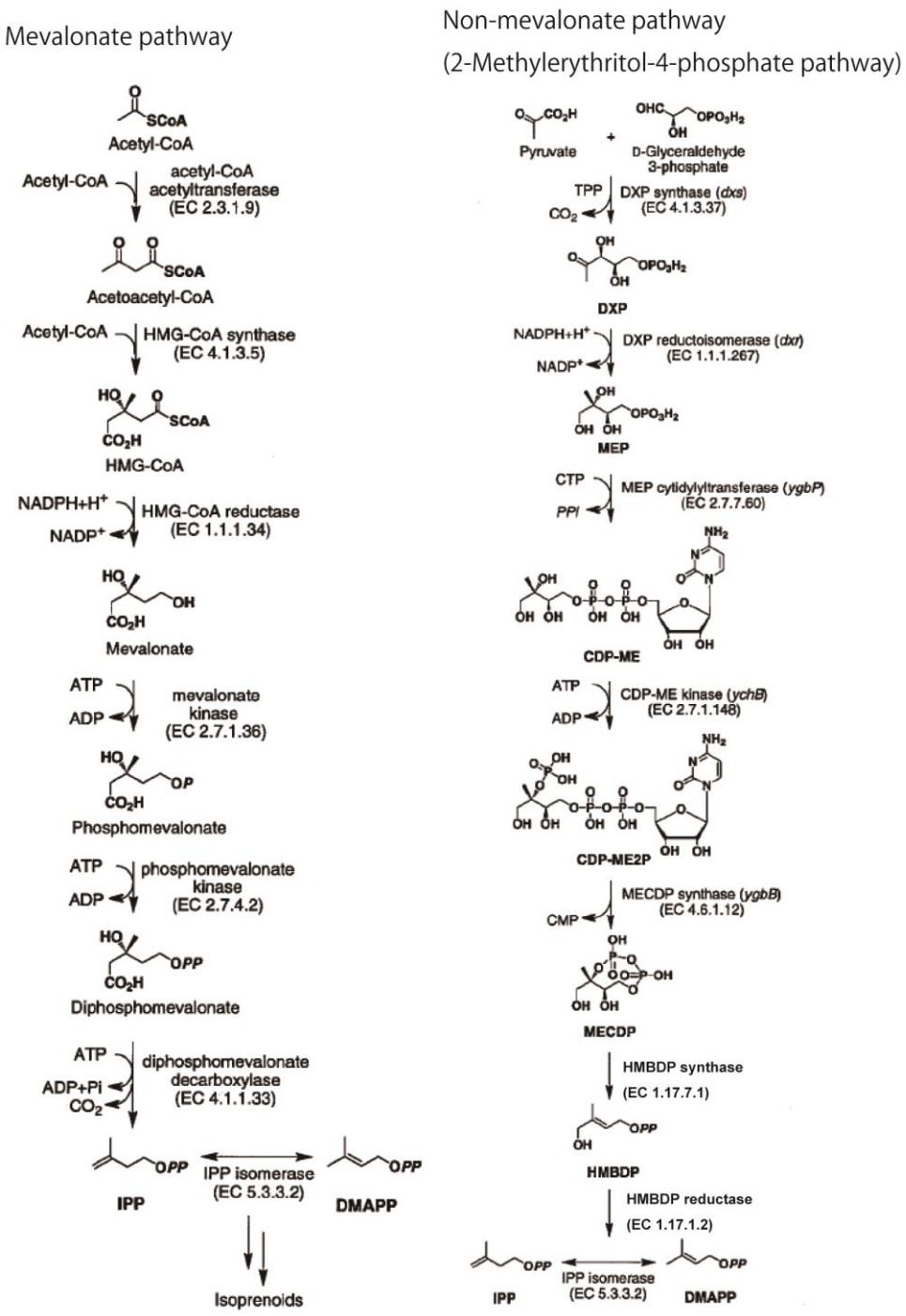


Fig. GI-2 Two types of biosynthetic pathways, mevalonate pathway and non-mevalonate pathway (MEP pathway), for supply of DMAPP and IPP. Modified from (Kuzuyama, 2002).

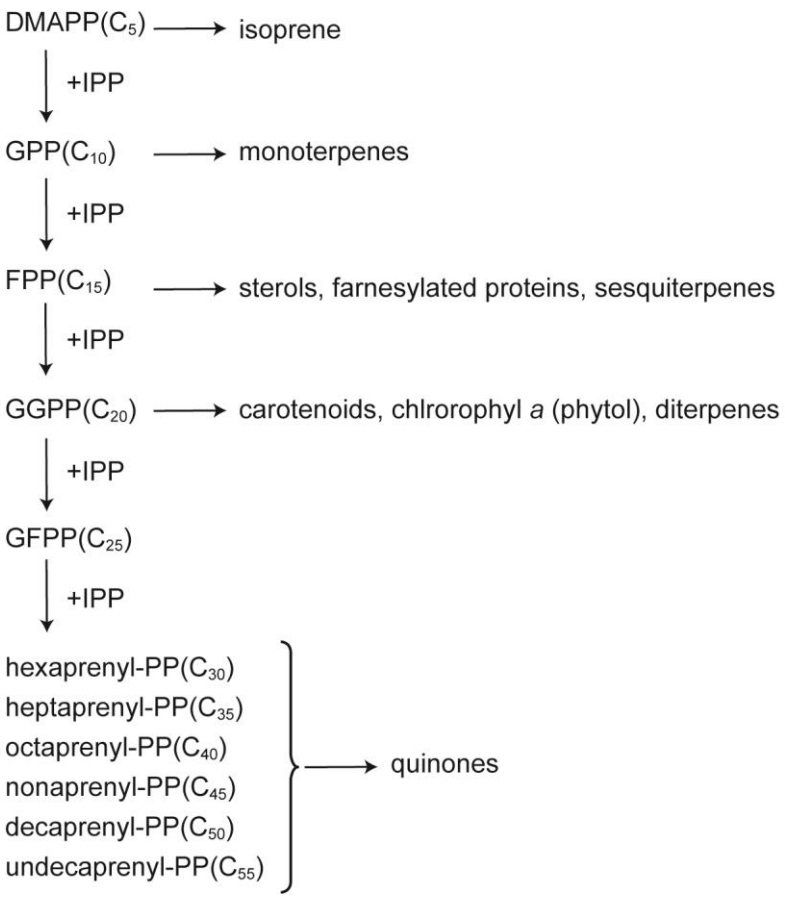


Fig. GI-3 Elongation of various lengths of prenyl chains. Isoprenoid compounds derived from these prenyl pyrophosphate species are shown.

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 μg·L culture^{-1}·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 μg·L culture^{-1}·day^{-1} at 712 hours. The overall production achieved was 1 mg·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 Overexpression of mevalonate pathway

Eukaryotes and some prokaryotes biosynthesize isoprenoids from acetyl-CoA via the mevalonate pathway. In Martin et al. (2003), isoprenoid production was dramatically improved by introducing foreign mevalonate pathway into *E. coli*. I expected the introduction of the mevalonate pathway is effective in cyanobacteria. I introduced yeast genes for the whole mevalonate pathway, which were assembled for expression in *E. coli* (Martin et al., 2003) into a neutral site of the *Synechocystis* genome under control of

trc and *lac* promoters. Complete segregation of the overexpression mutant was confirmed by PCR. The limonene production was improved (68 μg·L culture^{-1}·day^{-1}) (Fig. I-10).

I.3.10 Production other isoprenoids

Besides limonene, I tried photosynthetic production of geraniol (C_{10}) and amorpha-4, 11-diene (C_{15}). Geraniol is a monoterpene alcohol with a smell like roses, and often used in perfume. Amorpha-4, 11-diene is a precursor of artemisinin, which is an anti-malarial drug. I introduced genes for native geraniol synthase (*gs*) from *Perilla setoyensis* or codon-optimized amorpha-4, 11-diene synthase (*ads*) from *Artemisia annua* with the *trc* promoter into *Synechocystis*. The same gas stripping method was used for recovery of these products. GC-MS analysis revealed the increasing peaks in the course of photosynthetic growth (Fig. I-11, I-12). The molecular mass of the ads product $C_{15}H_{24}$ was identical to amorpha-4, 11-diene, though its identity was not fully confirmed because of lack of the authentic amorpha-4, 11-diene. On the other hand, the molecular mass of the major product of *gs* corresponded to 6-methyl-5-heptene-2-one ($C_8H_{14}O$), which is smaller than geraniol ($C_{10}H_{18}O$). Because this compound was predominant, geraniol may be converted to it by some other enzyme(s) intrinsic to *Synechocystis* or *gs* may produce it under heterologously expressed conditions.

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 μg·L culture^{-1}·day^{-1} and phytol ca. 600 μg·L culture^{-1}·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·L culture^{-1}·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.

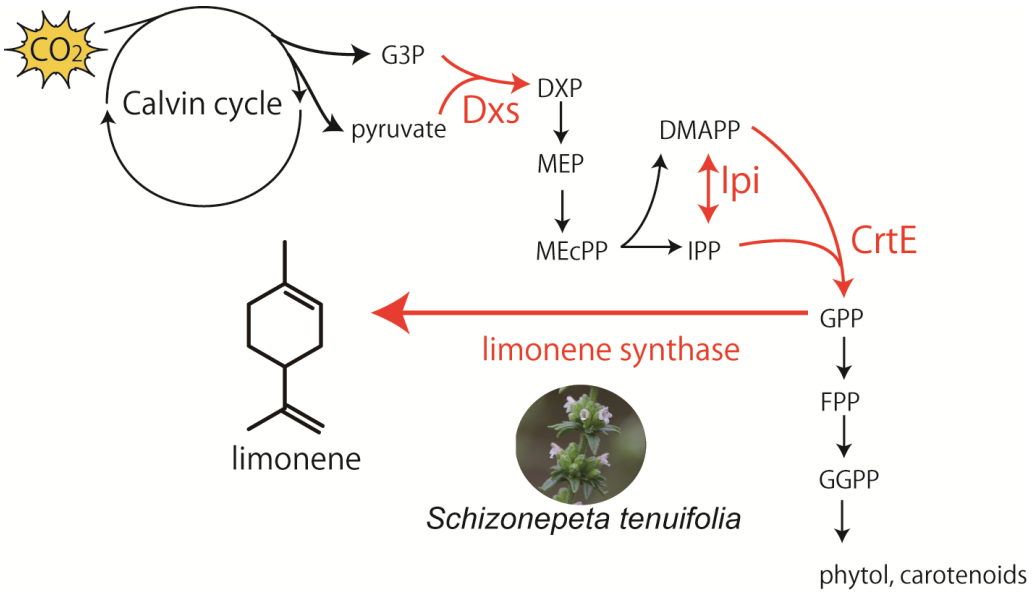


Fig. I-1. Biosynthesis of limonene from GPP by limonene synthase derived from *Schizonepeta tenuifolia.* GPP is a metabolic intermediate of the MEP pathway, which is supplied from CO_2 fixation via the Calvin cycle. Three engineered enzymes of the MEP pathway are highlighted in red.

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.

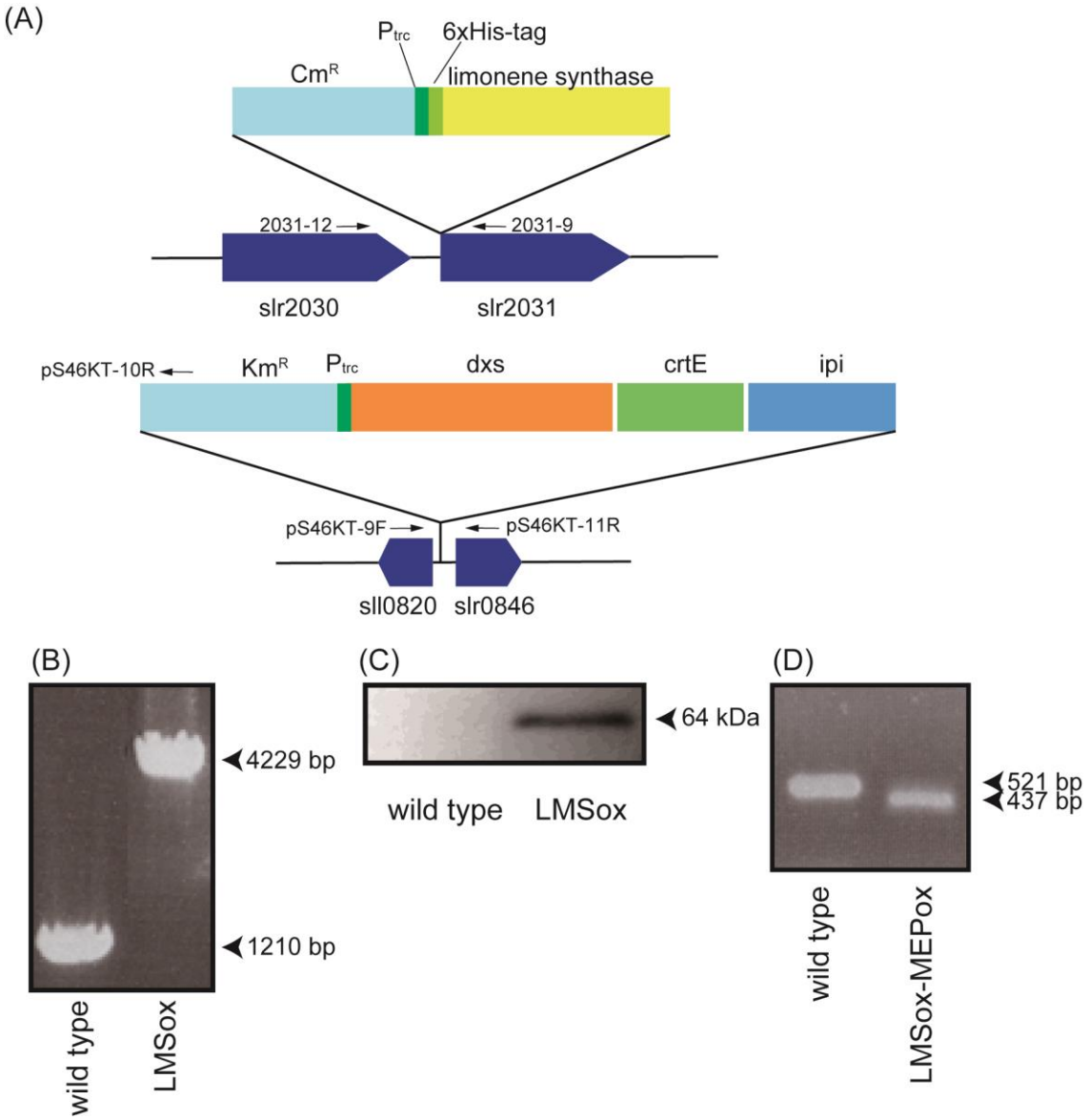


Fig. I-2. Integration and expression of engineered genes in the *Synechocystis* genome. A, Schematic representation of gene integration into the cyanobacterial chromosome by homologous recombination. P_{trc}, trc promoter; Cm^R, chloramphenicol resistance cassette; Km^R, kanamycin resistance cassette. 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.

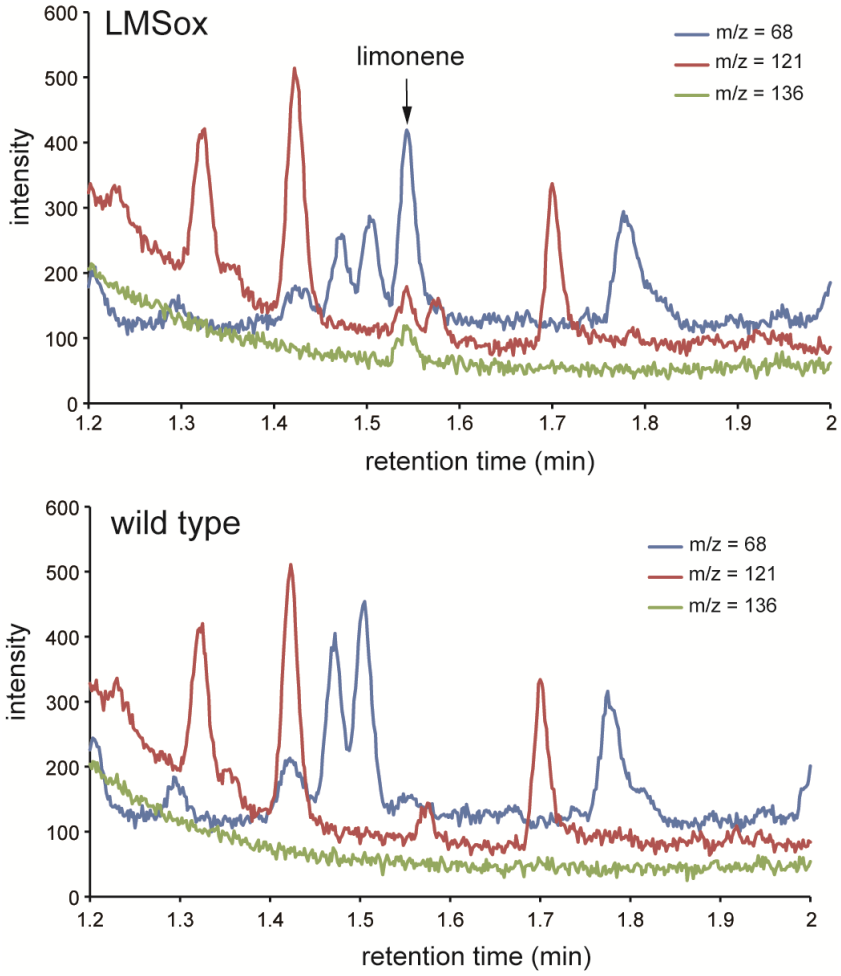


Fig. I-3. GC-MS analysis of extracts from LMSox and wild type cyanobacterial cells. Each line indicates a selected ion chromatogram.

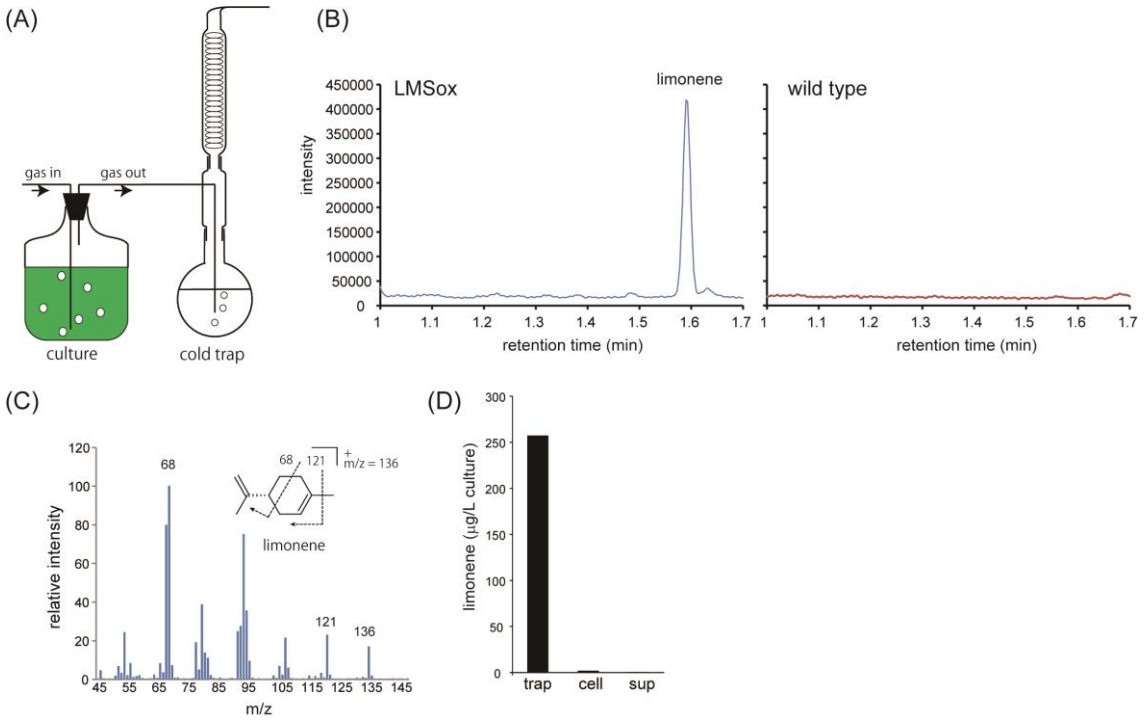


Fig. I-4. Extraction of limonene produced in cyanobacterial cultures using the gas-stripping method. A, Scheme for the gas-stripping system. B, GC-MS total ion chromatogram of the octane phase in the cold trap. C, mass spectrum of the limonene peak in panel B. D, distribution of the accumulated limonene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

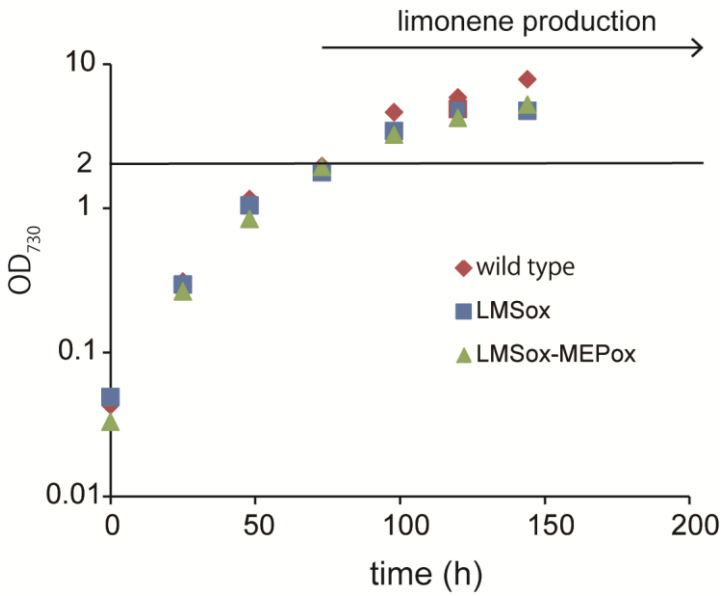


Fig. I-5. Growth of LMSox, LMSox-MEPox, and wild type cyanobacterial cells with bubbling under light at 50 μmol photons·m^{-2}·s^{-1}. The growth phase for the limonene production is denoted with an arrow.

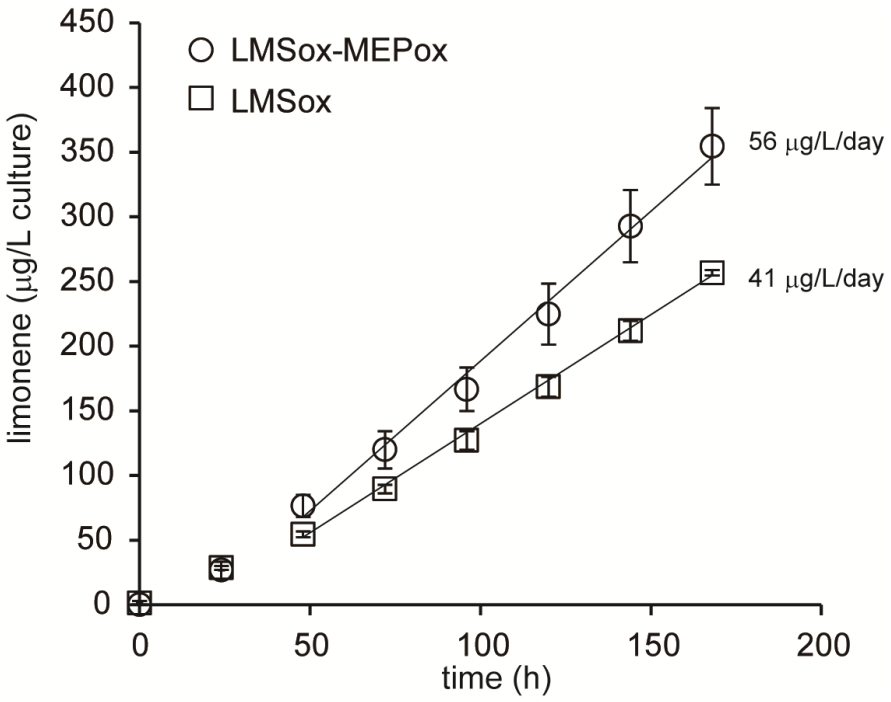


Fig. I-6. Limonene production in LMSox and LMSox-MEPox cyanobacterial cells. The standard error for three independent experiments is shown.

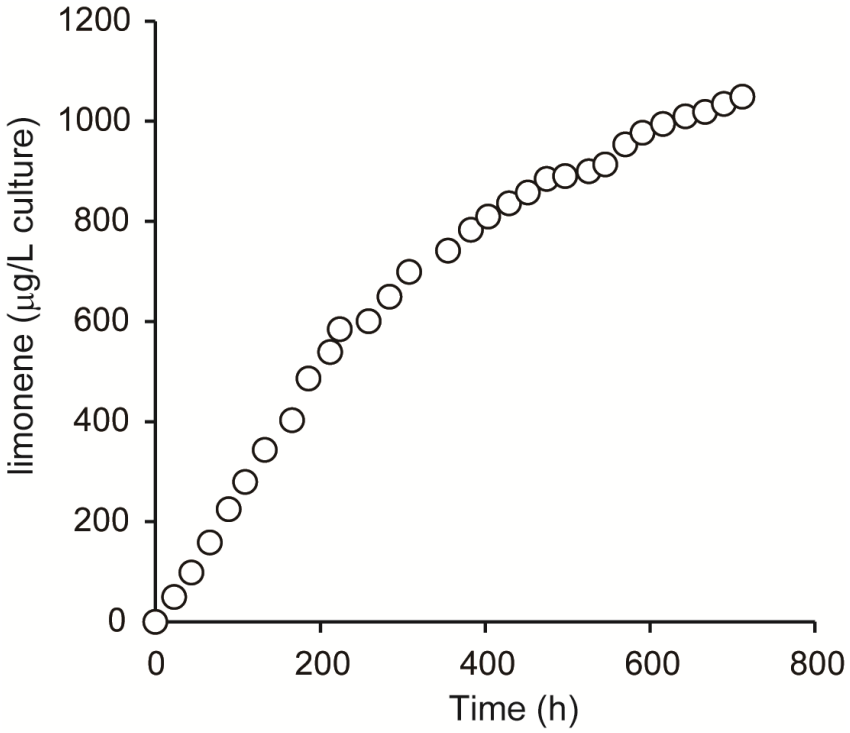


Fig. I-7. Augmented production of limonene in LMSox-MEPox cells.

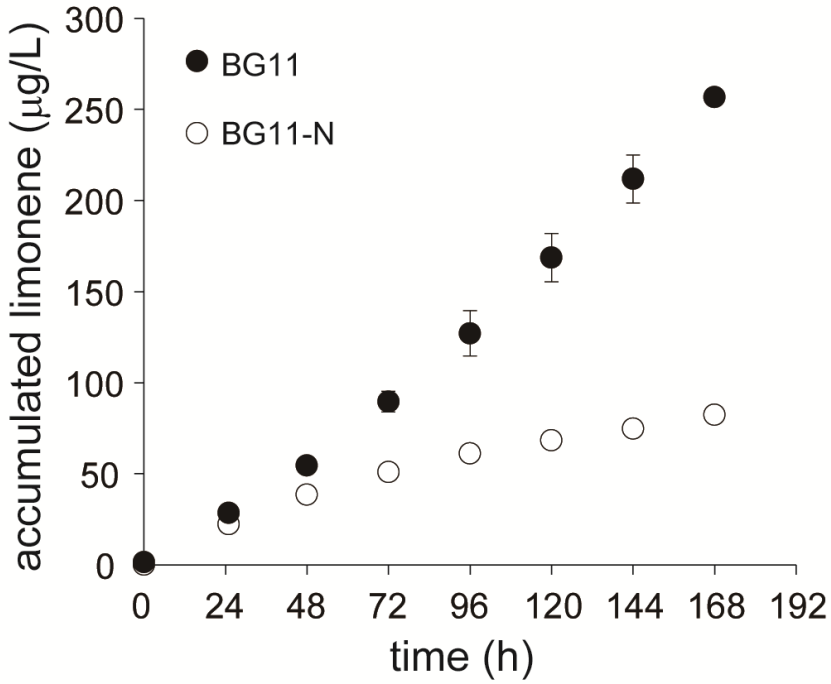
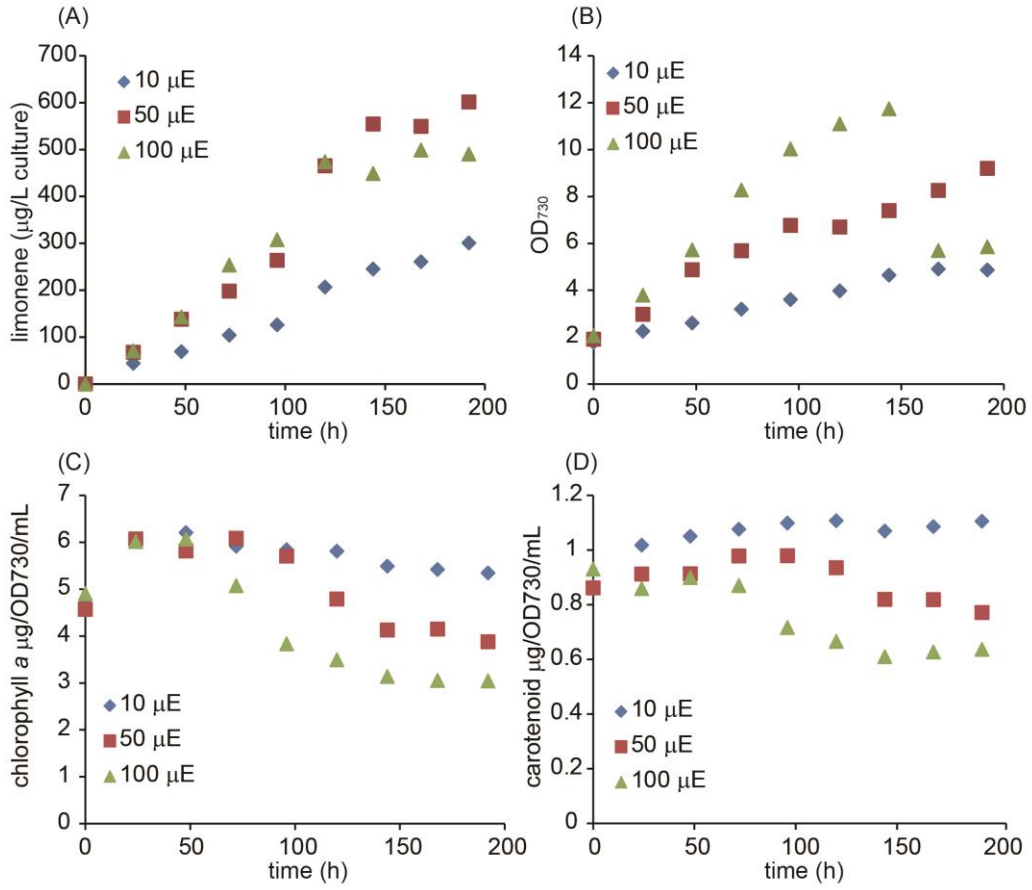


Fig. I-8. Limonene production in LMSox cyanobacterial cells under BG11-N (BG11 without nitrate) or BG11 condition.



(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).

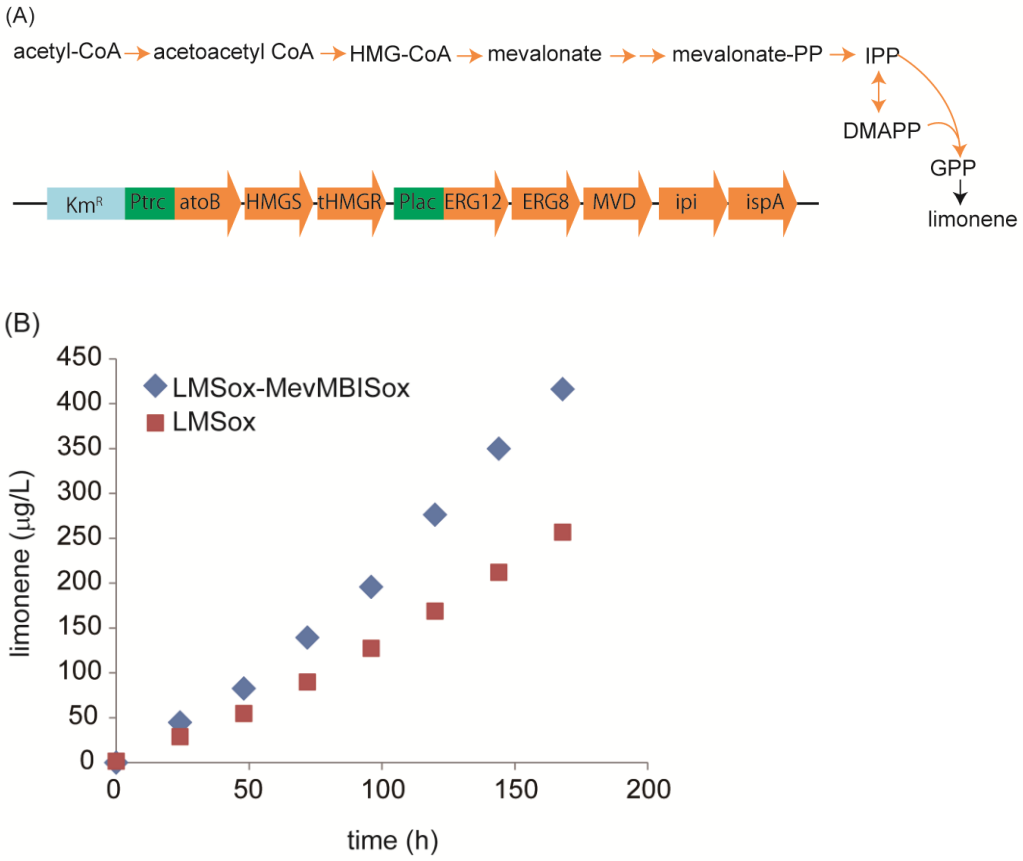


Fig. I-10. Limonene production in LMSox-MevMBIS and LMSox cyanobacterial cells. A, the mevalonate pathway and the construct for overexpression of enzymes for the pathway. B, limonene production in cyanobacterial cells with or without overexpression of the mevalonate pathway enzymes (MevMBIS). Km^R, kanamycin resistance cassette; Ptrc, trc promoter; atoB, acetoacetyl-CoA synthase; HMGS, hydroxymethylglutaryl-CoA synthase; tHMGR, hydroxymethylglutaryl-CoA reductase; Plac, lac promoter; ERG12,mevalonate kinase; ERG8, phosphomevalonate kinase; MVD, diphosphomevalonate decarboxylase; ipi, IPP isomerase; ispA, FPP synthase.

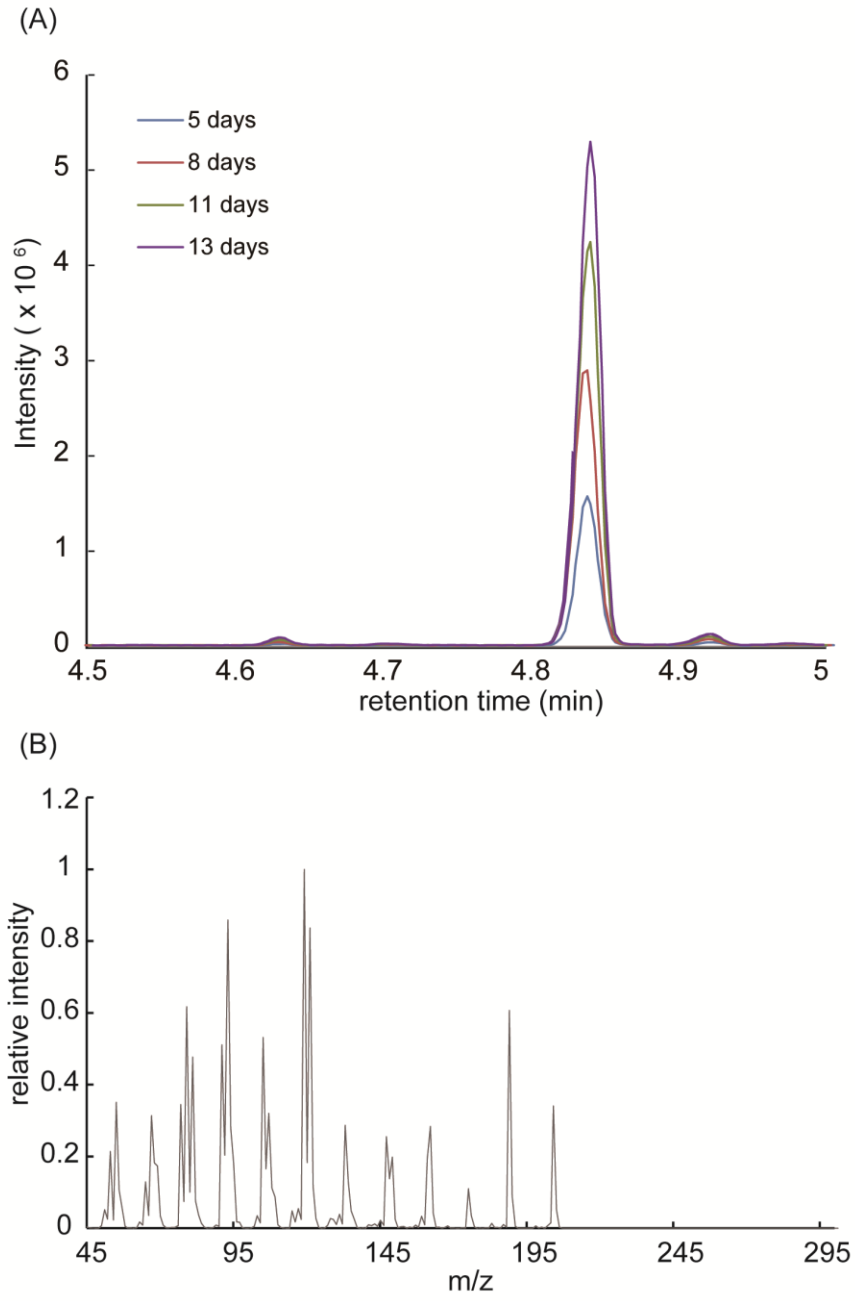


Fig. I-11. Production of amorpha-4, 11-diene in *ads*-overexpressed cyanobacterial cells. A, GC-MS total ion chromatogram of the octane phase in the cold trap. B, mass spectrum of the GC peak at 4.85 min (13days) in panel A.

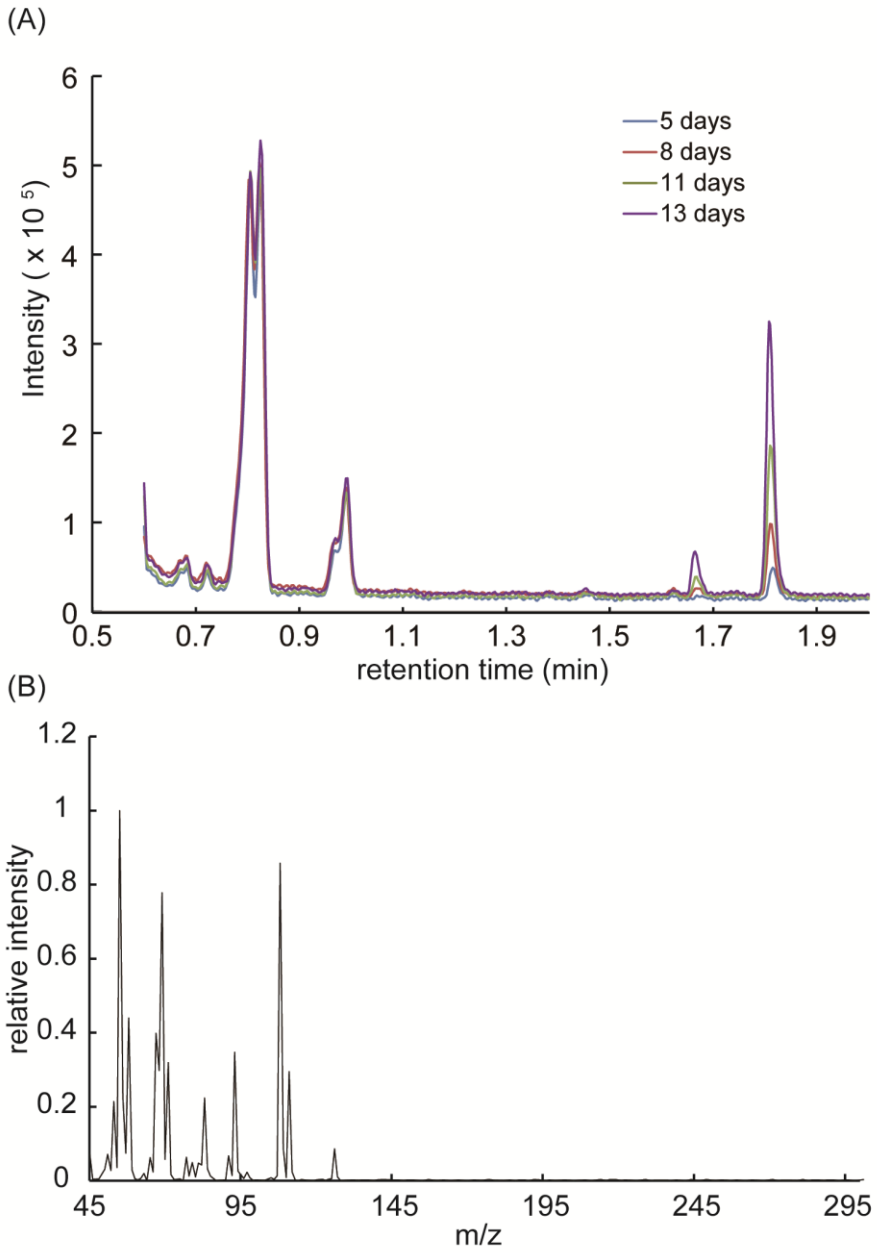


Fig. I-12. Production of geraniol-derived compounds in *gs*–overexpressed cyanobacterial cells. A, GC-MS total ion chromatogram of the octane phase in the cold trap. B, mass spectrum of the GC peak at 1.8 min (13days) in panel A.

Chapter II

Enzymatic analysis of *Synechocystis* GGPP synthase (Slr0739)

II.1 Introduction

Prenyltransferases are a class of enzymes that transfer prenyl groups to allylic acceptor molecules. These enzymes supply the isoprene unit to the isoprenoid biosynthesis. They can be classified into four groups according to the mode of subunit composition as well as the prenyl chain length of the final product. A short-chain prenyltransferase is a homodimeric and soluble enzyme, which belongs to Class I prenyltransferase; a medium-chain prenyltransferase is heterodimeric and soluble, which belongs to Class II prenyltransferase; and a long-chain prenyltransferase is a homodimeric enzyme that requires a carrier protein or lipid surfactant, which belongs to Class III or IV prenyltransferase, respectively (Ogura and Koyama, 1998).

Class I prenyltransferase includes GPP synthase, FPP synthase and GGPP synthase. FPP synthase provide farnesol chain of heme *a*. FPP can be converted to sterols and C_{30} carotenoids. GGPP is a precursor of the phytol chain of chlorophylls and C_{40} carotenoids. Many bacteria possess either GGPP synthase or FPP synthase: GGPP synthase is distributed to carotenoid-producing species such as *Corynebacterium glutamicum*, *Erwinia herbicola* and cyanobacteria, while FPP synthase is present in other species such as *Escherichia coli* and *Bacillus subtilis*, which do not produce chlorophylls nor carotenoids (Fujisaki et al., 1990; Heider et al., 2014; Math et al., 1992; Takahashi and Ogura, 1981). While chain-length specificity of FPP synthase is different from that of GGPP synthase, these prenyltransferases are phylogenically similar to each other. It is difficult to predict the specificity from the amino acid sequence correctly.

The gene for GGPP synthase (*crtE*) was first cloned from a carotenoid-producing proteobacterium *Erwinia* and its enzymatic activity was biochemically confirmed (Math et al., 1992). Since then, enzymatic activity of GGPP synthases has been studied in many organisms including bacteria, plant and fungi (Heider et al., 2014; Jiang et al., 1995; Orlova et al., 2009). Ohto and coworkers reported the *crtE* gene from a thermophilic *Synechococcus* and confirmed its GGPP synthase activity (Ohto et al., 1999). However, they studied the activity only qualitatively.

In many cyanobacteria, there are three putative prenyltransferase genes (*slr0739*, *slr0611*, and *sll0506* in *Synechocystis* sp. PCC 6803). The *crtE*-like gene *slr0739* may encode GGPP synthase to produce C_{20} diterpene, which can be utilized for production of carotenoids (C_{40}) and phytol chain (C_{20}) of chlorophyll *a* (Fig. II-1). The gene *slr0611* may encode a solanesyl diphosphate synthase to produce C_{45} terpene, which can be utilized for production of solanesol chain (C_{45}) of plastoquinone (Sadre et al., 2012). The gene *sll0506* may encode an undecaprenyl diphosphate synthase to produce C_{55} terpene, which can be used as a scaffold for peptidoglycan of the cyanobacterial cell wall (Lovering et al., 2012). I assume Slr0739 is the most important enzyme of the three for production of short-chain isoprenoids, because GGPP synthase catalyzes sequential reactions to produce GPP, FPP and GGPP (Fig. II-1).

Here, I prepared a CrtE-like protein, Slr0739, from *Synechocystis* using bacterial expression system and analyzed its kinetic properties as a basis of genetic engineering and metabolic manipulation of cyanobacterial photosynthesis.

II.2 Material and Methods

II.2.1 Plasmid and transformant for overexpression of Slr0739

The *slr0739* gene, which encodes a putative GGPP synthase of *Synechocystis*, was amplified by PCR using DNA polymerase (PrimeSTAR Max DNA polymerase、Takara Bio, Otsu, Japan) and directly cloned into pET28a expression vector (Merck, Darmstadt, Germany) using InFusion HD Cloning kit (Takara Bio). Resulting plasmid *slr0739*-pET28a was introduced into *E. coli* strain C41 (DE3).

II.2.2 Enzyme expression and purification

E. coli strain C41 carrying *slr0739*-pET28a was cultured in 1 liter of LB medium at 37°C. When the culture reached an optical density at 600 nm (OD_{600}) of 0.4 to 0.8, 1 mM isopropyl-β-D-1-thiogalactopyranoside was added (final concentration, 1 mM), and the cells were grown at 37°C for 3 h to achieve induction. The cells were then collected by centrifugation at 4,220 *g* for 15 min, suspended in 20 mM HEPES-NaOH (pH 7.5) containing 100 mM NaCl and 10% (w/v) glycerol, and homogenized three times with a French press at 1,500 kg/cm^2. Insoluble proteins were removed by centrifugation at 50,000 g for 30 min and soluble proteins were collected. His-tagged Slr0739 was purified by Ni-affinity column chromatography (HisTrap HP; GE Healthcare, Little Chalfont, United Kingdom), with the eluent consisting of a gradient of 30 to 430 mM imidazole in the aforementioned buffer system. Proteins in each fraction were subjected to SDS-PAGE, followed by Coomassie

brilliant blue staining. Low-molecular-mass calibration kit (GE Healthcare) was used as the molecular mass marker. Fractions enriched with the Slr0739 protein were pooled and dialyzed against the aforementioned buffer to remove imidazole. Protein concentration was assayed using the Bradford method (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

II.2.3 Assay for prenyltransferase activity

The reaction mixture contained, in final volume of 0.1 mL, 50 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM dithiothreitol, 5 $\mu g \cdot mL^{-1}$ bovine serum albumin, various concentrations of IPP, DMAPP, GPP and FPP and 0.2 μM of Slr0739. Reaction temperature was 31°C. After incubation, 0.1 mL of 0.4 M HCl was added to the reaction mixture, and the mixture was incubated at 31°C for 30 min to complete the hydrolysis of the products. Then, 10 μL of 100 ppm of cinnamyl alcohol was added to the hydrolysates as an internal standard. The hydrolysates were extracted with 0.1 mL of heptane. The extracts were subjected to gas chromatography-mass spectrometry (GC-MS) analyses.

II.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 = 93, 136, 204, 272). The GPP, FPP, and GGPP peaks were identified as the retention time in the selected ion chromatogram and quantified based on 10 ppm of the cinnamyl alcohol peak.

II.2.5 Simulation analysis of GGPP synthases

I constructed a model consisting of three sequential reactions, which obey Michaelis-Menten kinetics (Fig. II-6A). Reaction velocities (v_1~v_9) are represented by following equations.

$$v_1 = k_1[\mathrm{DMAPP}][\mathrm{E}_{free}]$$

$$v_2 = k_2[\mathrm{DMAPP\cdot E}]$$

$$v_3 = k_3[\mathrm{DMAPP\cdot E}]$$

$$v_4 = k_4[\mathrm{GPP}][\mathrm{E}_{free}]$$

$$v_5 = k_5[\mathrm{GPP\cdot E}]$$

$$v_6 = k_6[\mathrm{GPP\cdot E}]$$

$$v_7 = k_7[\mathrm{FPP}][\mathrm{E}_{free}]$$

$$v_8 = k_8[\mathrm{FPP\cdot E}]$$

$$v_9 = k_9[\mathrm{FPP\cdot E}]$$

Then variation of each substance is represented by the following simultaneous differential equations.

$$\frac{d[\mathrm{DMAPP}]}{dt} = -v_1 + v_2$$

$$\frac{d[\mathrm{DMAPP\cdot E}]}{dt} = v_1 - v_2 - v_3$$

$$\frac{d[\mathrm{GPP}]}{dt} = v_3 - v_4 + v_5$$

$$\frac{d[GPP \cdot E]}{dt} = v_4 - v_5 - v_6$$

$$\frac{d[FPP]}{dt} = v_6 - v_7 + v_8$$

$$\frac{d[FPP \cdot E]}{dt} = v_7 - v_8 - v_9$$

$$\frac{d[E_{free}]}{dt} = -v_1 + v_2 + v_3 - v_4 + v_5 + v_6 - v_7 + v_8 + v_9$$

Kinetic parameters in previous reports (Heider et al., 2014; Orlova et al., 2009) and this thesis (Table II-1) were used for the simulations (k_1~k_9). The values of k_{cat} for DMAPP, GPP and FPP were applied for k_3, k_6 and k_9. The values of K_m for DMAPP, GPP and FPP were applied for k_2/k_1, k_5/k_4 and k_8/k_7 and the absolute values of k_1, k_4 and k_7 were hypothesized as 100 $s^{-1} \cdot M^{-1}$ arbitrary. The initial concentration of [DMAPP] was fixed at 100 μM, of [E_{free}] was 5 μM (Fig II-6) and 0.2 μM (Fig. II-7). Simultaneous differential equations were solved numerically by MATLAB/SIMULINK (MathWorks, Massachusetts, US).

II.3 Results

II.3.1 Cloning, expression and purification of Slr0739

The putative *crtE* gene, *slr0739,* was cloned from the *Synechocystis* genome by PCR into pET28a vector (*slr0739*-pET28a) and confirmed by di-deoxy sequencing. Recombinant Slr0739 protein was expressed in *E. coli* strain C41 and purified with Ni-affinity chromatography. Purified protein migrated as a single band at 36 kDa in SDS-PAGE analysis (Fig. II-2).

II.3.2 GC-MS analysis for GPP, FPP and GGPP

Prenyl pyrophosphates have low vapor pressure and cannot be analyzed by GC. Prenylalcohols, which are obtained by hydrolysis of the prenyl pyrophosphates, are available for GC-MS analysis. To measure prenyltransferase activity of Slr0739, I examined conversion of prenyl pyrophosphates to prenyl alcohols by HCl treatment. Figure II-3 shows GC-MS chromatograms derived from hydrolysates of GPP, FPP and GGPP. In non-hydrolysis samples no peak was detected (Fig. II-3). Hydrolyzed GPP gave two peaks at 2.3 min (minor) and 3.2 min (major) (m/z =136). Hydrolyzed FPP gave one major peak at 5.4 min (m/z =136, 204). Hydrolyzed GGPP gave two peaks at 7.9 min (major) and 8.8 min (minor) (m/z = 136, 204, and 272). I used the major peaks to quantify these prenyl alcohols. (GPP, the peak at 3.2 min, m/z = 136; FPP, the peak at 5.4 min, m/z =136, GGPP, the peak at 7.9 min, m/z = 136)

II.3.3 Slr0739 synthesizes GGPP from DMAPP

First, I followed the whole time course of Slr0739 reaction until the enzyme reaction from IPP and DMAPP was nearly completed. Figure II-4 shows accumulation and consumption of GPP, FPP and GGPP. The initial product GPP accumulated after 10 min and then slowly decreased to very low level. The secondary product FPP also accumulated after 10 min and stayed at this level afterwards. The tertiary product GGPP also accumulated after 10 min and increased continuously up to 50 min. Any other product was detected in the GC chromatograms. Moreover, the final level of GGPP is nearly equivalent to the level of the initial substrate DMAPP. These facts

suggest that Slr0739 does not possess any transferase activity beyond C_{20}.

II.3.4 kinetic parameters for DMAPP and FPP

Next, I examined kinetic parameters of Slr0739 for each substrate by measuring the initial velocity at 5 min of incubation (Fig. II-5). Based on the Michaelis-Menten kinetics, I obtained Michaelis constants (K_m) and turnover rates (k_{cat}) from Lineweaver–Burk plot. The value of k_{cat} / K_m for FPP was higher than for DMAPP. Slr0739 may have higher activity for FPP than GPP. However, I could not obtain K_m or k_{cat} for GPP with unknown reason. Table 1 shows K_m, k_{cat} and k_{cat} / K_m values for DMAPP and FPP. K_m and k_{cat} for DMAPP were smaller than those for FPP. The value of k_{cat} / K_m for DMAPP was clearly higher than those for FPP. This may support the fact that intermediary FPP did not accumulate much throughout the whole reaction from DMAPP to GGPP (Fig. II-4).

II.4. *Discussion*

In general, GGPP synthase produces GGPP from DMAPP via GPP and FPP (Fig. II-1). Here, I partially characterized enzyme kinetics of *Synechocystis* GGPP synthase Slr0739. The kinetics values are compared with those of GGPP synthases from bacteria and plants (Table II-1) (Heider et al., 2014; Ohnuma et al., 1997; Orlova et al., 2009). The parameters of Slr0739 except GPP are more or less similar to them. I could not determine the parameters for GPP with unknown reason.

In order to estimate the kinetic properties of Slr0739 for GPP, I

adopted the simulation analysis of a multistep enzyme reaction deduced from the experimental parameters for DMAPP and FPP (Table II-1) and accumulation time course of intermediates and the end product (Fig. II-4). Before dealing with cyanobacterial Slr0739, I simulated the accumulation time course for two GGPP synthase enzymes (CgCrtE and CgIdsA) from *Corynebacterium glutamicum* based on kinetics parameters of these enzymes (Heider et al., 2014). The calculated results (Fig. II-6 B-1 and B-2) clearly show that the transient accumulation of GPP or FPP largely depends on k_{cat} / K_m values for GPP and FPP. In CgCrtE, there is practically no accumulation of GPP, which reflects its very high k_{cat} / K_m for FPP. Marked accumulation of GPP and FPP in CgIdsA is accounted for by differences in the ratio between the reaction from GPP to FPP and FPP to GGPP; the ratio of k_{cat} / K_m (GPP) over k_{cat} / K_m (GPP) of CgIdsA is nearly 40-fold lower than that of CgCrtE. Although I could not get reliable data for GPP in Slr0739, the reaction properties of Slr0739 may be somehow similar to CgIdsA.

Kinetics of plant GGPP synthases (*Nicotiana tabacum*, *Hevea brasiliensis* and *Taxus baccata*) has also been reported (Burke and Croteau, 2002b; Orlova et al., 2009; Takaya et al., 2003). These GGPP synthases have higher K_m for FPP than GPP. In order to see the impact of this high K_m, I simulated the accumulation profile of the tobacco GGPP synthase (GGPPS1), based on the assumption (k_{cat} = 1 s^{-1} for all DMAPP, GPP and FPP) in addition to the reported K_m values (Orlova et al., 2009). The simulated results (Fig. II-6 B-3) shows marked accumulation of FPP at early stage of the time course, which was not observed in the experimental accumulation profile of

Synechocystis Slr0739. When the value of 1/5 of K_m (GPP) was used for K_m (FPP), the simulation resulted in much less level of the transient FPP accumulation, resembling the experimental profile of Slr0739 (Fig. II-6 B-4). It is suggested that the ratio of K_m (GPP) to K_m (FPP) critically affects the accumulation profile of those intermediates.

The accumulation profile of Slr0739 was simulated by using experimentally determined kinetic parameters and assumption (K_m (GPP) = K_m (FPP), k_{cat} (GPP) = k_{cat} (FPP) (Fig. II-6 B-5) or K_m (GPP) = K_m (DMAPP), k_{cat} (GPP) = k_{cat} (DMAPP) (Fig. II-6 B-6)), because the kinetic parameters for GPP were not obtained. Evidently, the simulation results (K_m (GPP) = K_m (FPP), k_{cat} (GPP) = k_{cat} (FPP)) were more similar to the experimental accumulation profile (Fig. II-4) than the simulation (K_m (GPP) = K_m (DMAPP), k_{cat} (GPP) = k_{cat} (DMAPP)). This finding suggests that K_m and k_{cat} for GPP of Slr0739 are close to those for FPP.

Then, I compared the simulation profile with the experimental profile at the same Slr0739 concentration (Fig. II-7). It is obvious that the whole reactions including the transient accumulation of GPP and FPP proceeded two- or three-fold faster in the experiment than in the simulation, though the shape of the profile was more or less similar. This finding suggests that GPP produced by the first catalytic reaction could be efficiently channeled as a substrate to the second catalytic reaction on the same enzyme protein and FPP produced by the second catalytic reaction could be efficiently channeled as a substrate to the third catalytic reaction, too. In this case, the intermediary product (GPP or FPP) does not need to be diffused to the

catalytic site, so that the substrate concentration at the site should be much higher than that in the freely diffusable solution. The channeling of intermediates to enhance the catalytic reactions has been well documented in co-localized enzymes and chimeric proteins fused in nature or artificially (Bach, 1995; Brodelius et al., 2002). It should be mentioned that the channeling has been often found for sequential multi-step biosynthesis pathways such as isoprenoid biosynthesis. However, the channeling must be unfavorable if one of the intermediates is to be taken up by other enzyme for mass production besides the end product. In cyanobacterial cells, the endogenous GGPP synthase Slr0739 seems to be indispensable for production of FPP and GGPP that are precursors for carotenoids, chlorophyll *a*, phylloquinone and plastoquinone. Therefore, one possible way to overcome the channeling of the endogenous Slr0739 is to replace it with *Corynebacterium* CgIdsA, which allows relatively large accumulation of GPP as shown in Fig. II-6 B-2. Alternatively, introduction of GPP synthase in addition to the endogenous Slr0739 would provide additional production of GPP, which can be available for the limonene synthase described in Chapter I.

Table 1 Kinetic parameters of GGPP synthases for DMAPP, GPP, and FPP

	Synechocystis		*C. glutamicum**						*N. tabacum***		
	Slr0739		CgCrtE			CgIdsA			GGPPS1		
	DMAPP	FPP	DMAPP	GPP	FPP	DMAPP	GPP	FPP	DMAPP	GPP	FPP
K_m (μM)	15	55	8	8	20	7	0.1	6	22.81	3.63	28.82
k_{cat} (s^{-1})	0.17	1.2	1.1	0.1	0.4	0.02	0.2	0.1	–	–	–
k_{cat}/K_m (s^{-1}/mM)	11	22	137.5	12.5	20	2.9	2000	17	–	–	–

* Heider et al. (2014)
** Orlova et al. (2009)

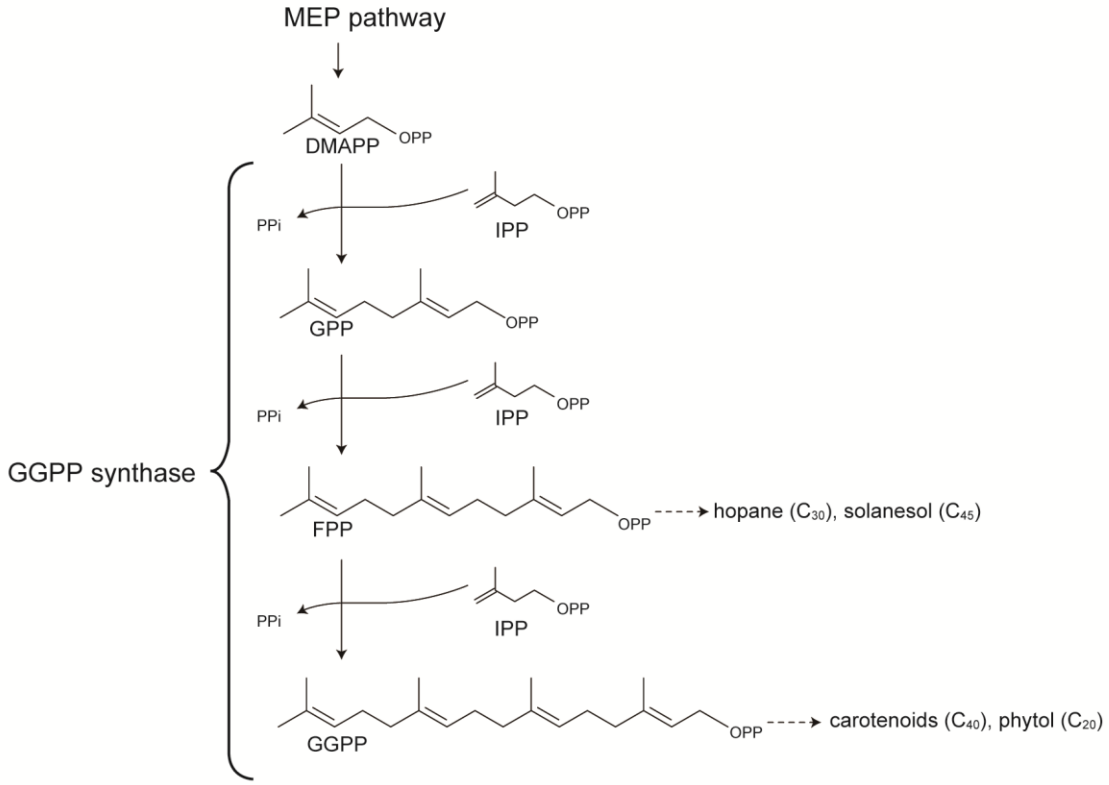


Fig. II-1 GGPP synthase catalyzes three sequential reactions. GGPP synthase condenses IPP into allylic substrates (DMAPP, GPP and FPP).

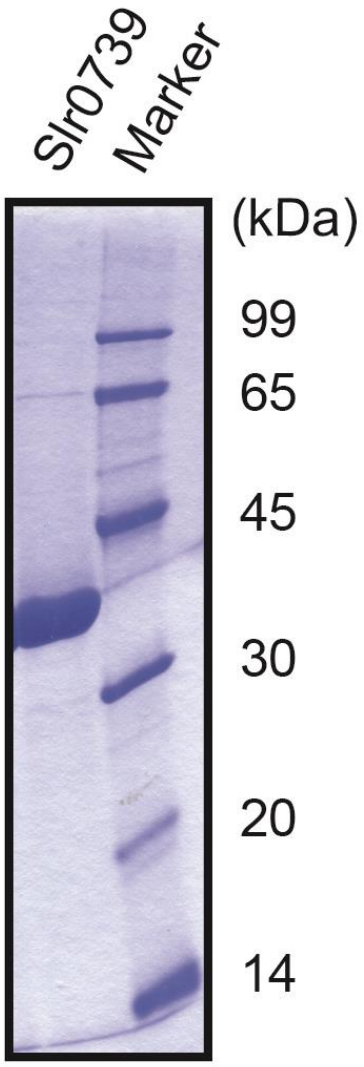


Fig. II-2 SDS-PAGE analysis of purified Slr0739. Purified Slr0739 (36 kDa) was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. Marker, marker proteins.

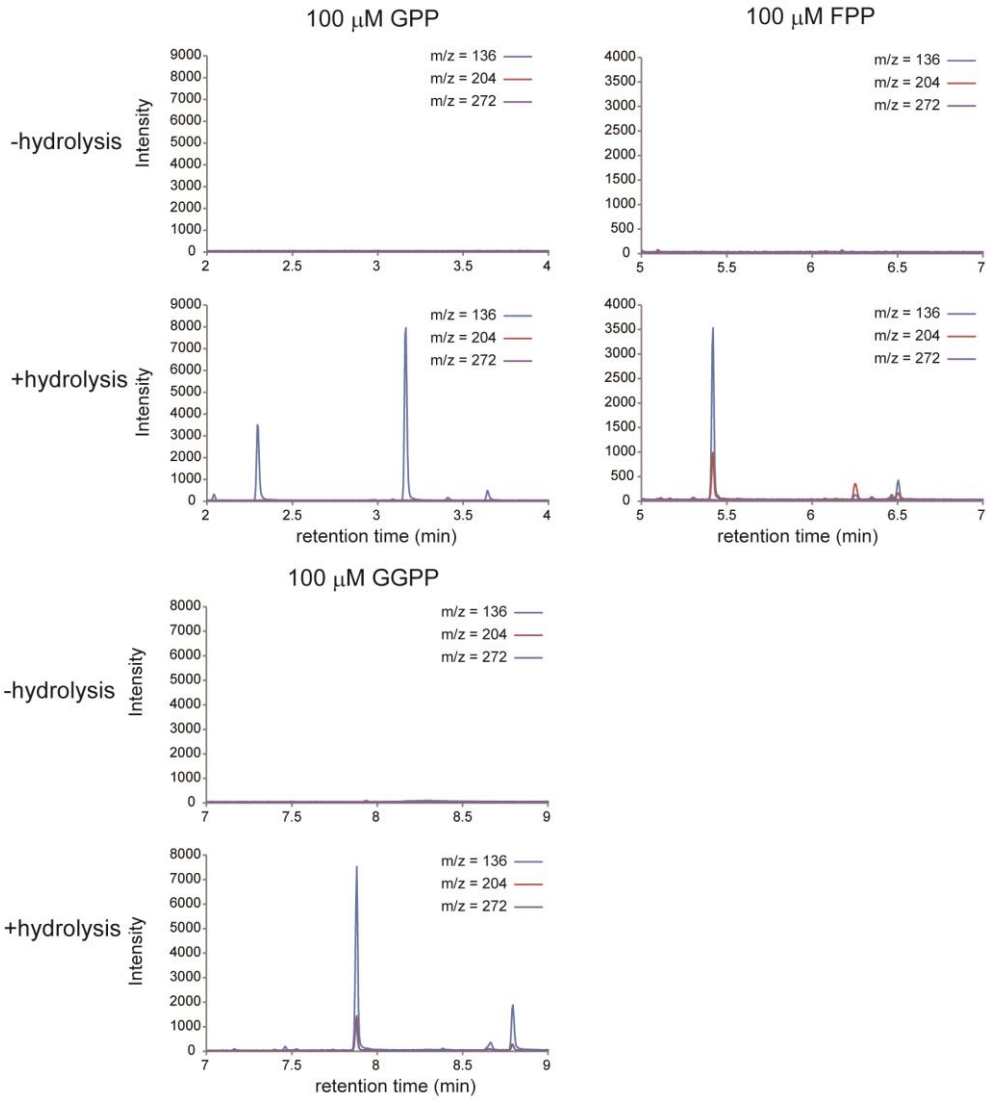


Fig. II-3 GC-MS analysis of GPP, FPP and GGPP before and after the hydrolysis treatment. Each line indicates a selected ion chromatogram.

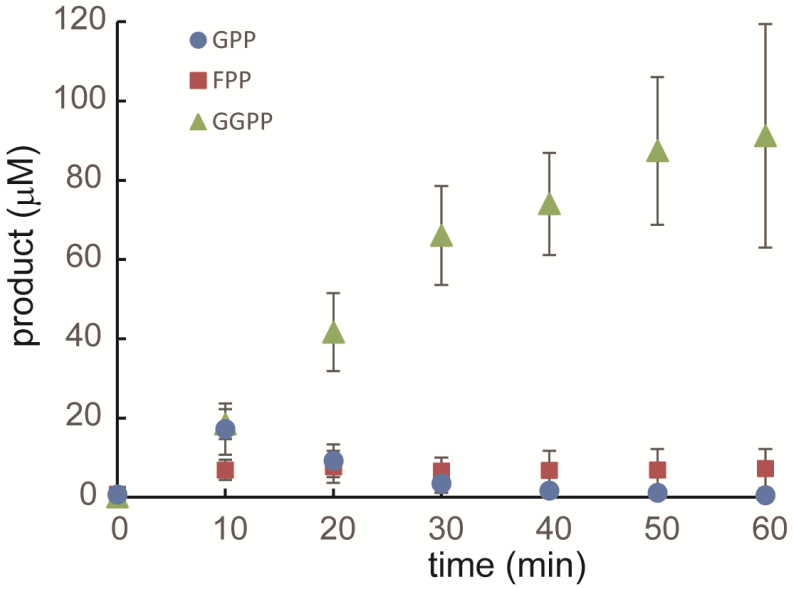


Fig. II-4 Accumulation profile of products in GGPP synthase reaction. Error bars represents standard deviations for three independent experiments.

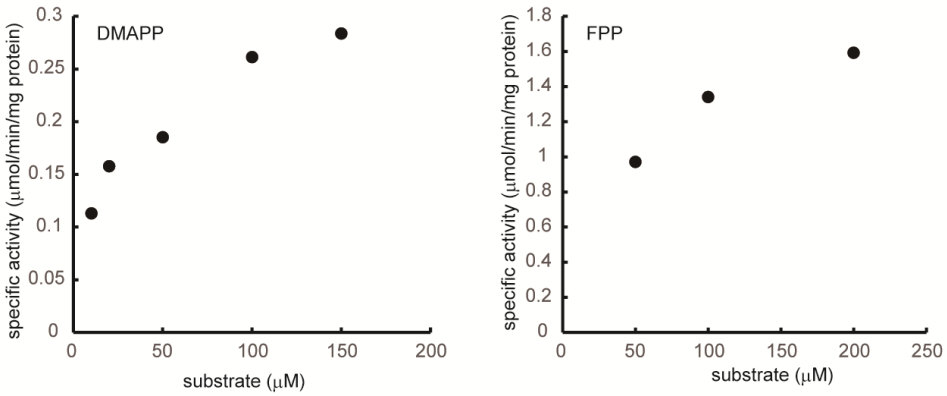


Fig. II-5 Concentration dependency of DMAPP or FPP for the Slr0739 activity. Enzyme concentration was used for 0.2 μM for FPP and 0.05 μM for DMAPP. The activity for DMAPP is expressed as summation of produced GPP and FPP.

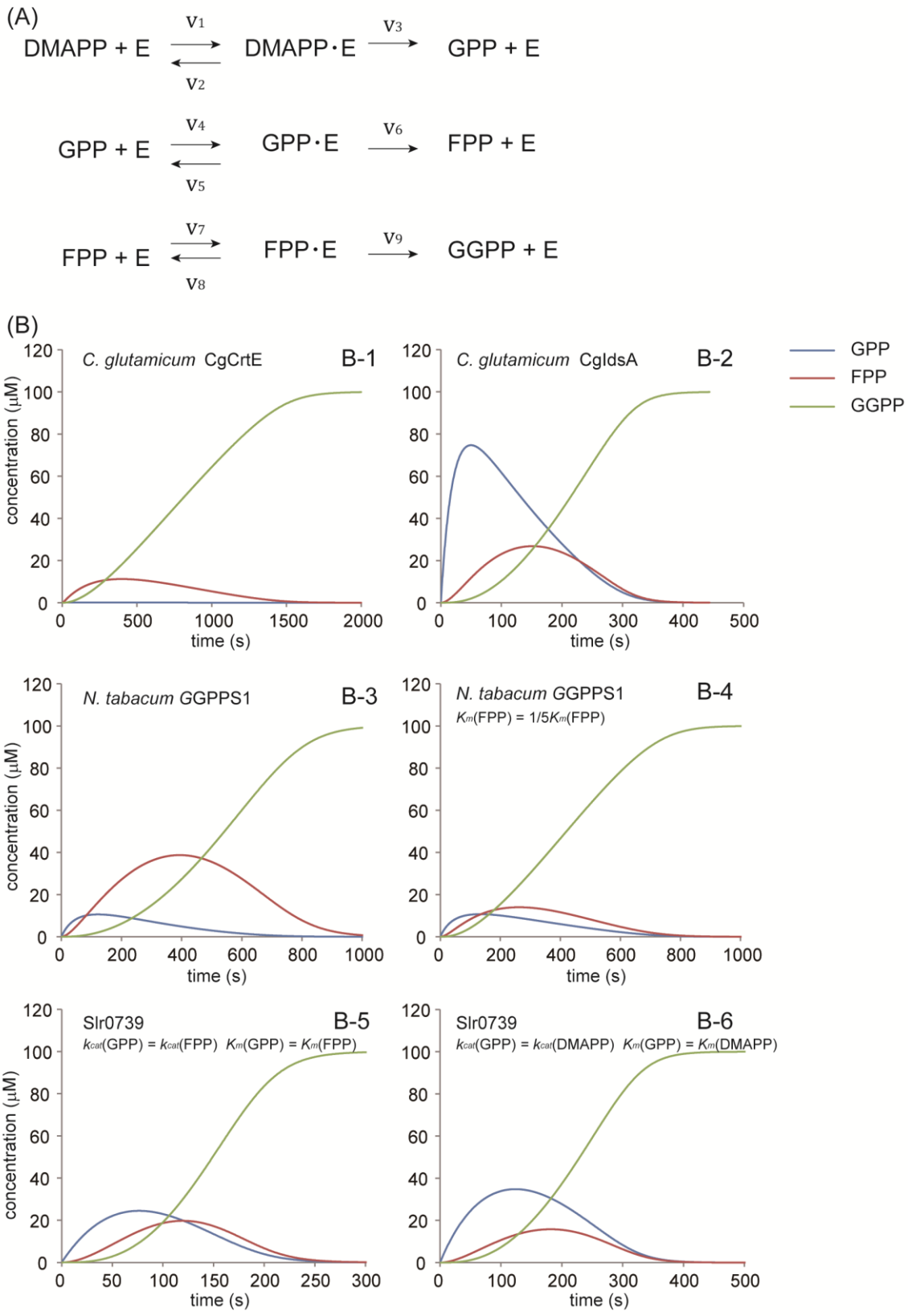

(A)
DMAPP + E
v1
v2
DMAPP·E
v3
GPP + E
GPP + E
v4
v5
GPP·E
v6
FPP + E
FPP + E
v7
v8
FPP·E
v9
GGPP + E
(B)
C. glutamicum CgCrtE
B-1
C. glutamicum CgIdsA
B-2
GPP
FPP
GGPP
N. tabacum GGPPS1
B-3
N. tabacum GGPPS1
Km(FPP) = 1/5Km(FPP)
B-4
Slr0739
kcat(GPP) = kcat(FPP) Km(GPP) = Km(FPP)
B-5
Slr0739
kcat(GPP) = kcat(DMAPP) Km(GPP) = Km(DMAPP)
B-6
concentration (μM)
time (s)

Fig. II-6 Simulation of the accumulation profile of GGPP synthases. A, Model of GGPP synthase reactions applied for the simulation. B, Simulated reaction profiles of GGPP synthases from *Corynebacterium glutamicum*, (B-1, CgCrtE and B-2, CgIdsA), *Nicotiana tabacum* (B-3, GGPPS1 with parameters reported in the original report (Orlova et al., 2009), and B-4, GGPPS1 with K_m (FPP) hypothesized as 1/5 of K_m (GPP)), and *Synechocystis* (B-5, Slr0739 with K_m (GPP) = K_m (FPP) and k_{cat} (GPP) = k_{cat} (FPP), B-6, Slr0739 with K_m (GPP) = K_m (DMAPP) and k_{cat} (GPP) = k_{cat} (DMAPP)). Enzyme concentrations are 5 μM. DMAPP･E, complex of DMAPP and enzyme; GPP･E, complex of GPP and enzyme; FPP･E, complex of FPP and enzyme. Blue, GPP; red, FPP; green, GGPP.

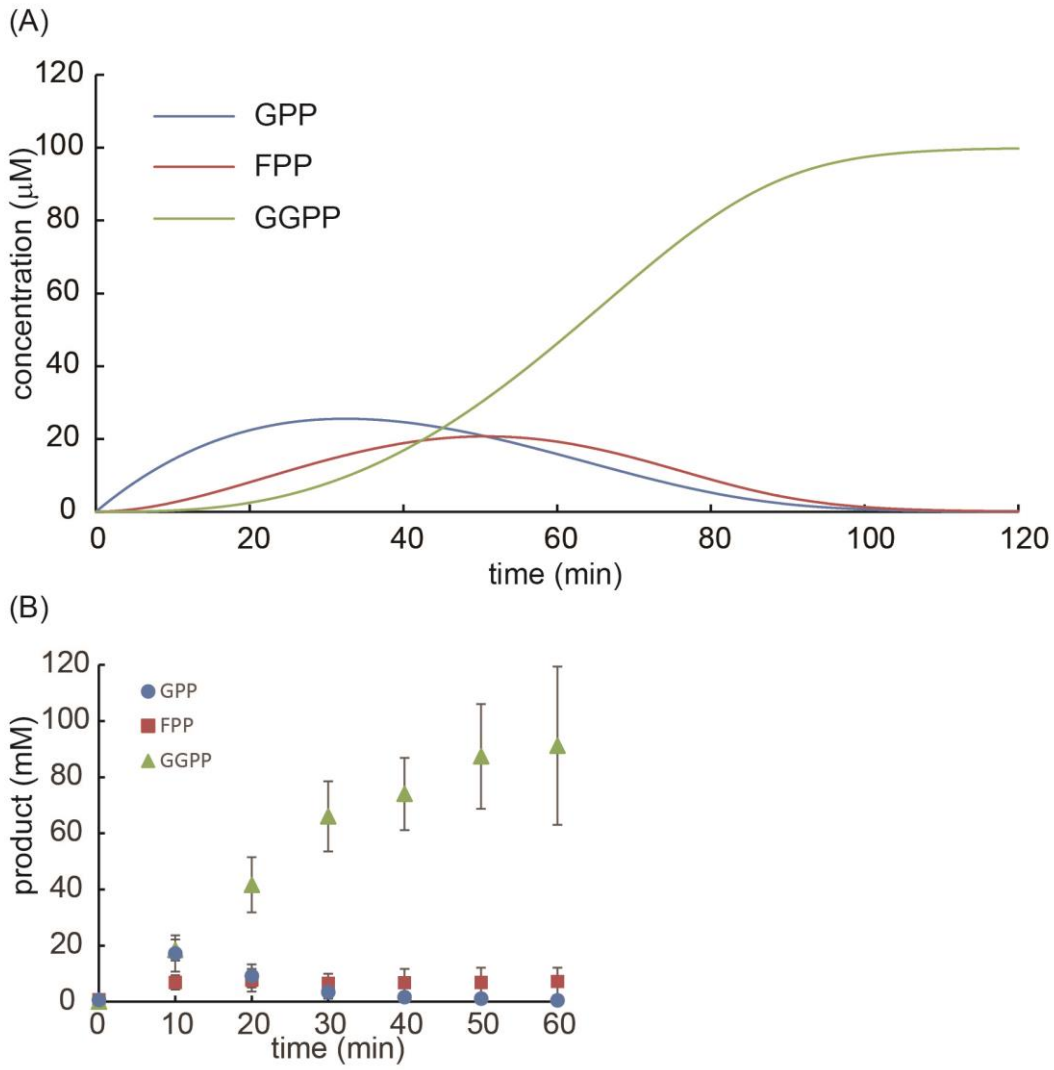


Fig. II-7

Simulated accumulation profiles of *Synechocystis* Slr0739 (panel A). Concentration of Sl0739 was 0.2 μM, which is identical to the experiment (panel B). The kinetics parameters are the same as in Fig. II-6 B-5 except the enzyme concentration. Blue, GPP; red, FPP; green, GGPP. The experimental profile is the reproduction of Fig. II-4 and shown here for comparison with the simulation.

General Discussion

In this thesis, I studied the photosynthetic production of isoprenoid using cyanobacteria as a model for the development of metabolic engineering and biomass production using photosynthetic organisms. I succeeded in continuous production and collection of the volatile limonene. The easy recovery of the product from the culture medium is a great advantage compared with the production of nonvolatile biomass compounds such as polysaccharides, fatty acids and lipids. I believe that my achievement in this thesis and its further improvement will contribute to future application of the photosynthetic biomass production.

Currently, the production level of isoprenoids obtained in this thesis is very low compared with the production of alcohols, fatty acids, and sucrose in the literature (Atsumi et al., 2009; Ducat et al., 2012; Liu et al., 2011; Oliver et al., 2013). This is also true for production of isoprene and other isoprenoids in the literature (Englund et al., 2014; Savakis and Hellingwerf, 2014). It may be due to that carbon assimilation capabilities of cyanobacteria were not fully directed to the target isoprenoids. This problem may be due to two factors as follows. First, the introduced terpene synthases could not compete with endogenous enzymes for the pathway toward the intrinsic isoprenoids, such as carotenoids and phytol. Second, assimilated carbon is mainly directed to polysaccharides and fatty acids, and relatively small amount of carbon is directed to the isoprenoids via the MEP pathway at least in *Synechocystis* cells. As for the former factor, I discussed in Chapter II that prenyl transferase (CrtE) may compete with the introduced limonene synthase for the same substrate. For the latter factor, I could demonstrate that the

overexpression of the MEP pathway or the introduction of the mevalonate pathway improved the limonene production (Chapter I). However, this was not sufficient for predominant production of limonene. For the photosynthetic isoprenoid production, it is important to solve these problems. For future perspectives, I am taking several approaches such as (1) additional introduction of the GPP supply system, (2) creation of new acetyl-CoA supplying pathway for the acetyl-CoA-consuming mevalonate pathway, (3) separation of photosynthetic production and photosynthetic growth by artificial switches.

(1) K_m (GPP) of the limonene synthases is 0.1 to 50 μM (Lücker et al., 2002; Landmann et al., 2007), and may be comparable to K_m (GPP) of CrtE (Slr0739). Given that the limonene synthase and CrtE compete for GPP, overexpression of CrtE may interfere with the limonene synthase reaction (Chapter I). To outcompete the intrinsic CrtE, it must be effective to introduce a GPP synthase, which shares DMAPP as a substrate and produces GPP as a product (Burke and Croteau, 2002a). Currently, I am trying to co-overexpress the GPP synthase gene derived from a conifer, *Abies grandis* into LMSox strain (as a collaboration with Dr. D. Umeno).

(2) There are two types of biosynthetic pathways for supply of DMAPP and IPP: MEP pathway and mevalonate pathway. MEP pathway is mainly used in prokaryotes (including cyanobacteria) and plastids of plants and utilizes pyruvate and glyceraldehyde-3-phosphate as starting metabolites

(Hunter, 2007). Mevalonate pathway is mainly used in eukaryotes and utilizes acetyl-CoA as a starting metabolite (Miziorko, 2011). Introducing the mevalonate pathway improves productivity of isoprenoids more than overexpression of the intrinsic MEP pathway in *E. coli* (Martin et al., 2003). However, the carbon yield of the mevalonate pathway is lower than the MEP pathway: the mevalonate pathway synthesizes DMAPP and IPP (C_5) from three molecules of pyruvate (C_3) via three molecules of acetyl-CoA (C_2), releasing four molecules of CO_2, while the MEP pathway synthesizes DMAPP and IPP from pyruvate (C_3) and glyceraldehyde-3-phosphate (C_3), releasing single molecule of CO_2. Namely, the carbon yield of the mevalonate pathway is 5/9, whereas that of the MEP pathway is 5/6. Fig. GD-1 shows a likely pathway from photosynthesis to the isoprenoid precursors (DMAPP and IPP) via the mevalonate pathway in the recombinant *Synechocystis* cells (Chapter I). This figure illustrates that the major loss of carbon (three molecules of CO_2) takes place at the pyruvate dehydrogenase reaction (three molecules of pyruvate -> three molecules of acetyl-CoA).

Now, I am trying to create a new acetyl-CoA supplying pathway ("reverse glyoxylate pathway" in Fig. GD-1B) for the acetyl-CoA consuming mevalonate pathway. Here, the intrinsic phosphoenolpyruvate (PEP) carboxylase fixes bicarbonate to generate oxaloacetate and oxaloacetate can be converted to two molecules of acetyl-CoA by four enzymes (malyl-CoA synthetase, malyl-CoA lyase, isocitrate lyase and citrate-ATP lyase) in addition to two intrinsic citrate cycle enzymes (malate dehydrogenase and aconitase). This pathway is similar to the known glyoxylate pathway (Fig.

GD-1C), but in an opposite direction (Eastmond and Graham, 2001). Therefore, I designate this pathway “the reverse glyoxylate pathway”. Because the reverse glyoxylate pathway is incompatible with an intrinsic citrate cycle enzyme (citrate synthase), I am going to delete genes for this enzyme, after construction of the pathway. At the moment, I borrowed those four enzymes from different organisms, because no organism has all four enzymes. In summary, the intrinsic glycolysis pathway converts one molecule of PEP, which is derived from 3-phosphoglycerate, to acetyl-CoA and CO_2 via pyruvate, whereas the reverse glyoxylate pathway I proposed provides two molecules of the acetyl-CoA from one molecule of PEP by carboxylation. I constructed a strain of *Synechocystis* that expresses four enzyme genes and confirmed the expression of at least two enzyme activities (malyl-CoA synthetase and malyl-CoA lyase) using its crude extract. I expect that the reverse glyoxylate pathway would work to provide acetyl-CoA more efficiently than the intrinsic glycolytic pathway in photosynthetic *Synechocystis* cells. If so, this pathway can be applied not only to the isoprenoid production but also to other metabolic pathways that consume acetyl-CoA such as fatty acid biosynthesis.

(3) It is important to separate the photosynthetic biomass production from the photosynthetic growth, when microalgae including cyanobacteria are applied to the biomass production, because a large amount of nitrogen is needed for the growth. Now, I am working to contribute to a joint project for the light switching of the photosynthetic metabolisms in cyanobacteria among several laboratories (Univ. Tokyo, Toyohashi Univ. Technol., RIKEN,

Chiba Univ., and Denso Co.). The goal of the project is to control the photosynthetic biomass production pathway and other pathways by light-induced transcriptional regulation of certain key enzymes. So far, I constructed strains having a GFP gene with regulatory promoter under control of light switch (CcaS/R system (Hirose et al., 2008)). I evaluated the system as differential expression of GFP nearly three-fold between on and off conditions. Then, introduced the light switch into a key metabolic enzyme, pyruvate dehydrogenase or citrate synthase. I determined various metabolites under two different conditions and found the switch-dependent changes in certain metabolites.

It is well known that the Calvin cycle for CO_2 fixation is not so efficient, because the carboxylating enzyme RubisCO is notoriously slow and promiscuous in a sense of discrimination of the proper substrate CO_2 from the improper substrate O_2 (Andersson, 2008). There have been only a few theoretical studies that aimed for efficient CO_2 fixation under aerobic conditions by selecting oxygen-resistant enzymes from alternative CO_2 fixation pathways (Bar-Even et al., 2010; Shih et al., 2014). Here, I propose alternative carbon assimilation cycle (Fig. GD-3). In this proposal, I selected PEP carboxylase (PEPC) and acetyl-CoA carboxylase (ACC), which are intrinsic carboxylating enzyme resistant against O_2 (Ducat and Silver, 2012). The PEPC, which is predominantly used for C_4 photosynthesis, utilizes bicarbonate ion, and, therefore, has high activity and high specificity even under the oxygen-rich atmosphere. The ACC that utilizes bicarbonate ion to

produce malonyl-CoA is the key enzyme for biosynthesis of fatty acids (Polyak et al., 2012). These two enzymes are already present in cyanobacteria and are assumed to be resistant against O_2. I combined these two carboxylating enzymes with a part of "the reverse glyoxylate pathway" (shown in Fig. GD-3) and seven enzymes from one of the alternative carbon fixation cycles (3-hydroxypropionate bi-cycle) (Zarzycki et al., 2009).

In detail, the alternative carbon assimilation cycle I propose here consists of three distinct cycles (Fig. GD-3). One cycle (cycle #1) starting from acetyl-CoA and glyoxylate (boxed in Fig. GD-3) fixes one molecule of bicarbonate to produce pyruvate and acetyl-CoA. In this case, the product acetyl-CoA is, then, used as the entry acetyl-CoA (connected by dotted line in Fig. GD-3) (as a net reaction, glyoxylate is combined with one bicarbonate molecule to generate pyruvate). A second cycle (cycle #2) starting from pyruvate and acetyl-CoA (double boxed in Fig. GD-3) fixes two molecules of bicarbonate and generates pyruvate, acetyl-CoA and glyoxylate via isocitrate (as a net reaction, two bicarbonate molecules are converted to glyoxylate). A third cycle (cycle #3) starting from pyruvate fixes two molecules of bicarbonate to produce pyruvate and acetyl-CoA via malate, malyl-CoA, glyoxylate plus acetyl-CoA (as a net reaction, two bicarbonate molecules are converted to acetyl-CoA). These three cycle reactions are summarized as follows:

acetyl-CoA + glyoxylate + HCO_3^- –> acetyl-CoA +pyruvate (#1)

pyruvate + acetyl-CoA + 2 HCO_3^- –> pyruvate + acetyl-CoA +glyoxylate (#2)

pyruvate + 2 HCO_3^- –> pyruvate + acetyl-CoA (#3)

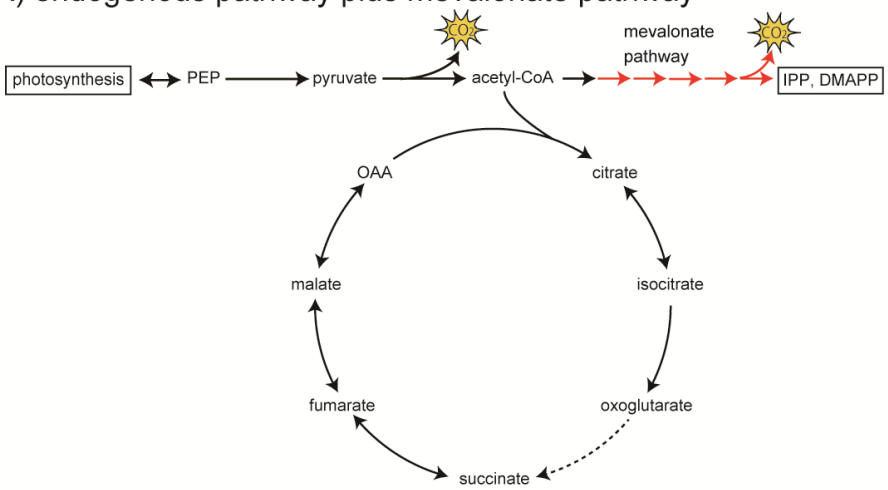
As a total of the cycles #1 plus #2,

3 HCO_3^- –> pyruvate (#1 + #2)

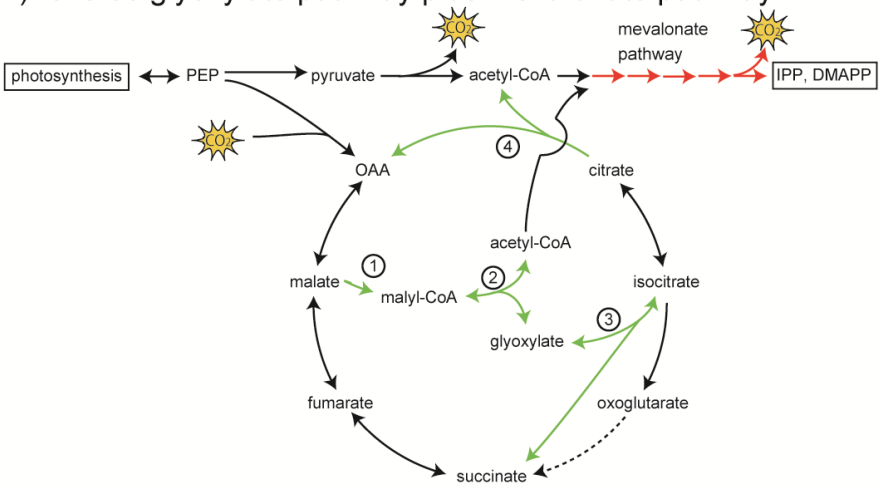
The cycle #3 provides net production of acetyl-CoA as a supplementary path for the cycle #1.

Thus, it is theoretically possible to avoid RubisCO from the whole carbon fixation reactions in cyanobacteria, enabling the construction of efficient carbon fixation system, which could be incorporated into eukaryotic algae and plants in distant future. As I mentioned earlier, I am now undertaking construction of the reverse glyoxylate pathway in *Synechocystis* (Fig. GD-1B). I already succeeded in introduction of malyl-CoA synthetase and malyl-CoA lyase in *Synechocystis*. Now I hope it is almost ready to demonstrate my proposal of the PEPC/ACC cycle in the *Synechocystis* cells, aiming for improved efficiency of photosynthetic biomass production in near future.

(A) endogenous pathway plus mevalonate pathway



(B) reverse glyoxylate pathway plus mevalonate pathway



(C) typical glyoxylate pathway of plants

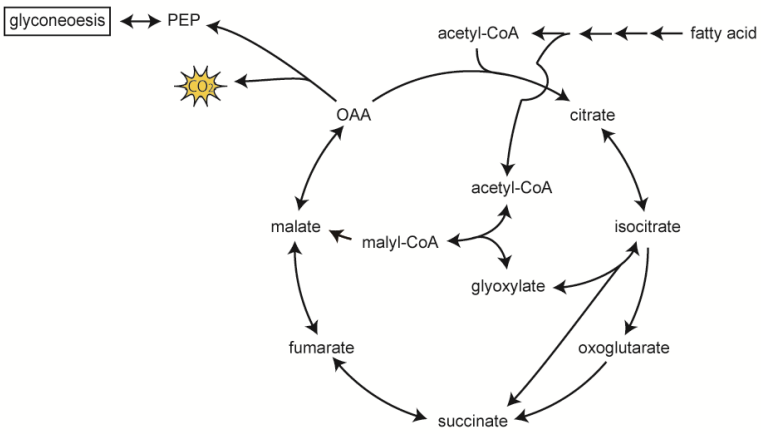


Fig. GD-1 A: An endogenous metabolic pathway (a part of glycolysis) from photosynthesis to acetyl-CoA and the introduced mevalonate pathway (red arrows) from acetyl-CoA to IPP and DMAPP. Endogenous pathway is shown with black arrows. B: The "reverse glyoxylate pathway (green arrows)" and the introduced mevalonate pathway (red arrows). The reverse glyoxylate pathway proposed in this study provides two molecules of acetyl-CoA from one molecule of PEP. ① malyl-CoA synthetase, ② malyl-CoA lyase, ③ isocitrate lyase, ④ ATP citrate lyase. Note that one molecule of a photosynthetic product, PEP, can be converted to two molecules of acetyl-CoA without releasing CO_2. C: The typical glyoxylate pathway of plants. Acetyl-CoA, which is derived from β-oxidation of fatty acids, is converted to phosphoenolpyruvate via glyoxylate pathway into gluconeogenesis. PEP, phosphoenol pyruvate; OAA, oxaloacetate.

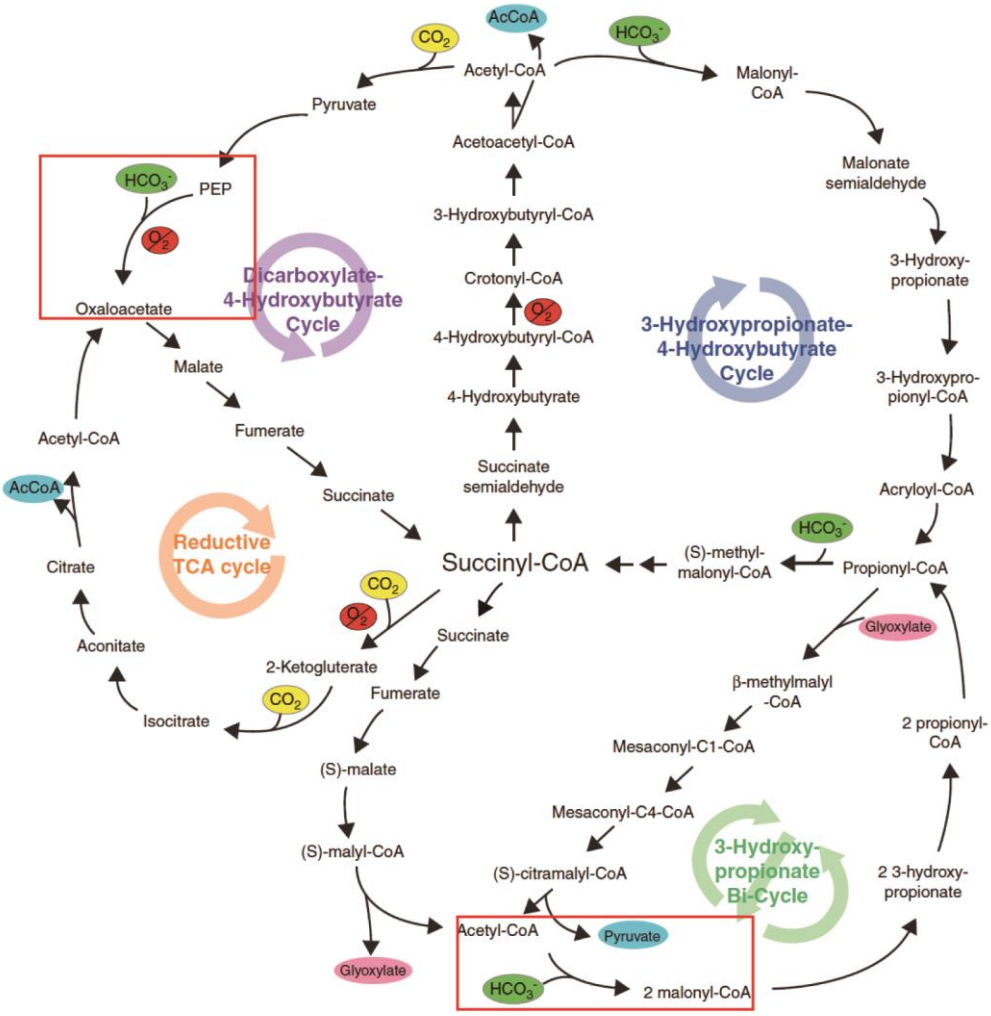


Fig. GD-2 Alternative carbon fixation pathway and reverse glyoxylate pathway. Two carbon fixating reactions are highlighted (PEPC and ACC). Modified from Fig. 2 in (Ducat and Silver, 2012).

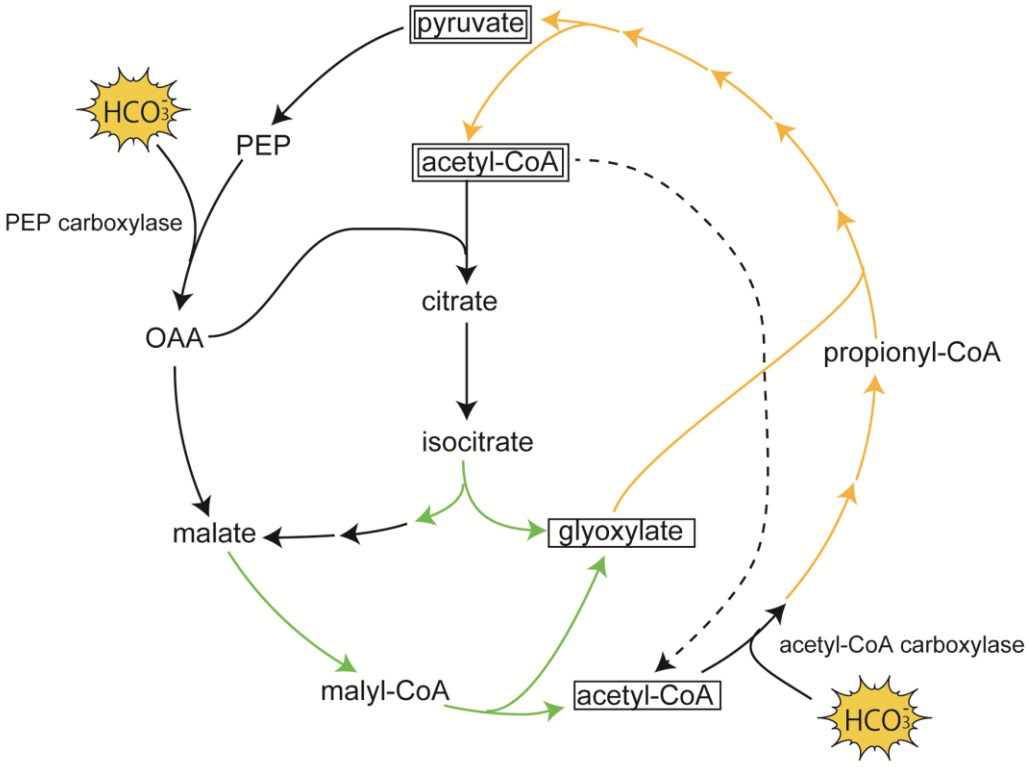


Fig. GD-3 The proposed carbon fixation cycle, "PEPC/ACC cycle". Orange arrows, enzyme steps of a part of 3-hydroxypropionate bi-cycle; green arrows, enzyme steps of a part of reverse glyoxylate pathway; black arrows, endogenous metabolic pathways in *Synechocystis*. The dotted arrow indicates entry of acetyl CoA, the product of the first cycle, into the second cycle.

ATGCGGCGCTCCGGCAATTATAAACCCAGTCGGTGGGACGTGGATTTTATGCAGAGCC
TGAACTCCGACTACCAAGAAGAACGCCATCGCACCAAAGCCTCCGAATTGATTACCCA
AGTGAAAAATTTGTTAGAAAAAGAAACCAGTGATGACCCCATTCGCCAATTGGAATTA
ATTGATGACTTACAGCGTCTGGGGTTGAGCGATCATTTTGAACACGAGTTTAAAGAAG
TGCTGAACTCCATCTATTTGGATAACAAATACTACAATATTAACATTATGAAAGAAAC
CACTTCCAGTCGGGATTTGTACTCCACCGCCTTAGCTTTTCGTCTGTTGCGGGAACAT
GGATTTCAAGTGGCCCAGGAAGTTTTTGATTGTTTTAAAAACGAAGAAGGAGAGTTTA
AAGCCAGCTTATCTGATGACCCCCGTGGCTTACTGCAACTGTATGAAGCTTCCTTTTT
GTTTAAAGAAGGTGAAAACACTTTGGAAATTGCCCGGGAATTTGCTACCAAATTGTTA
CAGGAAAAAGTGAATAGCTCTGATGAAATTGATGACAACCTGTTGTCCAGTATTCGGT
ATTCCTTAGAAATTCCCACCTACTGGTCTGTGATTCGCCCCAATGTGTCCGTTTGGAT
TGATGCCTATCGCAAACGTCCCGACATGAATCCCGTGGTTTTAGAACTGGCCATTTTG
GATGCTAACATTATGCAAGCCCAGTTGCAACAGGAATTAAAAGAAGCTCTGGGTTGGT
GGCGCAATACCTGGTTTGTGGAAAAATTACCCTTTGCCCGGGATCGCCTGGTGGAATC
CTACTTTTGGAGTACTGGGATGGTTCCCCGGCGCCAACATAAAACCGCTCGTCAGTTG
ATGGCCAAAGTGATTGCTCTGATTACTGTTATGGATGATATATATGATGTGTACGGCA
CTTTAGAAGAATTGGAATTGTTTACCGACGCCTTTCGTCGGTGGGATGTTAGCTCTAT
TGACCACTTGCCCACCTACATGCAACTGTGTTTTCTGAGTATTAATAACTTTGTGGTT
GATACTGCCTACAACATTCTGAAAGAAACCGGAGTGAACGTTACCACTTACTTGGAAA
AATCCTGGGTGGATCAGGCTGAAAACTATTTGATGGAAAGCAAATGGTTTTACTCTGG
CCATAAACCCTCCTTGGATGAATATTTGGAAAACAGTTGGATTTCCGTGAGTGGTCCC
TGCGTTTTAACCCATGAATTTTTCGGGGTGACTGATAGTCTGGCCAAAGATACCTTAG
ACAGCCTGTATGAATACCACGACATTGTTCGCTGGTCCAGTTATTTGCTGCGTCTGGC
CGATGACTTGGGAACCAGTGTGGAAGAAGTTAGCCGGGGCGATGTGCCCAAATCTATT
CAATGTTACATGAACGACAATAACGCCAGTGAAGAAGAAGCTCGTGAACACGTGAAAG
GTTTAATTCGGGTTATGTGGAAAAAGATGAATGCCGAACGCGTGAGCGAAGATTCTCC
CTTTTGCAAAGACTTTATTCGGTGTTGCGAAGATTTGGGGCGCATGGCCCAGTTTATG
TATCATTACGGCGACGGTCACGGGACTCAGCACGCTAAAATTCACCAGCAGATTACCG
ACTGTTTGTTTCAACCCTTTGCCTAAGGATCC

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

ATGAGTCTGACCGAAGAAAAACCCATTCGCCCCATTGCTAACTTTCCCCCCTCCATTT
GGGGTGACCAATTTCTGATTTACGAAAAACAGGTGGAACAGGGGGTTGAACAAATTGT
GAATGATTTGAAAAAAGAAGTGCGCCAATTGTTAAAAGAAGCCTTAGATATTCCCATG
AAACATGCTAACCTGTTGAAACTGATTGACGAAATTCAGCGGCTGGGCATTCCCTATC
ATTTTGAACGCGAAATTGACCACGCCTTGCAATGTATTTACGAAACCTACGGAGATAA
CTGGAACGGCGACCGTTCCAGTCTGTGGTTTCGTTTGATGCGGAAACAGGGTTATTAC
GTTACCTGCGATGTGTTTAACAACTACAAAGACAAAAACGGTGCCTTTAAACAAAGCT
TAGCTAACGATGTTGAAGGGTTACTGGAACTGTATGAAGCCACCTCTATGCGTGTGCC
CGGAGAAATTATTTTAGAAGATGCTCTGGGCTTTACTCGTAGCCGGTTATCTATTATG
ACTAAAGACGCCTTTTCCACCAATCCCGCTCTGTTTACTGAAATTCAGCGGGCCTTGA
AACAACCCTTATGGAAACGCCTGCCCCGTATTGAAGCCGCTCAGTACATTCCCTTTTA
CCAACAGCAAGATAGTCATAACAAAACCTTGCTGAAACTGGCCAAATTGGAATTTAAC
CTGTTGCAAAGCCTGCATAAAGAAGAATTGTCTCATGTGTGTAAATGGTGGAAAGCCT
TTGATATTAAGAAAAATGCTCCCTGTCTGCGCGACCGTATTGTTGAATGCTATTTTTG
GGGGTTGGGATCCGGCTACGAACCCCAATATTCCCGGGCCCGGGTGTTTTTCACCAAA
GCCGTGGCTGTTATTACTCTGATTGATGACACCTATGATGCCTACGGCACCTATGAAG
AATTGAAAATCTTTACTGAAGCTGTGGAACGGTGGTCCATTACTTGTTTAGATACCCT
GCCCGAATACATGAAACCCATTTACAAATTGTTTATGGACACTTACACCGAAATGGAA
GAATTTCTGGCCAAAGAAGGTCGCACCGATTTGTTTAACTGCGGGAAAGAATTTGTTA
AAGAATTTGTGCGTAACCTGATGGTGGAAGCCAAATGGGCTAACGAAGGACATATTCC
CACCACTGAAGAACACGATCCCGTGGTTATTATTACCGGCGGTGCCAATTTACTGACC
ACTACCTGTTACTTGGGTATGAGTGACATTTTTACTAAAGAATCCGTTGAATGGGCCG
TGAGTGCTCCCCCCTTGTTTCGGTATAGCGGTATTTTAGGGCGTCGGTTGAACGATTT
AATGACCCATAAAGCCGAACAGGAACGCAAACACAGCTCTTCCAGTCTGGAAAGTTAC
ATGAAAGAATACAACGTGAACGAAGAATACGCCCAAACCTTGATTTACAAAGAAGTGG
AAGATGTTTGGAAAGACATTAACCGTGAATACCTGACTACCAAAAACATTCCCCGTCC
CTTGTTAATGGCCGTTATTTACTTGTGCCAGTTTTTAGAAGTGCAATATGCTGGCAAA
GATAATTTTACCCGCATGGGTGATGAATACAAACACTTGATTAAAAGCCTGCTGGTTT
ATCCCATGTCCATTTAG

Supplementary Fig. I-S2 Transit-peptide truncated codon optimized sequence of amorpha-4, 11-diene synthase

ATGCGACGCAGTGGAAACTACCAACCTTCCATTTGGGATTTCAACTACGTTCAATCTC
TCAACACTCCCTATAAGGAAGAGAGGTATTTGACAAGGCATGCTGAGTTGATTGTGCA
AGTGAAACCGTTGTTGGAGAAAAAAATGGAGGCTACTCAACAGTTGGAGTTGATTGAT
GACTTGAACAATCTCGGATTATCTTATTTTTTTCAAGACCGTATTAAGCAGATTTTAA
GTTTTATATATGACGAGAACCAATGTTTCCACAGTAATATTAATGATCAAGCAGAGAA
AAGGGATTTGTATTTCACGGCTCTTGGATTCAGACTTCTCAGACAACATGGCTTTAAT
GTCTCTCAAGAAGTATTTGATTGTTTCAAAAACGACAAGGGTAGTGATTTTAAGGCAA
GCCTTAGTGGCAATACCAAAGGATTGTTACAACTGTACGAGGCATCTTTCCTAGTGAG
AGAAGGTGAAGACACGCTGGAGCTAGCTAGACAATTCGCCACCAAATTTCTGCGGAGA
AAACTTGATGAAATTGACGACAATCATCTATTATCACGCATTCACCATTCTTTGGAGA
TCCCACTTCACTGGAGAATTCAAAGGCTGGAGGCAAGATGGTTCTTAGATGCTTACGC
GACGAGACACGACATGAATCCAATCATTCTTGAGCTCGCCAAGCTCGATTTCAATATT
ATTCAAGCAACACACCAAGAAGAACTCAAGGATGTCTCAAGGTGGTGGCAGAATACAC
GGCTGGCTGAGAAACTCCCATTTGTGAGGGATAGGCTTGTAGAAAGCTACTTTTGGGC
CATTGCGCTGTTTGAGCCTCATCAATATGGATACCAGAGAAGAGTGGCAGCCAAGATT
ATTACTCTAGCAACATCTATAGATGATGTTTACGATATCTATGGTACCTTAGATGAAT
TGCAGTTATTTACAGACAACTTTCGAAGATGGGATACTGAATCACTAGGCGGACTTCC
ATATTCCATGCAATTATTTTATATGGTAATCCACAACTTTGTTTCCGAGCTGGCATAC
GAAATTCTCAAAGAGAAGGGTTTCATCGCTATCCCATATTTACAGAGATCGTGGGTAG
ATTTGGCGGAATCATTTTTAAAAGAAGCAAATTGGTACTACAGTGGATATACACCAAG
CCTGGAAGAATATATCGACAACGGCAGCATTTCAATTGGGGCAGTTGCAGTATTATCC
CAAGTTTATTTCACATTAGCAAACTCCATAGAGAAACCTAAGATCGAGAGCATGTACA
AATACCACCACATTCTTCGCCTCTCCGGATTGCTCGTAAGGCTTCATGATGATCTAGG
AACATCACTGTTTGAGAAGAAGAGAGGCGACGTGCCGAAAGCAGTGGAGATTTGCATG
AAGGAAAGAAATGATACCGAGGAAGAGGCTGAAGAACACGTGAAATATCTGATTCGGG
AGGCGTGGAAGGAGATGAACACAGCGACGGCGGCGGCCGGTTGTCCGTTTATGGATGA
GTTGAATGTGGCCGCAGCTAATCTCGGAAGAGCGGCGCAGTTTGTGTATCTCGACGGA
GATGGTCATGGCGTGCAACACTCTAAAATTCATCAACAAATGGGAGGCCTAATGTTCA
AGCCATATGTCTGA

Supplementary Fig. I-S3 Transit-peptide truncated geraniol synthase

CAATATTCTGAAATGAGCTGTTGACAATTAATCATCCGGCTCGTATAATGTGTGGAAT
TGTGAGCGGATAACAATTTCACACAGGAAACAGCGCCGCTGAGAAAAAGCGAAGCGGC
ACTGCTCTTTAACAATTTATCAGACAATCTGTGTGGGCACTCGACCGGAATTATCGAT
TAACTTTATTATTAAAAATTAAAGAGGTATATATTAATGTATCGATTAAATAAGGAGG
AATAAACCCATATGCACATCAGCGAACTGACCCACCCCAATGAGTTAAAGGGTTTATC
TATCCGTGAGCTGGAGGAAGTTTCCCGGCAAATCCGAGAAAAACATCTCCAAACTGTG
GCCACCAGCGGCGGTCATCTGGGGCCTGGGTTGGGAGTGGTGGAACTGACGGTGGCCC
TATATTCCACTCTTGATTTAGACAAAGACCGGGTGATTTGGGATGTGGGACACCAAGC
CTATCCCCACAAAATGTTGACGGGACGTTACCATGATTTCCATACCCTGCGGCAAAAA
GATGGGGTGGCTGGTTACCTCAAACGCTCTGAAAGTCGTTTTGACCATTTTGGTGCTG
GGCACGCTTCCACAAGTATTTCCGCTGGTTTGGGCATGGCTTTAGCTCGAGATGCCAA
GGGGGAAGATTTTAAAGTAGTTTCCATCATTGGCGATGGTGCTTTAACCGGGGGCATG
GCCTTGGAGGCAATCAACCATGCCGGGCATTTGCCCCATACCAGACTAATGGTGATTC
TCAATGACAATGAGATGTCCATTTCCCCCAACGTGGGAGCCATTTCCCGTTATTTAAA
TAAAGTCCGCCTCAGCAGTCCGATGCAGTTTTTGACGGACAATCTGGAGGAACAGATC
AAACACTTGCCCTTTGTGGGGGATTCCCTCACCCCGGAGATGGAAAGGGTCAAGGAGG
GAATGAAACGTCTTGTTGTCCCCAAAGTGGGAGCGGTGATTGAAGAACTAGGATTTAA
ATATTTTGGTCCCATTGACGGTCATAGTTTACAGGAATTAATCGATACCTTTAAACAA
GCGGAAAAAGTGCCTGGTCCGGTGTTTGTCCATGTTTCCACCACCAAGGGCAAGGGTT
ATGACTTAGCCGAAAAGGATCAAGTAGGCTACCACGCCCAAAGTCCTTTCAACCTTTC
CACCGGTAAGGCCTATCCTTCTAGTAAGCCGAAACCCCCCAGCTATTCCAAGGTTTTT
GCCCATACGTTGACTACCCTGGCCAAGGAAAATCCCAACATTGTCGGTATCACGGCTG
CTATGGCGACGGGGACAGGGCTAGATAAACTCCAGGCCAAGCTACCCAAGCAGTATGT
GGACGTGGGCATTGCGGAACAACACGCAGTTACTTTGGCGGCGGGCATGGCCTGCGAG
GGCATTCGTCCAGTGGTGGCCATCTATTCCACCTTTTTGCAACGGGGTTACGACCAGA
TTATCCATGACGTTTGTATTCAAAAATTACCCGTATTTTTCTGCCTAGACCGGGCGGG
CATTGTGGGGGCGGATGGGCCCACCCACCAAGGGATGTATGACATTGCCTATCTCCGT
TGCATTCCCAATCTGGTGCTGATGGCTCCCAAGGATGAAGCAGAGTTACAACAAATGC
TGGTTACTGGGGTTAACTACACCGGTGGGGCGATCGCCATGCGTTATCCCCGGGGTAA
TGGCATCGGGGTGCCCCTGATGGAGGAAGGTTGGGAACCGCTGGAAATTGGTAAAGCT
GAAATTCTCCGGTCGGGGGATGATGTGCTTCTGTTGGGCTACGGCTCCATGGTCTATC
CCGCTCTGCAAACAGCGGAATTACTCCACGAACACGGCATCGAAGCAACGGTGGTTAA
TGCCCGCTTTGTTAAACCACTAGATACGGAATTAATTTTGCCCTTGGCCGAGCGCATC
GGCAAAGTGGTGACCATGGAAGAAGGTTGCCTCATGGGGGGCTTTGGCTCAGCAGTGG
CGGAGGCCCTGATGGATAACAACGTGTTGGTGCCCTTGAAACGTTTAGGGGTGCCCGA
CATACTTGTGGACCATGCCACTCCGGAACAATCCACCGTTGACCTTGGTTTAACCCCA
GCCCAGATGGCGCAGAATATTATGGCTTCACTGTTCAAAACTGAAACTGAATCAGTTG
TCGCTCCTGGAGTTAGTTAGTCGAACTGCCGCTCCCGGCCATTGCCCAACCCCGTTAT
TTAGTATCCAGTGACAGTATGGTTGCCCAACAAACACGAACCGACTTTGATTTAGCCC
AATACTTACAAGTTAAAAAAGGTGTGGTCGAGGCAGCCCTGGATAGTTCCCTGGCGAT
CGCCCGGCCGGAAAAGATTTACGAAGCCATGCGTTATTCTCTGTTGGCGGGGGGCAAA
CGATTGCGACCGATTTTATGCATTACGGCCTGCGAACTGTGTGGCGGTGATGAAGCCC
TGGCCTTGCCCACGGCCTGTGCCCTGGAAATGATCCACACCATGTCCCTCATCCATGA
TGATTTGCCCTCCATGGATAATGACGATTTCCGCCGGGGTAAACCCACTAACCACAAA
GTGTACGGGGAAGACATTGCCATTTTGGCCGGGGATGGACTGCTAGCCTATGCGTTTG
AGTATGTAGTTACCCACACCCCCCAGGCTGATCCCCAAGCTTTACTCCAAGTTATTGC
CCGTTTGGGTCGCACGGTGGGGGCCGCCGGTTTAGTGGGGGGACAAGTTCTAGACCTG
GAATCGGAGGGGCGCACTGACATCACCCCGGAAACCCTAACTTTTATCCATACCCATA
AAACCGGGGCATTGCTGGAAGCTTCCGTGCTCACAGGCGCAATTTTGGCCGGGGCCAC
TGGGGAACAACAACAGAGACTGGCCCGCTATGCCCAGAATATTGGCTTAGCTTTTCAA
GTGGTGGATGACATCCTCGACATCACCGCCACCCAGGAAGAGTTGGGTAAAACCGCTG
GTAAAGATGTCAAAGCCCAAAAAGCCACCTATCCCAGTCTCCTCGGTTTGGAAGCTTC
CCGGGCCCAGGCCCAAAGTTTGATTGACCAGGCCATTGTCGCCCTGGAACCCTTTGGC
CCCTCCGCCGAGCCCCTCCAGGCGATCGCCGAATATATTGTTGCCAGAAAATATTGAT

CGCTCCAGTTGATGGCAAAATTGCTTGCCGGTATTTGTAATGTAATTCACTGATGGAT
AGCACCCCCCACCGTAAGTCCGATCATATCCGCATTGTCCTAGAAGAAGATGTGGTGG
GCAAAGGCATTTCCACCGGCTTTGAAAGATTGATGCTGGAACACTGCGCTCTTCCTGC
GGTGGATCTGGATGCAGTGGATTTGGGACTGACCCTCTGGGGTAAATCCTTGACTTAC
CCTTGGTTGATCAGCAGTATGACCGGCGGCACGCCAGAGGCCAAGCAAATTAATCTAT
TTTTAGCCGAGGTGGCCCAGGCTTTGGGCATCGCCATGGGTTTGGGTTCCCAACGGGC
CGCCATTGAAAATCCTGATTTAGCCTTCACCTATCAAGTCCGCTCCGTCGCCCCAGAT
ATTTTACTTTTTGCCAACCTGGGATTAGTGCAATTAAATTACGGTTACGGTTTGGAGC
AAGCCCAGCGGGCGGTGGATATGATTGAAGCCGATGCGCTGATTTTGCATCTCAATCC
CCTCCAGGAAGCGGTGCAACCCGATGGCGATCGCCTGTGGTCGGGACTCTGGTCTAAG
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GGCTGGAGCTGGGGGCACCAGTTGGAGTGAAGTGGAAGCCCATCGACAAACCGATCGC
CAAGCGAAGGAAGTGGCCCATAACTTTGCCGATTGGGGATTACCCACAGCCTGGAGTT
TGCAACAGGTAGTGCAAAATACTGAGCAGATCCTGGTTTTCGCCAGCGGCGGCATTCG
TTCCGGCATTGACGGGGCCAAGGCGATCGCCCTGGGGGCCACCCTGGTGGGTAGTGCG
GCACCGGTATTAGCAGAAGCGAAAATCAACGCCCAAAGGGTTTATGACCATTACCAGG
CACGGCTAAGGGAACTGCAAATCGCCGCCTTTTGTTGTGATGCCGCCAATCTGACCCA
ACTGGCCCAAGTCCCCCTTTGGGACAGACAATCGGGACAAAGGTTAACTAAACCTTAA

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*.

CAATATTCTGAAATGAGCTGTTGACAATTAATCATCCGGCTCGTATAATGTGTGGAAT
TGTGAGCGGATAACAATTTCACACAGGAAACAGCGCCGCTGAGAAAAAGCGAAGCGGC
ACTGCTCTTTAACAATTTATCAGACAATCTGTGTGGGCACTCGACCGGAATTATCGAT
TAACTTTATTATTAAAAATTAAAGAGGTATATATTAATGTATCGATTAAATAAGGAGG
AATAAACCCATATGAAAAATTGTGTCATCGTCAGTGCGGTACGTACTGCTATCGGTAG
TTTTAACGGTTCACTCGCTTCCACCAGCGCCATCGACCTGGGGGCGACAGTAATTAAA
GCCGCCATTGAACGTGCAAAAATCGATTCACAACACGTTGATGAAGTGATTATGGGTA
ACGTGTTACAAGCCGGGCTGGGGCAAAATCCGGCGCGTCAGGCACTGTTAAAAAGCGG
GCTGGCAGAAACGGTGTGCGGATTCACGGTCAATAAAGTATGTGGTTCGGGTCTTAAA
AGTGTGGCGCTTGCCGCCCAGGCCATTCAGGCAGGTCAGGCGCAGAGCATTGTGGCGG
GGGGTATGGAAAATATGAGTTTAGCCCCCTACTTACTCGATGCAAAAGCACGCTCTGG
TTATCGTCTTGGAGACGGACAGGTTTATGACGTAATCCTGCGCGATGGCCTGATGTGC
GCCACCCATGGTTATCATATGGGGATTACCGCCGAAAACGTGGCTAAAGAGTACGGAA
TTACCCGTGAAATGCAGGATGAACTGGCGCTACATTCACAGCGTAAAGCGGCAGCCGC
AATTGAGTCCGGTGCTTTTACAGCCGAAATCGTCCCGGTAAATGTTGTCACTCGAAAG
AAAACCTTCGTCTTCAGTCAAGACGAATTCCCGAAAGCGAATTCAACGGCTGAAGCGT
TAGGTGCATTGCGCCCGGCCTTCGATAAAGCAGGAACAGTCACCGCTGGGAACGCGTC
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CATTGATGGGTATGGGGCCAGTACCTGCCACGCAAAAAGCGTTACAACTGGCGGGGCT
GCAACTGGCGGATATTGATCTCATTGAGGCTAATGAAGCATTTGCTGCACAGTTCCTT
GCCGTTGGGAAAAACCTGGGCTTTGATTCTGAGAAAGTGAATGTCAACGGCGGGGCCA
TCGCGCTCGGGCATCCTATCGGTGCCAGTGGTGCTCGTATTCTGGTCACACTATTACA
TGCCATGCAGGCACGCGATAAAACGCTGGGGCTGGCAACACTGTGCATTGGCGGCGGT
CAGGGAATTGCGATGGTGATTGAACGGTTGAATTAAGGAGGACAGCTAAATGAAACTC
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GTCCCTAACTGTTTTGTCTAAGTTGATCAAGAGTTACAACATCGACACCAACAAAATT
GGTAGATTAGAAGTCGGTACTGAAACTCTGATTGACAAGTCCAAGTCTGTCAAGTCTG
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CTGTTACGGTGGTACCAACGCGTTGTTCAACTCTTTGAACTGGATTGAATCTAACGCA
TGGGATGGTAGAGACGCCATTGTAGTTTGCGGTGATATTGCCATCTACGATAAGGGTG
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AAGCCATTCCACAAAGAGAGAGTTGCCCAATCTTTGATTGTTCCAACAAACACAGGTA
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TCTCTATATTCTTGCAAAATTGTTGGTGACGTCCAACATATTATCAAGGAATTAGATA
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CGAATTGAGAGAAAATGCCCATTTGAAGAAGAACTTCAAACCTCAAGGTTCCATTGAG
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GCTAAGAATTTGGTTGGATCTGCAATGGCTGGGTCTGTTGGTGGATTTAACGCACATG
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CAACGCACGTCAATTAGCAAGAATAGTTGCCTGTGCCGTCTTGGCAGGTGAATTATCC
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TGGCCTACTTGGGGGGGTTAATAGGATCTAATGACTTGGAAAAGCTGTCAGAAAACGA
TAAGCATATAGTGAATCAATGGGCCTTCATAGGTGAAAAGTGTATTCACGGTACCCCT
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CACATAATGGAACAATAAACACAAACAATTTTAAGTTCTTAGATGATTTCCCAGCCAT
TCCAATGATCCTAACCTATACTAGAATTCCAAGGTCTACAAAAGATCTTGTTGCTCGC
GTTCGTGTGTTGGTCACCGAGAAATTTCCTGAAGTTATGAAGCCAATTCTAGATGCCA
TGGGTGAATGTGCCCTACAAGGCTTAGAGATCATGACTAAGTTAAGTAAATGTAAAGG
CACCGATGACGAGGCTGTAGAAACTAATAATGAACTGTATGAACAACTATTGGAATTG
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TGGCGGCGGTTGCTCTTTGACTTTGTTACGAAGAGACATTACTCAAGAGCAAATTGAC
AGCTTCAAAAAGAAATTGCAAGATGATTTTAGTTACGAGACATTTGAAACAGACTTGG
GTGGGACTGGCTGCTGTTTGTTAAGCGCAAAAAATTTGAATAAAGATCTTAAAATCAA
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AGAGTTGAGAGCCTTCAGTGCCCCAGGGAAAGCGTTACTAGCTGGTGGATATTTAGTT
TTAGATACAAAATATGAAGCATTTGTAGTCGGATTATCGGCAAGAATGCATGCTGTAG
CCCATCCTTACGGTTCATTGCAAGGGTCTGATAAGTTTGAAGTGCGTGTGAAAAGTAA

ACAATTTAAAGATGGGGAGTGGCTGTACCATATAAGTCCTAAAAGTGGCTTCATTCCT
GTTTCGATAGGCGGATCTAAGAACCCTTTCATTGAAAAAGTTATCGCTAACGTATTTA
GCTACTTTAAACCTAACATGGACGACTACTGCAATAGAAACTTGTTCGTTATTGATAT
TTTCTCTGATGATGCCTACCATTCTCAGGAGGATAGCGTTACCGAACATCGTGGCAAC
AGAAGATTGAGTTTTCATTCGCACAGAATTGAAGAAGTTCCCAAAACAGGGCTGGGCT
CCTCGGCAGGTTTAGTCACAGTTTTAACTACAGCTTTGGCCTCCTTTTTTGTATCGGA
CCTGGAAAATAATGTAGACAAATATAGAGAAGTTATTCATAATTTAGCACAAGTTGCT
CATTGTCAAGCTCAGGGTAAAATTGGAAGCGGGTTTGATGTAGCGGCGGCAGCATATG
GATCTATCAGATATAGAAGATTCCCACCCGCATTAATCTCTAATTTGCCAGATATTGG
AAGTGCTACTTACGGCAGTAAACTGGCGCATTTGGTTGATGAAGAAGACTGGAATATT
ACGATTAAAAGTAACCATTTACCTTCGGGATTAACTTTATGGATGGGCGATATTAAGA
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GCCAGAAAGCTTGAAAATATATACAGAACTCGATCATGCAAATTCTAGATTTATGGAT
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TTGAGTCTCTTGAGAGGAATGACTGTACCTGTCAAAAGTATCCTGAAATCACAGAAGT
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GCCGATATCGAACCTCCCGTACAAACTAGCTTATTGGATGATTGCCAGACCTTAAAAG
GAGTTCTTACTTGCTTAATACCTGGTGCTGGTGGTTATGACGCCATTGCAGTGATTAC
TAAGCAAGATGTTGATCTTAGGGCTCAAACCGCTAATGACAAAAGATTTTCTAAGGTT
CAATGGCTGGATGTAACTCAGGCTGACTGGGGTGTTAGGAAAGAAAAAGATCCGGAAA
CTTATCTTGATAAATAGGAGGTAATACTCATGACCGTTTACACAGCATCCGTTACCGC
ACCCGTCAACATCGCAACCCTTAAGTATTGGGGGAAAAGGGACACGAAGTTGAATCTG
CCCACCAATTCGTCCATATCAGTGACTTTATCGCAAGATGACCTCAGAACGTTGACCT
CTGCGGCTACTGCACCTGAGTTTGAACGCGACACTTTGTGGTTAAATGGAGAACCACA
CAGCATCGACAATGAAAGAACTCAAAATTGTCTGCGCGACCTACGCCAATTAAGAAAG
GAAATGGAATCGAAGGACGCCTCATTGCCCACATTATCTCAATGGAAACTCCACATTG
TCTCCGAAAATAACTTTCCTACAGCAGCTGGTTTAGCTTCCTCCGCTGCTGGCTTTGC
TGCATTGGTCTCTGCAATTGCTAAGTTATACCAATTACCACAGTCAACTTCAGAAATA
TCTAGAATAGCAAGAAAGGGGTCTGGTTCAGCTTGTAGATCGTTGTTTGGCGGATACG
TGGCCTGGGAAATGGGAAAAGCTGAAGATGGTCATGATTCCATGGCAGTACAAATCGC
AGACAGCTCTGACTGGCCTCAGATGAAAGCTTGTGTCCTAGTTGTCAGCGATATTAAA
AAGGATGTGAGTTCCACTCAGGGTATGCAATTGACCGTGGCAACCTCCGAACTATTTA
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TGAAAAAGATTTCGCCACCTTTGCAAAGGAAACAATGATGGATTCCAACTCTTTCCAT
GCCACATGTTTGGACTCTTTCCCTCCAATATTCTACATGAATGACACTTCCAAGCGTA
TCATCAGTTGGTGCCACACCATTAATCAGTTTTACGGAGAAACAATCGTTGCATACAC
GTTTGATGCAGGTCCAAATGCTGTGTTGTACTACTTAGCTGAAAATGAGTCGAAACTC
TTTGCATTTATCTATAAATTGTTTGGCTCTGTTCCTGGATGGGACAAGAAATTTACTA
CTGAGCAGCTTGAGGCTTTCAACCATCAATTTGAATCATCTAACTTTACTGCACGTGA
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GGCCCACAAGAAACAAACGAATCTTTGATTGACGCAAAGACTGGTCTACCAAAGGAAT
AACTGCAGCCCGGGAGGAGGATTACTATATGCAAACGGAACACGTCATTTTATTGAAT
GCACAGGGAGTTCCCACGGGTACGCTGGAAAAGTATGCCGCACACACGGCAGACACCC
GCTTACATCTCGCGTTCTCCAGTTGGCTGTTTAATGCCAAAGGACAATTATTAGTTAC
CCGCCGCGCACTGAGCAAAAAAGCATGGCCTGGCGTGTGGACTAACTCGGTTTGTGGG
CACCCACAACTGGGAGAAAGCAACGAAGACGCAGTGATCCGCCGTTGCCGTTATGAGC
TTGGCGTGGAAATTACGCCTCCTGAATCTATCTATCCTGACTTTCGCTACCGCGCCAC
CGATCCGAGTGGCATTGTGGAAAATGAAGTGTGTCCGGTATTTGCCGCACGCACCACT
AGTGCGTTACAGATCAATGATGATGAAGTGATGGATTATCAATGGTGTGATTTAGCAG
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GGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGAGGAGGAATGAGTAATGGACT
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TTAGGTGGTAAGCGCCTGCGACCTTTCCTGGTTTATGCCACCGGTCATATGTTCGGCG
TTAGCACAAACACGCTGGACGCACCCGCTGCCGCCGTTGAGTGTATCCACGCTTACTC
ATTAATTCATGATGATTTACCGGCAATGGATGATGACGATCTGCGTCGCGGTTTGCCA
ACCTGCCATGTGAAGTTTGGCGAAGCAAACGCGATTCTCGCTGGCGACGCTTTACAAA
CGCTGGCGTTCTCGATTTTAAGCGATGCCGATATGCCGGAAGTGTCGGACCGCGACAG
AATTTCGATGATTTCTGAACTGGCGAGCGCCAGTGGTATTGCCGGAATGTGCGGTGGT
CAGGCATTAGATTTAGACGCGGAAGGCAAACACGTACCTCTGGACGCGCTTGAGCGTA
TTCATCGTCATAAAACCGGCGCATTGATTCGCGCCGCCGTTCGCCTTGGTGCATTAAG
CGCCGGAGATAAAGGACGTCGTGCTCTGCCGGTACTCGACAAGTATGCAGAGAGCATC
GGCCTTGCCTTCCAGGTTCAGGATGACATCCTGGATGTGGTGGGAGATACTGCAACGT
TGGGAAAACGCCAGGGTGCCGACCAGCAACTTGGTAAAAGTACCTACCCTGCACTTCT
GGGTCTTGAGCAAGCCCGGAAGAAAGCCCGGGATCTGATCGACGATGCCCGTCAGTCG
CTGAAACAACTGGCTGAACAGTCACTCGATACCTCGGCACTGGAAGCGCTAGCGGACT
ACATCATCCAGCGTAATAAATAA

Fig. I-S5. Combined sequence of trc promoter and genes for mevalonate pathway. Purple, trc promoter; green, pBAD33MevT derived sequence containing three genes for mevalonate pathway (acetoacetyl-CoA synthase, hydroxymethylglutaryl-CoA synthase, and hydroxymethylglutaryl-CoA reductase); orange, 5'-UTR region of pMBIS containing lac promoter; red, pMBIS derived sequence containing five genes for mevalonate pathway (mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentenyl pyrophosphate isomerase, and farnesyl pyrophosphate synthase).

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