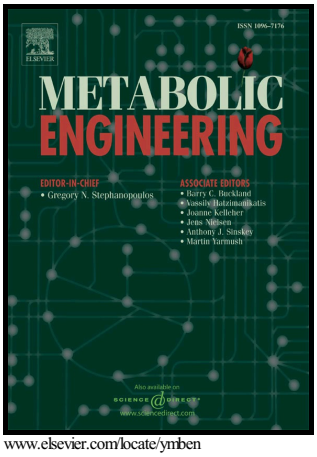

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Precise precursor rebalancing for isoprenoids production by fine control of *gapA* expression in *Escherichia coli*

Juyoung Jung[a,1], Jae Hyung Lim[b,1], Se Yeon Kim[c], Dae-Kyun Im[d], Joo Yeon Seok[c], Seung-Jae V. Lee[a,c], Min-Kyu Oh[d], Gyoo Yeol Jung[b,c*]

[a]Department of Life Sciences, Pohang University of Science and Technology, 77 Cheongam-ro, Nam-gu, Pohang, Gyeongbuk 37673, Korea

[b]Department of Chemical Engineering, Pohang University of Science and Technology, 77 Cheongam-ro, Nam-gu, Pohang, Gyeongbuk 37673, Korea

[c]School of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology, 77 Cheongam-ro, Nam-gu, Pohang, Gyeongbuk 37673, Korea

[d]Department of Chemical and Biological Engineering, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul 02841, Korea

[*]To whom correspondence should be addressed: Department of Chemical Engineering, Pohang University of Science and Technology, 77 Cheongam-ro, Nam-gu, Pohang, Gyeongbuk 37673, Korea. Tel.: +82 54 279 2391; fax: +82 54 279 5528. gyjung@postech.ac.kr

ABSTRACT

Biosynthesis of isoprenoids via the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway requires equimolar glyceraldehyde 3-phosphate and pyruvate to divert carbon flux toward the products of interest. Here, we demonstrate that precursor balancing is one of the critical steps for the production of isoprenoids in *Escherichia coli*. First, the implementation of the

[1] These authors contributed equally to this work.

synthetic lycopene production pathway as a model system and the amplification of the native DXP pathway were accomplished using synthetic constitutive promoters and redesigned 5′-untranslated regions (5′-UTRs). Next, fine-controlled precursor balancing was investigated by tuning phosphoenolpyruvate synthase (PpsA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The results showed that tuning-down of *gapA* improved the specific lycopene content by 45% compared to the overexpression of *ppsA*. The specific lycopene content in the strains with down-regulated *gapA* increased by 97% compared to that in the parental strain. Our results indicate that *gapA* is the best target for precursor balancing to increase biosynthesis of isoprenoids.

Keywords: Precursor balancing; Isoprenoid; Lycopene; *gapA*; *ppsA*; G3P

1. INTRODUCTION

The ultimate goal of metabolic engineering and synthetic biology is the redesign of cellular metabolism to construct efficient biological processes in terms of yield and productivity for the production of value-added chemicals from renewable biomass. Isoprenoids form one of the most diverse groups of natural products (Ajikumar et al., 2008), and over 70,000 different isoprenoids have been identified till date (Vickers et al., 2015). These are considered as the compounds with high commercial potential due to their broad applicability as fuels (Peralta-Yahya et al., 2012), fine chemicals (Herrero et al., 2008; Lindberg et al., 2010), and nutraceuticals (Alper et al., 2005c; Ye et al., 2000), as well as their applicability as pharmaceuticals important for human health (Ajikumar et al., 2010; Martin et al., 2003; Ridley, 2002). From an engineering standpoint, these diverse compounds are easily obtainable through cellular metabolism by modulating the downstream biosynthetic pathways, as the diversity of isoprenoids is derived from two universal precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). These building blocks are synthesized via the mevalonate (MVA) pathway in archaea and eukaryotes or the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway in eubacteria (Kirby and Keasling, 2009).

During the past decade, the study of isoprenoid synthesis has been focused on carotenoids, which are structurally composed of eight isoprene units, due to their diverse applications that range from pharmaceuticals to nutraceuticals and the property of imparting color. Lycopene, a carotenoid, is currently used as a colorant and nutraceutical antioxidant. Moreover, beneficial effects of lycopene in several cancers and chronic diseases (Agarwal and Rao, 2000; Khan et al., 2008) have been proposed, creating great potential for their use in relation to human health. Out of its many properties, the property to impart color enabled colorimetric screening for high lycopene producers, and combinatorial approaches for

metabolic engineering could be devised (Ajikumar et al., 2008). For example, *in silico* stoichiometric modeling and random transposon mutagenesis were employed to identify systematic and combinatorial targets, and these approaches succeeded in increasing the production of target carotenoids (Alper et al., 2005b; Alper et al., 2005c; Kang et al., 2005; Stephanopoulos et al., 2004). More recently, the new genome-editing tool, multiplex automated genome engineering (MAGE), facilitated isoprenoid overproduction via simultaneous modification of identified targets (Wang et al., 2009). Although many studies demonstrated successful increase of carotenoids via genetic modifications, precise refinement by using a rational approach is required for further improvement.

One of the important issues for metabolic engineering is balancing precursor pools; this can be solved by a rational approach that beneficially minimizes the efforts to search for the optimum balance among the various expression levels at the critical nodes. The biosynthesis of isoprenoids via the DXP pathway, for example, the equimolar glyceraldehyde 3-phosphate (G3P) and pyruvate (PYR) requires for basic units of isoprenoids; IPP and DMAPP. Previously, precursor balancing between G3P and pyruvate has been examined by Farmer and Liao (2001) to enhance the production of lycopene in *Escherichia coli*. In their work, redirecting flux from pyruvate back to G3P through overexpression of gluconeogenic enzymes, such as PEP synthase (encoded by *ppsA*) and PEP carboxykinase (encoded by *pck*), or deletion of major PEP consuming enzymes; pyruvate kinase-I and –II (encoded by *pykF* and *pykA*), increased the lycopene production. Conversely, dissipating the G3P pool into TCA cycle by overexpression of PEP carboxylase (encoded by *ppc*) had the opposite effect due to the shortage of available G3P than pyruvate in *E. coli* (Chassagnole et al., 2002; Farmer and Liao, 2001). This clearly showed that increase in the G3P pool is necessary to divert carbon flux toward isoprenoid pathways. However, when flux between G3P and pyruvate was

controlled only by enhancing the gluconeogenic activity through up-modulation of *ppsA* or *pck*, futile cycles causing the inefficient flux control could be occurred. On the other hand, expression control of *gapA* gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) can be more effective target for engineering the flux between G3P and pyruvate to achieve the optimal rebalancing the precursors of isoprenoids. In addition, enhanced flux toward the isoprenoid pathway should reduce the flux to TCA cycle and consequence the decreased growth rate. For this reason, fine control of flux toward isoprenoid synthesis pathway is essential to optimize the isoprenoid production and this can be accomplished by controlling the G3P and pyruvate pools as well.

In this study, we explored a novel and efficient engineering target, *gapA*, for precursor balancing and demonstrated that the importance of precise precursor balancing to divert carbon flux toward isoprenoids via the DXP pathway in *E. coli*. As a model system, we initially constructed a lycopene-overproducing *E. coli* strain by sequential amplification of the DXP pathway using synthetic expression cassettes, such as a constitutive strong promoter and redesigned 5′-untranslated regions (5′-UTRs), to allow targeted gene expression to be maximized at both transcription and translation levels. Furthermore, we investigated the effect of precise precursor balancing with PpsA and GAPDH through fine-tunable control at both transcription and translation levels. Taken together, the results showed that the tuning-down of *gapA* is a more efficient approach for precursor balancing than the overexpression of *ppsA* to optimize biosynthesis of isoprenoids. Our strategy could be broadly applied to increase production of numerous high-value isoprenoids in the field of metabolic engineering and synthetic biology.

2. MATERIALS AND METHODS

2.1 Bacterial strains, reagents, and plasmids

A list of the *E. coli* strains and plasmids used in this study has been presented in Table 1. Oligonucleotides, synthesized by Macrogen (Daejeon, Korea), are listed in Supplementary Table 1. The *rpsL-neo* template DNA was obtained from the counter selection BAC modification kit (Gene Bridges, Heidelberg, Germany). Restriction endonucleases and Phusion DNA polymerase were purchased from New England Biolabs (Ipswich, MA, USA), and T4 DNA ligase was supplied by Takara Bio Inc. (Shiga, Japan). The DNA fragments and plasmids were prepared using the GeneAll® Expin™ Gel SV kit (GeneAll Biotechnology, Seoul, Korea) and AccuPrep Nano-Plus Plasmid Mini Extraction kit (Bioneer, Daejeon, Korea), respectively. All cell culture reagents were purchased from BD Biosciences (Sparks, MD, USA), and 5× M9 salts and authentic lycopene were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The native DXP and heterologous lycopene synthesis pathways were amplified with a synthetic expression cassette to control gene expression at both transcriptional and translational levels. Specifically, a strong synthetic constitutive promoter (P_{BBa_J23100}) and a synthetic terminator (T_{BBa_B1005}) from the Registry of Standard Biological Parts (http://partsregistry.org) were commonly used, unless otherwise stated. Additionally, 5′-UTRs were redesigned for each gene by using UTR Designer (Seo et al., 2013b) (http://sbi.postech.ac.kr/utr_designer) to maximize gene expression and simultaneously avoid an unpredictable regulatory mechanism (Seo et al., 2013a). The DNA templates containing the synthetic expression cassette and unique restriction sites for plasmid construction were synthesized by Bioneer (Daejeon, Korea) after codon optimization of structural genes to

allow for maximization of translational efficiency at both initiation (Seo et al., 2013b) and elongation steps (Rocha, 2004) (Supplementary Table 2). First, the heterologous polycistronic gene *crtEBI* (from *Pantoea agglomerans*) was inserted into a plasmid pCDFDuet-1 by digestion with HindIII and NcoI, creating pCDF_*crtEBI*. Subsequently, pCDF_*ispA_crtEBI* was constructed by ligating the *ispA* fragment digested with BglII and HindIII into the matching sites of pCDF_*crtEBI*. Finally, *idi* fragment was inserted at the BglII site, creating pCDF_*idi_ispA_crtEBI*. The *dxs* gene was amplified from *E. coli* W3110 genomic DNA using dxs_F and dxs_R and digested with NcoI and BamHI. The pACYC_*dxs* was created by incorporating the digested fragment into the corresponding sites of the pACYCDuet-1 vector as presented in Table 1.

2.2 Modulation of *ppsA* and *gapA* expression levels

Replacement of nascent chromosomal genetic elements of *ppsA* and *gapA* with the synthetic promoter and synthetic 5′-UTR was conducted by the Red recombination system with pKD46 according to previous studies (Datsenko and Wanner, 2000; Lim et al., 2013b). To construct a synthetic expression cassette for the overexpression of *ppsA*, the PCR products were amplified with ppsA_F and ppsA_R by using FRT-Km^R-FRT-P_{BBa_J23100}::$synUTR_{ppsA(Vn)}$ as templates (Kim et al., 2015) and subsequently integrated upstream of the *ppsA* coding sequence, resulting in the *ppsA* UTR variants: V1 (0.11, relative activity), V3 (0.69), V4 (0. 82), and V5 (1). The downregulation of g*apA* was introduced through a scar-less recombination method by using the *rpsL-neo* counter-selection system, as described in a previous study (Heermann et al., 2008). Briefly, a point mutation within the *rpsL* gene was generated first using rpsL_A128G oligo to confer a streptomycin-resistant phenotype (Table S1). The resulting strain JYJ4 was then subjected to the insertion of a *rpsL-neo* cassette

containing P_{BBa_J23105} and synthetic 5′-UTR, amplified by sequential PCR with 1st_gapA_F/R and 2nd_gapA_F/R. Finally, oligo recombination using gapA_V (1 to 5) that have different synthetic promoters resulted in promoter strength variations: P_{BBa_J23101} (0.70, compared to P_{BBa_J23100}), P_{BBa_J23108} (0.51), P_{BBa_J23107} (0.36), P_{BBa_J23105} (0.24), and P_{BBa_J23115} (0.15). Final constructions of *ppsA* and *gapA* variations were identified by ppsA_check and gapA_check primers, respectively.

2.3 Media and culture conditions

The production of lycopene was analyzed in 2× M9 medium (13.56 g Na_2HPO_4, 6 g KH_2PO_4, 2 g NH_4Cl, 1 g NaCl, 2 ml of 1 M $MgSO_4$, 0.1 ml of 1 M $CaCl_2$ per liter) supplemented with 4 g/L of glucose. Multiple plasmids were maintained by including 40 μg/mL of spectinomycin and 27.2 μg/mL of chloramphenicol; the single cloDF13 origin-derived plasmid was maintained by including 50 μg/mL of spectinomycin. Overnight culture broth from a fresh colony in Luria-Bertani (LB) medium was subsequently cultured in 3 ml of 2× M9 medium until the optical density (OD_{600}) reached 0.8–1.0. This seed culture was inoculated into 20 ml of fresh 2× M9 medium in a 300 ml flask at an initial OD_{600} of 0.05 and incubated aerobically at 37°C for 24 hours in a rotary shaker (250 rpm), unless otherwise stated.

2.4 GAPDH activity assay

GAPDH activity was measured using a colorimetric GAPDH assay kit according to the manufacturer's instructions (ScienCell Research Laboratories, CA, USA) with slight modifications. Briefly, 1 ml of cell lysate (OD_{600} ~1) was prepared with cell lysis buffer, and GAPDH activity was quantified with 1.17 mM of β-NADH by measuring the absorbance

periodically at 340 nm using a VICTOR^3 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA), which indicated the reduction of β-NADH. The amount of total protein was measured by the Bradford assay (Bio-Rad, Hercules, CA, USA), and the activity was calculated as specific enzymatic activity (units/mg total protein) as described in our previous study (Lim et al., 2013a).

2.5 Quantification of lycopene production

To determine the lycopene content, 1 ml of culture broth was harvested by centrifugation at 15,493 ×g for 5min at 4°C, and the cell pellet was washed once with phosphate buffered saline (PBS). The cell pellet was suspended in acetone (1 ml) and incubated at 55°C for 15 min with intermittent vortexing for efficient extraction. The samples were centrifuged at 15,493 ×g for 15min at room temperature (RT), and subsequently the absorbance of the supernatant was measured at 475 nm using a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan). The concentration of lycopene was quantified using a standard curve prepared by using authentic lycopene (Cat. L9879, from Sigma-Aldrich; Fig. S1). Dry cell weight (DCW) was calculated from 10 ml of culture broth harvested by centrifugation at 4,713 ×g for 10 min at 4°C and washed twice with water. The cells were dried completely at 65°C and weighed. One unit of OD_{600} corresponds to a cell dry weight of 0.252 ± 0.032 g. The data have been represented by measurements from two independent experiments.

3. RESULTS AND DISCUSSION

3.1 Pathway amplification for native DXP and heterologous lycopene synthesis pathways

Several enzymes are involved in the production of the two universal isoprenoid building blocks, IPP and DMAPP, from the condensation of glycolytic intermediates, G3P and pyruvate (Fig. 1). As an initial attempt to produce lycopene in *E. coli*, we introduced the heterologous lycopene synthesis pathway (*crtEBI* from *Pantoea agglomerans*) (Cunningham et al., 1994), and the resulting strain JYJ0 produced 1.81 mg lycopene/g DCW, whereas lycopene was not detectable in the native strain W3110 (Fig. 2).

Next, we further engineered the strain JYJ0 with the native DXP pathway to increase carbon flux toward lycopene. In previous studies, *ispA*, *idi*, and *dxs* were identified as rate-limiting steps in DXP pathway (Choi et al., 2010; Kim and Keasling, 2001; Lemuth et al., 2011; Wang et al., 2009; Yuan et al., 2006). Initially, formation of farnesyl pyrophosphate (FPP), the precursor of the heterologous lycopene pathway and ubiquinone biosynthesis (Lu et al., 2014), was accomplished by overexpression of farnesyl diphosphate synthase (encoded by *ispA*). The engineered strain JYJ1, harboring pCDF_*ispA_crtEBI*, produced 3.29 mg lycopene/g DCW (increased by 81% compared to the control strain JYJ0) (Fig. 2). Additionally, isopentenyl diphosphate isomerase (encoded by *idi*), catalyzing interconversion between IPP and DMAPP, was targeted. IPP isomerization plays an important role in amplification of the isoprenoid flux, probably due to the different requirement of IPP and DMAPP at a 3:1 molar ratio per mole of lycopene (Fig. 1) (Kirby and Keasling, 2009). The additional overexpression of *idi* (JYJ2) resulted in 88% improvement in lycopene production (6.20 mg lycopene/g DCW) than that obtained with the overexpression of *crtEBI* and *ispA*.

Finally, *dxs* (encoding 1-deoxy-D-xylulose-5-phosphate synthase) was overexpressed to efficiently convert DXP from pyruvate and G3P. The resulting strain JYJ3, carrying *crtEBI*, *ispA*, *idi*, and *dxs*, represented more enhanced lycopene production (7.84 mg lycopene/g DCW), with an increase of up to 27% and 332% compared to JYJ2 and JYJ0, respectively (Fig. 2).

The lycopene production in these strains was superior or comparable to that in the previously reported strains with an engineered DXP pathway (Alper et al., 2005b; Wang et al., 2009). This indicates that DXP pathway was effectively amplified to check the effect of precursor balancing (Fig. S2).

3.2 Balancing the precursor pool by fine-tuning *ppsA* expression

To further enhance the lycopene production in the recombinant *E. coli*, the effect of balancing the precursors between G3P and PYR was examined via *ppsA* to increase the metabolic flux toward lycopene (Fig. 3A). To explore the optimal level of *ppsA*, we exploited the strongest constitutive promoter and synthetic UTR variants with various translational efficiencies identified previously, whose activities were in the range of an order of magnitude (Kim et al., 2015).

As shown in Fig. 3B, all PpsA variants showed improved specific lycopene production compared to the parental strain JYJ5, consistent with the previous studies demonstrating the increase in isoprenoid production via *ppsA* overexpression (Farmer and Liao, 2001; Zhang et al., 2015). Notably, the specific content of lycopene gradually increased up to 37% (9.90 mg/g DCW) in the V4 variant (JYJ8) along with increase in the activity of

ppsA. However, further amplification of *ppsA* reduced the production of lycopene (8.99 mg/g DCW in JYJ9, approximately 9% decrease compared to JYJ8). The observed optimum expression level for biosynthesis of isoprenoid is consistent with that reported previously by investigating the effects of varying 1-deoxy-D-xylulose-5-phosphate synthase (DXS) expression levels on lycopene production (Alper et al., 2005a). Despite the cellular growth were relatively unchanged among the *ppsA* overexpressed strains (Fig. S3), further increase of *ppsA* expression beyond the optimum level was detrimental for lycopene production probably due to the deterioration of futile cycle or abnormal perturbation of the PEP level that regulates the activities of PEP-consuming enzymes, such as phosphofructokinase and phosphoenolpyruvate carboxylase, involved in central carbon metabolism (Lau and Fersht, 1987; Xu et al., 2012).

3.3 Balancing the precursor pool by fine-tuning of *gapA* expression

In contrast to the overexpression of *ppsA*, we further investigated that reducing the activity of GAPDH (encoded by *gapA*) can directly increase G3P pools for the production of lycopene (Fig. 4A). To reduce the activity of GAPDH, we substituted the native promoter and UTR region with the synthetic parts and controlled the expression level through the synthetic constitutive promoter family from part-registry (see Section 2.2). Through the downregulation of *gapA*, we observed that the specific activity of GAPDH is controlled in the range of an order of magnitude similar to the controlled level of *ppsA*; however, the production of lycopene significantly improved up to 97% in JYJ11 (14.34 mg/g DCW) compared to the parental strain. Notably, the lycopene production increased with decrease in the specific activity of GAPDH, which indicates that tuning-down the expression level of

gapA directly increased G3P pools and diverted carbon flux toward lycopene (Fig. 4B). It is also important to note that the biomass yield is critical parameter to evaluate lycopene titer because the carotenoids are incorporated to cellular membrane (Das et al., 2007). Using our approach, the final biomass relatively unchanged among the GAPDH variants (Fig. S4A), even though specific growth rate decreased up to 43% along with the reduction of GAPDH activity (Fig. S4B) (Cho et al., 2012). Therefore, all GAPDH variants observed higher titer of lycopene than that of the parental strain (Fig. S4C).

Collectively, our results showed the importance of precursor balancing to divert carbon flux toward lycopene, as a model compound for isoprenoids. Specifically, we explored the engineering targets, *ppsA* and *gapA*, for precursor balancing and identified the tuning-down of *gapA* expression as an efficient approach to increase G3P pool from sugars, rather than the overexpression of *ppsA*. Indeed, the quantification of intracellular metabolites revealed that the G3P pool (DHAP + G3P) directly controlled depending on the specific activity of GAPDH and its ratio to pyruvate increased about 20-fold than that of the parental strain (Fig. 5). The ratio of (DHAP+G3P)/PYR in JYJ11, 3-fold higher than the stoichiometric requirement of G3P and PYR, is responsible for enhanced lycopene production. This indicates that two-substrate enzyme kinetics requires optimal concentration of each substrates depending on substrate affinity, rather than a stoichiometry (Cleland, 1963).

The changes in the ratio also showed a strong correlation with the production of lycopene depending on the GAPDH activity. However, it was found that the overexpression of *ppsA* caused the accumulation of 2-phosphoglycerate (2PG) and 3-phosphoglycerate (3PG) mostly rather than control of G3P/PYR ratio as aforementioned it redirect flux from pyruvate back to G3P in the route of gluconeogenesis (Fig. 5). These results clearly support that our approach

to rebalance the precursor pool through *gapA* affects the production of isoprenoids more directly. Traditionally, the improvement of carotenoid production has been facilitated by combinatorial approaches (Alper et al., 2005b; Alper et al., 2005c; Kang et al., 2005; Stephanopoulos et al., 2004). Exploitation of this valuable knowledge based on our rational approach will synergistically facilitate the efficient production of lycopene and also isoprenoids. Additionally, redox rebalancing for NADPH-consuming lycopene production system also enables the optimization of lycopene production in *E. coli* (Lim et al., 2015; Lim et al., 2013a; Martinez et al., 2008; Ng et al., 2015).

4. CONCLUSIONS

In this study, we demonstrated that precursor balancing is one of the most crucial steps for diverting carbon flux toward isoprenoids via the DXP pathway in *E. coli*. As a model system, we first constructed a lycopene-overproducing *E. coli* strain by the amplification of the DXP pathway. Subsequently, we compared the effect of precursor balancing between PpsA and GAPDH through a fine-tunable control at both transcriptional and translational levels by using synthetic expression cassettes. As a result, the specific lycopene content in optimal *gapA* and *ppsA* variants increased up to 97% and 37% respectively, compared to that of the parental strain. This corresponds to 45% improvement in the optimal *gapA* variant (14.34 mg/g DCW) compared to the optimal *ppsA* variant (9.90 mg/g DCW) in glucose-minimal medium, suggesting that controlling flux through *gapA* affects the production of isoprenoids more directly. Collectively, we propose *gapA* as an engineering target for precursor balancing to enhance the production of isoprenoids. Our strategy could be broadly utilized in the field of metabolic engineering and synthetic biology to increase the production of numerous high-value isoprenoids.

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Fig. 1. A schematic diagram of the synthetic metabolic pathway for the production of lycopene from two glycolytic intermediates, G3P and pyruvate (PYR). The overexpression of *ppsA* indirectly increased G3P pools, while the downregulation of *gapA* directly supported G3P pools for the biosynthesis of isoprenoids. Red arrows indicate the overexpressed targets for heterologous and endogenous genes. Blue arrow indicates the downregulated target for *gapA*. Abbreviations for metabolites and enzymes: *dxs*, gene encoding 1-deoxy-D-xylulose-5-phosphate (DXP) synthase; *dxr*, gene encoding DXP reductoisomerase; *ispD*, gene encoding 2-C-methyl-D-erythritol 4-phosphate (MEP) cytidylyltransferase; *ispE*, gene encoding 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) kinase; *ispF*, gene encoding 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP) synthase; *ispG*, gene encoding 4-hydroxy-3-methylbut-2-enyl-diphosphate (HMBPP) synthase; *ispH*, gene encoding HMBPP reductase; *idi*, gene encoding isopentenyl-diphosphate (IPP) delta-isomerase; *ispA*, gene encoding farnesyl diphosphate (FPP) synthase; *crtE*, heterologous gene coding geranylgeranyl pyrophosphate (GGPP) synthase; *crtB*, heterologous gene encoding phytoene synthase; *crtI*, heterologous gene encoding phytoene desaturase.

Fig. 2. Amplification of the DXP pathway for the production of lycopene. Sequential and additive overexpression of *ispA* (JYJ1), *ispA-idi* (JYJ2), and *ispA-idi-dxs* (JYJ3) resulted in production of 3.29, 6.20, and 7.84 mg/g DCW lycopene, respectively. Wild-type *E. coli* W3110 did not produce detectable lycopene (N.D.). Comparison experiments for plasmids

were conducted in 3-ml tube culture using glucose-supplemented minimal medium. The lycopene produced was quantified after 24 h of cultivation. The error bars indicate standard deviations of measurements from three independent cultures.

Fig. 3. The effect of precursor balancing via fine-tuning of *ppsA* for the production of lycopene. (A) A schematic diagram of *ppsA* fine-tuning for redirection of carbon flux indirectly toward lycopene. (B) Specific lycopene content depending on the specific activity of *ppsA*. The specific activities of PpsA are from our previous study (Kim et al., 2015) and each value was normalized to that of the V5 variant (JYJ9). All the data shown were obtained after 24-h cultivation in glucose-supplemented minimal medium. The error bars indicate standard deviations of measurements from three independent cultures.

Fig. 4. The effect of precursor balancing via fine-tuning of *gapA* for the production of lycopene. (A) A schematic diagram of *gapA* downregulation for the redirection of carbon flux directly toward lycopene. (B) Specific lycopene content depending on the specific activity of *gapA*. Each value was normalized to that of the wild-type variant (JYJ5). A positive correlation was observed between specific lycopene content and specific activity of *gapA*. All the data shown were obtained after 24-h cultivation in glucose-supplemented minimal medium. The error bars indicate standard deviations of measurements from three independent cultures.

Fig. 5. Quantification of intracellular metabolites for *ppsA* and *gapA* variants. The strains

selected depending on the extent of enzyme activity and lycopene production to identify the pattern of intracellular metabolites among variants. (A) and (B) (DHAP+G3P) / PYR. The sum of DHAP and G3P is represented here to describe G3P pool as triose phosphate isomerase can interconverts both intermediates in *E. coli*. (C) and (D) Intracellular 2-Phosphoglycerate (2PG) concentration. (E) and (F) Intracellular 3-Phosphoglycerate (3PG) concentration. The diamond symbol represents mean value and lower and upper whiskers denotes minimum and maximum value, respectively (n=3).

Table 1. Bacterial strains and plasmids used in this study

Name	Relevant characteristics	Source
Strains		
Mach1-T1^{R}	F^{-} φ80(*lac*Z)ΔM15 Δ*lac*X74 *hsd*R(r_K^- m_K^+) Δ*rec*A1398 *end*A1 *ton*A	Invitrogen
W3110	F^{-} λ^{-} rph-1 IN(*rrnD*, *rrnE*)1	ATCC27325
JYJ0	W3110/pCDF_*crtEBI*	This study
JYJ1	W3110/pCDF_*ispA_crtEBI*	This study
JYJ2	W3110/pCDF_*idi_ispA_crtEBI*	This study

JYJ3	W3110/pCDF_*idi_ispA_crtEBI*/ pACYC_*dxs*	This study
JYJ4	W3110 *rpsL**$_{A128G}$	This study
JYJ5	JYJ4/pCDF_*idi_ispA_crtEBI*/ pACYC_*dxs*	This study
JYJ6	JYJ5 FRT-Km^R-FRT::P_{BBa_J23100}::synUTR$_{ppsA(V1)}$::*ppsA*	This study
JYJ7	JYJ5 FRT-Km^R-FRT::P_{BBa_J23100}::synUTR$_{ppsA(V3)}$::*ppsA*	This study
JYJ8	JYJ5 FRT-Km^R-FRT::P_{BBa_J23100}::synUTR$_{ppsA(V4)}$::*ppsA*	This study
JYJ9	JYJ5 FRT-Km^R-FRT::P_{BBa_J23100}::synUTR$_{ppsA(V5)}$::*ppsA*	This study
JYJ10	JYJ5 P_{BBa_J23101}::synUTR$_{gapA}$::*gapA*	This study
JYJ11	JYJ5 P_{BBa_J23108}::synUTR$_{gapA}$::*gapA*	This study
JYJ12	JYJ5 P_{BBa_J23107}::synUTR$_{gapA}$::*gapA*	This study
JYJ13	JYJ5 P_{BBa_J23105}::synUTR$_{gapA}$::*gapA*	This study
JYJ14	JYJ5 P_{BBa_J23115}::synUTR$_{gapA}$::*gapA*	This study

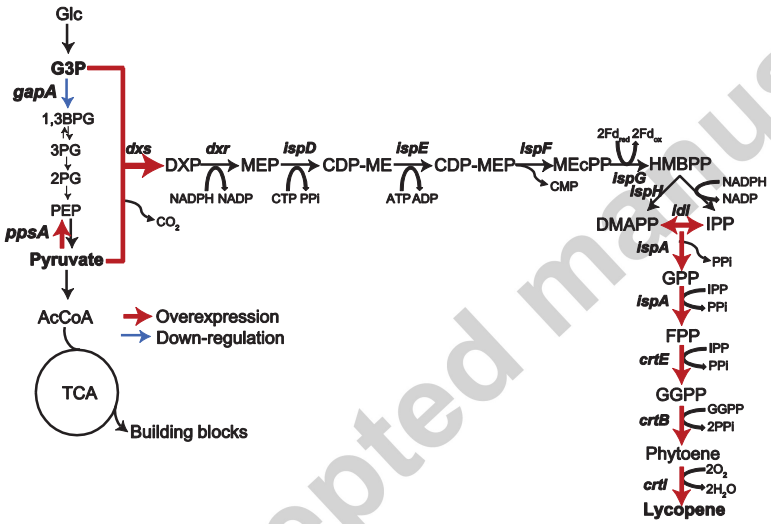
Plasmids

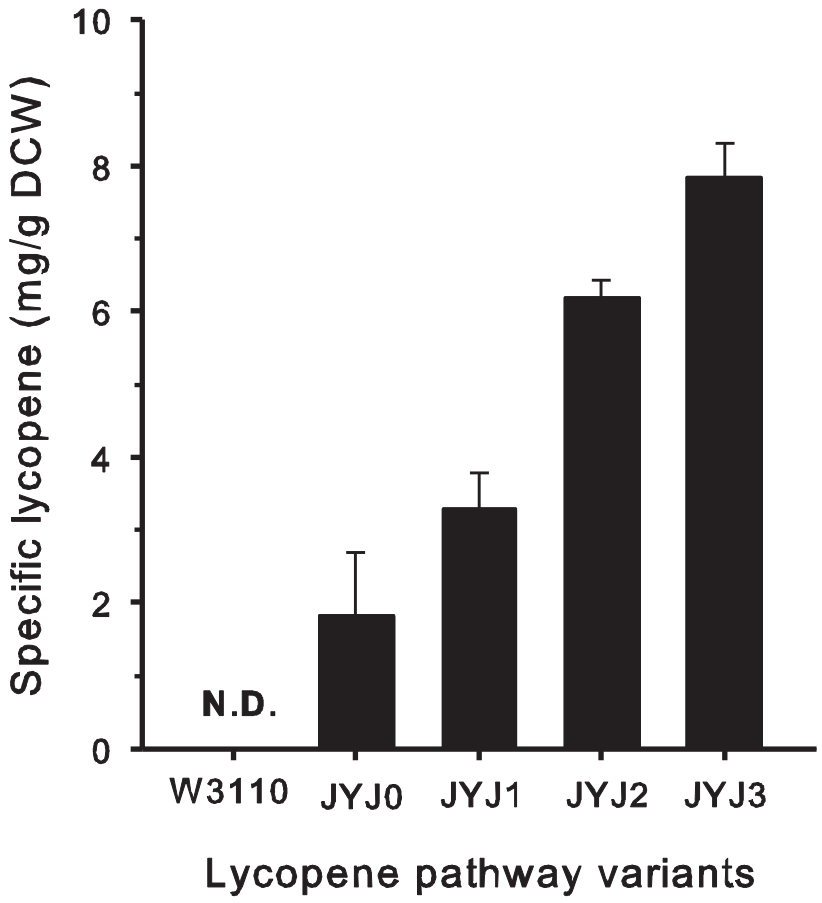
pKD46	Red recombinase expression vector, Amp^R	(Datsenko and Wanner, 2000)
pACYCDuet-1	Expression vector, Cm^R, p15A ori	Novagen

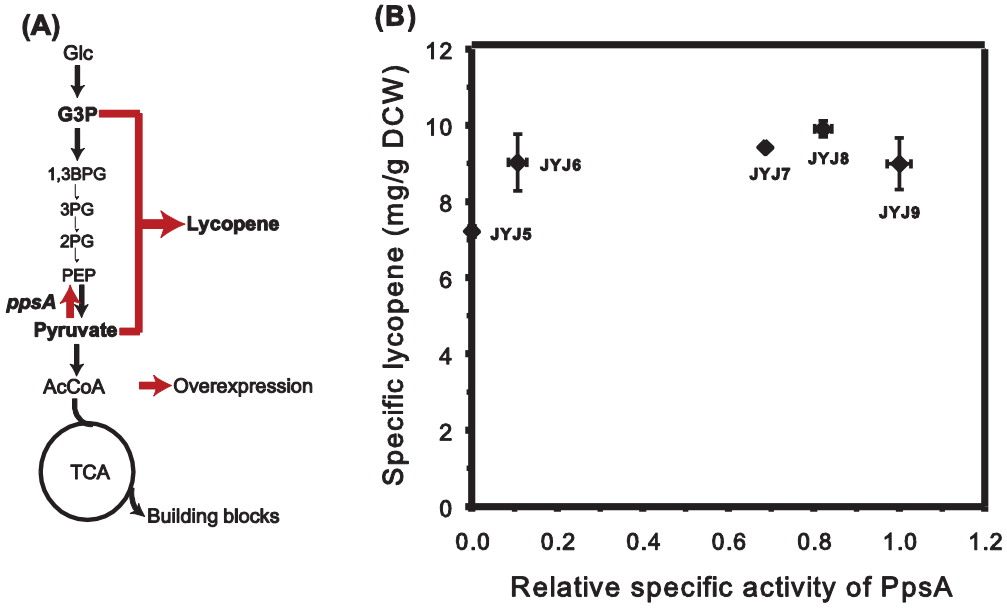
pCDFDuet-1	Expression vector, Sm^R, cloDF13 ori	Novagen
pCOLADuet-1	Expression vector, Km^R, ColA ori	Novagen
pMD20-T	Cloning vector, Amp^R	Takara
pPpsA-V1	pMD20-T/FRT-Km^R-FRT-P_{BBa_J23100}::$synUTR_{ppsA(V1)}$	(Kim et al., 2015)
pPpsA-V3	pMD20-T/FRT-Km^R-FRT-P_{BBa_J23100}::$synUTR_{ppsA(V3)}$	(Kim et al., 2015)
pPpsA-V4	pMD20-T/FRT-Km^R-FRT-P_{BBa_J23100}::$synUTR_{ppsA(V4)}$	(Kim et al., 2015)
pPpsA-V5	pMD20-T/FRT-Km^R-FRT-P_{BBa_J23100}::$synUTR_{ppsA(V5)}$	(Kim et al., 2015)
pCDF_*crtEBI*	pCDFDuet-1/HindIII_P_{BBa_J23100}::*crtE-crtB-crtI*::T_{BBa_B1005}_NcoI	This study
pCDF_*ispA_crtEBI*	pCDF_*crtEBI*/BglII_P_{BBa_J23100}::*ispA*::T_{BBa_B1005}_HindIII	This study
pCDF_*idi_ispA_crtEBI*	pCDF_*ispA_crtEBI*/BglII_P_{BBa_J23100}::*idi*::T_{BBa_B1005}_BglII	This study
pACYC_*dxs*	pACYCDuet-1/NcoI::P_{BBa_J23100}::*dxs*::T_{BBa_B1005}::BamHI	This study

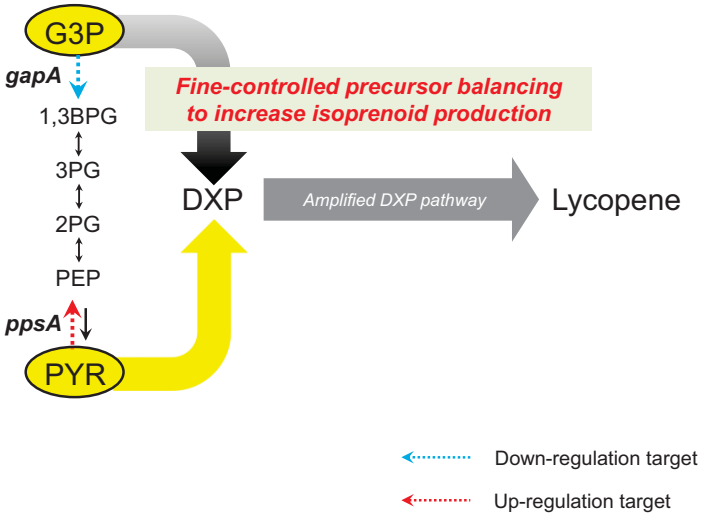
Highlights

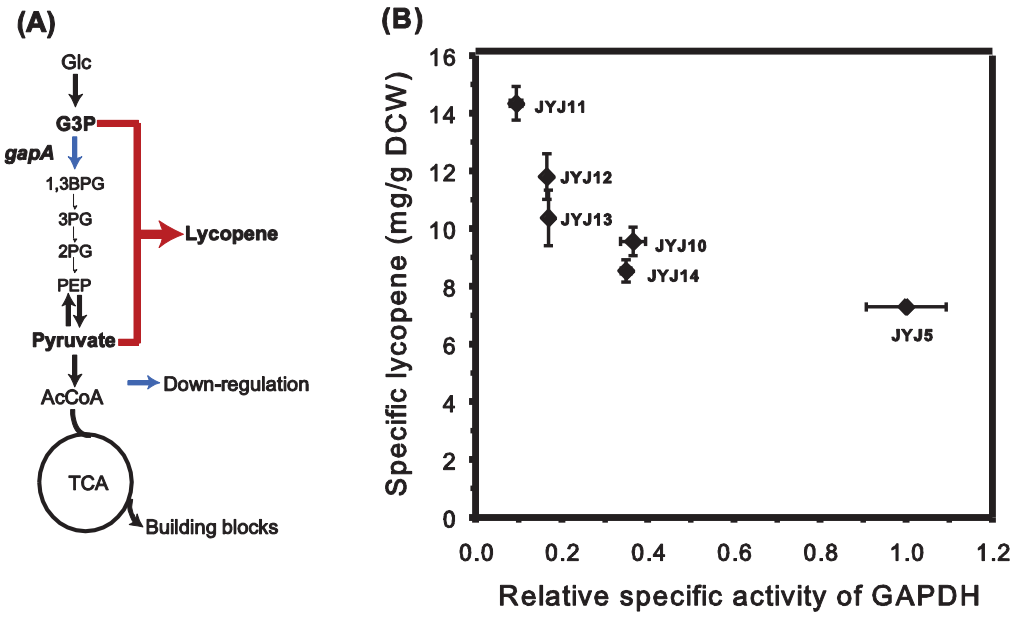
- The effect of precursor balancing for the biosynthesis of isoprenoids was explored through fine-control of *ppsA* and *gapA*.
- Precise tuning-down of *gapA* resulted in 14.34 mg lycopene/g DCW while 9.90 mg lycopene/g DCW showed in the optimal *ppsA* overexpression.
- A novel target (*gapA*) to optimize biosynthesis of isoprenoids was firstly demonstrated and showed the best effect on the precursor rebalancing.

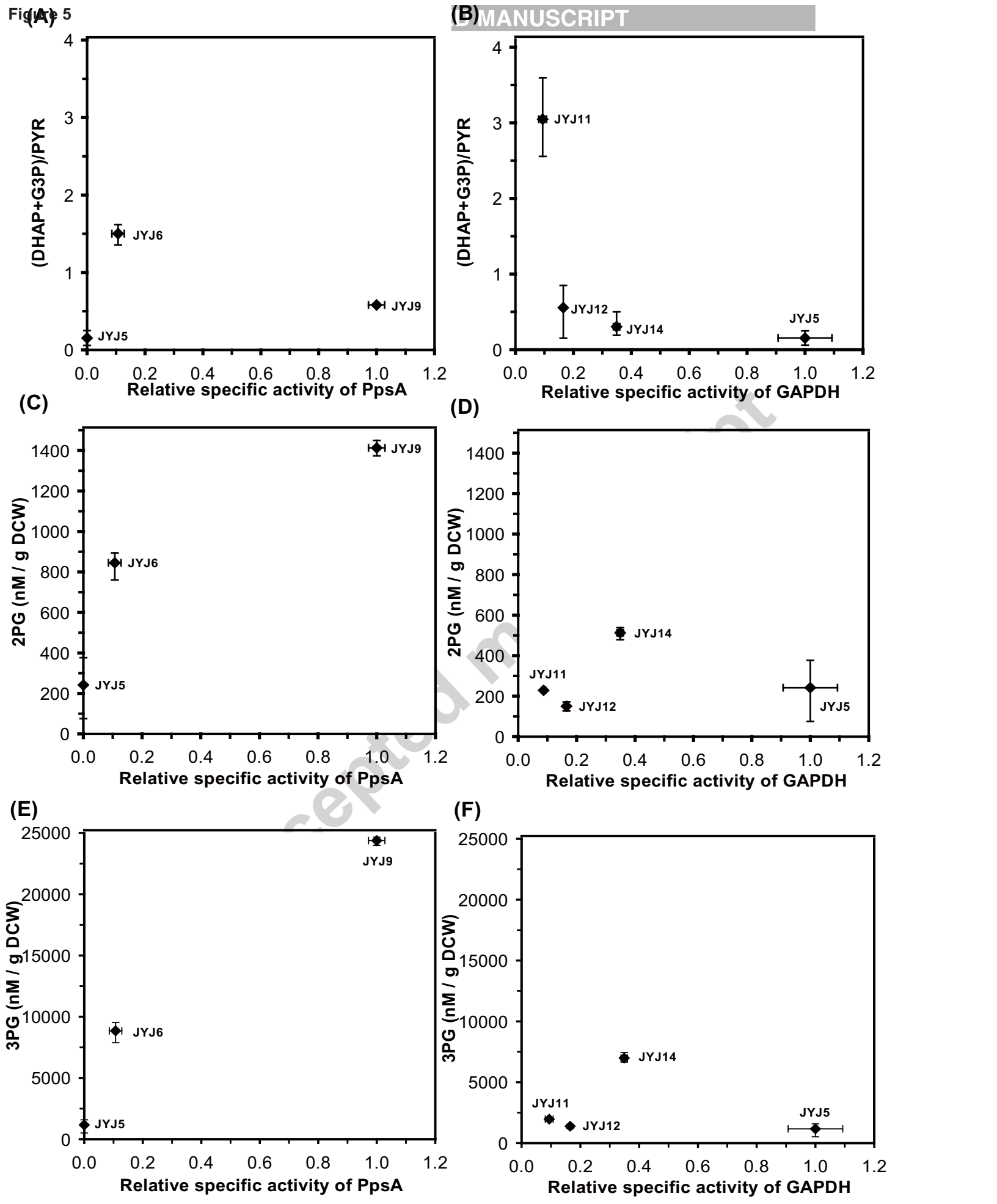



Specific lycopene (mg/g DCW)
10
8
6
4
2
0
N.D.
W3110
JYJ0
JYJ1
JYJ2
JYJ3
Lycopene pathway variants


(A)
Glc
G3P
1,3BPG
3PG
2PG
PEP
ppsA
Pyruvate
AcCoA
TCA
Lycopene
Overexpression
Building blocks
(B)
Specific lycopene (mg/g DCW)
Relative specific activity of PpsA
JYJ5
JYJ6
JYJ7
JYJ8
JYJ9
0
2
4
6
8
10
12
0.0
0.2
0.4
0.6
0.8
1.0
1.2




(A)
Glc
G3P
gapA
1,3BPG
3PG
2PG
PEP
Pyruvate
AcCoA
TCA
Lycopene
Down-regulation
Building blocks
(B)
Specific lycopene (mg/g DCW)
Relative specific activity of GAPDH
JYJ11
JYJ12
JYJ13
JYJ10
JYJ14
JYJ5
0
2
4
6
8
10
12
14
16
0.0
0.2
0.4
0.6
0.8
1.0
1.2


(A)
(DHAP+G3P)/PYR
Relative specific activity of PpsA
JYJ6
JYJ9
JYJ5
(B)
(DHAP+G3P)/PYR
Relative specific activity of GAPDH
JYJ11
JYJ12
JYJ14
JYJ5
(C)
2PG (nM / g DCW)
Relative specific activity of PpsA
JYJ9
JYJ6
JYJ5
(D)
2PG (nM / g DCW)
Relative specific activity of GAPDH
JYJ14
JYJ11
JYJ12
JYJ5
(E)
3PG (nM / g DCW)
Relative specific activity of PpsA
JYJ9
JYJ6
JYJ5
(F)
3PG (nM / g DCW)
Relative specific activity of GAPDH
JYJ14
JYJ11
JYJ12
JYJ5