

Review

Metabolic engineering for the production of clinically important molecules: Omega-3 fatty acids, artemisinin, and taxol

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Driven by requirements for sustainability as well as affordability and efficiency, metabolic engineering of plants and microorganisms is increasingly being pursued to produce compounds for clinical applications. This review discusses three such examples of the clinical relevance of metabolic engineering: the production of omega-3 fatty acids for the prevention of cardiovascular disease; the biosynthesis of artemisinic acid, an anti-malarial drug precursor, for the treatment of malaria; and the production of the complex natural molecule taxol, an anti-cancer agent. In terms of omega-3 fatty acids, bioengineering of fatty acid metabolism by expressing desaturases and elongases, both in soybeans and oleaginous yeast, has resulted in commercial-scale production of these beneficial molecules. Equal success has been achieved with the biosynthesis of artemisinic acid at low cost for developing countries. This is accomplished through channeling the flux of the isoprenoid pathway to the specific genes involved in artemisinin biosynthesis. Efficient coupling of the isoprenoid pathway also leads to the construction of an *Escherichia coli* strain that produces a high titer of taxadiene – the first committed intermediate for taxol biosynthesis. These examples of synthetic biology demonstrate the versatility of metabolic engineering to bring new solutions to our health needs.

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1 Introduction

Metabolic engineering harnesses the intrinsic metabolic machinery of cells for the manufacture of useful molecules. One approach for metabolic

engineering is to tune existing biochemical pathways within cells to maximize synthesis of desired molecular entities. Another approach for metabolic engineering is to create entirely new biosynthetic pathways de novo within cells to enable production of valuable molecules. Both techniques have been successfully implemented in microbial cells, plant cells, and mammalian cells. Metabolic engineering can be advantageous over traditional organic synthesis for several reasons: metabolic engineering may be more cost-effective than chemical synthesis, particularly for production of pharmaceuticals and fine chemicals; metabolic engineering may be more environmentally friendly and sustainable than chemical manufacture; and metabolic engineering can be superior to traditional organic synthesis for the reliable production of complex biological molecules [1].

Novel technologies in biochemical and biomedical engineering can have the highest impact when these technologies address pressing clinical needs

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Abbreviations: ACT, artemisinin combination therapy; ADS, amorphadiene synthase; ALA, alpha linolenic acid; CPS, β -caryophyllene synthase; DBR2, artemisinic aldehyde $\Delta^{11}(13)$ double-bond reductase; DHA, docosahexaenoic acid; DMPP, dimethylallyl diphosphate; EDA, eicosadienoic acid; EPA, eicosapentaenoic acid; ETrA, eicosatrienoic acid; FPP, farnesyl diphosphate; GGDP, geranylgeranyl diphosphate; GGPP, geranylgeranyl pyrophosphate; GGPPS, GGPP synthase; GLA, gamma linolenic acid; GRAS, generally recognized as safe; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HMGR, HMG-CoA reductase; LA, linolenic acid; MEP/DXP, mevalonate-independent pathway; OCT, 5(12)-Oxa-3(11)-cyclotaxane; SDA, stearidonic acid; TS, taxadiene synthase

[2]. Metabolic engineering is increasingly being recognized as a valuable tool for producing therapeutics, including antibiotics, antivirals, antiparasitics, anti-cancer agents, anti-hypertensive agents, cholesterol-lowering drugs, antioxidants, vitamins, and hormones [3]. Medicines synthesized via metabolic engineering are progressively reaching clinical practice. Currently, the products of metabolic engineering are utilized to treat many global diseases, including cardiovascular and cerebrovascular diseases, infectious diseases, nutritional deficiencies, and cancer. Metabolic engineering has therefore grown into a technology of immense clinical relevance, with the potential to alleviate disease worldwide. This review will discuss three such examples of the clinical relevance of metabolic engineering: the production of omega-3 fatty acids for the prevention of coronary artery disease; the biosynthesis of artemisinic acid, an anti-malarial drug precursor, for the treatment of malaria; and the production of the complex natural molecule taxol, an anti-cancer agent.

2 Metabolic engineering of yeast and plants for omega-3 biosynthesis

2.1 Clinical relevance of omega-3 fatty acids

Omega-3 fatty acids are naturally occurring unsaturated fats which are vital for normal metabolism. Omega-3 fatty acids have numerous biological targets; long-chain omega-3 fatty acids have been shown to lower triglycerides [4]; prevent major coronary events [5–8]; reduce risk of stroke [9]; prevent Alzheimer's disease and cognitive decline [10], and alleviate rheumatoid arthritis [11]. Omega-3 fatty acids are therefore important in treating and preventing several diseases.

2.2 Biosynthetic pathways of omega-3 fatty acids

Omega-3 fatty acids (Fig. 1) include α -linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). The starting substrates for biosynthesis of long-chain unsaturated fatty acids are linoleic acid (LA) and ALA (Fig. 2). Although both molecules contain 18 carbons, LA is an omega-6 fatty acid, while ALA is an omega-3 fatty acid.

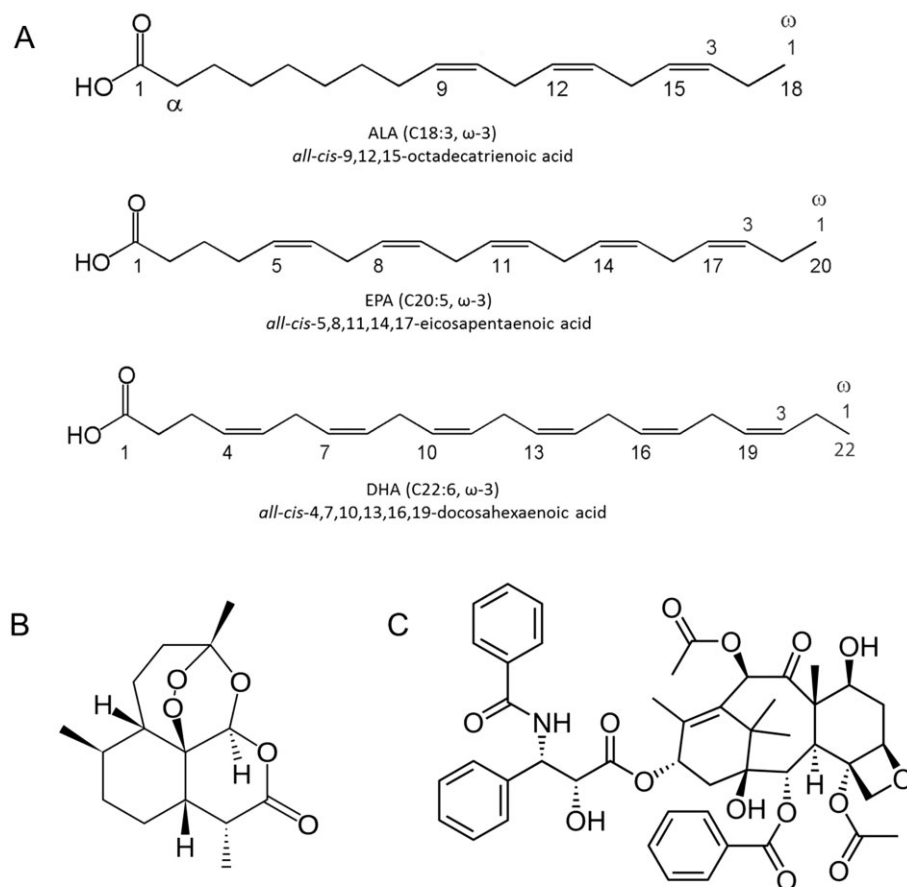


Figure 1. Chemical structures of (A) omega-3 fatty acids; (B) artemisinic acid; (C) taxol.

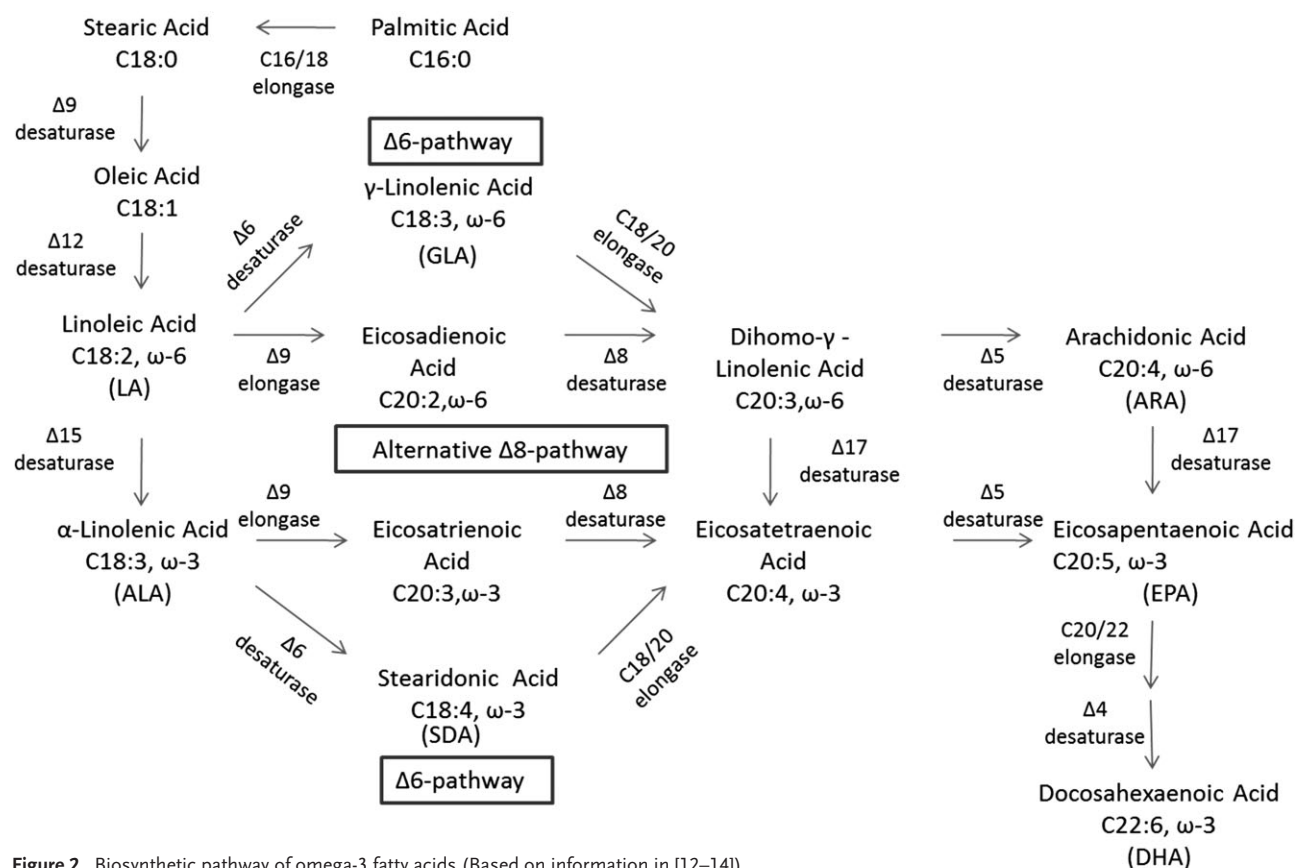


Figure 2. Biosynthetic pathway of omega-3 fatty acids (Based on information in [12–14]).

acid. LA and ALA can be converted to EPA and DHA by adding more carbon atoms and unsaturated bonds. These reactions are catalyzed by a series of elongases and desaturases [12–14]. There are two possible pathways to synthesize EPA from LA and ALA. The “Δ6-pathway” starts with the Δ6-desaturase which converts LA to γ-linolenic acid (GLA) or ALA to stearidonic acid (SDA). Subsequent elongation and desaturation steps lead to EPA formation. The “Δ9-pathway” starts with Δ9-elongase, which converts LA into eicosadienoic acid (EDA) or ALA to eicosatrienoic acid (ETra). This is followed by reactions carried out by Δ8-, Δ5-, or Δ17-desaturases. Once EPA is synthesized, C20/22 elongase and Δ4-desaturase convert EPA into DHA.

In the mammalian system, conversion of ALA starts with Δ6-desaturase, which is rate-limiting. Once EPA is formed, its conversion to DHA is more complex. The current hypothesis is that conversion involves two elongation steps to lengthen the acyl chain to 24 carbons, followed by a desaturation step carried out by Δ6-desaturase [15, 16]. The last step is a β-oxidation reaction that reduces the number of carbons to 22; β-oxidation occurs in the peroxi-

some and all elongation and desaturation steps take place in the endoplasmic reticulum.

2.3 The need for a bioengineering solution for omega-3 fatty acid production

Plants synthesize the omega-3 fatty acid ALA [17]. In humans, the ability to convert dietary ALA to the long-chain omega-3 fatty acids EPA and DHA is limited [18]. Currently, fish are the major dietary source of EPA and DHA. Notably, omega-3 fatty acids from fish or fish-oil supplements, but not ALA, benefit cardiovascular disease outcomes [19]. Fish are not a sustainable source of omega-3 fatty acids; fish populations are sensitive to climate change and over-fishing. Furthermore, many fish are contaminated by toxins and pollutants [20, 21]. Yeast and oil plants are thus being investigated as alternative sources for omega-3 fatty acids.

2.4 Bioengineering of *Yarrowia lipolytica* for omega-3 fatty acid production

Oleaginous yeast *Y. lipolytica* can produce and store 40% of its dry cell weight as fatty acids [22, 23].

Y. lipolytica is a GRAS (generally recognized as safe) organism with robust fermentation performance. Another advantage of *Y. lipolytica* is its genetic system. Genes can be easily integrated into chromosomes through transformation with linear DNA. Integration events are predominantly random, and favorable integration events can be selected [24]. The general strategy of pathway engineering in this organism is to clone genes related to omega-3 biosynthesis with appropriate promoters and terminators and express these genes in the chromosome [14]. Three methods are used to increase gene expression: (i) selection of promoters with appropriate strength; (ii) optimization of codon usage for each foreign gene; and (iii) integration of multiple copies of genes in the chromosome. To identify promoters with appropriate strength, the *E. coli* β -glucuronidase reporter system is used. A suite of promoters, including GPM, GPD, and FBA are useful for gene expression. Fusion of the FBA promoter along with the first 23 amino acids and the intron (FBAin) results in a much stronger promoter. For integration of multiple genes, the cassettes with promoter and terminator are constructed using *URA3* gene as the selection marker. The *URA3* gene is then recycled before the next round of integration. Using this method, constructed strains have multiple copies of desaturases and elongases, to achieve high-level expression of pathway genes.

EPA biosynthesis can be accomplished using the “ $\Delta 6$ -pathway” (Fig. 2). Starting with $\Delta 6$ -desaturase, both LA and ALA can be converted to EPA with a combination of $C_{18/20}$ -elongase, $\Delta 5$ -desaturase, and/or $\Delta 17$ -desaturase. Multiple copies of genes encoding these elongases and desaturases were inserted into the *Y. lipolytica* chromosome [14]. In addition, two copies of $C_{16/18}$ -elongase and 3 copies of $\Delta 12$ -desaturase were added to increase the flux from palmitic acid. The strain constructed, Y2097, produced oil with 40% EPA. To produce DHA, the $C_{20/22}$ -elongase from *Ostreococcus tauri* and $\Delta 4$ -desaturase from *Thraustochytrium aureum* were expressed. The DHA-producing strain had an oil composition of 18% DPA and 6% DHA.

To achieve higher EPA content, the “ $\Delta 9$ -pathway” was explored. The genes include $\Delta 9$ -elongase, $\Delta 8$ -desaturase, and the common $\Delta 5$ -desaturase (Fig. 2). During fermentation, a two-stage fed-batch process increases the oil content from the EPA-producing strain [25]. The first stage generates biomass. In the second stage, the yeast are deprived of nitrogen and fed glucose to promote oil accumulation. The EPA-enriched oil from the yeast strain is extracted with food-grade isohexane. The oil contains 55% EPA and <10% saturated fatty acids [25, 26]. The EPA-enriched oil from *Y. lipolytica* has

been marketed under the brand name NewHarvest™ Omega-3. The safety profile of EPA-enriched oil is comparable to that of fish oil [27, 28]. Furthermore, this source of EPA is free of environmental contaminants.

2.5 Bioengineering of soybean and other oilseeds as sources of omega-3 fatty acids

Production of omega-3 fatty acids from soybean and canola uses sustainable resources, while avoiding the toxic contaminants in fish oil. Metabolic engineering of plants to direct flux toward EPA and DHA biosynthesis requires more effort compared to *Y. lipolytica*. One solution is to produce SDA [29–31]. The conversion ratio of SDA to EPA is 3.3 to 1.0, which is greater than the 14 to 1 ratio of ALA to EPA. By bypassing the rate-limiting step of $\Delta 6$ -desaturase, oils containing high amounts of SDA are more effective than oils containing ALA in increasing serum EPA levels.

Conventional soybean oil contains high amounts of the omega-6 fatty acid LA. To metabolically engineer soybeans to produce SDA, two desaturases are expressed [32]. The $\Delta 6$ desaturase is obtained from the flower plant, *Primula juliae*. This enzyme catalyzes the formation of a double bond between the $\Delta 6$ and $\Delta 7$ carbons, leading to the formation of GLA from LA, and SDA from ALA (Fig. 2). By expressing a second desaturase, the $\Delta 15$ -desaturase from red bread mold *Neurospora crassa*, the intermediate LA is pushed to ALA, increasing the carbon flux to SDA. The transgenic soybean has 15–30% SDA (Table 1). LA levels in the transgenic soybean are reduced to 15–30% from 48 to 65%. In clinical trials, the SDA-enriched soybean oil has demonstrated efficacy in raising serum EPA levels [33, 34]. In 2009, the Food and Drug Administration approved GRAS status for SDA-enriched soybean oil [35]. The product contains 20% SDA and is marketed as Soyomega™ SDA Soybean Oil (Solae).

The next challenge is to engineer oilseeds to produce both EPA and DHA at a similar ratio as that in fish oil [12, 36]. Another necessary improvement is the optimization of oil accumulation, with

Table 1. Fatty acid content of regular and SDA-soybean oil by weight.

Fatty acids	Soybean oil (%)	SDA-enriched oil (%)
Oleic acid	19–30	10–20
LA	48–65	15–30
ALA	5–10	9–12
GLA	n/a	5–8
SDA	n/a	15–30

n/a, not applicable, [32]

little undesirable omega-6 fatty acids. In this regard, the metabolic capability of microalgae provides a rich source of genes for oilseed crops [12]. BASF and Dow Agrosciences are working on metabolic engineering of canola to produce plant-based EPA and DHA.

3 Metabolic engineering for production of the anti-malarial artemisinin

3.1 Clinical relevance of artemisinin

Artemisinin is an anti-malarial drug produced in the leaves of *Artemisia annua*. Structurally, artemisinin is an endoperoxide sesquiterpene lactone (Fig. 1). Artemisinin activity depends on hemoglobin digestion by the parasite, and involves reductive cleavage of endoperoxide bridges by ferrous iron, producing reactive intermediates which destroy the malarial parasite [37–40]. Artemisinin also exhibits anti-angiogenic and anti-tumor activity [41].

Annually, 300 million people are afflicted with malaria, and one million will die [42]. To effective-

ly treat the parasite and avoid development of resistance, the World Health Organization recommends artemisinin combination therapies (ACT) for malaria. Yet, access to ACTs is limited in malaria-endemic countries [43]. Given the large number of cases, world demand for artemisinin is 130 tons year⁻¹ [44]. The development of transgenic production platforms for artemisinin, including microbes and plants, is essential to lower artemisinin prices and stabilize supply for millions of people who depend on the drug.

3.2 Biosynthetic pathway of artemisinin

Sesquiterpenes are a class of terpenes that consist of 15 carbons made from three isoprene units in the isoprenoid pathway (Fig. 3). The common sesquiterpene precursor farnesyl diphosphate (FPP) is generated via either the mevalonate-dependent or mevalonate-independent (MEP/DXP) pathway [45, 46]. The committed step of artemisinin biosynthesis is the cyclization of FPP to amorpha-4,11-diene (amorphadiene). This step is carried out by the enzyme amorphadiene synthase (ADS; [47]). Amorphadiene is then oxidized by the cytochrome P450

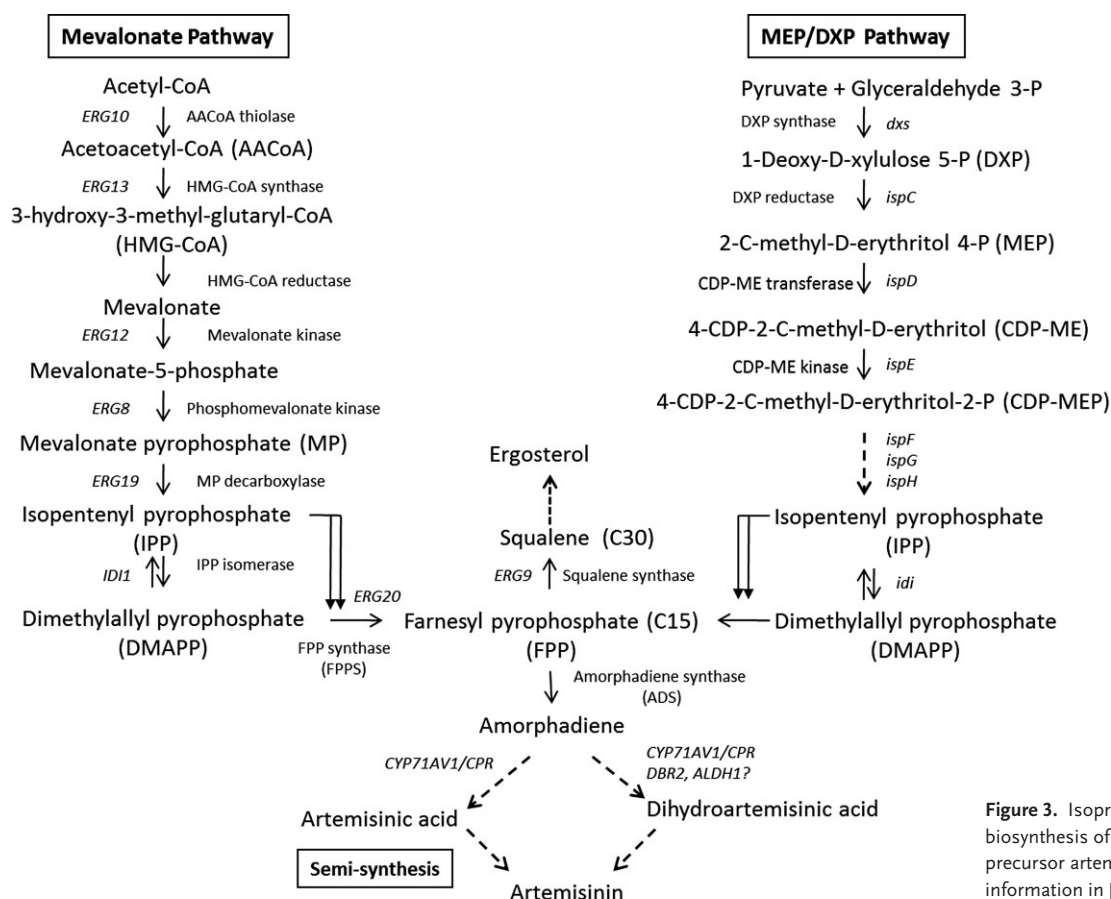


Figure 3. Isoprenoid pathways and biosynthesis of artemisinin and its precursor artemisinic acid (Based on information in [48–51]).

monooxygenase, CYP71AV1, to artemisinic aldehyde [48, 49]. Its reduction to dihydroartemisinic aldehyde is catalyzed by artemisinic aldehyde $\Delta 11(13)$ reductase, BDR2 [50]. A specific aldehyde dehydrogenase is likely involved in the oxidation step to form hydroartemisinic acid [51]. Remaining reactions in the pathway could be non-enzymatic. The side product artemisinic acid, formed by reactions of CYP71AV1, may not be relevant in artemisinin biosynthesis in vivo, but it can be efficiently converted to artemisinin through chemical reactions. This is the basis for semi-synthesis of artemisinin via metabolic engineering.

3.3 Engineering *E. coli* to produce a high level of artemisinin precursor

Instead of using the native MEP/DXP pathway, Keasling expressed the heterologous mevalonate pathway from *S. cerevisiae* in *E. coli* to improve isoprenoid flux [52]. When coupled with the expression of ADS, amorphaadiene (amorpha-4,11-diene) was produced, demonstrating the usefulness of this approach. However, a high-level of expression of the mevalonate pathway resulted in accumulation of pathway intermediates and inhibition of cell growth [53]. Metabolite analysis revealed the accumulation of HMG-CoA, suggesting that the bottleneck was HMG-CoA reductase (HMGR). By modulating the expression level of this enzyme via addition of another copy of the truncated HMGR (tHMGR), the growth rate was restored. To further alleviate this rate-limiting step, bacterial HMGR genes, *mvaA* from *S. aureus* and *mvaE* from *Enterococcus faecalis*, were investigated [54]. The strain containing *mvaA* from *S. aureus* produced the highest titer, compared to those containing the yeast or *E. faecalis* HMGR gene. In light of this success, another strain was constructed to replace the yeast HMG synthase (HMGS) gene with the *S. aureus* HMGS gene *mvaS*. The strain containing both *mvaS* and *mvaA* had an even higher amorphaadiene titer. Under fermentation conditions where carbon and nitrogen were strictly controlled, this strain had a high titer with commercial potential (27.4 g L⁻¹).

3.4 Metabolic engineering of *S. cerevisiae* for artemisinic acid production

As demonstrated in *E. coli*, the key to a high titer is to optimize the isoprenoid pathway flux. In yeast, this was accomplished by the up-regulation of several key genes in the mevalonate pathway and the down-regulation of genes for sterol biosynthesis [48]. The HMG-CoA reductase in the mevalonate pathway is the principal target of complex regula-

tion. Deletion of the N-terminal regulatory region of HMG-CoA reductase increases the carbon flux to isopentenyl diphosphate [55]. Over-expression of tHMGR increased amorphaadiene production by fivefold in yeast. When tHMGR over-expression was combined with reduction in the expression of squalene synthase using the methionine-repressible *MET3* promoter, another twofold increase in titer was obtained. In yeast, the sterol biosynthesis competes for the common precursor FPP (Fig. 3). To further down-regulate the sterol pathway, the *upc2-1* mutant allele was over-expressed. The transcriptional factor UPC2 is a key regulator of yeast sterol uptake [56]. Expression of the mutant *upc2-1* allele allows uptake of exogenous steroids, which inhibits endogenous sterol biosynthesis. After integration of another copy of the tHMGR gene, the final strain produced 150 mg L⁻¹ amorphaadiene, nearly 500-fold higher than previously reported levels.

Further strain improvement [57] and optimization of the fermentation process are in progress to reduce cost. There will be additional cost associated with the chemical process to convert artemisinic acid to artemisinin. Improvement in the chemical process is needed to reach the economic target [58]. The current goal is to produce artemisinin at a price close to the market value of \$350–400/kg. While the microbial source currently is insufficient to meet global demand, microbe-derived artemisinin offers several advantages. Unlike plants which take months to cultivate, fermentation and semi-synthesis can produce artemisinin within weeks in a process independent of field conditions. The microbial fermentation process could therefore supplement and stabilize the artemisinin supply chain [59]. Through the collaboration of Amyris and Sanofi-Aventis, it is projected that by 2012, artemisinin produced by semi-synthesis will be incorporated into ACT for malaria [58].

3.5 Metabolic engineering of plants for artemisinin production

Efforts are underway to increase artemisinin levels in *A. annua* through metabolic engineering. Similar to strategies used in yeast, attempts have been made to increase carbon flux through the isoprenoid pathway for artemisinin biosynthesis in *A. annua*. One example is the expression of HMG-CoA reductase gene. The HMGR gene from *Catharanthus roseus* (L.) G. Don was integrated into *A. annua* using Agrobacterium-mediated transformation [60]. Stable integration of multiple copies of the gene was confirmed by PCR and Southern hybridization. One transgenic line showed a 22.5% in-

A. 2A peptide sequence with conserved amino acids underlined



B. Construct design

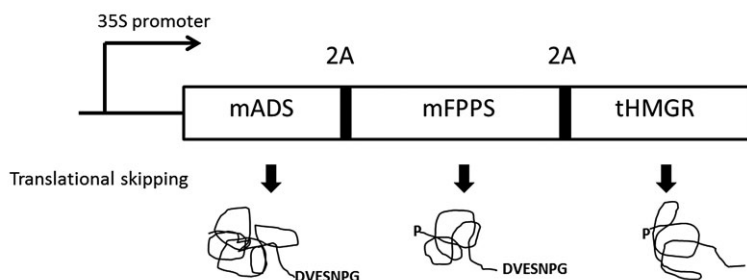


Figure 4. Use of 2A sequence to express multiple proteins as a single open reading frame (Based on information in [65, 67]). mADS, mitochondrial targeted ADS; mFPPS, mitochondrial targeted FPP synthase.

crease in artemisinin, compared to wild-type plants. Subsequent studies combining the expression of *HMGR* and *ADS* genes led to the production of a transgenic line that had a sevenfold higher (1.73 mg g^{-1} dried weight) artemisinin level than the non-transgenic plant [61]. Another example of metabolic engineering in *A. annua* is the down-regulation of the β -caryophyllene synthase (CPS) gene, which competes with ADS for FPP [62]. CPS catalyzes the conversion of FPP to β -caryophyllene. Using *Agrobacterium*-mediated transformation, the antisense fragment (750 bp) of CPS cDNA was transformed into *A. annua*. One transgenic line showed a 54.9% increase in artemisinin, demonstrating the potential of anti-sense technology in metabolic engineering of *A. annua*. Hairpin-RNA-mediated gene silencing of squalene synthase, to reduce competition for FPP, has also been reported [63]; artemisinin levels reached as high as 31.4 mg g^{-1} dry weight.

Expression of a few known genes in the artemisinin biosynthesis pathway was explored in tobacco plants [64]. Unlike *E. coli* or yeast systems, expression of the *ADS* and *CYP71AV1* genes together resulted in production of amorphaadiene and artemisinic alcohol only. No accumulation of artemisinic acid was detected. When these two genes were expressed along with artemisinic aldehyde $\Delta 11(13)$ double-bond reductase (DBR2), dihydroartemisinic alcohol was produced. Again, no accumulation of its acid (dihydroartemisinic acid) was found. Native enzymes in the tobacco plant may favor reduction of intermediates to alcohols. Metabolic engineering in *Nicotiana benthamiana*, a close relative to tobacco, revealed interesting findings [65]. In *N. benthamiana*, expression of the ADS protein was targeted to mitochondria, which is a better

environment for sesquiterpene synthase in *Ara-bidopsis thaliana* [66]. In addition, key genes in the mevalonate pathway, *FPPS* and *tHMGR*, were expressed to improve carbon flux. All three genes were constructed as a single open reading frame under control of a single 35S promoter. The individual proteins were separated by a viral peptide 2A (Fig. 4). During translation, the 2A peptide leads to ribosomal skipping and production of individual proteins [67]. This construct was introduced into leaves by agro-infiltration. The amorphaadiene concentration in the leaves reached 6.2 mg kg^{-1} fw. In addition to mitochondrial expression of ADS protein, the major contributor to the improvement in amorphaadiene concentration was the expression of tHMGR, which was not targeted to mitochondria. This suggests that transport of isoprenoid intermediates to mitochondria is not rate-limiting. Surprisingly, when the three-protein construct was co-introduced to leaves along with the plasmid harboring the *CYP71AV1* gene, amorphaadiene was almost completely converted to artemisinic acid in the form of artemisinic acid-12- β -diglucoside. Apparently, the native glycosyl transferases were very active; the glycosides can be extracted with hydrophilic solvent. After hydrolysis, the desired product can be recovered in an organic phase. The extent to which this extraction will increase costs remains to be determined.

Based on information gathered from *N. benthamiana*, Plant Research International and Dafra Pharma International NV are collaborating to engineer chicory (*Cichorium intybus*) to produce the artemisinin precursor dihydroartemisinic acid in the roots [68, 69]. Dihydroartemisinic acid, like artemisinic acid, can be chemically converted to artemisinin at low cost. As a member of the Aster-

acea family, chicory already produces considerable amounts of sesquiterpene lactones through the isoprenoid pathway. Chicory is a well-established plant for industrial non-food applications, and the entire chain of large-scale agricultural production is already in place. Experiments are underway to evaluate the expression of *HMGR*, *FPS*, and *ADS* in chicory.

4 Metabolic engineering for the production of the anti-cancer agent taxol

4.1 Clinical relevance of taxol

Taxol, generically named paclitaxel, is one of the most important and active chemotherapeutic agents for the clinical treatment of cancer [70]. Taxol is an anti-proliferative agent that stabilizes microtubules and inhibits cell division [71]. Currently, taxol is utilized for therapy of ovarian, breast, bladder, prostate, esophageal, head and neck, cervical, endometrial, and lung cancers as well as AIDS-related Kaposi's sarcoma [72]. Because taxol also blocks intracellular signaling and inhibits smooth muscle cell proliferation, the drug is additionally used in drug-eluting stents for prevention of coronary restenosis [73]. As of 2009, nearly 5 million Taxus® stents had been implanted in patients worldwide [74]. Moreover, taxol may have clinical applications for organ transplantation and autoimmune diseases [75]. Novel controlled release systems for taxol, including micelles [76] and liposomes [77], are being pursued to target delivery of taxol, highlighting the clinical importance of this potent drug.

4.2 Taxol and its sources

Taxol was originally isolated from the Pacific yew tree, *Taxus brevifolia* [78, 79]. Significant structural features of taxol are the taxane core, oxetane ring (D ring), C13-side chain, C2-O-benzoyl group, and other peripheral functional groups (Fig. 1). Taxol prevents cell division by binding to β -tubulin, thereby promoting microtubule assembly and stabilization [71]. This results in mitotic disruption and inhibition of cancer growth.

Clinical use of taxol was initially hampered by the drug's lack of availability. The natural concentration of taxol in Pacific yew trees is very low (0.02% dry weight) and extraction is inefficient. Destruction of thousands of Pacific yew annually would be required to meet taxol demand [80]; this is unsustainable. Chemical synthesis of taxol is not commercially viable due to low yield and high cost

[81–83]. The English yew tree *Taxus baccata* is a rich source of 10-deacetylbaccatin, which can be converted to taxol semi-synthetically [84]. The current commercial source of taxol is the *Taxus* cell line, from which taxol is extracted [85].

4.3 Taxol biosynthetic pathway

Since a microbial process offers higher productivity and flexibility, metabolic engineering of *E. coli* and yeast for taxol production could meet high demand and lower manufacturing costs. Bioengineering of taxol production requires a detailed knowledge of relevant biochemical steps and genes. Three discrete processes are involved in taxol biosynthesis: formation of the taxane core from isoprenoid intermediate, additions/modifications of functional groups on the core, and side chain formation (Fig. 5A; [86, 87]). The first step of the pathway is a committed reaction, carried out by the taxadiene synthase (TS). TS catalyzes the formation of the taxane core, taxa-4(5), 11(12)-diene, using the substrate geranylgeranyl diphosphate (GGDP). GGDP is a universal intermediate from the MEP/DXP pathway (Fig. 3).

Functionalization of the taxadiene core with regiospecific oxygenation is carried out by a series of cytochrome P450 taxoid hydroxylases. The cytochrome P450 taxadiene 5 α -hydroxylase is responsible for the first oxygenation step, yielding taxa-4(20),11(12)-dien-5 α -ol [88]. The acyl CoA-dependent transferases are responsible for acylations at the C5-O- and C10-O-positions and benzoylation at the C2-O position. Several more reactions, including oxidation at C9 and formation of the oxetane ring, are required for biosynthesis of the advanced taxane diterpenoid intermediate baccatin III. Side chain formation starts with attachment of β -phenylalanine to the C13-O-position in baccatin III by the aminoacyl CoA *N*-transferase [89]. The side chain is further modified by hydroxylation at the 2'-position, followed by *N*-benzoylation to complete the pathway.

4.4 Taxol pathway engineering in yeast

Initial engineering of *S. cerevisiae* to produce a taxol precursor was carried out by expressing five genes on plasmids to reconstitute the first five steps of the pathway [90]. These genes were GGPP synthase (GGPPS), TS, cytochrome P450 taxadiene 5 α -hydroxylase, taxadien-5 α -ol-O-acetyl transferase, and cytochrome P450 taxoid 10 β -hydroxylase. Analysis of the product from strains with all five genes shows only the presence of taxadiene, the committed intermediate. The experiment demon-

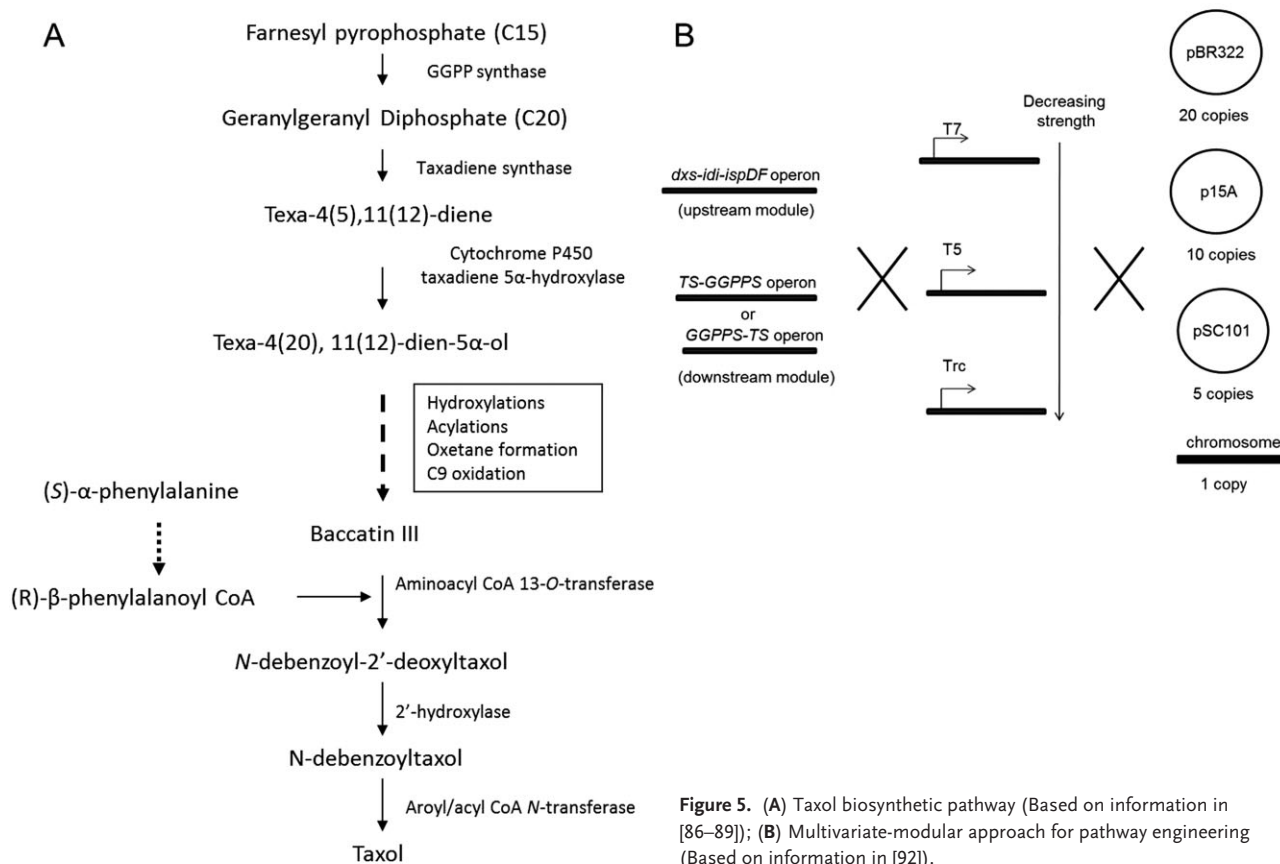


Figure 5. (A) Taxol biosynthetic pathway (Based on information in [86–89]); (B) Multivariate-modular approach for pathway engineering (Based on information in [92]).

strates coupling of the isoprenoid pathway and taxol precursor production, but also reveals a bottleneck step catalyzed by the first cytochrome P450 taxoid hydroxylase. Taxadiene production is low, at 1.0 mg L⁻¹.

Subsequent attempts to engineer the taxol precursor pathway in yeast focused on increasing the carbon flux and the efficient expression of TS [91]. As described in the metabolic engineering of yeast for artemisinic acid production, the HMG-CoA reductase in the mevalonate pathway is the principal target of complex regulation. Deletion of the N-terminal regulatory region of HMG-CoA reductase increases the carbon flux to isopentenyl diphosphate,

which can be used for taxol biosynthesis. Indeed, the strain expressing a truncated HMG1 (tHMG1) has a 50% increase in taxadiene (Table 2). To reduce the impact of sterol biosynthesis, two strategies are taken. The first strategy involves the introduction of the mutant *upc2-1* allele. The second strategy is the expression of a GGPPS that does not use FPP as the substrate. The squalene synthase in the sterol biosynthetic pathway, as well as the GGPPS enzyme from *T. chinensis*, use FPP. However, GGPPS from *S. acidocaldarius* synthesizes GGPP through the sequential addition of dimethylallyl diphosphate (DMPP). Replacing *T. chinensis* GGPPS with *S. acidocaldarius* GGPPS reduces the

Table 2 Metabolic engineering of yeast for taxadiene biosynthesis.

Enzymes or Genes	Taxadiene (mg L ⁻¹)	Geranylgeraniol (mg L ⁻¹)	Total
TS + GGPPS	0.204	n/a	n/a
TS + GGPPS + tHMG1	0.306	0.253	0.559
TS + GGPPS + tHMG1 + <i>upc2-1</i>	0.306	0.283	0.589
TS + GGPPS(Sa) + tHMG1 + <i>upc2-1</i>	0.32	27.6	27.92
TS(o) + GGPS(Sa) + tHMG1 + <i>upc2-1</i>	8.7	33.1	41.8

n/a, not applicable, [91]

competition of the sterol pathway for the isoprenoid intermediate. These two strategies enable a significant increase in geranylgeraniol but not taxadiene, suggesting an increase in flux from the MVA pathway but a possible bottleneck in the expression of TS. Upon optimization of the TS gene from *T. chinensis*, the taxadiene level in the final strain is 8.7 mg L⁻¹, a 40-fold increase. Further improvements will be required.

4.5 Taxol pathway engineering in *E. coli*

Great headway has been made to produce the taxol intermediate taxadiene in engineered *E. coli* [92]. This is accomplished by balancing gene expression, through a “multivariate-modular” approach (Fig. 5B). In this approach, the synthetic pathway is split into two modules. The first module consists of an operon *dxs-idi-ispDF*. Genes in this operon are rate-limiting. The second module is the operon with *TS* and *GGPS* genes. Expression levels of these two operons are controlled by copy numbers (1, 5, 10, or 20 copies) as well as promoters, *Trc*, *T5*, or *T7*, which have different strengths. The impact of gene order for *TS* and *GGPS* in the operon is also tested. A series of strains with different combinations of these two modules at various expression levels are constructed and evaluated for taxadiene production. The best combination has been found in strain 26 where the operon *dxs-idi-ispDF* is under the control of the *Trc* promoter and the entire operon is integrated in the chromosome. In this strain, the downstream module, the *TS-GGPS* operon, is controlled by the *T7* promoter and expressed on pSC101, a low copy number plasmid (5 copies). Under fermentation conditions, the taxadiene concentration in this strain reaches 1 g L⁻¹, over 100-fold higher than that achieved in yeast.

In addition to taxadiene, other intermediates and byproducts in constructed strains are analyzed. Metabolomic analysis indicates the accumulation of an inhibitor that has an inverse relationship with taxadiene biosynthesis. This inhibitor was determined to be indole. Interestingly, strain 26 shows minimal accumulation of this compound. Exogenous addition of >100 mg L⁻¹ indole to strain 26 also severely inhibits taxadiene synthesis; the mechanism remains to be clarified.

Taxol biosynthesis requires up to 19 steps (Fig. 5A). After taxadiene, the next series of reactions are the oxygenation on the taxane core. These reactions are carried out by cytochrome P450 taxoid hydroxylases. In the yeast strain, expression of the first cytochrome P450 hydroxylase, taxadiene 5 α -hydroxylase, proves to be difficult [90]. In general, functional expression of plant cytochrome

P450 in *E. coli* is also challenging. Successful expression of plant cytochrome P450 requires trans-membrane engineering and the construction of a chimeric enzyme containing an additional component, the cytochrome P450-reductase [93, 94]. Such a chimeric enzyme is designed for the taxadiene 5 α -hydroxylase for expression in *E. coli*. One of the constructs is highly efficient in converting the taxadiene to taxadien-5 α -ol and the byproduct 5(12)-Oxa-3(11)-cyclotaxane (OCT). Formation of undesirable OCT is due to non-specificity of the chimeric hydroxylase. Accumulation of OCT complicates the pathway engineering by decreasing flux to later steps. Furthermore, productivity of strain 26 is significantly reduced after the introduction of the chimeric enzyme, leading to an increased indole level. The delicate pathway balance in strain 26 was thus disturbed by the introduction of a new gene. There will be several challenges to engineer other pathway genes. Given the complexity of the pathway for taxol biosynthesis, the balancing act for flux optimization may have to be carried out repeatedly. A much higher selectivity for taxoid hydroxylases and other remaining enzymes in the pathway will be required to optimize the production of desirable intermediates. Despite these challenges, the same multivariate-modular approach can be applied to future pathway engineering in *E. coli* or yeast. The high titer achieved for the taxol precursor taxadiene in the engineered *E. coli* demonstrates the potential of this microbial platform.

5 Conclusion

The biosynthetic pathways for omega-3 fatty acids, artemisinin, and taxol involve complicated biochemical reactions. Natural sources of these molecules are scarce, and total synthesis of these molecules through conventional chemistry is difficult and costly. Metabolic engineering of microbes and plants has enabled the synthesis of all three products, demonstrating the versatility of metabolic engineering to bring economical and sustainable solutions to nutritional and pharmaceutical needs.

These examples of metabolic engineering highlight important factors to be considered for such a project [3]. Characteristics of the host organism have a profound impact on ultimate commercial success. These characteristics include metabolic capabilities, genetic systems, and scale-up potential. *E. coli* grows fast and is easy to manipulate genetically, but may not accommodate enzymes from eukaryotic sources as yeast does. It may be desirable to pursue both hosts in parallel initially. De-

pending on the product of interest, other hosts may be more suitable. In the case of omega-3 fatty acid production, *Y. lipolytica* has proven to be an excellent host due to its friendly genetic system, easy fermentation scale-up, and ability to accumulate oil [14]. As for plant hosts, the competition of native enzymes for pathway intermediates could reduce the carbon flux and lead to formation of unintended products [64, 65]. For plants or even microbes, limitations of the genetic system could present challenges.

Once the optimal host is determined, the strategy for metabolic engineering may involve choosing a specific biosynthetic pathway and enzymes to target. A detailed understanding of the biochemistry and gene regulation networks is essential for pathway construction. Optimization of pathways entails balancing the upstream and downstream steps as well as elimination or reduction of competing reactions. Bottlenecks can be caused by gene expression, protein folding, cofactor availability, and the intrinsic biochemistry of the pathway enzymes. These issues are resolved by codon optimization, tuning of promoters, or copy numbers, and exploration of alternative enzymes. For the biosynthesis of the taxol precursor taxadiene in *E. coli*, optimal balance of upstream and downstream pathways is achieved through a combinatorial approach [92]. For the expression of the heterologous mevalonate pathway in *E. coli*, the bottleneck step, HMG-CoA conversion, is overcome by replacement of the key enzymes HMG-CoA synthase and HMG-CoA reductase with those from *Staphylococcus aureus* [54]. Another reason to choose the appropriate enzyme for pathway construction is that a highly specific enzyme can reduce byproduct formation. For example, some $\Delta 6$ -desaturases in the omega-3 biosynthetic pathway use both ALA and LA as substrates. On the other hand, the desaturase from *Primula vialii* is specific for ALA but not LA. As a result, the engineered linseed which utilizes this enzyme accumulates SDA [30]. When an appropriate enzyme cannot be identified from nature, protein engineering is required.

Technological developments have greatly contributed to metabolic engineering. Large gene constructs can now be synthesized at reasonable cost and speed, which simplifies cloning and codon optimization. Gene shuffling technology coupled with high-throughput screening provides an efficient way to obtain novel enzymes with desired properties. The application of next-generation sequencing expedites the analysis of the genome and transcriptomes of both plants and microbes. Systems biology approaches based on bioinformatics and

biomathematics are increasingly being refined to allow design and optimization of strains. Metabolic engineering techniques will continue to expand, and benefit many areas of medicine.

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6 References

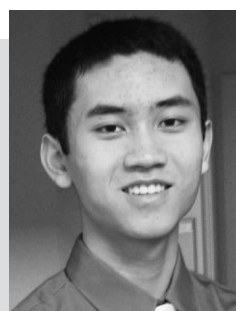
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