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Cloning and enzymology analysis of farnesyl pyrophosphate synthase gene from a superior strain of *Artemisia annua* L.

ZHAO Yujun[1,2], YE Hechun[1], LI Guofeng[1], CHEN Dahua[1] & LIU Yan[1]

1. Molecular Biological Key Laboratory of Photosynthesis and Environment, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China;
2. National Key Laboratory of Biomacromolecule, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

Correspondence should be addressed to Ye Hechun (e-mail: hcye@ns.ibcas.ac.cn)

Abstract **A cDNA(af1) encoding farnesyl pyrophosphate synthase AaFPS1 (FPS, EC2.5.1.1/EC2.5.1.10) from a high yield *Artemisia annua* strain 025 has been cloned from its cDNA library. Sequence analysis showed that the cDNA encoded a protein of 343 amino acid (aa) residues with molecular weight of 39 kD. Deduced aa sequence of the cDNA was similar to FPS from other plants, yeast and mammals, containing 5 conserved domains found in both prenyl transferase and polyprenyl synthase. The expression of the cDNA in *Escherichia coli* showed measurable specific activity of FPS *in vitro*. The enzyme was purified by ion exchange chromatography and its kinetics was measured. These results would further promote the molecular regulation of artemisinin biosynthesis.**

Keywords: artemisinin, farnesyl pyrophosphate synthase (FPS).

Artemisia annua L. (Aa), as a traditional Chinese medicine for treatment of fever, has been used for many centuries in China. Also it is the only source of the most important and clinically useful antimalarial agents called artemisinin (Art), an endoperoxide sesquiterpene lactone. Like Art, many other secondary metabolites of plant isoprenoids are drawing much attention, such as phytoalexins and yewol in human cancer resistance[1]. However, the low level of Art in natural plants, limited resources and high cost of chemical synthesis aroused the scientists' interest to increase the Art content by genetic manipulation of gene encoding enzymes involved in the biosynthetic pathway. So far, it has been known that three enzymes: farnesyl diphosphate synthase, sesquiterpene synthase, squalene synthase are related to the biosynthesis of artemisinin in plants. The transformation of heterologous FPS cDNA to Aa has been studied. The results showed that Art content in transgenic Aa was significantly higher (5-fold—7-fold) than that in wild plants[2—4]. Although the content in transgenic Aa plants has been greatly increased, it is still far from satisfactory[5]. We report here the isolation and analysis of the AaFPS1 cDNA from a high yield strain 025 of Aa and the AaFPS1 activities of products expressed in bacteria.

1 Materials and methods

(i) Materials. Leaves used for RNA extraction were from a high yield strain 025 of *A. annua* L. in Sichuan Province. 4-^{14}C-IPP (57.5 mCi/mmol) was purchased from NEN^{TM} (Boston. MA). Regents and all other biochemicals were bought from Sigma Chemical Co., Gibco Brl.

(ii) cDNA library construction. Total RNA was isolated from *in vitro* leaves of the high yield Aa strain by Trizol reagent (GIBCO BRL) and was refined using Oligo(dT) cellulose column. Double stranded cDNA was synthesized according to manufacture's instructions and purified through spun column. The cDNA and the λgt11 vector was ligated by T4 DNA ligase. Reconstructed phage was packaged by protein and used to transfect *E. coli*. Y1090.

(iii) Cloning of cDNA encoding FPS. The 025 Aa library was screened using PCR method. Primers were designed according to the sequences conserved in many FPS as follows: FPS-R, 5′-ACT ATG TGG ATC TTG TGG AC-3′; FPS-F, 5′-GGA GCA CCA AAA CAG TCT AG-3′, then separately partnered with λgt11F and λgt11R for PCR amplification. The full-length cDNA of AaFPS1 was finally amplified with primer pair F: 5′-TAC AAA TAC CCC CCA ACA CAC ACA CA-3′ and R: 5′-TGC ACC ACC AAA ACT ACA TAC GA-3′. The insert was sequenced to confirm that no unwanted mutation occurred during PCR.

(iv) Construcion of expression vector of AaFPS1 cDNA and induction in *Escherichia coli*. Firstly the primers were designed according to the multi-cloning site of expression vector and encoded region of AaFPS1. Encoded region of AaFPS1 cDNA was obtained by PCR from pGEM-T-Easy vector inserted the AaFPS1 cDNA. The PCR products were digested by endonuclease and then ligated into pET-30a. Transformed *E.coli* BL21 and control cells (not transformed) were placed in 200 mL flask containing LB medium and grown to an A_{600} = 0.6. Isopropyl β-D-thiogalactopyranoside was then added to a final concentration of 1 mmol/L. After 12 h induction at 20℃, the cells were pelleted, washed with buffer A (20 mmol/L Tris, 1 mmol/L EDTA, pH 7.0) and resuspended in 0.5 mL of the same buffer. The cell suspensions were subjected to sonication prior to centrifugation at 12000 *g* to remove unbroken cells and debris. The supernatants were frozen in aliques for protein determination and SDS-PAGE[6].

(v) Farnesyl pyrophosphate synthase assay. Farnesyl pyrophosphate synthase activity was measured by modification of the method described by Reed and Rilling[7]. Assays were performed in 20 mmol/L Tris-HCl, pH 7.0, containing 5 mmol/L $MgCl_2$, 20 mmol/L KF, 10 mmol/L iodoacetamide, 6.75 μmol/L geranyl pyrophosphate, and 3.47 μmol/L isopentenyl pyrophosphate (4-^{14}C-IPP, 1.85 KBq, 57.5 mCi/mmol). Reaction mixtures (100 μL) were preincubated at 37℃ for 2 min prior to addition of bacterial cell extracts (5 μL containing 2—5 μg of protein). 5 min later, the reactions were terminated by addition of 100 μL of 6 mmol/L HCl, and radiolabeled products were extracted with 500 μL hexane and quantitated by liquid scintillation counting. Farnesyl pyrophosphate synthase specific activity was expressed as nanomoles of product formed per minute per milligram of protein[8].

(vi) Purification of AaFPS1 and initial velocity measurements. All protein purification steps were conducted at 4℃. Clarified supernatant of sonicated expressed cells was subjected to ammonium sulfate fractionation, and the fraction precipitation between 35%—65% saturation was collected and dissolved in 10 mL of 0.02 mol/L Tris-HCl buffer pH 7.0 containing 0.1 mol/L NaCl and desalted by dialysis to the same buffer. The resultant solution was applied to a Q-sepharose FF column (16 × 24) for FPLC. The elution was performed with a linear gradient of NaCl from 0.1 mol/L to 0.6 mol/L in 0.02 mol/L Tris-buffer, pH 7.0. AaFPS1 fractions was recovered in fraction 35—44 and eluted proteins were detected at 280 nm. Fractions were analyzed by 12% stacking SDS-PAGE after staining with Coomassie blue R250. Protein concentrations were determined by the Bradford procedure with bovine serum albumin as a standard[9]. Kinetic runs were in duplicate, and the average of the two values was used for calculations. All determinations were made in the linear region of the progress curve where <15% of substrate was consumed. A blank containing buffer was run for each treatment at the same time. Kinetic constants were determined by nonlinear curvefitting of plots of initial velocity versus substrate concentration using a softerware of Origin 6.

2 Results

(i) Cloning and sequence analysis of AaFPS1 cDNA. Two DNA fragments, 850 and 800 bp, were obtained using subsection cloning from 025 cDNA library. Sequencing analysis showed that the two fragments were partly overlapped AaFPS cDNA. So the whole AaFPS cDNA was amplified using a pair of specific primers designed according to the 3′ and 5′ ends.

Sequencing of the AaFPS1 cDNA revealed a 1333 bp fragment. The nucleotide sequence presents 96% identity with a native Aa FPS cDNA[11], encoding a protein of 343 aa, 39022 D. The protein sequence presents 98, 93, 45 identity with those from the native Aa, *Helianthus annus* and human respectively. The amino acid sequence also contained five distinct high similarity regions among all the FPS known[10—12]. The five conserved domains are 46—52, 88—105, 61—65, 91—92, 226—256 (boxed) (see Fig. 1). Compared with the native Aa FPS cDNA sequence, AaFPS1 revealed several single aa residue differences in encoded region (shadow): 4-T(ACC)→I (ATC); 113 P(CCC)→L (CTC); 212-V(GTC)→M(ATG); 268-P (CCC)→A(GCC); 275-T(ACC)→V(GTC); 322-H (CAC) →R(CGC).

These changes may suggest the DNA polymorphisms associated with different *Artemisia annua* L. genotypes used[13].

(ii) Expression and confirmation of the af1. *In vitro* incubation was conducted using transformed bacteria cell extracts. As shown in Table 1, extracts from *E. coli* BL21-af1 afforded easily measurable levels of FPS activity whilst *E. coli* BL21 (no insert) and *E. coli* BL21-af1 (uninduced) gave rise to little detectable FPS catalytic activity. The observed activity was dependent on the addition of GPP (gerenyl pyrophosphate) or DMAPP (dimethylallyl diphosphate)(data not shown) to the reaction mixture, since assays carried out in the absence of this allylic diphosphates showed little activity. Further analysis of the reaction products confirmed that the cell extracts of *E. coli* BL21-af1 have no activity to convert FPP with IPP (isopentanyl diphosphate) into GGPP (geranyl geranyl diphosphate) in the presence of Mn^{2+}. Therefore, the protein encoded by the cDNA has the activity of FPS but not GGPPS (GGPP synthase). These data confirm the identity of the cDNA as encoding FPS of *Artemisia annua*[11].

(iii) Kinetic studies. The results of a typical purification are shown in Fig. 2. 1 μg refined protein was used for 100 μL reaction buffer. Kinetic studies were conducted by using the acid-lability assay to measure the formation of 4-^{14}C-FPP from 4-^{14}C-IPP and unlabeled GPP. Some basic kinetics parameters of the enzyme were evaluated. Initial speed of different concentrations of GPP and FPP was calculated by solving the slope of the time-velocity curve function. The substrate-velocity curve for GPP and IPP (Fig. 3(a) and (b)) fit for the Michaelis-Menton equation (r = 0.99). An apparent K_m 29.4 μmol for IPP and 17.2 μmol for GPP were calculated from the curve founction (Fig. 3(c)).

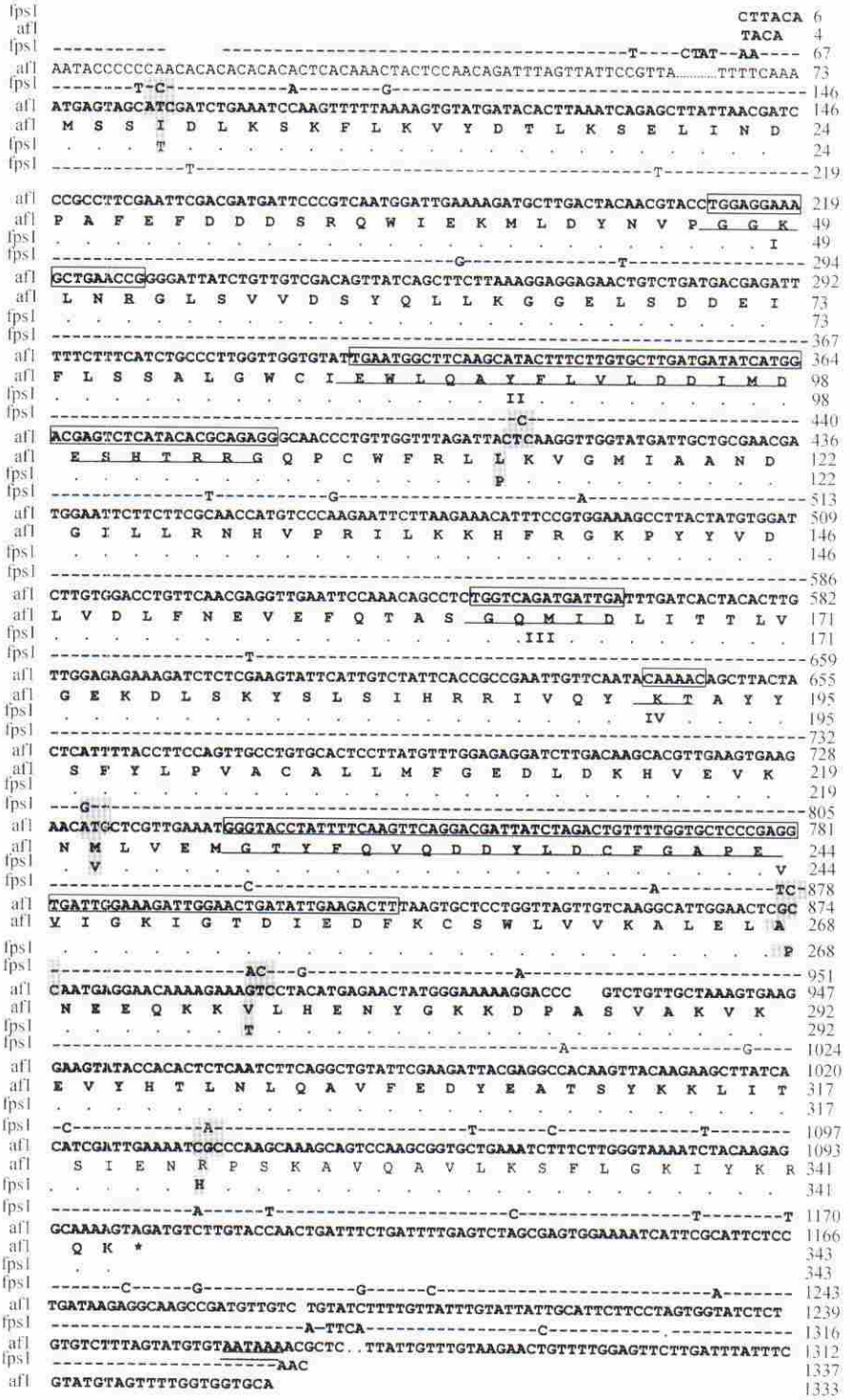

Fig. 1. The complete nucleotide sequence of AaFPS1 cDNA (af1) and alignment with a native Aa FPS cDNA (fps1)[11]. A putative polyadenylation signal is double underlined. Stop codon is denoted by an asterisk. Identical residues are represented by dots. The conserved five regions presented in many prenyltransferase are boxed. The sequence has been submitted to the GenBank/EMBL with accession No. AF112881.

Table 1 Farnesyl pyrophosphate synthase (AaFPS1) activity comparison of whole extracts of *E. coli* cell[a)]

Cell	Plasmid	GPP	IPP	IPTG	FPP	Specific activity /nmol • min^{-1} • mg^{-1}	Standard deviation
BL21	No	+	+	+	–	0.64	0.86
BL21	artfps(+)	+	+	–	–	0.98	0.92
BL21	artfps(+)	+	+	+	–	46.0	0.68
BL21	artfps(+)	–	+	+	+	0.76	0.88

a) Farnesyl pyrophosphate synthase activities were measured in bacterial cell extracts from control cells and cells transformed with plasmids as indicated. GGPPS was judged through the presence (+) and absence (–) of FPP.

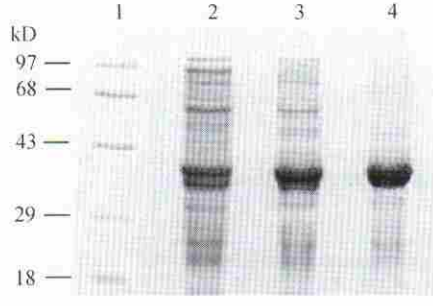

Fig. 2. SDS-PAGE of AaFPS1 samples purified by different methods. 1, Molecular markers; 2, cell-free extract from *E. coli* BL21; 3, ammonium sulfate fraction; 4, recovered active fractions from Q-Sepharose FF.

3 Discussion

Despite its pivotal role in artemisinin biosythesis, little is known about farnesyl pyrophosphate synthase from *Artemisia annua*. A native Aa FPS cDNA sequence was reported in 1996, but its nucleotide sequence changes among different ecotypes, especially the enzymological character, have not been studied.

Our data suggest that the cDNA sequence of the FPS has great changes not only in different species, but also among different varieties in the same species. In this study, many nucleotide sequence changes happened between 025 and native Aa, both in coded region and untranslated region (Fig. 1). Was it suggested that there was a gene family in Aa plant as in *Arabidopsis*? Did the two different cDNAs represent different genes or just differences in different strains[14]? Although it needs further experiments to answer, the lower conservatism certainly reflects the complexity and particularity of FPS.

Table 2 shows that, compared with the reported yeast FPS[10], K_m value for AaFPS1 was 3-fold (K_m^{GPP}) to 9-fold (K_m^{IPP}), however, turnover number (k_{cat}) for AaFPS1 is only 2.2-fold lower at 0.7 μmol • s^{-1}μmol^{-1}. Based on the relationship between K_m value and enzyme-substrate affinity, k_{cat} and enzyme activity, apparently, the activity of AaFPS is significantly lower than the yeast FPS. The affinity of AaFPS for GPP is higher than that for IPP. Because turnover number is mainly determined by disintegrating velocity of enzyme-substrate, conclusion that activity decrease of AaFPS1 is more influenced by lower affinity than by disintegrating of enzyme-substrate. The combination of AaFPS1 with IPP is a bottleneck. The data may tell us why artemisinin content is lower in natural Aa plant. In plants, the FPS gene is crucial for plants to keep adaptability to environment and to enhance disease and adversity resistance. So as an enzyme involved in the secondery metabolism of plants, its adaptable changes may play an important role in systematic evolution[15,16].

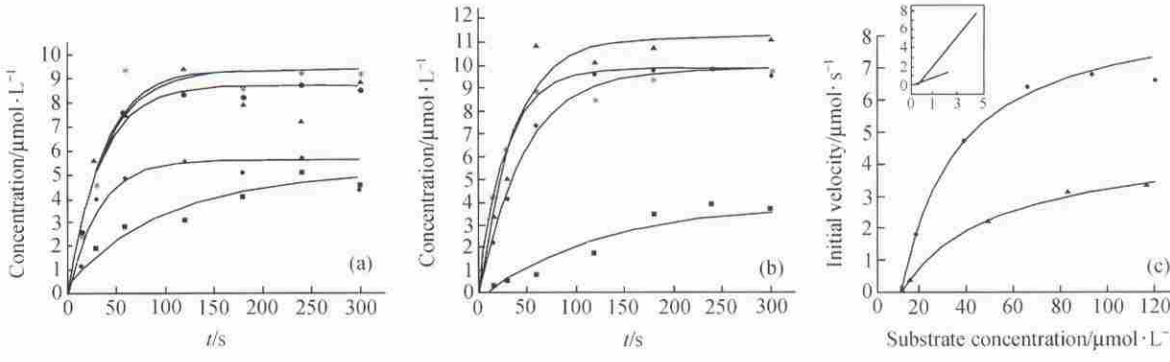

Fig. 3. Kinetics of AaFPS1. (a) Exponential decrease of time-velocity for GPP. *, ▲, ●, ○, ■ represent different GPP concentrations; (b) that for IPP. ■, *, ▲, ● represent different IPP concentrations, (c) Michaelis-Menton curve for GPP and IPP. Buffer for each sample contained 20 mmol/L Tris-HCl (pH 7.0), increasing amounts of GPP or IPP. The inset shows the double-reciprocal plot of $1V^{-1}$ against $1[S]^{-1}$ for GPP and IPP (r = 0.99). ● GPP; ▲ IPP.

Table 2 FPS kinetic constants comparison of different FPS

	K_m^{IPP} /μmol • L^{-1}	K_m^{GPP} /μmol • L^{-1}	k_{cat} /s^{-1}	k_{rel}
AaFPS1	29.4	17.2	0.7	194
YeastFPS	3.4	6.7	1.6	444

k_{cat} was calculated by assuming maximal velocities with 120 μmol IPP and 81 μmol GPP.

Field experiment also showed that the growth rate, growth tendency and resistance to adverse the environment of high yield strain are significantly higher than those of others. Many compounds need FPP as their substrate, so we cannot say that the enhanced activity or overexpressed level of FPS surely leads to the increase of artemisinin content. In fact, the data showed that the discrepancy between different plants and different situations was huge[17]. For Aa, overexpression of FPS, in deed, enhanced the Art level[2,5]. But, whether the high level of artemisinin in 025 strain means the favorable mutation of FPS gene through natural evolution and adaptation selectively survived, or, if the mutation enhanced the FPS activity need to be further studied. Above all, although the secondary metabolism is an important defensive system for plants, over-accumulation of products also is harmful[17]. As an important enzyme for the secondary metabolism of Aa, its lower activity and limited regulation on metabolism might be the main obstacle for biosynthesis of artemisinin[2]. But then, we could attempt to regulate the synthesis of artemisinin through various gene engineering means; for example, transfer a highly active FPS gene, like yeast FPS, into Aa plant, increase the substrate concentration, especially IPP through various means; on the other hand, put the 025 cDNA under a strong promoter and then deliver them into itself, for homologous gene might be easily regulated by its own promoter and translation systems. Transformation of homologous gene is under way. Overexpression of highly active enzymes regulating the artemisinin formation would hold a great potential to improve the production of artemisinin.

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