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A *corpora allata* farnesyl diphosphate synthase in mosquitoes displaying a metal ion dependent substrate specificity

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Abstract

Farnesyl diphosphate synthase (FPPS) is a key enzyme in isoprenoid biosynthesis, it catalyzes the head-to-tail condensation of dimethylallyl diphosphate (DMAPP) with two molecules of isopentenyl diphosphate (IPP) to generate farnesyl diphosphate (FPP), a precursor of juvenile hormone (JH). In this study, we functionally characterized an *Aedes aegypti* FPPS (AaFPPS) expressed in the *corpora allata*. AaFPPS is the only FPPS gene present in the genome of the yellow fever mosquito, it encodes a 49.6 kDa protein exhibiting all the characteristic conserved sequence domains on prenyltransferases. AaFPPS displays its activity in the presence of metal cofactors; and the product condensation is dependent of the divalent cation. Mg^{2+} ions lead to the production of FPP, while the presence of Co^{2+} ions lead to geranyl diphosphate (GPP) production. In the presence of Mg^{2+} the AaFPPS affinity for allylic substrates is $GPP > DMAPP > IPP$. These results suggest that AaFPPS displays “catalytic promiscuity”, changing the type and ratio of products released (GPP or FPP) depending on allylic substrate concentrations and the presence of different metal cofactors. This metal ion-dependent regulatory mechanism allows a single enzyme to selectively control the metabolites it produces, thus potentially altering the flow of carbon into separate metabolic pathways.

Abstract

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Keywords

Farnesyl diphosphate synthase; mosquito; juvenile hormone; prenyltransferases; metal dependence; substrate specificity

INTRODUCTION

Terpenes are the largest class of natural products with more than 55,000 structurally and stereochemically diverse compounds, all of which ultimately originate from the universal 5 carbon precursors dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) (Christianson, 2006). Terpenoids are abundant throughout nature and serve a multitude of functions in plants, animals, bacteria and fungi. They mediate antagonistic and beneficial interactions among organisms, defending them against predators, pathogens and competitors, as well as carrying messages to conspecifics and mutualists regarding the presence of food, mates and predators (Aharoni et al., 2005; Tholl, 2006). Terpenoid synthases or prenyltransferases are ubiquitous enzymes that catalyze the formation of isoprenoids (Vandermoten et al., 2009). They can be divided in two classes characterized by different initiation of catalysis mechanisms. Class I activity is triggered by metal ionization and Class II by protonation of the epoxide ring. Class I enzymes can be further subdivided into three categories, 1) chain elongation enzymes (head-to-tail), 2) irregular isoprenoid condensation (non-head-to-tail) and 3) cyclization enzymes (Lesburg et al., 1998). The chain elongation enzymes from Class I, can be as well divided into enzymes producing short-chain (C_{10} - C_{20}), medium-chain (C_{25} - C_{35}), and long-chain (C_{40} - C_{50}) isoprenoid products (Gershenzon and Kreis, 1999).

Farnesyl diphosphate synthase (FPPS, E.C. 2.5.1.1/2.5.1.10) is a Class I short-chain prenyltransferase. It catalyzes the head-to-tail condensation of DMAPP with two molecules of IPP to generate farnesyl diphosphate (FPP); a C_{15} essential precursor of cholesterol in vertebrates (Kellogg and Poulter, 1997). FPP is also a very important precursor of juvenile hormone (JH), a sesquiterpene synthesized by the *corpora allata* (CA), a pair of endocrine glands connected to the brain, which plays vital roles in insect development and reproduction (Goodman and Cusson, 2012). Despite their importance in insect metabolism, only a few insect prenyltransferases have been functionally characterized (Koyama et al., 1985; Sen et al., 2002; Sen et al., 2007ab; Taban et al., 2009; Vandermontent et al., 2008); with most studies focusing on molecular cloning and functional expression (Castillo-Gracia and Couillaud, 1999; Cusson et al., 2006), and fewer reports on enzymatic characterization.

All insect FPPSs are homo-dimeric enzymes, which are active in the presence of divalent cations, such as Mn^{2+} and Mg^{2+} (Sen et al., 2002; Sen et al., 2007a; Koyama et al., 1985). They have a conserved α -helical prenyltransferase fold and display aspartate rich motifs (DDXXD) that are implicated in metal recognition, as well as initiation of catalysis (Christianson, 2006). Although the metal-dependence of FPPS catalysis has been known for decades (Robinson and West, 1970), it was not until 2013 that a study of an insect prenyltransferase demonstrated a new regulatory mechanism that controls product specificity of FPPS on the basis of the local concentrations of particular metal ions, resulting

in the production of either defense compounds or developmental hormones (Frick et al., 2013; Snyder and Qi, 2013).

A species-specific diverse number of FPPS genes are present in insect genomes (Zhang and Li, 2008; Cusson et al., 2006). The genome of the yellow fever mosquito *Aedes aegypti* contains a single FPPS gene (*AaFPPS*) that is highly expressed in the CA (Nouzova et al., 2011). FPPS catalytic activity in the nanomolar range was described in mosquito CA extracts, with changes in activity that were concurrent with increases and decreases of JH synthesis (Rivera-Perez et al., 2014).

Given the critical role of FPP as a JH precursor, we set out to establish the functional characterization of *AaFPPS*, with a special emphasis on the role of metal ions on product specificity. The bases of our studies consisted of a series of *in vitro* assays testing a diversity of allylic substrates (IPP, DMAPP and GPP) and divalent cations (Co^{2+} , Mg^{2+}). Our results revealed that *AaFPPS* synthesizes farnesyl diphosphate from IPP and DMAPP, as well as from GPP and IPP. The enzyme requires metal cofactors for its activity and produces preferentially FPP in the presence of Mg^{2+} , revealing a switch of product specificity toward GPP in the presence of Co^{2+} . In addition, we showed that in the presence of an equimolar mix of both metal cofactors, *AaFPPS* synthesized a 1:1 proportion of both products, but in significant lower concentrations compared to reactions who only contained one cofactor, suggesting a potential impeding competition of both ions for the metal binding motif (DDXXD).

MATERIAL AND METHODS

Chemicals

Isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), farnesyl diphosphate (FPP), geranyl diphosphate (GPP), farnesol (FOL) and geraniol (GOL) were obtained from Echelon Biosciences (Salt lake City, UT). Geranylgeraniol (GGO) was obtained from Sigma (St. Louis, MO).

Expression and purification of recombinant *AaFPPS*

The full length *AaFPPS* (AAEL003497) was obtained from an *A. aegypti corpora allata-corpora cardiaca* library, constructed and sequenced as previously described (Noriega et al., 2006). The coding region of the *AaFPPS* cDNA was ligated into the pET-28a(+) expression vector (Novagen, Gibbstown, NJ). *E. coli* strain BL21 (DE3) were transformed with the construct and expressed as previously described (Mayoral et al., 2009). Recombinant protein containing a C-terminal His-tag was purified with a cobalt column (Pierce, Rockford, IL) and desalted using a PD-10 column (Amersham, Pharmacia Piscataway, NJ) (Mayoral et al., 2009). Glycerol was added to the isolated protein solution (final concentration 50%), and then aliquoted and stored at -80°C until further analysis. The purity of *AaFPPS* was verified by SDS-PAGE and Western blot using a mouse anti-His antibody as described by Mayoral et al. (2009). Protein concentration was determined by the bicinchonic acid protein assay reagent (BCA) (Pierce, Rockford, IL). Bovine serum albumin was used as a standard.

Phylogenetic analysis and homology modelling

Sequence similarity searches were performed using the alignment tool BLAST (Altschul et al., 1997). FPPS amino acid sequences were obtained from the National Center of Biotechnology Information and Vector Base. Analyses of degrees of similarity among sequences were performed using the ClustalW tool (Larking et al., 2007). *AaFPPS* secondary structure was predicted using PSIPRED version 2.5 (Jones, 1999). Amino acid sequence alignments were performed using Muscle (Edgar, 2004), and maximum-likelihood trees were built using MEGA software version 5.1 (Tamura et al., 2011), with a bootstrapping value of 1000. A pairwise deletion method was selected for the gap/missing data. *AaFPPS* was modeled using the protein structure homology-modeling server Swiss v. 8.05 and avian FPPS (PDB code 1UBX) as template.

Enzyme assays

Purified recombinant *AaFPPS* was used to test substrate specificity and cofactor requirements. *In vitro* *AaFPPS* enzymatic assays were performed in 50 mM Tris-HCl pH 7.5, containing 5 mM MgCl₂ as cofactor, and 100 μM allylic substrate (DMAPP and IPP) in a volume of 100 μL. Reactions were incubated for 1 h at 37 °C, subsequently 50 μL of 2.5 N HCl was added and samples were incubated for 10 min to convert the diphosphates into the corresponding alcohols. Afterward, 500 μL of hexane was added, samples were vortexed for 1 min and centrifuged at 14,000 rpm for 10 min at 4 °C. Organic phases (containing the alcohols) were recovered, filtered through a 0.2 μm nylon filter, dried under nitrogen and stored at -20 °C until use.

Reactions products were analyzed by Reverse-phase HPLC on a Dionex Summit System (Dionex, Sunnyvale, CA) equipped with a UVD 170U detector, 680 HPLC pump, TCC 100 column oven and Chromeleon software. HPLC was performed on an analytical column Acclaim 120 C₁₈ (250 × 2.1 mm ID, particle size 5 μm; Dionex). Products were separated using a isocratic elution from 0 to 20 min (acetonitrile-water, 1:1 v/v), followed by a linear gradient from 20 to 50 min (acetonitrile-water, 50-95% v/v); held at 95% acetonitrile for 15 min at a flow rate of 0.2 mL/min. The eluate was monitored at 214 nm. Water was used in place of recombinant enzyme in negative controls.

Effect of divalent metal ion cofactors

The effect of divalent cations (Co²⁺, Mg²⁺) on FPPS activity and product formation was tested *in vitro* using different concentration of metal cofactor (0 – 10 mM) in reaction mixtures containing IPP and DMAPP (100 μM each) and 200 ng of recombinant protein. The competitive effect of two metal cofactors on *AaFPPS* activity was tested by adding optimum concentrations of each metal ion into reaction mixtures containing IPP and DMAPP (100 μM each) and IPP and GPP (100 μM each). Reaction conditions and product analysis were as described above.

Kinetic studies

The K_m values for IPP, DMAPP and GPP were obtained by double reciprocal Lineweaver-Burk plots of the amount of total allylic diphosphate product formed at increasing

concentrations of substrate. K_m values for DMAPP and GPP were in the range of 10-100 μM , with a fixed IPP concentration of 100 μM . The K_m value for IPP was determined for concentrations between 10-100 μM with fixed 100 μM GPP. Assays were conducted with 200 ng of recombinant protein in a final volume of 100 μL as described above. Samples were stored at -20°C until analysis. Reaction products were analyzed by HPLC.

Statistical analysis

Data were analyzed for statistical significance using GraphPad Prism (GraphPad Software, San Diego CA). The results were expressed as means $\pm$ S.E.M. Significant differences ($P < 0.05$) were determined with one tailed students t-test performed in a pairwise manner.

RESULTS

Molecular characterization of *A. aegypti* FPPS

The full-length *AaFPPS* ORF is 2263 bp long and encodes a 340-aa protein with a calculated molecular mass of 49.6 kDa and a pI of 8.41. Analysis of the *AaFPPS* sequence revealed the presence of several critical domains characteristic of isoprenyl diphosphate synthases (Koyama et al., 1993; Wang and Ohnuma, 2000), including the two aspartate rich motifs named FARM (DDAMD, D₁₈₃-D₁₈₇) (Fig. 1) and SARM (QDDFLD, Q₃₁₈-D₃₂₃) (Supplementary Fig. 1).

AaFPPS shares ~50% identity to other insect prenyltransferases, and 36.9% identity to the human FPPS (P14324). A phylogram of selected insect and vertebrate prenyltransferases revealed the existence of a clade of type-I FPPSs lepidopteran species, displaying a distinct FARM motif, where NDXXE replaces the classical DDXXD (Fig. 1) (Koyama et al., 1993). The remaining insect sequences analyzed, including *AaFPPS*, are FARM type II enzymes, exhibiting at least one aromatic amino acid at the -5 or -4 positions adjacent to the FARM motif, indicative of a potential role as a short-chain prenyltransferase (Wang and Ohnuma, 1999). Additional non-conservative substitutions were observed among insect prenyltransferases, such as the presence of flexible amino acids (S, A, L or V) at the -5 and -4 positions. These substitutions suggest that these FPPSs could also act as geranylgeranyl diphosphate synthases (GGPPS) (Wang and Ohnuma, 2000). *A. aegypti* possesses a distinctive GGPPS (AAEL004900); distinguished from *AaFPPS* by the two amino acids adjacent to the FARM domain, which are **VFLICDDAMD** (FPPS) and **SSLIDDIED** (GGPPS).

Structural analysis of the active site of *AaFPPS*

The molecular model of *AaFPPS* was built by homology modelling using as template the avian FPPS (1UBX) that exhibits a 43.87% identity to *AaFPPS* (Fig. 2A). Major differences were observed on the -4 and -5 amino acids adjacent to the FARM motif, with *AaFPPS* having V₁₇₈ and F₁₇₉ and the avian protein A₁₁₂ and S₁₁₃ (Fig. 2B). FPPSs' product chain length is determined by the size of the hydrophobic pocket in the active center, therefore these amino acid substitutions define product chain length specificity (Wang and Ohnuma, 1999). The avian FPPS shows a larger cavity in the vicinity of the pocket, which facilitates the processing of longer chain products (Tarshis et al., 1996), while in the mosquito, the

presence of F₁₇₉ likely contributes to prevent the elongation of long chain products, as it has been established for the human FPPS (Wilkin et al., 1990).

Functional characterization of AaFPPS

The recombinant AaFPPS was identified from *E. coli* extracts using an anti-His antibody (Supplementary Fig. 2). AaFPPS synthetic activity was measured by RP-HPLC. AaFPPS condensed IPP and DMAPP in the presence of Mg²⁺ to produce FPP; however in the presence of Co²⁺, IPP and DMAPP produced almost 3 times more GPP than FPP (Fig. 3). In an attempt to establish if under optimal conditions there was a preference for the use of Mg²⁺ or Co²⁺, we measured enzyme activities in the presence of an equimolar quantity of both metals (5 mM) (Fig. 3). Having equal access to both metal cofactors resulted in a 1:1 proportion of both products, but in significantly lower concentrations compared to reactions which only contained one metal cofactor. As described for other insect FPPSs, the mosquito enzyme was inactive in the absence of metal cofactors (Frick et al., 2013).

In order to better understand the ratios of product accumulation (GPP versus FPP) in the presence of Mg²⁺ or Co²⁺, recombinant AaFPPS was incubated with different cofactor concentrations (0 – 10 mM) (Fig. 4). Concentrations of Mg²⁺ as low as 0.001 mM were sufficient to efficiently synthesize FPP (239.8 ± 14.8 µmol/mg/min). AaFPPS exhibited optimum catalytic activity at 0.05 mM Mg²⁺ (328.1 ± 79.8 µmol/mg/min), with a 4:1 FPP/GPP product ratio. A complete different reaction product profile was observed when Co²⁺ was used as cofactor. A very low cobalt concentration (0.001 mM) allowed the enzyme to produce small amounts of FPP (52.4 ± 7.2 µmol/mg/min); as Co²⁺ concentrations increased, FPPS started to produce higher concentrations of GPP than FPP, showing almost exclusive production of FPP with cobalt concentrations over 0.2 mM, and optimum catalytic activity at 5 mM (842.4 ± 45.3 µmol/mg/min).

Alternatively, we tested FPPS activity at a constant Mg²⁺ or Co²⁺ concentrations (5 mM), while increasing substrate concentrations (IPP and DMAPP). AaFPPS produced almost exclusively FPP in the presence of Mg²⁺ at all concentrations tested. On the contrary, in the presence of Co²⁺, both products (FPP and GPP) were produced, with an increased specificity for GPP as substrate concentration increased, to attain higher levels of GPP at a substrate concentration of 100 µM (430.0 ± 52.2 µmol/mg/min) (Fig. 5). Remarkably, in the presence of IPP and GPP, AaFPPS was able to synthesize FPP as well as traces of GGPP (Fig. 6).

Kinetic analysis of AaFPPS

The kinetic parameters measured for AaFPPS are shown in Table 1. In the presence of Mg²⁺ and a fixed concentration of IPP, AaFPPS had a K_m of 55 ± 12 for DMAPP and a K_m of 17 ± 8 for GPP; suggesting that GPP is a preferred substrate compared with DMAPP. Significant V_{max} differences were observed between GPP (72 ± 8 µmol/mg/min) and DMAPP (666 ± 65 µmol/mg/min). When GPP was fixed in the presence of Mg²⁺, the K_m was 24 ± 3 µM. Our results indicated that in the presence of Mg²⁺ the preference for substrate is GPP>IPP>DMAPP (Supplementary Fig. 3).

DISCUSSION

Short-chain isoprenyl diphosphate synthases are a class of prenyltransferases that includes geranyl diphosphate synthase (GPPS), farnesyl diphosphate synthase (FPPS) and geranylgeranyl diphosphate synthase (GGPPS), which synthesize geranyl diphosphate (GPP) (C₁₀), FPP (C₁₅), and GGPP (C₂₀), respectively. While FPPS and GGPPS are ubiquitous in nature, GPPS is largely restricted to plant species (Sommer et al., 1995; Burke et al., 1999), but has been also described in insects (Gilg et al., 2005). Isoprenyl diphosphate synthases catalyze the head-to-tail condensation of DMAPP with two molecules of IPP to sequentially generate GPP, FPP and GGPP (Kellogg and Poulter, 1997). The specificity and ratio of products generated depends on the interactions established in the catalytic pocket between allylic substrates (IPP, DMAPP, GPP and FPP) and divalent metal cofactors (Co²⁺, Mg²⁺ or Mn²⁺), with the reaction proceeding and terminating precisely at a specific carbon length (10, 15 or 20) according to the enzyme's product chain length specificity (Kellogg and Poulter, 1997).

There are several examples of insect diphosphate synthases displaying “catalytic promiscuity”, and changing the type and ratio of products released (GPP or FPP) depending on allylic substrate concentrations (Sen et al., 2007a; Vandermoten et al., 2008) or the presence of different metal cofactors (Sen et al., 2007b; Frick et al., 2013). In insects, FPPSs are typically present as a single-copy gene, with exceptions in Hymenoptera, Homoptera and Lepidoptera (Vandermoten et al. 2009). In the Lepidoptera, FPPSs have long been suspected of exhibiting structural features allowing them to accommodate the bulkier homologous substrates and products used as precursors of ethyl-branched JHs (Cusson et al. 2012).

We set out to establish the molecular bases of substrate specificity for *AaFPPS*, as well as the role of substrate concentration and metal ions on its catalytic specificity. Sequence analysis and homology modelling indicated that *AaFPPS* is a homodimeric protein, with each subunit containing a single site for chain elongation. *AaFPPS* showed a conserved alpha structure with the avian FPPS (Fig 1A), with a catalytic pocket located in a central cavity formed by a bundle of 10 α -helices. The two critical aspartate rich sequences (DDXXD) were positioned on opposite sides of the cavity, and two hydrophobic amino acids (V₁₇₈ and F₁₇₉) adjacent to the first aspartate rich motif were sitting at the bottom of the pocket. These two aromatic amino acids would limit the access of long chain substrates to the hydrophobic pocket (Wang and Ohnuma, 1999, 2000), defining structurally *AaFPPS* as a Class I short-chain FPPS. The dimension of the cavity suggests that there is enough space to incorporate GPP into the binding pocket and elongate it to FPP; this assumption was corroborated in our *in vitro* assays, where recombinant *AaFPPS* under different conditions produced GPP, FPP and even traces of GGPP.

In the FARM motifs of GGPPSs, hydrophobic amino acids are replaced by aliphatic amino acids, which are more flexible residues and allow the entrance of longer substrates into the pocket (Wang and Ohnuma, 2000). It has been reported that the lepidopteran *Manduca sexta* CA possesses GGPP synthase (GGPPS) activity; significant amounts of GGPP were produced by larval and adult *M. sexta* CA homogenates when DMAPP was used as the

allylic substrate (Sen et al., 2007b). AaFPPS was also able to synthesize traces of GGPP in the presence of IPP and GPP.

The function of the divalent metal cation is to anchor the diphosphate moieties and to facilitate ionization of allylic substrate (Brems and Rilling, 1977). Sen et al. (2007b) reported metal- dependent changes of product chain length in CA crude homogenates of *M. sexta*; showing that FPP formation was stimulated by adding Mg^{2+} , whereas GPP formation increased in the presence of Mn^{2+} . Mosquito FPPS, also produced preferentially FPP in presence of Mg^{2+} , and the affinity for GPP in the presence of Mg^{2+} was similar to those described in other insect species, such as *Drosophila melanogaster* (7 μM) (Sen et al., 2007a), *M. sexta* (0.8 μM) (Sen and Sperry, 2002), *Myzus persicae* (25.4 μM and 15.4 μM) (Zhang and Li, 2012), *Aphis gossypii* (12.6 μM) (Ma et al., 2010) and *Phaedon cochleariae* (1.1 μM) (Frick et al., 2013). AaFPPS only displayed a switch on the product released when Co^{2+} was used as divalent cofactor, as described in the horseradish leaf beetle *P. cochleariae* (Frick et al., 2013). Interestingly, the simultaneous presence of both cofactors, Mg^{2+} and Co^{2+} , did not lead to increased production of a single product; instead it reduced the synthesis of FPP and GPP, which suggests that a competition of both ions for the metal binding motif might interfere with catalysis.

Substrate concentration and metal ion might regulate product specificity through changes of the conformation of the catalytic pocket. Frick et al. (2013) reported that the quaternary structure of *P. cochleariae* FPPS is different when the protein is coordinated with Co^{2+} or Mg^{2+} ; resulting in different abilities to accommodate and process allylic substrates. This metal ion-dependent regulatory mechanism allows a single enzyme to selectively control the metabolites it produces, thus potentially altering the flow of carbon into separate metabolic pathways. Whether this phenomenon is of any functional significance in mosquitoes *in vivo* remains to be determined, however this type of 'flexible' enzyme may provide insects faster mechanisms for the generation of the chemical diversity critical to adjust to developmental or environmental changes (Snyder and Qi, 2013).

In summary, mosquito *A. aegypti* possess a single FPPS, which is able to synthesize GPP and FPP in different proportion depending on the metal cofactor; since the mosquito does not possesses a specific GPPS in its genome, it is not surprising that AaFPPS has evolved to synthesize GPP and FPP under different cellular environmental conditions, since these two metabolites are important precursors of the mevalonate and juvenile hormone pathway in insects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A farnesyl diphosphate synthase is expressed in the mosquito *corpora allata* (CA).
- It synthesizes farnesyl-PP (FPP), a precursor of juvenile hormone.
- In the presence of Mg^{2+} synthesizes FPP from isopentenyl-PP (IPP) and dimethylallyl-PP (DMAPP).
- In the presence of Co^{2+} synthesizes geranyl-PP (GPP) from IPP and DMAPP.
- In the presence of an equimolar mix of metals synthesizes lower concentrations of GPP and FPP in a 1:1 proportion.

Fig. 1. Phylogenetic analysis of prenyltransferases from selected insects and vertebrates
Two main cluster are displayed (FPPS and GGPPS). The first cluster includes FPPS type-I enzymes from lepidoptera: *Agrotis ipsilon* (CAA08918), *Bombyx mori* (BAF62113) and *Choristoneura fumiferana* (AAY26575), and FPPS type-II from coleoptera: *Ips pini* (AAX55631), *Phaedon cochleariae* (AGE89831) and *Tribolium castaneum* (NP_001164089), heteroptera: *Rhodnius prolixus* (RPRC014226), diptera: *Drosophila melanogaster* (NP_477380), *Aedes aegypti* (XP_001663796) and *Anopheles gambiae* (AGAP007104), hymenoptera: *Bombus terrestris* (NP_001267835) and *Nasonia vitripennis*

(XP_001604464), hemiptera *Acyrtosiphon pisum* (XP_001950423) and vertebrate: *Homo sapiens* (P14324) and *Gallus gallus* (P08836). The second cluster includes GGPPS from similar species: *B. mori* (XP_004931181), *C. fumiferana* (AGW99945), *T. castaneum* (XP_971444), *R. prolixus* (RPRC000062), *D. melanogaster* (NP_523958), *A. aegypti* (AAEL004900), *An. gambiae* (AGAP006894), *B. terrestris* (NP_0012678471), *N. vitripennis* (XP_001605679), *A. pisum* (XP_008184262), *H. sapiens* (NP_001032354) and *G. gallus* (XP_424685). The first aspartic rich motif is shown for each sequence. Blue amino acids delimitates the specificity and red amino acids are involved in metal binding.

Fig. 2. A) Overall structure of AaFPPS

AaFPPS homology model (light blue) with the superimposed avian model *G. gallus* FPPS (1UBX, with A₁₁₂/S₁₁₃F) (dark blue). B) Detailed view of the first and second aspartic rich motifs. FARM and SARM interactions with the metal cofactor (red residues) and farnesyl diphosphate (FPP) (blue residues).

Fig. 3. *AaFPPS* activity depends on the metal cofactor available

Recombinant *AaFPPS* was incubated in the presence of IPP and DMAPP (100 μM each) and either Mg²⁺, Co²⁺ or an equimolar mixture of both metals (5 mM). The production of GPP or FPP was monitored by HPLC. Bars represent the means ± S.E.M. of three replicates (unpaired t-test, ***P*<0.01).

Fig. 4. Effect of cofactor concentration on product specificity

Recombinant AaFPPS was incubated in the presence of IPP and DMAPP (100 μM each), and increasing concentrations of Mg^{2+} or Co^{2+} . The production of GPP or FPP was monitored by HPLC. Bars represent the means $\pm$ S.E.M. of three replicates.

Fig. 5. Effect of substrate concentration on AaFPPS product specificity

Recombinant AaFPPS was incubated in the presence of increasing concentrations of IPP and DMAPP (10 - 100 μM each), in the presence of optimal concentrations of Mg²⁺ or Co²⁺ (5mM). The production of GPP or FPP was monitored by HPLC. Bars represent the means ± S.E.M. of three replicates.

Fig. 6. Activity of AaFPPS using IPP or GPP as substrate

Recombinant AaFPPS was incubated in the presence of IPP and GPP (10 - 100 μM each), in the presence of either Mg²⁺ or Co²⁺ or an equimolar mixture of both metals (5 mM). The production of FPP and GGPP was monitored by HPLC. Bars represent the means ± S.E.M. of three replicates.

Table 1Kinetic constants for *Aa*FPPS with different allylic substrates.

Substrate 1	Substrate 2 (fixed concentration)	Vmax ($\mu\text{mol/mg/min}$)	Km (μM)
IPP	GPP (100 μM)	120.6 $\pm$ 4.1	24.5 $\pm$ 2.7
DMAPP	IPP (100 μM)	665.9 $\pm$ 65.1	54.8 $\pm$ 11.7
GPP	IPP (100 μM)	71.8 $\pm$ 8.0	17.3 $\pm$ 7.8