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In Silico Analysis of Ent-Kaurene Synthase shows Similar Functional Domain with Levopimaparadiene Synthase Involved in Biosynthesis of Ginkgolide

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Abstract

Ginkgo biloba is popularly known as living fossil. This plant is very popular due to presence of its novel phyto-constituents. Its posses very high amount of flavonoid and terpene trilactones. Ginkgolide and bilobalide are diterpenes and popularly known as terpene trilactone. The biosynthetic pathways for synthesis of terpene trilactone is yet to elucidate, but little and debatable information is present so far. The major gene levopimaradiene synthase is considered as the precursor enzyme for synthesis of ginkgolide molecule. Chloroplast precursor ent-kaurene synthase share common domain structure and conserved amino acid motif with levopimaradiene synthase, involved in diterpene synthesis. So, levopimaradiene synthase may evolved as chloroplast ent-kaurene synthase in higher plants and involved in diterpene synthesis.

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Introduction

G. biloba is popularly known as living fossil and still exist since the period of Mesozoic and Cenozoic era of evolutionary history. This plant is resistance against wide variety of biotic and abiotic stress (Mohanta, 2012). This is due to presence of wide variety of secondary phytoconstituents (Mohanta *et al.,* 2012). The plant has been well investigated chemically for various classes of novel phyto-constituents. However terpene trilactones (ginkgolide and bilobalide) and flavonoids are considered the main, novel and innovative bioactive constituents (Ding *et al,* 2007). Ginkgolides were first isolated by Furukawa (1932) from *G.biloba* root bark. Later, their structures were determined by Maruyama *et al.,* (1967 a-d) and called ginkgolides A, B, C and M (figure 1). The only differences between these compounds are the number and the position of hydroxyl groups which may be present at C1, C3 or C7 of the spirononane frame work (table 1). Bilobalide is closely related to the ginkgolides and the structure was elucidated by Nakanishi and Habaguchi (1971). Ginkgolides are the diterpenes having cage like structure consisting of six 5- membered carbocyclic rings, three lactones, a tetrahydrofuran and a tetra butyl group in their structure. Bilobalide is a sesquiterpene and differs by the absence of tetrahydrofuran ring to that of ginkgolides. Terpene trilactones are extraordinarily stable despite the presence of the multiple oxygen functional groups (Stomgaard and Nakanishi, 2004). The quantities of terpene trilactones can vary greatly with small changes in parameters such as collection site, harvest time and plant growth stage (Ding *et al.,* 2007). Analysis of terpene trilactones, including bilobalide and ginkgolides A, B, C and J has long been difficult because of poor UV absorption due to the lack of good chromospheres and relatively low concentrations in leaves (Van Beek, 2002).

Biosynthesis of Ginkgolide

The biosynthetic pathways of synthesis of ginkgolide and bilobalide is yet to elucidate due to complex framework of these molecules (Schepmann *et al.,* 2001). Previously it was thought that the ginkgolide biosynthesis occurs via mevalonate pathways and, dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) are the precursor molecules (Nakanishi and Habaguchi, 1971). In this process three molecules of acetyl coenzyme A react and are reduced to give mevalonic acid, which then phosphorylated followed by elimination of phosphate and CO_2 to give DMAPP and IPP. But later, it has been found that, non-mevalonate pathway plays the role for synthesis of ginkgolid (figure 3) (Rohdich *et al.,* 2001; Eisenreich *et al.,* 1998; Schwarz and Arigoni, 1999). Non-mevalonate pathway is commonly known as deoxyxylulose phosphate pathway, in which pyruvate and glyceraldehyde 3-phosphate (GAP) react to produce 2C-methyl-D-erythritol 2,4-cyclodiphosphate and ultimately DMAPP and IPP (Stomgaard and Nakanishi, 2004). The first enzyme, 1-deoxy-D-xylulose 5-phosphate (DXP) synthase (DXS), catalyzes the condensation of pyuruvate and GAP to yield DXP (Schepmann *et al.,* 2001). The DXS-catalyzed step is under regulation by multiple copies of the enzymes. 1-hydroxy-2-methyl-2-(E)-butenyl 4-

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diphosphate (HMBPP) is reduced by HMBPP reductase / isopentenyl diphosphate/dimethylally diphosphate synthase to produce isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP react to produce the universal diterpene precursor geranylgeranyl pyrophosphate in presence of levopimaradiene synthase, which is converted into tricyclic intermediate levopimaradiene (Schepmann *et al.,* 2001; Stomgaard and Nakanishi, 2004). This leads to synthesis of dihydroabietane which is transported from plastid to cytoplasm. Dehydroabietane then converted to ginkgolide through complex series of reactions involving several oxidation steps (Kim *et al.,* 2008).

Materials and Methods

Levopimaradiene synthase gene of *Ginkgo biloba* was downloaded from uniprot (Q947C4) and *Oryza sativa* ent-kaurene (LOC_Os02g17780.1) gene was downloaded from rice genome database and *Arabidopsis thaliana* ent-kaurene (At1g79460.1) gene was downloaded from TAIR (The Arabidopsis information resource). Amino acid sequence alignment was carried out using Multalign software. To understand the presence of conserved functional domain, SWISS model was used to find out potential conserved domain of LPS, ent-kaurene synthase gene of rice and *Arabidopsis* respectively.

Results and Discussion

A gene popularly known as ent-kaurene synthase (ent-copalyl-diphosphate diphosphate lyase) present in chloroplast is involved in cyclization process thereby transforming it to diterpene (McGarvey and Croteau, 1996). Major substrate of ent-kaurene synthase is ent-copalyl diphosphate, which converted into ent-kaurene and diphosphate. Ent-copalyl diphosphate produces from geranylgeranyl pyrophosphate in presence of ent-copalyl synthase. Lin *et al.,* (2011) reported the involvement of geranygeranyl-diphosphate in ginkgolide/bilobalide biosynthesis. Molecular analysis shows chloroplast ent-kaurene synthase gene of angiosperm shows similar domain feature with *Ginkgo biloba* levopimaridiene synthase (figure 4). Each of these gene contains conserved terpene synthase like domain (N-terminal terpene synthase domain) and metal binding domain (C-terminal metal binding domain) (figure 5). The terpene synthase/metal binding domain of rice ent-kaurene synthase is smaller than that of LPS terpene synthase/metal binding domain. Amino acid alignment of *Ginkgo biloba* levopimaradiene synthase with rice ent-kaurene synthase (OsENTK) and Arabidopsis ent-kaurene synthase (AtEntK) shows more than 60% homology with conserved domains (figure 5). The comparative protein model of ent-kaurene synthase and *Ginkgo bilob*. The proposed phosphorylation motif of ent-kaurene synthase and levopimaradiene synthase are conserved G-S-X-X-X-S-P-A (G-S-L-H-S-S-P-A for ent-kaurene synthase and G-S-F-L-S-S-P-A for levopimaradiene synthase) (figure 5). This suggests that, a common protein kinase reversibly phosphorylates ent-kaurene synthase and LPS suggesting their common function. So, it can be speculated that ent-kaurene synthase and levopimaradiene synthase leads to synthesis of ginkgolide either independently or collectively and role of ent-kaurene synthase can't be ruled out (figure 6). Ent-kaurene synthase gene from Ginkgo biloba did not reported so far. Either levopimaradiene synthase gene of gymnosperm evolved as ent-kaurene synthase gene of angiosperm during the course of evolution.

Earlier it was also reported that, the concentration of terpene trilactone content decreases from spring to autumn and again its level increases in summer reaching highest terpene level in month of August (Van Beek, 2002; Ding *et al.,* 2007). Concentration of Ginkgo biloba ent-kaurene / kaurenoic acid also decreased from spring and reaches maximum in summer, a similar trends as found in case of terpene trilactone (Faccio *et al.,* 2004). Kaurenoic acid synthesis occurs from oxidation-reduction process of ent-kaurene in presence of NADP oxido-reductase. So, the role of ent-kaurene synthase gene in ginkgolide biosynthesis cannot be ruled out.

Conclusion

Great effort has been given by different scientist to elucidate the biosynthetic route for synthesis of gingkgolide and bilobalide, but still no exact biosynthetic pathway has been elucidated. Common functional domain of ent-kaurene synthase with that of LPS can provide some more depth into its biosynthetic route. Biochemical study including over expression of ent-kaurene synthase in LPS mutated cell line can answer this question.

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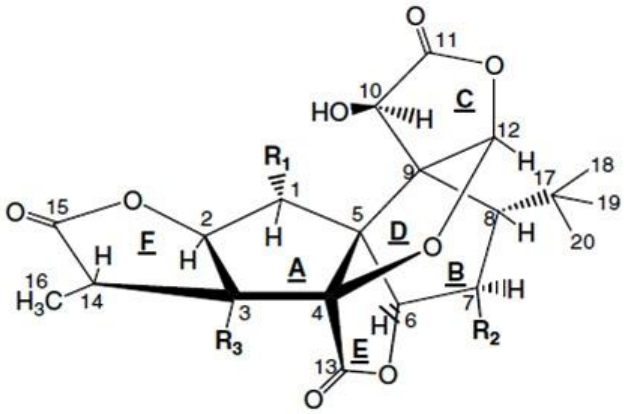
Figure 1. Structural back bone of ginkgolide molecule. R1, R2, R3 represents corresponding side chain functional groups of different ginkgolide molecule.



Ginkgolide	R^1	R^2	R^3
Ginkgolide A (GA)	H	H	OH
Ginkgolide B (GB)	OH	H	OH
Ginkgolide C (GC)	OH	OH	OH
Ginkgolide J (GJ)	H	OH	OH
Ginkgolide M (GM)	OH	OH	H

Figure 2. Chemical structure of bilobalide molecule

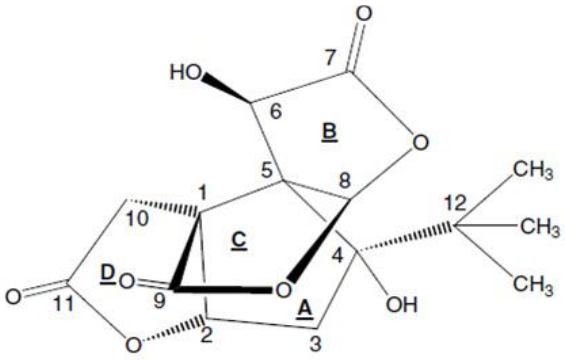


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Figure 3. Biosynthetic pathway of synthesis of ginkgolide molecule (old model) where levopimaradiene synthase leads to synthesis of ginkgolide molecule.

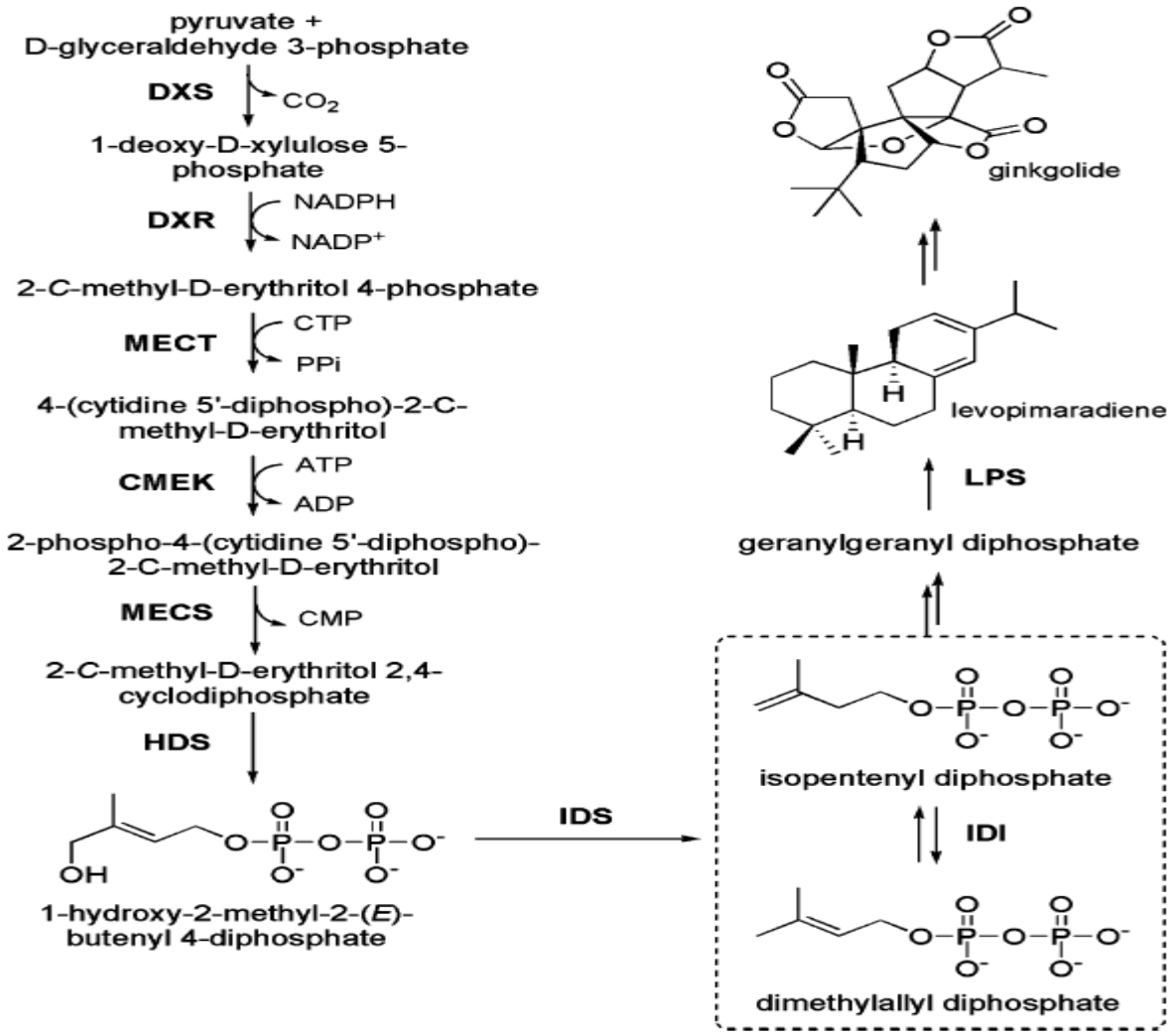

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Figure 4. Comparative domain structure of levopimaradiene synthase and rice ent-kaurene synthase shows common terpene synthase domain and metal binding domain. Figure 4A demonstrate the domain structure of rice ent-kaurene synthase and figure 4B deomonstrates domain structure of Ginkgo biloba levopimaradiene synthase. Domain structure shows close homology.

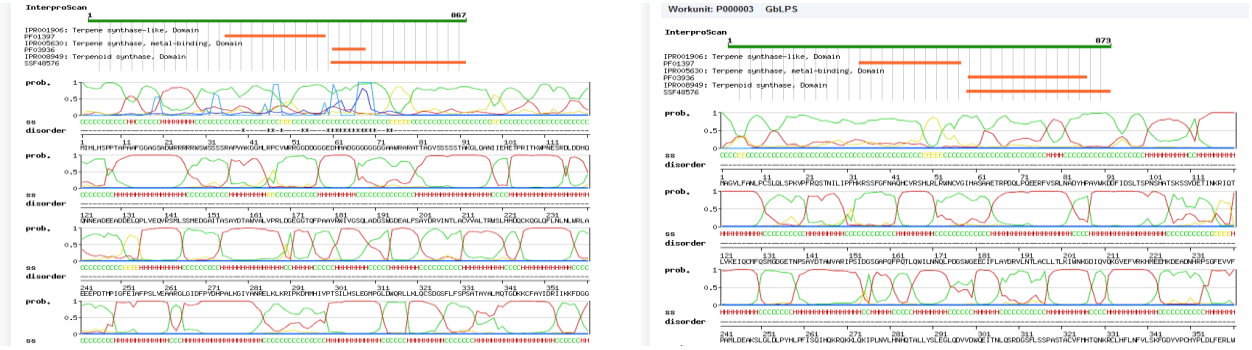

Figure 5. Amino acid sequence alignment of levopimaradiene synthase with ent-kaurene synthase of rice and Arabidopsis thaliana respectively shows conserved amono-acid sequences. This shows both the gene may share common functionalities.

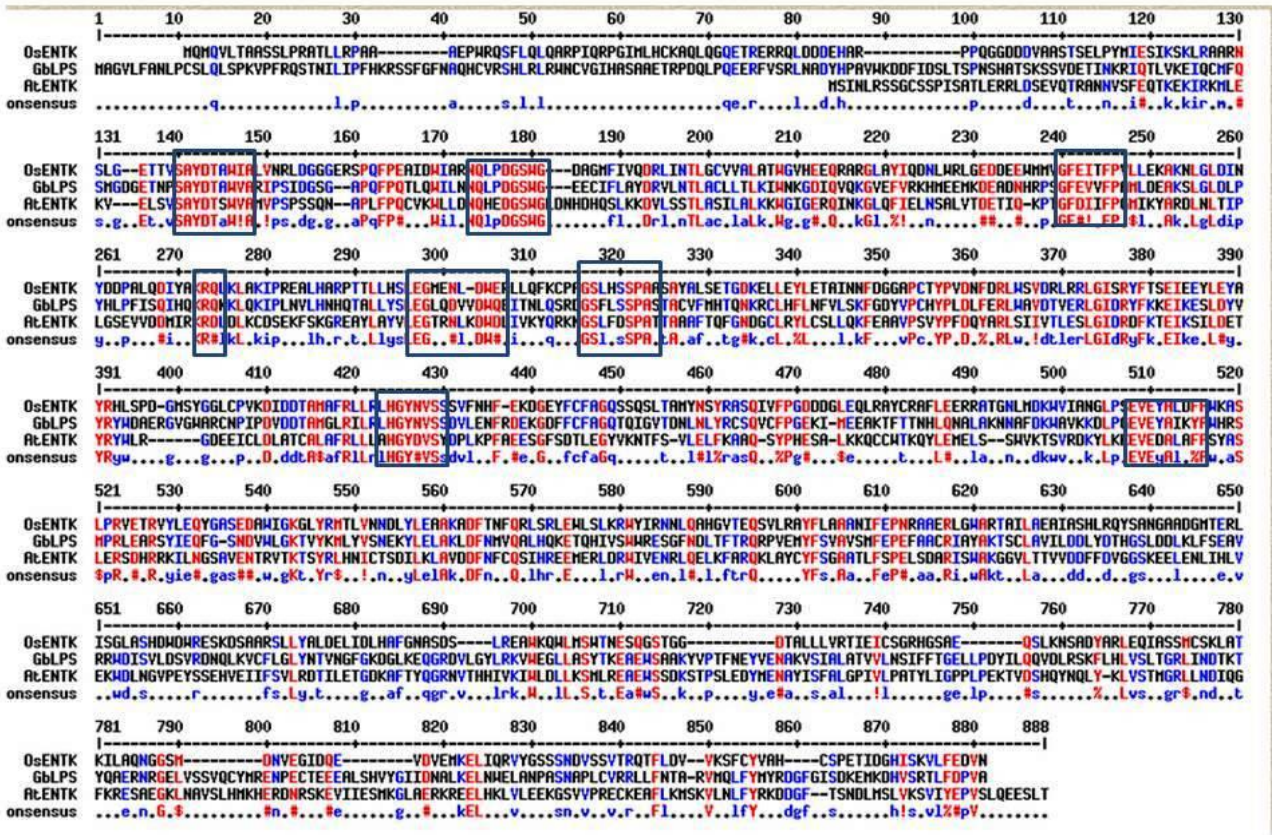

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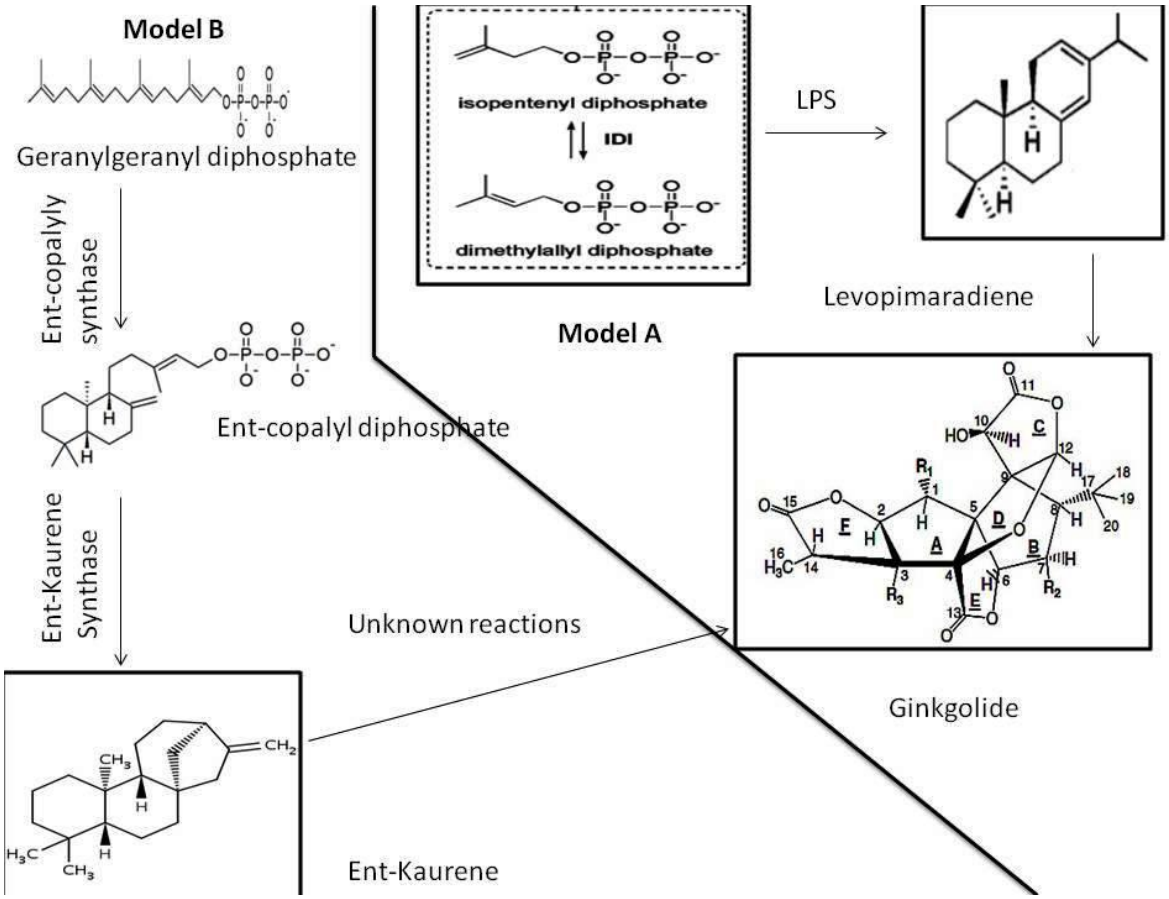
Figure 6. New model for biosynthetic pathway for synthesis of ginkgolide molecule. In model B Geranylgeranyl diphosphate gives rise to entcopalyl diphosphate and entcopalyl diphosphate transform tp ent-kaurene synthase in presence of enzyme ent-kaurene synthase. Several unknown oxidation reduction steps leads to synthesis of ginkgolide molecule from ent-kaurene.



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