Biosynthesis pathways of ginkgolides

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ABSTRACT

The ginkgolides, acting as anti-platelet-activating factors, have been studied for many years. The biosynthetic pathway of ginkgolides is still far away from unveiling at the level of molecular genetics and biochemistry. There are at least 11 kinds of enzymes having been cloned from *Ginkgo biloba* L., which catalyze the formation of ginkgolides via a series of reactions. Some researchers have indicated that the addition of precursors and elicitors can influence the accumulation of ginkgolides in the suspension cell cultures of *G. biloba.* There are also other factors that can influence the production of ginkgolides. This review focuses on the aforementioned aspects to discuss the biosynthetic pathways of the ginkgolides.

Key words: Biosynthetic pathways, cell culture, enzymes, genes, *Ginkgo biloba*, ginkgolides

INTRODUCTION

Ginkgo biloba L. (ginkgo), the only remaining species of the order Ginkgoales, is a living fossil plant as it has been existing on the earth for more than 200 million years. The leaf extract of ginkgo has been employed for treating cerebrovascular and cardiovascular diseases for centuries. It has been well studied in recent years as it contains many bioactive constituents, such as flavanoid compounds, diterpene lactone compounds, polysaccharides, etc.[1-3] The diterpene lactones contain ginkgolides A, B, C, J, and M and bilobalide compounds [Figure 1].[1] Recently, two new diterpenoid compounds, ginkgolides *P* and Q, have been isolated from the leaves of ginkgo.[2] Bilobalide exhibits neuroprotective effects by decreasing the release of excitotoxic amino acids, particularly glutamate and aspartate.[3] Ginkgolides have many pharmacological activities such as acting as specific platelet-activating factor antagonists,[4] selective antagonists of glycine receptors,[5] etc., The commercial ginkgolides are produced merely from *G. biloba* plants, especially from the ginkgo leaves. But the contents of ginkgolides in the native ginkgo plants are very low. Furthermore, the native ginkgo plant materials are limited. Some studies have been made on ginkgo cell and tissue cultures with the aim of producing ginkgolides, but the concentrations of ginkgolide and bilobalide in ginkgo cells and tissues were even lower than that of natural plants.[6] The chemical synthesis of ginkgolides has been accomplished, but the procedures were too complicated to fulfill commercial-scale production of ginkgolides.[7] Therefore, detailed understanding of the biosynthetic pathway and the involved enzymes would contribute to improve the biological production yield. On the basis of molecular genetics of ginkgolide biosynthesis, ginkgolides might be obtained alternatively through metabolic engineering, including breaking the committed step, blocking the branch ways of ginkgolide biosynthesis, and fluxing the secondary metabolite pools to ginkgolide biosynthesis.[8]

This review focuses on the biosynthetic pathways of ginkgolides, the enzymes related to the biosynthesis of the ginkgolides, the genes encoding the enzymes cloned from the ginkgo, and the factors influencing the production of ginkgolides in cell cultures.

BIOSYNTHETIC PATHWAYS OF GINKGOLIDES

Terpenoids such as ginkgolides are biosynthesized from a universal 5-carbon building block: Isopentenyl diphosphate (IPP).[9] IPP can be derived from two pathways: One is the classical cytosolic mevalonic acid (MVA) pathway and the other is the plastidial methylerythritol 4-phosphate (MEP) pathway, which is mevalonate independent. The MVA pathway in the cytosol, starting from 3 acetyl-CoA to finally yield IPP, is responsible for

synthesizing sesquiterpenoids and sterols. The MEP pathway producing IPP and dimethylallyl diphosphate (DMAPP) from pyruvate and D-glyceraldehyde 3-phosphate (GAP) is mainly responsible for forming monoterpenoids, diterpenoids constituents.

The ginkgolides biosynthetic pathway can be described in three major stages: (1) The plastid MEP pathway providing universal isoprenoid precursors; (2) the condensing steps producing direct precursors; and (3) the ensuing modifying procedures yielding ginkgolides.[10]

There are seven enzymes involved in the MEP pathway to biosynthesize the GAP into IPP and DMAPP.[11] Among the seven enzymes in ginkgo MEP pathway, condensation of pyruvate and GAP into 1-deoxy-D-xylulose 5-phosphate (DXP) is catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (GbDXS), which initiates the biosynthesis of ginkgolides.[12] 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) is the second enzyme of the MEP pathway for ginkgolide biosynthesis, which catalyzes the conversion of DXP into MEP.[13] 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase (MECT), the third enzyme, catalyzes the formation of 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol from MEP.[14] The following two steps are then catalyzed by 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase (CMEK)[15] and 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECS), respectively.[16] Then, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate is catalyzed by 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (HDS), forming 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBPP).[17] In the final step of the MEP pathway, HMBPP is reduced by HMBPP reductase (HDR) to produce both IPP and its isomer DMAPP.[10,18,19]

In plastids, the key universal diterpene precursor geranylgeranyl diphosphate (GGDP) is biosynthesized from IPP and DMAPP by catalysis of geranylgeranyl diphosphate synthase (GGPPS).[8] Levopimaradiene is biosynthesized from GGDP via a series of reactions, and levopimaradiene synthase (LPS) performs the first committed step of cyclization of GGPP in ginkgolide biosynthesis.[20] C ring is further oxidized and rearranged to produce the ginkgolides. The plastid MEP biosynthetic pathway of ginkgolide is shown in Figure 2.

GINKGOLIDES BIOSYNTHETIC-RELATED ENZYMES AND GENES

Many genes encoded enzymes have been isolated and cloned from the *G. biloba*, including 1-Deoxy-D-xylulose 5-phosphate synthase (DXS), DXR, MECT, CMEK, MECS, HDS, HDR, 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), GGPPS, LPS, and mevalonate diphosphate decarboxylase (MVD), which are involved in the biosynthesis of ginkgolides.

1-Deoxy-D-xylulose 5-phosphate synthase
The initial step of the MEP pathway is the formation of DXP catalyzed by DXS, which may be considered the first committed step of the MEP pathway for ginkgolides biosynthesis. The full-length cDNA of DXS was isolated and characterized from the *G. biloba*.[12] The full-length cDNA sequence of GbDXS with 2,795 bp was deduced and subsequently confirmed by sequencing, containing a 2,154 bp open reading frame (ORF) encoding a protein of 717 amino acids. Comparative and bioinformatic analyses indicated that GbDXS had high homology with DXSs sequences from other plant species, containing a conserved transit peptide for plastid import, histidine residue, a putative thiamine diphosphate-binding site, and a transketolase motif. GbDXS could be expressed in roots, stems, leaves, and other tested tissues.

The accumulation of ginkgolide B was increased after induction of methyl jasmonate, arachidonic acid, acetylsalicylic acid, and ceric ammonium sulfate in tissue culture of *G. biloba*. RT-PCR analyses indicated that the expression of GbDXS was also strongly increased with elicitor treatments. The increase in the DXS mRNA accumulation correlates with ginkgolide accumulation, which suggested that DXS might influence the biosynthesis of ginkgolides.

1-Deoxy-D-xylulose 5-phosphate reductoisomerase
DXR, catalyzed the formation of 2-C-methyl-D-erythritol 4-phosphate from DXP in the presence of NADPH,[21] may be considered the second step of MEP pathway, catalyzing in the earlier step of ginkgolide biosynthesis. The full-length cDNA sequence of GbDXR was cloned and characterized.[13] The full-length cDNA was 1720 bp containing a 1431 bp ORF encoding a peptide of 477 amino acids with a calculated molecular mass (M_r) of 52 kDa and a isoelectric point (pI) of 6.58. GbDXR had high homology with DXRs from other plant species and can be expressed in all tissues including roots, stems, leaves, pericarps, and seeds.

2-C-methyl-D-erythritol 4-phosphate cytidyltransferase
MECT, the third enzyme of the MEP pathway, catalyzes the formation of 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol from MEP.[14] *GbMECT* was cloned and characterized from *G. biloba* embryonic roots presumably, which is involved in ginkgolide biosynthesis. The full-length cDNA of GbMECT was 1,411 bp, consisting of a 984-bp ORF, which was obtained by combining the sequence information of 3'-and 5'-RACE fragments. The deduced amino acid sequence of GbMECT consisted of 327 residues, with theoretical M_r and pI values of 36.3 kDa and 9.08, respectively. Transcription levels of GbMECT remained generally constant in embryonic roots and leaves for 1 month. As shown by protein-targeting analysis with GFP as a reporter protein in *Arabidopsis thaliana* protoplasts,
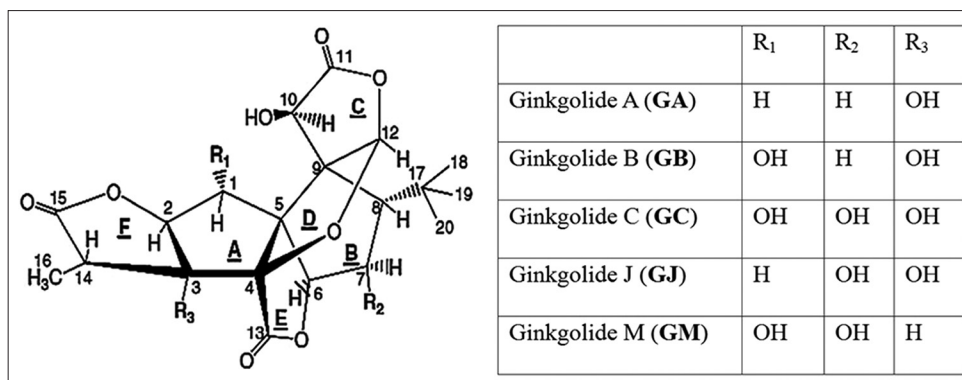
		R$_1$	R$_2$	R$_3$
Ginkgolide A (**GA**)		H	H	OH
Ginkgolide B (**GB**)		OH	H	OH
Ginkgolide C (**GC**)		OH	OH	OH
Ginkgolide J (**GJ**)		H	OH	OH
Ginkgolide M (**GM**)		OH	OH	H

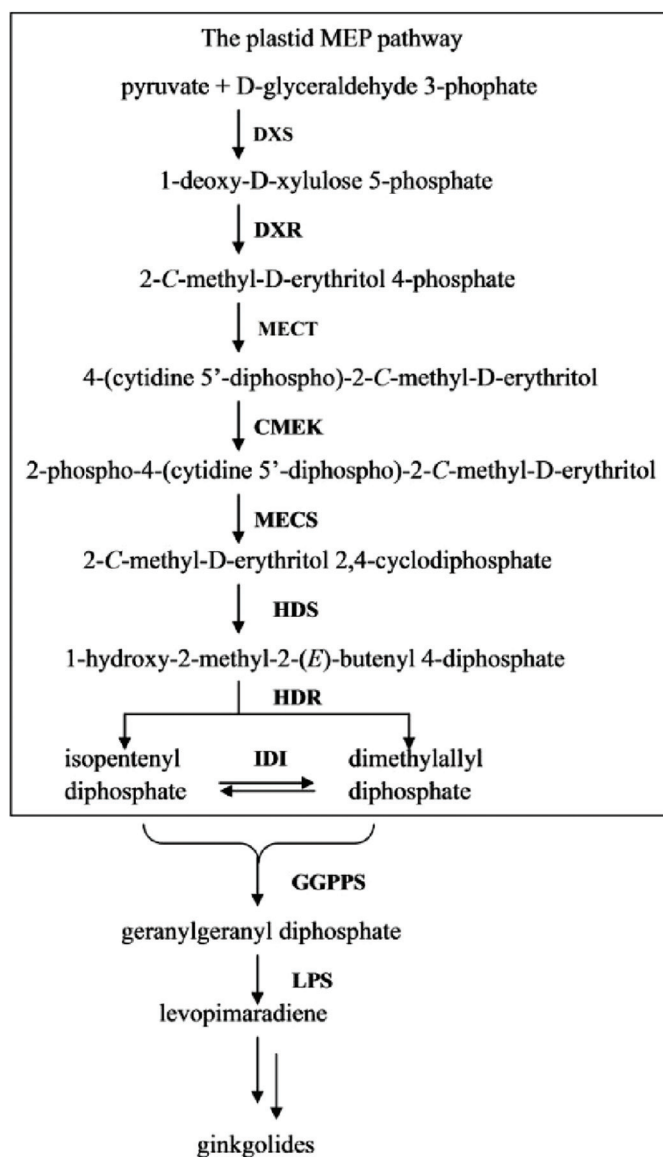**Figure 1:** Ginkgolide structures from *Ginkgo biloba*[1]

Figure 2: Methylerythritol 4-phosphate pathway in the isoprenoid biosynthesis of *Ginkgo biloba*. DXS = 1-deoxy-D-xylulose 5-phosphate synthase, DXR = 1-deoxy-D-xylulose 5-phosphate reductoisomerase, MECT = 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase, CMEK = 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase, MECS = 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, HDS = 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase, HDR = 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase, IDI = Isopentenyl diphosphate isomerase, GGPPS = Geranylgeranyl diphosphate synthase, and LPS = Levopimaradiene synthase

the full 88 N-terminal residues were necessary to deliver the protein into the chloroplast.

4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase

CMEK, the fourth enzyme of the 2-C-methyl-D-erythritol 4-phosphate pathway, phosphorylates the 2-hydroxyl group of 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol in the presence of ATP.[15] The classes of genes encoding CMEK (*GbCMEK1* and *GbCMEK2*) were cloned and characterized from *G. biloba*. GbCMEK1 and GbCMEK2 were comprised of 433 and 428 amino acid residues, and their theoretical M_r and p*I* values were 39.2 kDa and 5.12, and 39.9 kDa and 5.19, respectively. They showed high amino acid sequence identities with other CMEKs. ChloroP program predicted that GbCMEKs had the chloroplast transit peptides.

2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase

MECS from *G. biloba* (*GbMECS*), the fifth enzyme in the MEP pathway sequence, transforming 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol into 2-C-methyl-D-erythritol 2,4-cyclo-diphosphate, had been cloned and characterized.[16] The full-length GbMECS cDNA was 935 bp, consisting of 717 bp long ORF and 3'-and 5'-untranslated regions (UTR). The deduced protein contained 238 amino acid residues. The N-terminal chloroplast transit peptide in the deduced GbMECS, which consisted of 59 residues, was predicted by the ChloroP program. The theoretical M_r and p*I* of GbMECS were 19.3 kDa and 6.47, respectively. Furthermore, the quantification of GbMECS transcript indicated that, the transcription level in the embryo roots was at least 2 times higher than that in the embryo leaves throughout the 1-month period of embryo culture.

1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase

HDS converts 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP) into HMBPP, which is the sixth enzyme of the MEP pathway, supplying building blocks for plant isoprenoids of ginkgolides. The full-length cDNA encoding HDS (*GbHDS*) had been isolated from *G. biloba*.[17] The full-length cDNA of GbHDS was 2,763 bp containing 164 bp 5'-UTR and 193 bp 3'-UTR. This cDNA contained an ORF of 2,226 bp encoding a protein consisting of 741 amino acids. The deduced amino acid sequence of GbHDS contained 679 residues, with theoretical M_r of 75.6 kDa and p*I* of 5.5. The copy number of GbHDS transcript in the 1-week-old embryo roots was slightly lower than that of the leaves. But from the week 2 onward, the level in the roots was always higher than that in the leaves, and at week 3, it reached 2 times higher level. The ChloroP program predicted that GbHDS had a chloroplast transit peptide consisting of 62 amino acid residues, suggesting its putative localization in the plastids.

1-Hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase

HDR is proved to be the terminal-acting enzyme in the plastid MEP pathway which provides isoprenoid precursors for the biosynthesis of ginkgolides. The full-length cDNA encoding HDR (*GbHDR*) was isolated from *G. biloba* and characterized.[10] The full-length cDNA of GbHDR was 1,827 bp containing 1,422-bp ORF, with a 3'-UTR of 374 bp downstream from the stop codon and a 5'-UTR of 28 bp upstream of the start codon. There were 474 amino acid residues for the deduced GbHDR, with a calculated M_r of 53.2 kDa and p*I* of 5.76. The deduced GbHDR protein showed high identity to HDRs of other plant species, and four conserved cysteine residues were found in all plant HDRs. It was predicted by the targetP algorithm to possess a chloroplast transit peptide. Transcription pattern analysis revealed that GbHDR had high transcription in roots, and low in leaves and stems.

Geranylgeranyl diphosphate synthase

GGPPS catalyzed the biosynthesis of GGPP, which was a key precursor for ginkgolides biosynthesis. The full-length cDNA encoding GGPPS from *G. biloba* (designed as *GbGGPPS*) had been cloned.[8] The full-length cDNA of GbGGPPS was 1657 bp long. It contained 1176 bp ORF encoding a 391 amino acid residues, with M_r of 42.5 kDa and theoretical p*I* of 5.98. GbGGPPS had high homology with other plant GGPPSs and the PSI-BLAST results indicated that GbGGPPS belonged to the GGPPS family. Moreover, the TargetP had shown that there was a 79-amino acid transit peptide at the N-terminal of GbGGPPS, for targeting to the plastids, in which ginkgolides were biosynthesized.

Levopimaradiene synthase

LPS, which catalyzes the initial cyclization step in ginkgolide biosynthesis, was cloned and characterized.[20] The cDNA library from *G. biloba* was prepared from cultivated seedling roots and degenerate primers were designed based on conserved sequence regions in gymnosperm terpene synthases. The sequencing revealed a similar 2,681 bp cDNA similar to other diterpene synthases, but LPS lacked the initiation codon. The full-length cDNA encoded an 873 amino acid ORF with a predicted M_r of 100,289 Da. Compared to other terpenoid synthases, the predicted LPS polypeptide sequence had high sequence identity to *A. grandis* abietadiene synthase. It maintained most amino acid residues that were highly conserved in mono-, sesqui-, and diterpene synthases.

3-Hydroxy-3-methylglutaryl coenzyme a reductase

HMGR catalyzes the NADP-dependent synthesis of MVA from 3-hydroxy-3-methylglutaryl-CoA, which is the first committed step in MVA pathway for biosynthesis of isoprenoids.[22] The full-length cDNA encoding GMGR (GbHMGR) had been isolated from *G. biloba*. The full-length GbHMGR cDNA was 2,237 bp with partial

poly (A), containing 1716 bp ORF encoding a protein with 571 amino acid. There was a 5'-UTR of 120 bp upstream from the start codon with G as the putative transcript start site. The coding region of GbHMGR was followed by 3'-UTR (401 bp) downstream from the stop codon. The M_r and pI of the deduced GbHMGR protein were predicted to be 60.87 kDa and 6.43, respectively. There was a high homology between the GbHMGR and other plant HMGRs, while GbHMGR diverged earlier than other plant species. The Southern blot and RT-PCR assay results indicated that GbHMGR belonged to a small gene family, and expressed in a tissue-specific manner with a low level expression being only found in root.

Mevalonate diphosphate decarboxylase

MVD catalyzes the conversion of mevalonate diphosphate to IPP, which is important for the biosynthesis of secondary metabolites. A full-length cDNA of MVD from *G. biloba* (GbMVD) was isolated and characterized.[23] The full-length cDNA of GbMVD was 1,958 bp with a poly (A) tailing, containing an ORF of 1,290 bp encoding 430 amino acids. There was a 5'-UTR of 291 bp upstream from the start codon with G as putative transcription start, and the coding region was followed by 3'-UTR that was 345 bp-long downstream from the stop codon. It was predicted that the calculated M_r and pI of the deduced GbMVD protein were 48 kDa and 5.83, respectively. It also showed a high homology with other MVDs from some species. Transcript accumulation analysis revealed that GbMVD was transcribed in root, stem, and leaf tissues.

Up to now, the genes encoded whole enzymes in the MEP pathway have been cloned from *G. biloba*, but the enzymes and genes involved in the MVA pathway still need further research. Furthermore, how levopimaradiene transforms into ginkgolides is still under investigation.

INFLUENCING FACTORS IN ACCUMULATION OF GINKGOLIDES

Some elicitors, including fungal and bacterial elicitors, have been studied and investigated the influences on the accumulation of ginkgolides in *G. biloba* cell cultures. Kang found that native *Staphylococcus aureus* KCTC 1916 and *Candida albicans* KCTC 7121 as biotic elicitors produced more of ginkgolide A (GA) and ginkgolide B (GB) than the control groups.[24] Effect of mycelium extract of *Rhizopus japonicus* on the production of GB was the most significant in the studied 10 kinds of fungi.[25]

The precursors involved in the terpenoids biosynthesis had also been investigated. Kang *et al*. found that treatments with HMG-CoA, GPP, and IPP, which were precursors related to MVA and MEP pathways, had different promoting effects on GA and GB productions, with 2.7 folds to 4.25 folds

compared to the control groups.[26] Dai had investigated the effects of isoprene and geraniol on the GB production in suspension cultured cells of *G. biloba*.[25] Compared with the control, the total GB yields were enhanced by 69%, 13.8%, and 11.4% when adding 100 mg/L of isoprene, 10 mg/L isoprene, and 50 mg/L isoprene in the media, respectively.

Furthermore, strains in the fermentation liquid of endophytic fungi influenced the ginkgolide accumulation.[27] The production patterns in the leaf, stem bark, and stem of female and male trees were also different.[28]

FUTURE PERSPECTIVES

As demonstrated above, many researches about *G. biloba* have been made involved extraction, purification, structural elucidation, and bioactive determination. The investigations of ginkgolide biosynthetic pathways have also made in some progresses, for example, 11 kinds of enzymes having been cloned from *G. biloba*, which catalyze the formation of ginkgolides via a series of reactions. However, the biosynthetic pathway of ginkgolides is still far away from unveiling at the level of molecular genetics and biochemistry: The biosynthesis of ginkgolides was mainly regulated by MEP pathway or MVA pathway, or both the two pathways, and the pathways of the GGPP is transformed into ginkgolides, etc., are still under investigation. Those need scientists to find out the enzymes and genes included in the pathways which are still not clear. Moreover, it provides us an opportunity to find out a way to improve the accumulation of ginkgolides in the cultured cells which would be hard to determine them in natural *G. biloba*.

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