African Journal of Biotechnology Vol. 11(93), pp. 15936-15944, 20 November, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.627
ISSN 1684–5315 ©2012 Academic Journals

Review

Advances in *Ginkgo biloba* research: Genomics and metabolomics perspectives

Tapan Kumar Mohanta

National Institute of Plant Genome Research (Lab. No. 102), Aruna Asaf Ali Marg, New Delhi 110067, India.
E-mail: nostoc.tapan@gmail.com.

Accepted 19 October, 2012

The maiden hair tree*, Ginkgo biloba* is very much resistant to a wide spectrum of biotic and abiotic stress conditions. It hardly seems to be attacked by any herbivore or microbe. In spite of its strong resistant nature to wide stress conditions, only little research has been carried out at genomics and metabolomics level to understand its mechanism of stress resistance. Ginkgolide and bilobalide, the unique terpene trilactone present in *G. biloba* were found to play an important role during stress conditions. Massive parallel sequencing of *G. biloba* leads to generation of 64057 expressed sequence tags (EST's). Beside this, chloroplast genome (cpDNA) of *G. biloba* has been sequenced. This massive parallel sequencing of ESTs, along with cpDNA genome, will open the door for high throughput genomic research.

Key words: *Ginkgo biloba*, cycad, ginkgolide, bilobalide, bilobol, proton nuclear magnetic resonance spectroscopy (^{1}H NMR).

INTRODUCTION

Ginkgo biloba, popularly known as living fossil, is considered as missing link between gymnosperm and angiosperm (Zhou and Zheng, 2003). This taxon is distinguished from the coniferophyta (Conifers) on the basis of its reproductive structures, most notably its multi flagellated sperm cells, and from the cycadophyta (Cycads) on the basis of its vegetative anatomy (Wang and Chen, 1983). Recent molecular analysis of the *Ginkgo* genome (incomplete), suggests a much closer relationship to the cycads than to the conifers (Hasebe, 1997). The genus *Ginkgo* appears to have reached the peak of its diversity during the lower Cretaceous (141 to 98 myr) (Del Tredici, 2000). The fossil record of *Ginkgo* shows a decline in diversity and distribution, particularly towards the end of the period when worldwide temperatures decreased dramatically. The diminution of *Ginkgo*'s range continued into the tertiary, and was particularly striking from the Oligocene (38 to 26 myr) (Del Tredici, 2000). The genus disappeared from polar areas, through the end of the Miocene (24 to 7 myr). These dramatic changes were most likely the result of the extensive cooling that occurred throughout the Northern hemisphere during these time periods. The genus *Ginkgo* disappeared from Europe by the end of the Pliocene (1.8 myr) as temperatures dropped down and the rainfall regimen gradually shifted from summer wet to summer dry. The only known Pleistocene (1.8 myr to present) occurrences of the genus *Ginkgo* are from South Western Japan (Uemura, 1997).

As a wild species, *G. biloba* is native to China (Uemura, 1997). One of *Ginkgo*'s last wild refugia is thought to be in Zhejiang Province, China, on the west peak of Tianmu mountain (Xitianmu Shan). There are also reports of "wild" *Ginkgo* populations in other parts of China, including Guangxi, Guizhou and Sichuan Provinces (Liang, 1993). *Ginkgo* was introduced into Western Japan from Eastern China about 800 years ago. From Japan, *Ginkgo* was introduced into Europe at the Botanic Garden in Utrecht, The Netherlands, around 1730, and into Kew Gardens, near London, England, around 1754. From England, the tree was imported into North America in 1784 (Del Tredici, 1991).

Ginkgo possesses a high degree of resistance to fungal, viral, and bacterial diseases, as well as to ozone

and sulfur dioxide pollution, making it an excellent choice for planting in urban areas (Sinclair et al., 1987; Honda, 1997). It also shows tolerance to air pollution (Rensselaer, 1969). Major and Tietz (1962) observed that Japanese beetles died of starvation rather eating fresh leaves of *G. biloba*. Investigations have been carried out to study the insecticidal properties of the tree. It has been shown that the leaves are very acidic and an aldehyde, 2-hexenal is produced when *Ginkgo* leaves are damaged in the presence of oxygen (Major et al., 1963). Bevan et al. (1961) have reported that this aldehyde is an insect repellent. Ginkgolide A has been found the most active of several compounds which included bilobalide and ginkgolic acids, possessing antifeedant activity against larvae of the cabbage butterfly *(Pieris rapae crucivora)* (Matsumoto and Sei, 1987). Roots and stems of *G. biloba* are also toxic to insects (Major, 1967).

GENOMICS OF *Ginkgo biloba*

Genus *Ginkgo* itself goes back to Jurassic period, approximately 170 million years ago (Zhou and Zheng, 2003). Although it is widely believed that the survival of *G. biloba* depended upon Buddhist monks who venerated the tree cultivated in their temple grounds. Molecular evidence suggests that some stands in China (Wuchuan, Guizhou) are of natural origin (Del Tredici, 2000).

Fan et al. (2003) reported genetic diversity and differentiation of nine population of *G. biloba* from China using random amplified polymorphic DNA (RAPD) method. They got 47 clear and repeatable RAPD bands, 46 were polymorphic (overall polymorphism 97.9%). Genetic diversity and differentiation of Chinese populations were higher than those of Korean and North American populations, which were ultimately descended from China as reported previously (Fan et al., 2003). To understand inferences about the glacial refugia that harbored *G. biloba*, Shen et al. (2005) examined the genetic structure of eight potential refugial populations and plantations using chloroplast DNA (cpDNA) with eight size variants in the trnK-1 and trnK-2 fragment. Majority of the cpDNA haplotypes are restricted to minor portions of the geographical range. They suggested that, *G. biloba* were located in south western China. This area is currently biodiversity hotspot of global importance, and may have been protected from the extremes of climatic fluctuations during the Pleistocene (Shen et al., 2005).

Molecular sequence data shows *Ginkgo* is potentially and closely related to *Cycas* and distantly related to conifers (Zhou and Zheng, 2003). Interestingly, *Ginkgo* and *Cycads* share certain plesiomporphic characters (Brenner et al., 2005; Friedman, 1987). Brenner et al. (2005) studied comparative analysis of *Ginkgo* unigene (3830) data set with that of *Cycas rumphii* unigene (4706). These gymnosperm specific genes show homology to *Ginkgo* genes. *Ginkgo* unigene (GinkgoA2411, GinkgoA3214, GinkgoA325) match to non seed plant and other gymnosperm specific genes. *Ginkgo* unigene (GinkgoA2273) matches non-seed plant with similarity to gene in *Chlamydomonas*.

Despite the presence of early seed plant characteristics, surprisingly little work has been performed on *Ginkgo.* Some recent molecular and genomic research on *Ginkgo* has been initiated as well, however, no complete genomic work on *G. biloba* has been completed to date. Brenner et al. (2005) also created three distinct cDNA library from reproductive structures (microsporangiate, megasporangiate) and vegetative organs (leaves) and performed expressed sequence tags (EST) analysis of *G. biloba* for assessment of conserved developmental regulators and gymnosperm specific genes. From these three cDNA libraries, they generated 6434 ESTs and clustered into 3830 unigene. Comparison of these unigene against fully annotated genomes of rice and *Arabidopsis* and all available ESTs in genebank revealed that 256 *Ginkgo* unigene matches genes only among the gymnosperms and non-seed plants, many with multiple matches to genes in non-angiosperm plants.

Conversely, another group of unigene in *Ginkgo* had highly significant homology to MADS box genes as well as post transcriptional regulators. MADS box genes are key regulators of plant development. They appear as a large multigenic family whose history is complex. Jager et al. (2003) were able to retrieve 33 different MADS-box sequences from genomic DNA of *G. biloba*. They found GBM5 to be potential orthologous gene with that of *Arabidopsis thaliana* agamous gene.

To discover novel functional genes efficiently and to understand the development of living fossil *G. biloba*, massive parallel pyrosequencing has been done to generate 64057 ESTs (NCBI accession number: SRX022356) (Lin et al., 2011). These ESTs were combined with the 21590 *Ginkgo* ESTs of genebank and assembled into putative transcripts, in which 13922 novel unique putative transcripts were identified by 454 sequencing. After being assigned to putative functions with gene ontology term, they found detailed view of *Ginkgo* biological systems including key enzymes and transcription factors involved in ginkgolide/bilobalide and flavonoid biosynthetic pathways, as well as unique putative transcripts related to response to disease and defense (Table 1). They also cloned three full length *Ginkgo* genes phenylalanine ammonia lyase (Gb_PAL), chalcone synthase isomerase (Gb_CHSI), 1-hydroxy-3-methyl-2-butenyl 4-diphosphate reductase (Gb_IDS) encoding key enzymes from their EST database.

Due to lack of Gene chip of *G. biloba* for microarray experiment, heterologous microarray (with *A. thaliana* gene chip) experiment was carried out to understand the induced gene expression strategy of *G. biloba* against insect feeding and mechanical damage. Data analysis and its validation demonstrated significant up and down

Table 1. Number of putative unique putative transcripts and ESTs involved in ginkgolide/bilobalide biosynthesis. BLAST against the SWISSPROT and KEGG databases.

Enzyme name	E.C[a]	Number of unique putative transcripts	Number of ESTs	Number of 454 ESTs	Number of Gene Bank ESTs
DXP synthase (DXS)	2.2.1.7	2	6	6	0
DXP reductoisomerase (DXR)	1.1.1.267	1	18	11	7
MEP cytidylyltransferase (MCT)	2.7.7.60	1	2	2	0
CDP-ME Kinase (CMK)	2.7.1.148	2	2	1	1
MECDP synthase (MECPS)	4.6.1.12	2	2	2	0
4-Hydroxy-3-methylbut-2-en-1-yl disphosphate synthase (HDS)	1.17.7.1	4	15	14	1
1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (IDS)	1.17.1.2	3	119	119	0
Isopentenyl-diphosphate delta-isomerase (IDI)	5.3.3.2	1	8	7	1
Acetyl-CoA C-acetyltransferase (AACT)	2.3.1.9	2	10	9	1
HMG-CoA synthase (HMGS)	2.3.3.10	1	5	0	5
HMG-CoA reductase (HMGR)	1.1.1.34	2	3	3	0
Phosphomevalonate kinase (PMK)	2.7.4.2	2	2	2	0
Mevalonate diphosphate decarboxylase (MVD)	4.1.1.33	1	5	2	3
Geranyl-diphosphate synthase (GPPS)	2.5.1.1	3	19	15	4
Farnesyl-diphosphate synthase (FPPS)	2.5.1.10	2	7	3	4
Geranylgeranyl-diphosphate synthase (GGPPS)	2.5.1.29	2	13	13	0
Levopimaradiene synthase (LPS)	4.2.3.32	2	2	2	0

regulation of different genes involved in plant stress response, including transcription factor regulator (Mohanta et al., 2012). Some other study includes phenylpropanoid metabolism pathway and reactive oxygen species scavenging system. These studies closely relate to stress response strategy of modern angiospermic plant *A. thaliana* (Conklin et al., 1996; Davison et al., 2002; De Vos et al., 2005). Important genes involved phenylalanine ammonia lyase, chalcone synthase, flavonol synthase, superoxide dismutase, ascorbate peroxidase, catalase etc. This suggests plant defense response genes were evolved since the evolutionary origin of plant life.

Recently, Lin et al. (2012) determined the complete chloroplast genome (cpDNA, accession number: AB684440) sequencing project of *G. biloba* and reported mechanism of inverted repeat (IR) contraction. The cpDNA molecule of *G. biloba* (Figure 1) is quadripartite and circular, with a length of 156,945 bp with a pair of IRs separated by large single-copy (LSC) and small single copy region (SSC) (Figure 2). cpDNA genome is 6,458 bp shorter than that of *Cycas taitungensis*. This short cpDNA is due to complete loss of ycf2 from the IR_A (Figure 2). *Ginkgo* cpDNA has at least five editing sites and one copy of ycf2. This ycf2 is a duplicate of ancestral ycf2, and ancestral one has been lost from inverted repeat A (IR_A). This loss event should have occurred and led to contraction of IRs after *Ginkgo* diverged from other gymnosperm. They identified 120 unique genes in *Ginkgo* cpDNA, 81 protein coding genes, 35 tRNA genes and 4 rRNA genes. A total of 14 genes duplicated including three protein coding genes, six tRNA gene and four rRNA genes in the IR as well as one tRNA gene in the LSC region. Thirteen protein coding genes and eight tRNA genes have introns.

Comparison of *Ginkgo* and *Cycas* cpDNA shows, they are very much similar except that *Ginkgo* has only one single copy of ycf2 and its rpl23 become pseudo, and *Cycas* lost the trnT-GGU originally located between psbD and trnE-UUC in the LSC region. These events lead to downsizing of *Ginkgo* cpDNA. In addition, *Ginkgo* cpDNA contains a specific cluster of three novel tRNA genes (trnSeC-UCA, trnC-ACA, and trnY-AUA)

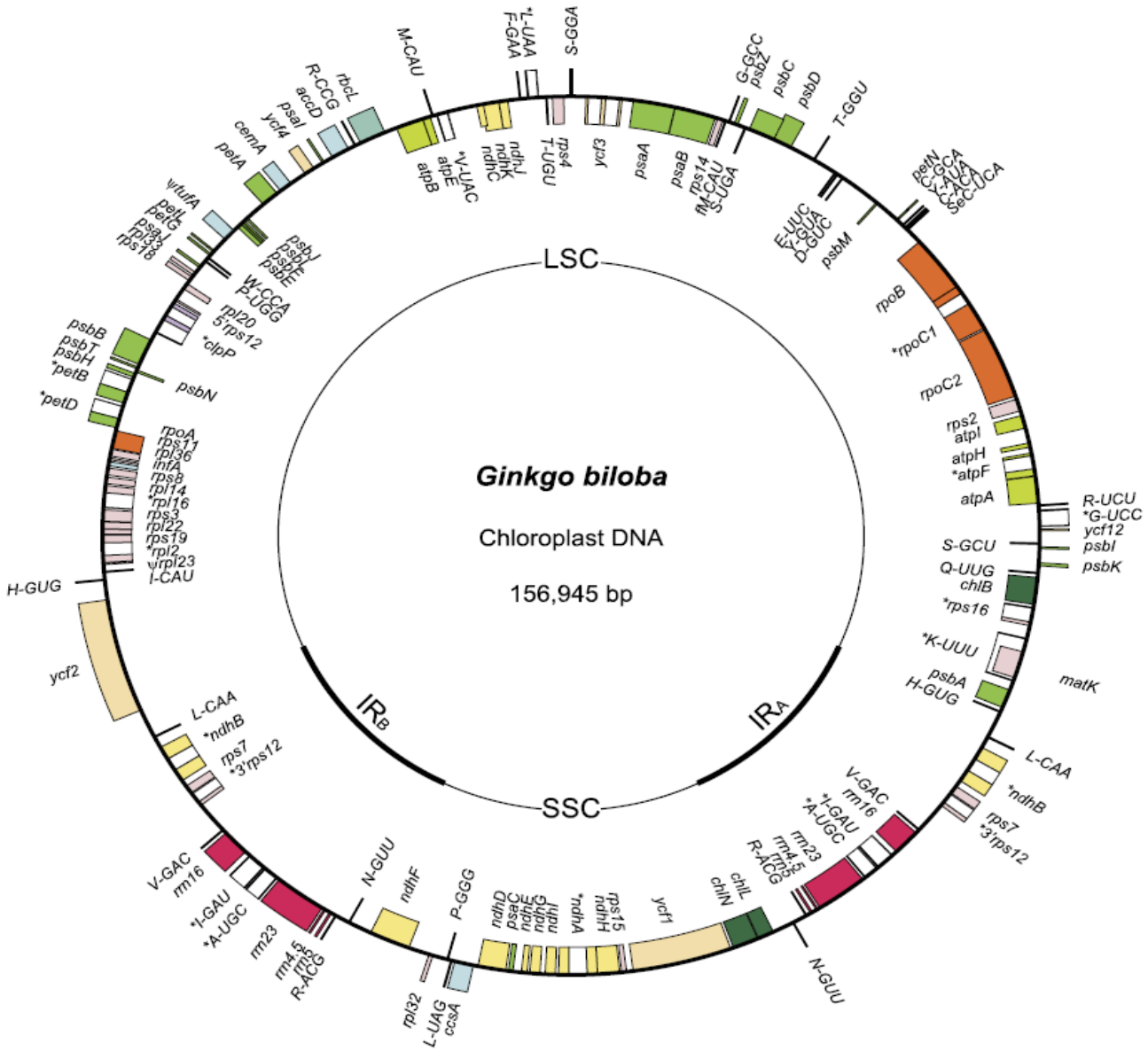


Figure 1. Complete cpDNA map of *Ginkgo biloba*. Genes inside and outside the circle are transcribed clockwise and counterclockwise, individually. *Genes with introns; Ψ, pseudogenes (Photograph adopted from Lin et al., 2012).

that are located between the rpoB and the trnC-GCA of the LSC region.

METABOLOMICS OF *Ginkgo biloba*

G. biloba is well known for its unique chemical constituents and secondary metabolite contents (Cheng et al., 2009; Eckhard and Christel, 2009; Flesh et al., 1992; Hasler and Meier, 1993). Many secondary phytoconstituents from *G. biloba* has been identified till date and their pharmacological evaluation has been well studied. In a standardize *Ginkgo* extract EGb 761, flavonoid glycoside was present (24%) whereas terpene trilactones (TTLs) was found to be 6% of dry weight.

The novel molecule found in *G. biloba* is terepene trilactone (Nakanishi, 1967; Van Beek, 2002). It collectively constitutes ginkgolides (Figure 3) and bilobalide (Figure 4). Ginkgolide contains ginkgolide A (GA), ginkgolide B (GB), ginkgolide C (GC), ginkgolide J (GJ), ginkgolide M (GM), ginkgolide K (GK) and ginkgolide L (GL) (Van Beek, 2002). Two new diterpenoid compounds, ginkgolide P (GP) and ginkgolide Q (GQ) were isolated from the leaves of *G. biloba* L. (Figure 5). Ginkgolides are diterpenes containing a spirononane carbocyclic ring, three lactones, a tetrahydrofuran ring

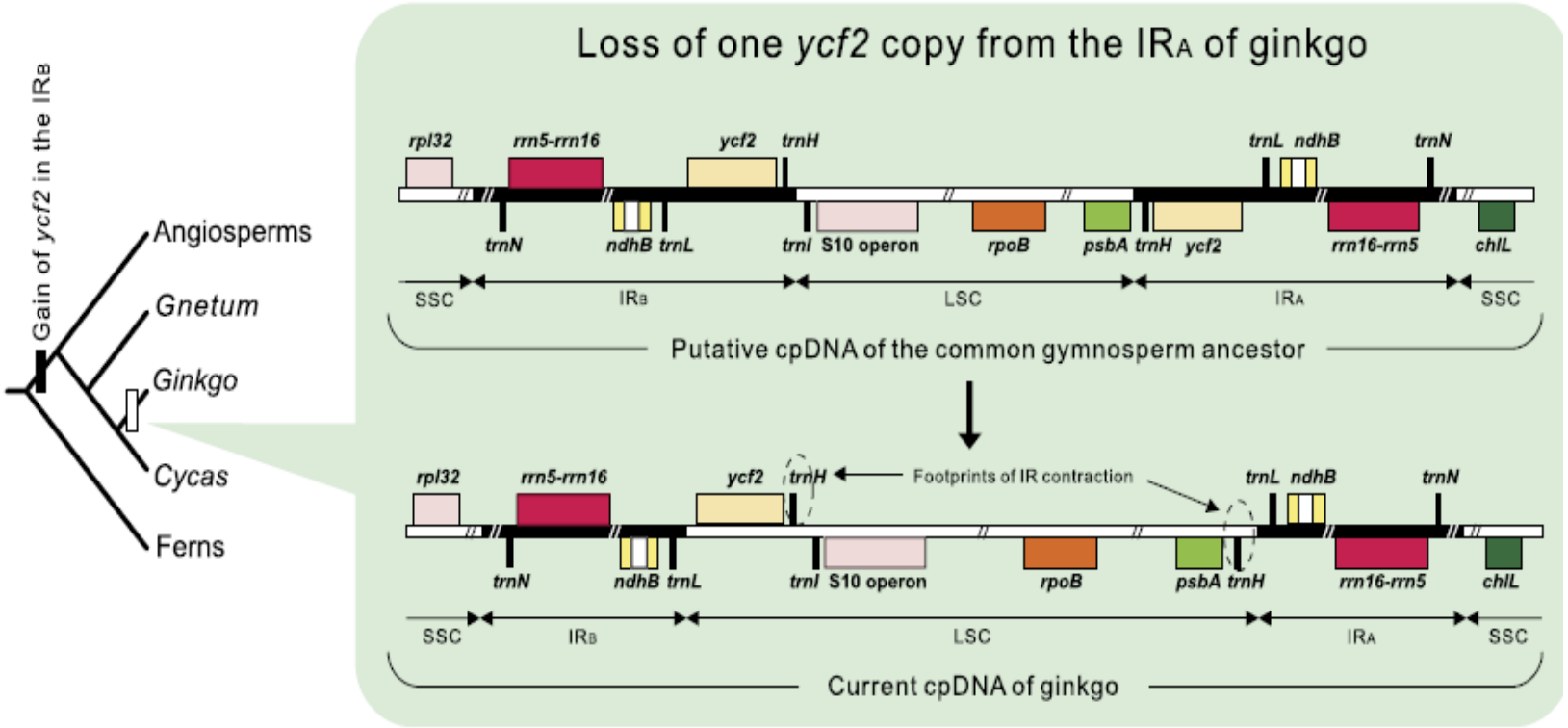
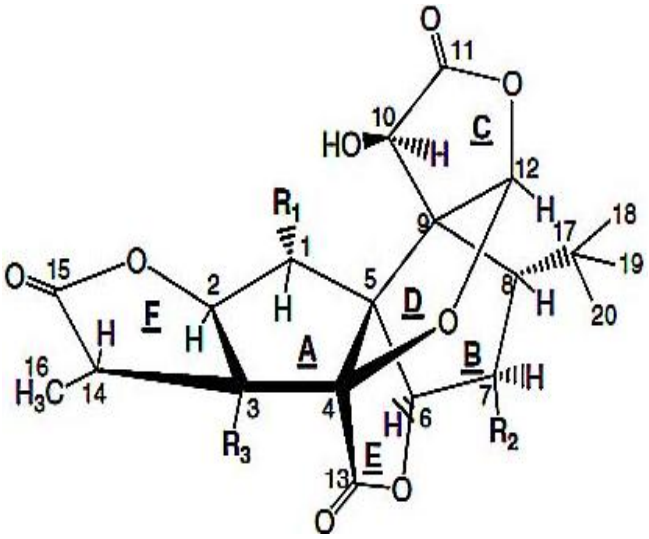


Figure 2. Hypothetical scenario illustrating IR contraction in *Ginkgo* cpDNA. ycf2 was lost from ancestral IR_A during *Ginkgo* evolution. Photograph adopted from Lin et al. (2012).

and tetrabutyl group (Figure 3) (Liao et al., 2011). The only differences between these compounds are the number and the position of hydroxyl groups which may be present on C1, C3 or C7 of the spirononane framework (Liao et al., 2011). Bilobalide (Figure 4) is a sesquiterpene and closely related to the ginkgolides. It differs from the ginkgolides by the absence of tetrahydrofuran ring. TTLs are extraordinarily stable despite the presence of multiple oxygen functional groups (Stomgaard and Nakanishi, 2004).

Terpene trilactone is neither a polar nor a non polar, and very complex molecule. Due to this property, it became very difficult to isolate pure ginkgolide or bilobalide molecule. A lot of research has been carried out to detect terpene trilactone by high-performance liquid chromatography ultra violet (HPLC UV) diode array (Van Beek and Montoro, 2009). Though, analysis of terpene trilactone from leaf and EGb 761 using LC-MS system greatly facilitates its detection and quantification. Grata et al. (2009) used ultra-high-pressure liquid chromatography at elevated temperature coupled to time-of-flight mass spectrometry to analyze complex chemical mixture. Later, Agnolet et al. (2010) used proton nuclear magnetic resonance spectroscopy (^{1}H NMR) -based metabolomics combined with high performance liquid chromatography, photo-diode-array, mass spectrometry, solid-phase extraction and nuclear magnetic resonance (HPLC-PDA-MS-SPE-NMR) for investigating standardize *G. biloba* extract collected from 16 different



Ginkgolide	R1	R2	R3
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 3. Chemical structure of ginkgolide back bone. Ginkgolides differ from each other in presence or absence of H or OH group on side chain R^1, R^2 and R^3.

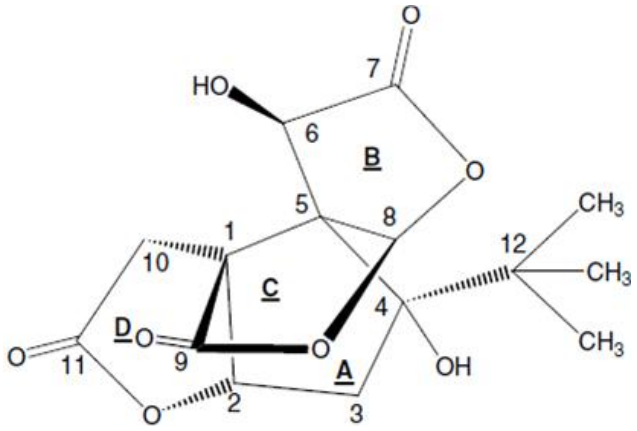


Figure 4. Structure of bilobalide molecule.

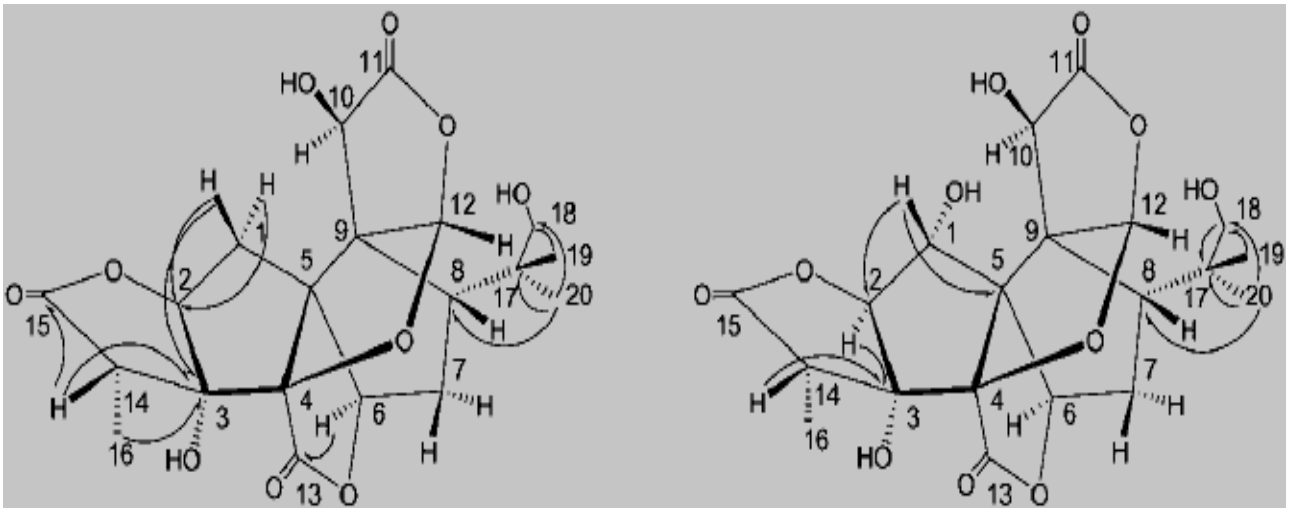


Figure 5. Structure of ginkgolide P (left) and Q (right) molecule.

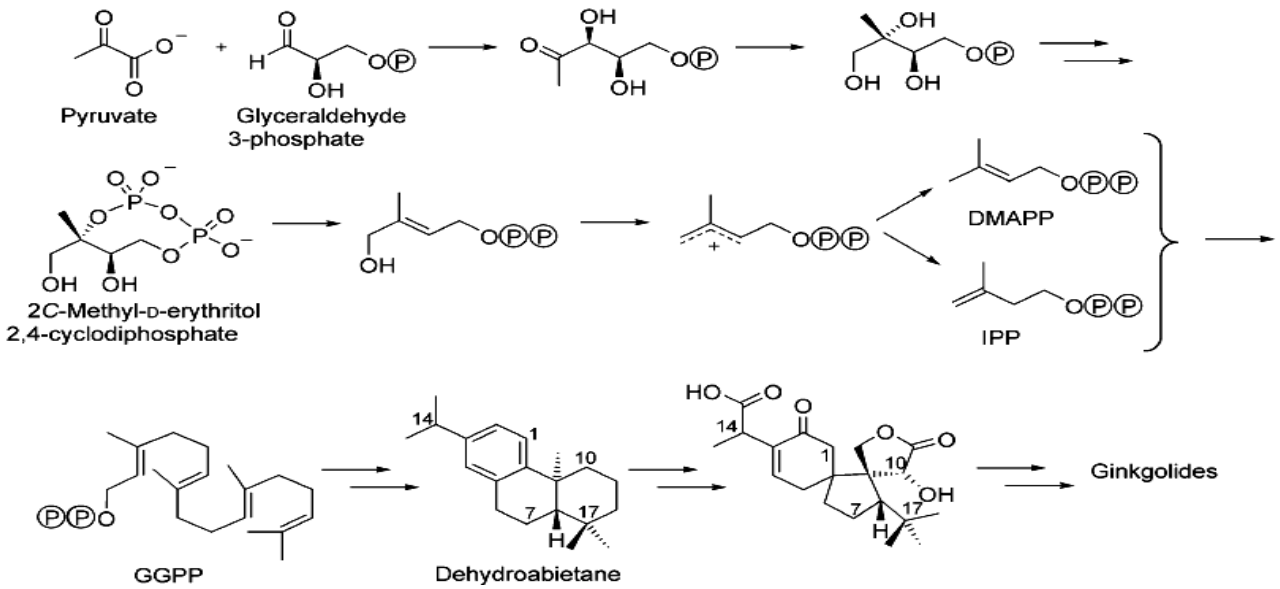


Figure 6. Biosynthetic pathway of synthesis of ginkgolide molecule.

commercial preparations from Europe (Denmark, Italy, UK, Sweden).

Comprehensive and global characterization by alternative technology is necessary for analysis of *G. biloba* preparations. Results show that ^{1}H NMR spectra allow simultaneous assessment of the content as well as identity of flavonoid glycosides and TTLs based on very simple sample-preparation procedure consisting of extraction, evaporation and reconstitution in acetone-d_6. Unwanted extract constituents were easily identified by ^{1}H NMR spectra, which contrast traditional method that depends on UV absorption or MS ionizability and usually require availability of reference standards. Automated integration of ^{1}H NMR spectral segment provides relative distribution plots TTLs based on their H-12 resonance. This study shows that ^{1}H NMR based metabolomics is an attractive method for non-selective and comprehensive analysis of *Ginkgo* extracts.

BIOSYNTHESIS OF GINKGOLIDE

Due to their complex framework, it was difficult to categorize which structural category of natural products ginkgolide belongs (Van Beek and Montor, 2009). Earlier, it was thought that ginkgolides were synthesized through conventional mevalonate pathway, but biosynthetic study led to the surprising discovery of non-mevalonate or deoxyerythritol phosphate pathway (Stomgaard and Nakanishi, 2004). In deoxyerythritol phosphate pathway, pyruvate and glyceraldehyde and 3-phosphate react to produce 2C-methyl-D-erythritol 2,4-cyclodiphosphate and ultimately dimethylallyl pyrophosphate (DMAPP) and isopentynyl pyrophosphate (IPP). Initially, IPP and DMAPP react to produce the universal diterpene precursors, geranylgeranyl pyrophosphate (GGPP), which was converted into tricyclic intermediate, levopimaradiene. This leads to synthesis of dehydroabietane, which is transported from plastids into cytoplasm and then converted into ginkgolides through a complex series of reactions involving several oxidations steps (Figure 6) (Stomgaard and Nakanishi, 2004). Schwarz and Arigoni (1999) also independently studied biosynthesis of ginkgolide and showed involvement of non-mevalonate pathway for ginkgolide biosynthesis.

A cDNA that encodes *G. biloba* levopimaradiene synthase (GbLPS), a diterpene synthase involved in ginkgolide biosynthesis, was isolated and characterized (Schepmann et al., 2001). *G. biloba* levopimaradiene synthase is responsible for a multistep reaction sequence that converts GGPP into levopimaradiene, a double-bond positional isomer of abietadiene. The cloning and isolation of this enzyme, together with cloning of other biosynthetic genes, could provide a route for the large-scale production of ginkgolides (Figure 4). ^{14}C-Labelled ginkgolide and bilobalide experiments show that GA undergoes *in situ* bioconversion into GC.

Previously, it was reported that biosynthetic steps to the synthesis of ginkgolide took place in root, and products are then translocated to the leaves (Liao et al., 2011). To assess the organ-specificity and developmental characteristics of GbLPS expression, Kim et al. (2012) carried out GbLPS promoter-driven β-glucuronidase (GUS) expression in transgenic *Arabidopsis* plant. Histological analysis of transgenic *Arabidopsis* plant showed that the GUS accumulation was mainly localized in the epidermis of leaves, phloem of shoots, ovaries and stamens of flowers and

vasculature of roots. These studies also correlated with the occurrence of LPS transcripts in roots and male strobili of *G. biloba*. Treatment of methyl jasmonate on the transformant exhibited significant up-regulation of the reporter gene in the root with little change in leaves and flowers. This finding supports biosynthesis of ginkgolide in the root of *G. biloba* plant, but, on the other hand, studies carried out by Carrier et al. (1998) confirmed biosynthesis of ginkgolide in aerial parts of plants (Stomgaard and Nakanishi, 2004).

ROLE OF *Ginkgo biloba* METABOLITE UPON DIFFERENT STRESS CONDITION

Major metabolite of *G. biloba* constitutes flavonoids and terpenoids (Nakanishi, 1967). Most important flavonoid includes quercetin, kaempferol, apigenin, anthocyanin etc. whereas terpenoids are of TTLs that constitutes ginkgolide and bilobalide. The role of different flavonoids such as quercetin, kaempferol etc. has been well studied with regards to plant biotic and abiotic stress. Usually, they play important role as antioxidant, anti-feeding, antinutritive agent against insect herbivores. It has been demonstrated that upon feeding *G. biloba* to *Spodoptera littoralis*, significant differential levels of quercetin and kaempferol were observed, compared to mechanically damaged *G. biloba* (Mohanta et al., 2012).

Tanaka et al. (2011) reported the role of bilobol and found that it plays significant role in plant stress response. Cell cultures of *G. biloba* with biotic elicitors *Candida albicans* resulted in enhanced accumulation of bilobalide and ginkgolide as compared to control. Bilobalide content was significantly increased (30.5 mg/gm) as compared to control 7.0 mg/gm after 48 h of treatment (Kang et al., 2009). He et al. (2009) investigated dynamic responses of secondary metabolites in the leaves of *Ginkgo* to elevated O_3. The results showed that secondary compounds from *Ginkgo* leaves responded to elevated O_3 exposure in different ways. Elevated O_3 increased the concentration of quercetin, but decreased the concentration of isorhamnetin and condensed tannin (He et al., 2009). They also found decreased phenolic content in *Ginkgo* leaves. At elevated O_3 level, concentration of terpenes increased significantly. The total mean terpenes concentration was 23% higher than control for elevated O_3. This was probably related to higher level of reactive oxygen species and declined activities of anti-oxidative system in *Ginkgo* leaves due to elevated O_3.

It has been observed that increase in terpene concentration was absolutely season-dependent, with largest difference occurring in September compared to control. At elevated O_3, concentration of bilobalide increased by 220%, ginkgolide C by 69.6%, ginkgolide A by 34.1% and ginkgolide B by 34.3% ($P < 0.01$) (He et al., 2009). When the anti-oxidant system in *Ginkgo* leaves could not resist the long-term O_3 exposure, the increased concentrations of terpene might play significant role in scavenging the high level of oxygen species caused by ozone at the end of O_3 fumigation (He et al., 2009).

FUTURE PERSPECTIVES OF *Ginkgo biloba* RESEARCH

G. biloba is resistant to wild variety of biotic and abiotic stress condition and thus can be a model plant for studying plant disease resistance as well as other stresses. Researches on *G. biloba* increase recently. Although, the research field has been more or less pharmacological based and little work efforts was directed towards genomic and metabolomics aspects. Nevertheless, these days have seen more than 80 thousands EST sequences available for *G. biloba* research. Among them, 21590 EST sequences present in genebank. Besides the EST sequences, more than 500 cDNA sequences are also present in genebank. Although this much information is not enough to carry out research work at genomic level, the accumulated data is sufficient enough to initiate genomic work. It was expected that *G. biloba* research can decode the mechanism of multiple stress resistance and evolution of plant stress tolerance.

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