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# Ginkgolide production in relation to organogenesis in *Ginkgo biloba*

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**Abstract** The contents of diterpenoids, ginkgolides A, B, and C, in seeds, embryos, and plantlets of *Ginkgo biloba* were analyzed to clarify the relations between organogenesis and terpene contents in *G. biloba*. There is so far no published report on the contents and changes in such terpenes in seeds and very young plantlets of *G. biloba*. Ginkgolides were present in seeds and embryos. Plantlets cultured in both the dark and under illumination contained substantial amounts of ginkgolides, more abundant than in seeds and embryos. It is concluded that ginkgo yields ginkgolides in its early stage of development regardless of light.

**Key words** *Ginkgo biloba* · Ginkgolide · Organogenesis

## Introduction

*Ginkgo biloba* L. produces specific diterpenoid ginkgolides, which were isolated by Nakanishi[1] and Okabe et al.[2] As reviewed by Braquet,[3] ginkgolides possess medicinal properties for the treatment of septic shock and inflammatory reactions due to their antagonistic effects on platelet-activating factor.

Ginkgolides have a cage-type molecular structure with six five-membered rings and a *tert*-butyl group (Fig. 1). According to Corey et al.,[4] because of their complex structure chemical synthesis of these compounds is possible but difficult. Therefore, the main source of ginkgolides is the leaves of the *G. biloba* tree. Several studies have been done using tissue cultures of ginkgo to clarify the biosynthetic pathway and establish alternative methods for the production of ginkgolides, including those by Chauret et al.,[5] Huh and Staba,[6] and Jeon et al.[7] Seasonal variation of the ginkgolide contents and the influence of growth and light level on the yields of ginkgolides in the ginkgo tree were also reported by van Beek and Lelyveld[8] and Flesch et al.[9]

Ginkgo seeds and embryos are known to accumulate substantial amounts of ginkgolides. As reported by Inoue et al.,[10] ginkgo seeds, immediately after becoming detached from the tree in autumn, are immature but gradually mature until March of the next year. It is speculated that the ginkgolide content in seeds may change during this maturing process. Although it is known that young plantlets of ginkgo yield ginkgolides, there is no detailed report of the alteration of ginkgolide contents in relation to the growth of the plantlets. In the present study, we investigated the alteration of the content of ginkgolides (A, B, and C) during the maturation of seeds, germination, and growth of seedlings. The effects of the light level on the yields of ginkgolides in seedlings and plantlets obtained from embryos cultured *in vitro* were also studied.

## Materials and methods

### Standards

Ginkgolides A, B, and C were supplied by Dr. H. Schick, Department of Chemistry, Heidelberg University, Heidelberg, Germany.

### Plant materials

Seeds of *Ginkgo biloba* were collected around a ginkgo tree at the University of Tokyo in November 1996. These seeds were used immediately for the quantitative and qualitative analysis of ginkgolides or were stored at 3°C until February and April 1997. Seedlings and embryo cultures were obtained using the seeds stored until April.

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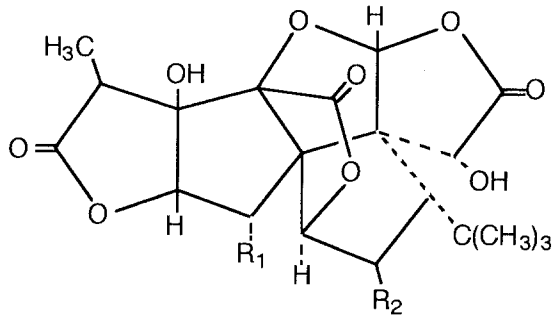


| | |
|---|---|
| Ginkgolide A | R1=R2=H |
| Ginkgolide B | R1=OH, R2=H |
| Ginkgolide C | R1=R2=OH |

**Fig. 1.** Chemical structures of ginkgolides

### Culture conditions

The seeds were sown in vermiculite and cultivated in a greenhouse in natural daylight or in a climate chamber in the dark. In the greenhouse, the temperature during the day was maintained at 25°C and during the night at 20°C; the temperature in the chamber was maintained at 25°C. Seedlings were harvested 1 month after germination.

Embryos were excised aseptically from the seeds and inoculated on a solid medium with half-strength Linsmaier and Skoog's mineral salts,[11] thiamine chloride 1.0 mg/l, myo-inositol 100 mg/l, sucrose 30 g/l, and gellan gum 2 g/l (pH 5.9). They were incubated at 25°C under 16 h/day illumination with fluorescent light (about 1000 lux) and in the dark. Most of the embryos soon germinated, and plantlets were harvested 1 month after initiation of the culture. About 30 seeds or embryos were used for each culture condition.

### Sample preparation

In November 1996 and February and April 1997 a total of 20 seeds were chosen, and stony layers were removed. The albumens and embryos were collected separately, mixed, and used for ginkgolide analysis. Among the seedlings and plantlets cultured *in vitro*, 20 plants were selected at random for each culture condition, mixed, and used for analysis.

Each of the mixed samples was lyophilized; the dry weight was measured, and the samples were then pulverized mechanically with a coffee mill. The aliquots of the resultant powder were weighed (about 500 mg) and then extracted and fractionated according to the method of Huh and Staba.[6] The purified extracts were dissolved in an appropriate amount of methanol and successively analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

### Analysis of ginkgolides

For the analysis by GC, an appropriate amount of purified methanolic fraction was evaporated to dryness and trimethylsilylated by adding 50 μl of a silylating agent (Tri-Sil BSA formula D, Pierce Chemical, USA). This mixture was vortexed and heated for 1 h at 80°C. Analyses by GC-flame ionization detection were performed with a Shimadzu GC-14A (Shimadzu, Japan) equipped with a 30 m × 0.25 mm TC-1 capillary column (GL Sciences, Japan). The temperatures of the column, injector, and detector were maintained at 285°C, 300°C, and 300°C, respectively. $N_2$ was used as the carrier gas at a flow rate of 2.6 ml/min. For quantitative determination of ginkgolides, standard curves were prepared with known amounts of authentic ginkgolides A, B, and C after silylation. The concentration of each ginkgolide was determined using the standard curves. The individual retention times of ginkgolides were compared with those of standards. The retention times of ginkgolides A, B, and C were 8.1, 9.4, and 9.9 min, respectively. Each ginkgolide peak was also identified by comparing the fragmentation pattern of each peak with that of authentic ginkgolides in GC-MS (DX-303; JEOL, Japan).

## Results and discussion

Ginkgolide contents of various samples are presented in Table 1, and the alterations in the amounts of total ginkgolides (the sum of ginkgolides A, B, and C) and in the maturation of seeds, germination, and growth of seedlings are shown in Table 2. As shown in Table 1, ginkgolides were detected in all the samples examined. In embryos, ginkgolide A (GA) was the most abundant of ginkgolides, and the amount of ginkgolide B (GB) was about one-third of that of GA. Ginkgolide C (GC) was not clearly detected. In albumens, GB was the principal ingredient, and GA and GC were present in much lower amounts. Both seedlings cultivated on vermiculite and plantlets cultured *in vitro* yielded GA and GB, regardless of light. In seedlings grown in the dark, the amounts of GA and GB were almost the same, whereas light-grown seedlings produced much higher amounts of GA than GB. In both dark- and light-grown plantlets, GA was somewhat more abundant than GB. Seedlings and plantlets did not yield detectable amounts of GC.

The total amount of ginkgolides per sample are shown in Table 2; that is, the amount of ginkgolides per sample (dry weight) is divided by the number of the samples equivalent to the dry weight. Here, the dry weight per sample is the mean of 20 samples. The amounts of ginkgolides in seeds (i.e., the sum of ginkgolides in embryos and albumens) were almost constant from November to April. Dark- and light-grown seedlings contained almost the same amount of ginkgolides, and the levels were much higher than those in the seeds. These results indicate that ginkgo seedlings do produce significant amounts of ginkgolides regardless of light during the early stage of their growth. Almost the same

**Table 1.** Alteration of ginkgolide contents in relation to storage period of seeds and culture condition for seedlings and plantlets

| Sample | Content of ginkgolides (μg/g dry weight)[a] | | |
|---|---|---|---|
| | GA | GB | GC |
| Embryo | | | |
| November 1996 | 1290 | 475 | – |
| February 1997 | 1650 | 440 | – |
| April 1997 | 925 | 338 | – |
| Albumen | | | |
| November 1996 | 9.36 | 59.6 | 8.43 |
| February 1997 | 9.03 | 119 | 15.8 |
| April 1997 | 8.14 | 113 | 12.5 |
| Seedling[b] | | | |
| Dark | 1170 | 1180 | – |
| Light[c] | 1370 | 762 | – |
| Plantlet[d] | | | |
| Dark | 1250 | 844 | – |
| Light[e] | 1100 | 789 | – |

GA, GB, GC, ginkgolides A, B, and C, respectively; –, trace amount.
[a] Each value is a mean of 20 samples.
[b] Seedlings were obtained from the seeds sown in vermiculite and successively cultured for a month before harvest.
[c] Natural daylight in a greenhouse.
[d] Plantlets were obtained from excised embryos in vitro and harvested 1 month after initiation of culture.
[e] With 1000 lux of fluorescent light

**Table 2.** Alteration of total ginkgolide contents in relation to storage period of seeds and culture condition for seedlings and plantlets[a]

| Sample | Content of total ginkgolides[b] (μg/sample) |
|---|---|
| Seed | |
| November 1996 | 62.5 |
| February 1997 | 72.8 |
| April 1997 | 76.5 |
| Embryo | |
| November 1996 | 10.9 |
| February 1997 | 19.3 |
| April 1997 | 13.4 |
| Albumen | |
| November 1996 | 51.6 |
| February 1997 | 53.5 |
| April 1997 | 63.1 |
| Seedling | |
| Dark | 436 |
| Light | 441 |
| Plantlet | |
| Dark | 54.7 |
| Light | 51.0 |

[a] Sum of GA, GB, and GC in each sample group in Table 1, in units of μg/g sample (dry weight), is converted to the unit of μg/sample.
[b] Total ginkgolide amount (μg/g dry weight) is divided by the number of the samples equivalent to the dry weight. Here the dry weight per sample is the mean of 20 samples

amounts of ginkgolides were detected in dark- and light-grown plantlets. The plantlets contained more ginkgolides than embryos but much less than soil-grown seedlings. The lower ginkgolide yields from *in vitro* plantlets may be due to the deficiency of ginkgolide precursors in albumens, the relatively insufficient growth owing to the loss of albumens, or both. The relative unsuitableness of *in vitro* culture conditions itself (e.g., high humidity, high concentrations of sugar and mineral salts in the medium) may affect ginkgolide production.

Our results indicate that light does not play an important role on the production of ginkgolides in very young plants. Dark-grown plants showed etiolation and no leaf expansion but contained ginkgolides in amounts almost equal to those in light-grown ones with green leaves. This finding indicates that leaves are not the sole site for biosynthesis of ginkgolides; and other organs, such as roots and stems, can biosynthesize ginkgolides, supporting the results reported by Huh and Staba.[6] According to Chinn and Silverthorne,[12] ginkgo is completely dependent on light for chlorophyll synthesis and chloroplast development. Therefore, it is obvious that in young ginkgo plants, chloroplast development is not necessary for ginkgolide biosynthesis. That is, such immature plastids as proplastids, etioplast, and leucoplast may contribute to ginkgolide biosynthesis. As shown in soil-grown seedlings, light seems to have some influence on the contents of individual GA and GB. Although the relation between GA and GB at the level of biosynthesis pathways is not yet known, the development of organs caused by light must affect the ginkgolide pathways. As ginkgolides have a similar chemical structures, conversions between the ginkgolides may exist. In albumens, it seems that GB and GC, which are more hydroxylated than GA, increased; and GA decreased as the period of storage became longer. In contrast, the alteration of total ginkgolide amounts was relatively slight. These results suggest that conversions between ginkgolides may be possible, and GA may be oxidized to GB or GC in seeds during storage.

This study demonstrates that the degree of biosynthesis of total ginkgolides in young ginkgo plants is not influenced by the level of light, but the development of organs probably caused by light affects the individual ginkgolide contents. Moreover, conversions between individual ginkgolides may occur in ginkgo plants. Further investigations are needed to confirm these theories.

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