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Effect of supplementing terpenoid biosynthetic precursors on the accumulation of bilobalide and ginkgolides in *Ginkgo biloba* cell cultures

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Abstract

The effect of precursor feeding on the production of bilobalide and ginkgolides was studied with suspension cell cultures of *Ginkgo biloba*. The precursors greatly influenced the productivity of bilobalide and ginkgolides. Precursor supplementation increased the accumulation of both bilobalide and ginkgolides, and with positive effect on cell growth. The GA accumulation by cell cultures was influenced by precursors upstream in the metabolism, whereas the BB accumulation was under the influence of downstream precursors of the terpenoid biosynthetic pathway. Furthermore, precursor feeding modified the ratios of the BB, GA and GB in cells and cell cultures of *G. biloba*. The studies also aid in understanding effect of precursor feeding on the bilobalide and ginkgolides biosynthetic pathway.

Keywords: Bilobalide; Ginkgolides; Mevalonate pathway; MEP pathway; *Ginkgo biloba*

Abbreviations: BB, bilobalide; GA, ginkgolide A; GB, ginkgolide B; MVA pathway, mevalonate pathway; MEP pathway, 2-methyl-D-erythritol-4-phosphate pathway; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MVA, mevalonate; FPP, farnesyl pyrophosphate; GA-3P, glyceraldehyde-3 phosphate; SP, sodium pyruvate; GPP, geranyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylally pyrophosphate

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1. Introduction

In higher plants, the biosynthesis of terpenoids involves common building block isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Lichtenthaler, 1999). Isoprenoids are derived from a common precursor, IPP. The two biosynthetic pathways that lead to synthesis of IPP are, the cytosolic mevalonate (MVA) and the plastidic 2-methyl-D-erythritol-4-phosphate (MEP) pathways (Lichtenthaler et al., 2002; Rodriguez-Concepcion, 2002). Most terpenoids could be constructed by a repetitive condensation of isoprene units derived from a common precursor IPP formed via MVA and MEP pathway (McGarvey and Croteau, 1995). The action of various prenyl-transferases then generates higher order terpenoid building blocks, geranyl pyrophosphate (GPP; C_{10}), farnesyl pyrophosphate (FPP; C_{15}), and geranylgeranyl pyrophosphate (GGPP; C_{20}). These branch point intermediates may then self-condense to C_{30} and C_{40} precursors of sterols and carotenoids, respectively (Fig. 1). Sesquiterpenes, triterpenes, sterols, and brassinosteroids (BRs) are biosynthesized via the MVA pathway, whereas gibberellins, abscisic acid, carotenoids, and chlorophyll side chains are formed through the MEP pathway (Kasahara et al., 2002).

The leaves and root barks of *Ginkgo biloba*, are listed in the traditional Chinese pharmacopeia. These are characterized by the presence of the bilobalide and ginkgolides (A, B, C, J). Bilobalide and ginkgolides (C_{20} compounds) are a unique family of phytochemicals with restricted occurrence in *G. biloba* tissues (Boralle et al., 1988). They have a cage like molecu-

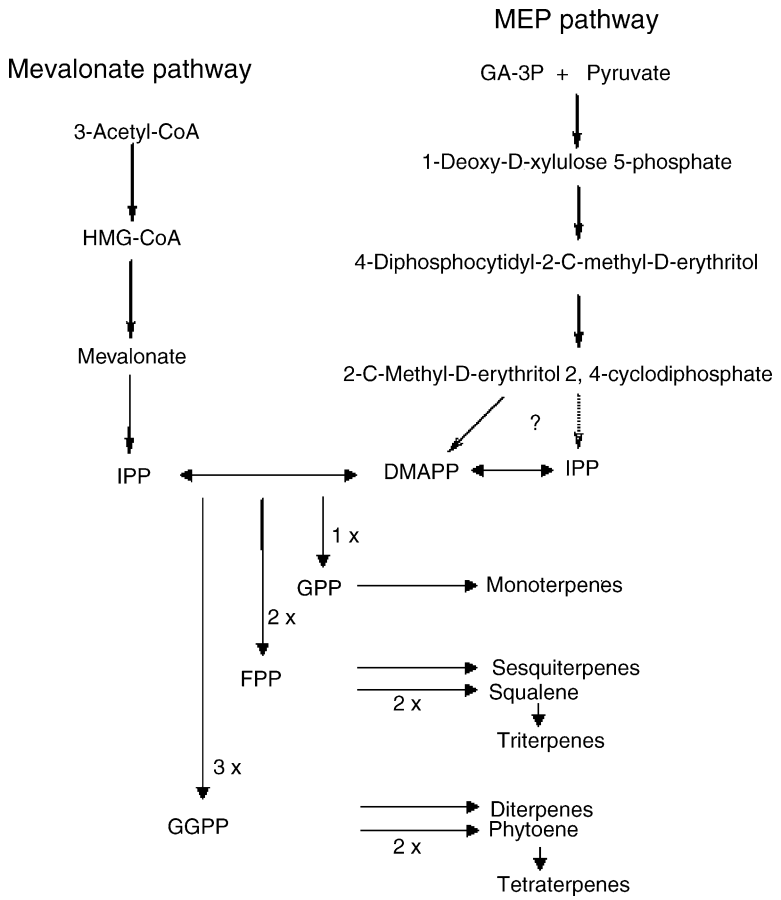

Fig. 1. Outline of the MVA and MEP and terpenoid biosynthesis pathways. 1×, 2× and 3× indicate the number of IPP units added.

lar structure with six five-member rings and a *ter*-butyl group. Bilobalide is sesquiterpene whereas ginkgolides are diterpenes (Carrier et al., 1998). Gingkolides show a significant pharmacological activity against the platelet aggregating factor (PAF). Ginkgolide B is one of the most efficient PAF antagonists (Braquet et al., 1987).

In spite of the pharmacological importance of bilobalide and ginkgolides, little is known about their metabolism and its regulation in *G. biloba*. Also, the influence of the MVA and MEP pathway-related precursor supplementations on production of BB, GA and GB in *G. biloba* has not been elucidated.

The principal goal of this work is to enrich the understanding of the biosynthetic pathway of bilobalide and ginkgolides from *G. biloba*. In this study we characterize the effects of feeding metabolic precursors on the production BB, GA and GB. The precursors related to MVA and MEP pathway have been employed to enhance the production of bilobalide and ginkgolides in cell suspension cultures of *G. biloba*.

2. Materials and methods

2.1. Callus and cell suspension cultures

The callus of *G. biloba* was induced from zygotic embryo. The seed surface was sterilized with 70% (v/v) ethanol for 1 min, 3% (v/v) NaClO for 15 min, and then rinsed with sterile distilled water repetitively for seven times. The zygotic embryo was separated from sterilized seed with knife and pincette. To induce callus, the embryos were surface wounded with knife and transferred to MS solid medium supplemented with 3% (w/v) sucrose, 0.3% (w/v) gelrite, and 3.5 mg/l NAA. The pH was adjusted to 5.8 before autoclaving at 121 °C for 15 min. The cultures were incubated in dark at 25 ± 1 °C, and subcultured after every 4 weeks. For proliferation, the induced calli were transferred into the MS liquid medium supplemented with 3% (w/v) sucrose and 3.5 mg/l NAA. The cell suspension cultures were incubated on a rotary shaker (100 rpm) under dark conditions at 25 ± 1 °C.

2.2. Precursor supplementations for suspension cell cultures

The precursors; geranyl pyrophosphate (GPP), geranylgeranyl pyrophosphate (GGPP), isopentenyl pyrophosphate (IPP), dimethylalyl pyrophosphate (DMAPP), farnesyl pyrophosphate (FPP) solutions were purchased from Sigma–Aldrich, USA. Acetyl-CoA, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), glyceraldehyde-3 phosphate (GA-3P), mevalonate (MVA), and sodium pyruvate (SP) were dissolved in distilled water and filtered through a syringe fitted with a filter (25 mm, Gelman Sciences). The precursors at concentrations of 0.01 mM were incorporated into liquid media. The precursors were treated after 2 weeks of initial cultures which are indicated linear phase of *Ginkgo* cell growth. The precursor fed cultures were harvested after 5 days. The flasks which did not receive precursors served as controls.

2.3. Measurement of cell growth

The extent of cell growth of *G. biloba* suspension cell cultures was measured by determining their fresh and dry weights. To find the fresh weight (FW) the cell were separated from the medium by filteration and washed repeatedly with distilled water and weighed. The dry weight (DW) of the cells was recorded after drying them to a constant weight at 50 °C for 24 h.

2.4. Extraction and quantification of bilobalide and ginkgolides

To quantify the cell extracts were prepared as per Park et al. (2004) method. Briefly, about 0.1 g of dry cells was ground into a paste with pestle and mortar. A 10 ml *n*-hexane was added to the cell paste and sonicated (JAC2010, Jinwoo, Korea). After 1 h of sonic treatment, the *n*-hexane layer was evaporated. Samples of bilobalide and ginkgolides were prepared by extraction of cell paste with 10 ml ethyl acetate for 2 h in ultrasonicator. The resulting extracts were centrifuged (6000 rpm for 10 min), and the supernatant was concentrated using a rotary vacuum evaporator. The bilobalide and ginkgolides in the culture media were extracted with equal volumes of ethyl acetate by intermittent vortexing over a period of 2 days. The combined ethyl acetate extract was dried with rotary vacuum evaporator.

The resulting residue was dissolved in 200 μl MeOH (HPLC grade), filtered through a pre-filter (0.2 μm Supelco) and analyzed by HPLC (Gilson, France) equipped with a Lichrospher 100 RP-18

(4.6 mm × 25 cm, 5 μm, Merck) column and a UV detector (Gilson, UV 3000). The isocratic mobile phase comprised of MeOH and H_2O (50:50 (v/v)). After the injection of 20 μl of the sample solution, the column was operated at a flow rate of 0.5 ml/min. The system was calibrated with standard bilobalide, ginkgolide A and ginkgolide B for quantitative analysis. The correlation coefficients (*R*) of the standards were 98.9% for bilobalide, 99.7% for ginkgolide A and 99.6% for ginkgolide B. Quantification of BB, GA and GB was achieved by comparison with the retention time and co-chromatogram of the standards and samples. Retention times of BB, GA and GB were 6.89, 9.92 and 10.26 min, respectively.

2.5. Statistical analysis

Data are expressed as an average of at least three separate experiments. The error bars in the charts indicate standard deviation (S.D.) from the mean of each replicate treatment.

3. Results and discussion

3.1. The effect of precursor feeding on the cell growth

The precursors feeding to cell suspension cultures generally increased the cell growth of *G. biloba* (Fig. 2). After 5 days of feeding various precursors, most of the cell cultures except for GA-3P treatment exhibited an increase in cell mass in comparison to that of control. The lag phase of suspension culture was observed up to 6 days, and the exponential phase and linier phase was observed between 6 and 18 days, respectively. The cell biomass increased slightly during the stationary phase between 18 and 30 days (data not shown). Treatment of cell cultures with acetyl-CoA showed 39% increase in cell growth. Also, GGPP, IPP, FPP, DMAPP and GPP feeding lead to an increase of 50, 35, 29, 28 and 28% in fresh weight, respectively. It has been observed usually that suspended cells are more sensitive than tissue to environmental change (here precursor feeding). The increase in cell mass however may be attributed to the fact that precursors resemble endogenous compounds which are produced in the cells. As precursors used in the study are terpenoid biosynthesis related, the positive effects are reflected in cell cultures. Bohm and Mack (2004) have indicated that precursor feeding is not negative on growth as endogenous precursors are being continuously formed during metabolism in plants.

3.2. The influence of precursors on the accumulation of bilobalide and ginkgolides in cell cultures

Fig. 3 shows the production of the BB, GA and GB in the cells after 5 days after feeding with various precursors. The supplementation of MVA pathway-related precursors to cell cultures increased the BB contents in cells. On HMG-CoA treatment, the BB content of cells increased to 2.3-folds. However, other treatments of MVA pathway-related precursors were ineffective for BB accumulation. When acetyl-CoA

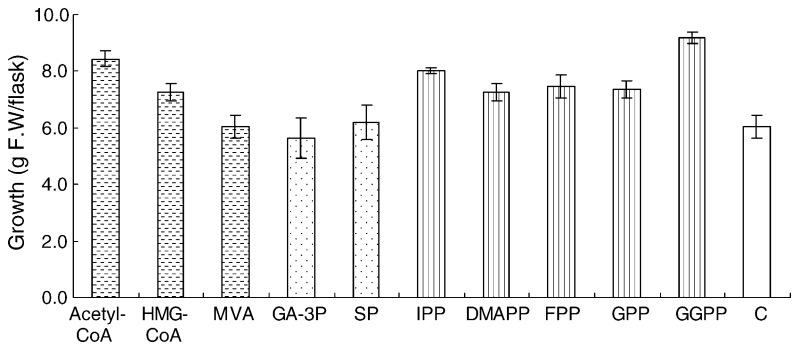

Fig. 2. Effects on cell growth by precursor feeding in cell suspension culture of *G. biloba* (C, control). A dotted line bars represent MVA pathway-related, doted bars relate to MEP pathway-related, and bars with vertical lines relate to IPP condensation-related precursors.

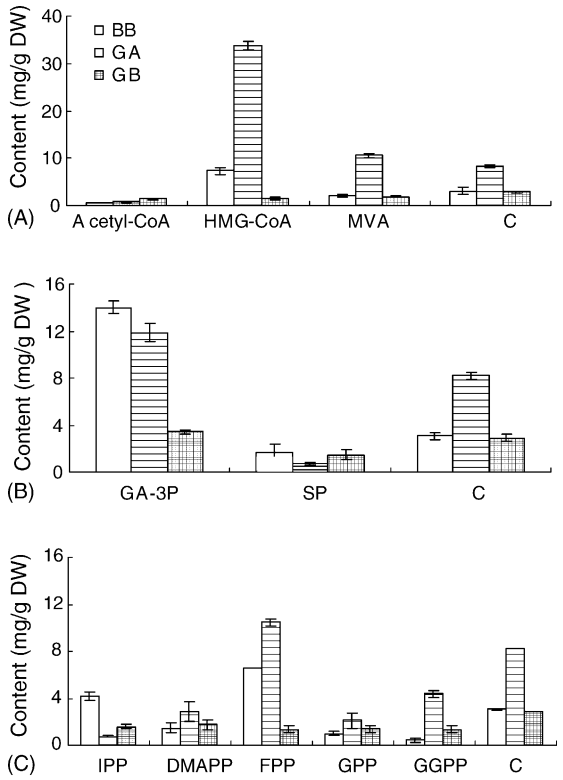

Fig. 3. Influence of precursor feeding on BB, GA and GB production in cell suspensions (C, control). (A) MVA pathway-related, (B) MEP pathway-related, and (C) IPP condensation-related precursors.

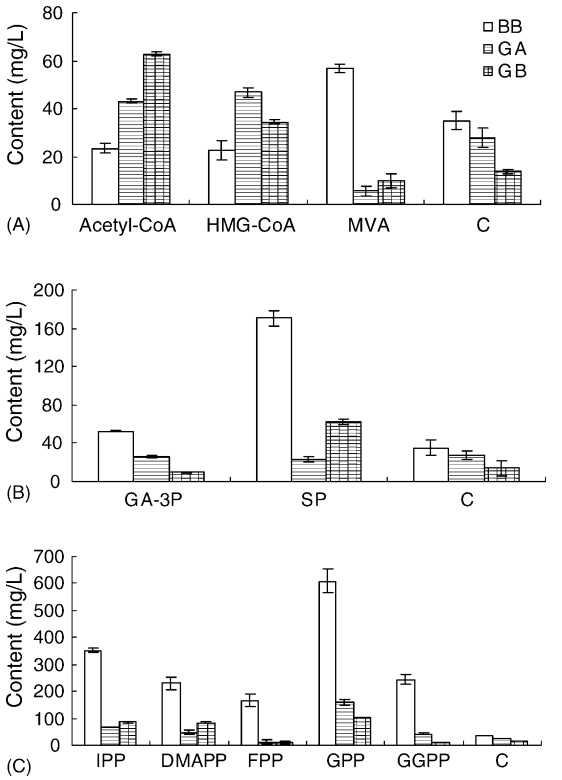

Fig. 4. Influence of precursor feeding on BB, GA and GB accumulation in culture medium of suspended cells treated with precursors (C, control). (A) MVA pathway precursors, (B) MEP pathway precursors, (C) IPP condensation precursors.

was fed to cell cultures, the bilobalide and ginkgolides contents decreased. The production of GA increased after cell cultures were supplemented with HMG-CoA. The HMG-CoA treatment increased the GA content by 4.25-folds, recording a maximum yield of 34.3 mg/g DW. However, GB content did not increase on feeding cell cultures with MVA pathway-related precursors (Fig. 3A). In the case of MEP pathway-related precursors feeding, except for GA-3P, most precursors did not improve BB and GA production. When GA-3P was the precursor, the BB and GA contents increased up to 4.6 and 1.5-folds, respectively. On a similar basis, the feeding of MEP pathway-related precursors did not enhance the GB content (Fig. 3B). Most of the IPP-related condensation precursors were ineffective towards production of BB, GA and GB in cell cultures. However, FPP feeding increased the BB content of the cell cultures by two-folds (Fig. 3C).

GB is the final compound in the secondary metabolism of *G. biloba*. In general, final metabolites do not easily increase by elicitor treatments. Kang et al. (2004) have shown that production of hyocyamine was markedly increased by treatment with signaling compounds. In elicitor treatment experiments to enhance production of tropane alkaloids in *Scopolia parviflora*, hyocyamine contents increased whereas scopolamine, the final product after hyoscyamine increased insignificantly.

3.3. Influence of precursors on accumulation of bilobalide and ginkgolides in the culture medium

Fig. 4 shows the production of the BB, GA and GB in the culture medium on fifth day after feeding variety of precursors. After treatment with MVA, a MVA pathway-related precursor, the BB production in

culture medium increased 1.6-folds. However, MVA treatment was ineffective in production of GA and GB.

The production of the GA and GB were increased by inclusion of acetyl-CoA and HMG-CoA. After acetyl-CoA and HMG-CoA treatments, the GA content in the cells increased to 1.5 and 1.6-folds. The maximum yield of GA was 43 and 47 mg/l, respectively. Also, GB contents were increased by 4.5 and 2.4-folds on feeding acetyl-CoA and HMG-CoA (Fig. 4A). In culture medium, the BB production was owing to downstream metabolism related precursor such as MVA in MVA pathway, whereas the GA and GB accumulation were effective by upstream metabolism related precursors, such as acetyl-CoA and HMG-CoA.

In the case of feeding MEP pathway-related precursors, SP supplementation in culture medium did not improve the production of the GA. However, SP increased both BB and GB accumulations up to 4.8 and 4.4-folds (Fig. 4B).

IPP and DMAPP feeding increased the production of not only BB but also GA and GB. After providing DMAPP, the BB and GB content in the culture medium increased by 6.5–6.0-folds. Also, the content of the GA increased, 1.75-folds on feeding DMAPP. In case of IPP treatment enhanced BB, GA and GB by 10, 2.3 and 6.2-folds, respectively. The IPP treatment stimulated the excretion of the GA and GB by cell cultures and the production of GA and GB reached the highest level of 5.7 and 7.2-folds, respectively. The BB production also markedly increased to 17.4-folds. The production of the BB in culture medium markedly increased after FPP feeding. FPP was able to increase BB content up to 4.7-folds. The GGPP feeding enhanced the BB production up to 6.9-folds compared with the control. However, treatment of this precursor did not have definite effects on the GA and GB accumulation (Fig. 4C). The most effective treatment for production of BB and ginkgolides in cultured medium was due to GPP. Treatment with GPP not only enhanced the production of the BB in the culture medium but also of GA and the GB. As shown in Fig. 4C, the excretion of the BB, GA and GB into the culture medium was markedly increased by GPP treatment. Nakanishi and Habaguchi have demonstrated that the ginkgolide biosynthetic pathway in seedlings involves geranylgeranyl pyrophosphate (GGPP) as a precursor (Nakanishi and Habaguchi, 1971). Also, the ginkgolide and bilobalide pathway has been reexamined by Arigoni's laboratory (Schwarz, 1994). They established the role of GGPP as a precursor, further they also demonstrated unequivocally that bilobalide is not a sesquiterpene, as presumed so far, but a pentanorditerpene. The bilobalide and ginkgolides share the same metabolic pathway to a common precursor with 20 carbon atoms (Neau et al., 1997). However, we in our study confirm that GPP is an important precursor related the BB, GA and GB biosynthesis.

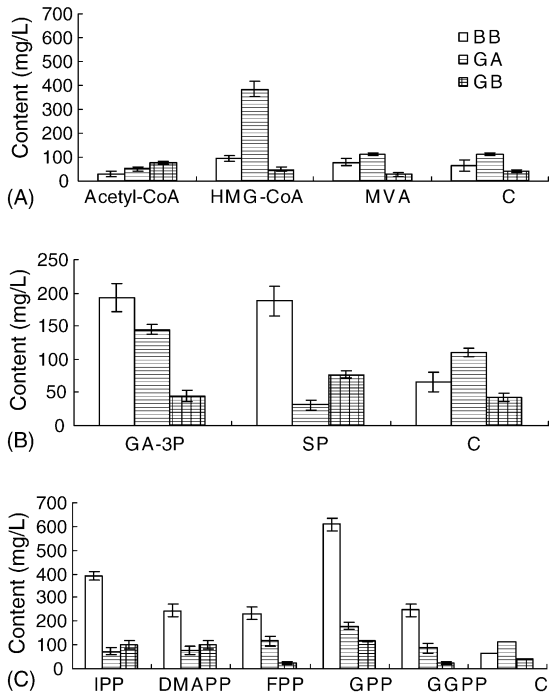

Fig. 5. Total content of BB, GA and GB in cells and cell culture medium after precursor feeding (C, control). (A) MVA pathway precursors, (B) MEP pathway precursors, (C) IPP condensation precursors.

3.4. The effect of precursors on the total production of bilobalide and ginkgolides in cells and cultured medium

Fig. 5 shows the total content of the BB, GA and GB in the cells and culture medium after various precursor supplements. The GA content was maximal in HMG-CoA fed cultures. The HMG-CoA feeding did not improve the production of the GB, whereas it increased the GA contents up to 3.5-folds. The GA-3P

and GPP feeding although enhanced GA contents but only slightly.

The BB accumulation was effective by GPP, IPP, DMAPP, GGPP, and FPP in that sequence. Namely, precursors formed by repetitive joining of isoprene units, increased the BB accumulation. The HMG-CoA, MVA, GA-3P, and SP increased the BB accumulation slightly. The BB content was maximal in GPP fed culture. Treatment of GPP not only increased BB accumulation but also GA and GB in the culture. As shown in Fig. 5C, the contents of the BB, GA and GB markedly increased up to 9.3, 1.7, and 2.7-folds, respectively on treatment of GPP.

The IPP and DMAPP treatments were also simulative for GB and BB accumulation. The accumulation of BB by IPP and DMAPP feeding increased 6.0 and 3.7-folds (Fig. 5C).

In the present study, we confirm that HMG-CoA feeding was very effective in GA production in cells, whereas GPP feeding was most effective for GA accumulation in culture medium. Maximal BB content in cells was when GA-3P was fed. However, for the production of the BB and GB in culture media, feeding of GPP as a precursor was most effective. That is, GA accumulation was enhanced by precursors upstream in terpenoid biosynthesis, whereas BB and GB accumulation was effective by downstream related precursors derived from IPP. Moreover, the ratio of BB, GA and GB contents was modified on precursor feeding. For instance, IPP and DMAPP treated cultures showed similar levels of GA and GB whereas in control cultures the GA content was about twice the GB content. Furthermore, the GA is not anymore the major compound in treated cultures compare with control where the GA content was much more than BB and GB. The biosynthetic steps leading to the formation of BB and GB seemed therefore more sensitive to the addition of IPP and DMAPP than GA biosynthesis. Bohlmann et al. (1997) have reported that all diterpenes are derived from geranylgeranyl diphosphate (GGDP) and are prevalent throughout the plant kingdom. In this study, we confirm that IPP derived precursors, especially GPP were effective on the accumulation of both BB and GB. Up to now, there is little information on their metabolism and its regulation in *G. biloba*. There are several reasons for this. An overwhelming problem with the terpenoids is their sheer number. A given plant may synthesize (and catabolized) many different terpenoid types (from C_5 to C_{40} and higher) at different times and locations for different purposes throughout the course of plant development (Endo and Suga, 1992). Because all terpenoids arise from a common biosynthetic pathway (McCaskill and Croteau, 1995), sophisticated control mechanisms must exist to ensure the production of appropriate levels of these often structurally related complex compounds in the proper metabolic, developmental, and environmental context.

The BB, GA and GB accumulation and cell growth did not agreed in *Ginkgo* cell cultures. Acetyl-CoA enhanced on cell growth, whereas it was decreased BB, GA and GB accumulation. On the other hand, GA-3P decreased cell growth, but enhanced on BB, GA and GB accumulation. In general, secondary metabolites were negative to cell growth.

Treatment of precursors was enhanced BB, GA and GB accumulation in cultured cells and medium. Precursors feeding stimulated the secretion of BB, GA and GB in culture medium. However, we could not confirm to why some precursors increase the accumulation in cells, while others increase the accumulation in the cultured medium. Further work is required to investigate the relation on BB, GA and GB accumulation in cells and cultured medium.

In our experiments, we confirm that HMG-CoA is an important precursor for GA production (Fig. 5A). IPP is thought to be strictly derived from the MVA pathway, and the enzyme HMG-CoA reductase was considered to be a rate-limiting enzyme for this biosynthetic route (Dimster-Denk et al., 1994; Chappell, 1995). Hence, HMG-CoA feeding probably lead to activity increase of HMG-CoA reductase by an affluent substate response resulting finally to increase GA production. In case of MEP pathway related precursor, GA-3P and SP (Fig. 5B), were effective for BB production. In general, sesquiterpene production relates to MVA pathway. However, the results (Fig. 5B) suggest that MEP pathway is also affected with respect to sesquiterpene production. Furthermore, our experiments showed that IPP derived precursors increased the production of BB and GB in cell cultures of *G. biloba* (Fig. 5C). These results suggest that IPP is important in stimulating the biosynthesis for enhanced-production diterpene and most other terpenoids (McGarvey and Croteau, 1995). Also, our results suggest that high accumulation of BB, GA and GB in both cells and

culture medium can be useful for the large scale production. Especially, secretion in medium of BB, GA and GB should be made to allow the industrialization economically by continuous culture system. Further work is required to investigate the related enzymes involved in the enzymatic biosynthesis of diterpene. In addition, the detailed mechanisms of diterpene production and expression of key enzymes during the treatment of precursor is to be further investigated.

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