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Role of Jasmonic Acid in Alteration of Ginsenoside Heterogeneity in Elicited Cell Cultures of *Panax notoginseng*

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The efficient manipulation of ginsenoside heterogeneity of *Panax notoginseng* cells using a recently synthesized elicitor, 2-hydroxyethyl jasmonate (HEJ, at 200 μM), has been reported. In this work, the activities of two enzymes related to ginsenoside heterogeneity (distribution), protopanaxdiol 6-hydroxylase (P6H) and UDPG-ginsenoside Rd glucosyltransferase (UGRdGT), were examined in cell cultures of *P. notoginseng* elicited by HEJ. P6H and UGRdGT activities were increased by HEJ with corresponding changes in Rb/Rg ratio and Rb1/Rd ratio. Endogenous jasmonic acid (JA) seemed to mediate the induction of UGRdGT activation, but was not involved in P6H activation. The results suggest that JA, as a signal transducer, may play an important role in the alteration of ginsenoside heterogeneity in elicited *P. notoginseng* cells.

[**Key words:** *Panax notoginseng*, enzyme activity, ginsenoside heterogeneity, secondary metabolite, synthetic jasmonate derivative, plant cell culture]

Plant cell and tissue culture technology is useful for obtaining valuable plant secondary metabolites, and various developments have been made in this research direction (1–4). Molecular diversity is a widely existing phenomenon in plant secondary metabolites, and rational manipulation of the heterogeneity or distribution of structurally similar secondary metabolites in cell cultures is an interesting and important issue (3). Elicitation as a useful strategy does not only enhance the production of secondary metabolites, but also regulate changes in the molecular diversity of plant secondary metabolites (*e.g.*, Refs. 5, 6).

Ginseng plants (*Panax* spp.) have been used as a healing drug and health tonic in China, Japan, and other Asian countries since ancient times, and ginseng saponins (ginsenosides) are among its principal bioactive ingredients. Ginsenosides, as glycosylated triterpenes, are biosynthesized through the isoprenoid pathway by cyclization of 2,3-oxidosqualene (Fig. 1). This cyclization event occurs at the branch point of two pathways. One pathway leads to cycloartenol via sterol biosynthesis while the other produces various triterpene products including oleanane and dammarane triterpenes (7). There are two major types of ginsenoside, *i.e.*, protopanaxdiol and protopanaxtriol. Different ginsenosides have different biological activities (5). For example, protopanaxadiol saponins (Rb group, *e.g.*, Rb1 and Rd) have antitumor activity (8), while protopanaxatriol saponins (Rg group, such as Rg and Re) affect the spontaneous motility of the intestine (9). Furthermore, some ginsenosides have opposite biological activities. For example, Rg_1 has the effect of stimulating the central nervous system, while Rb_1 has antitumor activity and tranquilizing effects on the central nervous system (9). Therefore, an engineering approach to manipulating ginsenoside heterogeneity in cell cultures has a significant impact on practical applications. The alteration of ginsenoside distribution (heterogeneity) by elicitation with jasmonate was observed in cell cultures of *P. notoginseng* and adventitious root cultures of *P. ginseng* (5, 6, 10). However, the mechanism underlying the alteration of ginsenoside heterogeneity in such cases is as yet unclarified.

Our recent work indicated that external calcium regulates ginsenoside heterogeneity by affecting calmodulin (CaM), calcium-dependent protein kinase, and UDPG-ginsenoside Rd glucosyltransferase (UGRdGT) (11). A recently synthesized jasmonate, 2-hydroxyethyl jasmonate (HEJ), was also found to effectively alter ginsenoside heterogeneity and UGRdGT activity to a greater degree than methyl jasmonate (MJ), in cell cultures of *P. notoginseng* (6). However, it is yet uncertain whether endogenous JA, the important signal transducer in plant cells, is involved in the induction of UGRdGT in ginsenoside biosynthesis.

Protopanaxdiol 6-hydroxylase (P6H), a cytochrome P450 enzyme, catalyzes the biotransformation from protopanaxdiol to protopanaxtriol in *P. notoginseng* cells (Yue, C. J., Ph.D. thesis, ECUST, Shanghai, 2005). To date, no studies have been reported about the role of the P450 enzyme in regulating the ginsenoside heterogeneity of plant cells.

In this study, HEJ was taken as a typical example of novel jasmonate analogues, and the activities of two enzymes related to ginsenoside heterogeneity, *i.e.*, UGRdGT and P6H, were investigated in suspension cultures of *P. notoginseng* cells under HEJ elicitation. Endogenous JA level was exam-

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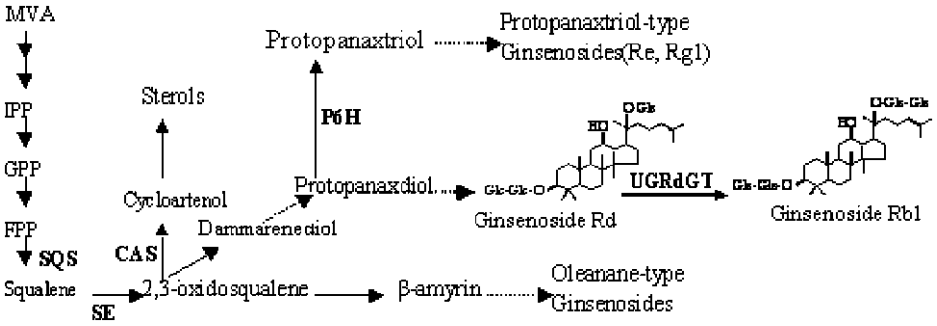

FIG. 1. Proposed ginsenoside biosynthetic pathway modified from Refs. 11 and 18. Abbreviations: MVA, mevalonate; FPP, farnesyl diphosphate; SS, squalene synthase; SE, squalene epoxidase; CAS, cycloartenol synthase; P6H; protopanaxadiol 6-hydroxylase; UGRdGT, UDPG-ginsenoside Rd glucosyltransferase.

ined. In all experiments, MJ, which is an HEJ analogue and a conventional but powerful elicitor, was also applied to the cell cultures for comparison and discussion.

MJ and HEJ were obtained as described earlier (12). DTT, EGTA, PMSF, NADPH, FMN, and FAD were purchased from Sigma (St. Louis, MO, USA). Sodium diethydithiocarbamate (DIECA) was purchased from Sigma-Aldrich. Protopanaxtriol and protopanaxdiol of 98% purity were obtained from Jianfu Company (Chengdu, China). Other chemicals were purchased from Shanghai Chemical (Shanghai, China).

Suspension cells of *P. notoginseng* were grown in a 250-ml Erlenmeyer flask containing 50-ml Murashige and Skoog (MS) medium and subcultured every 2 weeks. MJ and HEJ were added (at 200 μM) as described elsewhere (6). In experiments, a JA biosynthetic inhibitor, DIECA, was added to the cell cultures (at 500 μM) 20 min before the elicitation treatment.

For sampling, three identical shake flasks were used for each data point. The samples collected from flasks were filtered under vacuum and washed with several volumes of distilled water to remove residual medium. The analytical procedures for cell dry weight (DW) and ginsenoside content were the same as those described previously (5). UGRdGT, which catalyzes the formation of Rb_1 from Rd in *P. notoginseng* cells, was detected as described previously (8, 13).

For the assay of P6H activity, P6H was extracted at 4°C. *P. notoginseng* cells were elicited with 200 μM MJ or HEJ on day 4, and the cells were harvested by vacuum filtration and prechilled at 4°C. Prechilled cells were ground rapidly on ice using a prechilled mortar and pestle, and 2-ml buffer containing 100 mM sodium phosphate (pH 7.8), 1.0 mm DTT, 250 mM sucrose, 2.0 mM EGTA, and 1.0 mM PMSF was added. The cell homogenate was centrifuged at 20,000×*g* for 15 min at 4°C. The supernatant was used as a crude enzyme extract for assay. Enzyme activity was estimated on the basis of the conversion of protopanaxdiol to protopanaxtriol. The reaction mixture (0.5 ml) contained 100 mM sodium phosphate (pH 7.5), 0.2 mM protopanaxdiol, 5 mM NADPH, 0.0025 mM FMN, 0.0025 mM FAD, and a crude enzyme extract. The reaction mixture without protopanaxdiol was used as the control. The reaction was started by adding 5 mM NADPH. After 90 min at 30°C, the reaction was stopped by adding 0.5 ml dichloromethane. The added dichloromethane was extracted twice and volatilized, and then the residue was redissolved in methanol for protopanaxtriol assay by HPLC, using a Shimadzu LC-10AT_{VP} HPLC apparatus equipped with a variable-wavelength UV detector (SPD-10A_{VP}; Shimadzu, Kyoto). A Shimadzu VP-ODS column (250×4.0 mm; 5 μm) was used at 25°C. The mobile phase consisted of acetonitrile (solvent A) and water (solvent B) and the following gradient protocol was used: 0–15 min with 20–25% A and 80–75% B, 15–37 min with 25–32% A and 75–68% B, 37–60 min with 32–46% A and 68–54% B, 60–65 min with 46–100% A and 54–0% B, and 65–70 min with 100–20% A and 0–80% B. The flow rate was kept constant at 0.8 ml/min. Protopanaxtriol was monitored and identified at 203 nm by comparing it with its authentic sample. Total protein was quantified by the Bradford method (14).

Quantification of JA in *P. notoginseng* cells was performed as described earlier (12). Gas chromatography/mass spectrometry (GC/MS) on a Micromass GCT unit (UK) with a HP-5 fused silica capillary column of 30 m×0.32 mm ID and 0.25 μm film thickness was carried out.

All data represent mean values with standard deviations from three independent samples. Data were analyzed using Student's *t*-test. The difference between treatments was considered significant when $P<0.05$ in a two-tail analysis.

The application of HEJ resulted in an increase in endogenous JA level and accumulation of ginsenosides in *P. notoginseng* cells (6), which was re-confirmed in this work. In order to determine whether JA, as a signal transductor, mediates ginsenoside biosynthesis in cell cultures elicited by HEJ, a JA biosynthetic inhibitor was used. Wang *et al.* (6) reported that ibuprofen (Ibu), a JA biosynthetic inhibitor, decreased endogenous JA level. In this case, JA level was 116.4 ng/g FW after a 12-h elicitation by HEJ, which was much higher than that of control cells (5.4 ng/g FW). When Ibu was added together with HEJ, the content of JA was nearly the same as that of control cells. However, IBU had negligible effect on ginsenoside biosynthesis. To use a JA biosynthetic inhibitor to depress both JA production and ginsenoside accumulation, another JA biosynthetic inhibitor, DIECA (15), was applied here. As shown in Table 2, following elicitation with HEJ, the biosynthesis of each ginsenoside was enhanced, and ginsenoside heterogeneity also changed as the content of the Rb group increased to a greater extent than that of the Rg group compared with the control. The effects of DIECA on JA production (Table 1) and the content of each ginsenoside (Table 2) were simultaneously