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Efficient induction of ginsenoside biosynthesis and alteration of ginsenoside heterogeneity in cell cultures of *Panax notoginseng* by using chemically synthesized 2-hydroxyethyl jasmonate

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Abstract Chemically synthesized 2-hydroxyethyl jasmonate (HEJA) was for the first time employed to induce the ginsenoside biosynthesis and to manipulate the product heterogeneity in plant cell cultures. The dose response and timing of HEJA elicitation were investigated in cell suspension cultures of *Panax notoginseng*. The optimal concentration and timing of HEJA addition for both cell growth and ginsenoside accumulation was identified to be 200 μM added on day 4. It was interestingly found that HEJA could stimulate ginsenosides biosynthesis and change their heterogeneity more efficiently than methyl jasmonate (MJA), i.e., the total ginsenoside content and the Rb/Rg ratio increased about 60 and 30% with HEJA elicitation than that by MJA, respectively. The activity of Rb_1 biosynthetic enzyme, i.e., UDPG-ginsenoside Rd glucosyltransferase (UGRdGT), was also higher in the former case. A maximal production titer of ginsenoside Rg_1, Re, Rb_1, and Rd was 47.4±4.8, 52.3±4.4, 190±18, and 12.1±2.5 mg/l with HEJA elicitation, which was about 1.3-, 1.3-, 1.7-, and 2.1-fold than that using MJA, respectively. Early signal events in plant defense response, including oxidative burst and jasmonic acid (JA) biosynthesis, were also examined. Levels of H_2O_2 and NO in medium and L-phenylalanine ammonia lyase activity in cells were not affected by addition of MJA and HEJA. On the other hand, the JA content in cells was increased with external jasmonates elicitation, and it was inhibited with the addition of JA biosynthesis inhibitors. The results suggest that oxidative burst might not be involved in the jasmonates-elicited signal transduction pathway, and MJA and HEJA may induce the ginsenoside biosynthesis via induction of endogenous JA biosynthesis and key enzymes (such as UGRdGT) in the ginsenoside biosynthetic pathway of *P. notoginseng* cells. The information is useful for hyperproduction of plant-specific heterogeneous products.

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Introduction

Plant cell culture is an alternative to whole plant extraction for obtaining valuable secondary metabolites. Molecular diversity is a widely existing phenomenon in plant secondary metabolites (Sticher 1998), and intentional manipulation of the heterogeneity of secondary metabolites in cell cultures is a very important issue (Wang and Zhong 2002). Exogenously applied methyl jasmonate (MJA) could enhance production of secondary metabolites by a variety of plant species (Dong and Zhong 2001; Yu et al. 2002; Tabata 2004). Changes of the molecular diversity of ginsenosides in ginseng cell and root cultures were also observed (Wang et al. 2005; Yu et al. 2005). Recently, Qian et al. (2004a,b) reported that the hydroxylation of MJA at its C-1 position resulted in a higher stimulatory activity on taxane biosynthesis in cell cultures of *Taxuschinensis*. However, it remains unknown whether the newly synthesized jasmonates (Qian et al. 2004a,b) could change the heterogeneity of secondary metabolites as much as, or even more than, MJA.

Elicitation could induce the formation of defense compounds in plant cell cultures. This induced synthesis requires a series of signal molecules transmitting the message between the elicitor plant cell wall receptor and gene ac-

tivation. The octadecanoid pathway leading to the synthesis of jasmonic acid (JA) has been identified as an integral part of the signaling cascades triggered by elicitation (Creelman and Mullet 1997). The oxidative burst, which corresponds to a rapid and transient production of active oxygen species (AOS) such as H_2O_2, is a typical early event in plant defense responses (e.g., Chen et al. 1993; Hu et al. 2003b; Zhao and Sakai 2003). It was reported that pathogens or elicitors could induce a rapid production of H_2O_2 and finally act on gene expression that caused programmed cell death and the activation of L-phenylalanine ammonia lyase (PAL) (Desikan et al. 1998). The rapid production of H_2O_2 could induce jasmonate signals and finally act on the biosynthesis of various secondary metabolites including ginsenosides (Hu et al. 2003a; Zhao et al. 2005). In other cases, JA induced by wounding could act on early signaling genes and the rapid production of H_2O_2, and H_2O_2 then induced the expression of late defense genes, but NO in this signal cascade inhibited the defense genes expression by inhibiting H_2O_2 accumulation (Orozco-Cárdenas and Ryan 2002). Qian et al. (2004a) investigated the response of H_2O_2 to 2,3-dihydroxypropyl jasmonate, a chemically synthesized elicitor, in cell cultures of *T. chinensis*. However, no detailed information is available about the studies on signal cascade for such synthetic elicitors, not to mention for the case of cell cultures of ginseng species.

Panax notoginseng (Sanchi-ginseng) is one of the most valuable traditional Chinese medicinal herbs. Cell culture of *P. notoginseng* is a promising technology to obtain ginsenoside, one of its major bioactive secondary metabolites (Woragidbumrunge et al. 2001; Zhang and Zhong 2004). Because different ginsenosides have different or even opposite pharmacological activities, engineering approach for manipulation of ginsenoside heterogeneity in cell cultures has a significant impact on practical application (Wang and Zhong 2002). We have demonstrated that an addition of 200 μM MJA could increase the content of individual ginsenosides and change their heterogeneity in cell cultures of *P. notoginseng* for both shake flask and bioreactor cultivations (Wang and Zhong 2002; Wang et al. 2005).

The present work investigates the effects of self-synthesized hydroxyl-containing jasmonate elicitor, 2-hydroxyethyl jasmonate (HEJA), on the biosynthesis and heterogeneity of ginsenosides in cell cultures of *P. notoginseng*. To understand the signal transduction from jasmonate elicitation to ginsenoside biosynthesis, early signal events in plant defense response (including oxidative burst and JA biosynthesis) were also studied together with the investigation on the activity of UDPG-ginsenoside Rd glucosyltransferase (UGRdGT), a new and important enzyme in the ginsenoside biosynthetic pathway (Yue and Zhong 2005a,b). In all experiments, MJA was also used for comparison and discussion.

Materials and methods

Chemicals

MJA was purchased from Tokyo Kasei Co. Ltd. (Tokyo, Japan). Silica gel (200–400 mesh, 60 Å, for column chromatography) was obtained from Aldrich. Organic solvents were obtained from commercial suppliers and were of the highest purity available; they were dried over 3Å molecular sieves for at least 48 h prior to use. Reagents for synthesis of HEJA were purchased from Sigma and used without further purification.

Chemical synthesis of HEJA

HEJA was synthesized and purified as described earlier (Qian et al. 2004b), and both MJA and HEJA contain the same ratio of stereoisomers (Qian et al. 2004b).

Cell subcultures

Suspension cells of *P. notoginseng* were grown in Murashige and Skoog (MS) medium and subcultured every 2 weeks. The details were described elsewhere (Woragidbumrunge et al. 2001; Wang and Zhong 2002).

Cultivation conditions

P. notoginseng cells (2 g of fresh weight) were incubated in a 250-ml Erlenmeyer flask containing 50-ml medium with the same culture conditions as in subcultures. MJA, HEJA, or JA biosynthesis inhibitors was dissolved in ethanol and sterilized by filtering through 0.22 μm polyvinylidenedifluoride (PVDF) syringe filters (Millipore) before adding to the cell cultures. The final ethanol concentration in cultures was 1 ml/l. Equal volumes of ethanol were added to all flask cultures. For all cultures, multiple flasks were run under each condition, and the cultivation data represent the mean values with the standard deviations from three independent samples.

Measurement of cell weight and ginsenoside content

For sampling, three identical shake flasks were used for each data point. The samples 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 described previously (Zhang and Zhong 2004; Wang et al. 2005).

Enzyme extraction and assay of UGRdGT activity

UGRdGT, which catalyzes the formation of Rb_1 from Rd in *P. notoginseng* cells (Yue and Zhong 2005a,b), was detected as described in Wang et al. (2005).

Analysis of H_2O_2

H_2O_2 produced by the cells and released into the medium was determined by the scopoletin fluorescence oxidative quenching method (excitation wavelength, 350 nm; emission, 460 nm) according to Cazalé et al. (1998). To measure the H_2O_2 concentration, samples were taken at various intervals after the elicitation. Aliquots of 3 ml cell-free broth were mixed with 90 μl 1 mM stock solution of scopoletin (Fluka) in DMSO (Sigma) and 90 μl 1 mg/ml stock solution of peroxidase (Sino-American Biotechnology Co., Shanghai, China), respectively. Scopoletin was oxidized, and the concentration of H_2O_2 was calculated from the fluorescence decrease monitored by a spectrofluorimeter (Varian Cary Eclipse, CA, USA) using a calibration curve established in the presence of H_2O_2. A standard curve by adding scopoletin to the solutions at different H_2O_2 concentrations was prepared by using cell-free medium.

The effects of MJA and HEJA on peroxidase-dependent assay for H_2O_2 determination were tested. Various chemicals were added to cell-free medium to obtain final concentrations of 50, 100, or 200 μM, respectively. In usual conditions of assay (see above), the addition of jasmonates had no obvious effect on the decrease of scopoletin fluorescence due to the addition of H_2O_2.

Assay of NO

NO in the medium was quantified by spectrophotometric measurement of the conversion of oxyhemoglobin (HbO_2) to methemoglobin (metHb) (Murphy and Noack 1994; Delledonne et al. 2001). HbO_2 was prepared according to Murphy and Noack (1994). Twenty-five milligrams of hemoglobin (Hb) (Sigma) was dissolved in 1 ml of phosphate buffer (50 mM, pH 7.4), and 1–2 mg of sodium hydrosulfite powder was added to the solution. Then, a light stream of O_2 was continuously blown to the solution for about 15 min, and Hb was converted into HbO_2 by reacting with O_2. The resulting HbO_2 solution is desalted and purified by passing it through a Sephadex G-25 (Pharmacia) column. HbO_2 should be prepared fresh each day and stored on ice in dim light. NO concentration in the medium was analyzed as describe by Delledonne et al. (2001). Briefly, cell suspensions were filtrated, and HbO_2 was added to the medium to a final concentration of 10 μM. After 2 min, the changes in absorbance of the medium at 421 nm and 401 nm were measured, and the NO levels were calculated by using an extinction coefficient of 77 mM^{-1} cm^{-1} [A_{401} (metHb)–A_{421} (HbO_2)].

Enzyme extraction and assay of PAL activity

Fresh cells from the suspension culture were extracted for the assay of PAL as described by Qian et al. (2004b). Fresh cells of 1 g were frozen in liquid nitrogen and grounded with mortar and pestle. Enzyme in frozen powder was extracted by adding 50 mg polyvinylpolypyrroridone, 2-ml prechilled buffer of pH 7.2 (0.1 M phosphate buffer, 2 mM ethylenediaminetetraacetic acid, 4 mM dithiothreitol), and then homogenized at 4°C. The mixture was centrifuged at 10,000×*g* for 30 min at 4°C. The supernatant was used directly for PAL assay, using a method slightly modified from the one described by Heide et al. (1989). The enzyme extracts (200 μl) were incubated with 120 μl 0.1M L-phenylalanine (dissolved in 0.1 M borate buffer of pH 8.8) and 280 μl 0.1 M borate buffer of pH 8.8 at 30°C for 60 min. The reaction was stopped by adding 50 μl 5 N trichloroacetic acid. After centrifugation at 10,000×*g* for 3 min, 20 μl supernatant was analyzed by HPLC, using a Shimadzu LC-10AT_{VP} HPLC apparatus equipped with a variable wavelength UV detector (Shimadzu, SPD-10A_{VP}). A Shimadzu VP-ODS column (250×4.0 mm^2; 5 μm) was used at 25°C. The mobile phase consisted of water, methanol, and acetic acid at a ratio of 40:60:1 (v/v/v). The flow rate was kept constant at 1 ml/min. Trans-cinnamic acid was monitored at 275 nm identified by comparison with its authentic sample (Sigma). One unit (U) of enzyme activity is defined as the amount of enzyme forming 1 pmol of trans-cinnamic acid from the substrate L-phenylalanine per minute. Protein was quantified with Bradford method.

Detection of JA

The cell suspensions were filtrated, and 10 g cells by fresh weight (FW) were extracted for the assay of JA using a method slightly modified from that of Gundlach et al. (1992). For the assay of JA, 10 g FW cells were shock-frozen in liquid N_2, thawed in 20 ml of ethanol, and to which 9,10-dihydrojasmonic acid was added as internal standard. Samples were grinded, and the mixture was separated and extracted a second time with 20 ml ethanol. The ethanolic extracts were evaporated at 40°C to dryness, and the residue was dissolved in 40 ml water. The solution was acidified with 2.4 ml of 12 M HCl and was extracted with 40 ml $CHCl_3$ for three times. The organic phase was dried over Na_2SO_4 and evaporated. The residue was dissolved in 1 ml of *n*-hexane and was applied to a silica solid-phase extraction column (Supelclean LC-Si SPE Tubes, 500 mg, 3 ml, Supelco, USA). The column was washed with 5 ml of *n*-hexane and eluted with 7 ml of *n*-hexane/diethyl ether, 2:1 (vol/vol). The sample was taken to dryness, dissolved in 100 μl methanol for analysis. JA was analyzed by gas chromatography/mass spectrometry (GC/MS) on a Micromass GCT unit (England), with an HP-5 fused silica capillary column of 30 m×0.32 mm ID and 0.25-μm film thickness. The column temperature was ini-

tially held at 80°C for 0.5 min, then shifted to 200°C at 4°C/min, and then increased to 280°C at 30°C/min, and the injector and detector temperature was set at 270 and 280°C, respectively. Hydrogen was used as the mobile phase at a flow rate of 1 ml/min.

Statistical analyses

Experiments were done in a completely randomized layout. Each experiment was carried out in triplicate, and three different sets of experiments reproduced the same result. Data were analyzed by one-way analysis of variance (ANOVA). Means of an experiment were analyzed using Tukey's Honestly Significant Difference multiple-comparison test with a family error rate of 0.05. All differences are significant unless otherwise indicated.

Results

Optimization of HEJA elicitation conditions

Exposure time and dosage of an elicitor are two main factors that affect cell growth and product yield for a specific culture system and elicitor (Wang and Zhong 2002; Ketchum et al. 1999; Tabata 2004). Thus, experiments on the timing of HEJA addition and dose response were carried out at first.

For the investigation of elicitation time, 100 μM HEJA was added to the cultures of *P. notoginseng* cells at different growth stages, i.e., at day 0 (lag phase), day 4 (beginning of log phase), day 7 (middle of log phase), and day 10 (end of log phase). Maximum DW and ginsenoside content on day 13 are shown in Table 1. DW on day 13 for the elicited cultures exposed at different log phases (day 4, 7, and 10) was almost the same as that of the control (without addition of elicitors). Whereas, cell growth was highly inhibited when HEJA was added just after inoculation, and there appeared to be about 20% decrease in DW on day 13 compared with the control. HEJA could increase each individual ginsenoside biosynthesis after its addition to the cell cultures at various growth stages. On the other hand, the cells showed different stimulatory responses of ginsenoside biosynthesis to elicitation at different growth stages. As shown in Table 1, relatively higher individual ginsenoside content was obtained with the cells exposed to the elicitor at early stage of cultivation (day 0 and 4). On the contrary, when HEJA was added on day 7 and 10, the stimulating effect on ginsenoside content was a little lower compared with the elicitation on day 0 and 4. With HEJA elicitation, the ginsenoside content of Rb group increased much more than Rg group compared to the control. In particular, Rb_1 content increased much more than Rg_1 and Re, whereas Rd was also detected in this case. Similar to MJA (Wang and Zhong 2002), HEJA could manipulate the ginsenoside heterogeneity in cell cultures of *P. notoginseng*.

For the study on elicitation dosage, 4-day-old cell suspension cultures of *P. notoginseng* were elicited with different levels (100–500 μM) of HEJA. Maximum DW and ginsenoside content on day 13 are shown in Table 2. A lower HEJA concentration seemed to have no obvious effect on DW. The respective DW for 100 and 200 μM of HEJA on day 13 was 8.88±0.20 and 8.71±0.21 g/l, nearly the same as the control (9.11±0.18 g/l). Whereas, a higher HEJA concentration (i.e., 500 μM) resulted in a decreased DW (7.62±0.25 g/l) compared with the control. An increase of HEJA concentration from 100 to 200 μM could enhance the content of individual ginsenosides, for example, Rb_1 content was increased from 1.86±0.05 to 2.31±0.05 mg/100 mg DW. However, an even higher HEJA concentration (i.e., 500 μM) showed a slight inhibition on ginsenoside content, and the optimum HEJA elicitation condition was identified to be 200 μM added on day 4.

Comparison of HEJA with MJA

A comparison of the effects of MJA and HEJA on cell growth, ginsenoside biosynthesis, and UGRdGT activity was done. Both MJA and HEJA were added on day 4 to the cell cultures at 200 μM.

Time profiles of DW (a), total ginsenoside (Rg_1+Re+Rb_1+Rd) content (b), and the ratio of Rb to Rg group of ginsenosides (c) are shown in Fig. 1. Compared with the

Table 1 Effects of HEJA addition time on DW and the content of individual ginsenosides on day 13

Cultivation conditions	DW (g/l, day 13)	Ginsenoside content (mg/100 mg DW)					Rb:Rg[2]
		Rg_1	Re	Rb_1	Rd	Total[1]	
Control	8.94±0.20[a]	0.24±0.02[a]	0.25±0.02[a]	0.27±0.02[a]	0[a]	0.75±0.06[a]	0.55±0.00[a]
Added on day 0	7.21±0.35[b]	0.47±0.01[b]	0.44±0.02[b]	2.03±0.04[b]	0.14±0.03[b]	3.07±0.10[b]	2.39±0.02[b]
Added on day 4	8.68±0.14[a]	0.48±0.01[b]	0.45±0.03[b]	2.07±0.04[b]	0.14±0.03[b]	3.14±0.11[b]	2.37±0.04[b]
Added on day 7	9.19±0.33[a]	0.36±0.02[c]	0.37±0.02[c]	1.67±0.05[c]	0.11±0.02[c]	2.49±0.11[c]	2.46±0.05[b]
Added on day 10	8.99±0.21[a]	0.31±0.01[d]	0.30±0.01[d]	1.25±0.04[d]	0.09±0.02[c]	1.94±0.08[d]	2.18±0.01[c]

The same letters all noted in a single column are not significantly different according to Tukey's Honestly Significant Difference multiple-comparison test with a family error rate of 0.05

[1]Total content=(Rg_1+Re+Rb_1+Rd)

[2]Rb:Rg=(Rb_1+Rd)/(Rg_1+Re)

Table 2 Effects of HEJA concentration on DW and the content of individual ginsenosides on day 13

HEJA level (μM)	DW (g/l, day 13)	Ginsenoside content (mg/100 mg DW)					Rb:Rg[2]
		Rg_1	Re	Rb_1	Rd	Total[1]	
0	9.11±0.18[a]	0.21±0.03[a]	0.19±0.01[a]	0.22±0.02[a]	0[a]	0.62±0.04[a]	0.54±0.03[a]
100	8.88±0.20[a]	0.47±0.02[b]	0.54±0.02[b]	1.86±0.05[b]	0.14±0.02[b]	3.00±0.13[b]	1.99±0.01[b]
200	8.71±0.21[a]	0.53±0.01[c]	0.56±0.01[b]	2.31±0.05[c]	0.15±0.03[b]	3.54±0.13[c]	2.25±0.03[c]
500	7.62±0.25[b]	0.51±0.01[c]	0.53±0.02[b]	2.12±0.06[d]	0.15±0.03[b]	3.30±0.02[d]	2.19±0.10[c]

The same letters all noted in a single column are not significantly different according to Tukey's Honestly Significant Difference multiple-comparison test with a family error rate of 0.05

[1]Total content=(Rg_1+Re+Rb_1+Rd)

[2]Rb:Rg=(Rb_1+Rd)/(Rg_1+Re)

control, no obvious difference on cell growth was observed for both MJA and HEJA elicitation. The cells all reached their maximum DW on day 13, and the respective DW for control, MJA elicitation, and HEJA elicitation was 9.40±0.31, 9.01±0.37, and 8.94±0.46 g/l, respectively.

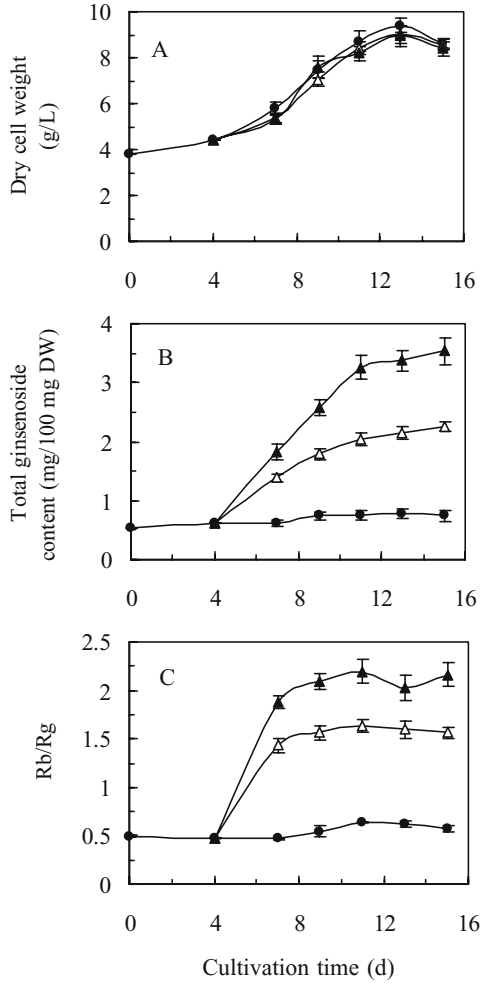

Fig. 1 Time courses of dry cell weight (**a**), total ginsenoside (Rg_1+Re+Rb_1+Rd) content (**b**), and the ratio of Rb to Rg (**c**) of *P. notoginseng* cells with 200 μM of MJA or HEJA added on day 4. Control (*dark circle*), 200 μM MJA added on day 4 (*open triangle*), 200 μM HEJA added on day 4 (*dark triangle*)

From Fig. 1b, we could see that for both MJA and HEJA elicitation, the content of total ginsenosides increased from day 4 to about day 11 after the addition, and then a slight fluctuation was observed. The stimulating effect of HEJA on ginsenoside biosynthesis was at a higher level than MJA during cultivation. Maximum ginsenoside content was 0.76±0.06 (day 11), 2.26±0.09 (day 15), and 3.54±0.23 (day 15) mg/100 mg DW for the control, MJA elicitation, and HEJA elicitation, respectively. The Rb/Rg ratio (Fig. 1c) of the control maintained at about 0.5 during the cultivation, whereas it increased sharply with MJA or HEJA elicitation. During the cultivation, the Rb/Rg ratio with HEJA elicitation was about 30% higher than that with MJA elicitation.

Time profiles of UGRdGT activity (a) and the content of ginsenoside Rb_1 (b), and Rd (c) with addition of MJA and HEJA are shown in Fig. 2. This enzyme catalyzed the glucosylation of ginsenoside Rd into Rb_1 in *P. notoginseng* cells (Yue and Zhong 2005a,b). The enzyme activity increased sharply after the addition of the elicitors on day 4 and reached a maximal level on day 7. Although a little decrease was seen after day 7, the enzyme activity still remained at a relatively higher level until day 15 compared with the control. In comparison to MJA, for HEJA, the increase of UGRdGT activity coincided with the higher content of ginsenoside Rb_1 in its case. The maximum production of individual ginsenosides on day 13 is shown in Table 3. With HEJA elicitation, a maximal production titer of ginsenoside Rg_1, Re, Rb_1, and Rd was 47.4±4.8, 52.3±4.4, 190±18, and 12.1±2.5 mg/l, about 1.3-, 1.3-, 1.7-, and 2.1-fold than that with MJA elicitation, respectively.

Effects of MJA and HEJA on oxidative burst in P. notoginseng cell cultures

Effect of the jasmonates (MJA and HEJA) on oxidative burst was studied by analyzing H_2O_2 concentration in the medium with 200 μM of the jasmonates added to the *P. notoginseng* cell cultures 4 days after inoculation. Both NO concentration in medium and PAL activity in cells related to oxidative burst were measured. Both MJA and HEJA had no obvious effects on H_2O_2 and NO levels in

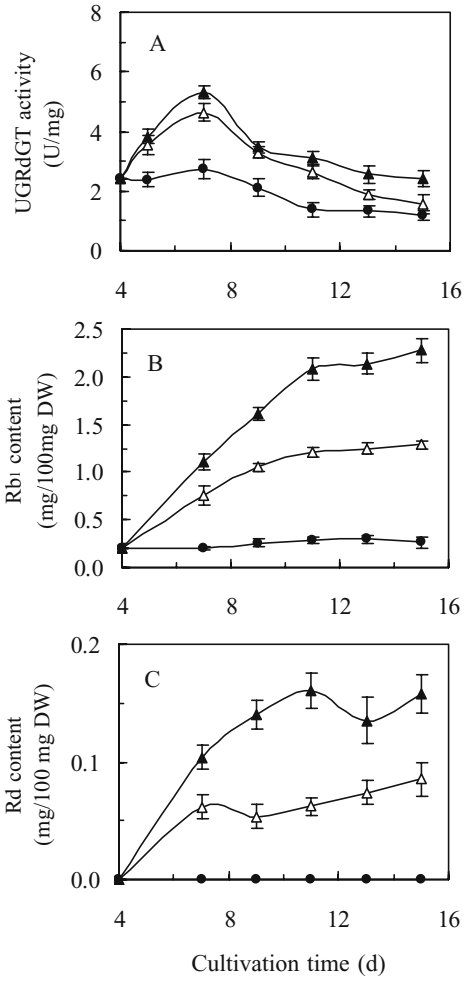

Fig. 2 Dynamic changes of UGRdGT activity (**a**) and the content of ginsenoside Rb_1 (**b**) and Rd (**c**) of *P. notoginseng* cells with 200 μM of MJA or HEJA elicited on day 4. The symbols are the same as in Fig. 1

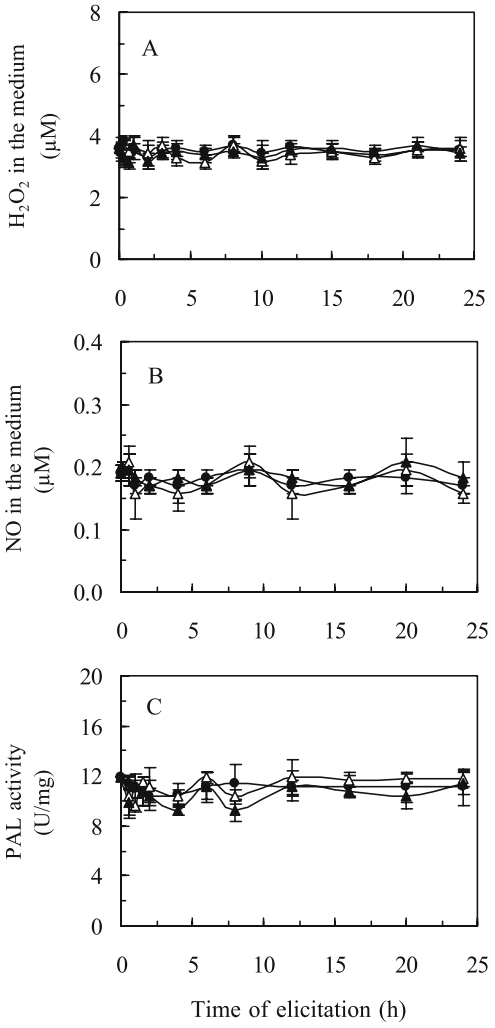

Fig. 3 Effect of 200 μM of MJA or HEJA on H_2O_2 (**a**) and NO (**b**) concentration in medium and PAL activity in cells (**c**) of *P. notoginseng*. The symbols are the same as in Fig. 1

medium and PAL activity in cells until 24 h after elicitation (Fig. 3a–c).

Effects of MJA and HEJA on JA biosynthesis

Three JA biosynthesis inhibitors, i.e., ibuprofen (Ibu), *n*-propylgallate (Pro), and phenylbutazone (Phyb) (Staswick et al. 1991; Farmer 1994), were tested for their possible effects on the jasmonate signaling pathway that led to enhanced ginsenoside biosynthesis (Table 4). Each inhibitor (at 100 μM) was added together with 200 μM of the jasmonates to the cell cultures of *P. notoginseng* on day 4, and the cells were sampled on day 13. Here, 100 μM of each inhibitor added alone on day 4 was proved to have no effects on cell growth and ginsenoside content (data not shown).

When MJA was added simultaneously with the inhibitors, all three inhibitors (Ibu, Pro, and Phyb) did not affect MJA-mediated enhancement of ginsenoside biosynthesis, and the content of each ginsenoside was very similar to that of MJA-elicited cells. On the other hand, they could partially reduce the eliciting activity of HEJA on ginsenoside biosynthesis when added together with HEJA. For example, when Ibu was added with HEJA, total ginsenoside content was 2.44±0.13 mg/100 mg DW. It was lower than that of HEJA-elicited cells (2.89±0.15 mg/100 mg

Table 3 Effects of MJA and HEJA on the production of individual ginsenosides on day 13

Cultivation conditions	Ginsenoside production (mg/l)			
	Rg_1	Re	Rb_1	Rd
Control	22.1±1.3[a]	23.0±1.2[a]	28.1±3.1[a]	0[a]
MJA	35.2±4.0[b]	39.2±3.5[b]	113±10[b]	5.87±0.88[b]
HEJA	47.4±4.8[c]	52.3±4.4[c]	190±18[c]	12.1±2.5[c]

The same letters all noted in a single column are not significantly different according to Tukey's Honestly Significant Difference multiple-comparison test with a family error rate of 0.05

Table 4 Effects of JA biosynthesis inhibitors on the eliciting activities of MJA and HEJA in ginsenosides biosynthesis (day 13)

Cultivation condition	DW (g/l, day 13)	Ginsenoside content (mg/100 mg DW)					Rb:Rg[2]
		Rg_1	Re	Rb_1	Rd	Total[1]	
Control	9.02±0.11[a]	0.19±0.01[a]	0.22±0.03[a]	0.20±0.01[a]	0[a]	0.61±0.06[a]	0.49±0.02[a]
MJA	8.88±0.20[a]	0.35±0.02[b]	0.34±0.01[b]	1.29±0.05[b]	0.09±0.02[b]	2.06±0.06[b]	2.00±0.12[b]
MJA+Ibu	8.75±0.27[a]	0.36±0.03[b]	0.35±0.03[b]	1.24±0.06[b]	0.09±0.02[b]	2.03±0.14[b]	1.86±0.03[b]
MJA+Pro	8.61±0.21[a]	0.34±0.04[b]	0.37±0.01[c]	1.30±0.07[b]	0.11±0.03[b,d]	2.12±0.16[b]	1.99±0.02[b]
MJA+Phyb	8.32±0.11[a]	0.35±0.02[b]	0.34±0.03[b]	1.23±0.11[b]	0.09±0.03[b]	2.00±0.18[b]	1.92±0.06[b]
HEJA	8.77±0.13[a]	0.42±0.02[c]	0.44±0.03[c]	1.88±0.07[c]	0.15±0.03[c]	2.89±0.15[c]	2.37±0.12[c]
HEJA+Ibu	8.92±0.25[a]	0.35±0.02[b]	0.36±0.01[b]	1.61±0.07[d]	0.13±0.02[d]	2.44±0.13[d]	2.46±0.11[c]
HEJA+Pro	8.78±0.28[a]	0.37±0.01[b]	0.39±0.01[d]	1.70±0.06[d]	0.12±0.02[b,d]	2.57±0.11[d]	2.38±0.13[c]
HEJA+Phyb	8.83±0.18[a]	0.37±0.02[b]	0.37±0.02[b]	1.71±0.09[d]	0.12±0.03[b,d]	2.55±0.16[d]	2.49±0.12[c]

The same letters all noted in a single column are not significantly different according to Tukey's Honestly Significant Difference multiple-comparison test with a family error rate of 0.05

[1]Total content=(Rg_1+Re+Rb_1+Rd)

[2]Rb:Rg=(Rb_1+Rd)/(Rg_1+Re)

Table 5 JA content in the cells under different cultivation conditions

Cultivation conditions	JA content (ng/g FW)	
	2 h	12 h
Control	7.3±2.5[a]	9.2±2.4[a]
MJA	7.4±2.1[a]	20.8±3.9[b]
HEJA	9.2±3.3[a]	74.4±7.6
Ibu	7.9±2.1[a]	8.7±2.4[a]
MJA+Ibu	7.3±2.5[a]	14.7±2.4[c]
HEJA+Ibu	7.9±2.3[a]	23.2±2.1[b]

The same letters all noted in a single column are not significantly different according to Tukey's Honestly Significant Difference multiple-comparison test with a family error rate of 0.05

DW), but still much higher than that of control (0.61± 0.06 mg/100 mg DW).

Muller et al. (1993) reported that JA level reached the maximum between 0.5 and 2 h after yeast cell wall elicitation and kept at a relatively high level until 12 h after elicitation. Thus, in this study, the endogenous JA content at 2 and 12 h after the elicitation was analyzed, and the results are shown in Table 5. Both MJA and HEJA significantly increased the endogenous JA content at 12 h after the addition. HEJA addition could increase JA content to a higher level than MJA addition (74.4±7.6 vs 20.8±3.9 ng/g FW). Ibu had no effects on JA content compared with the control, when it was added together with HEJA or MJA, whereas it could inhibit the increase of JA content induced by the jasmonates.

Discussion

The optimal condition for HEJA elicitation was identified to be 200 μM added on day 4. The same phenomenon was observed for the MJA elicitation on *P. notoginseng* cultures (Wang and Zhong 2002). The inhibitory effect of high concentrations of elicitors on metabolites biosynthesis was also observed in JA-induced indole alkaloids biosynthesis (Rijhwani and Shanks 1998) and in MJA-induced taxol biosynthesis (Ketchum et al. 1999). The existence of an optimal dose of elicitors suggests that, at elicitor doses smaller than the optimum, the elicitor-binding sites in cells were still not fully utilized for activating the secondary metabolite synthesis, whereas excessive doses caused a deleterious effect on the cells' biosynthetic activity.

Stronger elicitation of ginsenoside biosynthesis by HEJA than MJA was demonstrated. It was confirmed that esterification did not change the distribution of jasmonate stereoisomers, as compared with the starting material (MJA) (Qian et al. 2004a,b). Therefore, it might be reasonable to neglect the effect of stereo configuration and take into account the difference in chemical structure. Compared with MJA, HEJA also had a higher stimulating activity on taxane biosynthesis in cell cultures of *T.chinensis* (Qian et al. 2004a,b). These results suggest that the esterification of the jasmonates at the C-1 position with glycol is essential to their stimulating activity on secondary metabolite biosynthesis.

A higher Rb/Rg ratio with HEJA elicitation was observed than that with MJA elicitation. The results indicate that compared to MJA, HEJA could lead to higher amounts of Rb group ginsenosides. This means that it can alter the distribution of heterogeneous ginsenosides more efficiently than MJA.

The increase of UGRdGT activity coincided with the higher content of ginsenoside Rb_1. A higher UGRdGT activity with HEJA elicitation than that with MJA elicitation suggests that HEJA had higher stimulating activity than MJA on ginsenoside biosynthesis at a molecular level. In addition, by using HEJA, the Rd content increased more than Rb_1. The fact implies that the jasmonate analogue might also strongly activate certain site(s) from 2,3-oxidosqualene to Rd in ginsenoside biosynthetic pathway (Fig. 4) besides its effect on UGRdGT. Future progress in

the elucidation of the detailed ginsenoside biosynthetic pathway will help us to investigate and understand this phenomenon.

Exogenously applied MJA could induce the lipoxygenase and lead to endogenous JA biosynthesis as reported (Melan et al. 1993; Zhao and Sakai 2003). In tobacco Bright Yellow-2 cells, the exogenously applied MJA was hydrolyzed to JA and then metabolized to its glucose and gentiobiose esters (Swiatek et al. 2004). In this work, we found that the jasmonates could increase the endogenous JA level, and JA biosynthesis inhibitors could inhibit the stimulated JA level. It is possible that the exogenously applied jasmonates could stimulate JA biosynthesis in *P. notoginseng* cells, or they may be hydrolyzed to JA. JA biosynthesis inhibitors were found to only partially inhibit ginsenoside biosynthesis induced by HEJA, and they had no effects on MJA-induced ginsenoside biosynthesis. The results imply that in *P. notoginseng* cells, both exogenously applied jasmonates and endogenously elicited JA could induce ginsenoside biosynthesis via the induction of key enzymes involved in the ginsenoside biosynthetic pathway such as UGRdGT.

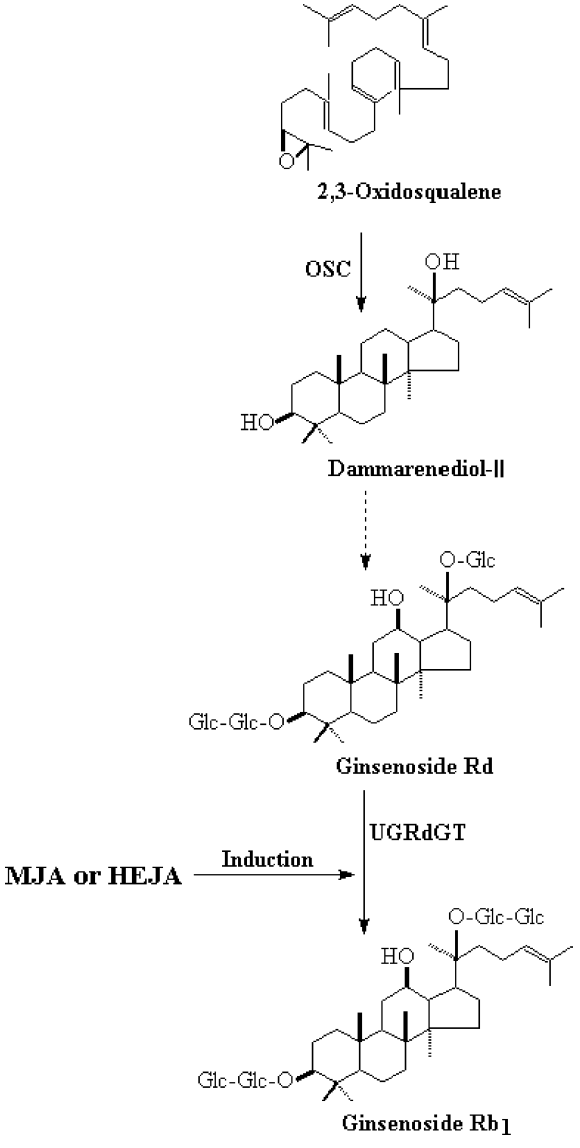

Fig. 4 Proposed acting point of jasmonates on the biosynthetic pathway of ginsenoside Rb_1 in *P. notoginseng* cells

In this work, no obvious effects of both HEJA and MJA on H_2O_2, NO, and PAL were observed until 24 h after elicitation, although the UGRdGT activity was already enhanced (Fig. 3). This suggests that the AOS generation pathway might have no relationship with the jasmonates-induced ginsenoside biosynthesis in our *P. notoginseng* cell cultures. In cell cultures of *P. ginseng* as reported, oligosaccharide induced jasmonate signal through a rapid production of H_2O_2 and finally enhanced ginsenosides production, whereas JA itself had no effects on H_2O_2 (Hu et al. 2003a). Such a signal transduction was also shown in other systems like in β-thujaplicin production induced by a yeast elicitor (Zhao and Sakai 2003). However, different results have also been reported in some other cases. For example, in root cultures of *P. ginseng* and *P. quinquefolium*, the induction of antioxidant system and H_2O_2 accumulation was observed after MJA elicitation (Ali et al. 2005). In tomato leaves, JA or systemin induced early signaling genes and the rapid production of H_2O_2, and H_2O_2 then induced the expression of late defense genes, but NO in this signal cascade inhibited the defense genes expression by inhibiting H_2O_2 accumulation (Orozco-Cárdenas and Ryan 2002). Recently, Wang and Wu (2005) reported that NO inhibitors could suppress MJA-induced taxol accumulation, but enhance H_2O_2 and PAL. In suspension cultures of *T. chinensis*, an increased level of H_2O_2 followed by higher PAL activity and taxoid overproduction was observed after jasmonates elicitation (Qian et al. 2004a). Hu et al. (2003b) reported that NO mediated elicitor-induced saponin synthesis in cell cultures of *Panax ginseng*. All these reports including this work suggest that the signal transduction pathway from elicitation, gene activation to secondary metabolite synthesis, is a very complicated system. We think that even for the same elicitor, the signal cascade may be different in different cell culture systems. The information obtained here is considered helpful to further understand the signal transduction network in our case.

It is reported that different jasmonate elicitors in chemical structure might induce different levels of plant defense responses (Qian et al. 2004a,b; Tabata 2004; Staniszewska et al. 2003; Miersch et al. 1999; Koda et al. 1991). Lobler and Lee (1998) proposed that the exogenous jasmonate is recognized by a plasma membrane receptor. In this work, we found that the cells performed similar signal responses to the elicitation by MJA and chemically synthesized HEJA. The different levels of ginsenoside accumulation by their elicitation might be due to the structure relationship between the jasmonates and their receptors. However, no such receptors have been reported. Studies on the structural requirements for the stimulating activity of chemical elicitors may help to the illumination of the receptors and the rational design and synthesis of more potent elicitors.

Conclusions

Chemically synthesized HEJA was shown to stimulate ginsenosides biosynthesis and manipulate their heterogeneity more efficiently than MJA by leading to higher amounts of Rb group ginsenosides in cell cultures of *P. notoginseng*. Studies on the signal events, including JA biosynthesis and oxidative burst, confirmed that the cells had similar defense response to HEJA elicitation as to MJA elicitation. This is the first report on efficient manipulation of the heterogeneity of plant secondary metabolites by using chemically synthesized elicitor. The information obtained here is useful for the large-scale manipulation of the heterogeneity of valuable ginsenosides in plant cell cultures.

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References

Ali MB, Yu KW, Hahn EJ, Paek KY (2005) Differential responses of anti-oxidants enzymes, lipoxygenase activity, ascorbate content and the production of saponins in tissue cultured root of mountain *Panax ginseng* CA Meyer and *Panax quinquefolium* L. in bioreactor subjected to methyl jasmonate stress. Plant Sci 169:83–92

Cazalé AC, Rouet-Mayer MA, Barbier-Brygoo H, Mathieu Y, Laurière C (1998) Oxidative burst and hypoosmotic stress in tobacco cell suspensions. Plant Physiol 116:659–669

Chen Z, Silva H, Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science 262:1883–1886

Creelman RA, Mullet JE (1997) Biosynthesis and action of jasmonates in plants. Annu Rev Plant Physiol Plant Mol Biol 48:355–381

Delledonne M, Zeier J, Marocco A, Lamb C (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc Natl Acad Sci U S A 98:13454–13459

Desikan R, Reynolds A, Hancock JT, Neill SJ (1998) Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defence gene expression in *Arabidopsis* suspension cultures. Biochem J 330:115–120

Dong HD, Zhong JJ (2001) Significant improvement of taxane production in suspension cultures of *Taxus chinensis* by combining elicitation with sucrose feed. Biochem Eng J 8:145–150

Farmer EE (1994) Fatty acid signaling in plants and their associate microorganisms. Plant Mol Biol 26:1423–1437

Gundlach H, Muller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc Natl Acad Sci U S A 89:2389–2392

Heide L, Nishioka N, Fukuki H, Tabata M (1989) Enzymatic regulation of shikonin biosynthesis in *Lithospermum erythrorhizon* cell cultures. Phytochemistry 28:1873–1877

Hu XY, Neill S, Cai WM, Tang Z (2003a) Hydrogen peroxide and jasmonic acid mediate oligogalacturonic acid-induced saponin accumulation in suspension-cultured cells of *Panax ginseng*. Physiol Plant 118:414–421

Hu XY, Neill S, Cai WM, Tang Z (2003b) Nitric oxide mediates elicitor-induced saponin synthesis in cell cultures of *Panax ginseng*. Funct Plant Biol 30:901–907

Ketchum REB, Gibson DM, Croteau RB, Shuler ML (1999) The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate. Biotechnol Bioeng 62:97–105

Koda Y, Kikuta Y, Tazaki H, Tsujino Y, Sakamura S, Yoshihara T (1991) Potato tuber-inducing activities of jasmonic acid and related compounds. Phytochemistry 30:1435–1438

Lobler M, Lee J (1998) Jasmonate signaling in barley. Trends Plant Sci 3:8–9

Melan MA, Dong X, Endara ME, Davis KR, Ausubel FM, Peterman TK (1993) An *Arabidopsis thaliana* lipoxygenase gene can be induced by pathogens, abscisic acid, and methyl jasmonate. Plant Physiol 101:441–450

Miersch O, Ksamell R, Parthier B, Wasternack C (1999) Structure activity relations of substituted, deleted or stereospecifically altered jasmonic acid in gene expression of barley leaves. Phytochemistry 50:353–361

Muller MJ, Brodschelm W, Spannagl E, Zenk MH (1993) Signaling in the elicitation process in mediated through the octadecanoid pathway leading to jasmonic acid. Proc Natl Acad Sci U S A 90:7490–7494

Murphy ME, Noack E (1994) Nitric oxide assay using hemoglobin method. Methods Enzymol 233:240–250

Orozco-Cárdenas ML, Ryan CA (2002) Nitric oxide negatively modulates wound signaling in tomato plants. Plant Physiol 130: 487–493

Qian ZG, Zhao ZJ, Xu Y, Qian X, Zhong JJ (2004a) Novel chemically synthesized hydroxyl-containing jasmonates as powerful inducing signals for plant secondary metabolism. Biotechnol Bioeng 86:809–816

Qian ZG, Zhao ZJ, Tian WH, Xu Y, Zhong JJ, Qian X (2004b) Novel synthetic jasmonates as highly efficient elicitors for taxoid production by suspension cultures of *Taxus chinensis*. Biotechnol Bioeng 86:595–599

Rijhwani S, Shanks JV (1998) Effect of elicitor dosage and exposure time on biosynthesis of indole alkaloids by *Catharanthus roseus* hairy root cultures. Biotechnol Prog 14:442–449

Staniszewska I, Królicka A, Maliński E, Łojkowska E, Szafranek J (2003) Elicitation of secondary metabolites in in vitro cultures of *Ammi majus* L. Enzyme Microb Technol 33:565–568

Staswick PE, Huang JF, Rhee Y (1991) Nitrogen and methyl jasmonate induction of soybean vegetative storage protein genes. Plant Physiol 96:130–136

Sticher O (1998) Getting to the root of ginseng. Chemtech 28:26–32

Swiatek A, van Dongen W, Esmans EL, van Onckelen H (2004) Metabolic fate of jasmonates in tobacco Bright Yellow-2 cells. Plant Physiol 135:161–172

Tabata H (2004) Paclitaxel production by plant-cell-culture technology. Adv Biochem Eng Biotechnol 87:1–23

Wang JW, Wu JY (2005) Nitric oxide is involved in methyl jasmonate-induced defense responses and secondary metabolism activities of *Taxus* cells. Plant Cell Physiol (adv access)

Wang W, Zhong JJ (2002) Manipulation of ginsenoside heterogeneity in cell cultures of *Panax notoginseng* by addition of jasmonates. J Biosci Bioeng 93:48–53

Wang W, Zhang ZY, Zhong JJ (2005) Enhancement of ginsenoside biosynthesis in high density cultivation of *Panax notoginseng* cells by various strategies of methyl jasmonate elicitation. Appl Microbiol Biotechnol 67:752–758

Woragidbumrung K, Sae-Tang P, Yao H, Han J, Chauvatcharin S, Zhong JJ (2001) Impact of conditioned medium on cell cultures of *Panax notoginseng* in an air-lift bioreactor. Process Biochem 37:209–213

Yu KW, Gao WY, Hahn EJ, Paek KY (2002) Jasmonic acid improving ginsenoside accumulation in adventitious root culture of *Panax ginseng* CA Meyer. Biochem Eng J 11:211–215

Yu KW, Murthy HN, Hahn EJ, Paek KY (2005) Ginsenoside production by hairy root cultures of *Panax ginseng*: influence of temperature and light quality. Biochem Eng J 23:53–56

Yue CJ, Zhong JJ (2005a) Impact of external calcium and calcium sensors on ginsenoside Rb_1 biosynthesis by *Panax notoginseng* cells. Biotechnol Bioeng 89:444–452

Yue CJ, Zhong JJ (2005b) Purification and characterization of UDPG: ginsenoside Rd glucosyltransferase from suspended cells of *Panax notoginseng*. Process Biochem (in press)

Zhang ZY, Zhong JJ (2004) Scale-up of centrifugal impeller bioreactor for hyperproduction of ginseng saponin and polysaccharide by high-density cultivation of *Panax notoginseng* cells. Biotechnol Prog 20:1076–1081

Zhao J, Sakai K (2003) Multiple signaling pathways mediate fungal elicitor induced β-thujaplicin biosynthesis in *Cupressus lusitanica* cell cultures. J Exp Bot 54:647–656

Zhao J, Davis L, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv 23:283–333