Biotechnology and Bioprocess Engineering **2005**, 10: 162-165

Efficient Elicitation of Ginsenoside Biosynthesis in Cell Cultures of *Panax notoginseng* by Using Self-chemically-synthesized Jasmonates

Wei Wang[1], Zhen-Jiang Zhao[2], Yufang Xu[2], Xuhong Qian[2], and Jian-Jiang Zhong[1]*

[1] State Key Laboratory of Bioreactor Engineering,
[2] Shanghai Key Laboratory of Chemical Biology, Institute of Pesticides and Pharmarceuticals, East China University of Science and Technology, Shanghai 200237, China

Abstract A series of fluorine and hydroxyl containing jasmonate derivatives, which were chemically synthesized in our institute, were investigated for their effects on the biosynthesis and heterogeneity of ginsenosides in suspension cultures of *Panax notoginseng* cells. Compared to the control (without addition of elicitors), 100 μM of each of the jasmonate was added on day 4 to the suspension cultures of *P. notoginseng* cells. It was observed that, jasmonates greatly enhanced the ginsenoside content and the ratio of Rb group to Rg group (*i.e.* (Rb_1 + Rd)/(Rg_1 + Re)) in the *P. notoginseng* cells. Some of the synthetic jasmonates, such as pentafluoropropyl jasmonate (PFPJA), 2-hydroxyethyl jasmonate (HEJA) and 2-hydroxyethoxyethyl jasmonate (HEEJA), could promote the ginsenoside content to 2.55 ± 0.11, 3.65 ± 0.13 and 2.94 ± 0.06 mg/100 mg DW, respectively, compared to that of 0.64 ± 0.06 mg/100 mg DW for the control and 2.17 ± 0.04 mg/100 mg DW by the commercially available methyl jasmonate (MJA); and they could change the respective Rb:Rg ratio to 1.60 ± 0.04, 1.87 ± 0.01 and 1.56 ± 0.05, compared to that of 0.47 ± 0.01 for the control and 1.42 ± 0.06 by MJA. The results suggest that suitable esterification of MJA with fluorine or hydroxyl group could increase the elicitation activity to induce plant secondary metabolism. The information obtained from this study is useful for hyper-production of heterogeneous products by plant cell cultures.

Keywords: *Panax notoginseng*, chemically synthesized jasmonates, ginsenoside, heterogeneous secondary metabolites

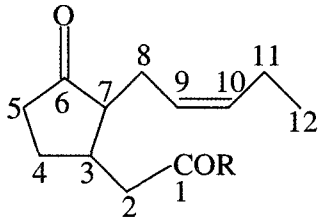
Plant cell culture is a stupendous technology to obtain valuable plant-specific metabolites [1]. Elicitation is one of the most efficient techniques to promote the productivity of useful secondary metabolites from plant cell cultures [2-5]. Jasmonic acid (JA) and methyl jasmonate (MJA) are widely used elicitors for inducing plant secondary metabolism [6-9], and they may also alter the molecular diversity of plant species [8-12]. It was reported that the chemical structure of the jasmonates could influence their stimulatory activity on secondary metabolism [6,13,14]. Recently, Qian *et al.* have developed chemically synthesized jasmonates to obtain a higher stimulatory activity on taxane biosynthesis than MJA in cell cultures of *Taxus chinensis* [15,16]. However, whether the structure of such jasmonates could influence the heterogeneity of secondary metabolites is still unclear.

Panax notoginseng (Sanchi-ginseng) is one of the most valuable oriental herbs. Cell culture of *P. notoginseng* is a promising alternative to obtain ginsenoside, one of its major bioactive secondary metabolites [17-19]. As different ginsenosides possess different or even opposite pharmacological activities, manipulation of ginsenoside heterogeneity in cell cultures is an important issue for not only the practical applications but also for theoretical studies [9,20]. Recently we have reported that MJA could increase the content of individual ginsenosides and change their heterogeneity in suspension cultures of *P. notoginseng* in both shake flask and bioreactor cultivations [9,21]. In this communication, the effects of self-chemically-synthesized fluorine and hydroxyl containing jasmonate derivatives on the ginsenoside biosynthesis and heterogeneity were studied.

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 3 Å molecular sieves for at least 48 h prior to use. Reagents for synthesis of the jasmonates were purchased from Sigma, Aldrich or Fluka.

The jasmonates (see Fig. 1) were synthesized and purified in our institute as described elsewhere [15,16], and

***Corresponding author**
Tel: +86-21-6425-2091 Fax: +86-21-6425-3904
e-mail: jjzhong@ecust.edu.cn



	R
Methyl jasmonate	OCH_3
Trifluoroethyl jasmonate	OCH_2CF_3
Pentafluoropropyl jasmonate	$OCH_2CF_2CF_3$
Pentafluorobenzyl jasmonate	$OCH_2C_6F_5$
2-Hydroxyethyl jasmonate	OCH_2CH_2OH
2-Hydroxyethoxyethyl jasmonate	$OCH_2CH_2OCH_2CH_2OH$
2,3-Dihydroxypropyl jasmonate	$OCH_2CHOHCH_2OH$
D-Glucosyl jasmonate	$OC_6H_{11}O_5$

Fig. 1. Structures of methyl jasmonate (MJA) and chemically synthesized trifluoroethyl jasmonate (TFEJA), pentafluoropropyl jasmonate (PFPJA), pentafluorobenzyl jasmonate (PFBJA), 2-hydroxyethyl jasmonate (HEJA), 2-hydroxyethoxyethyl jasmonate (HEEJA), 2,3-dihydroxypropyl jasmonate (DHPJA) and *D*-glucosyl jasmonate (GJA).

all the jasmonates had the same ratio of stereoisomers as MJA [16].

Suspended cells of *P. notoginseng* were grown in Murashige and Skoog (MS) medium and subcultured every 2 weeks. The details were described elsewhere [9,17].

P. notoginseng cells (2 g of fresh weight) were incubated in a 250-mL Erlenmeyer flask containing 50 mL of MS medium with the same culture conditions as in subcultures. All the elicitors were dissolved in ethanol and sterilized by filtering through 0.22 μm polyvinylidenedifluoride (PVDF) syringe filters (Millipore) before being added into 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. The cultivation data represented the mean values with the standard deviations from 3 independent samples.

For sampling, 3 identical shake flasks were taken 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 weight and ginsenoside content were the same as described previously [9,18].

Experiments were carried out in a completely randomized layout. Each experiment was done in triplicate and 3 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 were significant unless otherwise indicated.

One hundred μM of each elicitor was added to the cultures of *P. notoginseng* cells on day 4, and dry cell weight (DW) and individual ginsenoside content on day 13 were calculated (Table 1). The hydroxyl containing jasmonates had no obvious effects on cell growth, and the DW on day 13 was nearly the same as the control (without addition of any elicitors). Whereas, cell growth was slightly inhibited by the fluorine containing jasmonates, and there was about 10% decrease in DW on day 13, when compared with the control.

Similarly to MJA [9], all the chemically synthesized jasmonates could increase the content of individual ginsenosides and the ratio of Rb to Rg group (*i.e.* $(Rb_1 + Rd)/(Rg_1 + Re)$), when compared to the control. Among the fluorine containing jasmonates, pentafluoropropyl

Table 1. Effects of MJA and chemically synthesized jasmonates on DW and the content of individual ginsenosides of *P. notoginseng* cells (100 μM of each elicitor was added on day 4 and the cells were sampled on day 13)

Elicitor addition	DW (g/L)	Ginsenoside content (mg/100 mg DW)					Rb:Rg[2]
		Rg_1	Re	Rb_1	Rd	Total[1]	
Control	8.87 ± 0.25[a]	0.20 ± 0.01[a]	0.24 ± 0.02[a]	0.21 ± 0.02[a]	0[a]	0.64 ± 0.06[a]	0.47 ± 0.01[a]
MJA	8.19 ± 0.46[a,c]	0.36 ± 0.02[b,h]	0.54 ± 0.01[b]	1.21 ± 0.04[b]	0.07 ± 0.01[b]	2.17 ± 0.04[b]	1.42 ± 0.06[b]
TFEJA	7.88 ± 0.17[b,c]	0.30 ± 0.03[c,g]	0.46 ± 0.02[c]	0.89 ± 0.07[c]	0.06 ± 0.01[b]	1.70 ± 0.13[c]	1.25 ± 0.02[c]
PFPJA	7.59 ± 0.32[b,c]	0.45 ± 0.01[d]	0.53 ± 0.01[b]	1.44 ± 0.05[d]	0.13 ± 0.03[c]	2.55 ± 0.11[d]	1.60 ± 0.04[d]
PFBJA	7.94 ± 0.27[b,c]	0.29 ± 0.01[c]	0.49 ± 0.02[d]	0.87 ± 0.04[c]	0.06 ± 0.01[b]	1.70 ± 0.04[c]	1.19 ± 0.07[c]
HEJA	8.15 ± 0.62[a,c]	0.62 ± 0.02[e]	0.66 ± 0.02[e]	2.20 ± 0.06[e]	0.18 ± 0.03[d]	3.65 ± 0.13[e]	1.87 ± 0.01[e]
HEEJA	8.29 ± 0.38[a,c]	0.56 ± 0.02[e]	0.60 ± 0.02[f]	1.69 ± 0.04[f]	0.11 ± 0.02[c]	2.94 ± 0.06[f]	1.56 ± 0.05[f]
DHPJA	8.87 ± 0.51[a]	0.40 ± 0.01[f]	0.52 ± 0.01[b]	1.21 ± 0.07[b]	0.06 ± 0.01[b]	2.19 ± 0.11[b]	1.37 ± 0.04[b]
GJA	8.55 ± 0.17[a]	0.33 ± 0.03[g,h]	0.40 ± 0.02[g]	0.93 ± 0.03[c]	0.06 ± 0.00[b]	1.72 ± 0.08[c]	1.37 ± 0.05[b]

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

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

[a, b, c, d, e, f, g, h] Means with the same letter, 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 2. Effects of MJA and chemically synthesized jasmonates on the production of individual ginsenosides of *P. notoginseng* cells (100 μM of each elicitor was added on day 4 and the cells were sampled on day 13)

Elicitor addition	Ginsenoside production (mg/L)			
	Rg_1	Re	Rb_1	Rd
Control	17.8 ± 2.0^a	20.9 ± 2.7^a	18.2 ± 2.6^a	0^a
MJA	29.1 ± 0.6^b	$44.4 \pm 4.7^{b,f}$	101 ± 8^b	5.37 ± 1.00^b
TFEJA	23.6 ± 2.9^c	35.7 ± 2.7^c	69.9 ± 7.6^c	4.32 ± 0.68^b
PFPJA	34.5 ± 3.0^d	40.6 ± 3.3^b	110 ± 10^b	10.0 ± 2.7^c
PFBJA	22.9 ± 2.1^c	38.3 ± 0.0^c	68.8 ± 6.3^c	4.36 ± 0.75^b
HEJA	52.1 ± 4.3^e	55.5 ± 4.5^d	186 ± 14^d	15.3 ± 3.13^d
HEEJA	46.5 ± 0.9^f	49.9 ± 4.6^e	141 ± 11^e	8.85 ± 2.28^e
DHPJA	36.1 ± 3.8^d	$46.9 \pm 4.5^{e,f}$	104 ± 6^b	4.98 ± 0.98^b
GJA	28.2 ± 3.2^b	33.8 ± 2.8^c	79.5 ± 4.7^c	5.13 ± 0.14^b

[a, b, c, d, e, f] Means with the same letter, 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.

jasmonate (PFPJA) showed a higher stimulatory activity than commercially available MJA. With PFPJA elicitation, the content of total ginsenosides and the Rb/Rg ratio on day 13 increased to about 18 and 13%, respectively than MJA elicitation. In cell cultures of *T. chinensis,* trifluoroethyl jasmonate (TFEJA) and pentafluorobenzyl jasmonate (PFBJA) were reported to have a higher stimulatory activity on taxuyunnanine C biosynthesis than MJA, whereas PFPJA showed lower activity than MJA [15]. For the hydroxyl containing jasmonates studied, 2-hydroxyethyl jasmonate (HEJA) and 2-hydroxyethoxyethyl jasmonate (HEEJA) could enhance ginsenoside biosynthesis and change the heterogeneity of different ginsenosides more efficiently than MJA. Among them, HEJA had the highest stimulatory activity on ginsenoside biosynthesis, and the content of total ginsenosides and the Rb/Rg ratio on day 13 increased to about 70 and 30%, respectively when compared to MJA. In cell cultures of *T. chinensis,* all four hydroxyl containing jasmonates studied had higher stimulatory activities against taxuyunnanine C biosynthesis than MJA; and it was DHPJA that had the highest stimulatory activity [16]. The above results indicate that the chemically synthesized jasmonates were powerful but they had different elicitation effects on different plant secondary metabolites. Therefore, a detailed investigation in individual cell culture systems is necessary to ensure a highly efficient elicitation effect on secondary metabolite biosynthesis.

The results suggest that the fluorine and the hydroxyl group could influence the stimulating activity of the jasmonates on plant secondary metabolites, and the optimum number of fluorine or hydroxyl group for different plant species was different. In other facet, till date, little information is available about the detailed elicitation mechanism of synthetic jasmonates. The relationship between the chemical structure of jasmonate elicitors and their stimulating activity needs further investigation towards rational design and synthesis of more potent elicitors.

The production of individual ginsenosides on day 13 is shown in Table 2. With the jasmonates elicitation, all the cultures resulted in a higher production of individual ginsenosides than the control. Among the jasmonates studied, HEJA elicitation resulted in the highest ginsenoside production. The production of ginsenoside Rg_1, Re, Rb_1 and Rd with HEJA addition was about 1.8, 1.3, 1.8 and 2.9-fold higher than MJA elicitation, respectively.

This work indicates that suitable chemical modification of MJA could efficiently promote its stimulatory activity on ginsenosides biosynthesis in cell cultures of *P. notoginseng.* The information obtained is considered useful for hyper-production of the heterogeneous products on large scale.

Acknowledgments Financial support from the National Natural Science Foundation of China (NSFC projects 30270038, 20236040 and 20376023), the Shanghai Science & Technology Commission (Project no. 04QMH1410), and the National Key Project for Basic Research, the Ministry of Science and Technology of China (2003CB114400) are gratefully acknowledged. J. J. Z. also thanks the National Science Fund for Distinguished Young Scholars (NSFC project no. 20225619).

REFERENCES

[1] Verpoorte, R., R. van der Heijden, H. J. G. ten Hoopen, and J. Memelink (1999) Metabolic engineering of plant secondary metabolite pathways for the production of fine chemicals. *Biotechnol. Lett.* 21: 467-479.

[2] Zhong, J. J. (2002) Plant cell culture for production of paclitaxel and other taxanes. *J. Biosci. Bioeng.* 94: 591-599.

[3] Palazón, J., R. M. Cusidó, M. Bonfill, A. Mallol, E. Moyano, C. Morales, and M. T. Piñol (2003) Elicitation of different *Panax ginseng* transformed root phenotypes for an improved ginsenoside production. *Plant Physiol. Biochem.* 41: 1019-1025.

[4] Wu, J. and L. Lin (2002) Elicitor-like effects of low-energy ultrasound on plant *Panax ginseng* cells: induction of plant defense responses and secondary metabolite production. *Appl. Microbiol. Biotechnol.* 59: 51-57.
[5] Zabetakis, I., R. Edwards, and D. O'Hagan (1999) Elicitation of tropane alkaloid biosynthesis in transformed root cultures of *Datura stramonium*. *Phytochemistry* 50: 53-56.
[6] Yukimune, Y., H. Tabata, Y. Higashi, and Y. Hara (1996) Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in *Taxus* cell suspension cultures. *Nature Biotechnol.* 14: 1129-1132.
[7] Dong, H. D. and J. J. Zhong (2001) Significant improvement of taxane production in suspension cultures of *Taxus chinensis* by combining elicitation with sucrose feed. *Biochem. Eng. J.* 8: 145-150.
[8] Yu, K. W., W. Y. Gao, E. J. Hahn, and K. Y. Peak (2002) Jasmonic acid improving ginsenoside accumulation in adventitious root culture of *Panax ginseng* C. A. Meyer. *Biochem. Eng. J.* 11: 211-215.
[9] Wang, W. and J. J. Zhong (2002) Manipulation of ginsenoside heterogeneity in cell cultures of *Panax notoginseng* by addition of jasmonates. *J. Biosci. Bioeng.* 93: 48-53.
[10] Ketchum, R. E. B., D. M. Gibson, R. B. Croteau, and M. L. Shuler (1999) The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate. *Biotechnol. Bioeng.* 62: 97-105.
[11] Plata, N., I. Konczak-Islam, S. Jayram, K. McClelland, T. Woolford, and P. Franks (2003) Effect of methyl jasmonate and *p*-coumaric acid on anthocyanin composition in a sweet potato cell suspension culture. *Biochem. Eng. J.* 14: 171-177.
[12] Sheludko, Y., I. Gerasimenko, M. Unger, I. Kostenyuk, and J. Stoeckigt (1999) Induction of alkaloid diversity in hybrid plant cell cultures. *Plant Cell Rep.* 18: 911-918.
[13] Yukimune, Y., Y. Hara, E. Nomura, H. Seto, and S. Yoshida (2000) The configuration of methyl jasmonate affects paclitaxel and baccatin III production in *Taxus* cells. *Phytochemistry* 54: 13-17.
[14] Staniszewska, I., A. Królicka, E. Maliński, E. Łojkowska, and J. Szafranek (2003) Elicitation of secondary metabolites in *in vitro* cultures of *Ammi majus* L. *Enzyme Microbiol. Technol.* 33: 565-568.
[15] Qian, Z. G., Z. J. Zhao, Y. Xu, X. Qian, and J. J. Zhong (2004) Novel chemically synthesized hydroxyl-containing jasmonates as powerful inducing signals for plant secondary metabolism. *Biotechnol. Bioeng.* 86: 809-816.
[16] Qian, Z. G., Z. J. Zhao, W. H. Tian, Y. Xu, J. J. Zhong, and X. Qian (2004) Novel synthetic jasmonates as highly efficient elicitors for taxoid production by suspension cultures of *Taxus chinensis*. *Biotechnol. Bioeng.* 86: 595-599.
[17] Hu, W. W., H. Yao, and J. J. Zhong (2001) Improvement of *Panax notoginseng* cells for production of ginseng saponin and polysaccharide by high cell density cultivation in pneumatically agitated bioreactors. *Biotechnol. Prog.* 17: 838-846.
[18] Han, J. and J. J. Zhong (2002) High density cell culture of *Panax notoginseng* for production of ginseng saponin and polysaccharide in an airlift bioreactor. *Biotechnol. Lett.* 24: 1927-1930.
[19] Zhang, Z. Y. and J. J. Zhong (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.
[20] Zhong, J. J. (1999) High-density cell cultivation and manipulation of heterogeneity of plant secondary metabolites. *APBioChEC*. plenary lecture, Thailand.
[21] Wang, W., Z. Y. Zhang, and J. J. Zhong (2005) Enhancement of ginsenoside biosynthesis in high density cultivation of *Panax notoginseng* cells by various strategies of methyl jasmonate elicitation. *Appl. Microbiol. Biotechnol.* (in press).

[Received December 15, 2004; accepted April 13, 2005]