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# Quantitative Influence of Endogenous Salicylic Acid Level on Taxuyunnanine C Biosynthesis in Suspension Cultures of *Taxus chinensis*

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**ABSTRACT:** Intracellular signals are critical to secondary metabolite biosynthesis by plant cells, but their quantitative effects are not yet well understood in plant cell cultures. Using *Taxus chinensis* suspension culture, which has the potential to provide a sustainable supply of highly useful bioactive taxoids, this work investigated the impact of endogenous salicylic acid (SA) level on taxuyunnanine C (Tc) biosynthesis. It was observed that the Tc accumulation was strongly dependent on the endogenous SA level. Under elicitation with an endogenous SA of $97.1 \pm 11.8$ μg/g dry weight, a maximal Tc content of $10.3 \pm 0.52$ mg/g dry weight was obtained. The transcription levels of Tc biosynthetic genes of geranylgeranyl diphosphate synthase and taxadiene synthase were also analyzed, and they were increased with increase of internal SA level. The results demonstrated that manipulation of endogenous SA level could be an efficient strategy for improving secondary metabolite biosynthesis in plant cell culture.

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**KEYWORDS:** intracellular signal level; salicylic acid; plant cell culture; *Taxus chinensis*; secondary metabolite production; gene transcription

Cellular signals play an important role in mediating the response to environmental stresses during whole life of plants, such as salicylic acid (SA) in plant–pathogen interaction. Plant secondary metabolism is often stimulated by responding to biotic and abiotic stresses. Plant cell and tissue cultures as a unique source can provide a sustainable supply of some pharmaceuticals, food additives, and fine chemicals, but their production was generally limited by low yield for potential industrial application. To develop an optimal bioprocess, more attention has been paid to study the role of signal underlying various effective strategies leading to enhanced biosynthesis of target secondary metabolites (Zhao et al., 2005).

Influenced by the change of external conditions including elicitation, accumulation of secondary metabolites in plant cells often involves various intracellular signals and their mediated effects on biosynthetic genes expression. For example, intracellular jasmonic acid (JA) and reactive oxygen species (ROS) were proved to be involved in induction of taxoid biosynthesis under low-energy ultrasound treatment and elicitation (Hu et al., 2006; Wu and Ge, 2004). As an efficient elicitor, exogenous SA was applied to strongly induce secondary metabolite accumulation such as anthraquinone phytoalexin in *Rubia cordifolia* cultures (Bulgakov et al., 2002) and ginsenosides in *Panax ginseng* root cultures (Ali et al., 2006). The enhanced secondary metabolite biosynthesis upon exogenous SA elicitation was probably due to induction of genes involved in secondary metabolism (Taguchi et al., 2001).

Intracellular SA is an important messenger involved in the signal transduction of plant cells responding to various biotic and abiotic stresses. An essential role of intracellular SA signal in fungal elicitor-induced flavonol glycoside biosynthesis was suggested by using SA accumulation deficient *Ginkgo biloba* cells (Xu et al., 2009). In the leaves of constitutive salicylic acid producing (CSA)-tobacco and non-transgenic tobacco plants, Nugroho et al. (2002a) found large variations in nicotine and related alkaloids accumulations, but there was no significant correlation between SA levels and alkaloid accumulation in CSA-

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tobacco plants. The constitutive production of SA resulted in the expression of genes involved in systemic acquired resistance (SAR), but it did not affect terpenoid biosynthesis in the CSA-tobacco plant (Nugroho et al., 2002b). To the best of our knowledge, there have been no reports on the quantitative relationship between intracellular signal molecule level and secondary metabolite biosynthesis by plant cells, in spite of the potential significance of such information for biotechnology application. Information regarding the quantitative effect of internal signal on gene transcription of plant secondary metabolism is also not yet available.

In this work, the quantitative response of taxuyunnanine C (Tc) biosynthesis by *Taxus chinensis* cells to endogenous SA concentration is reported. Tc is an interesting physiologically active diterpene, which has the effect in enhancing neuron growth factor (NGF) activity and facilitating the treatment of Alzheimer's disease, and could be produced by suspension cultures of *T. chinensis* (Zhou and Zhong, 2009). The transcription levels of geranylgeranyl diphosphate synthase (GGPPS) and taxadiene synthase (TS), two important taxoid biosynthetic genes of *T. chinensis* under various internal SA levels were analyzed. This work aims to understand the quantitative effects of endogenous signal on secondary metabolite production and biosynthetic gene transcription in plant cell cultures.

To manipulate the endogenous SA level, a different concentration (0, 50, and 100 μM) of exogenous SA was added into *T. chinensis* cell cultures at day 7 of cultivation, respectively. Figure 1A shows that an increase of exogenous SA concentration from 0 to 100 μM resulted in a rapid increase of intracellular SA content. The higher the concentration of external SA, the higher the SA content of cells. The maximal endogenous SA of 97.1 ± 11.8 μg/g dry weight was observed at 1 h after addition of 100 μM exogenous SA, approximately 20-fold higher than intracellular SA before exogenous SA elicitation (4.53 μg/g dry weight, i.e., 170 ng/g fresh weight). The dose-dependent internal SA accumulation observed in our case was similar to that reported in *Phaseolus vulgaris* seedling (Palma et al., 2009). To understand the possible role of intracellular signal in secondary metabolites induction upon external stress response, treatment with signal biosynthesis inhibitor was proved useful (Peebles et al., 2009), and this approach was adopted in this work. Compared with that at 50 and 100 μM exogenous SA, addition of 100 μM paclobutrazol, the inhibitor of benzoic acid 2-hydroxylase (BA2H) which is responsible for SA biosynthesis (Leon et al., 1995), led to a lower level of internal SA content. The maximal endogenous SA accumulation was reduced to 67.1 ± 6.8 μg/g dry weight, 30% lower than that without the inhibitor (paclobutrazol) addition. Aminooxyacetic acid (AOA, at 100 μM), an inhibitor of phenylalanine ammonia lyase (PAL) which is

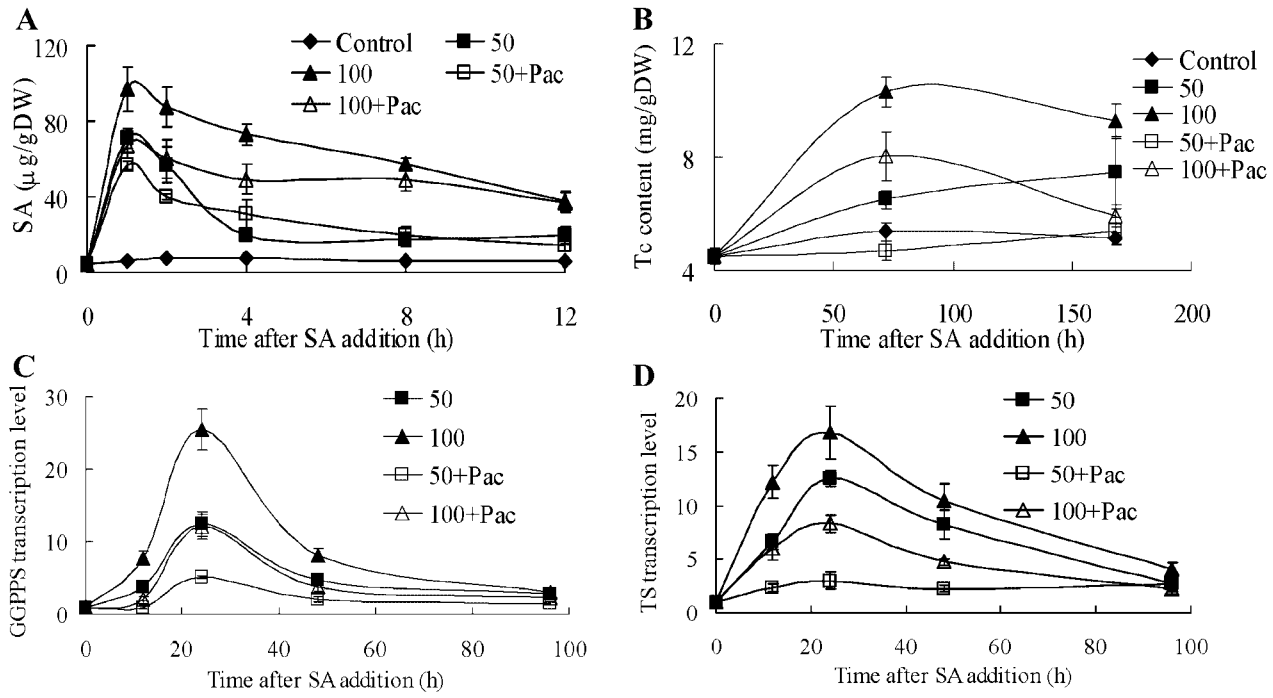


**Figure 1.** Intracellular salicylic acid (SA) accumulation (**A**), taxuyunnanine C (Tc) content (**B**), mean over control fold on GGPPS gene transcription level (**C**), and TS gene transcription level (**D**) in suspension cultures of *Taxus chinensis* treated with various concentrations of SA on day 7 of cultivation. Symbols for different levels of SA (μM): (◆) 0; (■) 50; (▲) 100; (□) 50 + 100 μM paclobutrazol; (△) 100 + 100 μM paclobutrazol. The control for gene transcription level was that of day 7 cells. Each data point represents the mean ± SD from three independent samples.

also involved in SA biosynthesis, had a similar decreasing effect on SA biosynthesis under 50 and 100 μM exogenous SA (Supplemental Fig. S1A). Besides, induction of PAL and BA2H (enzymes in SA biosynthesis pathway) and accumulation of benzoic acid (SA biosynthesis precursor) were also observed (Supplemental Fig. S1B–D). The results suggested that SA biosynthesis was activated in response to a higher external SA level, and the inhibition of the former reasonably decreased the intracellular SA level. But, intracellular SA induction was not completely inhibited by paclobutrazol treatment. This may be due to that there may be another pathway of SA biosynthesis in plant cells against defense response (Wildermuth et al., 2001).

Another source of intracellular SA increase is SA uptake from the medium as SA in medium significantly decreased (Fig. 2A). From Figure 2, it is considered that SA biosynthesis started after 5 min of exogenous SA addition as shown by benzoic acid accumulation and BA2H induction (Fig. 2C and D). The significant increase of intracellular SA level within 5 min of exogenous SA application contributed to about 20–30% of total intracellular SA increase (Fig. 2B), which was calculated by $(a)/(b) \times 100\%$, where $(a)$ was intracellular SA increase at 5 min before SA biosynthesis started and $(b)$ was maximal SA increase at 1 h. But not all absorbed SA existed as free SA as the increase of intracellular SA in 0–5 min after exogenous SA application was only 50–60% of the SA decrease in medium in the same period (Fig. 2A and B), where SA degradation was ruled out by SA detection in culture medium with removal of cells (cell-free medium). The results obtained here may be explained by a previous report, as largely (nearly 60%) uptaken SA was metabolized into SA metabolites and glucose conjugates such as methyl salicylate 2-*O*-β-D-glucoside and SA 2-*O*-β-D-glucoside (Dean et al., 2005) which were stored in cytoplasm or vacuole and considered as inactive in intracellular signal transduction. However, there was a possibility of the absorbed SA involved in signal transduction as glucosylation and vacuolar storage of SA could be reversible (Hennig et al., 1993).

The cellular Tc content was increased by increasing the internal SA level through exogenous SA application from 0 to 100 μM. The results agreed with earlier studies regarding taxoids induction upon exogenous SA application (Qian et al., 2006; Wang et al., 2007), but intracellular SA was largely overlooked in those studies. Paclobutrazol addition was also conducted (Fig. 1B), and an addition of 100 μM paclobutrazol decreased the induced Tc content after 72 h of exogenous SA application compared with that without paclobutrazol. The results indicated that Tc biosynthesis was probably dependent on internal SA concentration quantitatively, and inhibition of SA biosynthesis reduced Tc biosynthesis by *T. chinensis* cells.

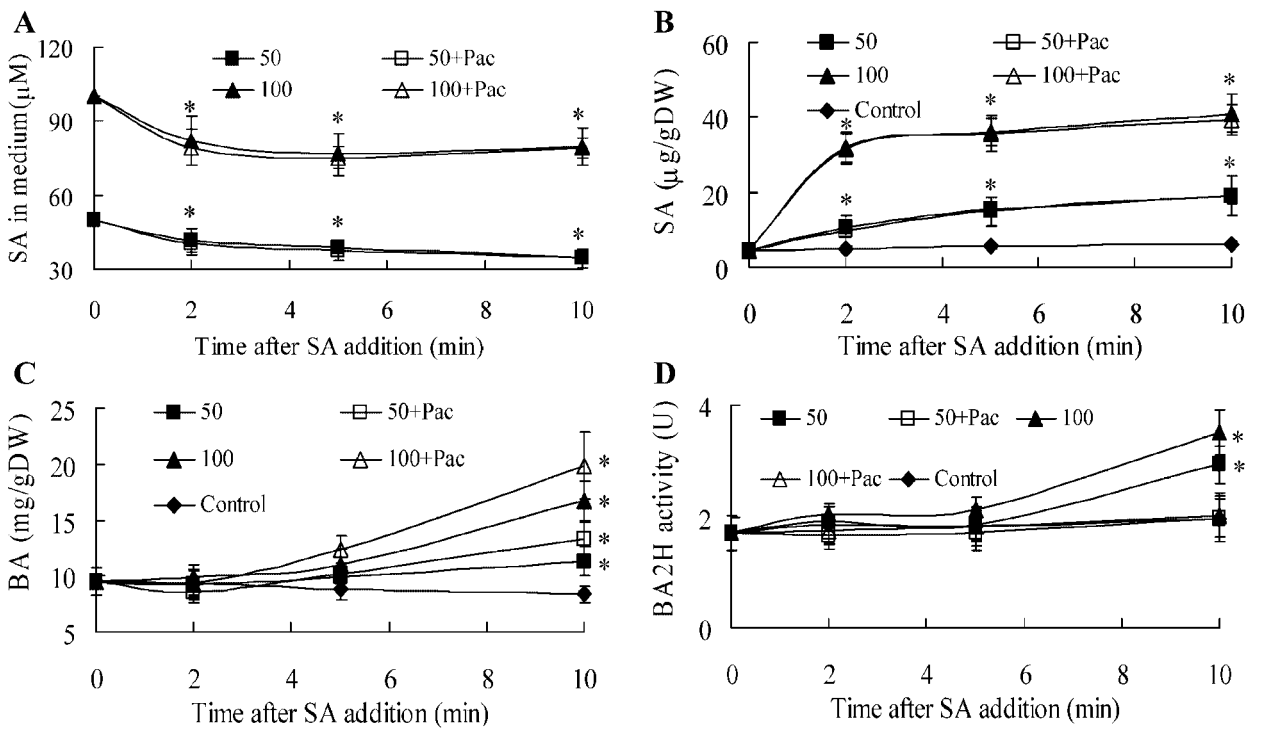


**Figure 2.** Medium salicylic acid (SA) (**A**), intracellular SA accumulation (**B**), intracellular benzoic acid (BA) accumulation (**C**), and benzoic acid 2-hydroxylase (BA2H) activity (**D**) in suspension cultures of *T. chinensis* treated with various concentrations of SA on day 7 of cultivation. Symbols for different levels of SA (μM): (◆) 0; (■) 50; (▲) 100; (□) 50 + 100 μM paclobutrazol; (△) 100 + 100 μM paclobutrazol. Each data point represents the mean ± SD from three independent samples. "*" in (A) indicates significant difference from 0 min while "*" in (B), (C), and (D) indicates significant difference among treatments and control ($P < 0.05$).

In order to understand how internal SA concentrations affected Tc biosynthesis, transcription level of important genes in Tc biosynthesis was investigated. Figure 1C and D showed GGPPS and TS transcription levels in the suspension cells responding to different levels of intracellular SA. At the maximal intracellular SA induction upon 100 μM SA elicitation, the transcription levels of GGPPS and TS reached highest ($25.5 \pm 2.89$ for GGPPS and $16.8 \pm 2.45$ for TS) at 24 h after SA application. Compared with that of SA application, SA-induced gene transcription was partially suppressed by treatment of *T. chinensis* cells with 100 μM paclobutrazol. The results (Fig. 1C and D) also indicated that the response of GGPPS and TS transcriptions to intracellular SA level was similar to that of Tc synthesis. This suggests that manipulation of intracellular SA level is very important for the genes transcription and the taxoid biosynthesis by *T. chinensis* cells.

Until now, the quantitative response of plant secondary metabolism to internal signal level has not yet been well understood. In order to identify potential relationships between intracellular SA and Tc biosynthesis, regression analyses were performed for maximum values of intracellular SA, Tc accumulation, and biosynthetic gene expression. Figure 3A showed that there was a linear correlation between maximal intracellular SA level and maximal-induced Tc biosynthesis ($P < 0.05$). Similarly, in case of internal calcium signal (within about 0.2−1.2 μmol/g fresh weight), its positive correlation with ginsenoside synthesis by *Panax notoginseng* cells was observed, but further increasing internal calcium content led to decreased ginsenoside content (Yue and Zhong, 2005). Different from the results obtained here, Nugroho et al. (2002a) reported no significant correlation between SA and alkaloids production by transgenic constitutive SA-producing tobacco plants. These different results might be due to different plant species and their compartmentation. As for gene expression, maximal induction of GGPPS transcription showed a good dose-dependent relation with the highest intracellular SA level ($P < 0.05$), but this trend was a bit weakly observed in case of TS transcription with maximal intracellular SA level. The results obtained may suggest a bit different transcription regulation of internal SA on GGPPS from that on TS. Until now, many details of SA signaling have not been worked out, including the mechanism of SA induced secondary metabolite biosynthetic gene transcription. SA could be stored in different cell compartments as absorbed SA was mainly in the cytosol and biosynthesized SA derived from phenylpropanoid pathway was reported in plastid. However, SA movement between cellular compartments is possible because (1) as a weak acid SA could diffuse through membrane as its lipophilic form and (2) pH-dependent carrier system of SA transport was reported in *Ricinus communis* (Rocher et al., 2009). It is clear that under stresses, internal SA induction may lead to ROS increase through SA-binding protein-1 (SABP1) which was proved to be catalase. Catalases belong to a group of enzymes

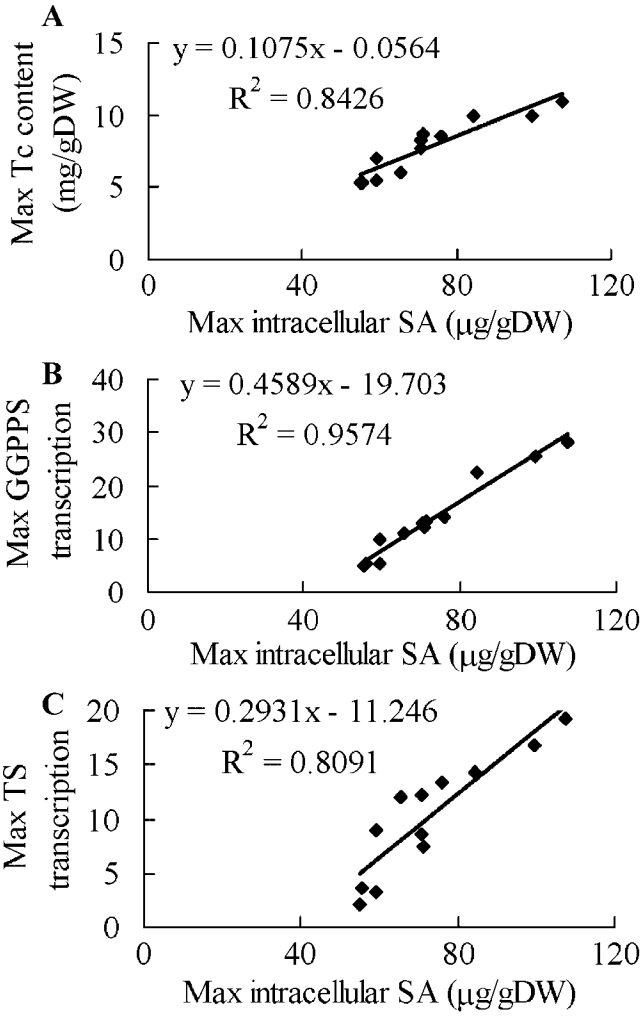


**Figure 3.** Regression analysis of intracellular SA with Tc content and Tc biosynthetic gene transcription levels. The correlations between maximum induced internal SA and (**A**) maximum induced Tc content ($R^2 = 0.84$, $P < 0.05$); (**B**) maximum induced GGPPS transcription ($R^2 = 0.96$, $P < 0.05$); and (**C**) maximum induced TS transcription ($R^2 = 0.81$) are presented.

involved in regulating the cellular levels of ROS and are responsible for converting $H_2O_2$ to $H_2O$ and $O_2$. Upon SA binding, the activity of catalase was inhibited, which resulted in more $H_2O_2$ accumulation (Durner and Klessig, 1996). As an important activator of defense gene, Non-expressor of pathogenesis related genes1 (NPR1) contains several cysteine residues that can be targets of redox regulation (Mou et al., 2003). The more reductive state of the cellular environment that follows the ROS burst phase in the presence of SA reduced NPR1 oligomer to its active monomer protein, which can translocate to the nucleus and interact with transcription factors to initiate target gene transcription. The extent of induction of genes for pathogenesis related-1 (PR-1) proteins was reported to almost directly relate to the endogenous levels of SA (Chen et al., 1995). So, a linear correlation between maximum

internal SA and expression of secondary metabolism genes as well as its product synthesis was possibly as a result of more endogenous SA accumulation, which allows "fresh" NPR1 to reinitiate the transcription cycle when the "exhausted" NPR1 is degraded by the proteasome after initiating transcription. Similarly, a linear increase in serine acetyltransferase (SAT) activity in *Arabidopsis* was also observed when SA accumulation in shoots increased, along with linear increase in its product *O*-acetyl-L-Ser and downstream metabolite glutathione (Freeman et al., 2005). But the SA regulated SAT activity post-translationally different from the results obtained in our case, which may be due to the different plant species and cultivation modes.

In conclusion, the results of this work showed the quantitative responses of Tc biosynthesis to intracellular SA signal. It is proposed that GGPPS and TS mediated the effect of internal SA signal onto Tc biosynthesis by *T. chinensis* cells. Regulation of internal signal level was demonstrated to be an efficient and powerful tool for manipulating taxoid biosynthesis by the cells. The information obtained here may be helpful to a large-scale cultivation process and other plant cell culture work.

## Materials and Methods

### Cell Culture

Suspended cells of *T. chinensis* were grown in modified Murashige and Skoog (MS) medium and subcultured biweekly. The cultures were grown at 25°C at 110 rpm in the dark. The details have been described previously (Hu et al., 2006).

### Salicylic Acid (SA) Elicitation and Inhibitor Addition

For all the treatments, *T. chinensis* cells of 2 g by fresh weight (FW) were inoculated into a 100-mL Erlenmeyer flask containing 20 mL of medium. For SA treatment, after cultivation for 7 days, various concentrations of filter-sterilized SA (0, 50, 100 μM) were added to the suspension cultures with or without inhibitor. One hundred micromolar of filter-sterilized paclobutrazol, the SA biosynthesis inhibitor, was added to the cultures approximately 30 min before exogenous SA addition. Cells were harvested 72 and 168 h after SA treatment for secondary metabolite analysis. For all cultures, multiple flasks were run under each condition and the data of each point represent the mean values with standard deviations from three independent samples.

### Taxuyunnanine C (Tc) Extraction and HPLC Detection

Tc, an intracellular bioactive taxoid, was extracted from dried *T. chinensis* suspended cells and analyzed by HPLC as reported previously (Qian et al., 2004; Zhou and Zhong, 2009).

### Quantification of Intracellular SA

After various periods (1, 2, 4, 8, and 12 h) of SA stimulation, cells from three samples of 20 mL for each were collected by filtration and cells were washed with distilled water to remove extracellular SA. Free SA was extracted as reported (Raskin et al., 1989) with certain modification. One gram of frozen tissue was ground in 3 mL of 90% methanol and centrifuged at 12,000*g* for 15 min. The pellet was resuspended in 2 mL of 100% methanol and reextracted at 12,000*g* for another 15 min. Supernatants from both extractions were combined and dried at 40°C under nitrogen. The residue was resuspended in 2.5 mL of 5% (w/v) trichloroacetic acid and centrifuged at 1,200*g* for 10 min. Free acids were then extracted three times with 2.5 mL of ether:petroleum (50:50, v/v). Organic phases were pooled, dried under nitrogen, and resuspended in 0.5 mL of the HPLC mobile phase (45% water/55% methanol, v/v, pH was adjusted to 4 by acetic acid). Samples were injected on a C18 column (Thermo Hypersil GOLD, 5 μm, 250 mm × 4.6 mm). The column was maintained at 25°C with a mobile-phase flow rate of 1 mL/min. SA was detected by a HPLC spectrofluorescence detector (Agilent, California) equipped with xenon lamp. The best quantification of SA was obtained with an excitation wavelength of 315 nm and an emission wavelength of 405 nm.

### RNA Isolation and Real-Time Quantitative RT-PCR (qRT-PCR)

Total RNA of *T. chinensis* cells was extracted, prepared for qRT-PCR and the primers and qRT-PCR conditions for GGPPS, taxa-4(5), 11(12)-diene synthase (TS), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were the same as previously reported (Zhou and Zhong, 2009). The gene transcription was quantified by qRT-PCR using Maxima$^{TM}$ SYBR Green chemistry on the realplex$^2$ epgradient Mastercylcer (Eppendorf). Transcript level was calculated and normalized against *T. chinensis* GAPDH gene (as an internal control gene), and the cells cultured in control medium on day 7 were used as reference. For each gene, the reference sample was defined as the expression level 1.0, and results were expressed as the fold increase of mRNA level over the reference sample. Post-qRT-PCR calculations to analyze relative gene expression were performed according to the $2^{-\Delta\Delta CT}$ method.

### Statistical Analyses

Data were analyzed with Student's *t*-test. The difference between treatments was considered significant when $P < 0.05$ in a two-tail analysis.

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