Responses of Defense Signals, Biosynthetic Gene Transcription and Taxoid Biosynthesis to Elicitation by a Novel Synthetic Jasmonate in Cell Cultures of *Taxus chinensis*

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Abstract: Recently novel synthetic jasmonate derivatives were shown to have very powerful stimulating effects on the biosynthesis of taxuyunnanine C (Tc) by *Taxus chinensis* cells (Biotech Bioeng, 86:595; ibid. 86: 809, 2004). To provide an insight into the elicitation mechanism of the newly synthesized elicitors, by taking 2-hydroxyethyl jasmonate (HEJ, at 100 μM) as a typical example, in this work the defense signals were detected and their influences on the expression of important genes in taxoid biosynthetic pathway were examined in cell cultures of *T. chinensis*. The oxidative burst (induced H_2O_2 production) was confirmed, and the induction of lipoxygenase (LOX) activity and intracellular jasmonate acid (JA) synthesis was found. The gene transcription of geranylgeranyl diphosphate synthase (GGPPs) and taxa-4(5),11(12)-diene synthase (TS) was up-regulated by HEJ elicitation compared to control. Inhibition of JA biosynthesis by a putative LOX inhibitor, ibuprofen (IBU), effectively depressed the HEJ-induced up-regulation of *GGPPs* and *TS* gene transcription levels. In contrast, the inhibition of H_2O_2 production by membrane NADPH oxidase inhibitor, diphenylene iodonium (DPI), did not affect the transcriptions of those genes. For the HEJ-induced Tc production, it was suppressed with addition of DPI or IBU. The results suggest that both H_2O_2 and JA signals were involved in HEJ-induced Tc biosynthesis, and JA mediated the induction of *GGPPs* and *TS* genes expression, but H_2O_2 was not essential to activate them. Finally, a signal transduction cascade from defense signal response to activated transcription of taxoid biosynthetic genes and enhanced Tc production is proposed.

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INTRODUCTION

Plant cell culture is a promising technology for mass production of valuable secondary metabolites, particularly those obtained from naturally rare plant species. The biosynthesis of many plant secondary metabolites is a part of plant defense response to the attack by insects and pathogens, or to other biotic and abiotic environmental stresses. Strategies with application of biotic and abiotic elicitors have been developed to improve the yield of plant secondary metabolites such as in cell cultures of *Taxus chinensis* (e.g., Qian et al., 2004b; Tabata, 2004; Ye et al., 2004). Biotic elicitors can be glucan polymers, glycoproteins, or fungal cell materials, while abiotic elicitors include electric field, light, ultrasonication, osmotic pressure, heavy metals, cold or heat shock, and various chemicals.

Jasmonic acid (JA), methyl jasmonate (MJ) and its derivatives are a family of important signal transducers, and can efficiently stimulate secondary metabolism in plant cells (e.g., Farmer and Ryan, 1990; Gundlach et al., 1992; Naill and Roberts, 2005; Wang et al., 2005). Since the first report regarding the effect of MJ on the accumulation of secondary metabolites in plant cell cultures (Gundlach et al., 1992), more than 100 plant species have been demonstrated to respond to the addition of MJ to the culture medium by accumulating secondary metabolites (Haider et al., 2000). Regarding the inducible mechanisms of jasmonates-stimulated secondary metabolite biosynthesis in plant cell cultures, to date, most

efforts have been focused on the induction of plant defense responses including oxidative and nitric oxide bursts by the addition of jasmonates (e.g., Chong et al., 2005; Wang and Wu, 2005). But, in those studies, there was no information on the alteration of secondary metabolic gene expression. To our knowledge, there is lack of reports on a causal link of signal cascade from defense signal response to gene activation and secondary metabolite accumulation in jasmonates-induced cell cultures.

Recently, synthetic jasmonate derivatives have received attention as they showed highly efficient induction of secondary metabolites production in plant cell cultures including those of *Eschscholzia californica*, *Panax notoginseng*, and *T. chinensis* (Haider et al., 2000; Qian et al., 2004a,b; Wang et al., 2005). Investigation on the mechanism of synthetic jasmonates-stimulated secondary metabolite biosynthesis remains at the level of early defense signal responses (Wang et al., 2005). The influence of synthetic jasmonates on the gene activation of secondary metabolism has not yet been revealed.

In *Taxus* cell cultures, various elicitors have been used to stimulate taxoid production (e.g., Huang et al., 2005; Naill and Roberts, 2005; Qian et al., 2004a,b; Tabata, 2004; Wu and Ge, 2004; Ye et al., 2004). Regarding the elicitation mechanism, there are reports on defense responses such as oxidative burst, NO burst and/or JA by cultured cells of Taxus spp. (e.g., Huang et al., 2005; Mueller et al., 1993; Wang and Wu, 2005), but the activation target of the transduction of those signals to secondary metabolic pathway was not detected. Several reports showed that elicitors activated gene transcriptions and/or enzyme activities of GGPP synthase (GGPPs) and taxadiene synthase (TS) in the taxoid biosynthetic pathway (Hefner et al., 1998; Tabata, 2004), however, those studies did not demonstrate what defense signals were stimulated by elicitors or how those signals affected secondary metabolite pathway. Until now, a systematic study on elicitor-induced signal transduction from defense response to gene activation and subsequent secondary metabolite accumulation in *Taxus* cells has not been reported.

In our recent work, novel chemically synthesized jasmonate derivatives could dramatically stimulate taxoid production in cell cultures of *T. chinensis* in comparison with MJ, which is a powerful, widely used and commercially available jasmonate (Qian et al., 2004a,b). Although the defense signal, H_2O_2, was detected following elicitation (Qian et al., 2004b), in view of the plant defense signal responses there have been no valid evidences regarding whether or not the new jasmonates-induced H_2O_2 was involved in the secondary metabolite (taxoid) accumulation. It is also unclear whether other defense signals (such as JA) were induced by the elicitation of the novel elicitors in *T. chinensis* cells.

In this study, 2-hydroxyethyl jasmonate (HEJ) is taken as a typical example of novel jasmonate elicitors (Qian et al., 2004a), and the signal transduction from defense response to gene activation and secondary metabolite accumulation in suspension cultures of *T. chinensis* cells by the HEJ elicitation is focused. Oxidative burst and JA biosynthesis were examined together with the investigation on genes transcription of GGPPs and TS, two important enzymes in the taxoid biosynthetic pathway. The characterization of elicitor-induced defense signal response and their relationship with secondary metabolic genes expression will provide an insight into the elicitation mechanism of the new elicitors, and it may be also helpful to design new strategies for hyperproduction of desired secondary metabolites by plant cells.

MATERIALS AND METHODS

Chemicals

The antioxidant *N*-acetyl cysteine (NAC), NADPH oxidase inhibitor diphenylene iodonium (DPI) and putative lipoxygenase (LOX) inhibitor ibuprofen (IBU) were purchased from Sigma (St. Louis, MO). MJ was purchased from Tokyo Kasei Co. Ltd (Tokyo, Japan). HEJ was synthesized and purified as described earlier (Qian et al., 2004a), and both MJ and HEJ contain the same ratio of stereoisomers (Qian et al., 2004a).

Cell Subcultures and Analyses of Dry Cell Weight and Taxuyunnanine C (Tc)

The cells were subcultured every 14 days in a 500-mL Erlenmeyer flask containing 200-mL modified Murashige and Skoog medium on a rotary shaker at 110 rpm and 25°C in the dark. Measurement of dry cell weight (DW) and taxuyunnanine C (Tc), a bioactive taxoid produced intracellularly, was the same as described elsewhere (Qian et al., 2004a,b).

Elicitation Experiments

For elicitation, 2 g of fresh cells was incubated in a 100-mL Erlenmeyer flask containing 20-mL medium with the same culture conditions as in subcultures. The type of closures used on the flasks was cotton plug, the same as in subcultures. Each elicitor at 100 μM was added to the cultures in 1 μL of ethanol per 1 mL of culture medium on day 7 after inoculation and sterilized by filtering through 0.22 μm polyvinylidenedifluoride (PVDF) syringe filters (Millipore). Here, 100 μM of MJ or HEJ (Qian et al., 2004a) was the best concentration of each elicitor for Tc production. In experiments with NADPH oxidase inhibitor DPI and LOX inhibitor IBU, each inhibitor was added to the cell cultures 20 min before the elicitation treatment.

Assay of Oxidative Burst

H_2O_2 production was assayed by scopoletin fluorescence according to a previous report (Cazale' et al., 1998). The details have been described previously (Qian et al., 2004a).

Quantification of JA

Extraction was performed as described by Koch et al. (1999). The cell culture samples were filtrated under vacuum, and

10 g cells by fresh weight (FW) were frozen and ground under liquid nitrogen. The resulting powder was suspended in a solution of acetone and 50 mM citric acid (70:30, v/v). 9,10-Dihydrojasmonic acid was added as an internal standard. Acetone was allowed to evaporate overnight at room temperature to avoid loss of jasmonic acid, which is a volatile fatty acid compound. The resulting aqueous solutions were filtered and extracted with 20 mL trichloromethane for three times. The organic phase was dried over sodium sulfate anhydrous and evaporated. The residue was dissolved in 800 μL of diethyl ether/methanol (9:1, vol/vol). Carboxylic acids were converted into methyl esters by the addition of 16 μL of a 2.0 M solution of trimethylsilyldiazomethane (Sigma) in hexane (Engelberth et al., 2004). The vials were then capped, vortexed, and allowed to sit at room temperature for 30 min. Excess trimethylsilyldiazomethane was then destroyed by adding an equivalent molar amount of acetic acid to each sample. Volatile metabolites were separated from the complex mixture by vapor-phase extraction as described (Engelberth et al., 2003). The trapped volatiles were then eluted with 1.5 mL of dichloromethane and were then evaporated by a constant airstream. The residue was dissolved in 50 μL of methanol and was applied to analyze JA by 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. The column temperature was initially held at 80°C for 0.5 min, and then shifted to 200°C at 4°C/min, and finally increased to 280°C at 30°C/min, and the injector and detector temperature was set at 270 and 280°C, respectively.

LOX Extraction and Activity Assay

The cell suspensions were filtrated under vacuums and then frozen immediately at −80°C. For lipoxygenase (LOX) activity assay, the cells were homogenized in an ice bath with 0.1 M Tris-HCl buffer (pH 8.0) containing 1% PVP (w/v), 1 mM $CaCl_2$, 5 mM DTT, and 10% (v/v) glycerol. The homogenate was centrifuged at 12,000*g* for 15 min at 4°C, and the supernatant was used as the enzyme extract. LOX was assayed according to a previous report (Zhao and Sakai, 2003). One milliliter of the enzyme reaction mixture contained 50 μL enzyme extract, 0.95 mL Na_2HPO_4 buffer (pH 7.0) and 5 μL of linolenic acid substrate solution. The increase of absorbance at 234 nm was monitored over 2 min after the extract addition. One unit of LOX is equivalent to an increase of 0.01 absorbance unit per minute. Protein was determined using the conventional Bradford method with bovine serum albumin (Sigma) as standard.

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

The gene transcription levels of *T. chinensis* cells were quantified by qRT-PCR using Tagman™ chemistry. Aliquots of 0.2 g FW cells were collected by filtration from the culture medium and frozen in liquid nitrogen. Total RNA was extracted by 1 mL of Trizol (Gibco-BRL) following the manufacturer's procedure. Residual genomic DNA was removed by incubating the RNA solution with 15 units of RNase-free DNase I (Takara) for 30 min at 37°C. The DNase reaction was stopped with a phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). Two micrograms of DNase-treated RNA was reverse-transcribed with SuperScript™ First-Strand Synthesis System for RT-PCR following the manufacturer's instructions.

The transcript levels were determined by real-time quantitative PCR on the PRISM ABI7700 sequence detection system (Applied Biosystems). Taq-Man probes and primers were designed using Primer Express software (Applied Biosystems) based on sequences present in databases. GGPPs (GenBank accession number L269221): forward primer, 5′-AGGCGGTAGAGC TGCTGGAT-3′; reverse primer, 5′-TCGGAAGCACATTCCAGTCA-3′; TaqMan probe, 5′-TCTAGCAGGAGGTAAGCGCGTCA-3′. TS (GenBank accession number BD241780): forward primer, 5′-TGGA-TGCGGCACTGGATAAG-3′; reverse primer, 5′-GCAGT-TGGCATGGCAAGGTC-3′; TaqMan probe, 5′-AAGGGA-GTGGCTTGACGCTGGGT-3′. Glyceraldehydes 3-phosphate dehydrogenase (GADPH) (GenBank accession number AY007207): forward primer, 5′-ACGTGACATGCTCGCT-CACA-3′; reverse primer, 5′-ACACGGTCCAAGGCCTAC-TG-3′; TaqMan probe, 5′-ACTGGAGCAGCTAA-GGTGT-TGGA-3′.

PCR reactions were carried out according to Platinum® Quantitative PCR SuperMIX-UDP (Invitrogen) manufacturer's procedure. After denaturation at 95°C for 5 min, amplification occurred in three steps: 30 s of denaturation at 95°C, 30 s of annealing at 55°C, 30 s of extension at 72°C with a total of 40 cycles. Identical thermal cycling conditions were used for all targets. Transcript level was calculated using the standard-curve method and normalized against *T. chinensis* GADPH gene as an internal control gene and untreated cells as a reference sample. 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 $2^{-\Delta\Delta C_T}$ method as described by Livak and Schmittgen (2001). The data for the gene expression are shown after normalization with the GADPH endogenous control.

Statistical Analyses

All data were generated in three independent experiments with two or three repeats. Data were analyzed with Student's *t*-test. The difference between treatments was considered significant when $P < 0.05$ in a two-tail analysis.

RESULTS

HEJ Induced Not Only Oxidative Burst But Also JA Biosynthesis in Cell Cultures

As mentioned above, in our experiments HEJ was used for investigating new elicitor-induced plant defense responses,

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whereas the cultures with MJ addition and without elicitation were used as controls. HEJ or MJ (100 μM) was added to the cultures on day 7 of cultivation (Qian et al., 2004a). The effects of HEJ on cell growth and Tc production were confirmed and similar results as reported for other synthetic jasmonates were obtained (data not shown) (Qian et al., 2004b).

We investigated how *T. chinensis* cells responded to HEJ in defense reactions. As shown in Figure 1A, the cells rapidly released H_2O_2 into the culture medium after the elicitation by HEJ. H_2O_2 production was considerably increased at 10 min after addition of HEJ, which level was interestingly even a bit higher than that by MJ (Fig. 1A). The H_2O_2 level reached its maximum at 30 min after exposure to HEJ or MJ, and dropped rapidly to a quite low level after 60 min of treatment. The H_2O_2 level of the control without jasmonates addition was almost not changed and always lower than that of elicited samples over the period as studied.

As shown in Figure 1B, the LOX activity of cells was induced by HEJ or MJ, which reached a maximum in 25 min, then dropping back to the control level after about 65 min. The JA level in the cells also showed a significant increase after 1 h of elicitation by HEJ (Fig. 1C), and reached the highest 3 h post-elicitation, peaking at approximately 30-fold that of control. Later it fell back to about six-fold over control (at 15 h).

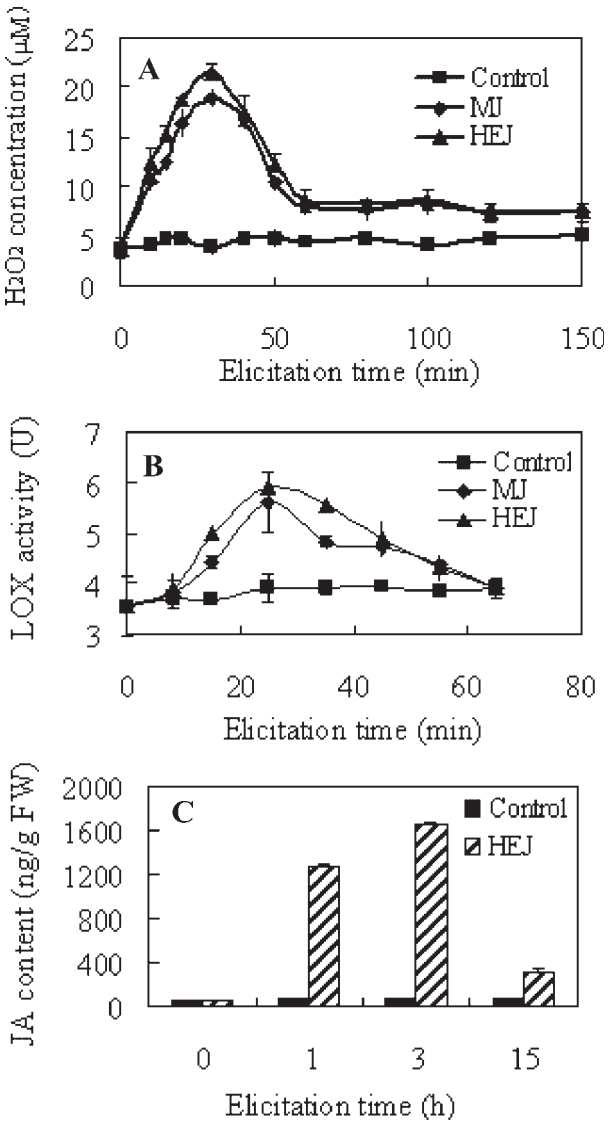

Figure 1. H_2O_2 production (**A**), LOX activity (**B**) and JA accumulation (**C**) in *T. chinensis* cell cultures treated with HEJ or MJ (at 100 μM) on day 7 of cultivation. Each data point represents the mean ± SD from three independent samples.

Effect of Inhibitor Addition on HEJ-Induced Oxidative Burst and JA Production

In order to confirm that both H_2O_2 production and JA accumulation were induced by HEJ and to observe whether oxidative burst and JA biosynthesis was interacted with each other, experiments using an inhibitor or an reactive oxygen species (ROS) scavenger were carried out. DPI, which is an inhibitor of membrane NADPH oxidase, has been shown to block oxidative burst in different plant systems (Desikan et al., 1996; Levine et al., 1994; Jih et al., 2003). Treatment of T. chinensis cells with 10 μM DPI effectively suppressed HEJ or MJ-induced H_2O_2 release (Fig. 2A). N-Acetyl-L-cysteine (NAC, at 100 μM), which is a putative ROS scavenger (Aruoma et al., 1989), had a similar decreasing effect on H_2O_2 production (data not shown). These facts confirmed that H_2O_2 was produced by the elicited cells and it was from the oxidative burst induced by the jasmonates. Effects of inhibitors on LOX activity were shown in Figure 2B, which was measured 25 min post-elicitation based on the results of Figure 1B. Data in Figure 2B showed that DPI (an oxidative burst inhibitor) had a slight inhibitory effect on LOX activity. Use of LOX inhibitor (IBU) almost completely inhibited the jasmonates-induced LOX activity (Fig. 2B). The intracellular JA accumulation was also greatly reduced by using IBU (Fig. 2C). The inhibition of JA production by IBU seems to have only a little inhibition on the HEJ-induced H_2O_2 production (Fig. 2A). The above results indicate that the oxidative burst and JA accumulation were induced by HEJ, and the responses of the two defense signals to HEJ in *T. chinensis* cells may be two independent events and they might be not closely interacted with each other.

Induction of Tc Biosynthetic Gene Expression by HEJ

Suspension cell cultures of *T. chinensis* were treated with HEJ or MJ 7 days after cultivation and harvested at 6, 24, 48, and 168 h after elicitation or immediately before induction. Untreated cells were taken as control at every time point sampled. During this 7 days induction period, the fresh cell mass increased by about 50% and Tc content in the cells rose from 4.30 to 11.91 mg/g DW and 4.30 to 9.91 mg/g DW under HEJ and MJ elicitation, respectively (Fig. 3C). The regulation of gene expression over the time course of

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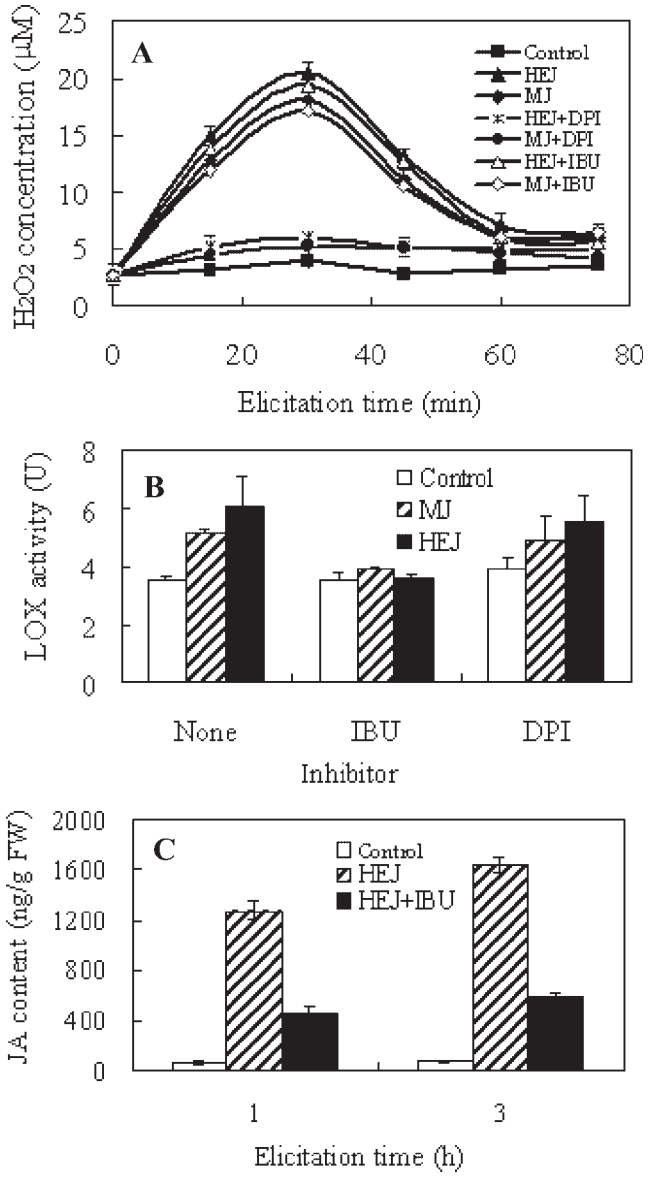

Figure 2. Effect of diphenylene iodonium (DPI, 10 μM) and ibuprofen (IBU, 100 μM) addition on HEJ-induced H_2O_2 production (**A**), LOX activity (**B**) and JA accumulation (**C**) by *T. chinensis* cells. Each inhibitor was added to the cultures 20 min before elicitation. Each data point represents the mean ± SD from three independent samples.

induction was examined by qRT-PCR. As shown in Figure 3A and B, the mRNA level of GGPPs and TS increased greatly within 6 h of elicitor treatment. With HEJ elicitation, it reached 11-fold on GGPPs and 20-fold on TS over control at 6 h, and later decreased progressively during next 6–168 h. At 168 h, the gene transcription level reduced to 4-fold on GGPPs and 15-fold on TS over control. The response of *TS* gene expression over the time course induced by MJ was almost the same as that by HEJ elicitation. However, the HEJ-induced *GGPPs* gene transcription level was higher than that of MJ-treated cells (Fig. 3A). Interestingly, compared to MJ elicitation, the HEJ induced cells with a higher GGPPs transcription level had a corresponding higher Tc content at the end of cultivation (Fig. 3C).

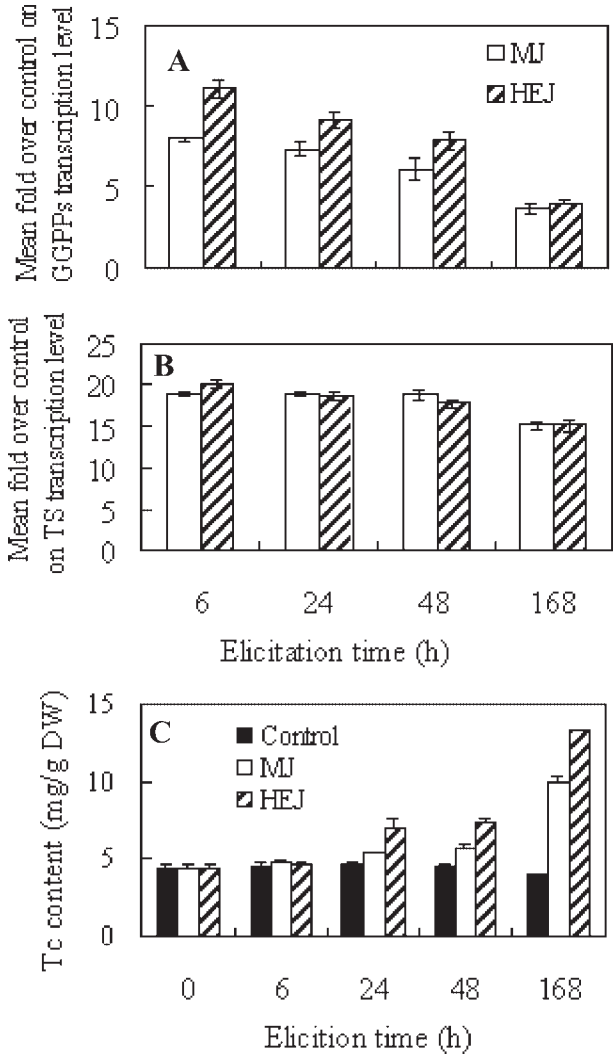

Figure 3. Mean over control fold on *GGPPs* and *TS* gene transcription levels in *T. chinensis* cells elicited with HEJ or MJ on day 7 of cultivation. An error bar in the figure indicates the mean ± SD from three independent samples.

H_2O_2 Was Not Essential to Activate Tc Biosynthetic Gene Expression in *T. chinensis*

The ROS inhibitor DPI had a significant depressing effect on H_2O_2 production induced by HEJ in *T. chinensis* cells (Fig. 2A). To test whether H_2O_2 is essential for elicitor-responsive gene expression, the effect of DPI addition on HEJ-induced *GGPPs* and *TS* gene expression was investigated. As summarized in Table I, the application of DPI to the suspended cells did not affect the expressions of both *GGPPs* and *TS* genes following the induction by HEJ at 6 h, although the HEJ-induced H_2O_2 production at 30 min and Tc production after 5 days were significantly suppressed. To evaluate whether H_2O_2 may be sufficient to induce the expression of biosynthesis genes involved in Tc production, 500 μM H_2O_2 was also added to the cell cultures. The results indicated that 30% stimulation of Tc content was observed after 5 days, but the *GGPPs* and *TS* gene expression (at 6 h) was not induced (data not shown).

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Table I. Effect of diphenylene iodonium (DPI) addition on H_2O_2 production, Tc biosynthetic gene expression and Tc production in HEJ-elicited cell cultures of *T. chinensis**.

Treatment	Signal	Gene transcription		Tc content
	H_2O_2 (μM)	GGPPs (fold)	TS (fold)	(mg/g DW)
Control	3.8 ± 0.5^a	1.0^a	1.0^a	4.15 ± 0.02^a
HEJ	22.5 ± 1.2^b	8.36 ± 0.80^b	17.74 ± 1.31^b	10.09 ± 0.41^b
DPI + HEJ	5.3 ± 0.7^c	8.42 ± 0.62^b	17.10 ± 1.0^b	5.65 ± 0.20^c

*The H_2O_2 production and genes transcription were detected at 30 min and 6 h post treatment according to the results of Figures 1 and 2 and Figure 3, respectively. Tc content was measured on day 5 after elicitation.

[a,b,c]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.

Involvement of Induced JA in Expression of *GGPPs* and *TS* Genes

In order to provide evidences whether JA acts as an intermediate in the HEJ-induced Tc biosynthetic gene expressions, cells were incubated with the octadecanoid pathway inhibitor IBU (Oikawa et al., 2001). Both JA accumulation and Tc content had a significant decrease upon IBU addition (100 μM) (Table II). qRT-PCR results showed that IBU (at 100 μM) significantly down-regulated the elicited expression levels of *GGPPs* and *TS* genes in HEJ-treated *T. chinensis* cells (Table II), while the mRNA level of family gene GADPH was not affected. Two repeated experiments showed similar data. From Table II, it is clear that inhibition of JA biosynthesis by IBU resulted in the inhibition of GGPPs and TS mRNA accumulation. The above results show that the elicitor-induced gene expression of GGPPs and TS in *T. chinensis* cells was mediated through the octadecanoid pathway, and the JA induced by HEJ was involved in the cellular Tc biosynthesis.

DISCUSSION

H_2O_2 from the elicitor-induced oxidative burst is a well-recognized early defense response in plant cell cultures (e.g., Zhao et al., 2005), which has been attributed to a membrane-bound NADPH oxidase (Aziz et al., 2004; Mithofer et al., 1997). Some chemical inhibitors of the enzyme NADPH oxidase found in mammalian neutrophils inhibit the pathogen-, elicitor-, and wound-induced accumulation of H_2O_2 derived from the oxidative burst in plants (Aziz et al., 2004; Orozco-Cárdenas et al., 2001; Levine et al., 1994). Previously the oxidative burst was detected in our HEJ-elicited cell cultures of *T. chinensis*. In this study it was confirmed with new evidences: the HEJ-induced H_2O_2 production was suppressed by the NADPH oxidase inhibitor DPI (Fig. 2A), and the H_2O_2 accumulation was also decreased with an addition of ROS scavenger NAC (data not shown). Furthermore, the Tc production induced by HEJ was simultaneously suppressed by DPI (Table I) or NAC (data not shown). The results suggest that the oxidative burst was from the HEJ induction and it was involved in the HEJ-induced Tc biosynthesis.

JA is regarded as another important defense signal in plant defense response, which has a close relationship with secondary metabolite accumulation induced by elicitors such as in cell cultures of *Oryza sativa* L, *Cupressus lusitanica* and *Panax notoginseng* (Nojiri et al., 1996; Wang et al., 2005; Zhao and Sakai, 2003). In this work, the results showed that JA was a signal mediator in the HEJ-induced taxoid biosynthesis by *T. chinensis* cells. HEJ could enhance the LOX activity and endogenous JA level. IBU, a JA biosynthetic pathway inhibitor, could significantly suppress the LOX activity, JA level and Tc production (Fig. 2 and Table II). The results suggest that the exogenously applied HEJ could greatly stimulate JA biosynthesis in *T. chinensis* cells, and the induced JA signal was critical to the over-production of Tc.

The transcription levels of both *GGPPs* and *TS* genes were examined by qRT-PCR and found to be up-regulated in response to exogenous HEJ or MJ. The results are in agreement with the observation that MJ induced significant increases of *GGPPs* and *TS* gene transcriptions in the paclitaxel biosynthesis pathway (Hefner et al., 1998). In our work, the *TS* gene expression over the time course induced by MJ was similar to that by the synthetic HEJ, whereas *GGPPs* gene transcription was induced to a higher level in the cells treated with HEJ than that by MJ (Fig. 3). Interestingly, compared to MJ, HEJ also caused stronger defense responses including higher levels of H_2O_2 production and LOX activity (Fig. 2). The results indicate that the new synthetic jasmonate had higher stimulating activity than MJ in both defense signals and Tc biosynthetic gene expressions. The information obtained here should be helpful in further understanding the superior inducing activity of new jasmonate analogs against MJ in Tc biosynthesis.

To investigate whether HEJ-induced H_2O_2 production regulated the expression of the *GGPPs* and *TS* genes, DPI was used to depress the oxidative burst by the elicitation. As seen in Figure 2A and Table I, the HEJ-induced H_2O_2 level was considerably decreased by DPI, and the HEJ up-regulation of *GGPPs* and *TS* genes transcription was not

Table II. Effect of ibuprofen (IBU) addition on HEJ-induced JA accumulation, Tc biosynthetic gene expression and Tc production in *T. chinensis* cell cultures*.

Treatment	Signal	Gene transcription		Tc content
	JA (ng/g FW)	GGPPs (fold)	TS (fold)	(mg/g DW)
Control	46.3 ± 4.3^a	1.0^a	1.0^a	4.15 ± 0.02^a
HEJ	1742 ± 30.1^b	8.36 ± 0.80^b	17.74 ± 1.31^b	10.09 ± 0.41^b
IBU + HEJ	412 ± 29.3^c	1.78 ± 0.3^c	2.25 ± 0.45^c	6.06 ± 0.20^c

*The JA level and genes transcription were detected at 3 and 6 h post treatment according to the results of Figures 1 and 3, respectively. Tc content was measured on day 5 after elicitation.

[a,b,c]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.

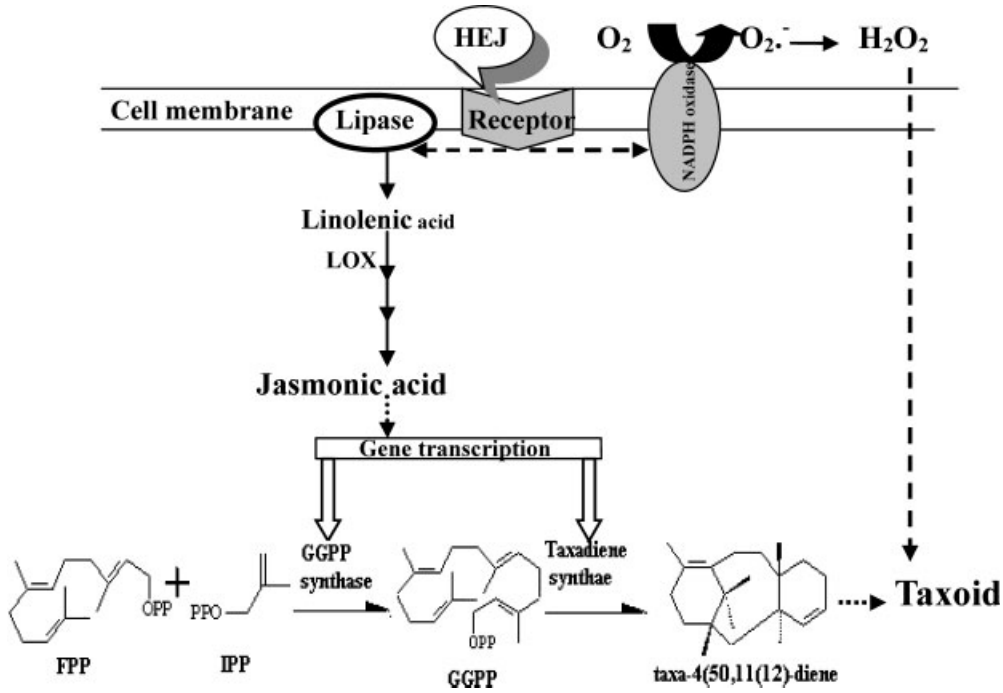

Figure 4. A proposed signal transduction cascade of *T. chinensis* cells induced by the novel elicitor HEJ. The involvement of jasmonic acid in HEJ-elicited taxoid biosynthetic gene expression and Tc production is shown. H_2O_2 signal is involved in the induced secondary metabolite biosynthesis but its targeting site is yet unclear.

inhibited (Table I). In addition, exogenous H_2O_2 was also directly added to the culture medium and it did not affect the two genes expression (data not shown). The results suggest that the HEJ-induced H_2O_2 production may be independent of the activation of *GGPPs* and *TS* gene transcription in *T. chinensis* cells. Similar to the results presented here, in soybean DPI was reported not to affect elicitor-induced chalcone synthase mRNA accumulation although the oxidative burst was inhibited simultaneously (Levine et al., 1994). Pauw et al. (2004) also showed that H_2O_2 from the oxidative burst had no effects on the activation of indole alkaloid biosynthetic gene expression by yeast extract.

For the HEJ-induced JA biosynthesis, inhibitor treatment results suggest that it was essential for enhanced transcription of *GGPPs* and *TS* genes and Tc accumulation by *T. chinensis* cells. TS activities are limited to formation of taxadiene, which is assumed as precursor of taxoids (Besumbes et al., 2005). The HEJ-elicited JA biosynthesis affected Tc production probably via induction of those two important biosynthetic genes. In tobacco cell suspensions, expression of the sesquiterpene cyclase gene was induced by a fungal elicitor but not by MJ (Rickauer et al., 1997), while the nicotine biosynthesis was elicited with jasmonate treatment (Imanishi et al., 1998). The facts reflect a general complex network from elicitor and defense signal to secondary metabolite gene expression in plant cells.

Until now, an abiotic elicitor induced-signal transduction pathway has not been well characterized by including all the information on defense signal, gene expression and secondary metabolite accumulation in plant cell cultures. In this work, based on the results of defense response, gene expression and Tc accumulation, a signal transduction cascade in *T. chinensis* cells induced by HEJ is proposed (Fig. 4). Elicitation of the expression of taxoid biosynthetic genes *GGPPs* and *TS* was mediated through the octadecanoid pathway leading to JA production. That is, the elicitor-stimulated JA production in *T. chinensis* cells was a prerequisite for secondary metabolite (i.e., Tc) genes activation and enhanced Tc accumulation. H_2O_2 signal was involved in jasmonates-induced Tc biosynthesis, but its acting site is yet unknown, which requires further investigation.

In conclusion, the work indicates that both defense signals of JA and H_2O_2 played an important role in the jasmonate derivatives-induced secondary metabolism. JA signal elicited by HEJ affected taxoid metabolism probably via induction of its biosynthetic genes, which suggests a causal relationship between early (i.e., defense signal response) and late (i.e., secondary metabolic gene activation and secondary metabolite biosynthesis) reactions of the cells to the novel abiotic elicitor HEJ. Since elicitation has been a successful strategy for enhancing the production of many bioactive secondary metabolites in plant cell/tissue cultures, the characterization of elicitor-induced defense signal response and their relationships with secondary metabolic genes expression is important to provide new insights into the elicitation mechanism as well as to have more effective strategies for over-production of desired plant secondary metabolites.

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