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Differential Induction of Protein Expression and Benzophenanthridine Alkaloid Accumulation in *Eschscholtzia californica* Suspension Cultures by Methyl Jasmonate and Yeast Extract

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Methyl jasmonate (MJ) and yeast extract (YE) induce protein expression and benzophenanthridine alkaloid accumulation in *Eschscholtzia californica* suspension cell cultures. One hundred μM MJ primarily induced dihydrosanguinarine (509.0±7.4 mg/l); 0.2 g/l YE induced sanguinarine (146.8± 3.8 mg/l) and an unknown compound. These results occur because dihydrobenzophenanthridine oxidase (DHBO) is induced by YE and not by MJ. YE and chitin (CHI) had similar effects on sanguinarine production and DHBO expression. Differential induction of secondary metabolites was shown in *E. californica* suspension cultures and the expression of proteins confirmed the metabolite results. Furthermore, treatment by various oligosaccharides helped us to understand the elicitation effect of YE in signal transduction pathways.

Keywords: Benzophenanthridine alkaloid, dihydrobenzophenanthridine oxidase, *Eschscholtzia californica*, methyl jasmonate, signal transduction, yeast extract

Methyl jasmonate (MJ) is a molecule within signal transduction pathways [38]. MJ induces defense genes and secondary metabolites in many plant species [12, 31, 35]. It induces geranylgeranyl diphosphate synthase, taxadiene synthase expression, and diterpene taxol accumulation in *Taxus canadensis* cell cultures [20], benzophenanthridine alkaloid production in *Eschscholtzia californica* cells [11], and ajmalicine production in *Catharanthus roseus* cell cultures [24]. It also enhances taxane production in suspension cultures of *Taxus chinensis* var. *mairei* [36]. In addition, octadecanoid precursors of jasmonic acid induce proteinase inhibitor gene expression in wounded tomato leaves [13].

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Elicitors of fungal origin have been employed successfully to induce a number of secondary metabolites in cell cultures; *i.e.*, cryptotanshinone in *Salvia miltiorrhiza* [6] and sesquiterpene in *Hyoscyamus muticus* [34]. Benzophenanthridine alkaloid production increases in suspension cultured *E. californica* treated with yeast extract (YE) [5]. Treatment with the fungus *Phytophthara megasperma* f. sp. *glycinea* increases the activity of phenylalanine ammonia lyase, the key enzyme induced by the elicitor, in *Glycine max* cells [15] and soybean suspension cells [9].

Cross-talk among multiple signaling pathways is an important mechanism in plant signal transduction networks [38]. MJ and fungus-derived elicitors often interact when inducing secondary metabolite accumulation. In tobacco cell cultures, elicitor treatment induces key enzymes of sesquiterpene biosynthesis, *i.e.*, sesquiterpene cyclase and aristolochene/deoxy-capsidiol hydroxylase, but MJ treatment does not [25]. In *Hyoscyamus muticus* root cultures, a fungal elicitor induces a higher level of lubin and a lower level of solavetivone production, whereas MJ primarily induces solavetivone production [34]. These results indicate that MJ and elicitors differentially regulate expression of proteins and biosynthesis of target compounds in the same plant.

This paper reports on the differential induction of protein expression and benzophenanthridine alkaloid (*i.e.*, dihydrosanguinarine and sanguinarine) accumulation by MJ and YE in *E. californica* suspension cultures, and tests several oligosaccharides derived from commercially available yeast extract (YEC) to understand the elicitation effect of YE. The effects of MJ and YE were correlated with expression of proteins as well as accumulation of secondary metabolites. We compared the influences of MJ and YE on expression of (*S*)-norcoclaurine-6-*O*-methyltransferase (6OMT), (*S*)-coclaurine *N*-methyltransferase (CNMT), 3-hydroxy-(*S*)-*N*-methylcoclaurine-4'-*O*-methyltransferase (4OMT), berberine bridge enzyme (BBE), and dihydrobenzophenanthridine

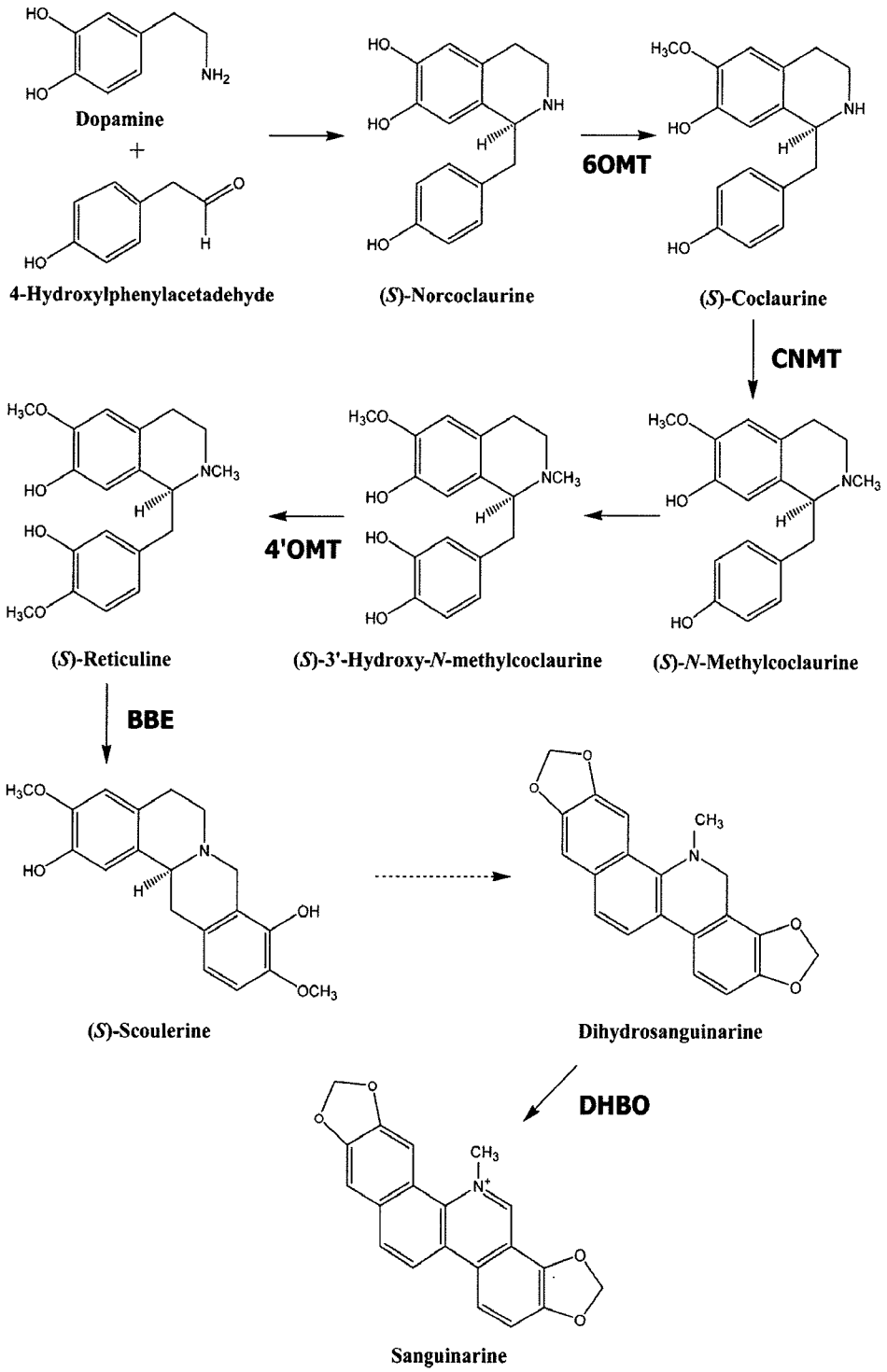

Fig. 1. Biosynthetic pathway leading to sanguinarine.
Enzyme abbreviations: 6OMT, (*S*)-norcoclaurine-6-*O*-methyltransferase; CNMT, (*S*)-coclaurine *N*-methyltransferase; 4OMT, 3'-hydroxy-(S)-*N*-methylcoclaurine-4'-*O*-methyltransferase; BBE, berberine bridge enzyme; DHBO, dihydrobenzophenanthridine oxidase.

oxidase (DHBO), which are all involved in the benzophenanthridine alkaloid pathway (Fig. 1) [7, 10, 21, 26, 33]. The results strongly indicate that MJ and YE induce different signal transduction pathways and that these pathways interact to increase metabolite production as well as protein expression in *E. californica*.

Materials and Methods

Plant Cell Line and Culture Conditions

Plant suspension cell cultures of *E. californica* were grown in 1,000-ml Erlenmeyer flasks containing 400 ml of Gamborg B5 medium [16] supplemented with 30 g/l sucrose, 0.5 mg/l 1-naphthalene acetic acid, and 1.0 mg/l 2,4-dichlorophenoxyacetic acid. These are

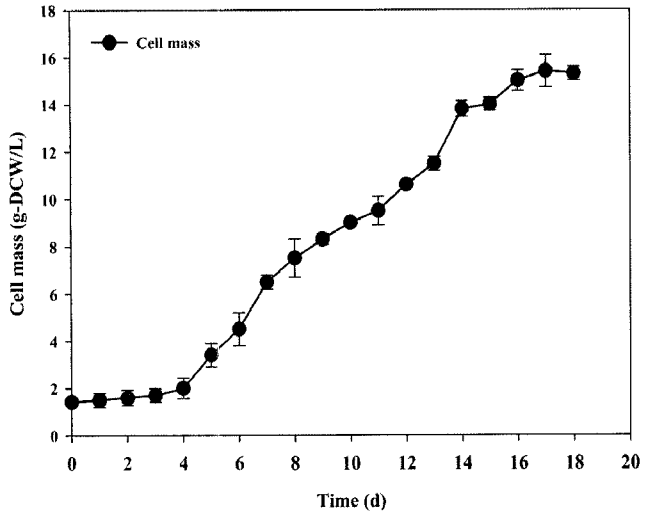

Fig. 2. Growth of *E. californica* in suspension cultures.
Data present means±standard deviations from triplicate samples in three independent experiments.

different from a previous report's cell lines [28]. The suspension was maintained on a continuous shaker (120 rpm) at 24°C, under a 16-h light period (1,000 lux). The cells were subcultured (24-ml packed cell volume in 400 ml of medium) biweekly and used for experiments on day 7. A typical growth curve of *E. californica* is shown in Fig. 2.

Elicitor Preparation

YE was isolated from YEC by ethanol precipitation [17]. The final precipitation was diluted with distilled water, autoclaved at 121°C for 15 min, and adjusted to various concentrations (Table 1). MJ (Sigma, St. Louis, U.S.A.) was dissolved in ethanol and prepared as a stock solution, which was then microfiltered (0.2 μm) and adjusted to various concentrations. YEC, chitin (CHI), β-glucan (GLU), ergosterol (ES), *N*-acetylglucosamine (N-AG), glycopeptide (GLY), and coronatine (COR) (Sigma, St. Louis, U.S.A.) were prepared according to Peltonen *et al.* [30]. Elicitors were diluted with distilled water, autoclaved for 15 min at 121°C prior to use, and adjusted to desired concentrations.

Elicitation

Each well of a 6-well plate was inoculated with 5 ml of 7-day suspension cultures. Various concentrations (Table 1) of elicitors were added to the cell cultures. The plates were incubated at 24°C, under a 16-h light period (1,000 lux) on a continuous shaker (120 rpm), and harvested at 6, 12, 24, 48, 72, 120, or 168 h after inoculation.

Table 1. Elicitors and concentrations used to test YE effect.

Elicitors	Unit	Concentration		
		1	2	3
Yeast Extract (YE)	g/l	0.02	0.2	2
Commercial Yeast Extract (YEC)	g/l	0.1	1	10
Chitin (CHI)	g/l	0.2	2	10
Glucan (GLU)	g/l	0.015	0.15	0.75
Ergosterol (ES)	g/l	0.1	1	5
N-Acetylglucosamine (N-AG)	g/l	0.2	2	10
Glycopeptide (GLY)	g/l	0.002	0.02	0.1
Methyl Jasmonate (MJ)	μM	10	100	500
Coronatine (COR)	mg/l	0.4	4	20

Analysis of Benzophenanthridine Alkaloids

Freeze-dried cell culture samples (0.01 g) were dissolved in 1 ml of a methanol solution containing 0.5% (v/v) HCl, extracted by sonication for 60 min, and centrifuged for 20 min at 15,000 ×*g*. The supernatant was collected and filtered.

Benzophenanthridine alkaloids were separated by HPLC on a C_{18} reverse-phase column (3.9×300 mm; μBondapak, Waters). The mobile phase was run at 1.0 ml/min flow rate using methanol:water [7:3 (v/v)] containing 0.2% (v/v) triethylamine. Benzophenanthridine alkaloids were detected at 284 nm with a photodiode array detector (996, Waters, Milford, MA, U.S.A.). Benzophenanthridine alkaloids were identified by their retention time and by UV spectral comparison with authentic standards of sanguinarine and dihydrosanguinarine.

Protein Extraction and Western Blot Analysis

Cells were frozen using liquid nitrogen and then mixed with ice-cold protein extraction buffer [60 mM Tris-HCl (pH 6.8), 1% (w/v) dithiothreitol (DTT), 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 10% glycerol] and homogenized. Samples were centrifuged at 15,000 ×*g* for 15 min. Protein concentration was determined using bovine serum albumin as the standard [3].

Thirty μg of protein from each sample was suspended in SDS Laemmli buffer, boiled 4 min at 95°C, and loaded on a 12% SDS-polyacrylamide gel (Mini-Protean II apparatus, Bio-Rad). After electrophoresis, proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.). Protein blots were blocked for 1 h with 5% nonfat dry milk in TBS (50 mM Tris-HCl, 150 mM NaCl) and incubated for 4 h with primary antibodies (diluted 1:1,000 in TBS), which were generated against each of the amino acid sequences [7, 10, 22, 26, 33]. After rinsing in TBS containing 0.2% Tween-20, the blots were incubated for 1 h in TBS containing goat anti-rabbit IgG conjugated to alkaline phosphatase (diluted 1:5,000 in TBS). Color development was performed in AP buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$) using the 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium liquid substrate system (Sigma, St. Louis, U.S.A.).

RESULTS AND DISCUSSION

Effects of MJ and YE on Metabolite Production

Suspension cultures were elicited on day 7 with 100 μM MJ and 0.2 g/l YE, and the cells were harvested 48 h after elicitation. The chromatograms show a difference in metabolites for untreated control cultures (Fig. 3A) and cultures treated with MJ (Fig. 3B) or YE (Fig. 3C). Only dihydrosanguinarine (R_t 32.7) was detected in control cultures. MJ induced a strong accumulation of dihydrosanguinarine but no other compounds. YE induced accumulation of sanguinarine (R_t 8.4), dihydrosanguinarine, and an unknown compound (R_t 26.3). These results suggest that MJ and YE mediate different steps in the benzophenanthridine alkaloid biosynthetic pathway.

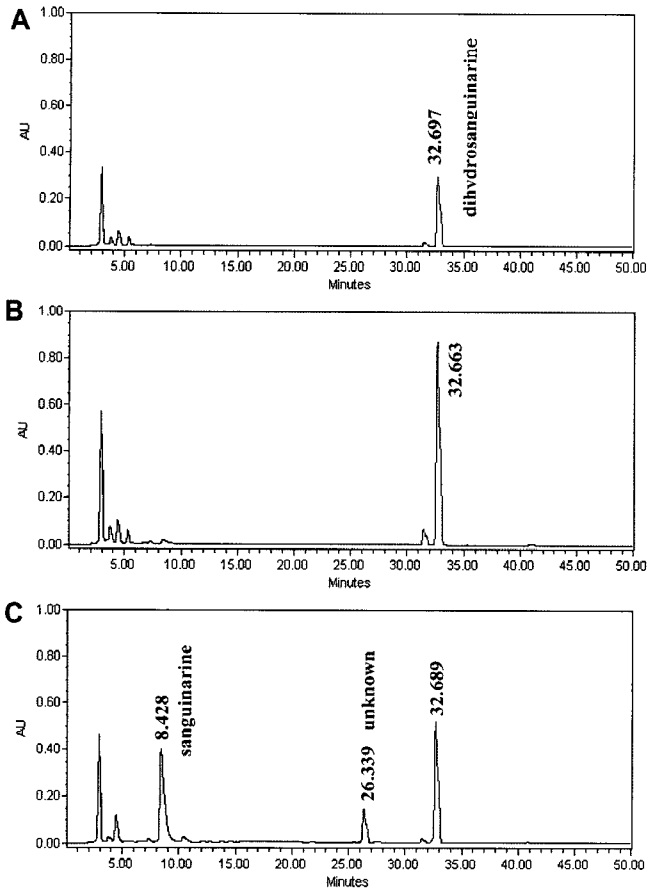

Fig. 3. Differential chromatogram profiling obtained from (**A**) control cultures, (**B**) cultures treated with 100 μM MJ, and (**C**) cultures treated with 0.2 g/l YE.
Retention times for the different compounds are shown on peaks: sanguinarine (R_t 8.4), unknown (R_t 26.3), dihydrosanguinarine (R_t 32.7).

Accumulation of dihydrosanguinarine and sanguinarine over time differed among treatments (Fig. 4). In control cultures, dihydrosanguinarine increased after 6 h, reached a maximum after 120 h (338.0±20.3 mg/l), and declined thereafter. Sanguinarine accumulation occurred after 120 h (Fig. 4A).

MJ treatment strongly elicited accumulation of dihydrosanguinarine (Fig. 4B). Dihydrosanguinarine accumulation started at 6–12 h after elicitation, increased until 72 h, and remained relatively constant afterwards. Maximum dihydrosanguinarine accumulation was 509.0± 7.4 mg/l, a 150% increase over that of the control. Sanguinarine production induced by MJ was little activated until 120 h. MJ has been previously used successfully to induce production of benzophenanthridine alkaloids in *E. californica* cell cultures [4, 11, 37]. However, those studies reported the total production of benzophenanthridine alkaloids but not separate related metabolites, and the highest total benzophenanthridine alkaloid accumulation in *E. californica* with 10–100 μM MJ was about 100 mg/l [37]. Even though this dosage of MJ was similar to ours, benzophenanthridine

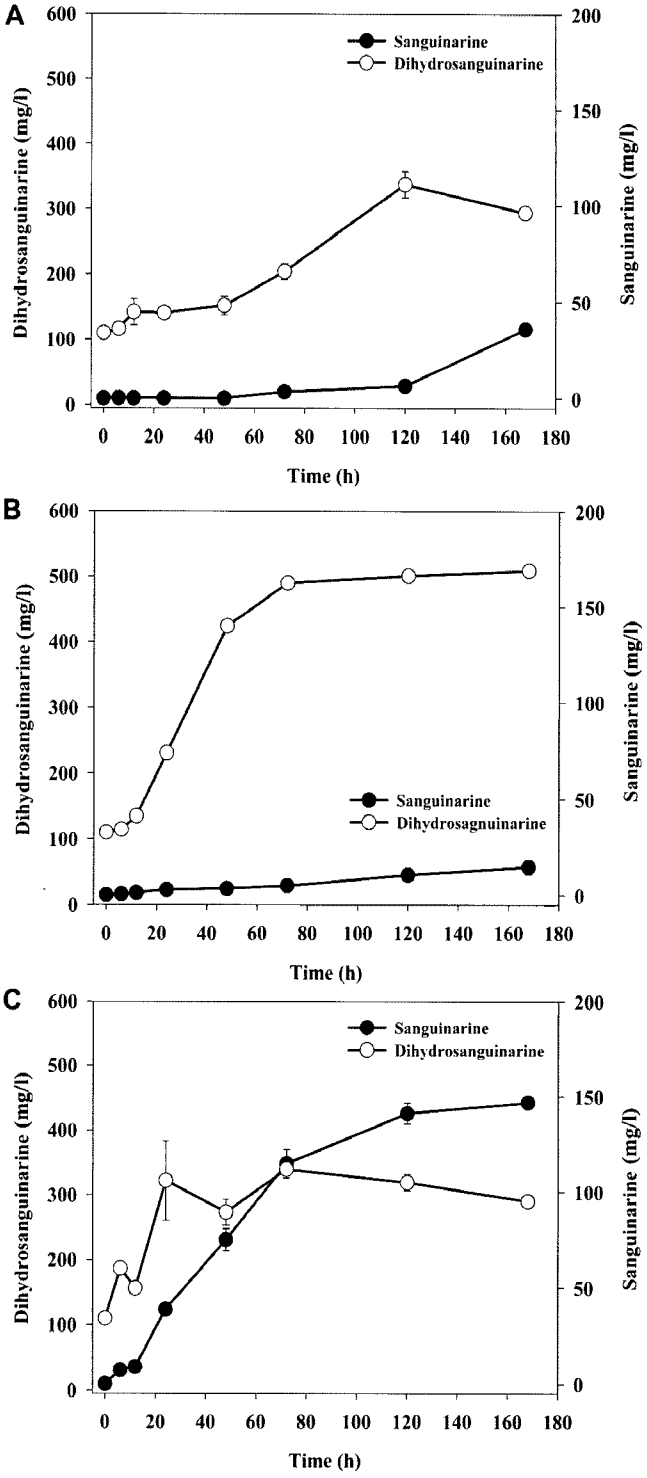

Fig. 4. Time-course of accumulation of dihydrosanguinarine (○) and sanguinarine (●) in *E. californica* suspension cultures.
Seven-day cultures were treated with (**A**) nothing, (**B**) 100 μM MJ, and (**C**) 0.2 g/l YE. Data present means±standard deviations from triplicate samples in three independent experiments.

alkaloid production was only 20% of that observed in the present study. Moreover, re-elicitation with 50 μM MJ in *E. californica* suspension cultures increased to 250 mg/l benzophenanthridine alkaloid accumulation [4].

YE treatment affected the time-course of production of dihydrosanguinarine and sanguinarine in different ways (Fig. 4C). Dihydrosanguinarine level increased immediately and exceeded that in the control until 120 h after treatment, but was similar to the control level thereafter. Sanguinarine production increased almost linearly until 72 h and reached a maximum measured accumulation (146.8±3.8 mg/l, 400% increase over that of the control) at 168 h after elicitation. Yeast elicitor has been used in suspension cultures of *E. californica* [5, 32]. Collinge and Brodelius [8] observed that the maximum level of sanguinarine occurred 6 h after yeast elicitor treatment, and that 12 h after elicitation, sanguinarine had been almost completely metabolized to other alkaloids. In contrast, in our cell cultures, sanguinarine accumulation continued for 168 h.

The signal transduction interaction between MJ and YE related to secondary metabolite production has been studied [34]. However, this is the first report that these treatments result in different time-courses of benzophenanthridine alkaloid accumulation. Differential induction of dihydrosanguinarine and sanguinarine in *E. californica* shows that MJ and YE mediate different signal transduction pathways.

Effects of MJ and YE on Protein Expression

Expression of 6OMT, CNMT, 4'OMT, BBE, and DHBO differed among the control, MJ-treated, and YE-treated cells (Fig. 5). Both MJ and YE increased expression levels of 6OMT, CNMT, 4'OMT, and BBE; the effect of MJ was less than that of YE. The accumulations of 6OMT, CNMT, and BBE were greater at 48 h than at 24 h after application of MJ or YE, but 4'OMT expression did not change during this interval.

Among these enzymes, only DHBO was induced by YE but not by MJ. YE treatment induced sanguinarine accumulation almost immediately, whereas MJ had little effect. DHBO catalyzes the synthesis of sanguinarine from dihydrosanguinarine in *E. californica* suspension cell cultures [21] (Fig. 1). This information suggests strongly that MJ and YE induce different pathways. YE is a mixture, and it clearly includes a substance or substances necessary for induction of DHBO, whereas MJ is a pure compound that does not have this capacity.

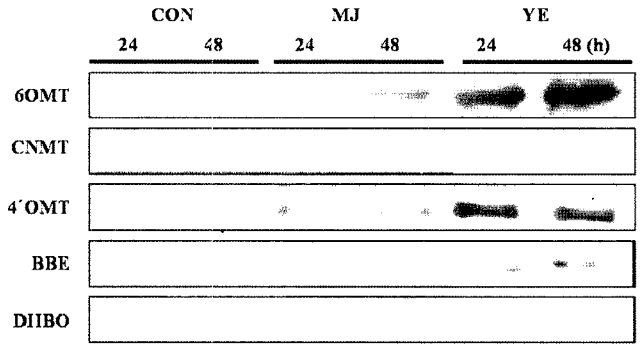

Fig. 5. Western blot analyses of 6OMT, CNMT, 4'OMT, BBE, and DHBO expressions in *E. californica* cultures treated with nothing, MJ, and YE.

One hundred μM MJ or 0.2 g/l YE was added to 7-day-old cultures. Blots are representative of three independent experiments.

MJ and YE have been used to induce expression of enzymes in this pathway (Fig. 1). Blechert *et al.* [1] observed strong induction of BBE and minimal induction of DHBO under the influence of 100 μM MJ or 0.25 g/l yeast cell wall elicitor. In contrast, our results showed strong induction of DHBO expression by 0.2 g/l YE. In the opium poppy, MJ induced lower BBE mRNA levels than did the fungal elicitor [10], as with our results. MJ has been frequently used to induce enzymes other than DHBO in the benzophenanthridine alkaloid pathway. In suspension cell cultures of *Thalictrum tuberosum*, the activities of 6OMT, CNMT, and 4'OMT in the formation of berberine were increased by MJ induction [14], and BBE transcripts were strongly induced by 100 μM MJ in *E. californica* [29].

In other plant species, MJ and YE induced different expressions of enzymes. In cultured *Glycyrrhiza glabra* cells, treatment with 100 μM MJ or 0.1% (w/v) YE increased expression of mRNA for enzymes related to soyasaponin biosynthesis. Expression of some enzymes was induced by MJ and others only by YE [19]. These results indicate that MJ and YE also have different effects on synthetic pathways in species other than *E. californica*.

Effects of Different Elicitors from YE

Various oligosaccharides derived from YEC were used as elicitors to understand which component of YE increased sanguinarine production. YE was prepared from YEC [17], and YEC contains several components that can elicit plant defense responses, including CHI, GLU, ES, N-AG, and GLY [2]. COR is an analog of the octadecanoid precursors of JA [37], so it was used to understand the dihydrosanguinarine increasing effect of MJ in *E. californica*. Table 1 shows the concentrations of elicitors used in this trial. Control cultures received no elicitor. These elicitors had different effects on the production of dihydrosanguinarine and sanguinarine in *E. californica* suspension cell cultures (Fig. 6).

Dihydrosanguinarine accumulation in the YE, YEC, and CHI treatments was similar to that in the control cultures, but decreased as the dosage increased (Fig. 6A). At a dosage of 0.2 g/l, dihydrosanguinarine accumulation was 246.3±7.0 mg/l under YE treatment and the same (*i.e.*, 250.0±24.0 mg/l) under CHI treatment. All other elicitors induced greater dihydrosanguinarine accumulation than did the control and the YE treatment, except for the highest dosage of MJ.

Sanguinarine accumulation was negligible in the control, but was measurable in all other treatments (Fig. 6B). Accumulation was greatest in the YE, YEC, and CHI

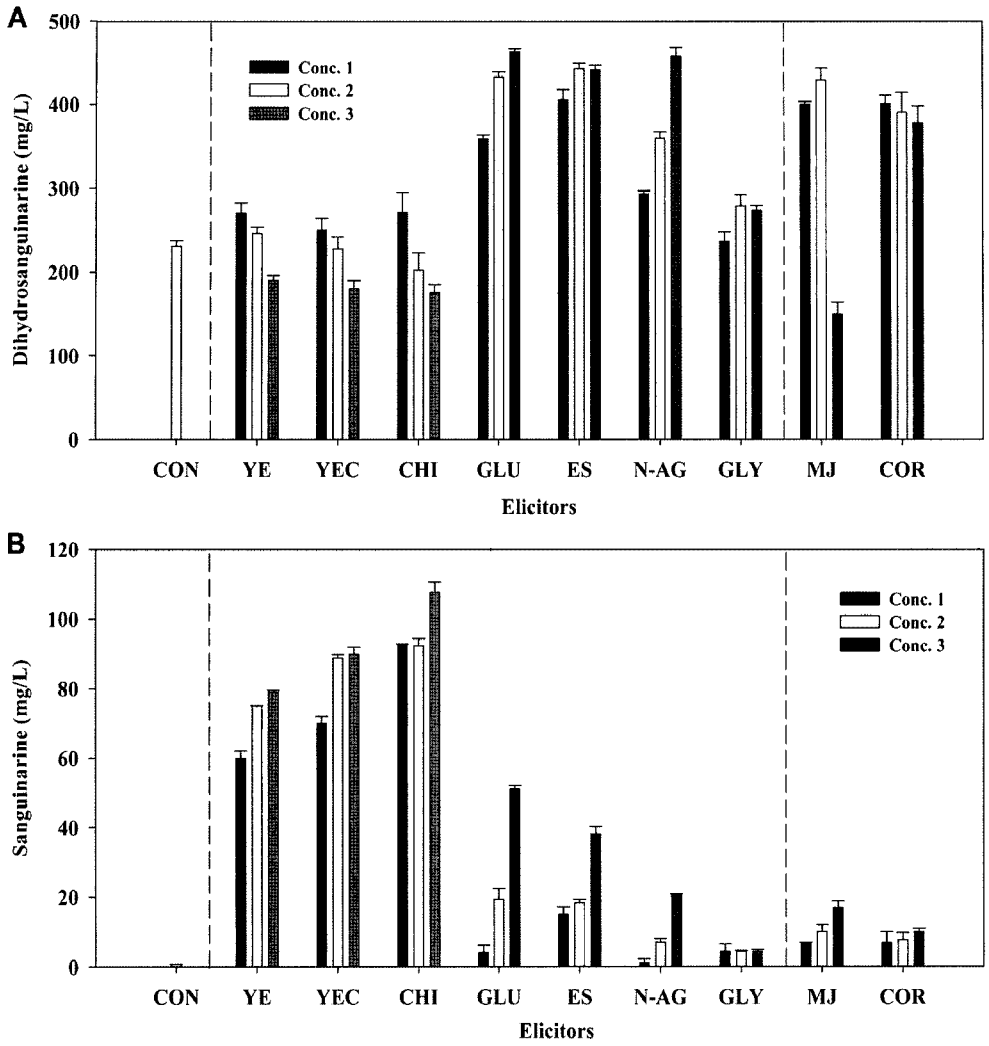

Fig. 6. Effects of different elicitors on (**A**) dihydrosanguinarine and (**B**) sanguinarine production in *E. californica* suspension cultures. The various concentrations of elicitors were added to 7-day cultures, and cells were harvested 48 h after elicitation. Data present means±standard deviations from triplicate samples in three independent experiments.

treatments, and increased with dosage. At a dosage of 0.2 g/l, sanguinarine accumulation was 74.9±0.2 mg/l under YE treatment and slightly higher (*i.e.*, 92.8±0.1 mg/l) under CHI treatment. All other treatments induced less sanguinarine accumulation than did the YE treatment.

All YEC components induced significant increases in accumulation of dihydrosanguinarine, but only CHI induced sanguinarine accumulation, exceeding that of YE. Dihydrosanguinarine accumulation decreased with increasing CHI dosage. These results strongly suggest that all of the YE components induce expression of enzymes responsible for steps leading to the synthesis of dihydrosanguinarine (Fig. 1), but that only CHI induces DHBO, which is necessary for conversion of dihydrosanguinarine to sanguinarine. GLU, ES, and N-AG induced a small amount of sanguinarine; these results suggest that these are lesser inducers than CHI. CHI has been added as an elicitor to increase the alkaloids production in *E. californica* cells [23] and plumbagin production in *Drosophyllum lusitanicum* suspension cultures [27]. COR induced dihydrosanguinarine and sanguinarine accumulation as did induction by 100 μM MJ. Other studies have also reported that COR induced benzophenanthridine alkaloid accumulation in *E. californica* [18, 37]. Contrary to our results, its ability was not as effective as MJ.

Consequently, we studied DHBO expression by 0.2 g/l YE, 1 g/l YEC, 0.2 g/l CHI, 0.75 g/l GLU, 5 g/l ES, 10 g/l N-AG, 0.02 g/l GLY, 100 μM MJ, or 4 mg/l COR (Fig. 7). CHI and YE treatments had similar effects on DHBO expression, paralleling their similar effects on sanguinarine metabolite accumulation. Forty-eight hours after elicitor treatment, the DHBO band was observed under YE, YEC, CHI, GLU, ES, and N-AG treatment. The level of DHBO expression with YE, YEC, or CHI was greater than the others, and DHBO expression was correlated with sanguinarine accumulation. GLY, MJ, and COR did not induce DHBO expression.

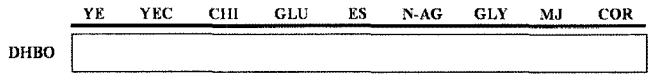

Fig. 7. Western blot analyses of DHBO in *E. californica* cultures treated with 0.2 g/l YE, 1 g/l YEC, 0.2 g/l CHI, 0.75 g/l GLU, 5 g/l ES, 10 g/l N-AG, 0.02 g/l GLY, 100 μM MJ, or 4 mg/l COR.
Blots are representative of three independent experiments.

The effect of YE on secondary metabolism is proving to be very complicated, probably because it is a mixture of numerous compounds. Our success in identifying CHI as a major component of the YE induction of sanguinarine accumulation indicates that further quantification of the effects of individual components will lead to a deeper understanding of the effects of YE.

MJ and YE had different effects on protein expression and benzophenanthridine alkaloid accumulation in *E. californica* suspension cell cultures. Using 7-day-old cultures, MJ induced the earlier metabolite (dihydrosanguinarine), whereas YE promoted conversion to the subsequent metabolite (sanguinarine) in the benzophenanthridine alkaloid biosynthetic pathway. The results suggest that these elicitors stimulate different signal transduction pathways. The metabolite results were confirmed by protein expression. Both MJ and YE increased the expressions of 6OMT, CNMT, 4'OMT, and BBE, but only YE increased DHBO expression. Of several components of YE, only CHI had comparable effects to YE on sanguinarine accumulation and on the level of DHBO expression. Our findings with *E. californica* cultures support that elicitors depending on different signal transductions induce different proteins and secondary metabolites.

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