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INVOLVEMENT OF PROTEIN KINASE AND G PROTEINS IN THE SIGNAL TRANSDUCTION OF BENZOPHENANTHRIDINE ALKALOID BIOSYNTHESIS

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Key Word Index—*Sanguinaria canadensis*; Papaveraceae; abscisic acid; calcium; benzophenanthridine alkaloids; signal transduction; protein kinase; GTP-binding proteins; tetrahydroberberine-*N*-methyltransferase; tetrahydrocoptisine-*N*-methyltransferase.

Abstract—We have previously reported that elicitor-induced benzophenanthridine alkaloid biosynthesis in suspension-cell cultures of *Sanguinaria canadensis* L. (SCP-GM) is mediated by a signal transduction system that involves calcium and possibly protein kinase(s). In this work, a number of exogenous agents were employed to further investigate the components of the signal transduction pathway involved in the induction of alkaloid biosynthesis by a fungal elicitor and abscisic acid (ABA). SCP-GM suspension-cells were treated with compounds that modify protein kinase activity, including phorbol esters, and 1-oleoyl-2-acetyl-rac-glycerol (OAG), a synthetic diacylglycerol analogue. Phorbol-12-myristate-13-acetate induced alkaloid accumulation by as much as 65-fold over control values, while the negative control, phorbol-13-monoacetate, had no effect. OAG also increased alkaloid production by approximately 25-fold as compared to controls. Likewise, pretreatment of the suspension-cell cultures with H-7 or staurosporine, significantly suppressed ABA- or fungal-induction of benzophenanthridine alkaloid biosynthesis.

Modulators of GTP-binding protein activity were also active in this system. Treatment of the suspension-cells with cholera toxin (CHX) induced alkaloid accumulation by 25-fold, which increased to 34-fold when CHX was combined with a fungal elicitor derived from *Penicillium expansum* (PE), and 32-fold when CHX was combined with ABA. Treatment of SCP-GM cells with CHX also enhanced the activities of two *N*-methyltransferases in the benzophenanthridine biosynthetic pathway namely, tetrahydroberberine-*N*-methyltransferase and tetrahydrocoptisine-*N*-methyltransferase, by six and seven fold, respectively. Furthermore, benzophenanthridine alkaloid biosynthesis was induced by treating the suspension-cells with the G-protein activators, mastoparan, mas-7 or melittin, while the inactive homologue, mas-17, did not. Suppression of alkaloid accumulation occurred when the suspension-cells were treated with GDPβS or pertussis toxin prior to treatment of the SCP-GM cells with either PE or ABA. The results support the hypothesis that one or more protein kinases, and putative G proteins are involved in the signal transduction pathway that mediates ABA and fungal-induced benzophenanthridine alkaloid biosynthesis.

INTRODUCTION

Benzophenanthridine alkaloid biosynthesis in suspension cell cultures of *Sanguinaria canadensis* L. may be induced by a variety of stimuli including fungal elicitation, hormonal manipulation, and a number of chemical stressors [1–4]. Furthermore, the induction of benzophenanthridine alkaloid biosynthesis has been demonstrated in cell cultures of other papaveraceous species, such as *Papaver bracteatum*, *P. somniferum* and *Eschscholzia californica*, in response to fungal elicitation, arachidonic acid, and polymyxin B [5–8]. The induction process is accompanied by an increase in biosynthetic enzyme activity, which is regulated at the level of transcription [5, 8]. Activation of gene transcription requires recognition of the stimulus at the plant cell surface and subsequent transduction of a signal to the nucleus [9]. A number of paradigmatic components for signaling in elicitor-induced secondary metabolism have been proposed [1, 5, 8–13], and it has become apparent that the signal transduction pathway is a complex and multicomponent system.

Previous work has demonstrated that the induction of benzophenanthridine alkaloid biosynthesis is

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mediated by a signal transduction system that involves both calcium ions and jasmonic acid [1, 5, 8]. It has further been suggested that a protein kinase(s) may also participate in the signaling process that mediates alkaloid biosynthesis [1, 8]. The involvement of protein kinase-like enzymes in other elicitor-induced responses has been previously reported. For example, a protein kinase C-like enzyme appears to be involved in the signal transduction of elicitor-induced phytoalexin synthesis in cultured carrot cells [10]. In addition, the protein kinase inhibitors, K-252a and staurosporine, suppressed some of the early responses to elicitors in tomato cells, such as medium alkalinization, ethylene biosynthesis, protein phosphorylation and the induction of phenylalanine ammonia-lyase activity [11, 12]. The involvement of a protein kinase in the induction of isoprenoid accumulation by arachidonic acid has also been reported [13]. More recently, staurosporine has been reported to inhibit elicitor-induced medium alkalinization and jasmonate formation in suspension-cell cultures of *E. californica* [8].

In 1990, Scheel and Parker proposed an hypothetical model that described the possible components involved in elicitor-mediated signal transduction [9]. This paradigm included the participation of GTP-binding proteins in the signaling pathway [9]. Since then, compelling evidence for the existence of GTP-binding proteins, and their involvement in the signal transduction pathways in higher plants has emerged [14]. Recently, there have been a few reports suggesting that G-proteins may be involved in the signal transduction of elicitor-induced responses. For example, Bolwell *et al.* have shown that cholera toxin and pertussis toxin, two potent modulators of G-protein activity, significantly enhanced the synthesis and activity of phenylalanine ammonia lyase in cultured French bean cells [15]. Furthermore, GTP-binding proteins have been implicated in early responses to elicitors, such as the rapid oxidative burst in cultured soybean cells [16], and induction of plasma membrane redox reactions [17]. However, to date, there are no reports directly associating the activation of protein kinase(s) and GTP-binding proteins with the induction of benzophenanthridine alkaloid biosynthesis.

In this work, several classical modulators of the activities of protein kinase and GTP-binding proteins in animal and plant cells were examined for their ability to induce benzophenanthridine alkaloid accumulation and biosynthetic enzyme activity in SCP-GM cells. Moreover, the effect of these modulators on fungal- and ABA-induction of benzophenanthridine alkaloid biosynthesis was investigated.

RESULTS AND DISCUSSION

Induction of benzophenanthridine alkaloid biosynthesis by ABA

ABA, a naturally occurring plant growth regulator and senescence-inducing agent, is known to modulate plant responses to adverse conditions by facilitating their adaptation to environmental stress [18, 19]. Inasmuch as the induction of secondary metabolite biosynthesis is well recognized as one of the many defense or stress-induced responses in plant cells, the effect of ABA on benzophenanthridine alkaloid biosynthesis in SCP-GM suspension-cells was tested. Dose-dependent induction of benzophenanthridine alkaloid accumulation was observed in SCP-GM suspension-cells treated with ABA (0.1–100 μM, for 24 h) (Fig. 1). Maximal alkaloid accumulation was observed in suspension-cells treated with 10 μM of ABA, increasing the concentrations of sanguinarine and chelerythrine by approximately 2.3 fold over control values. No increase in alkaloid concentrations were observed in control SCP-GM cells treated with sterile water only. ABA has been previously reported to induce indole alkaloid biosynthesis in *Catharanthus roseus* cell lines [20], and isoflavonoid biosynthesis in *Phaseolus vulgarus* [21].

The activities of two methyltransferases in the benzophenanthridine biosynthetic pathway, namely THB-NMT and THC-NMT [22, 23], were enhanced in SCP-GM cells treated with increasing concentrations of ABA (Fig. 2). Maximal induction of the activities of both *N*-methyltransferases was observed in SCP-GM cells treated with 10 μM of ABA for 16 h. No similar increase in the activity of either enzyme was observed in SCP-GM control cells treated with vehicle solvent. Induction of the activity of THB-NMT in SCP-GM cells by fungal elicitation, phosphate depletion and hormonal deprivation has previously been demonstrated [2, 3, 22, 23], and increased activity of this enzyme is directly associated with induction of benzophenanthridine alkaloid accumulation. This however, is the first report of the induction of THC-NMT activity, a novel enzyme isolated and characterized from *S. canadensis* [23], and further adds to the list of inducible enzymes in the benzophenanthridine biosynthetic pathway.

Evidence for protein kinase involvement in signal transduction

Participation of a protein kinase in the signal transduction pathway that mediates the induction of benzophenanthridine alkaloid biosynthesis in both *S. canadensis* and *E. californica* has been suggested [1, 2, 8]. To more fully investigate this hypothesis, SCP-GM suspension-cells were treated with staurosporine (1 μM) or H-7 (100 μM) for 1 h, and then PE-elicitor (35 μg/ml) or ABA (10 μM) for a further 24 h. Both staurosporine and H-7 suppressed PE-induced benzophenanthridine alkaloid accumulation by 55% and 42% respectively, and ABA-induced accumulation by 65% and 48%, respectively (Fig. 3a and b). ABA-induction of benzophenanthridine biosynthesis was also partially inhibited by treating the SCP-GM suspension-cells with EGTA and verapamil (Fig. 3a) suggesting that the process was calcium-dependent. Like-

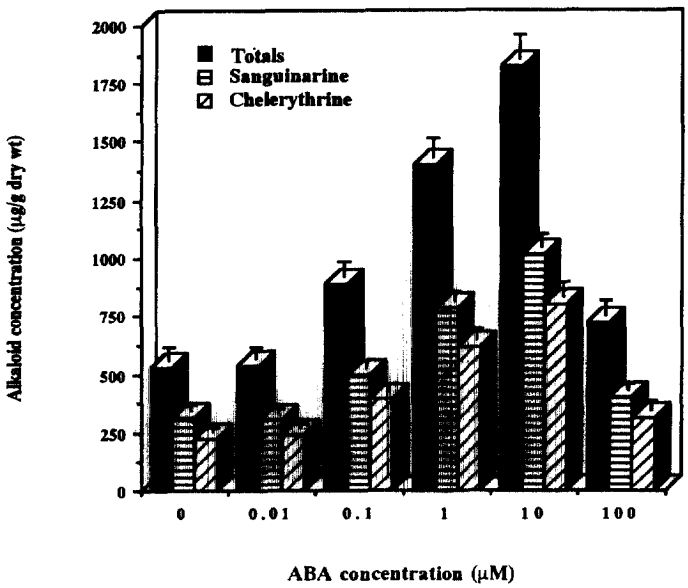

Fig. 1. Dose-dependent induction of benzophenanthridine alkaloid accumulation in suspension-cell cultures of *Sanguinaria canadensis* by abscisic acid. The points represent the mean ± s.e.m. (vertical bars, $n = 9$. $P < 0.05$).

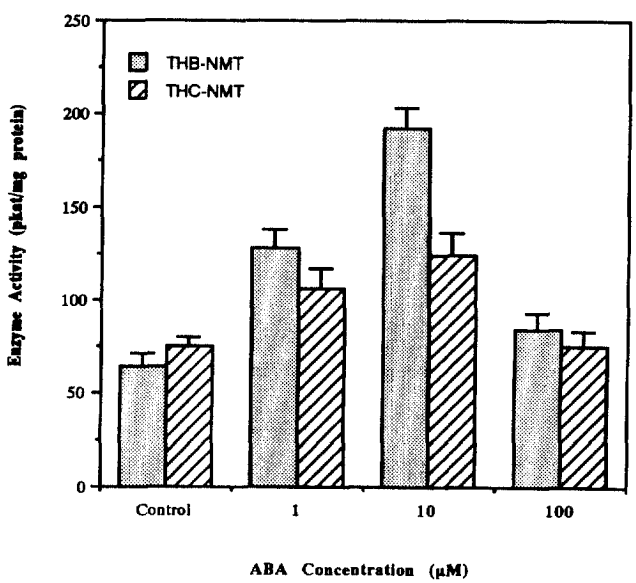

Fig. 2. Increase of SAM: tetrahydroberberine- and SAM: tetrahydrocoptisine-*N*-methyltransferase (THB-NMT and THC-NMT) activities in suspension-cell cultures treated with varying concentrations of abscisic acid (1, 10, 100 μM). Data are shown as mean ± standard error ($n = 9$).

wise, the activities of both THB-NMT and THC-NMT were significantly suppressed when the suspension-cells were pretreated with either EGTA, or EGTA plus staurosporine (2 h), and then ABA (10 μM, 16 h) (Fig 4). The results of these and previous experiments [1] suggest that induction of benzophenanthridine alkaloid accumulation and biosynthetic enzyme activity by both ABA and fungal elicitation is dependent on both the presence of calcium and protein kinase(s). Both staurosporine and H-7 have been effectively utilized in similar concentrations used in this study to ascertain the involvement of protein kinases in the signaling pathway that leads to the activation of defense-related responses in plant cells [10, 13, 24, 25].

Dose-dependent induction of benzophenanthridine alkaloid biosynthesis was observed in SCP-GM suspension-cells treated with phorbol-12-myristate 13-acetate (PMA), a potent activator of protein kinase C [26] (Fig. 5). Maximal benzophenanthridine alkaloid accumulation (up to 65-fold) was observed in suspension-cells treated with PMA (1 μM). In contrast,

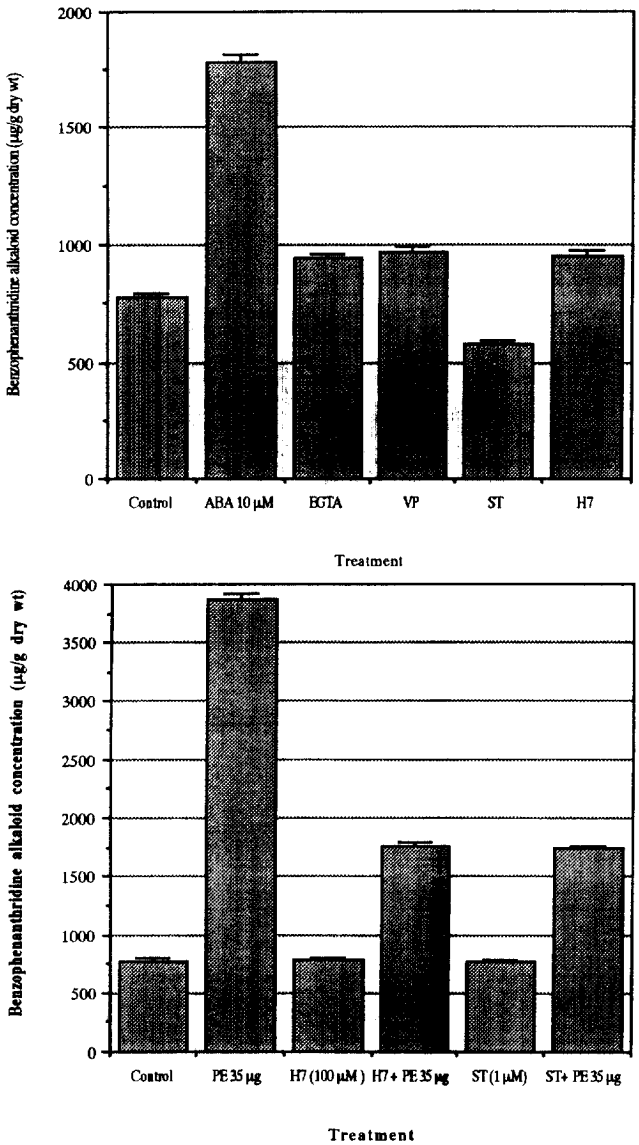

Fig. 3. (a) Effect of inhibitors on ABA-induced benzophenanthridine alkaloid biosynthesis in suspension-cell cultures of *Sanguinaria canadensis*. EGTA, verapamil (VP), staurosporine (ST) and H7. Cells were treated with inhibitors for 2 h prior to the addition of ABA (10 μM, 24 h). (b) Effect of protein kinase inhibitors, H-7 and staurosporine (ST) on elicitor (PE)-induced benzophenanthridine alkaloid biosynthesis in suspension-cell cultures of *Sanguinaria canadensis*. Negative controls were treated with vehicle solvent only. Positive controls were treated with PE 35 μg/ml or H-7 100 μM or ST 1 μM. Alkaloid biosynthesis represents the combination of sanguinarine and chelerythrine. The points represent the mean $\pm$ s.e.m. (vertical bars, $n = 9$, $P < 0.05$).

neither phorbol 13-monoacetate or 4-α-phorbol-12-myristate 13-acetate, derivatives of phorbol that do not activate protein kinase, induced alkaloid accumulation at concentrations up to 10 μM (Fig. 5). In further experiments, OAG, a synthetic diacylglycerol analogue that activates protein kinase C [26], induced benzophenanthridine alkaloid biosynthesis, in a dose-dependent manner at concentrations between 10–100 μM (Fig. 5), but was less effective than phorbol-12-myristate 13-acetate. The concentrations of phorbols and OAG used in these experiments were similar to those previously used to implicate protein kinase activity in plant cells [10]. Taken together, these data indicate that a protein kinase(s) forms part of the signal transduction pathway that mediates the induction benzophenanthridine alkaloid biosynthesis by ABA and the fungal elicitor.

Evidence for GTP-binding protein involvement in signal transduction

G-proteins are members of a large family of guanine nucleotide binding proteins found in all eukaryotes and mediate signal responses in a variety of pathways

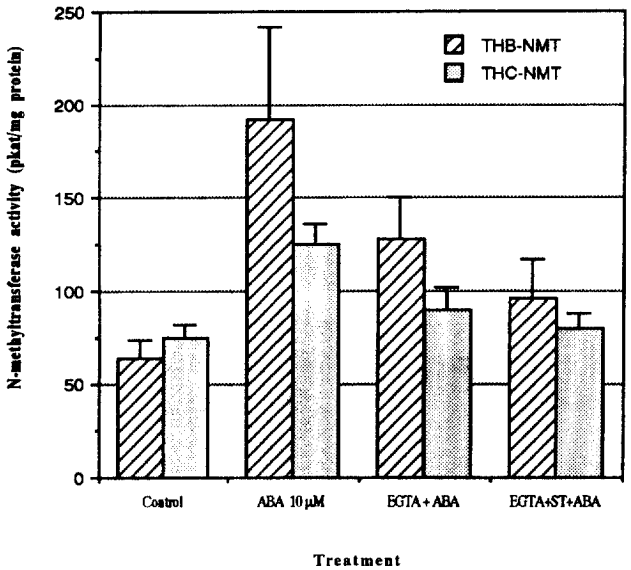

Fig. 4. Effect of EGTA and staurosporine on the ABA-induced activities of SAM: tetrahydroberberine- (THB-NMT) and SAM: tetrahydrocoptisine-*N*-methyltransferase (THC-NMT) biosynthesis in suspension-cell cultures of *Sanguinaria canadensis*. The points represent the mean ± s.e.m. (vertical bars, $n = 9$, $P < 0.05$).

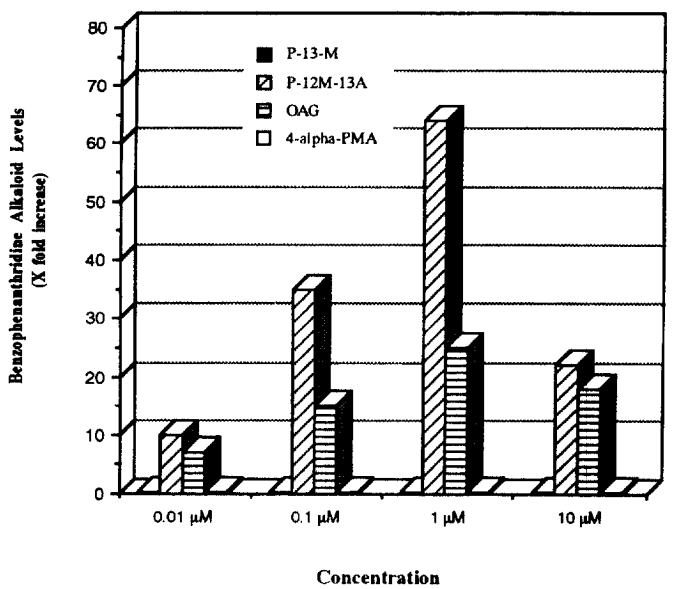

Fig. 5. Phorbol ester-induction of benzophenanthridine alkaloid accumulation in suspension-cell cultures of *Sanguinaria canadensis*. Phorbol-12-myristate 13 acetate (P-12M-13A), phorbol-13-monoacetate, (P-13-M) 4α phorbol-12-myristate 13-actetate (4-PMA) and 1-oleoyl-2-acetyl-rac-glycerol (OAG). Alkaloid biosynthesis represents the combination of sanguinarine and chelerythrine.

[14]. The involvement of GTP-binding proteins in the signal transduction pathway that mediates benzophenanthridine alkaloid biosynthesis was determined by treating SCP-GM suspension-cells with a series of specific agonist/antagonists of G proteins. The SCP-GM suspension-cell cultures were first treated with a series of guanidine nucleotide analogs. GTP(γ)S is a non-hydrolyzable GTP analog that directly activates G-proteins by eliminating the requirement for receptor-mediated activation [27], and has been used successfully to demonstrate the participation of GTP-binding proteins in plant cell functions [14]. Treatment of the SCP-GM suspension cells with GTP(γ)S (250 μM) induced the accumulation of benzophenanthridine alkaloids by approximately 3.5 fold over controls after 24 h (Fig. 6). An additive effect (approximately 10-fold) was observed when SCP-GM cells were treated with both GTP(γ)S (250 μM) and

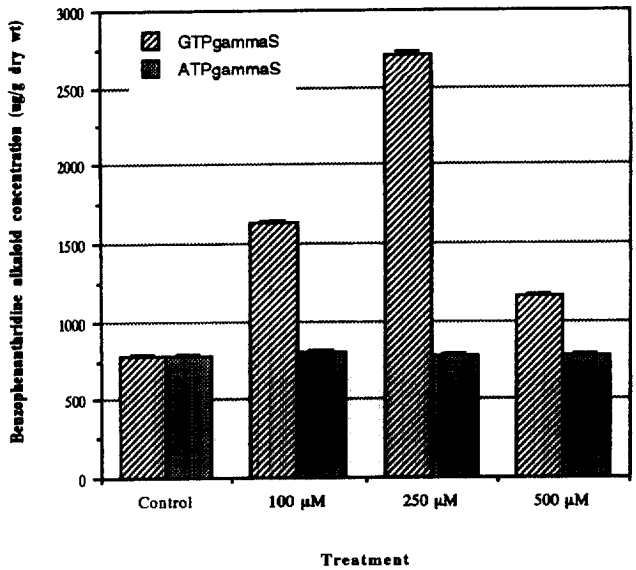

Fig. 6. Effect of GTPγS, and ATPγS on benzophenanthridine alkaloid biosynthesis in suspension-cell cultures of *Sanguinaria canadensis*. Data are shown as mean ± standard error ($n = 9$).

PE (35 µg/ml) (data not shown). Induction of benzophenanthridine alkaloid biosynthesis was not observed in SCP-GM suspension cells treated with similar concentrations of ATP(γ)S, thereby demonstrating nucleotide specificity.

In the next set of experiments, the suspension cells were treated with cholera toxin (CHX), a potent agonist of G protein which acts by preventing GTP hydrolysis. CHX has been shown to ADP-ribosylate proteins of the same molecular weight as common G-proteins in the monocot *Lemna* [28] and in *Pisum sativum* [29]. Treatment of the SCP-GM cells with the A subunit of cholera toxin (1 µg/ml, 24 h), induced benzophenanthridine alkaloid biosynthesis by approximately 25-fold over control. Alkaloid biosynthesis increased by approximately 34-fold when the cells were treated with a combination of CHX (1 µg/ml) and PE-elicitor (35 µg/ml), thereby demonstrating an additive effect (Fig. 7). Likewise, when SCP-GM suspension-cells were treated with CHX (1 µg/ml) in combination with ABA (10 µM), benzophenanthridine alkaloid accumulation increased 32-

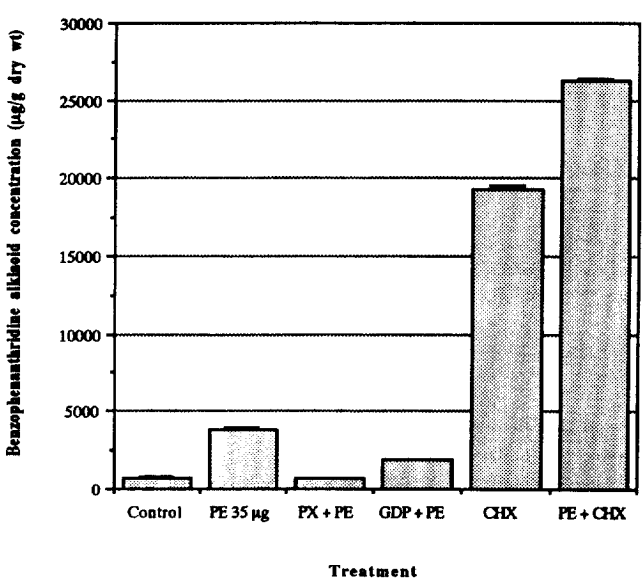

Fig. 7. Effects of GDPβS, cholera toxin (CHX), and pertussis toxin (PX) on elicitor (PE)-induced benzophenanthridine alkaloid biosynthesis in suspension-cell cultures of *Sanguinaria canadensis*. The points represent the mean ± s.e.m. (vertical bars, $n = 9$, $P < 0.05$).

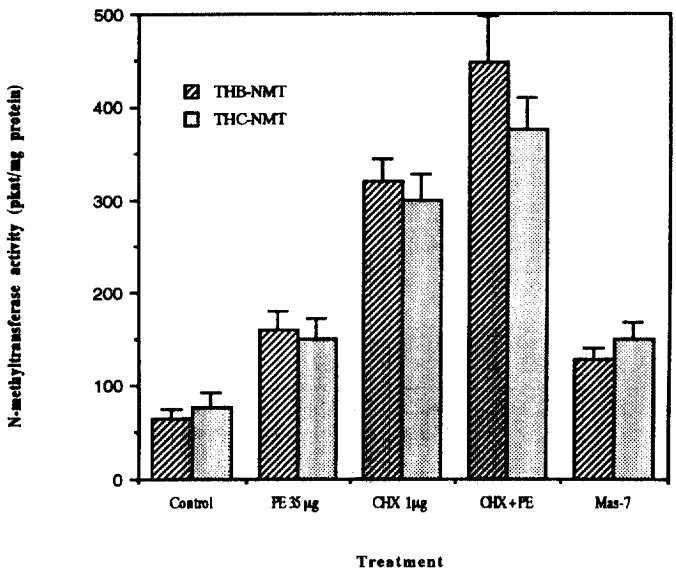

Fig. 8. Induction of the activities of tetrahydroberberine-*N*-methyltransferase (THB-NMT) and tetrahydrocoptisine-*N*-methyltransferase (THC-NMT) in suspension-cell cultures of *Sanguinaria canadensis* by cholera toxin (CHX), PE-elicitor and MAS-7.

fold over controls (data not shown). Moreover, the activities of both THB-NMT and THC-NMT were increased by approximately five fold in SCP-GM cells treated with CHX (1 µg/ml) alone, up to 7-fold when CHX (1 µg/ml) was combined with PE (35 µg/ml), (Fig. 8) and six and seven-fold respectively, when CHX (1 µg/ml) was combined with ABA (10 µM).

In related experiments, mastoparan (MP) and a series of related synthetic analogs (Mas-7 and Mas-17), which are cationic, amphipathic tetradecapeptides, and useful as cellular probes of G protein function [31, 32] were tested in this system. Mastoparan is a 14-residue oligopeptide from wasp venom and activates G-proteins by promoting nucleotide exchange by the G protein through a mechanism similar to that of the native receptors [21]. The ability of these peptides to activate G-proteins is related to their charge, hydrophobicity, and ability to form alpha helices in lipid bilayers [31]. Mas-7 is more hydrophobic and has a greater tendency to form an alpha helix than mastoparan, thereby making it more effective in activating G-proteins. In contrast, Mas-17 is less hydrophobic and has less of a tendency to form alpha helixes, therefore it does not activate G protein and thus can be used as a negative control [31]. Mastoparan can freely penetrate intact plant cells without an exogenous delivery system within minutes because of its low molecular weight and hydrophobic character [16, 17]. Addition of mastoparan (5 µM, 24 h) induced a 2-fold accumulation of the benzophenanthridine alkaloids (Fig. 9). However, Mas-7, the more potent synthetic mastoparan analog, induced a 4-fold increase in benzophenanthridine alkaloid accumulation at concentrations of 1 µM (Fig. 9). Furthermore, Mas-7 enhanced the activity of THB-NMT and THC-NMT (2 and 1.2-fold above control, respectively, Fig. 8). To demonstrate that the activity of the mastoparan analogs was not due to non-specific effects, the suspension-cell cultures were treated with the inactive analogue, Mas-17, that differs from Mas-7 by only one amino acid. Mas-17 did not induce alkaloid accumulation (Fig. 9), or biosynthetic enzyme activity in SCP-GM suspension-cells at any of the concentrations tested. Mastoparan analogues have been used successfully to demonstrate G-protein activation in plant cells at concentrations similar to those used in the current investigation. The half-maximal stimulation ($\sim$2.5 µM) was similar to that found for the mastoparan activation of the oxidative burst in soybean cells ($\sim$2 µM) [16]. The difference in the activities of mastoparan, Mas-7 and Mas-17 thus appears to be structure related.

In addition to the mastoparan analogues, melittin, an amphiphilic peptide reported to activate G proteins with a potency and efficacy similar to that of mastoparan [31], also enhanced benzophenanthridine alkaloid production in a dose-dependent manner to a maximum of 3.5 fold at 1 µM (Fig. 9).

Finally, SCP-GM cells were treated with GDPβS and pertussis toxin, both antagonists of GTP-binding proteins, to determine if the induction of benzophenanthridine alkaloid accumulation could be suppressed. GDPβS, blocks the activation of G proteins by competitively inhibiting GTP binding, and the A subunit of pertussis toxin, inactivates heterotrimeric GTP-binding proteins by uncoupling the receptor from its G protein [14]. SCP-GM suspension-cells were pretreated with GDPβS (100 µM, 4 h), and then with either the PE elicitor (35 µg/ml) or ABA (10 µM) for a further 24 h. In either case, GDPβS pretreatment

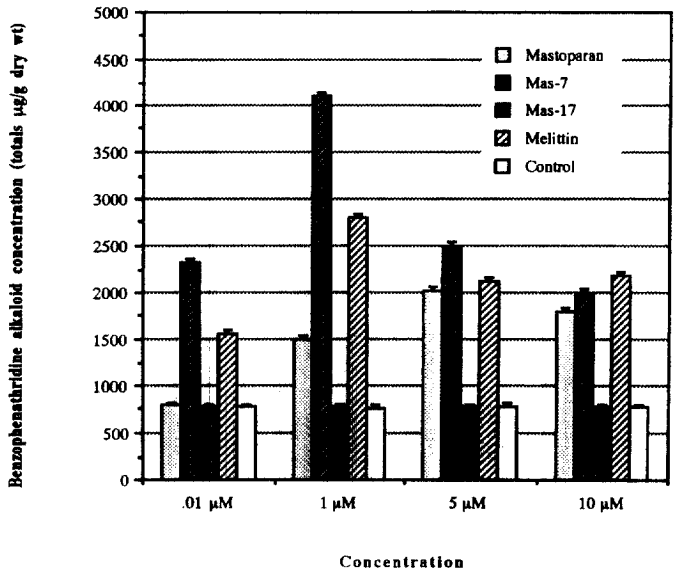

Fig. 9. Effects of mastoparan analogues and melittin on benzophenanthridine alkaloid biosynthesis. Control cells were treated with vehicle solvent only.

of the suspension-cells suppressed alkaloid accumulation by approximately 50% (Fig. 7). Conversely, treatment of SCP-GM suspension-cells with ADPβS failed to suppress the induction of benzophenanthridine alkaloid biosynthesis by ABA or the PE elicitor. Furthermore, when SCP-GM cells were preincubated with 1 µg/ml of pertussis toxin subunit A for 4 h and then treated with ABA or the PE-elicitor for a further 24 h, alkaloid biosynthesis was suppressed by 90 to 97%, respectively.

Data present in this study demonstrate that both ABA and a fungal elicitor can induce benzophenanthridine alkaloid accumulation and biosynthetic enzyme activity. The signal transduction system that mediates both processes appears to be quite similar. Previous studies have demonstrated that both fungal elicitors and ABA can induce wound and other stress signaling pathways, of which calcium ions, protein kinase and jasmonic acid may participate [1, 8, 35]. The data presented in this study is supportive of these works, and further suggests that along with calcium ions and jasmonic acid, the activation of a protein kinase(s) and putative GTP-binding proteins play a role in the signal transduction pathway that mediates the induction of benzophenanthridine alkaloid biosynthesis by ABA and fungal elicitors.

EXPERIMENTAL

Chemicals

Staurosporine, H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride), 1-oleoyl-2-acetyl-rac-glycerol (OAG), cholera toxin A subunit, pertussis toxin, phorbol 12-myristate 13-acetate, phorbol 13-monoacetate, 4α-phorbol 12-myristate 13-acetate, GTPγS, GDPβS and (±)ABA [*cis-trans* isomer] were purchased from Sigma Chemical Co., St Louis, MO. Mastoparan, mas-7, mas-17 and mellitin were obtained from Peninsula Laboratories, Inc., Belmont, CA. Radiolabeled *S*-adenosyl methionine (SAM)-[methyl-^{3}H] was obtained from ICN Biomedicals, Inc., Irvine, CA. Tetrahydroberberine was produced by sodium cyanoborohydride reduction of their quaternary equivalents prior to use. L-Tetrahydrocoptisine was kindly supplied by Prof. Shouxun Zhao, Division of Phytochemistry of China Pharmaceutical University, Beijing, China and Hélène Guinaudeau, University of Angiers, Angiers, France.

Cell cultures

Suspension cell cultures of *S. canadensis* (SCP-GM) were grown and subcultured on Gamborg B5 medium [34] supplemented with 2,4-D (0.5 mg/l) and sucrose (30 g/l) as previously described [1]. The cultures were maintained in 1 l roller bottles on a Wheaton Cell Production system (40 rpm), in walk-in environmental chambers, under fluorescent illumination (2000 lx, 12 h/day, 23°C ± 2°C). These cell lines produce the benzophenanthridine alkaloids only during the stationary phase of the cell growth cycle or upon induction [1, 3].

Elicitor preparation

The fungus *Penicillium expansum* (PE) Link. (Hyphomycetes) was grown on a LB liquid nutrient broth and harvested after 5 days of growth as previously described [2]. Harvested mycelium was collected by filtration, washed with deionized water, autoclaved for 30 min and then homogenized in a

0.1 M sodium acetate buffer, pH 5.5 (1:1 ratio). The homogenate was centrifuged (10,000 × *g*, 15 min), and the supernatant was decanted. Glucose equivalents of the filtrate were measured by the protocol described by Dubowski [36]. The extract was standardized by diluting the filtrate to 35 μg Glc equivalents per 100 μl with 0.1 M sodium acetate buffer and this solution was used as the PE-elicitor extract.

The SCP-GM suspension-cells were maintained on a 14 day growth and subculture schedule. Within this time frame, induction was initiated by the addition of 35 μg Glc equ/ ml of the PE-elicitor to the cell cultures in log-phase growth, as was previously described [1].

Treatment protocols

SCP-GM suspension cells were aseptically harvested and 0.1 g fr. wt ml^{-1} was added to each well of 24-well culture dishes containing 1 ml of freshly harvested sterile medium [1, 2]. (±)ABA [*cis-trans* isomer] was dissolved in sterile distilled water, filter-sterilized (0.22 μm, Millipore filter) and concentrations ranging from 0.1–100 μM (10 μl volume) were added to each well. Control cells received 1 ml of sterile medium and 10 μl filter-sterilized distilled water. SCP-GM suspension-cells were treated with staurosporine (1 μM) or H-7 (100 μM) for 2 h prior to the addition of 10 μM ABA. Negative control samples received either vehicle solvent, staurosporine or H-7 alone. For the ABA experiments, positive controls received ABA (10 μM). Other test compounds were added in 10 μl aliquots of freshly prepared stock solutions and control cells received either vehicle solvent alone or the agonist/antagonist only. Positive control cells received the PE-elicitor alone, and all experiments were performed in triplicate. The culture dishes were sealed to prevent evaporation and placed on an orbital rotary shaker (100 rpm, 23) for 24 h.

The protein kinase inhibitor, staurosporine, was prepared as a 1 mM stock solution in DMSO as described by Choi *et al.* [13]. Suspension-cells were treated with staurosporine in concentrations ranging from 0.01 to 10 μM. Control cells received vehicle solvent containing 1% DMSO. The SCP-GM suspension-cells were treated with the PE-elicitor as described previously [1]. All comparisons of inhibitor effects were made with respect to appropriate vehicle solvent controls. Phorbol esters and OAG were prepared in ethanol, and control cells received an equal volume of vehicle solvent. Stock solutions of cholera toxin A subunit (0.5 mg/ml) and pertussin toxin (0.05 mg/ml) were prepared in sterile water. Treated cells were incubated for 24 h in a shaker bath at 23 and then harvested and extracted as described [1].

Mastoparan, mas-7 and mas-17 stock solutions were prepared using sterile deionized water. An equivalent volume of deionized water was added as a control. After treatment, the medium was removed and the cells were harvested and the cells were analyzed for alkaloid content. For the enzyme experiments, 1 g of SCP-GM cells were added to 100 ml of medium in 1 l roller bottles, to which the test compounds were added. The cells were incubated as described above and the harvested at 16 h.

All experiments were performed in triplicate, and the data generated the result of at least three separate experiments. Statistical analysis was performed by using the Student's *t*-test and a probability of less than 0.05 was considered statistically significant.

Enzyme extraction and assay

The procedure for extraction of THB-NMT was as described by O'Keefe and Beecher [22]. Briefly, freshly harvested *S. canadensis* suspension cells were homogenized in a 50 mM sodium phosphate buffer (pH 7.5) containing 3 mM dithiothreitol, 5 mM $MgCl_2$, and 1% glycerol with a Beckman Polytron homogenizer and the homogenate was centrifuged (12,000 *g*, 15 min). Following centrifugation, the supernatant was precipitated in 75% ammonium sulfate and then centrifuged (20,000 *g*, 15 min). The protein pellet was brought up in a 10 mM sodium phosphate buffer (containing 3 mM dithiothreitol (DTT), 5 mM $MgCl_2$, and 1% glycerol, pH 7.5), concentrated and desalted by ultrafiltration in a YM-10 concentrator (Amicon Corp., Danvers, MA). The retentate was used in the enzyme assay.

The activities of the *N*-methyltransferases were assayed as described [22]. A standard assay solution consisting of 0.2 mM tetrahydroberberine (THB) or L-tetrahydrocopitisine, 0.28 μM *S*-adenosyl-L-methionine [^{3}H-methyl] (9.5 mCi $mmol^{-1}$), 3 mM DTT, 1% glycerol and 50 mM sodium phosphate buffer (pH 7.0) in a total volume of 100 μl was used. The reaction was initiated by the addition of 20 μl of enzyme, incubated for 1 h at ambient temperature. The reaction was stopped by the addition of 100 μl of saturated sodium chloride solution, and the reaction products were isolated by extraction in 3 ml of chloroform, followed by mechanical agitation and centrifugation (3000 *g*, 1 min). Following centrifugation, 100 μl of the organic phase was placed in a scintillation vial and subjected to liquid scintillation spectroscopy in a Beckman LS 5801 scintillation counter. Boiled enzyme served as the controls. Protein concentrations were determined by colorimetric assay using Coomassie Brilliant Blue system with BSA as a standard [37].

Alkaloid extraction and analysis

Harvested cells were briefly rinsed with 1 ml of 70% EtOH (60 s), and then the cells were extracted in acidified EtOH using the protocol described previously [2]. For quantitative determinations, the benzophenanthridine alkaloid concentrations were measured by HPLC on a Waters HPLC system including a WatersTM 717 plus Autosampler, WatersTM 486 Tunable Absorbance Detector and Waters 510

pumps. Briefly, *S. canadensis* suspension-cells were aseptically harvested by filtration and weighed. The fresh cells were extracted and applied to a cyano Prep-Sep column (Fisher Chemical Co., Itasca, IL), the columns were eluted with methanol, the eluant filtered through a Gelman Acro LC13 HPLC filter and the volume adjusted to 1 ml in a volumetric flask prior to HPLC analysis. Quantification was performed at ambient temperature on a 25 cm × 4.6 mm, C18 reversed phase column. The isocratic solvent system used was as described by Hashimoto *et al.* [38]. The column effluent (2 ml/min) was monitored at 284 nm. The spectrum of benzophenanthridine alkaloids was also monitored by HPLC/MS as previously described [3].

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