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TEMPORAL CORRELATION OF TYRAMINE METABOLISM WITH ALKALOID AND AMIDE BIOSYNTHESIS IN ELICITED OPIUM POPPY CELL CULTURES[1]

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

PETER J. FACCHINI

Department of Biological Sciences, University of Calgary, 2500 University Drive N.W., Calgary, Alta. Canada T2N 1N4

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Key Word Index—berberine bridge enzyme; dopamine; hydroxycinnamic acid amides; opium poppy; *Papaver somniferum*; Papaveraceae; peroxidase; sanguinarine; tyramine; tyramine hydroxycinnamoyl transferase; tyrosine/dopa decarboxylase

Abstract—Aromatic amines are involved in the biosynthesis of many plant secondary metabolites, including benzylisoquinoline alkaloids and hydroxycinnamic acid amides. Incorporation of tyramine and dopamine into both the antibiotic alkaloid sanguinarine and hydroxycinnamic acid amides was shown to occur in elicitor-treated opium poppy cell cultures. A temporal correlation was demonstrated between the biosynthesis of alkaloids and amides, the accumulation of tyrosine/dopa decarboxylase (TYDC) and berberine bridge enzyme (BBE) mRNAs, and the induction of tyramine hydroxycinnamoyl transferase (THT) activity. The induction of TYDC mRNAs occurred most rapidly, whereas the subsequent activation of BBE mRNAs and THT activity correlated with the synthesis and accumulation of sanguinarine and amides, respectively. In pulse-labeling experiments, a maximum of 1.2 and 0.6% of exogenous ^{14}C-tyramine and ^{14}C-dopamine, respectively, was incorporated into sanguinarine after elicitor treatment of cell cultures. In contrast, a maximum of 24 and 35% of exogenous ^{14}C-tyramine and ^{14}C-dopamine, respectively, was incorporated into an acid-insoluble fraction of the cell wall after elicitor treatment. Insolubilized radioactivity was released from cell walls after hydrolytic extraction and recovered mostly as hydroxycinnamic acid amides and free amines. The elicitor-induced incorporation of ^{14}C-amines into both sanguinarine and cell walls decreased during the cell culture growth cycle. The prolonged induction of THT activity in elicited cultures, coupled with high and constitutive peroxidase activity, suggests that the observed decrease in cell wall labeling 5 h after the addition of elicitor was due to hydroxycinnamoyl-CoA or H_2O_2 limitation.

INTRODUCTION

Plant defense responses to pathogen challenge involve a variety of physiological events that can be generally categorized as either physical or biochemical mechanisms. Lignin deposition in the cell wall, and the cross-linking of hydroxyproline-rich glycoproteins within the polysaccharide matrix of the cell wall, are two of the best-characterized examples of physical plant defense responses to reduce the susceptibility of plant cells to penetration by an invading pathogen [1, 2]. Induction of hydrolytic enzymes, such as chitinases and glucanases, and the production of low molecular weight antimicrobial compounds, known as phytoalexins, are well-characterized biochemical plant defense responses that increase the disease resistance of plants by directly inhibiting the growth of pathogenic organisms [3, 4]. Typically, a plant that recognizes a microorganism as a potential pathogen responds with a diverse and complex arsenal of defense strategies in an attempt to restrict the spread of the microorganism and, thus, prevent the development of disease.

Aromatic amino acids serve as the precursors to a number of metabolites that function in the defense response of plants to pathogen challenge. The role of L-phenylalanine as precursor to the general phenylpropanoid pathway, that supplies coumaroyl-CoA for the biosynthesis of lignin, flavonoids, and other phenolic compounds involved in plant defense responses, has been studied extensively at the biochemical and molecular levels [3]. One of the best

[1] Dedicated to Professor G. H. Neil Towers on the occasion of his seventy-fifth birthday.

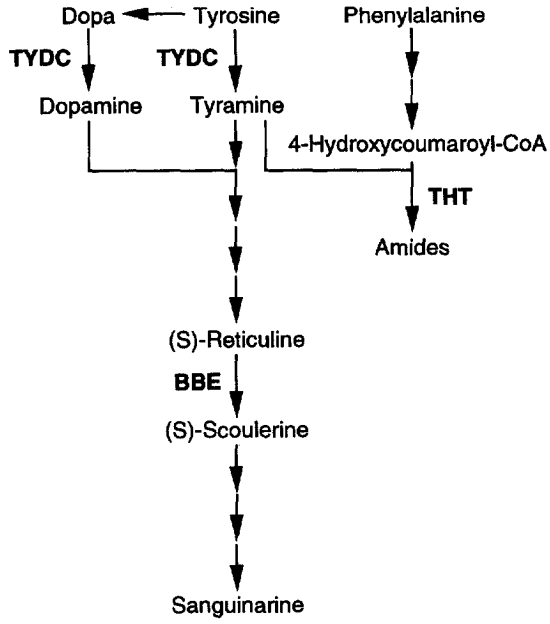

Fig. 1. Schematic representation showing the sites of action of tyrosine/dopa decarboxylase (TYDC), berberine bridge enzyme (BBE), and tyramine hydroxycinnamoyl transferase (THT) in biosynthetic pathways leading to the benzophenanthridine alkaloid sanguinarine and to hydroxycinnamic acid amides of tyramine in opium poppy.

studied examples of a plant defense response gene is that of phenylalanine ammonia lyase (PAL) which catalyzes the conversion of L-phenylalanine to *trans*-cinnamic acid. Studies in various plants have shown that the regulation of PAL in response to diverse stimuli, such as pathogen challenge, occurs largely at the transcriptional level [3, 5]. More recently, the rapid and transient activation of tyrosine decarboxylase (TYDC) at the transcriptional level has been reported in a variety of plant species [6–8]. In plants that produce benzylisoquinoline alkaloids, the decarboxylation of L-tyrosine and L-dopa to their respective amines represent the initial steps in the biosynthesis of the benzylisoquinoline alkaloid skeleton (Figure 1). More than 2500 known benzylisoquinoline alkaloids in five major plant families have been isolated, and all are derived from the common precursor (*S*)-norcoclaurine [9]. Sanguinarine is a benzylisoquinoline alkaloid, of the benzophenanthridine class, produced in select cultured plant cell species in response to treatment with fungal elicitors [10, 11]. Sanguinarine also exhibits potent antibiotic activity [12]; thus, it is considered to be a phytoalexin in these plants [13]. The accumulation of sanguinarine in opium poppy (*Papaver somniferum* L.) cell cultures follows the rapid induction of TYDC mRNA and enzyme activity levels [8].

The rapid and transient induction of TYDC mRNAs in plant species, such as parsley [6] and *Arabidopsis thaliana* [7], that do not accumulate tyrosine-derived alkaloids suggests that tyrosine serves as the precursor to a ubiquitous class of plant defense-response metabolites. Recent studies have suggested that the *de novo* biosynthesis of hydroxycinnamic acid amides of tyramine, and their subsequent polymerization in the cell wall by oxidative enzymes, is an integral and widespread component of the plant defense response to stimuli such as pathogen challenge [14–21]. These amides, and other cell wall phenolics, are believed to create a barrier against pathogens by reducing the digestibility of the cell wall and/or by directly inhibiting the growth of fungal hyphae. Hydroxycinnamic acid amides have been found in a variety of plants [22], and are formed by the condensation of hydroxycinnamoyl-CoA esters with various amines, such as polyamines (e.g. putrescine and spermidine) or tyramine. Tyramine hydroxycinnamoyl CoA:tyramine hydroxycinnamoyltransferase (THT) catalyzes the condensation of tyramine and derivatives of hydroxycinnamoyl-CoA [16]. THT is an elicitor-inducible enzyme that was first described in leaves of tobacco [16] and has since been purified to homogeneity from potato [23, 24].

This paper demonstrates the temporal correlation of tyramine synthesis, and its conversion to sanguinarine and cell wall-bound hydroxycinnamic acid amides, with TYDC and BBE gene expression, and THT activity in elicited opium poppy cell cultures. Incorporation of tyramine and dopamine into sanguinarine, and acid-insoluble cell wall material, are also compared to time-courses for the induction of TYDC and BBE mRNAs, and THT activity during a growth cycle of the cultures.

RESULTS

Temporal correlation between sanguinarine accumulation and the induction of TYDC and BBE mRNAs in opium poppy cell cultures

Opium poppy cell cultures elicited 3 d after subculture with a heat-solubilized cell wall fraction of *Botrytis ssp.* mycelia responded with the accumulation of sanguinarine beginning 5 to 10 h after the addition of elicitor. Sanguinarine levels increased to more than 100-fold above the level detected in control cultures after 80 h (Figure 2(A)). The growth rate of control and elicitor-treated cultures was identical for 80 h after elicitor treatment (Figure 2(A)). RNA gel blot analysis revealed only low levels of TYDC mRNAs in control cultures, whereas BBE mRNAs were not detected. In contrast, both TYDC and BBE mRNAs were rapidly, but only transiently, induced after treatment of cultures with fungal elicitor. TYDC mRNAs increased to a maximum level 2 h after elicitor treatment, and declined over the next 30 h to approximately 40% of the maximum (Figure 2(B)). In contrast, BBE mRNAs were induced to a maximum level 10 h after the addition of elicitor, and declined to near-baseline levels over the subsequent 70 h (Figure 2(B)).

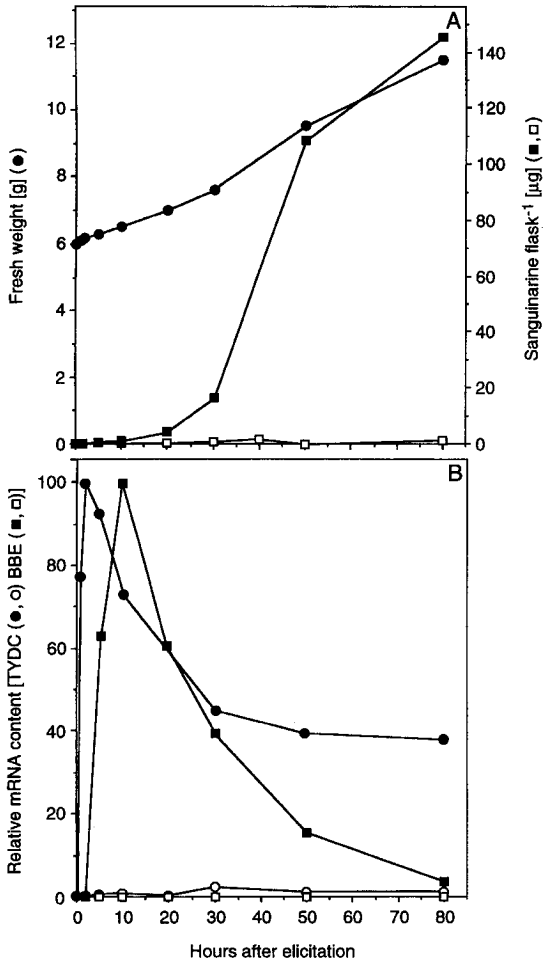

Fig. 2. Time-course of sanguinarine accumulation in relation to changes in (A) fresh weight and (B) levels of TYDC and BBE mRNAs in 3 d old control and elicitor-treated cell suspension cultures of opium poppy. Control cultures were mock-treated with sterile distilled water. The fresh weight of both control and elicited cultures was essentially identical at each time point. Alkaloid and total RNA extracts, prepared from cultures collected on the designated days after elicitor treatment, were used to measure sanguinarine levels, and the relative abundance of TYDC and BBE mRNAs. Triplicate experiments gave identical relative trends. Variation in growth rate and sanguinarine accumulation was <5% for any data point shown. The open symbols represent control cultures, whereas the closed symbols represent elicitor-treated cultures.

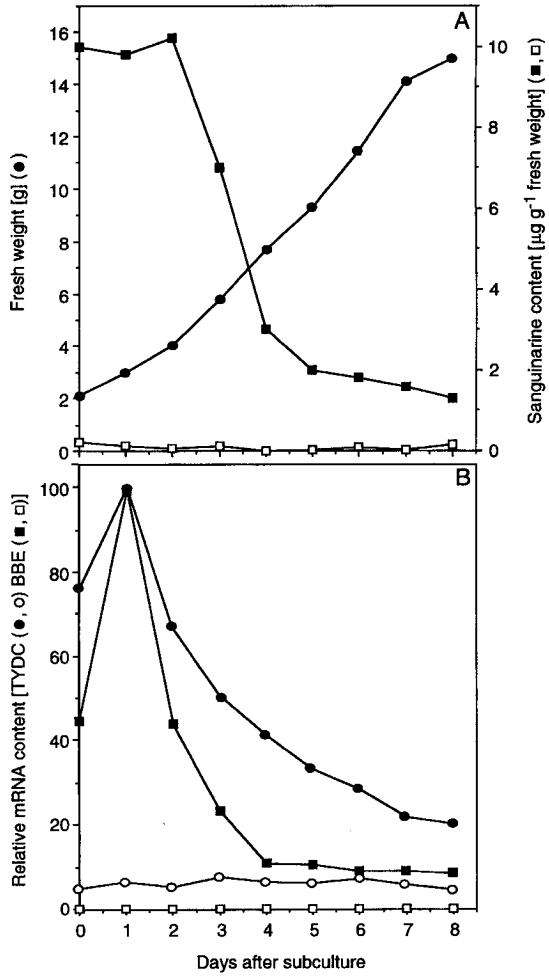

Fig. 3. Effect of culture age on the accumulation of sanguinarine in relation to changes in (A) fresh weight and (B) levels of TYDC and BBE mRNAs in control and elicitor-treated cell suspension cultures of opium poppy. Control cultures were mock-treated with sterile distilled water. On each of the days indicated, cultures were treated with elicitor and collected 24 h later. For any given day, the fresh weight (A) represents the mass of the cells at the start of the elicitation period, whereas the sanguinarine content (A), and relative TYDC and BBE mRNA abundance (B), show the levels at the end of the 24 h elicitation period. The fresh weight of both control and elicited cultures was essentially identical at each time point. Alkaloid and total RNA extracts, prepared from cultures collected on the designated days after elicitor treatment, were used to measure sanguinarine levels, and the relative abundance of TYDC and BBE mRNAs. Relative trends in triplicate experiments were identical. Variation in growth rate and sanguinarine accumulation was <10% for any data point shown. The open symbols represent control cultures, whereas the closed symbols represent elicitor-treated cultures.

Effect of culture age on the elicitor-induced accumulation of sanguinarine and induction of TYDC and BBE mRNAs

Opium poppy cell suspension cultures were routinely subcultured every 6 d to avoid a stationary growth phase. Generally, the opium poppy 2009 SPF cell culture line exhibited rapid browning at the onset of stationary phase, between 7 and 8 d after subculturing. To evaluate the effect of culture age on benzophenanthridine alkaloid and hydroxycinnamic acid amide biosynthesis, 2 g fresh weight of 6 d inoculum was transferred to 50 mL of fresh culture media. Over the course of the 8 d experimental period, the cells increased in mass by almost 8-fold (Figure 3(A)). However, the growth rate during the first 2 d after

subculturing (1.05 g day^{-1}) was slower than the average of the near-linear growth rate over the subsequent 5 d (2.0 g day^{-1}). Only trace amounts of sanguinarine were detected in control opium poppy cultures that were not treated with elicitor (Figure 3(A)). The induction of sanguinarine accumulation, per gram fresh weight, in cultures treated with elicitor for 24 h decreased during the 8 d growth cycle. Cultures treated with elicitor for 24 h between 0 and 2 d after subculture exhibited the highest levels of sanguinarine accumulation, whereas cultures treated with elicitor between 5 and 8 d after subculture responded with 4- to 5-fold lower levels of sanguinarine accumulation (Figure 3(A)).

BBE mRNAs were not detected at any time during the growth cycle of control cultures, but TYDC mRNAs were present at a consistent basal level. The decrease in the induction of sanguinarine accumulation during a typical growth cycle correlated with the decrease in the induction of BBE mRNAs in elicited cell cultures between 1 and 4 d after subculturing (Figure 3(B)). TYDC mRNA levels also decreased, although at a slower rate than the level of BBE mRNAs, during the course of an 8 d growth cycle (Figure 3(B)).

Pulse-labeling with ^{14}C-tyramine and ^{14}C-dopamine in elicitor-induced opium poppy cell cultures

Control opium poppy cell cultures incorporated both ^{14}C-tyramine and ^{14}C-dopamine into acid-insoluble cell wall material. Over the course of an 80 h culture period beginning 3 d after subculture, incorporation of both ^{14}C-tyramine and ^{14}C-dopamine decreased from 5.0 to 1.5% of the total radioactivity added to control cultures for the 2 h pulse-labeling period (Figure 4(A)). Elicited cells exhibited a rapid and transient increase in the incorporation of both ^{14}C-tyramine and ^{14}C-dopamine into the acid-insoluble cell wall fraction with a maximum at 5 h, and a return to near-baseline levels within 20 to 30 h after the addition of elicitor (Figure 4(A)). A maximum of 24 and 35% of the total exogenous ^{14}C-tyramine and ^{14}C-dopamine, respectively, was insolubilized in cell walls when added between 5 and 7 h after elicitor treatment.

Both ^{14}C-tyramine and ^{14}C-dopamine were incorporated into sanguinarine in elicitor-treated cultures. The incorporation rate for ^{14}C-tyramine was 2-fold greater than that for ^{14}C-dopamine (Figure 4(B)). Radiolabeled sanguinarine was not detected for the first 10 h after elicitor treatment. Maximum incorporation levels into sanguinarine 50 h after elicitor treatment were 1.2% and 0.6% for ^{14}C-tyramine and ^{14}C-dopamine, respectively. No incorporation of pulsed ^{14}C-tyramine and ^{14}C-dopamine into sanguinarine was detected in control cultures.

Effect of culture age on elicitor-induced pulse-labeling with ^{14}C-tyramine and ^{14}C-dopamine

Incorporation of ^{14}C-tyramine and ^{14}C-dopamine into acid-insoluble cell wall material and sanguinarine

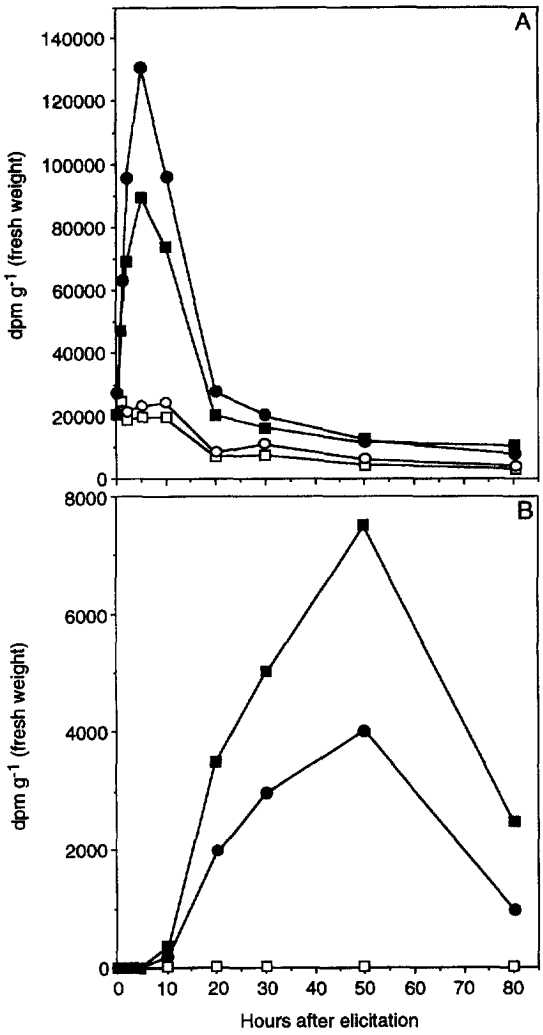

Fig. 4. Time-course for the pulse-labeled incorporation of [7-^{14}C]-tyramine (squares) and [8-^{14}C]-dopamine (circles) into acid-insoluble cell wall material (A) and sanguinarine (B) in control and elicitor-treated cell suspension cultures of opium poppy. Control cultures were mock-treated with sterile distilled water. The cell cultures were pulse-labeled for 2 h beginning at each of the indicated time points. Triplicate experiments gave identical relative trends. Variation for any data point shown was < 10%. The open symbols represent control cultures, whereas the closed symbols represent elicitor-treated cultures.

during a growth cycle in control and elicitor-treated cultures is shown in Fig. 5. The incorporation of ^{14}C-tyramine into the acid-insoluble cell wall fraction in elicitor-treated cells was generally about 30% lower than that of ^{14}C-dopamine at any point during a growth cycle (Figure 5(A)). In contrast, incorporation of ^{14}C-tyramine into sanguinarine was approximately 2-fold higher than that of ^{14}C-dopamine during the same growth cycle (Figure 5(B)). The increased radiolabeling of cell walls from cultures treated with elicitor for 24 h, relative to control cultures, was highest early in the growth cycle and decreased during the

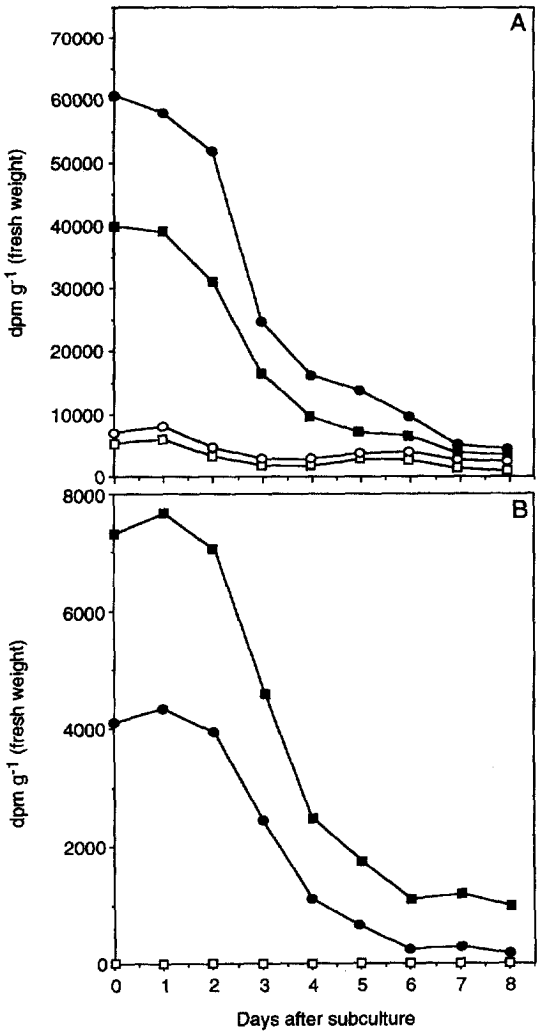

Fig. 5. Effect of culture age on the incorporation of [7-^{14}C]-tyramine (squares) and [8-^{14}C]-dopamine (circles) into acid-insoluble cell wall material (A) and sanguinarine (B) in control and elicitor-treated cell suspension cultures of opium poppy. Control cultures were mock-treated with sterile distilled water. On each of the days indicated, cell cultures were treated with elicitor and collected 24 h later. For any given day, the cultures were pulse-labeled for the last 2 h of the 24 h elicitation period. Relative trends in triplicate experiments were identical. Variation for any data point shown was $< 10\%$. The open symbols represent control cultures, whereas the closed symbols represent elicitor-treated cultures.

8 d culture period (Figure 5(A)). Seven to 8 d after subculture, elicitor-treated cells showed only a marginal increase in ^{14}C-tyramine and ^{14}C-dopamine insolubilization in cell walls compared to control cells.

The growth cycle-dependent decrease in elicitor-induced incorporation of ^{14}C-amines into cell walls was paralleled by their decreased incorporation into sanguinarine (Figure 5(B)). Labeling of sanguinarine with ^{14}C-dopamine was at least 14-fold lower than the incorporation of label into cell walls after 24 h of elicitation on any day of the growth cycle. In contrast, incorporation of ^{14}C-tyramine into sanguinarine was generally 5-fold lower than that into cell walls after 24 h of elicitation. The *in vivo* rate of sanguinarine synthesis after 24 h of elicitor treatment (Figure 5(B)) correlated with sanguinarine accumulation during the opium poppy cell culture growth cycle (Figure 3(B)).

THT and peroxidase activities in control and elicitor-treated opium poppy cell cultures

Both THT and peroxidase enzyme activities were detected in control opium poppy cell cultures (Figure 6). However, only THT activity was found to be inducible in elicitor-treated cultures. THT specific activity increased rapidly in cultures treated with elicitor, reached a maximum level 10 h after elicitation that was 18-fold higher than the level in control cultures, and then decreased slowly to approximately 60% of the maximum level after 80 h (Figure 6(A)). In contrast, peroxidase specific activity showed only a marginal increase for the first 10 h in elicitor-treated cultures (Figure 6(B)). The levels of THT and peroxidase specific activities in elicitor-treated cell cultures remained high even between 30 and 50 h, when the incorporation of ^{14}C-tyramine and ^{14}C-dopamine into cell walls had significantly declined (Figure 3(A)).

Hydrolytic extraction of cell walls from cultures pulse-labeled with ^{14}C-amines

Hydrolytic extraction of cell walls from opium poppy cultures pulse-labeled with ^{14}C-tyramine and ^{14}C-dopamine resulted in the recovery of most radioactivity as the original ^{14}C-labeled amines and *de novo* synthesized ^{14}C-labeled amides (Table 1). The maximum percentage of radioactivity recovered as ^{14}C-labeled amides from elicited cultures, relative to controls, was obtained when cells were pulse-labeled 5 h (data not shown) and 10 h Tab. 1 after elicitor-treatment. These samples also displayed the highest levels of ^{14}C-labeled amine insolubilization (Figure 4(A)) and THT activity (Figure 6(A)). In cell wall extracts from elicited cultures pulse-labeled with ^{14}C-tyramine, 13% of the total insolubilized radioactivity comigrated with a ^{14}C-coumaroyltyramine standard, whereas approximately 60% comigrated with ^{14}C-tyramine. Only 5% of the total insolubilized ^{14}C-tyramine remained bound to the pellet after hydrolytic extraction. The extracted ^{14}C-tyramine probably represents either the complete hydrolysis of amides, or free amine that was directly insolubilized in the cell wall, or both. Similar results were obtained for elicited cultures pulse-labeled with ^{14}C-dopamine Table 1.

DISCUSSION

The elicitor-induced biosynthesis of sanguinarine in cultured plant cells has been reported for various members of the Papaveraceae including *P. somniferum*

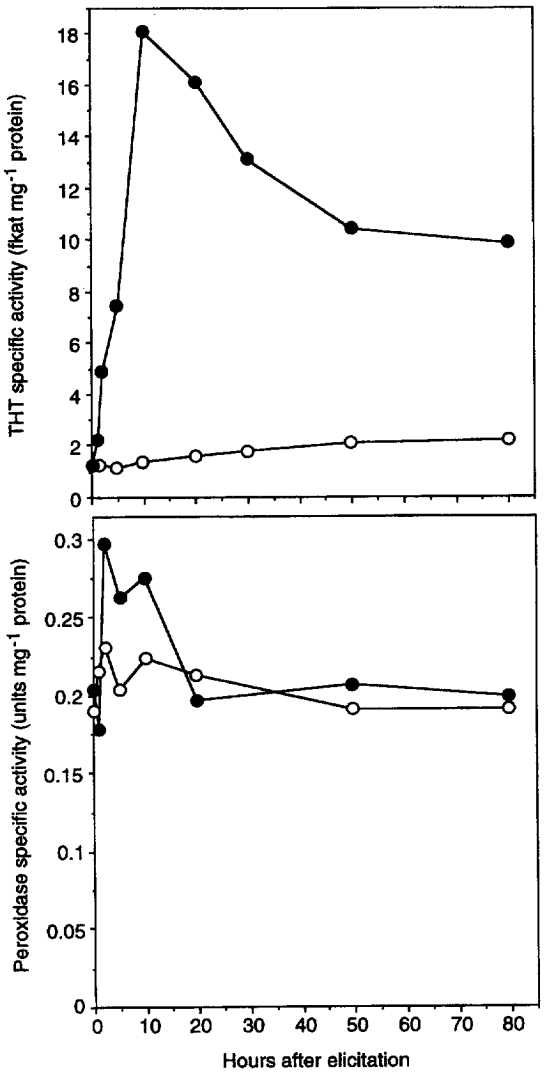

Fig. 6. Time-course for THT (A) and peroxidase (B) activity in control and elicitor-treated cell suspension cultures of opium poppy. Control cultures were mock-treated with sterile distilled water. After treatment of cultures for the indicated period of time, total soluble protein extracts were assayed for THT and peroxidase activity. Triplicate experiments gave identical relative trends. Variation for any data point shown was < 10%. The open symbols represent control cultures, whereas the closed symbols represent elicitor-treated cultures.

[8, 10], *P. bracteatum* [13], *Eschscholzia californica* [11], and *Sanguinaria canadensis* [25, 26]. The rapid accumulation of sanguinarine in response to elicitor treatment suggests that it plays an integral role in defense against pathogens. Sanguinarine, as a benzylisoquinoline alkaloid, is derived from two units of L-tyrosine. The first steps in benzylisoquinoline alkaloid biosynthesis are the TYDC-catalyzed decarboxylation of L-tyrosine and L-dopa to their corresponding amines, tyramine and dopamine, respectively. These amines are used, directly or indirectly, for the formation of (*S*)-norcoclaurine, the first committed benzylisoquinoline intermediate [9]. The branch-point enzyme BBE directs the benzylisoquinoline alkaloid intermediate (*S*)-reticuline toward sanguinarine biosynthesis Fig. 1. In opium poppy plants, (*S*)-reticuline also serves as precursor to the morphine branch pathway. However, this pathway is not active in dedifferentiated cultures [10].

In addition to opium poppy [27], TYDC cDNAs have been isolated from *Petroselinum crispum* [6] and *A. thaliana* [7], and used to show that TYDC mRNAs are induced in response to elicitor treatment, and around sites of attempted fungal infection [28]. TYDC enzyme activity has also been detected in *Nicotiana glutinosa* [29], *Syringa vulgaris* and *Hordeum vulgare* [30]. With the exception of opium poppy, none of these plants produce benzylisoquinoline alkaloids; thus, TYDC must serve a ubiquitous role in plants, in addition to providing substrates for the biosynthesis of alkaloids in opium poppy and related species. A number of studies have reported the condensation of tyramine with hydroxycinnamoyl-CoA esters by THT. The subsequent peroxidase-mediated integration of the resulting amides into cell walls [17], is considered a common defense response in many plant species [18, 20, 29, 31]. THT has been reported in *N. tabacum* [31], *N. glutinosa* [29], *E. californica* [29], and *Solanum tuberosum* [23]. Moreover, hydroxycinnamic acid amides of tyramine have been detected in members of the Solanaceae, Rosaceae, Leguminoceae, Hippocastanaceae, Compositae and Amaranthaceae [22].

Results presented here show that opium poppy cell cultures respond to elicitor treatment by the incorporation of tyramine and dopamine into two separate pathways. Molecular tools were used to confirm previous reports of the *de novo* activation of sanguinarine biosynthesis in elicited cell cultures [32, 33], and to show a temporal correlation between the accumulation of TYDC and BBE mRNAs, the induction of THT enzyme activity, and the incorporation of tyramine and dopamine into sanguinarine and insoluble cell wall products. The transcriptional activation of *tydc* genes began immediately upon exposure of the cultures to elicitor. It must be noted that the level of TYDC mRNAs shown in Fig. 2(B) represents the sum of numerous mRNAs for both *tydc*1- and *tydc*2-like genes. Opium poppy contains approximately 15 *tydc* genes that can be divided into two categories based on sequence identity [27]. TYDC1-like mRNAs are induced more rapidly and transiently than TYDC2-like mRNAs, but the isoforms are not catalytically different. For this study, it was more relevant to consider the accumulation of total TYDC mRNAs. Time-courses for the accumulation of sanguinarine (Figure 2(A)), and its *in vivo* synthesis rate (Figure 4(B)) better correlate with the accumulation of BBE, rather than TYDC, mRNAs (Figure 2(B)). These data suggest that BBE plays a more significant role in the elicitor-

Table 1. Hydrolytic extraction and analysis of opium poppy cell walls from control and elicited cultures pulse-labeled with [7-^{14}C]-tyramine and [8-^{14}C]-dopamine

Radiolabeled amine[a]	Treatment[b]	Radioactivity recovered from cell walls (dpm × 10^3/gdw)			
		total[c]	hydrolyzed amide[d]	hydrolyzed amine[d]	pellet
^{14}C-*dopamine*	control	20.0±1.9	1.1±0.3	11.3±1.3	2.7±0.5
^{14}C-*dopamine*	elicited	83.3±5.6	10.9±2.1	47.5±5.7	4.6±0.8
^{14}C-*tyramine*	control	25.1±2.1	0.9±0.2	15.2±3.9	6.0±0.8
^{14}C-*tyramine*	elicited	97.8±8.5	8.7±1.3	57.8±3.8	8.4±0.5

[a]Cell cultures were pulse-labeled for 2 h.
[b]Cultures were treated with elicitor for 10 h before addition of ^{14}C-*labeled amines*.
[c]Determined prior to extraction.
[d]Acid-insoluble cell wall material was hydrolyzed in 1.0 M NaOH. Amides were partitioned into ethyl acetate after acidification of aqueous extract.

inducible regulation of sanguinarine biosynthesis than TYDC.

Incorporation of tyramine, as a hydroxycinnamic acid amide, into plant cell walls involves its coupling with phenylpropanoid derivatives of L-phenylalanine, followed by oxidative polymerization of the product. Coumaroyl-CoA is formed from L-phenylalanine by the action of three enzymes: PAL, cinnamate 4-hydroxylase (C4H), and 4-coumaroyl-CoA ligase (4CL). The rapid and transient induction of PAL has been demonstrated in many elicited plant cell systems, including opium poppy [8]. TYDC induction in response to elicitor treatment has also been reported in other benzylisoquinoline alkaloid-producing species, such as *E. californica* and *Thalictrum rugosum* [34], and in those that do not produce tyrosine-derived alkaloids, such as parsley [6] and tobacco [29]. Data presented here are also in agreement with those reported previously that THT is inducible in many, but not all, elicited plant cell cultures including *E. californica* [29] and tobacco [16].

Although aromatic amines are necessary for the biosynthesis of sanguinarine, they appear to be more extensively incorporated into cell walls Fig. 4. A significant proportion (9 to 13%) of total cell wall-insolubilized radioactivity, from elicited opium poppy cultures pulse-labeled with ^{14}C-amines, was extracted as hydroxycinnamic acid amides Table 1. Recovery of most of the extracted radioactivity as the original ^{14}C-amines could represent the complete hydrolysis of amides after the strong alkaline treatment used to solubilize cell wall-bound phenolics. There was no evidence for any major radiolabeled product other than amides and amines in cell wall extracts. However, the possibility that ^{14}C-amines were converted to other insoluble derivatives cannot be completely ruled out. Clearly, the data confirm that hydroxycinnamic acid amides are a primary destination for aromatic amines in elicited opium poppy cultures. Similar experiments have shown that incubation of tobacco callus with ^{14}C-tyramine resulted in the insolubilization of radioactivity in cell walls only under conditions that induced high levels of THT activity [35].

The activation of TYDC mRNAs (Figure 2(B)) appears to correlate with the *in vivo* rate of tyramine and dopamine insolubilization in the cell wall (Figure 4(A)), and the induction of THT activity (Figure 6(A)). The *in vivo* incorporation of both ^{14}C-tyramine and ^{14}C-dopamine into cell walls increased rapidly following elicitor treatment, but was only maintained well-above baseline levels for about 20 h. THT activity remained elevated, at more than 50% of the maximum activity (i.e. at 10 h), in elicitor-treated cultures even after 80 h (Figure 6(A)). Peroxidase activity was not induced in response to elicitor treatment (Figure 6(B)); thus, the transient nature of the inducible incorporation of ^{14}C-amines into cell walls was possibly due to a limitation of hydroxycinnamic acids, or of the H_2O_2 required for peroxidase-dependent crosslinking in the cell wall. As components of the phenylpropanoid pathway, coumaric acid and other hydroxycinnamic acid derivatives are used extensively for the biosynthesis of phenolic compounds. An increase in phenolic metabolism is ubiquitous in elicitor-treated plant cell cultures [3, 4].

^{14}C-Tyramine was incorporated with twice the efficiency of ^{14}C-dopamine into sanguinarine, whereas ^{14}C-dopamine was more efficiently incorporated into cell walls than ^{14}C-tyramine Fig. 4. Labeling studies have shown that dopamine is less efficiently incorporated into the "lower" benzyl portion of benzylisoquinoline alkaloids than tyramine, whereas both amines are efficiently incorporated into the "upper" isoquinoline portion [36]; thus, the total incorporation of ^{14}C-tyramine into sanguinarine could be expected to be higher than that of ^{14}C-dopamine.

The occurrence of copious amounts of dopamine in cultured opium poppy cells [37] suggests that poppy THT might exhibit a lower K_m for dopamine relative to THT in species, such as tobacco, that do not accumulate this amine. The K_m of THT from tobacco [31] and potato [23] for dopamine was 10-fold higher than that for tyramine [23, 31] indicating that tyr-

amine is likely the *in vivo* substrate. But, THT from both species exhibited a relative activity (V_{max}) for dopamine that was 33% higher than that for tyramine. Data presented in Fig. 4(A) and Tab. 1 suggest that opium poppy THT can use both tyramine and dopamine as substrates *in vivo*, and that cell wall-bound amides in opium poppy could include both tyramine and dopamine derivatives. However, ^{14}C-dopamine, being a catecholamine, and its derivatives would be expected to incorporate more efficiently into cell walls than ^{14}C-tyramine and its derivatives; thus, the greater insolubilization of ^{14}C-dopamine could reflect factors other than the kinetic properties of THT. Moreover, it is not known whether an internal pool of each amine is available for both amide and alkaloid biosynthesis.

Of paramount significance in this study is the 10-fold greater incorporation of pulse-labeled ^{14}C-tyramine and ^{14}C-dopamine into cell walls as opposed to sanguinarine in elicitor-treated cultures Fig. 4. Based on their removal from the culture media, both ^{14}C-tyramine and ^{14}C-dopamine exhibited equal uptake rates into control and elicitor-treated cells (data not shown). The peak periods of amine incorporation into cell walls and sanguinarine are distinctly separate. These data suggest that the rapid induction of TYDC within 5 h in elicitor-treated cultures provides a pool of amines for the biosynthesis of hydroxycinnamic acid amides. Subsequently, the remaining cellular amine pool can be utilized for the biosynthesis of sanguinarine, which occurs between 10 and 80 h after elicitor treatment. Clearly, the flux of tyrosine into hydroxycinnamic acid amide metabolism supersedes that into benzylisoquinoline alkaloid biosynthesis.

The responsiveness of opium poppy cells to elicitor decreased during a growth cycle of the cultures. The levels of TYDC and BBE mRNAs, the incorporation of radiolabeled amines, and the accumulation of sanguinarine were inversely proportional to the culture fresh weight (Figures 3 and 5). However, it cannot be determined from this study whether the reduced response is due to an overall decline in the biosynthetic capacity of each cell, or to a decrease in the proportion of cells that are capable of either responding to the elicitor signal or synthesizing sanguinarine and hydroxycinnamic acid amides. The lower levels of *tydc* and *bbe* mRNAs suggest that the previously reported relationship between sanguinarine accumulation and growth in elicited opium poppy cell cultures [10] occurs at the level of gene regulation. Previous work has shown that *tydc* and *bbe* genes are also developmentally regulated in opium poppy plants [38, 39]. The relevance of these data in the context of the intact plant requires further study.

EXPERIMENTAL

Plant cell cultures

Opium poppy cell suspension cultures (*Papaver somniferum* cv Marianne; cell line 2009 SPF) were maintained in diffuse light at 23°C, on Gamborg 1B5C medium [40] consisting of B5 salts and vitamins plus 100 mg L^{-1} *myo*-inositol, 1 g L^{-1} hydrolyzed casein, 20 g L^{-1} sucrose, and 1 mg L^{-1} 2,4 dichlorophenoxyacetic acid (2,4-D). Cells were subcultured every 6 days using a 1:3 dilution of inoculum to fresh medium.

Fungal elicitor preparation

Fungal elicitor was prepared from *Botrytis spp.* according to [10]. A section (1 cm^2) of mycelia grown on potato dextrose agar was cultivated in 50 mL 1B5C medium, including supplements but excluding 2,4-D, on a gyratory shaker (120 rpm) at 22°C in the dark for 6 days. Mycelia and medium were homogenized with a Polytron (Brinkmann, Westbury, NY), autoclaved (121°C) for 20 min, and subsequently centrifuged under sterile conditions with the supernatant serving as elicitor. Elicitor treatments were initiated by the addition of 0.5 mL fungal homogenate per 50 mL cell culture. Cells were collected by vacuum filtration, frozen in liquid N_2, and stored at −80°C.

RNA gel blot hybridization

Total RNA for gel blot analysis was isolated according to [41], and 15 μg of each sample were fractionated on 1.0% formaldehyde agarose gels before transfer to nylon membranes [42]. RNA and DNA blots were hybridized with random primer ^{32}P-labeled probes [43] consisting of either the full-length open reading frame of the opium poppy *bbe*1 gene [39], or a combination of opium poppy TYDC1 and TYDC2 cDNAs [27]. Hybridizations were performed at 65°C in 0.25 M sodium phosphate buffer, pH 8.0, 7% (w/v) SDS, 1% (w/v) BSA, 1 mM EDTA. Blots were washed at 65°C, twice with 2 × SSC, 0.1% (w/v) SDS and twice with 0.2 × SSC, 0.1% (w/v) SDS [42] (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). RNA and DNA blots were autoradiographed with an intensifying screen at −80°C, and relative mRNA abundance was determined by scanning laser densitometry.

Sanguinarine extraction and analysis

For sanguinarine extraction, cultured plant cells were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The powdered tissue was extracted in methanol for 10 min at 100°C and insoluble debris was removed by filtration. Extracts were reduced to dryness under vacuum, redissolved in 1.0 M sodium carbonate/bicarbonate (3:2) buffer, pH 10.0, and extracted three times with ethyl acetate. The pooled ethyl acetate fractions were reduced to dryness and the residue was taken up in 1.0 mL methanol. Sanguinarine was quantified by high pressure liquid chromatography on a Beckman BioSys 500 liquid chromatography system (Beckman Instruments, Ful-

lerton, CA) and a Beckman System Gold 168 photodiode array detector using a Beckman Ultrasphere C18 reverse phase column (4.6 × 250 mm) at 1200 psi with an isocratic gradient of methanol: water (6:4) containing 0.1% triethylamine, and a flow rate of 0.5 mL min^{-1}. The sanguinarine peak was identified from its UV spectrum and by comparison of its retention time to that of an authentic standard. The identity of sanguinarine was confirmed by low resolution direct probe mass spectrometry (VG 7070F GC/MS System; VG Analytical, Manchester) in comparison to the spectrum of authentic standard as described previously [39].

Pulse-labeling experiments

The *in vivo* rates of incorporation of tyramine and dopamine into either cell walls or sanguinarine, were measured by pulse-labeling 50 mL samples of opium poppy cell suspension cultures with 0.2 μCi [7-^{14}C]-L-tyramine (0.027 μmol; 7.5 Ci mol^{-1}) or 0.2 μCi [8-^{14}C]-L-dopamine (0.013 μmol; 15.6 Ci mol^{-1}). Both isotopes were purchased from Sigma (St. Louis, MO). The culture samples were incubated for the specified period of time in the absence or presence of elicitor, before pulse-labeling the cells. After the addition of radiolabeled tyramine or dopamine, the cell cultures were incubated for an additional 2 h before the cells were collected and analyzed for incorporation of the isotopes into cell walls or sanguinarine. The incorporation of [7-^{14}C]-tyramine and [8-^{14}C]-dopamine was linear with respect to time under these conditions.

Sanguinarine was extracted from pulse-labeled cell cultures as described above for the extraction of cold sanguinarine. The final dried ethyl acetate fractions were taken up in 0.1 mL methanol and samples (20 μL) of the extracts were applied to silica gel 60 F_{254} thin layer chromatography (TLC) plates (EM Separations, Darmstadt) and developed in a solvent system consisting of ethylacetate:methanol (9:1). Spots corresponding to sanguinarine ($R_f = 0.8$) were scraped off the plate and the level of radioactivity was quantified by liquid scintillation counting. The cellular debris that remained after sanguinarine extraction was washed three times with 5% perchloric acid, by centrifugation. A fraction of this material, which contained both protein and acid-insoluble cell wall material, was counted for radioactivity.

THT and peroxidase assays

p-Coumaroyl-Co A was synthesized using a total protein extract from an *Escherichia coli* strain (pQE19) capable of directing the expression of a recombinant enzyme for *p*-coumarate:coenzyme A ligase (4CL) from tobacco [44]. The synthesis reaction consisted of 0.1 mM coenzyme A, 0.2 mM *p*-coumaric acid (Sigma, St. Louis, MO), 2.5 mM ATP, 1 mM DTT, and approximately 250–300 mg total bacterial protein extract [45]. After 1 h incubation, the synthesized *p*-coumaroyl-CoA was separated from the remaining reaction components using a disposable Sep-Pak C18 column (Waters, Milford, MA). The synthesized *p*-coumaroyl-CoA was concentrated, and its identity and purity were confirmed by TLC and comparison of the UV spectrum with that of the published standard. Tyramine hydroxycinnamoyl transferase (THT) activity was measured using a protocol modified from that described in [16]. Control and elicitor-treated cell cultures (2 g fresh weight) were ground to a fine powder under liquid nitrogen and extracted with 200 mM Tris–HCl, pH 7.8 (1 mL g^{-1} fresh weight) containing 14 mM β-mercaptoethanol (BME). The crude total protein extracts were desalted on PD-10 columns (Pharmacia Biotech, Uppsala) that were equilibrated with 50 mM Tris–HCl, pH 7.8 containing 14 mM BME. Ninety μL of each 2 mL desalted sample was incubated for 1 h with 0.5 μCi [8-^{14}C]-tyramine and 100 nmol *p*-coumaroyl-CoA. The reactions were stopped by the addition of 1.0 M HCl, and 20 μL was applied to a silica gel 60 F_{254} TLC plate that was subsequently developed in a solvent system consisting of chloroform:methanol:ammonia (5:4:1). The developed TLC plate was autoradiographed for 12 h. Radiolabeled spots corresponding to *p*-coumaroyltyramine ($R_f = 0.95$) were scraped off the plate and the level of radioactivity quantified by liquid scintillation counting.

Peroxidase activity was measured according to [35]. Control and elicitor-treated cell cultures (2 g fresh weight) were ground to a fine powder under liquid nitrogen and extracted in 100 mM sodium phosphate buffer, pH 6.0 (1 mL g^{-1} fresh weight) in the presence of activated charcoal (50 mg g^{-1} fresh weight). The crude protein extracts were desalted on PD-10 columns equilibrated with 100 mM sodium phosphate buffer, pH 6.0. Peroxidase assay reactions consisted of 5.0 μL of each 2 mL desalted sample in 20 mM guaiacol, 100 mM sodium phosphate buffer, pH 6.0, and 30 mM H_2O_2 in a total reaction volume of 1 mL. The reactions were incubated for 1 h at 30°C. The increase in absorbance was monitored at 420 nm. Specific peroxidase activity was expressed as units per mg protein, where one unit is defined as the amount of enzyme causing an increase in absorbance of 0.001 s^{-1}. Total protein was determined using the Bradford method [46].

Cell wall hydrolysis

Acid-insoluble cell wall material was hydrolyzed in 1.0 M NaOH for 4 h at 37°C. Insoluble debris was removed by centrifugation and washed with water. The supernatant was acidified to pH = 2 with 6.0 M HCl and extracted three times with equal volumes of EtOAc. The pooled EtOAc fractions were reduced to dryness and the residue recovered in methanol. The volume of the remaining aqueous phase was reduced under partial vacuum. Extracted samples were applied to a silica gel 60 F_{254} TLC plate. The TLC solvent

system consisted of chloroform:methanol:ammonia (5:4:1). After autoradiography, radiolabeled spots corresponding to tyramine (R_f=0.78), dopamine (R_f=0.74), and *p*-coumaroyltyramine (R_f=0.95) were recovered. Radioactivity was quantified by liquid scintillation counting.

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