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Uncoupled Defense Gene Expression and Antimicrobial Alkaloid Accumulation in Elicited Opium Poppy Cell Cultures[1]

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Treatment of opium poppy (*Papaver somniferum* L.) cell cultures with autoclaved mycelial homogenates of *Botrytis* sp. resulted in the accumulation of sanguinarine. Elicitor treatment also caused a rapid and transient induction in the activity of tyrosine/dopa decarboxylase (TYDC, EC 4.1.1.25), which catalyzes the conversion of L-tyrosine and L-dopa to tyramine and dopamine, respectively, the first steps in sanguinarine biosynthesis. *TYDC* genes were differentially expressed in response to elicitor treatment. *TYDC1*-like mRNA levels were induced rapidly but declined to near baseline levels within 5 h. In contrast, *TYDC2*-like transcript levels increased more slowly but were sustained for an extended period. Induction of *TYDC* mRNAs preceded that of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) mRNAs. An elicitor preparation from *Pythium aphanidermatum* was less effective in the induction of *TYDC* mRNA levels and alkaloid accumulation; however, both elicitors equally induced accumulation of *PAL* transcripts. In contrast, treatment with methyl jasmonate resulted in an induction of *TYDC* but not *PAL* mRNAs. The calmodulin antagonist *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide and the protein kinase inhibitor staurosporine partially blocked the fungal elicitor-induced accumulation of sanguinarine. However, only staurosporine and okadaic acid, an inhibitor of protein phosphatases 1 and 2A, blocked the induction of *TYDC1*-like transcript levels, but they did not block the induction of *TYDC2*-like or *PAL* transcript levels. These data suggest that activation mechanisms for PAL, TYDC, and some later sanguinarine biosynthetic enzymes are uncoupled.

Plants respond to pathogen challenge by the induction of numerous defense mechanisms that are typically activated by de novo transcription of multiple genes. Plant defense gene products function in diverse biochemical responses that include the biosynthesis of antimicrobial phytoalexins (Darvill and Albersheim, 1984; Ebel, 1986), the deployment of antimicrobial hydrolytic enzymes (Boller, 1985), and the putative reinforcement of the plant cell wall with structural glycoproteins (Showalter et al., 1985) and/or metabolic products such as lignin (Matern and Kneusel, 1988). Defense responses such as the synthesis of degradative enzymes and the activation of phenylpropanoid metabolism (Hahlbrock and Scheel, 1989) are generally ubiquitous, whereas other responses, such as the accumulation of specific phytoalexins, are more specialized (Dixon and Harrison, 1990; Dixon and Lamb, 1990). The structural diversity among phytoalexins in various plant species reflects their nature as secondary metabolites. For example, pathogen challenge results in the biosynthesis of flavonoid phytoalexins in the Apiaceae and Leguminoseae (Heller and Forkmann, 1988), whereas the formation of sesquiterpenoid phytoalexins occurs in the Solanaceae (Stoessl et al., 1976).

Members of the Papaveraceae and Fumariaceae synthesize benzophenanthridine alkaloids (Kutchan and Zenk, 1993), and select species respond to pathogen challenge or elicitor treatment with the accumulation of the antimicrobial alkaloid sanguinarine (Eilert et al., 1985; Cline and Coscia, 1988) as a putative phytoalexin (Grayer and Harbourne, 1994). The first steps in benzophenanthridine alkaloid biosynthesis involve decarboxylation of the aromatic amino acids L-Tyr and L-dihydroxyphenylalanine (Fig. 1). Two later branch-point enzymes in the biosynthesis of sanguinarine, BBE (Dittrich and Kutchan, 1991) and protopine-6-hydroxylase (Tanahashi and Zenk, 1990), have been shown to be induced in response to elicitor treatment in cell cultures of *Eschscholtzia californica* (California poppy; Papaveraceae). Cell cultures of *Papaver somniferum* (opium poppy; Papaveraceae) also respond to elicitor treatment by the rapid accumulation of sanguinarine (Eilert et al., 1985; Eilert and Constabel, 1986). The differential and tissue-specific expression of members from a gene family for TYDC may correlate with the biosynthesis of specific tetrahydroisoquinoline alkaloids, such as sanguinarine and morphine, in opium poppy plants (Facchini and De Luca, 1994, 1995a, 1995b) and may be a useful molecular marker with which to follow the activation of this pathway.

The induction of defense mechanisms involves recognition between pathogen and plant and the subsequent transduction of the recognition signal into the activation of various responses (Lamb et al., 1989). The specificity of the

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Abbreviations: BBE, berberine-bridge enzyme; JA, jasmonic acid; MeJA, methyl jasmonate; OA, okadaic acid; PAL, Phe ammonia lyase; ST, staurosporine; TFP, trifluoperazine; TYDC, Tyr/dopa decarboxylase; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

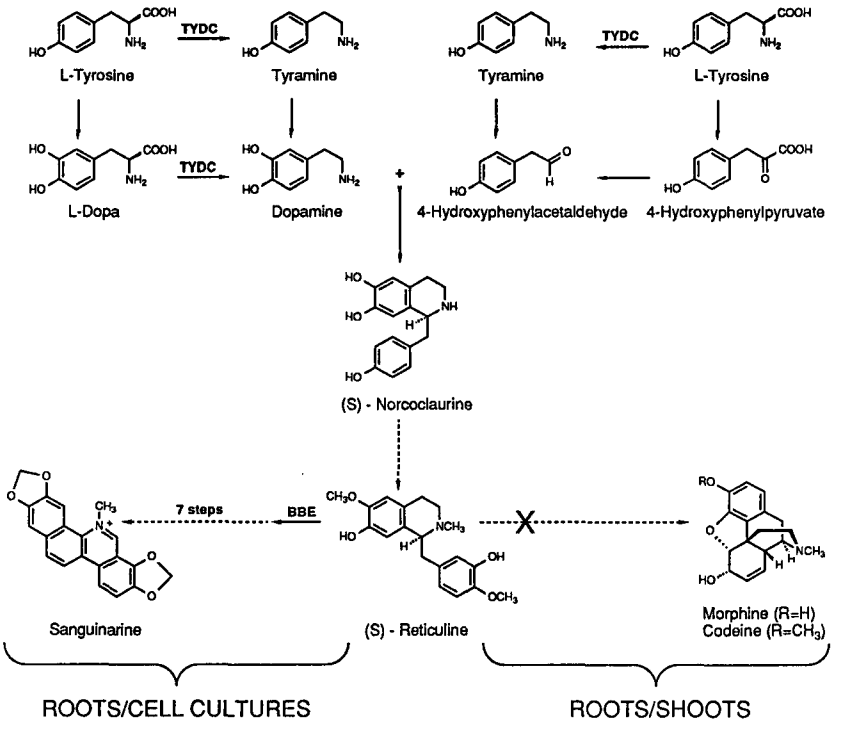

Figure 1. Biosynthesis of sanguinarine in opium poppy with a focus on the early steps from L-Tyr. The sites of action of TYDC and BBE are indicated. The branch pathway from (*S*)-reticuline to codeine and morphine is blocked in opium poppy cell cultures (as indicated by the X); thus, these metabolites do not accumulate.

recognition phenomenon has been demonstrated by the characterization of specific elicitors (Darvill and Albersheim, 1984) and receptors that bind a specific elicitor (Schmidt and Ebel, 1987; Cheong and Hahn, 1991). The mechanism by which recognition is coupled to signal transduction is currently unknown. However, a number of putative signal-response coupling mechanisms in plants have been described. Calcium has been implicated as a second messenger in a number of signal transduction pathways (Marmé, 1989). External stimuli often result in a transient increase in intracellular calcium levels that can bind to and activate calcium-binding proteins such as calmodulin. Despite a lack of direct evidence in plants, it is purported that the calcium-calmodulin complexes can activate other proteins, such as protein kinases, that are important for transcriptional regulation (Kapiloff et al., 1991). The use of Ca^{2+} chelators and channel blockers and calmodulin antagonists has demonstrated the role of calcium and calcium-binding proteins in the induction of sesquiterpenoid phytoalexin accumulation in potato (Zook et al., 1987) and tobacco (Vögeli et al., 1992) and in elicitor-induced, ethylene-dependent responses (Raz and Fluhr, 1992).

Treatment of plant tissues with elicitors has also been reported to cause changes in protein phosphorylation patterns that are considered to be early events in the activation of defense responses (Dietrich et al., 1990; Felix et al., 1991). For example, inhibitors of protein kinases have been shown to block the elicitor-induced activation of PAL and ethylene biosynthesis in tomato cells (Grosskopf et al., 1990), to suppress elicitor-induced Ca^{2+} uptake and K^+ efflux in parsley cells (Conrath et al., 1991), and to inhibit elicitor-induced ethylene production and cause changes in protein phosphorylation patterns in tomato cells (Felix et al., 1991). Recently, protein kinase and protein phosphatase inhibitors were used to demonstrate that the dephosphorylation of enzymes involved in redox processes, which transfer electrons from reducing agents to oxygen, regulate the elicitor-specific induction of redox activity (Vera-Estrella et al., 1994). Other recent evidence supports the possible role of GTP-binding proteins in the early transduction of elicitor signals, leading to the de novo transcription of a number of genes, including those involved in defense responses (Bowler and Chua, 1994; Vera-Estrella et al., 1994).

Results presented in this paper demonstrate that the elicitor-induced accumulation of sanguinarine in opium poppy cell-suspension cultures is associated with the differential and temporal-specific expression of *TYDC* genes. The differential expression of *TYDC* and *PAL* genes in response to different elicitor preparations, and the differential effects of various signal transduction inhibitors on the induction of these genes and on the biosynthesis of sanguinarine, suggest that the elicitor-induced regulation of PAL, TYDC, and, later, sanguinarine biosynthetic enzymes, is mediated by signal transduction mechanisms that are at least partially uncoupled. In addition, the ability of exogenous MeJA to induce *TYDC* but not *PAL* mRNA levels suggests that JA may be specifically involved in the transduction pathway for the activation of *TYDC* but not of *PAL* genes.

MATERIALS AND METHODS

[^{32}P]Deoxyribocytosine triphosphate was purchased from ICN and L-[*carboxyl*-^{14}C]Tyr was purchased from Amersham. TFP, W7, ST, OA, and sanguinarine chloride were

purchased from Sigma. (±)-MeJA was a kind gift from Dr. Normand Brisson (Université de Montréal, Canada).

Cell-Suspension Cultures

Cell-suspension cultures of opium poppy (*Papaver somniferum* cv Marianne; cell line 2009) were maintained on Gamborg 1B5C medium, which consisted of B5 salts and vitamins plus 100 mg L^{-1} *myo*-inositol, 1 g L^{-1} hydrolyzed casein, 20 g L^{-1} Suc, and 1 mg L^{-1} 2,4-D. Cells were subcultured using a 1:3 dilution of inoculum to fresh medium.

Elicitor and Inhibitor Preparation and Treatment

Fungal elicitors were prepared according to Eilert et al. (1985). Sections (1 cm^2) of mycelium grown on potato dextrose agar were used to inoculate 50 mL of 1B5C plant cell-suspension culture medium minus 2,4-D. Fungal mycelium cultures were grown on a gyratory shaker (120 rpm) at 22°C in the dark for 6 d. Mycelia and medium were homogenized with a Polytron (Brinkmann), autoclaved (121°C) for 20 min, and subsequently centrifuged under sterile conditions with the supernatant serving as elicitor. Opium poppy cell-suspension cultures in rapid-growth phase, corresponding to 4 d after subculture, were used for all experiments. Elicitor treatment was initiated by the addition of 1 mL of fungal homogenate per 50 mL of cell culture unless otherwise indicated. Inhibitors were added to the cell cultures 1 h before the initiation of elicitor treatment. TFP, W7, ST, and OA were prepared in DMSO as stock solutions of 100, 20, 1, and 300 mM, respectively. The effects of DMSO on the elicitor response were determined by the treatment of some cultures with 200 μL of the solvent alone 1 h before addition of the elicitor. Cells were collected by vacuum filtration and stored at −80°C. MeJA was diluted in ethanol and added to cell cultures to a final concentration of 100 μM 4 d after subculture.

Sanguinarine Extraction, Identification, and Quantification

Suspension-cultured cells were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Sanguinarine was extracted by mixing the powdered tissue with methanol for 10 min at 100°C. 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 mL of methanol. Samples (10 μL) of extracts were applied to Silica Gel 60 F_{254} TLC plates (EM Separations, Darmstadt, Germany) and developed in a solvent system consisting of ethyl acetate:methanol (9:1, v/v). Plates were dried at room temperature and photographed under UV illumination at 365 nm on Kodak Royal Gold 100 film. Sanguinarine was also quantified and its identity was confirmed by HPLC on a Waters 600E HPLC system and a Waters 991 photodiode array detector. Alkaloid extracts were separated on a Waters Nova Pak C_{18} reverse-phase column (3.9 × 300 mm) at 1200 p.s.i. with an isocratic gradient of methanol:water (6:4, v/v) containing 0.1% triethylamine. Alkaloid elution was monitored at 280 nm. The sanguinarine peak was identified from its UV spectrum and by comparison of its retention time to that of the authentic standard. The identity of sanguinarine was confirmed by low-resolution, direct-probe MS (VG 7070F GC/MS System, VG Analytical, Manchester, UK) in comparison with the spectrum of an authentic standard. Sanguinarine samples, as standard and from elicited cell cultures, were purified by TLC. Spots corresponding to sanguinarine were removed and alkaloids were redissolved in methanol. Insoluble silica was removed by centrifugation and the supernatants were subjected to MS. Low-resolution mass spectra obtained for sanguinarine from elicited poppy cells (*m/z* 332[100], 317[31], 194[39], 149[32]) were identical to those obtained for sanguinarine standard (*m/z* 332[100], 317[40], 194[19], 149[33]).

Tyr Decarboxylase Enzyme Assays

Total protein extracts from cultured cells were assayed for decarboxylase activity by measuring the release of $^{14}CO_2$ from L-[*carboxyl*-^{14}C]Tyr as described previously (Facchini and De Luca, 1994, 1995a). Enzymatically liberated $^{14}CO_2$ was trapped on quaternary ammonium-saturated GF/A filter disks (Whatman) suspended above the reaction solution in air-tight vials. Cultured cells were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The powdered cells were extracted with 200 mM Bis-Tris, pH 7.2, debris was removed by centrifugation, and the supernatant was desalted by passage through a PD-10 column (Pharmacia). The standard assay mixture for decarboxylase activity contained 50 mM Bis-Tris, pH 7.2, 1 mM EDTA, 25 μM pyridoxal-1-phosphate, 0.1 μCi (specific activity 55 mCi/mmol; 1 Ci = 37 GBq) L-[*carboxyl*-^{14}C]Tyr, and 250 μL of protein extract in a total volume of 1 mL. Reactions were incubated for 60 min at 35°C with constant agitation to liberate the released $^{14}CO_2$ from the aqueous solution. Reactions were stopped by the addition of 0.2 N HCl and agitated for an additional 1 h before scintillation counts from the GF/A filters were determined. Total protein concentration of plant cell extracts was determined by the method of Bradford (1976).

RNA Isolation and Analysis

Total RNA from cultured cells was isolated according to Logemann et al. (1987), and 15-μg samples were fractionated on 1.0% formaldehyde agarose gels before transfer to nitrocellulose membranes (Sambrook et al., 1989). RNA blots were hybridized with random-primer, ^{32}P-labeled (Feinberg and Vogelstein, 1984) complete inserts from cTYDC1, cTYDC2, or *PAL* H11–7 at 65°C in 0.25 M sodium phosphate buffer, pH 8.0, 7% SDS, 1% BSA, 1 mM EDTA. Blots were washed at 55°C, twice with 2× SSC, 0.1% SDS and twice with 0.2× SSC, 0.1% SDS (Sambrook et al., 1989) (1× SSC = 0.15 M NaCl:0.015 M sodium citrate, pH 7.0). RNA blots were autoradiographed with an intensifying screen on Fuji (Tokyo, Japan) RX100 film at −80°C. The

relative intensities of bands on autoradiograms were determined by scanning laser densitometry.

RESULTS

Induction of Sanguinarine and *TYDC* Genes with *Botrytis* Elicitor

Opium poppy cell-suspension cultures treated with the *Botrytis* sp. elicitor preparation exhibited an increase in intracellular sanguinarine content relative to control cultures (Fig. 2A). Sanguinarine levels began to increase 10 h after treatment with elicitor and were approximately 100-fold greater than control levels 80 h after elicitation. Elicitor treatment also resulted in a rapid 10-fold increase in TYDC enzyme activity, with maximum levels observed within 2 h after treatment was initiated (Fig. 2B). Subsequent to its peak at 2 h, TYDC activity decreased over the course of the experiment. However, levels remained elevated by 3-fold over controls even 80 h after the addition of elicitor.

The increase in TYDC activity in elicitor-treated cultures was accompanied by an increase in the levels of *TYDC* transcripts (Fig. 2C). Two different TYDC cDNA probes were used. cTYDC1 and cTYDC2 are representatives of two subgroups, based on sequence identity, within the *TYDC* gene family in opium poppy (Facchini and De Luca, 1994). Under the high-stringency hybridization conditions used, the cTYDC1 and cTYDC2 probes hybridize only to the approximately six members within the same *TYDC* subgroup, but they do not cross-hybridize (Facchini and De Luca, 1994, 1995b). As shown in Figure 2C, both *TYDC1*- and *TYDC2*-like transcripts increased rapidly and transiently after elicitor treatment. However, the two subgroups of *TYDC* genes exhibited differential- and temporal-specific patterns of expression. *TYDC1*-like transcripts increased more than 10-fold within 1 h after the initiation of elicitor treatment and reached maximum levels within 2 h. Subsequently, the level of *TYDC1*-like mRNAs decreased rapidly and returned to near-baseline levels within 5 h after addition of elicitor. In contrast, *TYDC2*-like transcripts increased to similarly high levels, although the rate of increase was significantly slower. *TYDC2*-like mRNAs reached maximum levels approximately 5 to 6 h after elicitation. *TYDC2*-like transcript levels remained elevated for the duration of the experiment to 80 h after elicitor treatment.

For comparison, the same RNA samples were probed with the *PAL* H11–7 clone from hybrid poplar (Subramaniam et al., 1993). As expected, *PAL* genes were also rapidly induced by elicitor treatment. However, the rate of increase of *PAL* mRNAs was approximately one-half of that exhibited by *TYDC2*-like genes and considerably

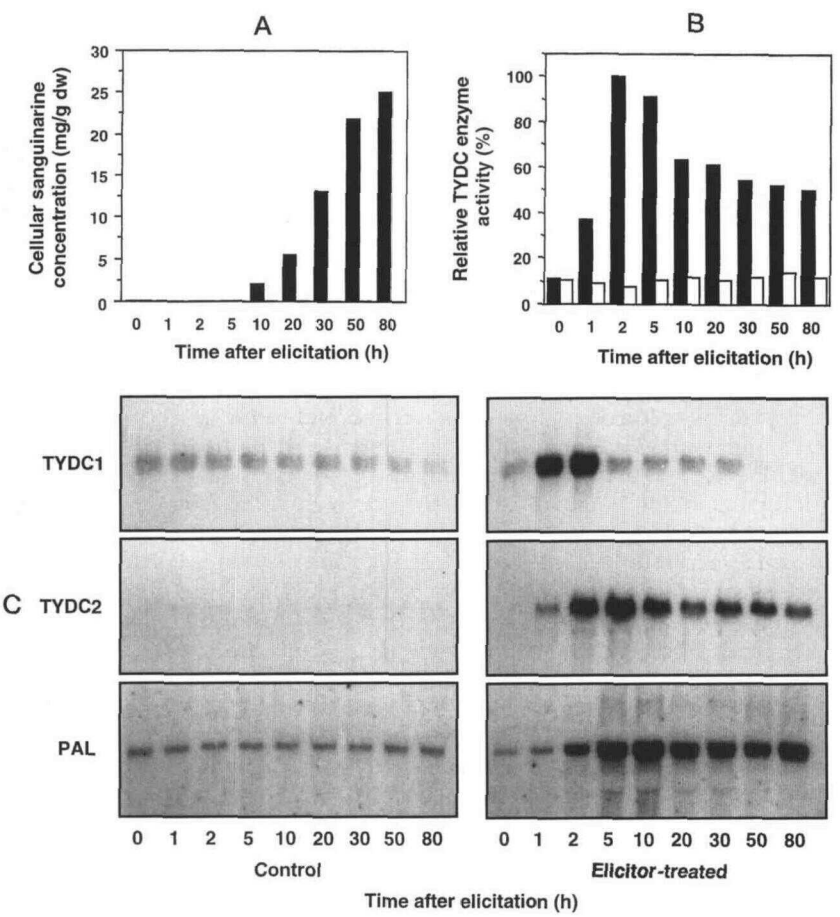

Figure 2. Induction time courses for the accumulation of sanguinarine, TYDC enzyme activity, and the level of *TYDC1*-, *TYDC2*-like, and *PAL* transcripts in control and induced opium poppy cell-suspension cultures treated with the *Botrytis* elicitor preparation. Cell cultures were treated with elicitor (1 mL/50 mL cell culture) or mock-treated with sterile water. Samples were collected at the time points indicated and divided into three aliquots for alkaloid, protein, and total RNA extraction. A, Cellular sanguinarine levels were quantified by HPLC using authentic sanguinarine as an internal standard. The identity of sanguinarine was confirmed by MS. B, Specific TYDC activity values in total protein extracts are expressed relative to the maximum value (2.5 nkat/mg protein). C, Each lane of replicate RNA gel blots contains 15 μg of total RNA fractionated on formaldehyde agarose gels, transferred to nitrocellulose membranes, and hybridized with ^{32}P-labeled cTYDC1, cTYDC2, or PAL H11–7 probes at high stringency. Gels were stained with ethidium bromide before blotting to ensure equal loading of samples. Black bars, Elicitor-treated cultures; white bars, control cultures.

slower than the induction of *TYDC1*-like transcripts. These data support a putative key role(s) for TYDC in plant defense responses.

Dose-Dependent Response to *Botrytis* Homogenate Demonstrates Elicitor Specificity

The magnitude of the elicitor response was dependent on the quantity of the homogenized and autoclaved *Botrytis* elicitor preparation added to opium poppy cell-suspension cultures (Fig. 3). In Figure 3A, the TYDC activity in cell-suspension cultures treated for 2 or 8 h with different volumes of the *Botrytis* elicitor preparation increased in a dose-dependent manner up to 1.0 mL of elicitor per 50 mL of cell culture. With larger volumes of elicitor, the decrease in TYDC activity probably resulted from the effects of toxic components in the crude elicitor preparation. The dose dependence of TYDC activity on elicitor concentration was also reflected in the levels of *TYDC* and *PAL* transcripts in total RNA isolated from the same cell cultures, as shown in Figure 3B. *TYDC1*- and *TYDC2*-like transcripts increased in a dose-dependent manner in total RNA from cells treated for 2 and 8 h, respectively, with various volumes of *Botrytis* elicitor. cTYDC1 and cTYDC2 were used to probe RNA gel blots of samples taken 2 and 8 h, respectively, after the addition of elicitor, since these time points corresponded to the peaks in *TYDC1*- and *TYDC2*-like transcript levels, respectively (Fig. 2C). *TYDC1*-, *TYDC2*-like, and *PAL* transcripts all exhibited maximum levels when 1.0 mL of elicitor per 50 mL of cell culture was used. Larger elicitor volumes resulted in a decreased response. These data suggest the presence of a specific elicitor(s) in homogenized and autoclaved *Botrytis* extracts that induced the expression of defense-response genes such as *TYDC* and *PAL* in opium poppy cell-suspension cultures. The empirically determined optimal volume of *Botrytis* elicitor preparation (1.0 mL/50 mL cell culture) was routinely used for all experiments reported in this paper.

Elicitors from Different Fungal Species Induce Similar Patterns of TYDC Gene Expression

The effectiveness of elicitors prepared from autoclaved homogenates of other fungal species in inducing TYDC activity and *TYDC* transcript levels is shown in Figure 4. An elicitor preparation from *Fusarium oxysporum* was equally as effective as that of *Botrytis* sp. in the induction of TYDC activity by 10-fold compared with controls (Fig. 4A). In contrast, the elicitor prepared from *Pythium aphanidermatum* resulted in only a 2-fold increase in the level of TYDC activity (Fig. 4A). However, despite the quantitative differences in the induced levels of TYDC activity, the patterns of induction in *TYDC* transcript levels were similar for both *Fusarium* and *Pythium* elicitor compared with that of *Botrytis* (Fig. 4B). *TYDC1*-like transcripts exhibited a rapid induction within 2 h after elicitor treatment, whereas *TYDC2*-like mRNAs were induced more slowly. Optimal volumes of *Fusarium* and *Pythium* elicitor preparations (1.0 mL/50 mL cell culture) were determined empirically (data not shown), as shown for the *Botrytis* elicitor (Fig. 3).

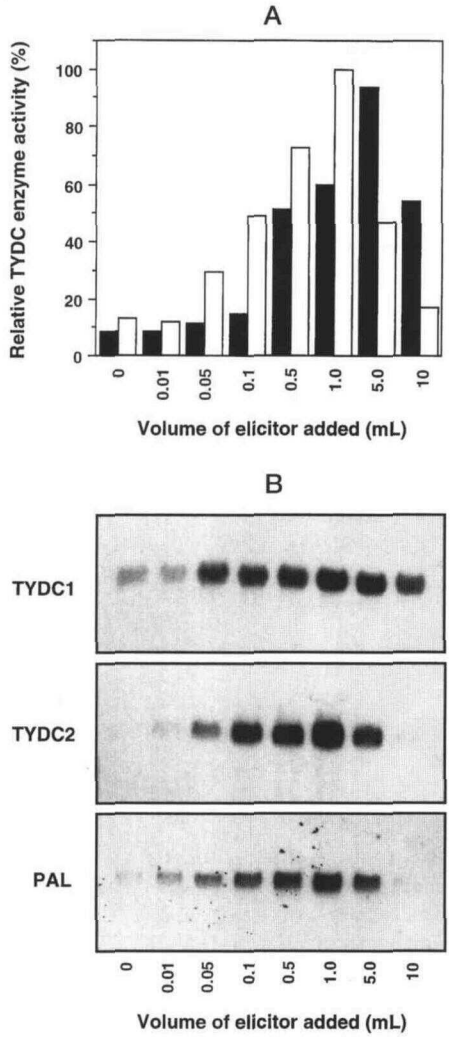

Figure 3. Dose response of TYDC enzyme activity and *TYDC1*-, *TYDC2*-like, and *PAL* transcripts in induced opium poppy cell-suspension cultures treated with different amounts of the *Botrytis* elicitor preparation. Cell cultures were treated with the volume of elicitor preparation indicated up to 10 mL/50 mL cell culture. Samples were collected 2 and 8 h after the addition of elicitor and divided into two aliquots for protein and total RNA extraction. A, Specific TYDC activity values in total protein extracts 2 and 8 h after elicitation are expressed relative to the maximum value (2.5 nkat/mg protein). B, Each lane of RNA gel blots contains 15 μg of total RNA fractionated on formaldehyde agarose gels, transferred to nitrocellulose membranes, and hybridized with ^{32}P-labeled probes at high stringency. RNA gel blots of samples collected 2 h after elicitation were hybridized with cTYDC1, whereas gel blots of samples collected 8 h after elicitation were hybridized with cTYDC2 and *PAL* H11–7. Gels were stained with ethidium bromide before blotting to ensure equal loading of samples. Black bars, Two hours after elicitation; white bars, 8 h after elicitation.

Sanguinarine Accumulation and the Differential Activation of *TYDC* and *PAL* Genes by *Botrytis* and *Pythium* Elicitors

Despite the qualitative similarity among TYDC mRNA induction kinetics in opium poppy cell-suspension cultures

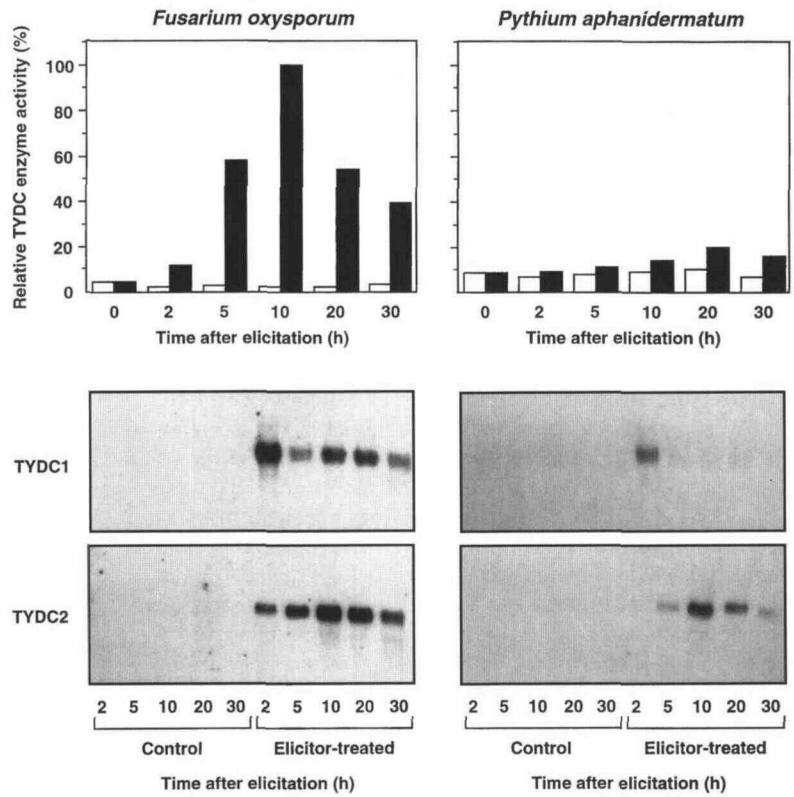

Figure 4. Induction time courses for TYDC enzyme activity, and the level of *TYDC1*- and *TYDC2*-like transcripts in control and induced opium poppy cell-suspension cultures treated with the *Fusarium* and *Pythium* elicitor preparations. Cell cultures were treated with elicitor (1 mL/50 mL cell culture) or mock-treated with sterile water. Samples were collected at the time points indicated and divided into two aliquots for protein and total RNA extraction. A, Specific TYDC activity values in total protein extracts are expressed relative to the maximum value (2.5 nkat/mg protein). B, Each lane of replicate RNA gel blots contains 15 μg of total RNA fractionated on formaldehyde agarose gels, transferred to nitrocellulose membranes, and hybridized with ^{32}P-labeled cTYDC1 or cTYDC2 probes at high stringency. Gels were stained with ethidium bromide before blotting to ensure equal loading of samples. Black bars, Elicitor-treated cultures; white bars, control cultures.

in response to different elicitors (Figs. 2C and 4B), the *Pythium* elicitor was much less effective than the *Botrytis* (Fig. 5A) or *Fusarium* (data not shown) elicitors as inducers of sanguinarine accumulation (Fig. 5A). The ineffectiveness of the *Pythium* homogenate as an elicitor of sanguinarine biosynthesis has been reported previously (Eilert et al., 1985) and is consistent with its inability to induce TYDC activity (Fig. 4A). Total RNAs from cells elicited for 2 and 8 h with *Botrytis* or *Pythium* homogenates were probed on the same blots with cTYDC1 and cTYDC2, respectively, to compare directly the levels of *TYDC* mRNA induction. In addition, a duplicate blot of 8-h RNA samples was probed with the *PAL* cDNA. As shown in Figure 5B, the *Botrytis* elicitor resulted in the induction of *TYDC1*- and *TYDC2*-like genes to levels that were 5- and 25-fold greater, respectively, than those levels in cells treated with the *Pythium* elicitor. In contrast, *PAL* mRNAs were induced to approximately equal levels with both *Botrytis* and *Pythium* elicitors (Fig. 5B). The ineffectiveness of the *Pythium* elicitor, compared with that of *Botrytis*, in inducing the expression of *TYDC* genes and other genes involved in the biosynthesis of sanguinarine, but the equal effectiveness of the two elicitors in inducing the expression of *PAL* suggests that *TYDC* and *PAL* are activated by mechanisms that are at least partially uncoupled.

Calmodulin and Protein Phosphorylation Inhibitors Block the Elicitor-Induced Accumulation of Sanguinarine

The possible role of calmodulin and protein phosphorylation in the induction of sanguinarine biosynthetic enzymes in elicitor-treated opium poppy cell cultures was determined by the pretreatment of cultures with various putative signal transduction inhibitors/antagonists 1 h before the addition of *Botrytis* elicitor (Fig. 6). Treatment of poppy cell cultures with 50 μM of the relatively nonspecific calmodulin antagonist TFP had no effect on the elicitor-induced accumulation of sanguinarine. Higher levels of TFP could not be used without causing rapid lysis and death of the cells. The same concentration (50 μM) of the relatively specific calmodulin antagonist W7 (Hidaka and Tanaka, 1983) resulted in less than one-tenth of the level of sanguinarine accumulation relative to cultures not exposed to the inhibitor before elicitor treatment. Pretreatment with the protein kinase inhibitor ST (Tamaoki et al., 1986) resulted in less than one-third of the sanguinarine levels relative to elicited controls, whereas sanguinarine levels were 1.2-fold higher in cultures pretreated with OA, a known inhibitor of protein phosphatases 1 and 2A (Ishihara et al., 1989). All samples were monitored 50 h after the addition of elicitor. Addition of TFP or W7 alone did not induce accumulation of sanguinarine. However, ST and OA added alone, without subsequent addition of elicitor, induced low levels of sanguinarine accumulation (data not shown).

Activation of *TYDC1*-Like But Not *TYDC2*-Like or *PAL* Genes Involves Protein Phosphorylation/Dephosphorylation

The possible roles of calmodulin and protein phosphorylation in the induction of *TYDC* and *PAL* gene expression in elicitor-treated opium poppy cell cultures

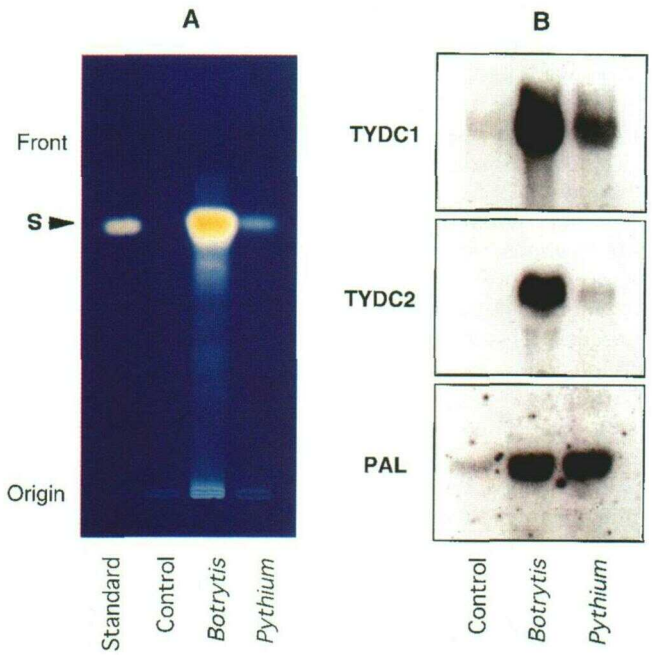

Figure 5. Relative accumulation of sanguinarine and *TYDC1*-, *TYDC2*-like, and *PAL* transcripts in induced opium poppy cell-suspension cultures treated with *Botrytis* or *Pythium* elicitor preparations. Cell cultures were treated with elicitor (1 mL/50 mL cell culture) or mock-treated with sterile water. Samples were collected 2, 8, and 80 h after the addition of elicitor. A, TLC of 80-h alkaloid extracts visualized at 365 nm. Identities of the sanguinarine standard and bands that migrated with the same R_F value (S) were confirmed by MS. B, Each lane of RNA gel blots contains 15 μg of total RNA fractionated on formaldehyde agarose gels, transferred to nitrocellulose membranes, and hybridized with ^{32}P-labeled probes at high stringency. RNA gel blots of samples collected 2 h after elicitation were hybridized with cTYDC1, whereas gel blots of samples collected 8 h after elicitation were hybridized with cTYDC2 and *PAL* H11–7. Gels were stained with ethidium bromide before blotting to ensure equal loading of samples.

was determined by pretreatment of cultures with various inhibitors/antagonists 1 h before the addition of elicitor. Treatment of poppy cell cultures with up to 50 μM TFP or W7 had no effect on the elicitor-induced increase in *TYDC1*-, *TYDC2*-like, or *PAL* transcripts monitored 3 h (TYDC1) and 10 h (TYDC2, PAL) after the addition of *Botrytis* elicitor (Fig. 7).

Pretreatment with up to 500 nM ST or 200 nM OA had little effect on the elicitor-induced levels of transcripts detected with the *TYDC2* or *PAL* probes. In contrast, treatment with 500 nM ST resulted in one-third of the level of *TYDC1*-like transcripts relative to elicited controls after 3 h (Fig. 7). Inhibition of the induction of *TYDC1*-like transcript levels by the *Botrytis* elicitor also resulted from pretreatment with 100 or 200 nM OA. The level of *TYDC1*-like mRNAs in cultures pretreated with ST or OA was about one-half the constitutive level of *TYDC1*-like transcripts in control cultures that were not exposed to elicitor. Addition of inhibitors/antagonists alone did not increase *TYDC* or *PAL* mRNA levels (data not shown).

MeJA Induces the Accumulation of TYDC But Not PAL Transcripts

Treatment of poppy cell cultures with 100 μM MeJA resulted in the induction of *TYDC1*- and *TYDC2*-like mRNAs. However, *PAL* transcript levels did not increase in response to MeJA treatment (Fig. 8). The kinetics of the MeJA-induced accumulation of *TYDC* transcripts were similar to those induced by fungal elicitors (Figs. 2 and 4). However, MeJA treatment did not induce the accumulation of sanguinarine (data not shown).

DISCUSSION

Results presented in this paper demonstrate that the elicitor-induced accumulation of sanguinarine in opium poppy cell-suspension cultures (Eilert et al., 1985; Eilert and Constabel, 1986) is accompanied by an induction of *TYDC* gene expression and enzyme activity (Fig. 2). Our previous work has shown the differential and tissue-specific expression of the *TYDC* gene family in intact

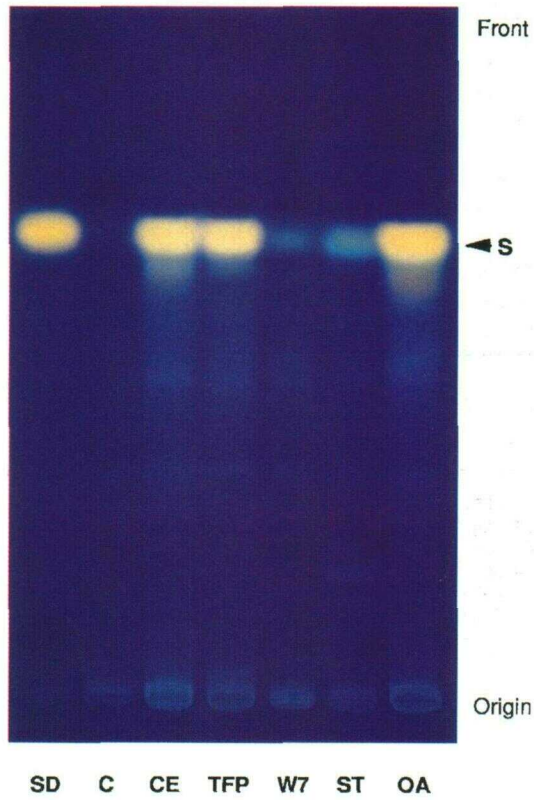

Figure 6. Effect of TFP, W7, ST, and OA on sanguinarine accumulation in cell-suspension cultures of opium poppy treated with the *Botrytis* elicitor preparation. Cell cultures were treated with 50 μM TFP, 50 μM W7, 500 nM ST, or 200 nM OA in DMSO 1 h before addition of elicitor (1 mL/50 mL cell culture). Control cultures were mock-treated with 200 μL of DMSO 1 h before the addition of 1 mL of sterile water (C) or elicitor (CE). Cells were collected 50 h after the addition of elicitor and total alkaloids were extracted. Alkaloids were separated by TLC and visualized at 365 nm. The identities of the sanguinarine standard (SD) and bands that migrated with the same R_F value (S) were confirmed by MS.

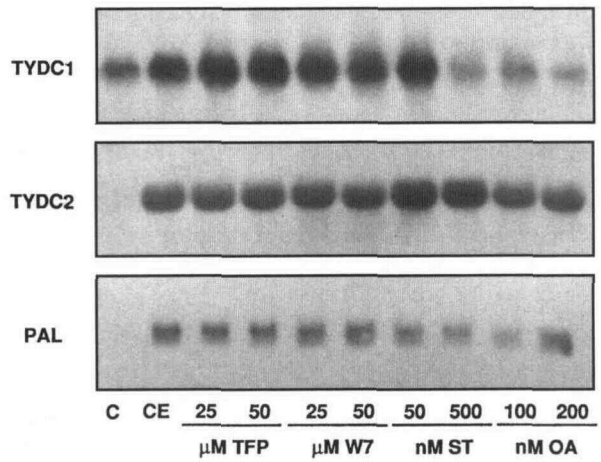

Figure 7. Effect of TFP, W7, ST, and OA on the level of *TYDC1*-, *TYDC2*-like, and *PAL* transcripts in cell-suspension cultures of opium poppy treated with the *Botrytis* elicitor preparation. Cell cultures were treated with the indicated concentration of inhibitor 1 h before addition of elicitor (1 mL/50 mL cell culture). Control cultures were mock-treated with 200 μL of DMSO 1 h before the addition of 1 mL of sterile water (C) or elicitor (CE). Cells were collected 3 (TYDC1) or 10 h (TYDC2 and PAL) after the addition of elicitor and total RNA was extracted. Each lane of replicate RNA gel blots contained 15 μg of total RNA fractionated on formaldehyde agarose gels, transferred to nitrocellulose membranes, and hybridized with ^{32}P-labeled cTYDC1, cTYDC2, or PAL probes at high stringency. Gels were stained with ethidium bromide before blotting to ensure equal loading of samples.

opium poppy plants (Facchini and De Luca, 1994, 1995). Our current work shows that the large *TYDC* gene family also exhibits differential and temporal-specific expression in cultured poppy cells in response to elicitor treatment. *TYDC1*-like genes are induced more rapidly and transiently than *TYDC2*-like genes (Fig. 2C). The rapid and transient increase in *TYDC* gene expression relative to that of *PAL* suggests that TYDC may play an important role in the defense response of opium poppy similar to the central role of PAL in the biosynthesis of antimicrobial phenolic compounds (Hahlbrock and Scheel, 1989). The rapid induction of *TYDC* gene expression in response to elicitor treatment has also been demonstrated for plants that do not accumulate Tyr-derived alkaloids, such as parsley (Kawalleck et al., 1993) and Arabidopsis (Trezzini et al., 1993). Moreover, the elicitor-mediated induction of TYDC enzyme activity has also been shown in *Thalictrum rugosum* and *E. californica* (Gügler et al., 1988; Marques and Brodelius, 1988), two plants that accumulate isoquinoline alkaloids.

Fungus-derived elicitors used in this study were crude autoclaved preparations from different species; thus, the concentration of active fractions in the crude preparations could not be measured. Although the chemical nature of the elicitor(s) from these preparations is not known, it is expected that hydrolyzed cell-wall glucans are the most likely candidates (Darvill and Albersheim, 1984). However, the presence of a specific elicitor(s) was demonstrated by the dose-dependence of the response (Fig. 3). The optimal volumes of all standardized elicitor preparations were determined (as shown for the *Botrytis* elicitor in Fig. 3), and these were used for the experiments reported in Figures 4 and 5. These data suggest that specific elicitors released from various fungi may be different. For example, the *Pythium* elicitor preparation was much less effective as an inducer of *TYDC* gene expression and sanguinarine accumulation, whereas both the *Botrytis* and *Fusarium* elicitor preparations induced strong responses (Figs. 4 and 5). However, both the *Botrytis* and *Pythium* elicitors were equally effective as inducers of *PAL* gene expression. The possibility that the perception of different elicitors may be coupled to specific gene-activation responses by at least partially distinct signal transduction pathways prompted us to speculate on the possible components of these putative induction pathways.

Experiments that use metabolic inhibitors or antagonists must be interpreted with caution, and all conclusions must consider the specificity of the inhibitors, since many are known to have secondary effects. However, antagonists of calcium/calmodulin and inhibitors of protein kinases and phosphatases have been used extensively in plant systems to initially demonstrate the role of calcium/calmodulin-dependent and protein phosphorylation/dephosphorylation-dependent processes (Saunders and Hepler, 1983; Raghothama et al., 1985; Grosskopf et al., 1990; Conrath et al., 1991; Vögeli et al., 1992; Bowler and Chua, 1994; Takeda et al., 1994). These studies have demonstrated that general physiological parameters are not indiscriminately impaired by the inhibitors/antagonists that were selected for use in our work. Data presented here suggest that a calcium-binding protein may play a role in the signal transduction mechanism for the elicitor-induced accumulation of sanguinarine in cultured opium poppy

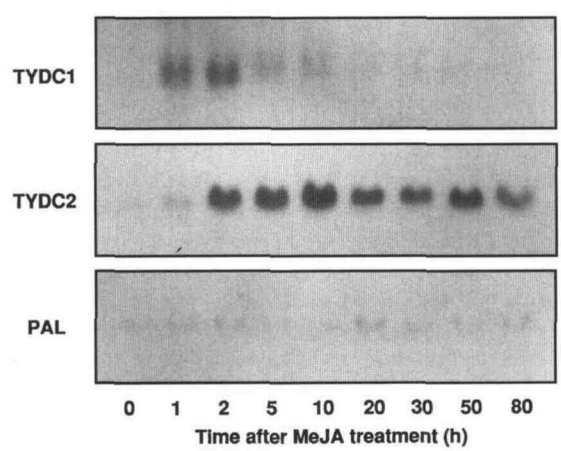

Figure 8. Induction time courses for the accumulation of *TYDC1*-, *TYDC2*-like, and *PAL* transcripts in opium poppy cell-suspension cultures treated with MeJA. Cell cultures were treated with 100 μM MeJA, and samples were collected at the time points indicated. Each lane of replicate RNA gel blots contains 15 μg of total RNA fractionated on formaldehyde agarose gels, transferred to nitrocellulose membranes, and hybridized with ^{32}P-labeled cTYDC1, cTYDC2, or PAL H11–7 probes at high stringency. Gels were stained with ethidium bromide before blotting to ensure equal loading of samples.

cells (Fig. 6). Two different classes of calmodulin antagonists were used: TFP, a phenothiazine, and W7, a naphthalenesulfonamide. It has been shown that the concentration of the relatively less specific antagonist TFP required to inhibit calmodulin-mediated processes is higher than that of the more specific W7 (Vögeli et al., 1992). At a concentration of 50 μM, W7 inhibited the induction of sanguinarine accumulation. In contrast, TFP was not effective at the same concentration. Concentrations of TFP higher than 50 μM could not be used because these resulted in rapid death of the cultured poppy cells.

Although W7 was effective as an inhibitor of sanguinarine accumulation, it did not inhibit the elicitor-mediated induction of *TYDC1*-, *TYDC2*-like, or *PAL* mRNA levels (Fig. 7). These data suggest that the induction of at least some genes later in the sanguinarine biosynthetic pathway (Fig. 1) may be activated by a signal transduction chain that is at least partially uncoupled from that/those involved in the induction of the *TYDC* gene family. It was anticipated that the elicitor-mediated regulation of *TYDC* and other alkaloid biosynthetic genes in opium poppy might be coordinately regulated. The induction patterns of *TYDC* mRNAs from opium poppy and *BBE* mRNAs from the related species *E. californica* (Dittrich and Kutchan, 1991) are strikingly similar. However, the differential sensitivity of *TYDC* gene expression and of sanguinarine biosynthesis to W7 suggests that the induction of at least some alkaloid biosynthetic genes involves calcium/calmodulin-dependent processes, whereas TYDC activation does not. The differential induction of *TYDC* and *PAL* gene expression by elicitors of different origin is demonstrated in Figure 5. Using TFP and W7, other investigators have shown that the elicitor-induced expression of *PAL* is uncoupled from the induction of a sesquiterpene cyclase involved in the biosynthesis of antibiotic sesquiterpenoids in tobacco (Vögeli et al., 1992). Thus, it is not surprising that the induction of *PAL* in poppy cultures was not affected by W7 (Fig. 7).

The reason for the apparent lack of a common mechanism of regulation for *TYDC* and other alkaloid biosynthetic genes may be that TYDC does not represent a dedicated step in alkaloid biosynthesis. In addition to its role as precursor to sanguinarine in opium poppy and related species, recent evidence also suggests that tyramine plays a critical role as a constitutive and pathogen- or wound-induced component of plant cell walls (Negrel and Lherminier, 1987; Borg-Olivier and Monties, 1993). The function of tyramine in a defense mechanism that involves cell-wall reinforcement is suggested by its accumulation, both as a free amine and as a ferulic-acid-conjugated amide, in the cell wall in response to tobacco mosaic virus infection and wounding (Negrel and Jeandet, 1987). Although there is little direct evidence, it is postulated that feruloyltyramine or hydroxycinnamoyltyramine moieties are insolubilized in the cell wall via peroxidase-mediated oxidative mechanisms (Negrel et al., 1993a, 1993b; Hohlfeld et al., 1995) similar to those that cross-link Tyr- and Hyp-rich glycoproteins in response to elicitation and wounding (Bradley et al., 1992). The importance of tyramine as an integral component of plant defense-response mechanisms is also suggested by reports on the isolation of elicitor-inducible *TYDC* genes in parsley (Schmelzer et al., 1989; Kawalleck et al., 1993) and Arabidopsis (Trezzini et al., 1993).

Although the induction of sanguinarine biosynthesis is somewhat sensitive to ST, only the induction of *TYDC1*-like mRNAs is inhibited by ST. It is interesting that *TYDC1*-like gene expression was also inhibited by OA, suggesting that both protein kinases and phosphatases may be involved in the activation of these genes. None of the signal transduction inhibitors/antagonists used in this study were effective as inhibitors of the elicitor-mediated induction of *TYDC2*-like and *PAL* mRNAs. These data provide additional support for the differential induction of *TYDC* and *PAL* genes and further demonstrate the differential regulation of the *TYDC* gene family in opium poppy. However, caution must be noted in these interpretations, since ST and OA treatment alone induced low levels of sanguinarine accumulation. The differential expression of a gene family by specific elicitors and signal transduction inhibitors has also been demonstrated for the differential induction of 3-hydroxymethylglutaryl-CoA reductase genes in potato (Choi et al., 1994). Differential induction of 3-hydroxymethylglutaryl-CoA reductase, which is involved in the biosynthesis of specific antimicrobial isoprenoids in solanaceous plants, is sensitive to JA and the fungal elicitor arachidonic acid. JA has also been shown to be involved as a putative signal transducer in numerous species of elicitor-induced plant cell cultures, including members of the Papaveraceae that accumulate sanguinarine and/or related benzylisoquinoline alkaloids (Gündlach et at., 1992). Treatment with MeJA was also shown to increase the level of *BBE* transcripts in cell cultures of *E. californica* (Kutchan, 1993).

In this paper we demonstrate that treatment of poppy cultures with MeJA results in increased levels of *TYDC* but not *PAL* mRNAs (Fig. 8). The differential induction of *TYDC* and *PAL* genes by MeJA further supports the uncoupled mechanisms for their activation. These data suggest that JA is a putative component of the transduction pathway involved in the activation of *TYDC* and at least some other (Kutchan and Zenk, 1993) alkaloid biosynthetic genes. The lack of sanguinarine accumulation suggests that one or more biosynthetic steps may not involve JA in the signaling pathway. Recent evidence also suggests that JA induction is sensitive to ST treatment (Blechert et al., 1995). However, the putative relationship between JA and calcium/calmodulin- and protein phosphorylation/dephosphorylation-dependent processes in the elicitor induction of *TYDC* gene expression and alkaloid biosynthesis in poppy cell cultures remains to be determined.

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