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Elicitor-mediated induction of phenylalanine ammonia lyase and tryptophan decarboxylase: Accumulation of phenols and indole alkaloids in cell suspension cultures of *Catharanthus roseus*

Dedicated to Dr. Friedrich Constabel on the occasion of his 60th birthday

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Abstract. Cell suspension cultures (cell line No 615) of *Catharanthus roseus* cv. Little Delicata responded to elicitor treatment by accumulating monoterpenoid indole alkaloids and phenolic compounds. The excretion of phenols into the culture medium resulted from the induction of the branch-point enzyme phenylalanine ammonia lyase. The accumulation of alkaloids, however, occurred several hours earlier than the elicitor-mediated induction of tryptophan decarboxylase through which shikimate pathway intermediates are channelled into tryptamine and related indole alkaloids. The results indicate that both pathways for phenol and indole alkaloid biosynthesis responded to elicitor treatment and that no obvious causal relationship between pathways could be deduced from this study.

Abbreviations: PAL — phenylalanine ammonia lyase; TDC — tryptophan decarboxylase

Introduction

Plant cell suspension cultures have recently been shown to accumulate alkaloids upon stimulation with homogenized and autoclaved fungal extracts [1–3]. The first time induction of alkaloid accumulation by substances of fungal origin was reported was for *Ruta graveolens* with regard to acridone epoxides [4, 5]. Cell suspension cultures of *Papaver somniferum* accumulated the antimicrobial alkaloid sanguinarine after cells were treated

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with fungal elicitors [6]. Monoterpenoid indole alkaloids accumulated in cell suspension cultures of *Catharanthus roseus* (L.) G. Don after treatment of cells with autoclaved fungal extracts from *Pythium aphanidermatum* [7]. In the latter two examples, alkaloid accumulation occurred as a result of de novo synthesis of alkaloids [8] and biosynthetic enzymes [9], respectively.

A common feature observed when treating cell suspension cultures with fungal elicitors is the vigorous browning of cells as well as of the culture medium. Browning may be due to intensive production of phenolics and/or polyphenolics [3]. Exposure of *Catharanthus roseus* cell suspension cultures to fungal elicitors resulted in rapid browning of cultures which coincided with alkaloid accumulation [7]. The precursors to monoterpenoid indole alkaloids and phenolics are tryptophan and phenylalanine (tyrosine), respectively, and both precursors are synthesized from the common intermediate chorismate. In this report we describe the effect of elicitors on the catalytic activity of the branch-point enzymes phenylalanine ammonia lyase (PAL) and tryptophan decarboxylase (TDC) during growth of *Catharanthus roseus* cell suspension cultures (cell line 615) and we compare the induction of these enzymes in relation to the time course of accumulation of phenols and monoterpenoid indole alkaloids.

Materials and methods

Cell suspension cultures and elicitor preparations

Catharanthus roseus (L.) G. Don cv. Little Delicata (cell line 615, alkaloid-producing) were grown in B5 medium [10] as described previously [11]. Elicitor preparations were isolated from *Pythium aphanidermatum* grown in B5 medium [10] without growth regulators as described previously [9].

Determination of phenols

Total phenolics in the culture medium were determined according to Swain & Hillis [12]. The culture medium (0.5 ml) was separated from cells by filtration (Whatman No. 1) under suction and the filtrate was diluted in 6 ml of distilled water and 0.5 ml of Folin reagent. After incubation for 3 min, 1 ml of saturated Na_2CO_3 solution was added. The mixture was diluted to a final volume of 10 ml with distilled water. The solution was allowed to stand for 1 h and absorbance at 725 nm was determined. An absorbance of 0.5 equals 100 μg of phenolics when calibration is carried out with caffeic acid as standard.

Determination of alkaloids

Cell samples were extracted following the protocol of Kurz & Constabel [11]. Alkaloid extracts were screened by TLC on silica gel containing fluorescent indicator at 254 nm (Baker S125OF) using methanol/ethyl acetate (10:90) as migration solvent. Alkaloids detected wtih ceric ammonium phosphate spraying reagent [13], were quantitated by high-performance liquid chromatography [14], and were compared to alkaloid standards in our reference collection.

Enzyme assays

Phenylalanine ammonia lyase was extracted and assayed as described previously [15]. The test volume was 1.3 ml containing 0.2 ml sample of crude extract. Tryptophan decarboxylase was extracted and assayed as described previously [9].

Protein determination

Protein was determined according to Bradford [16], with bovine serum albumin as standard.

Results

When 14-day-old cell suspension cultures from cell line 615 were transferred to fresh medium there was no induction of PAL enzyme activity (Fig. 1) nor were phenolic compounds detected in the medium (data not shown). When *Pythium* elicitor was added to the same cell cultures, a transient increase in PAL activity was observed which peaked 8 h after addition of elicitor (Fig. 1). This experiment was reproduced on four separate occasions and identical results were obtained. Browning of the medium was observed visually in elicitor-treated cultures 4–6 h after onset of elicitor treatment. Phenols appeared in the medium after a lag phase of 8 h, after which a large increase in phenol concentration occurred (Fig. 1). Cell suspension cultures from a non-alkaloid-producing cell line of *Catharanthus roseus* (line 916) [17] showed no browning and no phenol secretion subsequent to elicitor treatment.

Cell suspension cultures of cell line 615 were monitored over a longer period of time (52 h) for PAL activity and phenol production (Fig. 2). While PAL activity remained low and constant in control cells, elicitor-treated cells

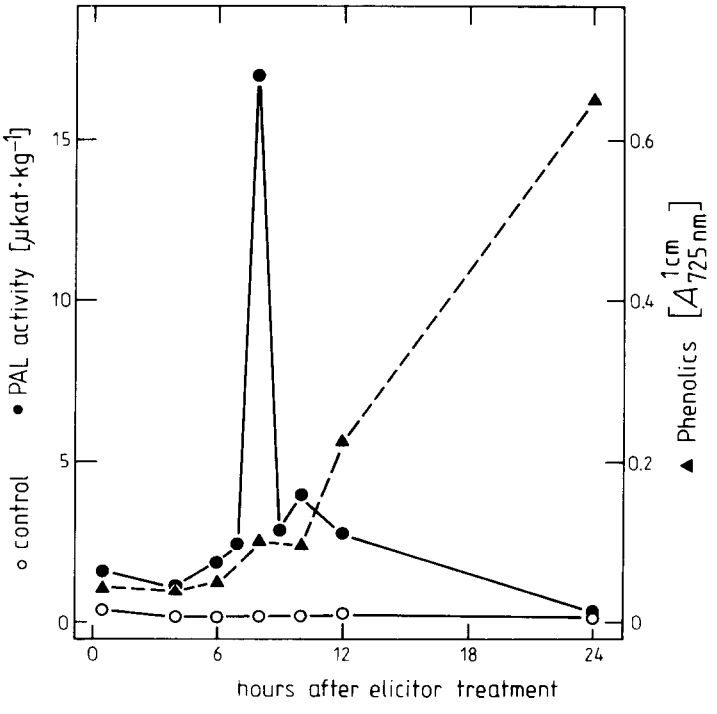

Fig. 1. Changes in the extractable activity of phenylalanine ammonia lyase (PAL) and excretion of phenolic compounds into the culture fluid in cell cultures of *Catharanthus roseus* (cell line No. 615) after treatment with a cell wall preparation from *Pythium aphanidermatum*. ●—● PAL activity after elicitor treatment, o—o PAL activity of control cells, ▲– – –▲ phenolic compounds (caffeic acid equivalents) in the culture medium after elicitor treatment.

showed a second increase in PAL activity which peaked 35 h after elicitor treatment (Fig. 2). When the pH of control and elicited cells was measured, elicited cells had a slightly higher medium pH (0.3 pH units) at the maxima of phenol accumulation (Fig. 2) whereas initial medium pH values were identical.

The above experiment was repeated and cell extracts were assayed for TDC enzyme activity as well as indole alkaloid accumulation. When 14-day-old cell suspension cultures were transferred to fresh medium, there was no induction of TDC enzyme activity, and indole alkaloids were not detected within the cells or in the medium (Fig. 3). Treatment of these cells with *Pythium* elicitor resulted in a transient increase in TDC enzyme activity after an initial lag phase of 8 h. The specific activity of TDC increased from 15 μkat kg^{-1} at time zero to 170 μkat kg^{-1} between 24–36 h after elicitor treatment. Elicitor-treated cell suspension cultures accumulated tryptamine,

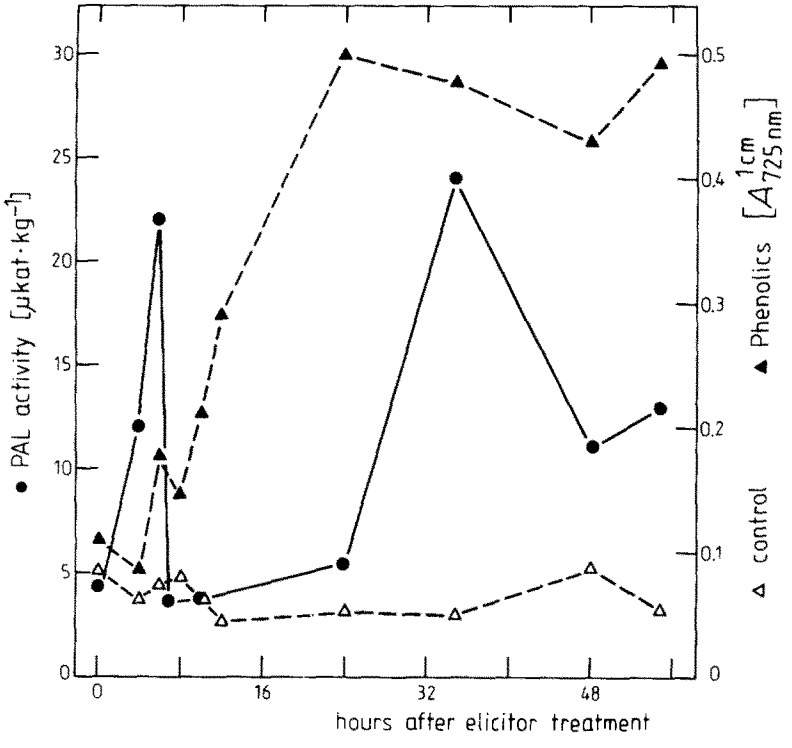

Fig. 2. Changes in the extractable activity of phenylalanine ammonia lyase (PAL) and excretion of phenolic compounds into the culture fluid in cell cultures of *Catharanthus roseus* after treatment with a cell wall preparation from *Pythium aphanidermatum*. ●—● PAL activity of elicitor-treated cells, ▲– – –▲ phenolic compounds in the culture medium after elicitor treatment, △– – –△ phenolic compounds in the culture medium of control cells.

strictosidine lactam, ajmalicine, catharanthine, and tabersonine as well as traces of other indole alkaloids as reported previously [7, 9]. Alkaloid accumulation peaked by 12 h after elicitor treatment and decreased slowly thereafter (Fig. 3).

Discussion

The stimulating effect of autoclaved culture homogenates of *Pythium aphanidermatum* on indole alkaloid accumulation [7] as a result of induction and de novo synthesis of biosynthetic enzymes, has been shown recently [9]. This report describes the elicitor-mediated induction of at least two related

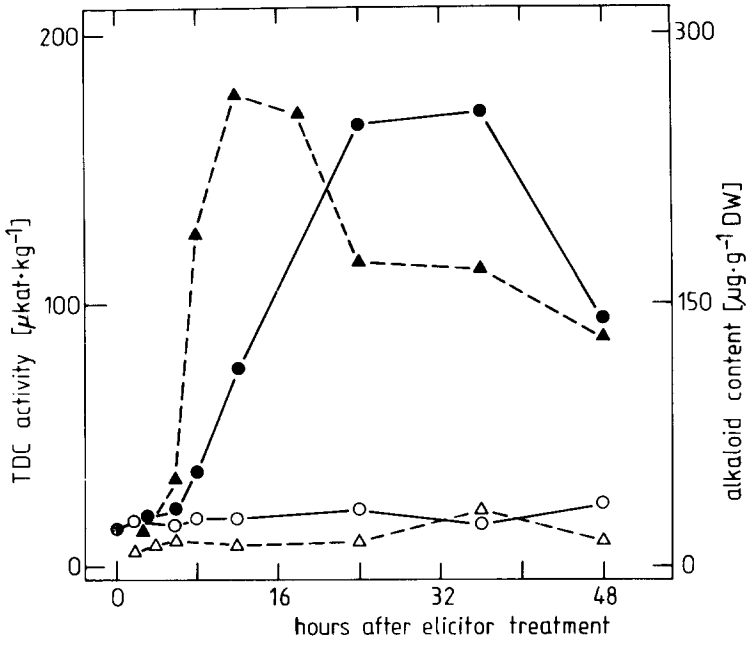

Fig. 3. Changes in the extractable activity of tryptophan decarboxylase (TDC) (○, ●) and total content of indole alkaloids (△, ▲) in cell cultures of *Catharanthus roseus* (cell line No. 615) with (▲, ●) or without (△, ○) elicitor treatment.

biosynthetic pathways which produce phenols and monoterpenoid indole alkaloids, respectively. The results illustrate the capability of the shikimate pathway to channel intermediates into both the phenylalanine and tryptophan branches of the pathway for subsequent biosynthesis to occur, and indicates that the chorismate pool is not rate-limiting.

When cell suspension cultures of cell line 615 were submitted to elicitor treatment, transient increases in PAL (Figs. 1 and 2) and TDC (Fig. 3) enzyme activities were observed. The elicitor-mediated induction of TDC enzyme activity, in contrast to that of PAL and phenol accumulation, lagged behind and only occurred after indole alkaloid accumulation had peaked (Fig. 3). Previous results have indicated that cell line 615 growing in 1B5 medium contains high levels of tryptamine and that elicitor treatment resulted in the depletion of tryptamine levels, presumably due to the channeling of this protoalkaloid into indole alkaloids [9]. Only after the tryptamine level was depleted by two-thirds did TDC activity begin to rise [9] and this suggested that tryptamine could hypothetically play a regulatory role in controlling the expression of TDC in cell line 615. These observations indicate that the early accumulation of monoterpenoid indole alkaloids

observed in elicited cells (Fig. 3) occurs as a result of a pre-existing tryptamine pool, which must be depleted before the need for TDC induction arises. This situation appears to be unique for this *Catharanthus roseus* cell suspension culture, since tryptamine was never detected in intact plants even after induction of TDC during development (unpublished observations).

The excretion of phenolic compounds and the accumulation of indole alkaloids in response to elicitor treatment occurred coincidentally (Figs. 2, 3). Elicitor treatment resulted in two peaks of the catalytic activity of PAL (Fig. 2) unlike the single but broad peak observed for TDC (Fig. 3). The major excretion of phenolic compounds into the culture fluid occurred after induction of the first maximum in the catalytic activity of PAL and several hours prior to the second PAL peak. The period between maximum PAL activity and the beginning of phenol excretion (Fig. 1) may be related to the occurrence of subsequent enzymic steps and excretion processes since phenol synthesis cannot be clearly assigned to either of the two PAL activity maxima (Fig. 2). Perhaps both activity maxima contribute to phenolic synthesis and accumulation. A nearly identical induction behaviour for PAL was observed after treatment of cell cultures of *Glycine max* with fungal cell wall fragments [18].

The accumulation and excretion of phenols and of indole alkaloids into the medium was a common feature of both biosynthetic processes. Indole alkaloids, however, accumulated transiently and turned over rapidly in the culture fluid [7], whereas phenolic compounds accumulated in the culture medium. A possible explanation of this phenomena may relate to the fact that the excreted phenolics are crosslinked by oxidative processes related to the hypersensitive response and are no longer available to the cell for utilization and turnover. The nature and fate of the phenolic compounds excreted into the medium, however, remain to be elucidated.

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