Pergamon

PII: S0031-9422(97)00849-2

Phytochemistry, Vol. 48, No. 1, pp. 113–117, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

INDUCTION OF FURANOCOUMARIN BIOSYNTHESIS IN *GLEHNIA LITTORALIS* CELL SUSPENSION CULTURES BY ELICITOR TREATMENT

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(*Received* 18 *August* 1997)

Key Word Index—*Glehnia littoralis*; cell culture; elicitor; furanocoumarin; bergapten; xanthotoxin; phenylalanine ammonia-lyase

Abstract—Cell suspension cultures were established from *Glehnia littoralis* plants belonging to two different geographic strains. When the cells were treated with yeast extract, they started to produce and excrete furanocoumarins into the culture medium; a major component, bergapten, and a minor one, xanthotoxin, were detected and identified by HPLC and GC/MS.

Changes in phenylalanine ammonia-lyase (PAL) activity and furanocoumarin production after elicitor treatment were traced, showing that PAL activity increased rapidly, reached a maximum after 24 h, and then declined to the normal level after 96 h which preceded the induced bergapten production. The induced-PAL activity of the cultured cells established from an *S*-type plant which accumulated trace amounts of furanocoumarins was about 50% of that in the cultured cells from an *N*-type plant that accumulated more than 0.1% furanocoumarins in the underground parts. However, the elicited production of bergapten was about six times higher in the cell cultures from the *S*-type plant. Addition of the PAL inhibitor 2-aminoindan-2-phosphoric acid (AIP) at 10 μM suppressed the induction of PAL activity and furanocoumarin production.

INTRODUCTION

Glehnia littoralis Fr. Schmidt ex Miquel, a perennial herb belonging to the Umbelliferae, is distributed along the coastline of northern Pacific countries. Hiraoka *et al.* [1–3] reported that there are two geographically different strains among *G. littoralis* plants growing in Japan. The *N*-type plants found in the northern parts of Japan contain in the underground parts more than 0.1% furanocoumarins of which imperatorin and isoimperatorin are major constituents. The *S*-type plants growing in the southern parts of Japan accumulate a polyacetylene compound, panaxynol, as a major secondary metabolite and the furanocoumarin content is very low.

We have established two strains of callus cultures from the plants belonging to the two different geographic strains in order to examine whether the difference of furanocoumarin production seen in the intact plant is also observed in the *in vitro* cultured cells. However, furanocoumarins found in the intact plants were not detected in the callus or cell suspension cultures, irrespective of the strains of the plants used for callus induction. Furanocoumarin production of cell suspension cultures of *Ammi majus* [4], *Petroselinum crispum* [5, 6] and *Ruta graveolens* [7] have been successfully induced by fungal elicitor treatment. Therefore, it seemed to be possible to induce furanocoumarin biosynthesis in *G. littoralis* cell suspension cultures using elicitors, although this has not been reported previously.

In the present investigation we examined the stimulative effect of yeast extract on the furanocoumarin production in cell suspension cultures of *G. littoralis*. We also compared the yeast extract-induced furanocoumarin biosynthesis between two cultured cell strains obtained from the plants of different geographic origins (*N*-type and *S*-type).

RESULTS

Induction of furanocoumarin biosynthesis by yeast extract treatment

Yeast extract was added to *G. littoralis* (*N*-type) cell suspension cultures 10 days after cell inoculation at a

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final concentration of 5 g l^{-1} and incubated for an additional day before analysis. Growth of the cell cultures was not affected by addition of yeast extract. The culture medium and the cells were separately extracted and analyzed by HPLC. Two peaks corresponding to bergapten and xanthotoxin were detected in the extract from the cultured medium. Their identities with bergapten and xanthotoxin were confirmed by GC/MS. Trace amounts of the furanocoumarins were found in the extract from the elicited cells. Imperatorin, isoimperatorin and 8-geranyloxypsoralen, which are the most abundant furanocoumarins present in the underground parts of the intact plants, were not accumulated in the cultured cells or in the medium. Unelicited cell cultures did not produce any furanocoumarins at a detectable level. Bergapten was a major furanocoumarin produced by the elicitor treatment and the xanthotoxin content was much lower than that of bergapten, so that only the bergapten content in the cultured medium was quantitatively determined by HPLC in the following experiments.

PAL activity and furanocoumarin production

Following yeast extract treatment, phenylalanine ammonia-lyase (PAL) activity started to increase rapidly and transiently, reaching a maximum 24 h after addition of yeast extract and then decreasing to the control level after 96 h. Following the increase of PAL activity, bergapten production in the yeast extract-treated cells was induced, reaching a maximum after 72 h (Fig. 1). The increases in PAL activity and in bergapten production by yeast extract-treatment were compared using 10-, 15- and 20-day-old cell cultures, which were representing the early, intermediate and later stages of growth, respectively (Fig. 2). Although a similar trend in PAL activity and bergapten production was observed, the extent of the increases was dependent on the culture stage of the cells, being highest in the 10-day-old cultures and lowest in the 20-day-old cultures. The extent of the increase in bergapten production correlated well with that of the increase in PAL activity. When various concentration of yeast extract (0.25–10 g l^{-1}) were added to the cell cultures, both PAL activity and bergapten production increased in a dose-dependent manner up to 5 g l^{-1} (data not shown).

To confirm that PAL works as one of the essential enzymes for furanocoumarin biosynthesis, the effect of a PAL inhibitor on yeast extract-elicitation of PAL activity and bergapten production was examined using a potent inhibitor 2-aminoindan-2-phosphoric acid (AIP). Treatment of yeast extract-elicited cell cultures with AIP at a final concentration of 10 μM in the culture medium effectively inhibited PAL induction and bergapten production (Fig. 3). When assayed *in vitro* using crude enzyme prepared from the yeast extract-treated cells, 10 μM AIP in the reaction mixture completely inhibited the PAL activity (data not presented).

Different responses to YE addition between two culture strains

Responses of the cell cultures to the YE treatment were compared between the cells from *N*-type plants and those from *S*-type plants (Fig. 4). PAL activity of both cell lines increased rapidly upon addition of YE and reached a maximum after around 12 h. However, the extent in the increase in the cell cultures from *N*-type plants was twice as high as that in the cells from *S*-type plants. On the other hand, the maximum bergapten production by the yeast extract-treated cells from *S*-type plants was six times higher than that by the cells from *N*-type plants in spite of the much lower increase in PAL activity in the cells from *S*-type plants.

DISCUSSION

Though cell suspension cultures of *G. littoralis* did not produce furanocoumarins found in the mother

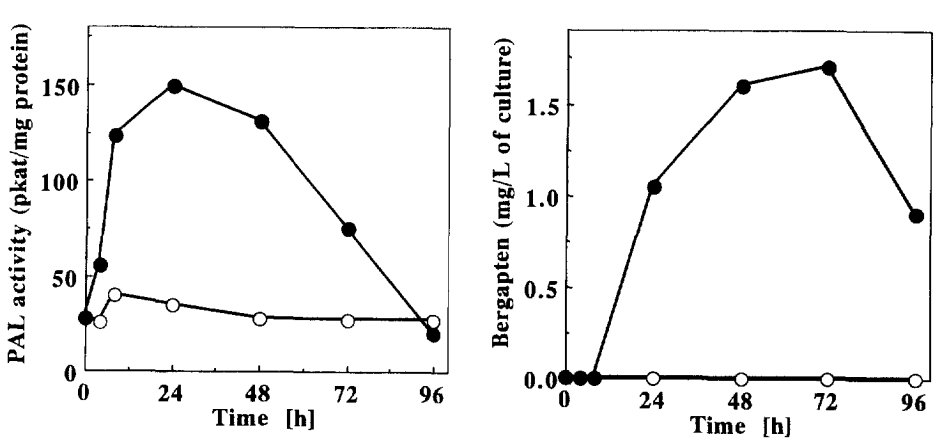

Fig. 1. Time course changes in PAL activity (left) and bergapten production (right) in the cell cultures derived from *N*-type plants of *G. littoralis* after addition of yeast extract (5 g l^{-1} of culture). Yeast extract (●) or water (○) was added to the cell suspension on day 10 and the cells were then incubated for a further 96 h.

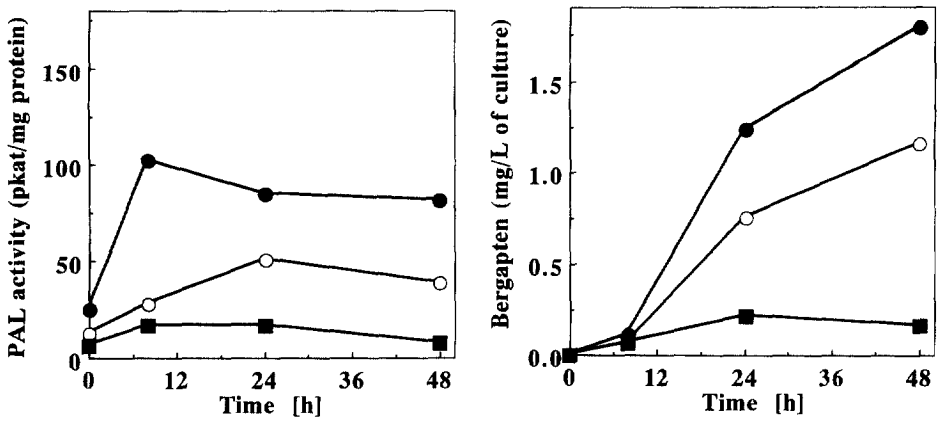

Fig. 2. Growth stage-dependent elicitation of PAL activity (left) and bergapten production (right) with yeast extract. Yeast extract was added to the cell cultures on day 10 (○), day 15 (●) or day 20 (■) and the cultures were incubated for an additional 48 h.

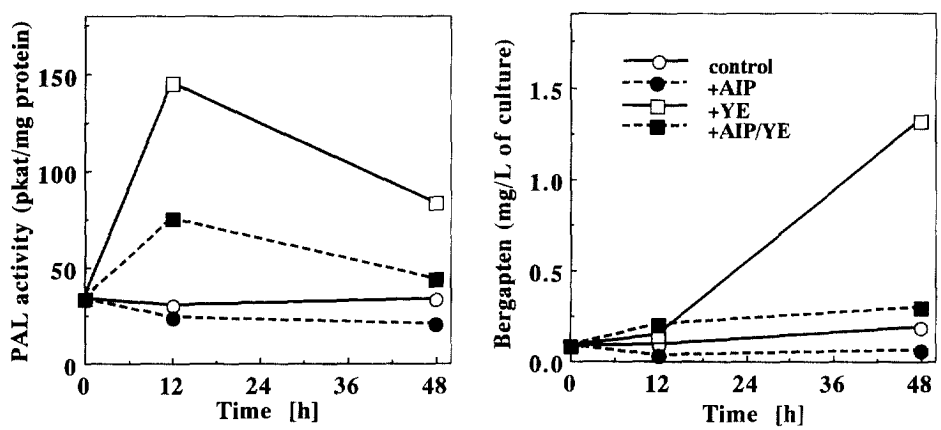

Fig. 3. The effect of the PAL inhibitor AIP on PAL activity (left) and bergapten production (right) in the elicitor-treated cell cultures. AIP was added at a final concentration of 10 μM to the cell cultures with or without yeast extract.

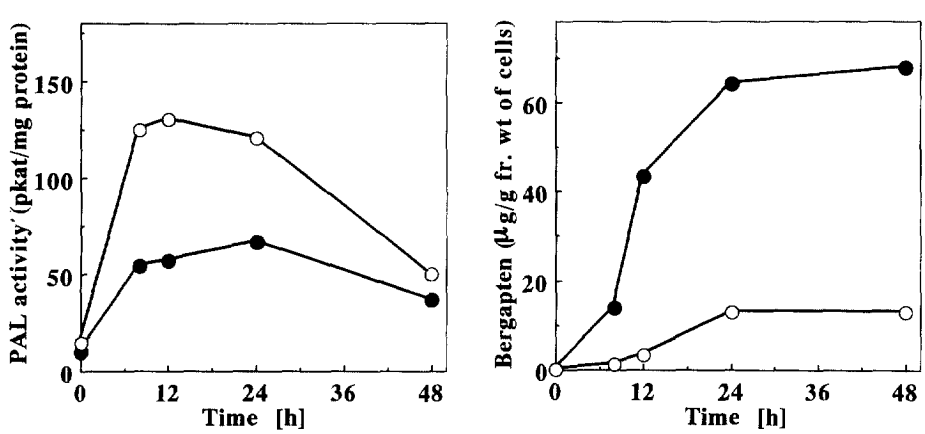

Fig. 4. Changes in PAL activity (left) and bergapten production (right) after addition of yeast extract (5 g l^{-1} of culture) to the cell cultures derived from the *S*-type plants (●) or *N*-type plants (○).

plants, furanocoumarin biosynthesis was successfully induced by yeast extract-treatment. This result is consistent with the elicitor induced furanocoumarin biosynthesis in the cell cultures of *Ammi majus* [4], *Petroselinum crispum* [5, 6], and *Ruta graveolens* [7]. Simple furanocoumarins such as bergapten and xan-

thotoxin were detected as elicitation compounds, whereas prenyloxy or geranyloxy derivatives of psoralen including imperatorin, isoimperatorin, 8-geranyloxypsoralen and bergamottin found as major components in the underground parts of *G. littoralis* [1–3] were not produced even by adding yeast extract to the cultures. In fact, diseased or wounded *G. littoralis* plants accumulated unusually large amounts of bergapten and xanthotoxin [3], and bergapten was identified as a stress compound together with xanthotoxin and psoralen [8].

The remarkable increase of PAL activity resulting from elicitation with yeast extract preceded and paralleled the change in furanocoumarin production. In addition, the treatment with AIP, a PAL inhibitor, suppressed yeast extract-induced furanocoumarin production. These results indicate that furanocoumarins are biosynthesized *de novo* following treatment with yeast extract, and that PAL works as one of the essential enzymes for furanocoumarin biosynthesis in *G. littoralis* cell cultures. The cultured cells derived from the *S*-type plants produced much more bergapten than those from the *N*-type plants after addition of yeast extract, though the increase in PAL activity in the former cell cultures was less than that in the latter cell cultures. This indicates that PAL is not the only enzyme determining the rate of furanocoumarin biosynthesis. It is also interesting to note that the cell cultures from the *S*-type plants produced four times higher amounts of bergapten than those from the *N*-type plants in response to elicitation with yeast extract in spite of the fact that the *S*-type plants contained a lower amount of furanocoumarin than the *N*-type plants.

EXPERIMENTAL

Chemicals

Xanthotoxin and bergapten were obtained from Sigma. [U-^{14}C]L-phenylalanine (specific activity, 204 GBq/mmol) purchased from Amersham was diluted with unlabelled L-phenylalanine (Nakarai Chemicals) to 7.4 MBq/mmol. AIP (2-aminoindan-2-phosphoric acid) was a generous gift from Prof. N. Amrhein, Institute fur Pflanzenwissenschaften, Switzerland. A dye reagent for protein assay was obtained from Bio-Rad and yeast extract from Difco.

Cell suspension culture

S-type and *N*-type plants of *G. littoralis* were collected in Nagasaki and Niigata, respectively. Callus cultures were induced from seedlings on Linsmaier and Skoog medium [9] containing 1 μM 2,4-D and 1 μM kinetin, at 25 C in the dark. Cell suspension cultures were established from the callus tissues in the liquid medium on a rotatory shaker (80 rpm) and were maintained by inoculating 5 ml cell suspension into 25 ml fresh liquid medium in a 100 ml Erlenmeyer flask every three weeks. Cultured cells were collected by vacuum filtration, ground into powder in liquid N_2 in a cold mortar and stored at $-70°$.

Furanocoumarin extraction

The powdered frozen cells (0.5 g) were extracted by shaking with MeOH (1.25 ml) at 70° for 60 min. The slurry was then centrifuged at 10,000 rpm for 5 min and the supernatant was analyzed by HPLC. Cultured medium (ca 30 ml) was extracted with EtOAc (40 ml × 3) and the combined EtOAc extract was evaporated to dryness below 40° under vacuum. The residue was redissolved in MeOH (1 ml) and subjected to HPLC analysis.

HPLC conditions

A Finepak SIL C 18T column was used and eluted with MeCN–H_2O (35:65) at a constant flow rate (1.5 ml min^{-1}). The eluent was monitored at 254 nm. R_t, bergapten 16.4 min, xanthotoxin 12.1 min.

GC/MS conditions

A Gas chrom Q column packed with 3% OV-17 was used at column temp. 215° and injection temp. 265°, with helium as carrier gas at a flow rate of 1.5 ml min^{-1}. For detection TIC detector was used and ionization was performed at 70 eV. R_t, bergapten 8.5 min, xanthotoxin 8.8 min. [M$^+$], bergapten 216, xanthotoxin 216.

Assay of PAL activity

PAL activity was determined by a radiochemical methods using [U-^{14}C]L-phenylalanine as a substrate. Crude enzyme preparation was carried out at 0–4°. Frozen cell powder (0.5 g) was mixed with polyvinylpolypyrrolidone (0.25 g) and 1.5 ml of 50 mM *tris*-HCl buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and thawed with occasional stirring for 30 min. After centrifugation at 10,000 rpm for 2 min, the supernatant was desalted (Sephadex G-25) and used as crude enzyme. A mixture of 100 mM potassium borate buffer (pH 8.7, 200 μl) containing 0.5 μmol [^{14}C]-phenylalanine (3.7 kBq) and crude enzyme soln. (50 μl) was incubated at 30° for 60 min. Reaction was terminated by adding 50 μl of 5 M HCl, 0.5 mM *trans*-cinnamic acid (10 μl) was added as a carrier, and the reaction product was extracted with toluene–EtOAc (1:1, 1 ml). After centrifugation (10,000 rpm, 30 s), radioactivity of the organic layer (0.4 ml) was measured by a liquid scintillation counter. The blank was obtained by the same method as described above using boiled enzyme. Protein was determined by Bradford's method [10], using bovine serum albumin (BSA) as a standard.

For *in vitro* experiments to examine PAL inhibition, AIP was added to the assay mixtures at a final con-

centrations of 2, 10, 50 and 100 μM. For *in vivo* experiments AIP was added to the cell cultures together with yeast extract at a final concentration of 10 μM.

Acknowledgement—We thank Prof. N. Amrhein, Institute fur Pfanzenwissenschaften, Switzerland, for his kind supply of AIP.

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