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Biosynthesis of carthamin in florets and cultured cells of *Carthamus tinctorius*

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Abstract

Phenylalanine labelled by ^{14}C was administered to the cultured cells and the intact flowers of *Carthamus tinctorius*, and the biosynthetic activity of carthamin in these two materials was compared. The cultured cells took up positively the fed substrate, but they could not incorporate the label into carthamin, while incorporation of the radioactivity from phenylalanine into the red pigment occurred in the intact flowers. The activities of polyphenol-oxidizing enzymes were screened in the cell cultures and the intact tissues from the herbal plant. Polyphenol-oxidizing enzymes were operative normally in the mother explant, whereas their activity patterns changed altogether in the cultured cells, where kurenamin, a new reddish pigment, is produced actively. The data are discussed in terms of the phenotypic changes in the polyphenol metabolism of the cultured cells propagated under restricted culture conditions.

Introduction

It has long been recognized that metabolic pattern often changes greatly in some explants when treated under specific culture conditions. Qualitative changes in a certain metabolite can occur, which may in extreme cases result in its total disappearance (Lee *et al.* 1972) and/or the formation of a completely new products (Forrest 1969, Alfermann and Reinhard 1971, Sugano *et al.* 1971). In spite of

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extensive investigations on the cultured explants and cells, many questions about the regulatory mechanism of the unique responses remain largely unknown. In particular, the extent of our knowledge of the metabolic change induced at early stage of the cell propagation is very limited. Even though some published data bring useful information, they are still almost all speculative and not conclusive.

C. tinctorius is an important source of the tinctorially useful dye-stuff, carthamin, which is accumulated in the tubular flowers through an enzyme process (Saito *et al* 1983). *In vitro* cultured cells of the flowering plant give no carthamin, however they retain a potential activity to produce another red pigment, kurenamin, at a considerably high level (Saito *et al.* 1988). This phenotypic difference seems to be helpful for us to find out a clear-cut distinction in the metabolic diversibility between intact tissues and cultured cells more precisely. Only through the comparative studies, some notable evidence can be obtained, showing that the imaginary biochemical modification is practically introduced into the cell cultures during the course of the special cultivation.

As an introduction to the studies, we performed here two parallel experiments, radiolabelled precursor feeding and enzyme activity estimation as presented in the following sections.

Materials and methods

Plant material: The seeds of *Carthamus tinctorius* L. were grown in the greenhouse at 28 ± 1 °C. After about three months' cultivation, small flower buds were collected randomly. For the feeding of radioactive precursor, ten buds, whose sizes are 1.21 ± 0.82 cm, were used. The cell cultures were originally derived from the meristem tissues. The explants were placed, if not specified otherwise, on LS medium and cultured at 25 ± 1 °C in the dark. About three weeks' cultivation, the excised segments had became callus. Relatively friable callus pieces were transferred to 300 cm^3 flasks containing 75 cm^3 liquid medium of the same composition as the callus medium, but with no agar. After a week, 15 to 20 cm^3 portions of the suspension were transferred to flasks containing fresh medium. This process was repeated every 3 to 4 d to enrich the rapidly growing cells. The cells thus obtained were subcultured routinely at 6- to 8-d intervals.

For inducing kurenamin, cell aggregates were transferred to 75 cm^3 of fresh liquid medium in 300 cm^3 flasks containing LS medium and additional components, D-phenylalanine (165.2 mg l^{-1}) and cellulose powder (40 g l^{-1}), and agitated on rotary shaker at 100 rpm in the dark at 25 ± 1 °C.

Feeding with radiolabelled phenylalanine: The buds with short stems were dipped upwards in small vials with aqueous solution (2 cm^3 each) that contained 0.74 MBq [U-^{14}C]phenylalanine and incubated for 6 d under white-light at 25 °C. Occasionally, distilled water was added to ensure the complete uptake of the fed compound. $^{14}CO_2$ resulting from the respiration was trapped continuously by scintillamine-OH during the feeding experiment.

For feeding of radioactive phenylalanine to cultured cells, two different media, LS and MS were used. The cultures were incubated for 14 d under white-light at 25 °C in 200 cm^3 fkasks with 50 cm^3 medium containing, if not indicated otherwise, 2,4-D (0.1 mg l^{-1}), BA (1 mg l^{-1}) and 2.96 MBq [U-^{14}C]phenylalanine. Released $^{14}CO_2$ was kept by scintillamine-OH solution as described above.

Determination of radioactivity: At the end of the feeding, the materials were subjected immediately to the process of the extraction and partial purification of radioactive carthamin through the method of Saito *et al.* (1983).

The contribution of ^{14}C-labels to the isolated carthamin was determined by scintillation counting. The concentration of carthamin was estimated spectrophotometrically with a double-beam UV/VIS spectrophotometer, *Shimadzu U-260*. The absorbance peak at 519 nm was used for the reading. The ratio of radioactivity in dpm to absorbance at 519 nm (A_{519}) was regarded as specific radioactivity, because carthamin was too difficult to be recrystallized.

Preparation of soluble protein extracts from intact florests and cultured cells: The younger and bright-yellow florets without ovaries (9.5 - 11.5 mm, 7.61 - 8.03 g) were collected randomly from the flower buds and frozen in liquid nitrogen, then they were ground with a pestle in a glass mortar. Thus obtained fine powders were thawed in an appropriate amount of maceration buffer containing 30 mM D-isoascorbic acid and 0.1 mM 2-mercaptoethanol in 50 mM citrate/phosphate buffer, pH 7.0. The suspension was poured into a pre-cooled beaker, allowed to stand for 10 min under continuous stirring, then passed through two layers of nylon-cloth and the filtrate centrifuged at 20000 *g* for 20 min. Solid $(NH_4)_2SO_4$ was added to the supernatant solution to obtain 35 % saturation. The precipitate was removed by centrifugation (5 min at 20000 *g*) and the supernatant was raised to 80 % $(NH_4)_2SO_4$ saturation. After stirring for 20 min in an ice-cold box, the precipitated protein was collected by centrifugation (5 min at 20000 *g*). The protein fraction was dissolved into a minimum volume of citrate/phosphate buffer, pH 7.0 and the solution passed through a *Sephadex G-25 gel* column. The transit protein solution was retained at 2 - 4 °C for using to the subsequent processes. An aliquot of the protein solution was charged onto a column (2.5 × 90 cm) of *Sephadex G-100* gel and chromatographed in citrate/phosphate buffer (50 mM, pH 7.0).

Protein extracts from the cell cultures were prepared by the same method as described above. For this process, cultured cells (10-15 g fresh mass) from the test batches were used. The protein fractions precipitating at 36 - 80 % $(NH_4)_2SO_4$ saturation were applied to the following enzyme assays after being desalted through a *Sephadex G-25* column.

Enzyme assays: CSE (carthamin-synthesizing enzyme), MOG (monophenol monooxygenase) and POD (peroxidase) were assayed by the standarized procedures (Saito *et al.* 1986, 1989a). CDE (carthamin-decomposing enzyme) activity was estimated spectrophotometrically by monitoring the decrease in absorbance at 519 nm when enzyme protein (0.5 cm^3, 1.0 - 1.3 μg protein) was added to 2 cm^3

citrate/phosphate buffer, pH 7.0 containing 55 μM carthamin and 0.00015 % (v/v) H_2O_2 (Kanehira and Saito 1990). Protein contents were determined by the method of Lowry *et al.* (1951).

Chemicals: [U-^{14}C]phenylalanine (15.0 GBq mol^{-1}) was purchased from *ICN Radiochemical* (Irvine, USA). Scintillamine-OH was supplied by *Dojindo Laboratories* (Kumamoto, Japan). Precarthamin used as CSE substrate was from our laboratory collection. Carthamin was prepared from the reddened florets of *C. tinctorius* according to the method of Saito *et al.* (1989a). BA and NAA were furnished by *Wako Pure Chemical* (Osaka, Japan). Cellulose and silica TLC plates were purchased from *Merck* (Darmstadt, FRG). Other chemicals used were all analytical grade of purity and obtained from several commercial sources. *Sephadex G-25, G-100, LH-20* and blue dextran were purchased from *Pharmacia Fine Chemicals* (Uppsala, Sweden). Avicel cellulose was a product of *Asahi Kasei Kogyo* (Tokyo, Japan).

Results

Intact florets and cell cultures took up the fed ^{14}C-phenylalanine (Table 1). Florets absorbed 99.7 % of the total radioactivity administered during the feeding of 6 d. Cultured cells also took up the amino acid markedly (98 %) in 14-d incubation. Only 0.3 % radioactivity taken up by the florets was detected in the K_2CO_3 fraction containing carthamin. The proportion of radioactivity of the final extract obtained by aqueous acetone (extracts of florets: 0.013 %, cultured cells: 0.02 %) was very small.

Table 1. Distribution of radioactivity in various fractions of florets and cultured cells after incubation with [U-^{14}C]phenylalanine.

	Floret radioactivity		Cultured cell radioactivity	
	[s^{-1}]	[%]	[s^{-1}]	[%]
1	4.44×10^7	100	1.77×10^8	100
2	4.43×10^7	99.7	1.73×10^8	98.0
3	1.33×10^5	0.3	-	-
4	5.69×10^3	0.013*	3.60×10^5	0.20*

1 - total radioactivity administered; 2 - radioactivity taken up; 3 - radioactivity extracted by 0.5 % K_2CO_3; 4 - radioactivity recovered with 65 % (v/v) aqueous acetone (recovery rate 80.3 %). *recovery rate was corrected.

Incorporation of labelled phenylalanine into carthamin was studied by one-dimensional chromatography on silica or cellulose TLC plates. The radioactivity on the air-dried plate was determined in the scraped powders. In the extracts from florets incubated with [U-^{14}C]phenylalanine a high level of the radioactivity was found in the region where authentic carthamin was located. In contrast, TLC of the extracts

from cultured cells exhibited a random distribution of radioactivity without any correlation to the position of carthamin (Fig. 1).

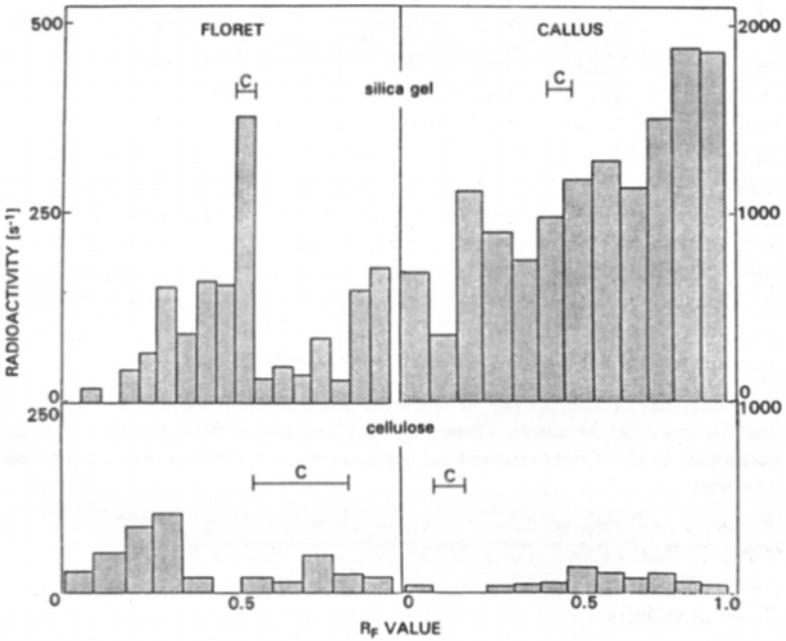

Fig. 1. TLC of extracts prepared from intact florets and cultured cells incubated with [U-^{14}C] phenylalanine. Both TLC plates were developed in *n*-butanol/acetic acid/water (4:1:2, v/v). The radioactive extract prepared from intact florets (0.288 g, total radioactivity 2654 s^{-1}) was applied to silica gel TLC and the region of authentic carthamin was scraped and extracted. The concentrated extract (radioactivity 421 s^{-1}) was applied again to cellulose TLC. The radioactive extract prepared from subcultured cells (6.38 g, total radioactivity 1726 s^{-1}) was applied to sillica gel TLC and the region of authentic carthamin was scraped and extracted. The concentrated extract (radioactivity 970 s^{-1}) was applied again to cellulose TLC. For further details of the chromatographic operation or others, see text. C - carthamin.

For corroborating net synthesis of carthamin from the fed substrate, column chromatography was repeated four times on *Sephadex LH-20* gel. Since carthamin is difficult to be recrystallized, we used here a conventional method, gel permeation in methanol/acetic acid/water (4:1:2, v/v). The ratio of radioactivity to absorbance at 519 nm was regarded as specific activity of carthamin. The specific radioactivity in carthamin recovered from florets is always constant even repeating the chromatographic treatment (Fig. 2). The specific value in carthamin from cell cultures, on the other hand, changes greatly during the repeated gel permeation. It decreases sharply to the bottom level only by the permeation. We performed similar experiments using cells cultured under different conditions as described in Table 2. However, no radioactive phenylalanine was incorporated into carthamin, which was observed after purification of an authentic carrier added (data not shown).

In order to confirm above data from the radiotracer feeding experiments, we carried out further enzyme activity screening both in intact florets and cell cultures

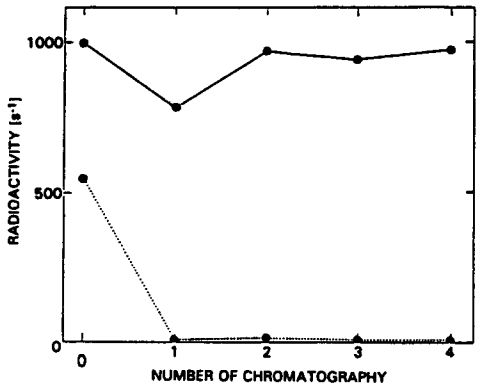

Fig. 2. Distribution of radioactivity in recovered fractions from repeated chromatography on a calibrated *Sephadex LH-20* column. Chromatography was done in methanol/acetic acid/water (4:1:2, v/v). For further details of rechromatographic operation, see text. Florets - *open circles*, cultured cells - *closed circles*

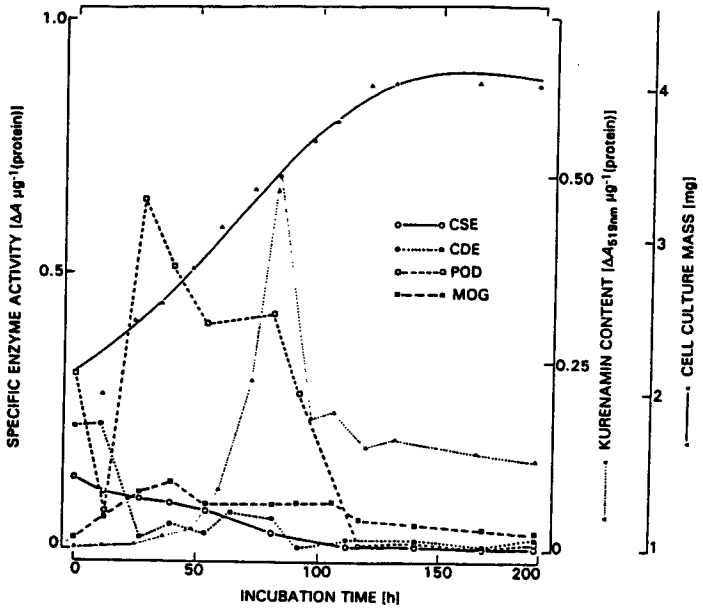

Fig. 3. Time-course of kurenamin accumulation, fresh mass increment and enzyme activity change in the cell cultures of *C. tinctorius*. Aliquots of cell cultures from a batch of Table 3 were maintained in a kurenamin inducing medium (Saito *et al.* 1988) for about 190 h. The resulting cultured cells (10 - 15 g fresh mass) were used for each determination. For details of protein extraction, enzyme assays or others, see text and a reference (Saito *et al.* 1988).

Table 2. Conditions of [U-14C]phenylalanine feeding to cultured cells.

	Cells used*	Culture condition medium	phytohormone [mg l^{-1}]	Time of feeding
I	small aggregated cells (white)	MS	2,4-D: 0.1, BA: 1.0	the beginning of culture
II	small aggregated cells (white)	LS	NAA: 0.002	the beginning of culture
III	small aggregated cells (white)	LS	NAA: 0.002	ten days after culture (log phase, latter)
IV	large aggregated cells (yellow)	MS	2,4-D: 0.1	the beginning of culture
V	large aggregated cells (yellow)	MS	BA: 1.0	the beginning of culture
VI	small aggregated cells (white)	LS	ABA: 0.26	seven days after culture (log phase, earlier)
VII	cells from the third generation after being transferred from MS medium	MS	2,4-D: 0.1, BA: 1.0	ten days after culture (log phase, latter)

*cells cultured in the MS liquid medium which contained 2,4-D (0.1 mg l^{-1}) and BA (1.0 mg l^{-1}) were transferred to each medium indicated and cultured for 14 d. Labelled phenylalanine was fed at the time indicated. Carthamin was extracted at 14 d after culture.

from *C. tinctorious*. The chromatographic patterns of polyphenol-oxidizing enzyme activities in the extracts from the younger and bright-yellow florets are resolved on a *Sephadex G-100* gel column as reported in our previous papers (Homma *et al.* 1985, Kanehira and Saito 1990). The patterns, however, are variable: they are changed during the course of cell proliferation in cultured media (Saito *et al.* 1989). The reliability of this fact is clear from the results of Table 3 which were obtained from a cell line maintained in LS medium for several weeks. In particular, CSE and CDE activities are reduced considerably through proliferating the floral meristematic cells in the culture medium, showing the ratios as follows: CSE (1/4.3), CDE (1/2.2), MOG (1/0.6), POD (1/0.6). These values are disordered again by transferring the cell cultures to a new proliferating medium. Fig. 3 presents the induction of a pigment synthesis and the changes in enzyme activities during cell propagation in a LS medium, whose composition was modified slightly (Saito *et al.* 1988). A red pigment, named kurenamin appears at a time of 24 h after cell inoculation (the end of lag phase) as a sharp increase, the content attains a maximum (after 82 h, the middle of log phase), and then it decreases quickly to a constant level at the stationary phase. With CSE, its activity went done hastily after cell cultures were inoculated into the medium. CDE activity also diminished progressively by cell cultivation. MOG activity rose by degree up to 35 h, then reached to a constant level. POD increased abruptly till 24 h after the cell inoculation and subsequently dropped in haste.

Table 3. Comparison of polyphenol-oxidizing enzyme activities in aqueous extracts from intact florets and cultured cells of *C. tinctorius*. The samplying of florets was done as described in Materials and methods. The cells used in this study were sampled from a batch subcultured two times in the LS agar-free medium at each time of 6- to 8-d intervals. For further details of enzyme preparation and activity screening, see Materials and methods.

Enzyme	Specific activity [ΔA μg^{-1}(protein)]	
	intact florets	cultured cells
CSE	0.525	0.123
CDE	0.472	0.217
MOG	0.0082	0.015
POD	0.184	0.313

Discussion

C. tinctorius is a suitable material for studying metabolic deversibility between intact tissues and cultured cells. It can proliferate a new type of a cell line which produces, kurenamin, instead of its inherent carthamin (Saito *et al.* 1988). The carthamin has already been indicated to be formed *via* the malonate-shikimate pathway (Fukushima *et al.* 1990). On the basis of above evidence, much attention has exclusively been centred here to the changes in pigment synthesis and enzyme activity induced in a living system under compulsorily defined culture conditions.

It has been well established in the current studies that the alteration in polyphenol metabolism is introduced into the explant cells promptly upon being propagated in culture media. These have been corroborated from the parallel two experiments, radioactive precursor administration and enzyme activity estimation. With regard to [U-^{14}C]phenylalanine feeding, the ratios of radioactivity of phenylalanine to A_{519}, which correspond to the specific activity of radioactive carthamin, were constant during repeated purification of the carthamin fraction from the intact florets of *C. tinctorius*. Similar attempts were also made with cell cultures, while no carthamin was detected in any cell lines which were derived from the same plant material. If carthamin could be accumulated only too small amount to be detected on TLC plates, it would be difficult to prove *de novo* synthesis of the pigment by this conventional chromatographic methods. Thus, by applying the same techniques as used in fresh florets, we examined the productivity of carthamin and the variability of [U-^{14}C]phenylalanine incorporation by various cell strains cultured under different conditions. Nevertheless, little [U-^{14}C]phenylalanine contributed to the carthamin synthesis in all test cell propagates. These data suggest that carthamin is not synthesized in the cultured cells used.

With an aim of corroborating above results from the radioactive precursor feeding, we screened the activity variation of phenol-oxidizing enzymes including CSE and CDE, the latter two of which are involved in carthamin turnover process in intact florets and cultured cells. The enzymes are active in florets, while they all tend to

decrease when the explant tissues are transferred to the culture medium, although no positive restriction is apparent on the cell growth (see Fig. 3). CSE was found to keep its high activity in the intact flowers. However, the activity of the enzyme is reduced conspicuously in the cell cultures (Table 3 and Fig. 3). With CDE, the activity goes done progressively upon proliferation of the cells in the culture media (Table 3 and Fig. 3). These support strongly the results from the radioisotope experiments. Both MOG and POD rise initially just after transferring the cells to a kurenamin inducible medium, whereas they also lost their activities before long (Fig. 3).

Variation in the patterns of metabolite productivity and enzyme reactivity has been reported in callus and cell cultures from various plant materials (Forrest 1969, Sugano *et al.* 1971. Alferman and Reinhard 1971, Lee *et al.* 1972, Saito *et al.* 1989a). Saito *et al.* (1988) propagated carthamin-free and kurenamin-producing cell strains from the floral meristems of *C. tinctorius* under a specific nutritional and hormonal conditions. These characteristic contrasts can be assumed to be the consequence of the totipotency of the *Carthamus* cells, whose inherited biochemical potential corresponding to the genome of the entire plant is prerequisite to certain biochemical diversity, the intermediate expression of the genetic information being determined by the interaction of the cells with both endogenous and exogenous conditions (Aitchinson *et al.* 1977). Thus, no production of carthamin in cell cultures can be regarded as a result of the differentiation process controlled by genetic factors together with environmental conditions (Kurz 1984). Accordingly, we supposed that in our currently used cell lines, an intermedial step(s) of the synthetic pathway which leads to the formation of precarthamin is perhaps temporarily blocked at the deamination step. This postulation came from the following two reasons mainly: (*1*) labelled phenylalanine is taken up by the cultured cells, while little of it is incorporated into carthamin, (*2*) CSE and CDE, both of which regulate initial steps of carthamin turnover process, are operative even in the cultured cells (Table 3 and Fig. 3), yet catalyze to produce no carthamin. It is likely that the deficient supply of the enzyme substrates to CSE and CDE reflects directly on generating the carthamin-free predisposition. The normal regulatory mechanism from the inherent genetic control must compulsorily be "switched" at the initial stage of cell differentiation and elongation to certain abnormal ways *via* supplying with of without nutritional and/or hormonal factors at various concentrations, some of which are too deficient and some are too excessive to keep normal metabolic processes with the living cells. The inability of the cultures to accumulate carthamin cannot be the result of the loss of the biochemical potential, the cells only indicate the impossibility of its realization under the particular conditions (Liau and Ibrahim 1973). Therefore, it may safely be said if carthamin formation is expected to be induced in cultured systems, it is advisable to re-examine closely the culture conditions, including the composition of phytohormones, nutrients and trace elements.

Thus, it can be summarized that the metabolic pattern of polyphenols in intact cells is changed promptly and easily just after being inoculated certain explant cells into culture media, and the vital changes are once introduced, the cells are kept propagating to grow into different phenotypes, inside of which an increased ability

can offer to produce polyphenols contrasting markedly with those of the original plant cells.

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