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ANTHOCYANIN-PRODUCING DANDELION CALLUS AS A CHALCONE SYNTHASE SOURCE IN RECOMBINANT POLYKETIDE REDUCTASE ASSAY

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Key Word Index—*Taraxacum officinale*; Compositae; *Glycyrrhiza echinata*; Fabaceae; callus cultures; anthocyanin; flavonoid biosynthesis; chalcone synthase; polyketide reductase.

Abstract—Purple-coloured dandelion (*Taraxacum officinale*) callus cultures producing anthocyanin pigments were established on a cytokinin-rich medium under the light. When the cells were placed in the dark, only grey cells proliferated. Anthocyanin productivity of these cells was partially restored in the light. The major pigment was identified as cyanidin 3-(6″-malonylglucoside). The lower stem of the original plant contained the same pigment. Chalcone synthase (CHS) activity was detected in the extracts of these purple cells, whereas no activity was observed in grey cells propagated in the dark. When the CHS-active cell-free extract was combined with the extract of *Escherichia coli* over expressing polyketide reductase (PKR) cDNA of licorice (*Glycyrrhiza echinata*), isoliquiritigenin (a 6′-deoxychalcone), in addition to naringenin (a 5-hydroxyflavanone), was detected as the reaction product from 4-coumaroyl-CoA, malonyl-CoA and NADPH. This result confirms the catalytic function of the PKR gene product. © 1997 Elsevier Science Ltd

INTRODUCTION

The chemical constituents of dandelion (*Taraxacum officinale*; Compositae) have been studied extensively, because the plant has long been used for medicinal purpose and as a foodstuff. It contains, for example, sesquiterpene and butyrolactone glycosides [1, 2], sterols [3] and triterpenes [4]. A recent paper [5] describes the phenolic constituents of dandelion tissues and medicinal preparations, and reveals their flavonoid and caffeoyl ester components. However, there has been no report on anthocyanin pigments from dandelion.

We have examined isoprenoid metabolism in cultured dandelion tissue [6–8] from the viewpoint of the expression of specific triterpenoid biosynthesis accompanied by organ-differentiation (especially of laticifer cells), taking advantage of the surprisingly high regeneration capacity of dandelion cultures [6, 9–11]. Reports from our and other laboratories have shown that dandelion tissue cultures contain triterpenoids [6, 7] and volatile metabolites [12]. In the first part of the present paper, we report on the anthocyanin-producing dandelion callus cultures established on a cytokinin-rich medium. The major anthocyanin was identified as cyanidin 3-(6″-malonylglucoside), and the production pattern of the callus pigments was compared to that of the lower stem of the original plant.

The anthocyanin-production in the cultured cells could be correlated with the extractable activity of chalcone synthase (CHS), a key enzyme of the flavonoid biosynthetic pathway (Fig. 1). In many leguminous plant cells, CHS and a polyketide reductase (PKR) co-act to perform 6′-deoxychalcone synthase (DOCS) activity (Fig. 1) [13]. DOCS activity is especially important in defence responses of leguminous plants against microbial infection as it leads to the production of 5-deoxyisoflavonoid-derived phytoalexins [14]. Theoretically, DOCS activity is not performed by sequential CHS and PKR reactions. Rather, the reductase should act on an unidentified substrate which is an intermediate of chain elongation steps of CHS reaction [15]. PKR cDNA has been cloned from soybean (*Glycine max*) [16, 17], kudzubean (*Pueraria lobata*) [18], alfalfa (*Medicago sativa*) [19, 20] and licorice (*Glycyrrhiza echinata*) [21]; but enzymatically active proteins overexpressed in heterologous systems have only been demonstrated for soybean reductase [16, 22]. This is due to the difficulty in the preparation of appropriate assay systems for

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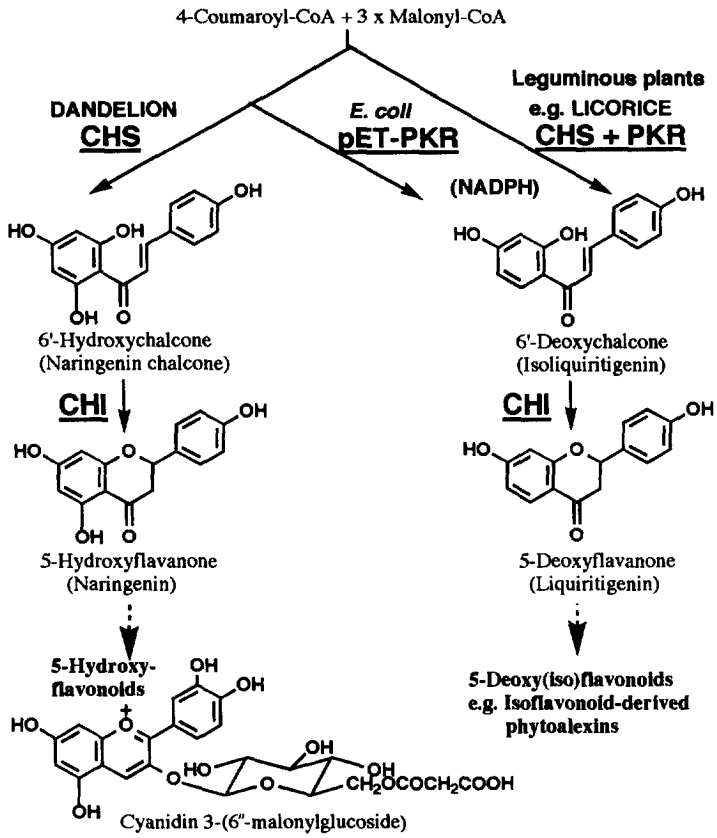

Fig. 1. Biosynthesis of 5-hydroxy- and 5-deoxy-flavonoids in plant cells and in combined cell-free systems of dandelion and *E. coli* harboring PKR cDNA of licorice.

the PKR activity. In the latter part of this report, enzyme activity of heterologously overexpressed licorice PKR protein is demonstrated by the use of cell-free extract of anthocyanin-producing dandelion callus as the donor of CHS proteins.

RESULTS AND DISCUSSION

Establishment of the pigment-producing cell line and the anthocyanin structure

Repeated selection of red-purple cells that appeared as spots on the surface of callus masses grown on a cytokinin-rich medium (1 mg l^{-1} kinetin) under light yielded a deep purple cell line, which stably proliferated under 12 hr light/12 hr dark conditions. When the cells were kept under continuous dark, only grey cells devoid of pigments proliferated. These dark-grown callus restored purple colour (although the extent was somewhat low compared to the original deep purple cell line) after they were returned to light. The colouration was thus strictly light-dependent.

To determine the structures of anthocyanins, 1% HCl extract was first analysed by TLC and HPLC, and, among the partially decomposed products, cyanidin 3-glucoside was identified. This was further confirmed by FAB-mass spectrum of the sample recovered from TLC. The main anthocyanin having cyanidin 3-glucoside as its partial structure was extracted with MAW (MeOH–HOAc–H_2O, 9:1:10) and purified by HP-20 and LH-20 resin column chromatography and preparative reversed-phase HPLC. Its M_r was determined to be 535 by FAB-mass spectrometry, indicating that the anthocyanin is composed of cyanidin 3-glucoside (M_r 449) and an additional organic acid (e.g. malonic acid; M_r 104) esterified to the sugar.

The complete structure was deduced from 2D NMR (see Experimental). Thus the major anthocyanin is cyanidin 3-(6″-malonylglucoside) (see Fig. 1). This compound is widespread in the plant kingdom [23], and has been isolated from cell cultures of *Centaurea cyanus* [24]. The anthocyanin content in the dandelion callus was estimated to be *ca* 0.05% of fresh weight by spectroscopic measurement of the MAW extracts at 530 nm. The original dandelion plants are red in the epidermis of the lower stem and petiole. MAW extracts of the stem were purified by successive resin column chromatography. HPLC analysis indicated that the major stem anthocyanin is also cyanidin 3-(6″-malonylglucoside).

CHS activity in anthocyanin-producing dandelion callus

The extractable activity of CHS, a key enzyme of flavonoid/anthocyanidin biosynthesis, was examined

in the coloured and colourless cells of dandelion. The reaction products from the incubation of 4-coumaroyl-CoA and [2-^{14}C]malonyl-CoA with 10000 *g* supernatant fractions of the light-grown (purple) and dark-grown (grey) dandelion cells were visualized by TLC-radiochromatography (data not shown). Because of the occurrence of chalcone isomerase (CHI) in the extract, a flavanone, naringenin, was the major radioactive product of the combined CHS/CHI reaction in the light-grown cells. Even when NADPH was incorporated in the enzyme reaction mixture, no 6′-deoxychalcone (isoliquiritigenin) or 5-deoxyflavanone (liquiritigenin) was detected, indicating that dandelion cells lack DOCS activity. In the dark-grown cells, no clear reaction product has been detected. Based on the recovered radioactivity in the naringenin spot, the CHS activity of the purple cells was estimated to be 153 nkat kg^{-1} protein.

Utilization of dandelion cell-free extract as CHS protein donor for DOCS assay with heterologously-expressed licorice PKR

The PKR cDNA of licorice has been cloned by plaque hybridization using soybean reductase as a probe (the nucleotide sequence of licorice PKR cDNA is found in DDBJ/GenBank/EMBL database under the accession number D83718) [21]. The identity of the cDNA has been estimated by a very high sequence homology with those from soybean [16] and alfalfa [19, 20]. Because the substrate for PKR has not yet been identified, the catalytic activity of PKR can only be measured as DOCS in combination with CHS protein. Thus, the purified soybean reductase has been combined with parsley CHS [25], and, more recently, soybean reductase and mustard CHS cDNAs have both been expressed in *E. coli* [22] to perform DOCS activity.

The presence of CHS lacking DOCS activity in coloured dandelion cells illustrated above satisfied the prerequisite for their utilization as the CHS donor in the demonstration of catalytic activity of the licorice PKR cDNA product (see Fig. 1). In order to overexpress the PKR cDNA, the sequence containing the coding region was excised from pBluescript and subcloned into the pET-21a vector, after the introduction of a new restriction (*Nde* I) site just before the start codon (ATG) by side-directed mutagenesis (see Experimental). *E. coli* cells harbouring pET-PKR thus produced were induced to translate the recombinant DNA. Immunoblot analysis of the protein extract using anti-(soybean reductase) antiserum displayed a band of expected M_r (*ca* 34 k, see [26]). The enzyme activity of the extract was then examined, and the typical results are shown in Fig. 2. Isoliquiritigenin (a 6′-deoxychalcone) was clearly produced in the combined dandelion—*E. coli* (pET-PKR) cell-free systems from 4-coumaroyl-and malonyl-CoAs and NADPH, although the major product was naringenin. In contrast, the dandelion extract alone and the combination with non-transformed *E. coli* produced only naringenin, and no radioactivity in the 6′-deoxychalcone could be detected. The reason for the formation of both 5-deoxy- and 5-hydroxy-type compounds in the reaction with combined extracts, and even with licorice extracts, is not known, but similar results have been reported in the assays with purified soybean reductase and parsley CHS [25] and with recombinant soybean reductase and mustard CHS [22]. In our experiments, the exact ratio of CHS/PKR proteins present in the assays has bot been determined, but an excess PKR could be estimated from the total protein in plant and bacterial extracts (see Experimental). Clearly, more studies will be needed to understand the nature of the protein interactions in DOCS reaction. Nevertheless, the catalytic activity of the protein translated from cloned licorice PKR cDNA has now been definitely demonstrated. The results also demonstrate the general association of PKR and CHS proteins beyond the original plant species.

Finally, the absence of liquiritigenin (a 5-deoxyflavanone) among the products of the combined incubation (Fig. 2) raised the possibility that CHI of dandelion does not employ 6′-deoxychalcone as a substrate. In fact, only naringenin chalcone could be used as the substrate of CHI in dandelion crude extract, and isoliquiritigenin failed to serve as the substrate (data not shown).

EXPERIMENTAL

Tissue cultures and selection of anthocyanin-rich cells. Callus cultures [7] established on Murashige–Skoog's agar (0.9%) medium containing 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg l^{-1} kinetin and 0.1% coconut H_2O were transferred onto the same basal medium with 0.1 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin, and maintained under *ca* 5000–8000 lux light (12 hr)/dark condition at 25°. The red-purple spots, which appeared on callus cultures were repeatedly transferred onto freshly prepd medium every 3 weeks, and after *ca* one year of subculture, totally purple callus cultures were established.

Extraction and purification of pigments. HCl extraction. The fresh purple callus cells (*ca* 3 kg) were extracted with 1% HCl at 4° twice. The combined anthocyanin extract (10 l) was adsorbed on a Diaion HP-20 resin column (50 × 300 mm), and after washing with 1% HCl (15 l) was eluted with 1% HCl in MeOH. From the concd eluate the anthocyanin mixt. (*ca* 8.1 g) was Et_2O-pptd. By TLC (see below), four bands could be sepd, and, among those, cyanidin 3-glucoside was identified.

Acetic acid extraction. The purple callus cells (4325 g) were soaked in MAW (MeOH–HOAc–H_2O, 9:1:10) and allowed to stand at 4° overnight and filtered twice. The reddish extract (10 l) was concd to *ca* 3 l, which was adsorbed on a Diaion HP-20 resin column (50 × 400 mm), washed with 3% HOAc (15 l), and then eluted with MeOH containing 3% HOAc to

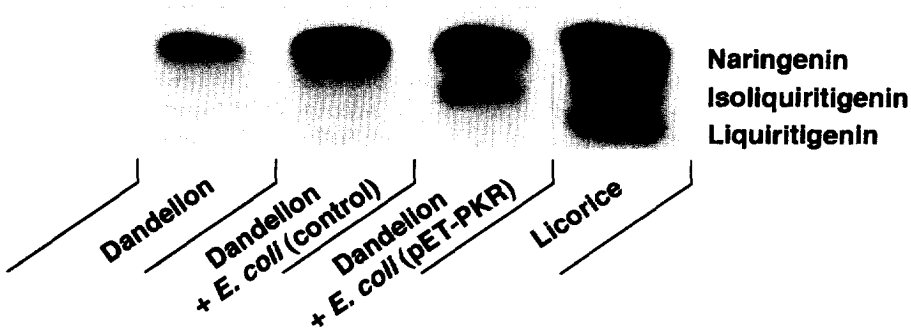

Fig. 2. DOCS assay using extracts from *E. coli* and dandelion cells. The products from the incubation of 4-coumaroyl-CoA, [2-^{14}C]malonyl-CoA and NADPH with indicated cell-free extracts were applied to silica gel TLC, and radioactive products were identified by autoradiography. DOCS activity is diagnosed by the formation of radioactive isoliquiritigenin and liquiritigenin. As a positive control for DOCS activity, TLC-autoradiogram of the products from the assay using the cell-free preparation of yeast extract-induced (12 hr) licorice cultures is shown (the track 'licorice'; for details, see Ref. [15]).

give 13.35 g of amorphous powder by Et_2O pptn of the condensed eluate. The ppts were dissolved in small amounts of MAW 136 (MeOH–HOAc–H_2O, 1:3:6) and passed though a Sephadex LH-20 column with the same solvent. The eluate containing anthocyanin was concd *in vacuo*, and the residue was Et_2O pptd to yield *ca* 2 g of anthocyanidin mixt. The dried pigment was dissolved in small amounts of MAW and were submitted to prep. HPLC. The eluted pigment was Et_2O pptd as above.

Anthocyanin analysis. TLC was performed on microcrystalline cellulose plates (Avicel SF, Funakoshi or Art. 5577, Merck) using solvent systems of AHW (HOAc–HCl–H_2O, 15:3:82) BAW415 (*n*-butanol–HOAc–H_2O, 4:1:5, upper layer), BuH (*n*-butanol–1N HCl, 1:1), and 15% HOAc. R_f values for cyanidin 3-glucoside were 0.29 (AHW), 0.31 (BAW415), 0.09 (BuH) and 0.45 (15% HOAc): and for cyanidin 3-(6″-malonylglucoside); 0.45 (AHW), 0.65 (BAW415), 0.44 (BuH) and 0.60 (15% HOAc). Analytical HPLC was run on a Shim-pack CLC-ODS column (6.0 × 150 mm) at 35° with a flow rate of 1 ml min^{-1} with linear gradient elution for 40 min from 25 to 100% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O). R_t's for cyanidin 3-glucoside and cyanidin 3-(6″-malonylglucoside) were 11.2 min and 16.2 min, respectively. Prep. HPLC was performed using a Shim-pack Prep ODS column (20 × 250 mm) at 35° with a flow rate of 6 ml min^{-1} by isocratic elution with the solvent 1.5% H_3PO_4, 10% HOAc and 12.5% MeCN in H_2O (R_t for cyanidin 3-glucoside, 7.0 min; R_t for cyanidin 3-(6″-malonylglucoside), 10.0 min). In both analytical and prep. HPLC's, the eluent was monitored at 530 nm. NMR spectra were recorded on a Bruker ARX-500 system either in DMSO-d_6 containing 10% DCl solution (20%) in D_2O or in DMSO-d_6 with 10% CF_3COOH. ^{1}H NMR of cyanidin 3-(6″-malonylglucoside) (DMSO-d_6 with DCl) δ: 8.66 (1H, *s*, H-4), 8.05 (1H, *dd*, J = 2.0, 8.7 Hz, H-6′), 7.91 (1H, *d*, J = 2.1 Hz, H-2′), 7.00 (1H, *d*, J = 8.8 Hz, H-5′), 6.93 (1H, *s*, H-8), 6.69 (1H, *s*, H-6). Glucose moiety: 5.24 (1H, *d*, J = 7.7 Hz, H-1), 4.28 (1H, *d*, J = 11.2 Hz, H-6_a), 4.03 (1H, *dd*, J = 7.9, 11.9 Hz, H-6_b), 3.69 (1H, *t*, J = 9.2 Hz, H-5), 3.44 (1H, *t*, J = 8.4 Hz, H-2), 3.35 (1H, *t*, J = 9.2 Hz, H-3), 3.16 (1H, *t*, J = 9.4 Hz, H-4). Malonyl CH_2: 3.22 (2H, *s*).

Preparation of dandelion and licorice cell-free extracts. Freshly harvested dandelion callus or elicitor-treated licorice cells [15] (10 g each) were homogenized in a chilled mortar with sea sand (2.5 g) and 10 ml of 0.1 M K-Pi buffer (pH 6.0) containing 20 mM sodium ascorbate and 25% glycerol. The resulting slurry was filtered through eight layers of cheesecloth and centrifuged at 10000 *g* for 10 min. The supernatant soln was mixed with 2.5 g of Dowex 1-X2 (equilibrated with 0.1 M K-Pi buffer, pH 6.0) and left standing for 20 min with occasional gentle stirring. The soln obtained by filtration on a glass-sintered filter was used as crude enzyme.

CHS and DOCS assay. The reaction mixts. for CHS assay consisted of [2-^{14}C]malonyl-CoA (Amersham, 55 mCi mmol^{-1}; 87000 dpm), 10 nmol 4-coumaroyl-CoA, and 1 ml of crude enzyme in a total vol. of 1.13 ml. Typically 250 μg ml^{-1} and 200 μg ml^{-1} proteins were present in dandelion and licorice crude enzymes, respectively. For DOCS assay an additional 1 mM NADPH was added to the above mixt. (total vol. 1.15 ml). After incubation at 30° for 1 hr, 2 ml of EtOAc were added and the mixt. was vortex-mixed. The EtOAc layer was sepd and co-chromatographed with standard compounds (naringenin, liquiritigenin, isoliquiritigenin) on silica gel TLC (Kieselgel F_{254}, Merck) by double development with toluene–EtOAc 3:2 or toluene–EtOAc–MeOH–petrol, 6:4:1:3, and autoradiography was carried out. The radioactive spots were scraped from the TLC plates and the radioactivity measured by liquid scintillation counting. Autoradiography film was Hyperfilm β-max (Amersham), and the exposure time was routinely one week.

Construction of an overexpression vector containing licorice PKR cDNA. Deletion of the 5′-flanking sequence of licorice PKR cDNA coding region in pBluescript [21] was carried out as follows. Three

nucleotides (AAC) upstream of the initiation codon ATG were converted to CAT by site directed metagenesis (Transformer™ Site-Directed Metagenesis Kit, Clontech) using synthetic oligonucleotide (CAACAACAACCAT*ATG*GCTGCTGCT; italicized correspond to the initiation codon) to introduce *Nde* I digestion site (CATATG). The mutated clone in pBluescript was digested with *Nde* I and *Xho* I, and the resultant fragment was subcloned into the same restriction sites of pET-21a expression vector (Novagen) to produce pET-PKR1, the sequence of which was verified by DNA sequence analysis.

Overexpression of PKR polypeptide. E. coli BL21 (DE3) transformed with pET-PKR1 was inoculated in 10 ml of LB medium containing ampicillin (100 $\mu g\ ml^{-1}$) and incubated at 37° until the $OD_{600} = 0.6$. Isopropyl β-D-thiogalactopyranoside was added to a final concn of 3 mM, and the cultures were further incubated for 6 hr at 30°. The medium was removed by centrifugation (5000 *g*, 10 min), and the cells were lysed in 500 μl of 50 mM Tris–HCl (pH 8.0) containing 2 mM EDTA, 1 mM PMSF, and 50 μg lysozyme for 15 min at room temp. The bacterial DNA was digested with 150 U of DNase I (Takara) containing a final concn of 50 mM $MgCl_2$ for 10 min at room temp. The soln was centrifuged (10 000 *g*, 10 min, 4°), and the supernatant was used for DOCS assay.

DOCS assay with E. coli *extract.* The bacterial (50 μl, 3 mg protein) and dandelion enzyme soln (1 ml, 250 μg protein) were mixed and assayed for DOCS activity as above.

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