Metabolic Activity of Hydroxycinnamic Acid Glucose Esters in Cell Suspension Cultures of *Chenopodium rubrum*

Dieter Strack and Maria Bokern

Botanisches Institut der Universität zu Köln, Gyrhofstr. 15, D-5000 Köln 41, Bundesrepublik Deutschland

Jochen Berlin and Sabine Sieg

GBF – Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-3300 Braunschweig, Bundesrepublik Deutschland

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Cell suspension cultures of *Chenopodium rubrum* accumulate high amounts of metabolically active glucose esters of *p*-coumaric and ferulic acid. Pulse-labelling experiments using [^{14}C]phenylalanine and application of the phenylalanine ammonia-lyase (PAL) inhibitor L-α-aminooxy-β-phenylpropionic acid (AOPP) revealed that the glucose esters are subject to high turnover. A considerable portion of the ferulic acid was found as an insoluble component, probably bound to cell wall material. Application of *m*-fluoro-DL-tyrosine (MFT), an effective inhibitor of tyrosine biosynthesis *via* L-arogenate, markedly increased the amount of 1-*p*-coumaroyl- and 1-feruloyl-glucose accumulated.

Introduction

There is increasing evidence that the widely occurring 1-O-acyl glucose esters of phenolic acids [1] are possible essential metabolically active intermediates in plant metabolism. Several reports describe their transient accumulation [2–4] and it is now well established that the enzymatic mechanism in the biosynthesis of conjugates can proceed *via* 1-O-glucose esters [5–9] as an alternative to the pathway *via* coenzyme A thioesters [10]. At present, involvement, regulation and specific role of glucose esters in secondary metabolism of plants are obscure. The appearance, steady-state conditions and disappearance of phenolic constituents in plants have to be considered as the results of the rates of synthesis and further metabolism, integrated into the developmental program and sensitive network of general metabolism. Thus there might be many cases of permanent flow of carbon through pools of secondary constituents [11], dependent on expression and control of the metabolic grid, into which they are integrated.

Techniques using pulse-labelling experiments with radioactive precursors (*e.g.*, [^{14}C]phenylalanine) [12] and the *in vivo* application of antimetabolite inhibitors, such as L-α-aminooxyphenyl-β-propionic acid (AOPP) [13, 14] or *m*-fluoro-DL-tyrosine (MFT) [15], are powerful tools in biochemical and physiological studies on phenylpropanoid metabolism. In the present study we applied these techniques to investigate the dynamics of the secondary phenolic metabolism in cell suspension cultures of *Chenopodium rubrum*.

The objectives of our studies were: i) to characterize the accumulation pattern of hydroxycinnamic acid glucose esters in the *Chenopodium rubrum* culture; ii) to reveal turnover rates and further metabolism of the esters; and iii) to illuminate regulatory interrelations between the metabolism of the phenolic acids and aromatic amino acids.

Abbreviations: AOPP, L-α-aminooxy-β-phenylpropionic acid; MFT, *m*-fluoro-DL-tyrosine.

Reprint requests to Dr. D. Strack.

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Material and Methods

Plant material

Cultures of *Chenopodium rubrum* (cell line CH, red coloured, betalain accumulating) were established by H. Harms from stem tissue of the intact plant. Suspension cultures were routinely maintained in 200-ml Erlenmeyer flasks, containing 70 ml MX-medium [16]. Every 14 d 2 g (fresh weight) of cell suspension were transferred to fresh medium. The CH cell line was incubated under a 16-h-day (fluorescent light, Osram L33W/77-2 Fluora) on a shaker operating at 110 rpm.

Determination of growth

Growth of the suspension culture was determined by following the increase in cell mass as a function of dry weight. Cells were collected by vacuum filtration and subjected to freeze-drying.

Isolation and identification of secondary products

Freeze-dried cells were extracted with 50% aqu. methanol. After centrifugation the extract was concentrated under reduced pressure at 30 °C. The hydroxycinnamic acid glucose esters were partially purified by polyamide CC 6 by elution with water [17] and 1-*p*-coumaroyl- and 1-feruloylglucose were identified by direct chromatographic comparison (TLC, HPLC) with those glucose esters, which had been prepared in a previous study [18]. The betalains were identified by HPLC analysis and comparison with authentic material [19].

Chromatography

Hydroxycinnamic acid glucose esters were separated and co-chromatographed with reference compounds on microcrystalline cellulose (Avicel) thin layers in BAW (*n*-butanol-acetic acid-water, 6 : 1 : 2), CAW (chloroform-acetic acid, 3 : 2, water saturated), and 5% acetic acid in water. Chromatograms were viewed under UV light (366 nm) with and without treatment with ammonia vapor. Free hydroxycinnamic acids were chromatographed on Avicel in TAW (toluene-acetic acid, 2 : 1, water saturated). High performance liquid chromatography was carried out on a Spectra-Physics (Santa Clara, Calif., USA) system, incorporating a computing integrator (System I, Spectra Physics), a Schoeffel UV/VIS-detector (Instrument Corp., Trapenkamp, FRG), and a HPLC radioactivity monitor (LB 503, Berthold, Wildbad, FRG) which was equipped with a 200 µl Cer-activated glass scintillator cell. (Yield of radioactivity was about 7%, dependent on flow rate and cell size applied.) Injection was performed *via* a Rheodyne rotary valve with a 20 µl loop. The elution system is described in Fig. 2.

Quantitative estimation of phenolic constituents and ^{14}C-incorporation

Freeze-dried tissues (50 or 100 mg) were treated with stirring for 30 min with 50% aqu. methanol. After centrifugation the pellets were reextracted twice and the combined supernatants were evaporated to dryness under reduced pressure at 30 °C. Residues were redissolved in defined volumes (200 or 500 µl). Prior to analyses of insoluble hydroxycinnamic acid, the pellets were extensively washed with water, methanol, acetone and finally ether and then dried in an exsiccator. The remaining white powder was treated with 1 N NaOH (80 °C) for 1 h and then allowed to stand at room temperature for approximately 12 h. The mixture was acidified with phosphoric acid and the liberated hydroxycinnamic acid was extracted with ether. The extract was evaporated to dryness and redissolved in a defined volume of methanol. Quantitative values were obtained with the aid of HPLC using 1-*p*-coumaroylglucose as the standard for UV-detection and [^{14}C]phenylalanine as the standard for ^{14}C-detection. ^{14}C-Chromatograms and calculation of dpm values were obtained by an Apple-computing system (Apple Computer International, France), equipped with a radio-HPLC processing program (Berthold).

Pulse-labelling experiments

Eight µCi L-[U-^{14}C]phenylalanine aliquots were added to the culture (per flask) immediately after transfer of cells into fresh medium. Samples were taken as indicated in Fig. 3.

Application of antimetabolite inhibitors

L-α-Aminooxy-β-phenylpropionic acid (AOPP) was added to the suspension culture at the 2nd and 3rd day of incubation to give a final concentration of 10^{-4} M; *m*-fluoro-DL-tyrosine (MFT) was added daily between the 5th and 9th day to give 4×10^{-4} M as final concentration. Sampling was performed as indicated in Figs. 4 and 5.

Results and Discussion

Fig. 1 shows the time course of the growth of the cell suspension culture of *Chenopodium rubrum,* cell line CH, together with the accumulation characteristics of soluble and insoluble hydroxycinnamic acids. Parallel to rapid cell growth, reaching almost 400 mg dry cell material per flask (corresponding to about 6 g l^{-1}) at day 10, there is a rapid accumulation of glucose esters of *p*-coumaric and ferulic acid.

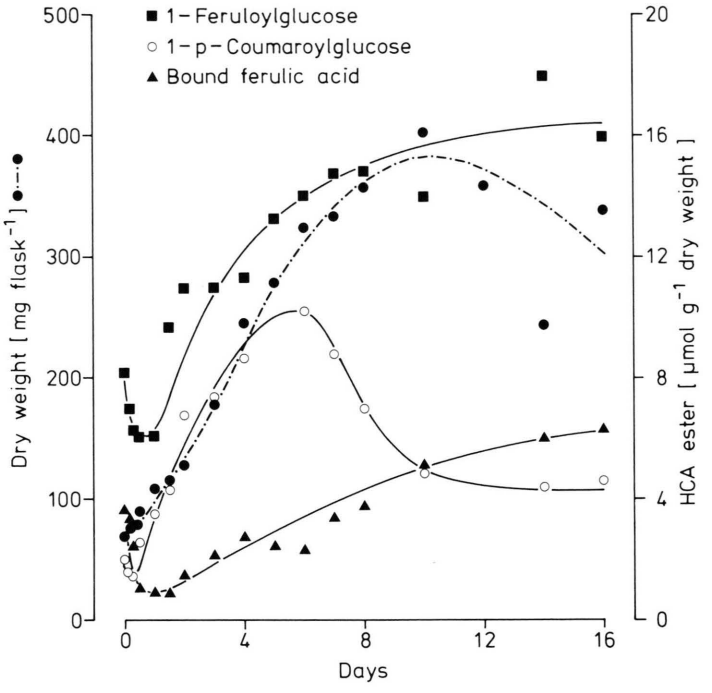

Fig. 1. Growth pattern of a cell suspension culture of *Chenopodium rubrum* and accumulation pattern of hydroxycinnamic acid (HCA) esters.

Quantification of the esters was performed by HPLC as shown in Fig. 2 (upper chromatogram). The esters were identified by their co-chromatographic (TLC and HPLC) behaviour and their appearance under UV light with and without ammonia vapour as compared to reference compounds [18]. The pool size of free 1-feruloylglucose is continuously increased to approximately 16 µmol g^{-1} dry weight in two-week-old tissue culture. 1-*p*-Coumaroylglucose exhibits a transient accumulation up to the maximal concentration of approximately 10 µmol at the 6th day of culturing and decreases during subsequent stages of growth to approximately 4.5 µmol.

A considerable amount of ferulic acid was found to be bound to insoluble cell material, which had been extensively extracted with a series of solvents (water, methanol, acetone, ether). Alkaline hydrolysis liberated ferulic acid from the insoluble cell material. Probably this portion of ferulic acid is bound to the cell wall of *Chenopodium*. The structure and possible functions of feruloylated cell wall material in cell cultures have recently been discussed in a study on *Spinacia* cell suspension culture [20].

Previous observations have shown that glucose esters of phenolic acids are accumulated transiently as essential intermediates in secondary metabolism, both in the intact plant [*e.g.*, 2, 17, 21] and in tissue culture [*e.g.*, 3, 22]. Thus accumulation of hydroxycinnamic acid glucose esters cannot always be considered merely as products of detoxification reactions [23]. Along with many other secondary phenolic plant constituents which are subject to turnover [11], phenolic acid glucose esters are candidates for possible involvement in metabolic processes because of their relatively high free energies of hydrolysis.

Turnover of an apparently accumulating metabolically inert end product can be detected by pulse-labelling experiments with a suitable radioactive precursor [12]. We administered ^{14}C-labelled phenylalanine, the pivotal amino acid in phenylpropanoid metabolism [24], immediately after transfer of *Chenopodium* cells into fresh medium. Within 3 h, 95% of the radioactivity was taken up from the medium and the incorporation of this into the hydroxycinnamic acids was followed in order to determine their metabolic role. This was accomplished with the aid of radio [^{14}C]HPLC as shown in Fig. 2. This technique, though relatively insensitive at present, allowed the rapid tracing of incorporated radioactivity. In all chromatograms we always found two unknown ^{14}C-labelled peaks (peak 3 and 4) with higher retention times than 1-feruloylglucose. Peak 4 which has to be considered as a non-phenylpropanoid constituent showed highest incorporation

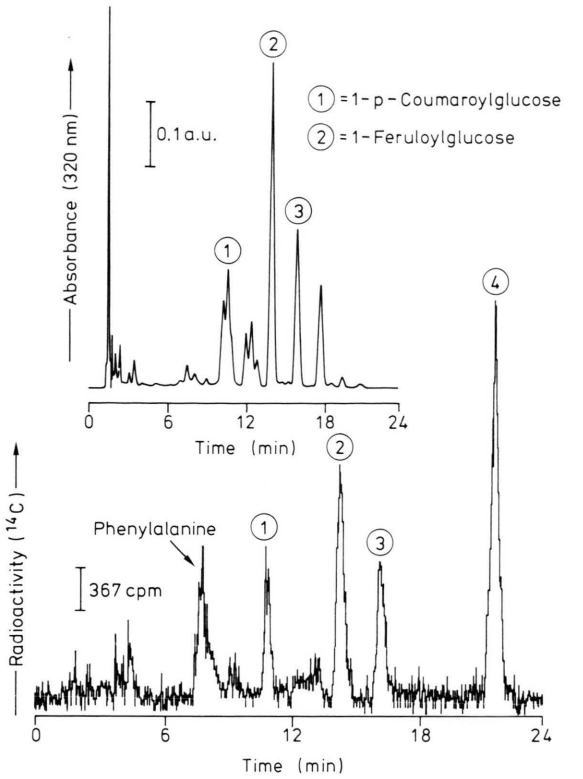

Fig. 2. High performance liquid chromatographic (HPLC) analyses of an extract from suspension culture of *Chenopodium rubrum* (UV/VIS and ^{14}C radioactivity monitors were connected in series). Twenty µl out of 200 µl extract from 50 mg (dry weight) tissue were injected onto the column (RP-18, 7 µm, 4×250 mm), developed linearily within 30 min from solvent A (1% *ortho* phosphoric acid in water) to 40% solvent B (1% *ortho* phosphoric acid, 20% *glacial* acetic acid, 25% acetonitrile in water) in A. Extract came from a pulse-chase experiment with [^{14}C]phenylalanine (12 h after pulse). Upper chromatogram: resolution of UV-absorbing components; lower chromatogram: resolution of ^{14}C-labelled components.

at day 4 (total activity of around 2×10^6 dpm per 100 mg dry weight), decreasing during subsequent stages to less than 50%. Peak 3, most probably a hydroxycinnamic acid conjugate, also showed a transient incorporation.

The time course of incorporation into the glucose esters of *p*-coumaric and ferulic acid along with labelled bound ferulic acid is shown in Fig. 3. The administered [^{14}C]phenylalanine is rapidly consumed and incorporated into the hydroxycinnamic acids. However, radioactivity in both the *p*-coumaric and ferulic acid glucose esters is drained off, indicating a high turnover rate. In contrast insoluble esterified ferulic acid seems to be a stable end product. At least a part of the feruloylglucose turnover seems to be due to the incorporation of ferulic acid into insoluble cell material, most probably cell wall material.

It would be of great interest to pursue the possible hypothesis that the soluble 1-feruloylglucose might be a suitable transport metabolite, used for the esterification of ferulic acid with a cell wall component of *Chenopodium* cells. This could be achieved by a simple transacylation reaction in which the 1-O-acyl glucoside serves as the donor molecule [*c.f.*, 6, 7, 9].

To substantiate our findings of the metabolic activity of the hydroxycinnamic acid glucose esters we administered L-α-aminooxy-β-phenylpropionic acid (AOPP) at the 2nd and 3rd day of cell growth. This potent and specific inhibitor of phenylpropanoid metabolism [13] blocks phenylalanine deamination *in vivo* [25], thus preventing carbon flow into the hydroxycinnamic acid pool. Fig. 4 shows that the application of AOPP resulted in a dramatic decrease, especially of the pool of 1-feruloylglucose. This result substantiates our findings from the pulse-chase experiments. The mutual support of these two methods allows an insight into the metabolic activity

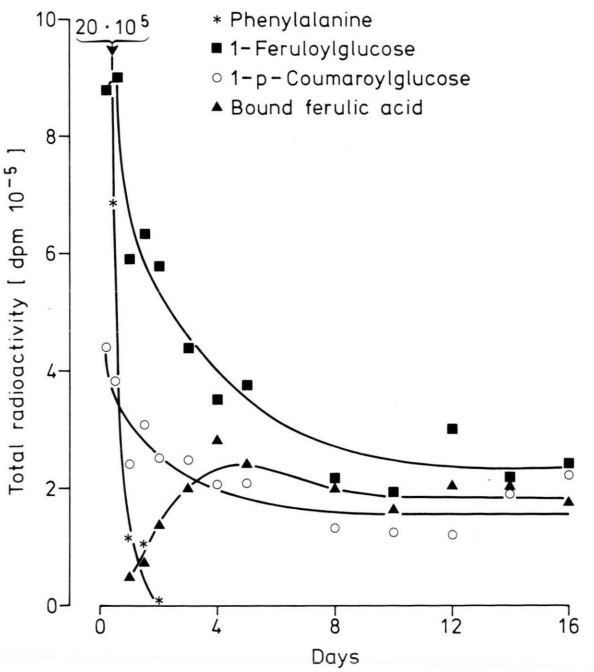

Fig. 3. Changes of total ^{14}C-activities of hydroxycinnamic acid esters after administration of [^{14}C]phenylalanine.

of a seemingly inert end product to be gained [14]. When we combined these techniques in an experiment in which we administered both [^{14}C]phenylalanine and 24 h later AOPP we observed that the specific radioactivity of the glucose esters remained rather constant at about 3.5×10^5 dpm μmol^{-1} (Fig. 4, right panel). This result was to be expected in the light of those described above. From this a half live time for feruloylglucose of 36 h during the first 5 days of culture was calculated.

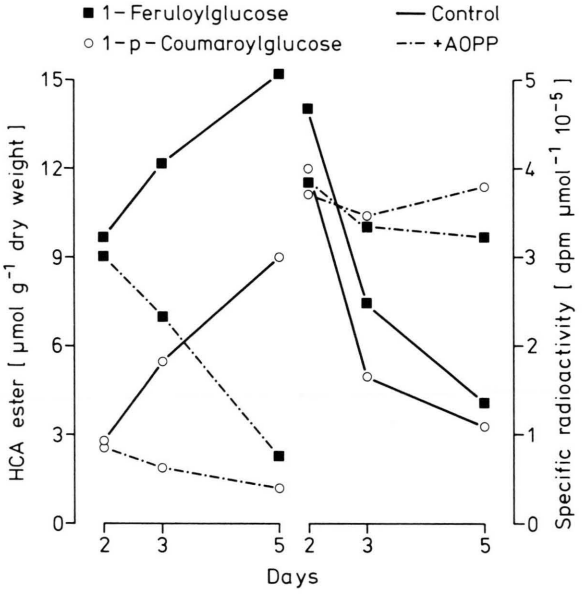

Fig. 4. Effect of L-α-aminooxy-β-phenylpropionic acid (AOPP) on the accumulation pattern and course of specific ^{14}C-labelling of 1-*p*-coumaroyl- and 1-feruloylglucose.

In recent studies [15, 26] it was shown, that L-tyrosine, an amino acid which is closely related to the phenylalanine-dependent phenylpropanoid metabolism, is synthesized *via* the arogenate pathway. There is increasing evidence that this pathway might be widespread in higher plants. This pathway seems to be present also in the *Chenopodium* cell culture. We were able to block synthesis of L-tyrosine by administration of *m*-fluoro-DL-tyrosine, an effective inhibitor of arogenate dehydrogenase [15]. Growth inhibition of *Chenopodium* cells by MFT was reversed by administration of L-tyrosine (Berlin *et al.*, unpublished). The effects of MFT on the secondary metabolism are illustrated in Fig. 5. There is a significant decrease in the pool of betalains (amaranthin) (Fig. 5 A), indicating that betalains might also be subject to turnover. Surprisingly the pool of hydroxycinnamic acid glucose esters markedly increased (Fig. 5 B) when biosynthesis of L-tyrosine is inhibited. 1-*p*-Coumaroylglucose reaches a level which is about 3 times higher than that reached by the control tissue. This result suggests that the arogenate-mediated synthesis of L-tyrosine in *Chenopodium rubrum* cell cultures obviously is closely interrelated with the prephenate pathway leading to L-phenylalanine. We assume that the arogenate pathway of the shikimic-acid pathway is inhibited or switched off by MFT and that the carbon flow into the prephenate branch is enhanced. Since the *Chenopodium rubrum* cultures synthesize metabolically active phenylpropanoids one does not find increased pools of phenylalanine but higher levels of phenylpropanoids. To prove this hypothesis

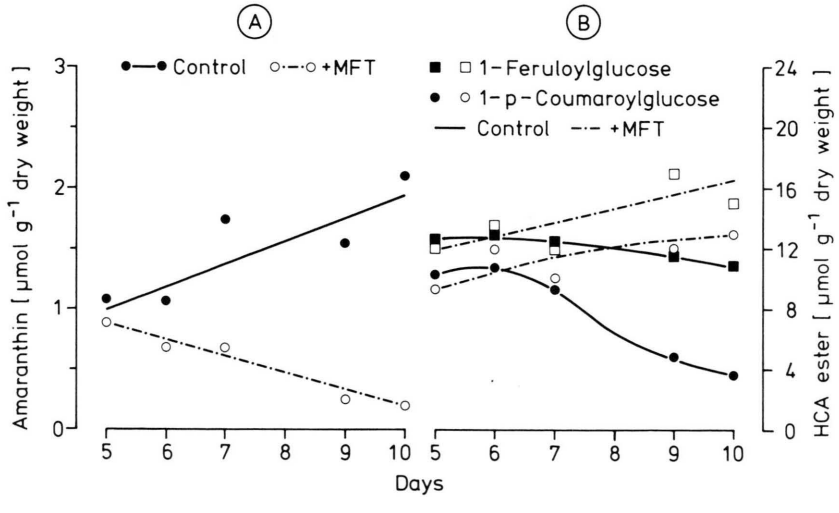

Fig. 5. Effect of *m*-fluoro-DL-tyrosine (MFT) on the accumulation patterns of betalains (amaranthin) (A) and 1-*p*-coumaroyl- and 1-feruloylglucose (B) in a cell suspension culture of *Chenopodium rubrum*.

we fed labelled shikimic acid to MFT treated and untreated cells. Unfortunately, the uptake of shikimic acid was rather poor (30% within 5 days). Thus, the labelling of betalains, tyrosine, phenylalanine and phenylpropanoids remained very low. Due to the high turnover, the kinetics (not shown) were not sufficiently accurate to clearly prove the detour of the carbon flow within the shikimic-acid pathway. On the other hand, there was no evidence that MFT affects the further metabolism of the glucose esters which could also have caused the observed increase of the ester pool.

Thus in conclusion we have shown that hydroxycinnamic acid glucose esters are highly metabolically active in cell suspension cultures of *Chenopodium rubrum*. This metabolism seems to respond sensitively to fluctuations in closely related pathways, such as the metabolism of tyrosine. Tissue cultures of *Chenopodium rubrum* seem to offer a powerful tool for the investigation of the functional role and regulatory aspects of phenylpropanoid metabolism. Elucidation of the specific role of the hydroxycinnamic acid glucose esters in this tissue must now await more detailed pulse-chase experiments and enzymatic studies, particularly on the localization and mechanism of the formation of insoluble conjugates.

Acknowledgement

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