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COUMARINS IN *HELIANTHUS TUBEROSUS*: CHARACTERIZATION, INDUCED ACCUMULATION AND BIOSYNTHESIS

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Key Word Index—*Helianthus tuberosus*; Compositae, Jerusalem artichoke; characterization; induction; biosynthesis; coumarins

Abstract—The biosynthesis of 7-hydroxylated coumarin phytoalexins has been examined in the tuber of *Helianthus tuberosus*. Ayapin and scopoletin where characterized by TLC, HPLC and GC-MS. Both compounds were excreted from sliced tuber tissues. They accumulated in response to treatment with chemical elicitors like $CuCl_2$ or sucrose, but not in response to $MnCl_2$. Scopoletin accumulation preceded ayapin build-up in the medium. Highest and earliest accumulation of both coumarins was measured after copper treatment. The formation of methylenedioxy bridges in several secondary metabolites is catalyzed by cyt P450 oxygenases. The changes in cyt P450 content, *trans*-cinnamate 4-hydroxylase and NADPH-cytochrome *c* reductase activities in microsomes from tuber tissues were, therefore, monitored for three days after wounding and elicitation. Cyt P450 content and NADPH-Cyt *c* reductase activity were not significantly modified in copper treated tissues, but increased three to five fold in response to sucrose. Copper elicitation resulted in a decrease in cinnamate-4-hydroxylase activity, while sucrose was a very strong and specific inducer of this Cyt P450 enzyme. No formation of ayapin was observed when radiolabelled scopoletin was incubated with NADPH and elicited plant microsomes, or fed to induced tuber tissues *in vivo*. No formation of methylenedioxy metabolite was obtained *in vitro* or *in vivo* from radiolabelled ferulate or feruloyl CoA.

INTRODUCTION

Ayapin (6,7-methylenedioxy-coumarin) and scopoletin (6-methoxy-7-hydroxy-coumarin) are phenolic compounds belonging to the family of the simple 7-hydroxylated coumarins. Scopoletin is found in fungi and in many botanical families which include important crops, for example cereals, Compositae, legumes and Solanaceae [1]. By contrast, the presence of ayapin has only been observed in a reduced number of plant species within the family of Compositae, i.e. in sunflower (*Helianthus annuus* L.)[1, 2], in *Eupatorium ayapana* Vent. [3], and in *Alomia fastigiata* Benth. [4].

Ayapin and scopoletin have been described as phytoalexins, their accumulation being correlated with resistance to microbial pathogens in tobacco [5] and sunflower [6, 7], as insect feeding deterrents [8] and as inhibitors or inducers of parasitic weed germination [9, 10]. In addition, physiological effects like promotion of stomatal closure in sunflower, or inhibition of bud growth in pea, have also been associated with scopoletin at very low concentrations [1]. The ecological role of these compounds has been recently reviewed [9].

Coumarin biosynthesis is tissue specific and regulated developmentally [1, 11, 12]. In addition, it is induced in response to an ample number of both biotic and abiotic stresses [6–8, 11], to nutritional deficiencies [13], and to chemical signals like plant hormones and metabolites as well as xenobiotics [12, 14].

It is well established that coumarins derive from *L*-phenylalanine via the phenylpropanoid pathway [1, 15] and that their synthesis involves the first three catalytic steps of the pathway leading to activated 4-coumaroyl CoA. This includes the 4-hydroxylation of cinnamic acid catalyzed by a cytochrome P450, the *trans*-cinnamate 4-hydroxylase (EC 1.14.13.11), which

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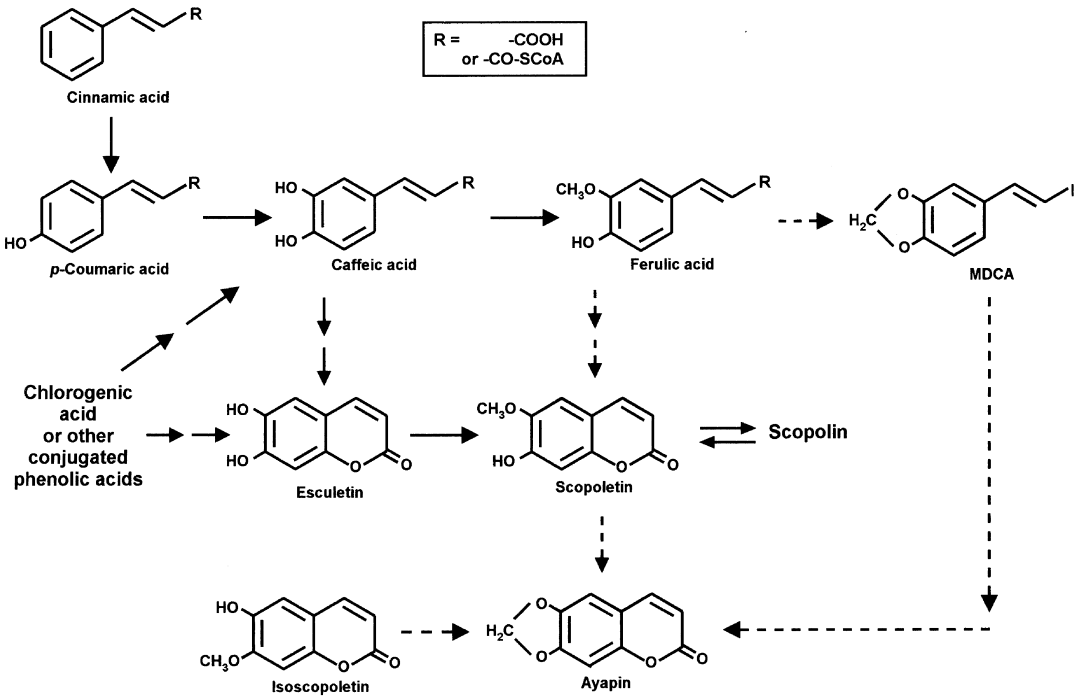

Fig. 1. Possible routes of ayapin and scopoletin biosynthesis. The putative Cyt P450-dependent reactions labelled with a dashed arrow have been assayed *in vitro* with radiolabelled substrates.

possibly constitutes a rate-limiting step in the first part of the pathway [16]. Despite intensive research almost nothing is known about the enzymes and genes specifically involved in coumarin biosynthesis, except that, based on *in vivo* experiments, scopoletin may be the direct precursor of ayapin [6].

We have undertaken a study of the role and biosynthesis of 7-hydroxylated coumarins in *Helianthus* spp. In this paper we show the presence of scopoletin and ayapin in *H. tuberosus* and their differential accumulation in response to wounding and environmental factors like metals and sucrose. In addition, attempts have been made to characterize the enzyme involved in ayapin biosynthesis in *H. tuberosus*. Based on theoretical and literature data [17–19], we postulated that the formation of the methylenedioxy bridge of ayapin, was catalyzed by a P450 monooxygenase. Scopoletin, isoscopoletin (6-hydroxy-7-methoxy-coumarin), ferulic acid and feruloyl CoA have been tested as potential precursors and substrates of the enzyme (Fig. 1). In addition to *in vitro* experiments with plant microsomes, *in vivo* feeding with radiolabelled scopoletin, cinnamate and ferulate have been performed. The results of our experiments question the previous data of Tal and Robeson [7], obtained with *H. annuus* hypocotyls, and suggest that, in *H. tuberosus*, scopoletin is possibly not the direct precursor of ayapin.

RESULTS

Ayapin and Scopoletin are synthetized by the tuber of H. tuberosus after wounding or treatment with chemical elicitors

Ayapin and scopoletin have previously been shown to accumulate in different proportions in the aerial part of *H. annuus* (leaves, stems, hypocotyls) in response to treatment with abiotic elicitors like copper or sucrose [11, 12]. Both free coumarins were mainly found excreted by these plant tissues, though scopoletin can also be incorporated into the cell wall or accumulated as scopolin, its glucosidic conjugated form, which was the main coumarin found in the sunflower plant cell. The tuber is the major reproductive organ of *H. tuberosus*. It is particularly exposed to soil-borne fungal pathogens. In addition, from a developmental point of view, the tuber is not a root but a modified stem. Therefore, we looked for the presence of coumarins in *H. tuberosus* tuber tissues, dormant, wounded (i.e. sliced and aged in water) or incubated in the presence of elicitors previously shown to induce the accumulation of phytoalexins. The ageing medium was also extracted and analysed to detect excreted compounds.

Coumarins were not found in untreated *H. tuberosus* tubers. TLC analysis revealed the presence of fluorescent material with R_F values corresponding to

Table 1. Accumulation of coumarins in the incubation medium of *H. tuberosus* tuber slices treated with manganese, copper or sucrose*

	Scopoletin		Ayapin	
Treatment	24h	72h	24h	72h
	pmol g^{-1} fresh weight		pmol g^{-1} fresh weight	
Control	9±1	8±1	30±2	32±3
Manganese	8±1	10±1	25±2	29±1
Copper	3405±218	540±51	235±6	1000±131
Sucrose	10±1	68±3	44±2	340±28

*Coumarin content is given as mean±S.D. of triplicates.

scopoletin and ayapin in the ageing medium of tuber tissues wounded and treated with sucrose and copper. Trace amounts of these fluorescent compounds were also detected in plant tissues after elicitation. Ayapin- and scopoletin-like material present in the incubation medium was further quantified by HPLC. Whereas very low and similar amounts were found excreted by wounded and manganese treated tuber slices, high levels of both compounds were found in tuber slices treated with copper or sucrose (Table 1). The highest levels were measured after copper treatment.

To confirm their identity, the two blue fluorescent compounds were extracted and purified from copper-treated *H. tuberosus* tuber incubation medium by consecutive chromatography on silica gel and HPLC, and analysed by GC-MS. By comparison with authentic samples, they were unambiguously identified as ayapin and scopoletin [1, 2]. Scopolin was not detected, or present in trace amounts, in the tuber tissues and ageing medium.

Timing of Coumarin Accumulation in Response to Wounding and Chemical Elicitors

The timing of the accumulation of the two coumarin phytoalexins in the ageing medium of tuber tissues after wounding and incubation in phosphate buffer, alone or with 0.4 M sucrose or 1 mM $CuCl_2$ is shown in Fig. 2. Following wounding, highest and similar levels of ayapin and scopoletin were found after 12 hours ageing. After longer incubations, the total coumarins detected in the medium decreased, with ayapin remaining three–four times higher than scopoletin.

A delayed coumarin accumulation was observed after sucrose treatment, highest levels of scopoletin (i.e. 27-fold the level measured in buffer alone) being measured at 36 hours. The time course of the accumulation of ayapin differed from that of scopoletin and reached a maximum after scopoletin had started to decrease. Ayapin detected in the medium after 72 hours ageing in the presence of sucrose was 11-fold the amounts measured in buffer alone. An increased level of scopoletin (6-fold the value in control) was observed as early as 12 hours treatment with copper. A maximum (342-fold the level in control) was reached around 24 hours. Scopoletin content then decreased rapidly, but remained about 50-fold higher than in the control. A constant increase in ayapin content of the medium was observed following copper treatment,

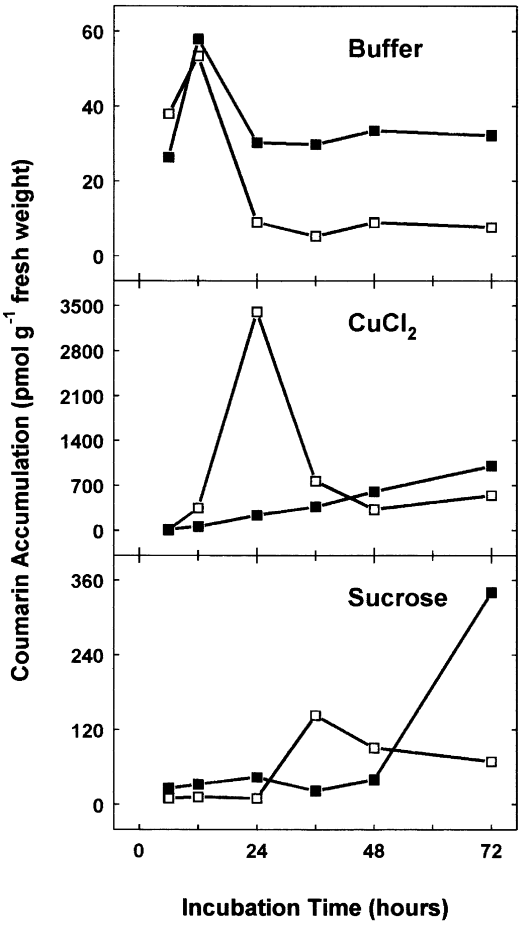

Fig. 2. Timing of ayapin and scopoletin accumulation in the incubation medium of *H. tuberosus* tuber tissue. Dormant tubers were sliced and aged up to 72 h in aerated Na phosphate 10 mM, pH 6.0 alone or containing 1 mM $CuCl_2$ or 0.4 M sucrose. The incubation media were extracted and analysed for ayapin (■) and scopoletin (□) coumarin content by HPLC. Data are means of three independent determinations.

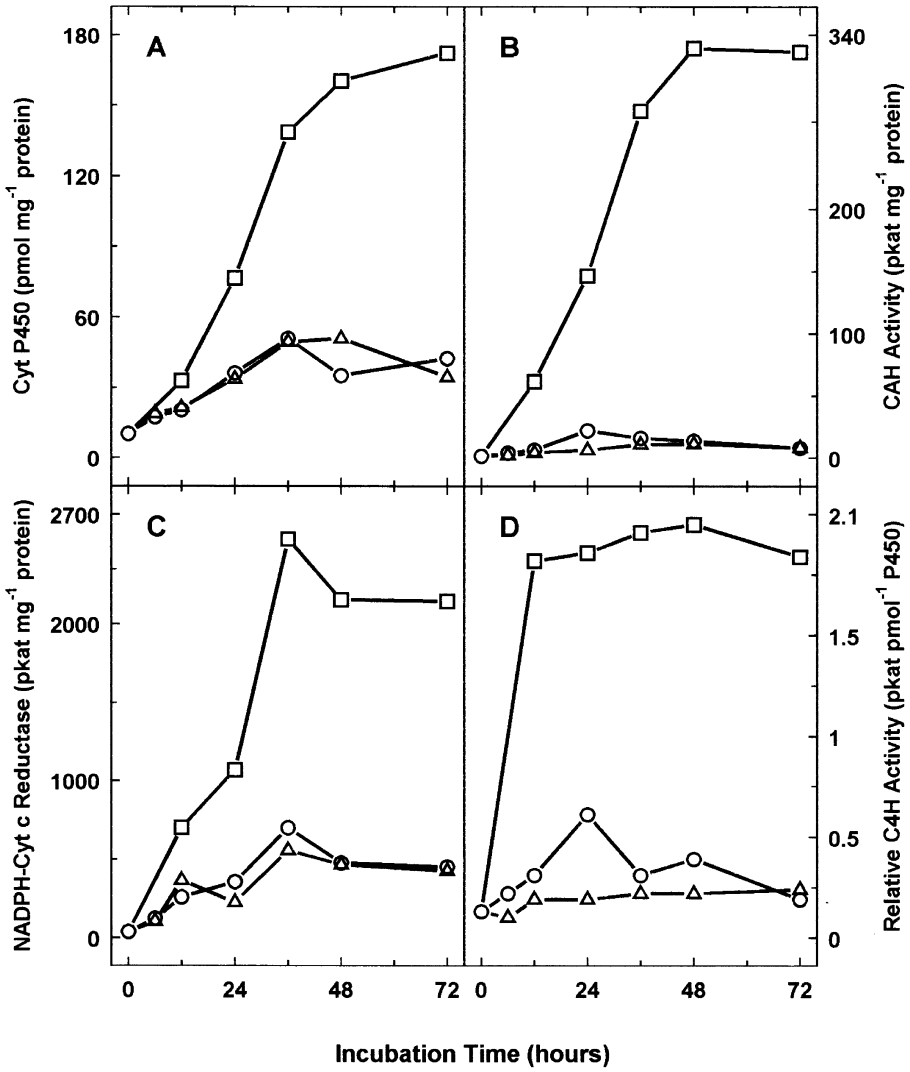

Fig. 3. Effect of $CuCl_2$ and sucrose on Cyt P450 content and related activities in *H. tuberosus* tuber. Cyt P450 content, NADPH-cytochrome *c* reductase and cinnamate 4-hydroxylase activities were measured in microsomes from tuber slices incubated in 10 mM Na phosphate buffer, pH 6,0, alone (○), or containing 1 mM $CuCl_2$ (△) or 0.4 M sucrose (□) up to 72 h.

highest level (31-fold the control) being reached at 72 hours.

*Cytochrome P*450 *content, NADPH-Cyt c reductase and cinnamate-*4*-hydroxylase activities in tuber tissues treated with copper or sucrose*

Changes in Cyt P450 content, NADPH-cytochrome *c* reductase (NADPH-Cyt *c* reductase; EC 1.6.2.4) and cinnamate-4-hydrolase activities in microsomes from tuber tissues exposed to $CuCl_2$ or sucrose are shown in Fig. 3. Before slicing, microsomal activities and Cyt P450 measured in *H. tuberosus* tuber were very low. As previously reported [20], they were increased in response to wounding. Ageing in the presence of copper resulted in little modification in Cyt P450 contents measured after wounding (except for a small 1.5-fold increase compared to control at 48 hour) and in a slight decrease in NADPH-Cyt *c* reductase activity. Cinnamate-4-hydroxylase induction, however, was clearly inhibited by $CuCl_2$. Reduced Cinnamate-4-hydroxylase activity did not seem to result from an inhibition of the catalytic activity related to the presence of Cu^{2+} ions or other inhibitory compounds since microsomes from $CuCl_2$-treated tissues had no inhibitory effect on the activity of the fractions prepared from sucrose-induced tuber. In addition, when the enzyme was directly quantified spectrophotometrically by substrate binding, the proportion of Cinnamate-4-hydroxylase was also found reduced from 0.59 to 0.23 Cinnamate-4-hydroxylase Cyt $P450^{-1}$. In contrast to $CuCl_2$, sucrose produced a strong induction of Cyt P450 (4.6-fold), of NADPH-Cyt *c* reductase (4.5-fold), and the highest induction of Cinnamate-4-hydroxylase ever reported in these tissues (24-fold).

Expression of Cinnamate-4-hydroxylase activity relative to total Cyt P450, content (Fig. 3D) emphasizes the specific effects of the chemical elicitors on this Cyt P450 isoform, i.e. a strong inhibition by copper and high selective induction by sucrose. In both cases, the response of Cinnamate-4-hydroxylase to the treatment was fast, changes in specific activity being observed before 12 hours of treatment.

Attempts to characterize the Methylenedioxy bridge forming enzyme

Microsomes from tuber tissues actively excreting ayapin, i.e. wounded and treated with copper or sucrose, with high Cyt P450 content or cinnamate hydroxylase activity, were incubated with NADPH and radiolabelled scopoletin. No formation of radiolabelled ayapin was detected. Radiolabelled ferulic acid, feruloyl-CoA, or isoscopoletin, other possible substrates of methylenedioxy forming enzyme (Fig. 1), were also tested for NADPH-dependent metabolism with microsomes from induced tuber tissues. No methylenedioxy or coumarin metabolite was obtained.

In vivo *metabolism of radiolabelled Scopoletin*

In an attempt to detect conversion of scopoletin into ayapin *in vivo*, radiolabelled scopoletin was fed to $CuCl_2$-induced tuber tissues. The radiolabelled molecule was directly applied onto the tuber slices or added to the ageing medium. Whatever the mode of feeding, similar results were obtained. No formation of radiolabelled ayapin could be detected, neither in the medium, nor in tuber tissues. Never more than 6% of the total radioactivity, including only trace amounts ($< 2\%$) of scopoletin, were recovered in the incubation medium after 24 hours. Most of the radioactivity was found in the tuber tissues, as highly hydrophylic or insoluble residues. Feeding experiments were also performed with radiolabelled cinnamic and ferulic acids. Ferulic acid was totally converted into highly polar metabolites and no radiolabelled coumarins were obtained. After feeding with [3-^{14}C] cinnamic acid, by contrast, a significant proportion of the label was recovered as scopoletin, but no radiolabelled ayapin was obtained.

DISCUSSION

The presence of ayapin and scopoletin in *H. tuberosus* has never been previously reported. Isolation and characterization of both coumarins from tuber tissues confirms their presence in several species from the *Helianthus* gender. It also shows that an active coumarin synthesis proceeds in tuberized stems and not only, as in *H. annuus* [11, 12], in the aerial parts of the plant. As expected for phytotoxic phytoalexins molecules, most of the coumarins were found excreted in the ageing medium.

The amounts of phytoalexins produced were dependent on the treatment of the plant tuber and on the molecule used as abiotic elicitor. $MnCl_2$ previously shown to promote plant defense, synthesis of phytoalexins and increase Cyt P450 content and activities in various plant cells and tissues, including *H. tuberosus* tuber [20–22], was ineffective for the induction of 7-hydroxylated coumarins (Table 1). By contrast, $CuCl_2$ and sucrose, known to stimulate coumarin accumulation in *H. annuus* [11, 12], were also good elicitors of scopoletin and ayapin synthesis in *H. tuberosus* tuber tissues. $CuCl_2$ is believed to induce a release of endogenous elicitors following cellular damage [23]. Carbohydrates, in particular sucrose, have been shown to positively modulate a number of pigments and defense genes, whose products mediate plant interactions with other organisms [24, 25]. As in *H. annuus*, $CuCl_2$ in *H. tuberosus*, elicited a stronger response than sucrose (Table 1) and primarily induced accumulation of scopoletin, while sucrose rather elicited excretion of ayapin (Fig. 2). It suggests that not only the coumarin pathway, but also its regulation are conserved among *Helianthus* spp.

The timing of ayapin and scopoletin accumulation differed after wounding or treatment with copper or sucrose (Fig. 2). After wounding, an early and simultaneous production of both coumarins was observed, with ayapin showing a greater stability over long incubations. After sucrose or $CuCl_2$ treatment, the accumulation of scopoletin was maintained for a longer time, and always preceded the accumulation of ayapin. A sequential induction of scopoletin and ayapin synthesis would be in concordance with the route of coumarins biosynthesis proposed in sunflower [7, 11]. However, the decrease in scopoletin measured in *H. tuberosus* tuber tissues (in terms of extent and timing) was not correlated to ayapin formation. A conversion of the excreted scopoletin into ayapin thus is very unlikely, and the biosynthesis of the two coumarins seems to be independently regulated.

The formation of methylenedioxy bridges in the biosynthesis of alkaloids and isoflavonoids have been shown to be catalysed by Cyt P450 monooxygenases [17–19]. In addition, the exact mechanism of the formation of the coumarin lactone cycle is still unknown, but is expected to be consecutive to the 2-oxygenation of phenolic acids [15], possibly as esterified or conjugated derivatives. We thus investigated the possible involvement of Cyt P450 enzymes in the synthesis of 7-hydroxylated coumarins in copper and sucrose-treated tuber tissues. Cinnamate 4-hydroxylase, an obligatory step for the synthesis of both coumarins was taken as reference of Cyt P450 induction and catalytic activity. Although copper treatment did not have a significant effect over total Cyt P450 content, except an increase at 48 hours when the synthesis of ayapin was maximal, a strong reduction in Cinnamate-4-hydroxylase activity and content was observed. It suggests an induction of specific Cu^{2+}-

responsive Cyt P450 isoforms. By contrast, sucrose rapidly and strongly increased total Cyt P450 and even more Cinnamate-4-hydroxylase activity (Fig. 3). Sucrose treatment thus seems to specifically induce Cinnamate-4-hydroxylase in *H. tuberosus* tuber tissues. This enzyme has been previously reported to represent 56% of the total Cyt P450 after 24 hours ageing in water [26]. Comparison of total Cyt P450 and Cinnamate-4-hydroxylase contents by spectrophotometric techniques (not shown) confirmed that in sucrose treated tissues the proportion of Cinnamate-4-hydroxylase was raised to 89%. Sucrose is thus, by far, the best specific inducer of CYP73A1 ever reported.

There is clearly no correlation between coumarin level and Cinnamate-4-hydroxylase activity: highest accumulation of coumarins was observed after $CuCl_2$ treatment, when Cinnamate-4-hydroxylase was decreased. Low levels of Cinnamate-4-hydroxylase and high induction of coumarins has also been observed in fragmented sunflower tissue incubated with the systemic fungicide metalaxyl (Gutiérrez-Mellado and colleagues, unpublished results). It suggests that only a very minor proportion of the cinnamate processed by the Cinnamate-4-hydroxylase after wounding or sucrose treatment is used for the synthesis of coumarins. This is in agreement with data showing that, in induced sunflower, the amount of coumarins never exceeds 0.5% of the total free phenolics (Gutiérrez-Mellado and colleagues, unpublished results). It must also be kept in mind that coumarins may be synthetized from a preexisting pool of metabolites, for example chlorogenic acid or related compounds. It has been proposed that chlorogenic acid could feed the phenylpropanoid pathway by a release of caffeic acid [27] and serve as a precursor for coumarin [11] or lignin [28] synthesis in sunflower.

No methylenedioxy forming activity was detected in tuber tissues aged up to 72 hours in the absence or presence of copper or sucrose, whether ferulic acid, ferulyl-CoA, isoscopoletin or scopoletin were used as substrates. Incubation of plant microsomes with ferulate or feruloyl-CoA did not lead to the formation of coumarins either. When *in vivo* feeding with labelled scopoletin was carried out, in order to confirm the biosynthetic sequence and the direct conversion of scopoletin into ayapin previously observed by Tal and Robeson [7] in pathogen infected sunflower stems, no radiolabelled ayapin was extracted from the tuber tissues. The radioactive scopoletin was completely converted into insoluble residues and polar metabolites, which probably results from the activity of a parietal and $CuCl_2$-inducible scopoletin peroxidase also observed in *H. annuus* [11, 29]. Such a peroxidase may prevent the uptake and biotransformation of exogenous scopoletin in $CuCl_2$-treated tuber tissues. However, when tissues were fed with exogenous radiolabelled cinnamic acid, label was found in scopoletin, but never recovered as ayapin. On the other hand, when [^{3}H] scopoletin feeding experiments were performed with sucrose-treated *H. annuus* stem segments and leaf discs, residual scopoletin was found in the ageing medium, but radiolabelled ayapin was never obtained.

Our results thus suggest that scopoletin is not the direct precursor of ayapin. They also indicate that $CuCl_2$-treated tissues, due to an increased oxidative metabolism, are probably not the best material to investigate coumarin biosynthesis. We did not observe Cyt P450-dependent formation of methylenedioxy bridge with any of the substrates tested in our experiments. Thus, either the proper substrate was not identified, or the reaction is catalyzed by another family of oxygenases. Best candidates would be soluble monoxygenases [30] or dioxygenases which were, in the last years, repeatedly found to catalyse the same reactions as Cyt P450 monoxygenases [31].

EXPERIMENTAL

Chemicals

Cinnamic acid, cytochrome *c*, FAD, FMN, NADPH and *S*-adenosyl-L-methionine were from Sigma (St. Louis, U.S.A.). Esculetin, isoscopoletin and scopoletin were from Carl Roth GmbH (Karlsruhe, Germany). Ferulic acid was from Fluka (Buchs SG, Siwtzerland), methylenedioxy-cinnamic acid (MDCA) from Aldrich-Chemie (Steinheim, Germany), *S*-adenosyl-*L*-[methyl-^{3}H]methionine from Amersham France (Les Ullis, France), and [3-^{14}C]cinnamic acid from Isotopchim (Ganagobie-Peyruis, France). [^{3}H]Feruloyl CoA was a gift from zeneca Agrochemicals (Bracknell, UK). Purified *O*-methyltransferase I from tobacco was a generous gift from Dr M Legrand, Institute of Plant Molecular Biology, Strasbourg, France.

Preparation of radiolabelled scopoletin, isoscopoletin and ferulic acid

[6-methoxy-^{3}H]Scopoletin was synthesised by methylation of esculetin with *S*-adenosyl-L-[methyl-^{3}H]methionine, using *O*-methyltransferase I purified from tobacco [32]. The reaction mixture (10 ml) contained *O*-methyltransferase I (around 270 pkat), 500 μM esculetin, 24 μM *S*-adenosyl-L-methionine, 1 μm *S*-adenosyl-L-[methyl-^{3}H]methionine (15 Ci.m-mol^{-1}). The mixture was incubated for 4 h at 37° before addition of carrier scopoletin and isoscopoletin (10 μmol each in 2 ml MeOH-, and extracted (3 ×) with an equal volume of EtOAc at −20°. The extract was evaporated under argon, resuspended in 3 ml EtOAc and spotted onto fluorescent silica TLC plates (G60 F254, Merck). The plates were developed in the organic phase of a toluene–AcOH–H_2O (6 : 7 : 3). Scopoletin ($R_f = 0.23$) and isoscopoletin ($R_f = 0.16$) were located under UV (364 nm), separately scraped off the plate, and eluted from the silica with Et_2O (3 × 10 ml). After extraction, the samples were dried under argon, redissolved in 1 ml MeOH and stored

at -20°. [6-methoxy-^{3}H]Scopoletin and [7-methoxy-^{3}H]isoscopoletin with specific activities of 20 mCi mmol^{-1}, were obtained with yields of 71% and 29%, respectively. MeOH was evaporated before using the ^{3}H-labelled molecules for the different experiments.

[5-methoxy-^{3}H]Ferulic acid was synthesised in the same way, by methylation of caffeic acid with *S*-adenosyl-L-[methyl-^{3}]methionine, using *O*-methyltransferase I from tobacco. After 4 h incubation at 37°, one volume of H_2O was added to the 2 ml reaction mixture. The mixture was extracted 3 × with an equal volume of cyclohexane-Et_2O (1 : 1). The extract was evaporated under argon, resuspended in 1 ml Et_2O and spotted onto fluorescent silica TLC plates (G60 F254, Merck). The plates were developed in the organic phase of a toluene–AcOH–H_2O (6 : 7 :3). Ferulic acid ($R_f = 0.41$) was located under UV (364 nm), scraped from the plate, and eluted from the silica with 3 × 2 ml Et_2O. The extract was dried under argon, dissolved in 0.1 M phosphate buffer pH 7.4, and stored at -20°. [5-methoxy-^{3}H]ferulic acid with a specific activity of 19.36 Ci mmol^{-1}, was obtained with a 40% yield.

Plant material and induction conditions

Jerusalem artichoke (*Helianthus tuberosus* L., var. blanc commun) tubers were grown in open field, harvested in November and stored in plastic bags at 4° in the dark. For ageing experiments, tubers were sliced (1.5 mm thick) and incubated in the dark at room temp in Na phosphate buffer 10 mM, pH 6,0 alone or containing $MnCl_2$ (25 mM), $CuCl_2$ (1 mM) or sucrose (0.4 M) for 72 h. The incubation medium was vigorously bubbled with a stream of hydrated and filtered air. Plant material was dried, weighed, frozen in liquid N_2, and stored at -80° prior to extraction or before use for the preparation of microsomes.

Coumarin extraction, analysis and characterization

Coumarins were extracted from the plant tissues and incubation medium, and analysed or quantified by TLC and HPLC-fluorescence as described by Gutiérrez-Mellado *et al.* [12].

Coumarins purified by consecutive TLC and HPLC were analysed by GC-MS (EI mode, 70 eV) on a DB5 column (30 m × 0.32 mm) coupled to a MD800 Fisons mass spectrometer. The oven temp program was as follows: 170° for 1 min, 170°–200° at 15°C min^{-1}, 200–300° at 2° min^{-1}.

Preparation of microsomal fractions

The frozen tuber slices (30 g) were directly homogenised and microsomes prepared as described by Reichhart *et al.* [20]. Microsomes were suspended in 10 mM Na phosphate buffer containing 1.5 mM 2-mercaptoethanol, 30% glycerol (5–10 mg protein ml^{-1}), frozen in liquid N_2 and stored at -80°.

Assay for methylenedioxy bridge formation from radiolabelled scopoletin or isoscopoletin

In a final volume of 200 μl of buffer, the reaction mixture contained 1.5 mM NADPH, 20 μM FMN, 20 μM FAD, 0.12–0.6 mg microsomal protein, 110 μM [7-methoxy-^{3}H]isoscopoletin or [6-methoxy-^{3}H]scopoletin (20 mCi mmol^{-1}-, and 90 μM isoscopoletin or scopoletin. Assays were performed in the presence of different buffers: 0.05 M or 0.1 M Na phosphate (pH 7.5) or Tris-HCl (pH 8.6). The reaction was initiated by addition of the coumarin after 5 min preheating at 30°. It was stopped after 45 min with 20 μl 4 N HCl, before addition of carrier ayapin and scopoletin (0.3 μmol each in 20 μl MeOH). Precipitated proteins were removed by centrifugation and 100 μl of the supernatant were directly spotted onto fluorescent silica TLC plates (G60 F254, Merck). The plates were developed in Et_2O. Ayapin and scopoletin or isoscopoletin were located under UV light (364 nm). Radioactivity associated with each of the coumarins was determined by scintillation counting of the fluorescent silica spots.

Assay for methylenedioxy bridge formation from radiolabelled ferulic acid or feruloyl-CoA

The reaction mixture, as above, was incubated 45 min with 200 μM [4-methoxy-^{3}H]ferulic acid (4.7 mCi mmol^{-1}) or 200 μM [4-methoxy-^{3}H]feruloyl-CoA (5 mCi mmol^{-1}) instead of scopoletin. When ferulic acid was used as a substrate, the reaction was stopped by the addition of 20 μl 4 N HCl before addition of carrier ferulic acid and MDCA (0.3 μmol each in 20μl MeOH). After 10 min on ice, precipitated proteins were removed by centrifugation, and 100 μl supernatant were directly spotted onto fluorescent silica TLC plates (G60 F254, Merck). The plates were developed in the organic phase of a toluene–AcOH–H_2O (6 : 7 : 3). Ferulic acid and MDCA were located under UV light (364 nm) and quantified by scintillation counting. In the case of the feruloyl-CoA assay, the reaction was stopped by addition of 200 μl 2 PrOH, and 200 μl of the supernatant obtained after protein precipitation were spotted onto cellulose TLC plates (Merck). The plates were developed with *n*-BuOH–AcOH–H_2O (5 : 2 : 3). The substrate and the reaction products were detected and quantified with a radiometer thin layer scanner (Berthold LB 2723).

In vivo *feeding experiments*

Studies of ayapin biosynthesis *in vivo* were carried out by feeding *H. tuberosus* tuber slices with radiolabelled scopoletin, as well as ferulic and cinnamic acids. The [^{3}H]scopoletin (21 nmol, 0.42 μCi for 2 g of tuber) was applied to slices prealably aged in 10 mM Na phosphate pH 6.0 containing 1 mM $CuCl_2$, in three different ways: (1) the radiolabelled scopoletin was layered onto the surface of the induced

slices, which were then floated on the inducing medium; (2) after application of scopoletin, the induced tuber slices were kept dry in Petri dishes; (3) radiolabelled scopoletin was added directly to the inducing medium. In all cases, plant tissues were kept in the dark at room temp and under agitation for 24 h. Coumarins were extracted from plant tissues and incubation media as above. The samples were analysed by TLC. Coumarins were detected under UV light (364 nm) and quantified with a radiometer thin layer scanner and by scintillation counting. The insoluble residue obtained after extraction of plant tissues was incubated in 2 ml KOH 4 N for 2 days in the dark at room temp, and radioactivity released was quantified by scintillation counting. Feeding with [5-methoxy-^{3}H]ferulic acid (3 nmoles, 0.36 μCi for 2 g of tuber) or [3-^{14}C]cinnamic acid (3 nmol, 0.21 μCi for 2 g of tuber) was performed as in experiment 1 for scopoletin.

Other analytical methods

Cytochrome P450 content, NADPH-cytochrome *c* reductase and cinnamate-4-hydroxylase activities were determined as described by Reichhart *et al.* [20]. Proteins were quantified using the BCA Protein Assay from Pierce.

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