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Coniferin dimerisation in lignan biosynthesis in flax cells

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Abstract

[$^{13}C_2$]-Coniferin was provided to a flax (*Linum usitatissimum* L.) cell suspension to monitor subsequent dimerisation by MS and NMR. The label was mainly incorporated into a 8–8′-linked lignan, lariciresinol diglucoside, a 8–5′-linked neolignan, dehydrodiconiferyl alcohol glucoside and a diastereoisomeric mixture of a 8-*O*-4′-linked neolignan, guaiacylglycerol-β-coniferyl alcohol ether glucoside. This latter compound is reported for the first time in flax. The strong and transient increase in these compounds in fed cells was concomitant with the observed peak in coniferin content. These results suggest (i) a rapid metabolisation of coniferin into lignans and neolignans and indicate the capacity of flax cells to operate different types of couplings, and (ii) a continuous synthesis and subsequent metabolisation of coniferin-derived dimers all over the culture period.

Keywords: *Linum usitatissimum*; Linaceae; Flax; LC-MS; MS/MS; NMR; Labelled coniferin; Lignan; Neolignan

1. Introduction

Monolignols are derived from cinnamic acids and supply precursors for various phenylpropanoid compounds such as lignins and lignans. The current view of the monolignol biosynthetic pathway envisages a metabolic grid leading to G and S units, through which the successive hydroxylation and *O*-methylation reactions may occur at different levels of side chain oxidation (Dixon et al., 2001). This general monolignol biosynthetic pathway occurring in most angiosperms is presented in Fig. 1 (adapted from Hoffmann et al., 2004 and from Shadle et al., 2007). The oxidative dimerisation of two coniferyl alcohol units leads to a great variety of secondary metabolites. When this dimerisation involves an oxidative linkage through the C-8 of the propenyl side chains of two coniferyl alcohol moieties, forming 8–8′ bonds, the resulting metabolites are called lignans. The term neolignan is used to define all the other types of linkage (Moss, 2000). Many of the dimers present in vascular plants have linkages other than 8–8′ and, of these, the bulk of the biochemical work has thus far been directed towards elucidating mechanisms of 8–5′, 8-*O*-4′ and 8–2′-linked neolignan formation (Davin and Lewis, 2003).

Lignans and neolignans, found in a wide range of plant species, display numerous pharmacological activities (Pool-Zobel et al., 2000; Arroo et al., 2002). The major lignans from flaxseed (*Linum usitatissimum*), secoisolariciresinol diglucoside and matairesinol, are converted into the "mammalian lignans" enterodiol and enterolactone by intestinal bacteria (Wang et al., 2000). The beneficial effects of these compounds on human health are well recognized (Westcott

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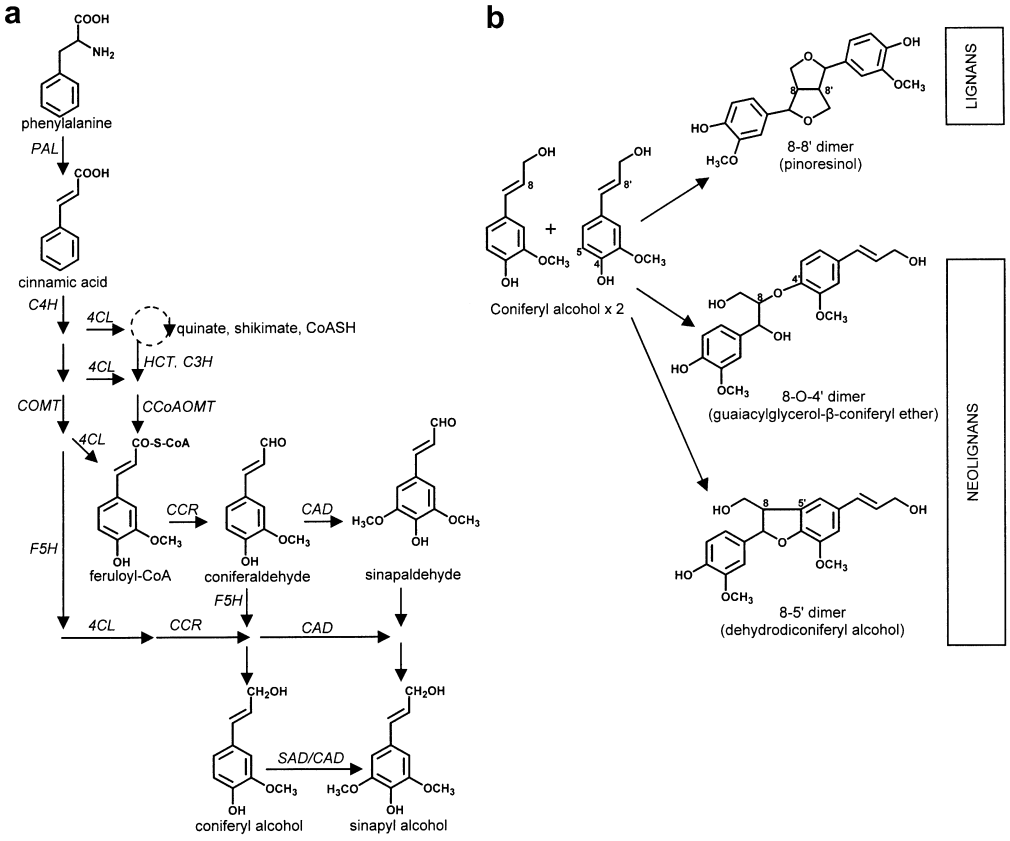

Fig. 1. Monolignol-derived products investigated in this study. (a) Monolignol biosynthetic pathway occurring in most angiosperms (from Hoffmann et al., 2004 and from Shadle et al., 2007). PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; HCT: hydroxycinnamoyltransferase; C3H: *p*-coumarate 3-hydroxylase; CCoAOMT: caffeoyl-CoA *O*-methyltransferase; COMT: caffeic acid *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H: ferulate 5-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase. (b) Coniferyl alcohol-derived products: pinoresinol (8-8′-linked lignan) guaicylglycerol-β-coniferyl ether (8-*O*-4′-linked neolignan) and dehydrodiconiferyl alcohol (8-5′-linked phenylcoumaran lignan) resulting from simple dimerisation of two coniferyl alcohols.

and Muir, 2003; McCann et al., 2005) and *in vitro* studies confirm the observed *in vivo* effect (Bylund et al., 2005). In particular, they were shown to reduce the incidence of breast and prostate cancers by modulating steroidal hormone synthesis (Adlercreutz and Mazur, 1997). 8-*O*-4′ neolignans are used as lead compounds for antifungal agents (Apers et al., 2003).

In view of the important pharmacological properties, numerous studies have been carried out in an attempt to get a better knowledge of the biological events linked to the biosynthesis and the accumulation of lignans and neolignans (Seidel et al., 2002; Sicilia et al., 2003). In flax, coniferyl alcohol was clearly evidenced to be the monolignol involved in lignan biosynthesis (Ford et al., 2001). Flax suspension cells are also known to accumulate neolignans (Attoumbre et al., 2006).

In an effort to study the biosynthesis and the accumulation of secondary metabolites, *in vitro* cultures have been established as a very useful tool because this system allows uniformity, accessibility and reduced complexity (Facchini, 2001; Mesnard et al., 2002; Verpoorte et al., 2002). In the present study, a recently established flax (*L. usitatissimum*) cell suspension (Hano et al., 2006; Attoumbre et al., 2006) was used in feeding experiments with ^{13}C-labelled coniferin to investigate monolignol dimerisation into both lignans and neolignans. In order to visualise its dimerisation by NMR and MS, doubly labelled (8,9-$^{13}C_2$)-coniferin was synthesised to offer easier detection (Beejmohun et al., 2006). Coniferin, considered as the putative storage form of coniferyl alcohol (Gross, 1985) was used to facilitate the solubilisation into aqueous culture media (Van Uden et al., 1995).

2. Results and discussion

2.1. Preliminary experiments

2.1.1. Growth kinetics

For the feeding experiments, a minimum concentration of 0.5 g/l (1.46 mM) of labelled coniferin was required due to the sensitivity of the analytical methods used. Such a concentration of an exogeneous precursor might be toxic for the flax suspension cells (Edahiro et al., 2005). Prior to experiments with labelled coniferin, the effect of unlabelled coniferin supplementation on cell growth was therefore

evaluated under the same conditions as in the feeding experiments (see Section 4.3).

Fig. 2 presents the *L. usitatissimum* cell growth kinetics in presence or absence of coniferin. Over the cultivation period of 14 days, the dry weight increased progressively until a maximum value measured at day 8 for both conditions. After one week of cultivation, the weight stabilised. In spite of a slight difference during the exponential growth phase (day 2 and day 4), both curves showed large similarities indicating that coniferin supplementation at 0.5 g/l in the culture medium did not affect cell growth.

2.1.2. Evolution of coniferin content in medium and cells

In order to verify that coniferin was incorporated into the cells, the evolution of coniferin amount in the culture media and inside the cells was evaluated by HPLC analysis. Two control experiments were performed by following the evolution of coniferin (i) in culture medium supplemented with coniferin but without cells and (ii) in cells (and their culture medium) untreated with coniferin.

As shown in Fig. 3a, the amount of coniferin remained stable in culture medium supplemented with coniferin without suspension cells over the 14 days period indicating that coniferin was not spontaneously degraded in the medium. The coniferin content rapidly decreased in coniferin supplemented culture media in presence of flax cells and was no longer detectable from day 2 (Fig. 3a). From the 2.5 mg coniferin added in the media in each well at the beginning of the feeding experiments, only 0.2 mg was remaining at day 1, suggesting that 2.3 mg were taken up by the cells or cleaved into coniferyl alcohol. Indeed coniferin could have been metabolised in coniferyl alcohol under the effect of external glucosidases. The occurrence of coniferyl alcohol was therefore sought in the media, but was not detected by HPLC, whatever the time of culture, which suggests that coniferin was rapidly incorporated into the cells, as confirmed in Fig. 3b. This graph shows that the flax cells fed with coniferin accumulated more coniferin in the first two

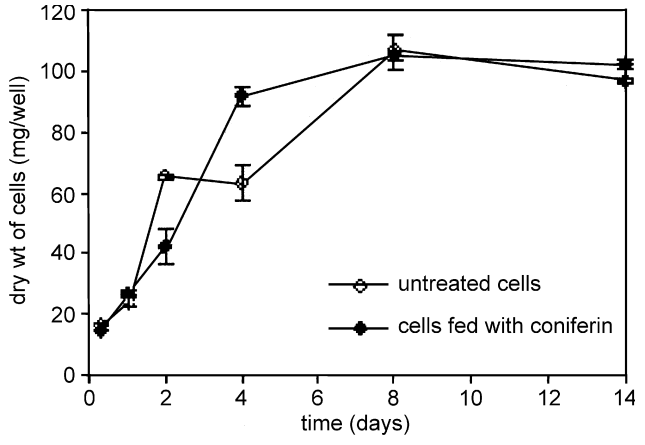

Fig. 2. Growth curves of cell suspensions of *L. usitatissimum* cultured with or without coniferin. Experiments were done in triplicate.

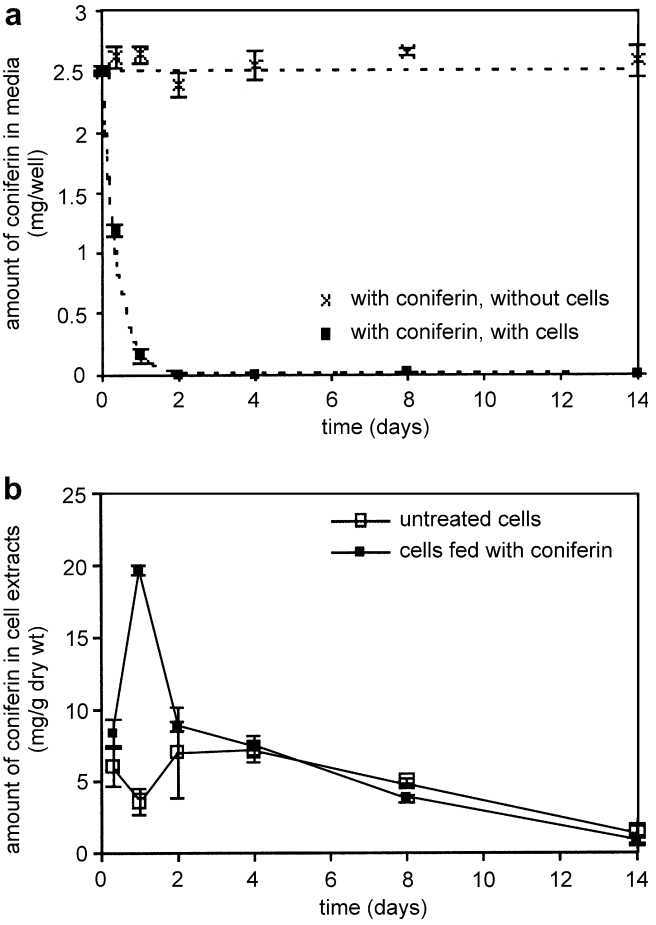

Fig. 3. Coniferin content in culture medium (a) and in *L. usitatissimum* cells extracts (b). Experiments were done in triplicate.

days of the feeding experiment as compared to untreated cells. Then, the coniferin content from day 2 evolved in the same manner with or without supplementation. At day 1, in treated cells, the coniferin concentration is 19.7 mg/g dry wt (Fig. 3b) which, for a total dry weight of ca 25 mg, corresponds to a coniferin amount of 0.5 mg. At the same time of culture, the coniferin amount in untreated cells was only 0.1 mg. Compared to untreated cells, the total intracellular coniferin amount increased therefore by 0.4 mg in treated cells, and it appears that an unrecovered fraction of 1.9 mg (2.3–0.4) of the supplemented coniferin was metabolised. Only traces of coniferyl alcohol were found in cell extracts showing that this compound is not accumulated in these flax cells. However, these data do not exclude a rapid conversion of coniferin into coniferyl alcohol before a subsequent metabolisation into other compounds, potentially lignans.

2.2. Metabolisation of coniferin

2.2.1. Identification of the labelled compounds

Feeding experiments were carried out with labelled and unlabelled coniferin. Since the aim of the study was the

investigation of (neo)lignan synthesis in flax cells, a classical methanolic extraction was performed before applying appropriate chromatographic analysis (Hano et al., 2006).

By comparison with feeding experiments with unlabelled coniferin, LC-MS analysis of the flax cells methanolic extracts revealed the incorporation of the label mainly into five compounds: peak 1 at M+2 and peaks 2, 3a, 3b, 4 at M+2 and at M+4 (Figs. 4 and 5), whatever the time of harvest. It is noteworthy that these five compounds were also accumulated in untreated cells, indicating that their biosynthesis is not specifically related to the supplementation.

Peak 1 with a retention time of 21.3 min was easily attributed to coniferin by comparison with a reference compound. This observation confirmed the incorporation of exogenous labelled coniferin into the intracellular coniferin pool before it is metabolised (Fig. 3b).

By comparison to a reference compound, peak 2 (Rt = 24.6 min) was assigned to lariciresinol diglucoside (Beejmohun, 2006). This tetrahydrofuran lignan is obtained from the reduction of pinoresinol – known to be formed by the dimerisation of two coniferyl alcohol moities through an 8–8′ bond (Davin and Lewis, 2003) – catalysed by pinoresinol-lariciresinol reductase. The same enzyme catalyses the formation of secoisolariciresinol from lariciresinol. Pinoresinol diglucoside was found labelled in trace amount (data not shown) but no secoisolariciresinol or secoisolariciresinol diglucoside was detected. This result is somewhat surprising but could be explained by a metabolisation of secoisolariciresinol at a rate higher than its biosynthesis from lariciresinol, thus leading to the accumulation of this intermediate product. The presence of the aglycone lariciresinol has already been reported at low amounts in a cell suspension of another *Linum* species (*L. album*, Smollny et al., 1998).

Peaks 3a and b showed similar positive and negative ESI-MS spectra ($[M+Na]^+$ m/z 561 and $[M-H]^-$ m/z 537) corresponding to the same molecular weight of 538. A formula of $C_{26}O_{12}H_{34}$ was proposed following accurate mass measurements (3a: $[M+Na]^+$ m/z 561, found 561.1960 for $C_{26}O_{12}H_{34}Na$ requires 561.1948, 3b: $[M+Na]^+$ m/z 561, found 561.1931 for $C_{26}O_{12}H_{34}Na$ requires 561.1948). The structure of these compounds was elucidated by MS/MS and NMR experiments. They are the *erythro* (a) and *threo* (b) forms of the glucoside of 1-(4-hydroxy-3-methoxyphenyl)-2-(4-(3-hydroxy-(*E*)-prop-1-enyl)-2-methoxyphenoxy)propane-1,3-diol, also called guaiacylglycerol-β-coniferyl alcohol ether glucoside (GGCG). Several glucosides formed from the same aglycone have been already described in several plants: *Arum italicum* (Della Greca et al., 1994), *Echinacea purpurea* (Li and Barz, 2006), *Citrus limon*, as citrusin A (Matsubara et al., 1991), *Glehnia littoralis*, as citrusin A (Yuan et al., 2002), *Angelica furcijuga* as hyuganoside III (Morikawa et al., 2004). However, they have not yet been observed in Linaceae. The MS/MS spectrum of these compounds isolated from the cell extracts cultured with unlabelled coniferin, obtained in the positive ion mode from the CID (collision induced dissociation, collision energy 37 V) process of the $[M+Na]^+$ ion (m/z 561), showed a fragmentation pattern similar to that described in the literature (Li and Barz, 2006). The identification of compounds 3a and 3b was confirmed by 1H NMR data which were in accordance with previous data reported in the literature (Takara et al., 2002; Morikawa et al., 2004).

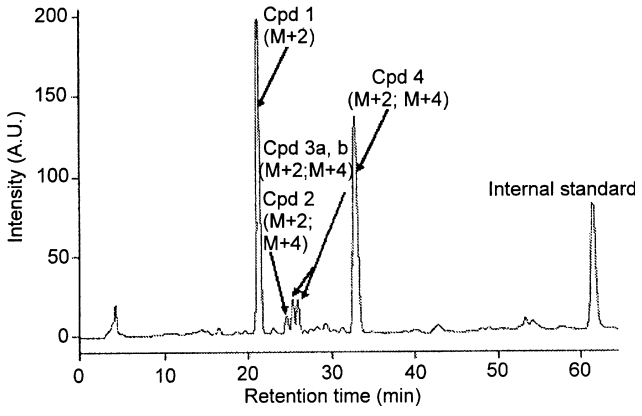

Fig. 4. HPLC chromatogram of an extract of *L. usitatissimum* cell suspension fed with coniferin for 8 h.

Peak 4 was eluted at 33.0 min. By comparison with an authentic standard, it was attributed to a 8–5′ neolignan (dehydrodiconiferyl alcohol-4-β-D-glucoside) which has already been characterised in *L. usitatissimum* cell cultures (Attoumbre et al., 2006). It was shown to accumulate at high amounts which is in agreement with the concentration values (5–30 mg/g dry wt) found during the experiments reported here. These labelling experiments showed that this compound was not released from the cell-wall but was produced directly from coniferin/coniferyl alcohol. This result is in agreement with previous labelling experiments reported by Orr and Lynn (1992) who showed the labelling of dehydrodiconyferyl alcohol when incubating *Vinca* cells with [^{3}H]-coniferyl alcohol. They proposed a direct biosynthetic pathway involving the 8–5′ coupling of two coniferyl alcohol moities. In our experiments, the M+4 enrichment of this compound revealing the incorporation of two doubly-labelled coniferin molecules might confirm this hypothesis but nothing is known about which carbons incorporated the label and consequently the mechanism of coupling. Fig. 6a represents the NMR spectrum of a methanolic extract of flax cells 24 h after feeding with (8,9-$^{13}C_2$)-coniferin. By comparison with a control performed in the same conditions with unlabelled coniferin (Fig. 6b), several doublets are clearly visible at $\delta = 55.5$ ppm ($v = 38.1$ Hz), $\delta = 64.0$ ppm ($v = 47.7$ Hz), $\delta = 65.1$ ppm ($v = 38.1$ Hz), and $\delta = 127.8$ ppm ($v = 47.7$ Hz). They respectively correspond to the C8′, C9, C9′ and C8 (coupled with their labelled neighbour carbon) of DCG, unambiguously confirming the biosynthetic pathway described above.

The biosynthesis of these four labelled molecules is highly likely to involve the same enzymatic systems as

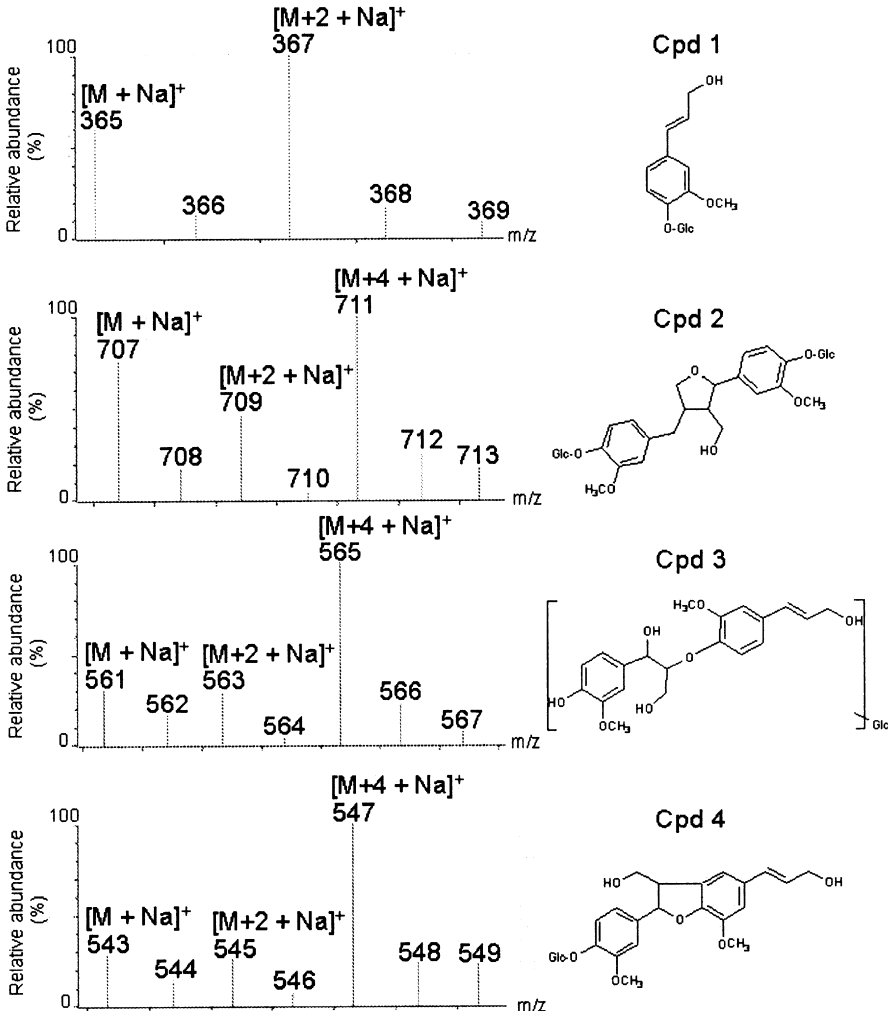

Fig. 5. ESI-MS spectra of the compounds 1, 2, 3 and 4 from the extract of a *L. usitatissimum* cell suspension cultured with $^{13}C_2$-coniferin for 48 h.

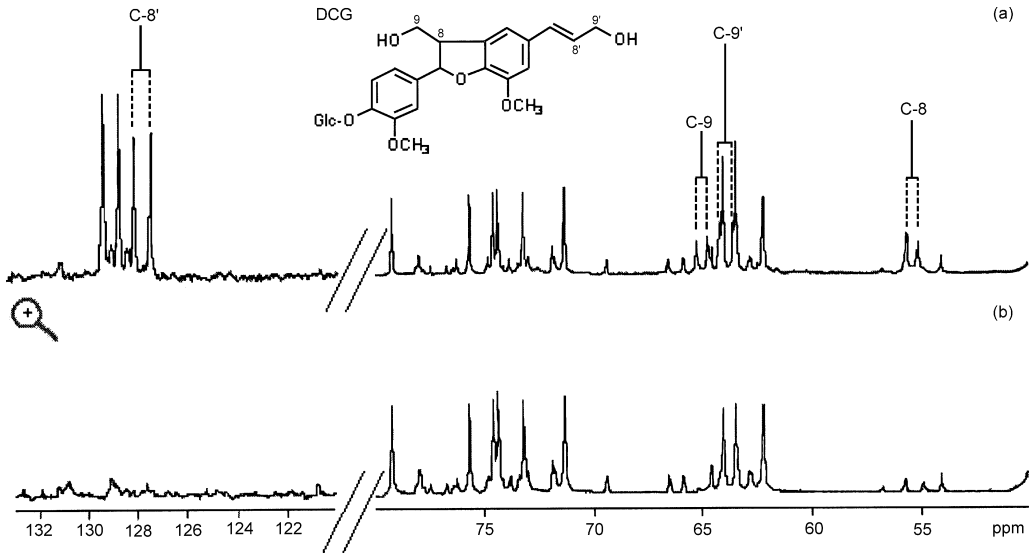

Fig. 6. NMR spectra of a flax cell extract after feeding of the cells with labelled (a) or unlabelled (b) coniferin for 24 h.

those involved in untreated cells since the coniferin supplementation did not induce the formation of any new compound (fed-cell extracts show the same HPLC profile as untreated cell extracts). Note that even isomeric forms of these labelled molecules would have been detected. Indeed, these molecules being glucosylated, enantiomers cannot be found (since it would involve the biosynthesis of L-glucose molecules) but only diastereoisomers which are detectable by LC-MS and NMR.

2.2.2. Kinetics of (neo)lignan biosynthesis

The kinetics of the accumulation of coniferin derived lignans and neolignans reported in Fig. 7 indicated a rapid increase in the first two days of culture after addition of coniferin, reaching a maximum amount at day 1 (from 3- to 6-fold increase when compared to untreated cells values). These increases (by 0.7 mg DCG, 0.1 mg LDG, 0.2 mg *erythro*-GGCG, 0.2 mg *threo*-GGCG) were concomitant with the observed increase in coniferin content in fed-cells; they correspond to the use of ca 80% of the 1.9 mg unrecovered coniferin (calculated in Section 2.1.2). After 2 days, lignan and neolignan contents in coniferin fed-cells progressively returned to that observed in untreated cells. These results suggest a rapid metabolisation of coniferin into lignans and neolignans, which would be quickly further transformed. This hypothesis is supported by the ratio of labelled versus unlabelled compounds (Table 1). Indeed labelled dimers were mainly accumulated at 48 h (maximum ratios labelled vs unlabelled), following the maximum ratio of labelled coniferin observed at day 1. Then these ratios decreased, suggesting a continuous synthesis and subsequent metabolisation of coniferin and coniferin-derived dimers all over the culture period. Coniferin and/or coniferin-derived dimers might be incorporated into higher polymers such as lignin; which might correspond to the remaining 20% of the unrecovered coniferin, at day 1.

Indeed, it is generally assumed that coniferin is a storage form of lignin and/or lignan precursors because this glycosylated form of coniferyl alcohol has a markedly higher water solubility and stability towards oxidation (Whetten et al., 1998). Our results seem to indicate the existence of a mechanism regulating the intracellular steady state levels of coniferin and coniferin-derived dimers in flax cells keeping them constant in both untreated and coniferin fed-cells.

Lignins and lignans share monolignols as common precursors and are both potentially involved in plant defence against pathogens (Gang et al., 1999). Elicitation of cell cultures with *Fusarium oxysporum* extracts triggered a strong incorporation of monolignols in the non condensed labile ether-linked lignin fraction concomitantly with a decrease in 8–8′ and 8–5′ lignan accumulation (Hano et al., 2006). One hypothesis to explain such a decrease in lignan and neolignan accumulation in elicited cell cultures was their possible incorporation in the lignin polymer to contribute to cell wall reinforcement. Such metabolisation

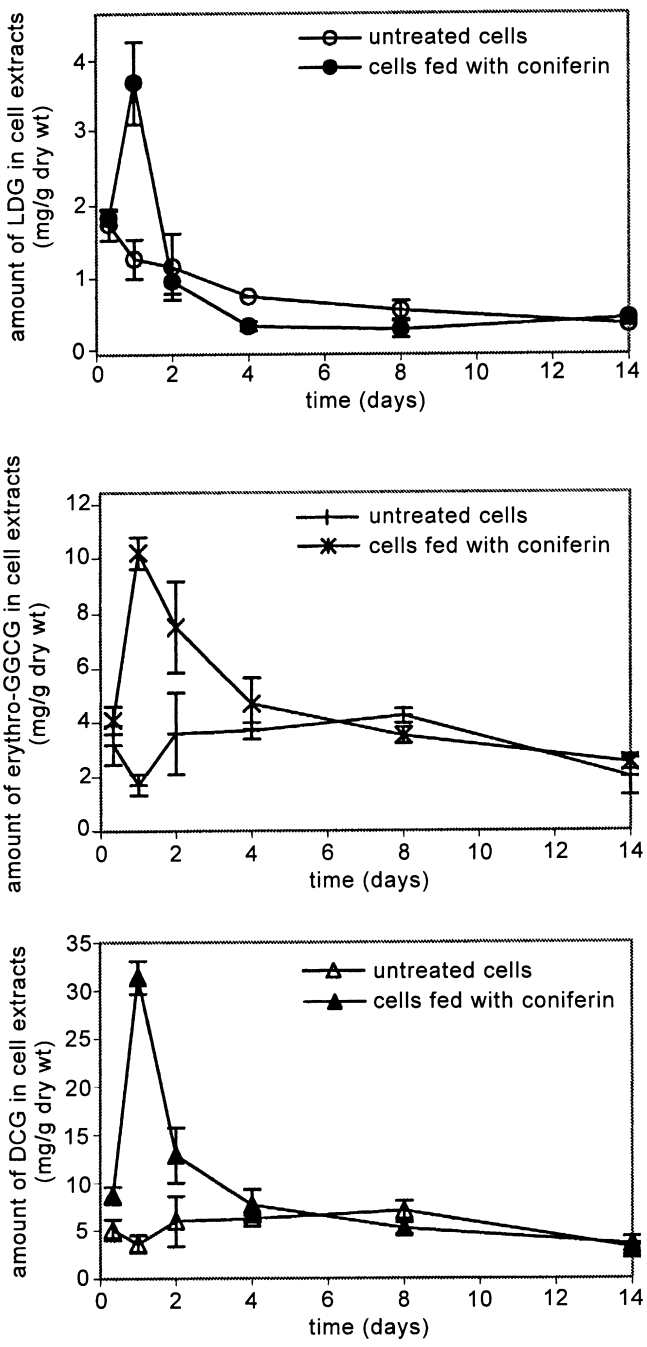

Fig. 7. Kinetics of accumulation of compounds 2 (LDG), 3 (GGCG) and 4 (DCG) over the 14-day experiment, in untreated cells and in cells fed with coniferin. Both forms (*erythro* and *threo*) of GGCG show similar accumulation kinetics. The kinetics of the *erythro* form is presented here. Experiments were done in triplicate.

of lignan and neolignan compounds has been already observed during tracheary element differentiation of isolated *Zinnia elegans* mesophyll cells (Tokunaga et al., 2005). These authors also reported the incorporation of pinoresinol, *erythro*- and *threo*-guaicylglycerol-β-coniferyl ether and dehydrodiconiferyl alcohol into the lignin polymer.

Table 1
Kinetics of label incorporation in compounds 1, 2, 3, and 4

	Cultivation time (h)					
	8	24	48	96	192	336
Coniferin						
Unlabelled	86	38	45	75	84	84
Labelled	14	62	55	25	16	16
LDG						
Unlabelled	87	60	36	29	52	41
Labelled	13	40	74	71	48	59
GGCG						
Unlabelled	93	47	20	26	42	44
Labelled	7	53	80	74	58	56
DCG						
Unlabelled	61	34	18	26	51	45
Labelled	39	66	82	74	49	55

Both forms (*erythro* and *threo*) of GGCG show similar incorporation kinetics. The kinetics of the *erythro* form is presented here. Results are given in percent

3. Concluding remarks

Four major labelled-dilignols were identified in coniferin-fed cell suspensions: lariciresinol diglucoside (8–8′-linked lignan), dehydrodiconiferyl alcohol glucoside (8–5′-linked neolignan) and finally the *erythro* and *threo* forms of guaicylglycerol-β-coniferyl ether glucoside (8-*O*-4′-linked neolignan). To our knowledge, this latter compound was evidenced for the first time in flax.

Limited information is available regarding lignan and neolignan biosynthesis accumulation and regulation. The system presented herein will be of interest in order to confirm or infirm the hypothesis of an incorporation of (neo)lignans into lignin under developmental or stress conditions. Indeed it will be possible to purify different ^{13}C-labelled (neo)lignans which will be provided to cell cultures to see whether the label is incorporated into lignin.

4. Experimental

4.1. Plant material

The 2-1B cell suspension culture of *L. usitatissimum* L. cv Barbara was established from hypocotyl-derived calli in MS-derived medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, 8.88 μM benzylaminopurine and 2.68 μM α-naphthalene acetic acid. All suspension cultures were incubated on a gyratory shaker at 120 rpm, with a 5 cm orbital size, in darkness at 25 °C and subcultured every 14 days. Usually, 100 ml MS-derived medium in a 250 ml Erlenmeyer flask were inoculated with 5 g of cells. Growth was evaluated by measuring dry weight of lyophilised cells.

4.2. Synthesis of [8,9-$^{13}C_2$]-coniferin

Conversion of vanillin to its corresponding β-glucoside was performed with tetra-*O*-acetyl-α-D-glucopyranosyl bromide and silver oxide in quinoline. The monolignol glucoside (8,9-$^{13}C_2$)-coniferin was then synthesised by condensation of the corresponding aldehyde with (1,2,3-$^{13}C_3$)-malonic acid as previously described (Beejmohun et al., 2006). The carboxylic acid was converted to the acyl chloride prior to its reduction to alcohol. Pure double labelled lignan precursors could only be obtained after separation from their contaminating *Z*-isomers and dihydro by-products by high-performance liquid chromatography.

4.3. Feeding experiments

All feeding experiments were performed in 6-well sterile plates. To each well was added 300 mg fresh weight of cells in exponential growth-phase and 5 ml of sterile culture medium containing labelled or unlabelled coniferin at 0.5 mg/ml. At 8, 24, 48, 96, 192 and 336 h following precursor addition, the cells were harvested, separated from the medium by filtration, washed with 5 ml water, frozen and lyophilised. Culture media and washings were pooled, frozen and lyophilised. Standard experiments were carried out under the same conditions but without coniferin addition in culture media.

4.4. Extraction

Each sample of dried cells was extracted twice with 20 ml MeOH 70% (v/v) with gentle shaking, at room temperature, for 24 h. The extracts were filtered and reduced to dryness under vacuum. Residues were resuspended in 500 μl MeOD for NMR analysis. 20 μl of *o*-coumaric acid solution (0.25 g/l) was added to 80 μl of the methanolic extract for LC analysis.

Lyophilised culture media were resuspended in 1100 μl of MeOH and 20 μl of *o*-coumaric acid solution (1 g/l) were added to 180 μl of the methanolic extract for LC analysis.

4.5. NMR analysis

^{13}C NMR spectra were recorded at 300 K on a Bruker Avance DMX 300 spectrometer, operating at 75.47 MHz using a 5 mm broad-band probe head. Spectra were accumulated using a 90° pulse angle, a recycle time of 4 s and an acquisition time of 1.08 s, for a spectral width of 15 kHz for 32 K data points. Before Fourier transformation, a zero filling to 64 K was applied, and a line broadening of 5 Hz was used to improve the spectral signal-to-noise ratio.

Spectra were acquired for 10,900 scans (meaning 15h 30 experiment time). All ^{13}C resonances were assigned through comparison with the chemical shifts of authentic reference compounds and with the literature data.

1H NMR spectra of compounds 3 were recorded at 300 K on a Bruker Avance DPX 300 spectrometer, operating at 300.13 MHz using a 5 mm selective probe head. Spectra were accumulated using a 30° pulse angle, a recycle

time of 1 s and an acquisition time of 2 s, for a spectral width of 3 kHz for 32 K data points.

4.6. HPLC and LC-MS analysis

4.6.1. HPLC

A Merck-Hitachi HPLC system (Fontenay sous Bois, France) equipped with a L7100 pump and a L7400 UV–Vis detector was used. The separation was performed at room temperature on a KROMASIL® (Macherey Nagel, Hoerdt, France) C18 column (250 mm × 4.6 mm i.d.; 5 μm). The mobile phase consisted of 0.2% acetic acid in water (solvent A) and acetonitrile (solvent B). The composition of the mobile phase varied during the run according to a nonlinear gradient as follows: A–B: 0–10 min (100:0, v/v), 10–30 min (82:18, v/v), 30–55 min (64:36, v/v) at a flow rate of 0.8 ml/min. Detection was performed at 280 nm. Lignan and monolignol glucosides were identified by comparison of their retention times to those of authentic standards and by mass spectrometry measurements.

4.6.2. LC-MS

LC-MS analyses were performed on a Waters 2695 Alliance coupled with a single quadrupole mass spectrometer ZQ (Waters-Micromass, Manchester, UK) equipped with an electrospray ion source (ESI-MS).

The chromatography was carried out using the conditions described in Section 4.6.1. Reference compounds and raw cell extracts were loaded on the KROMASIL® column using a sample injection volume of 20 μl (methanol solutions at 0.1 g/l for the reference compounds and at 1 g/l for the crude extracts). The effluent was flow-split via a peek tee with 1/5 of the flow directed toward the ESI source of the ZQ instrument and the residual 4/5 directed toward a PDA detector (Waters 2996). LC-ESI-MS data were recorded in the positive and negative ion mode. The source and desolvation temperatures were kept at 120 and 250 °C, respectively. Nitrogen was used as a drying and nebulizing gas at flow rates of 450 and 100 l/h, respectively. The capillary voltage was ±3.5 kV and a cone voltage range from ±20 to ±60 V was used (±ESI). Scanning was performed in the range 50–1950 Da at a scan rate of 1 s/scan. Data were collected in the centroid mode. Data acquisition and processing were performed with MassLynx V4.0 software.

4.7. HR-MS and MS/MS analysis

High-resolution electrospray mass spectra (HR-ESI-MS) in the positive and negative ion modes were obtained on a Q-TOF *Ultima Global* hybrid quadrupole/time-of-flight instrument (Waters-Micromass, Manchester, UK), equipped with a pneumatically assisted electrospray (Z-spray) ion source and an additional sprayer (Lock Spray) for the reference compound.

The purified compounds were dissolved in methanol and the solutions directly introduced (5 μl min^{-1}) through an integrated syringe pump into the electrospray source.

The source and desolvation temperatures were kept at 80 and 150 °C, respectively. Nitrogen was used as the drying and nebulizing gas at flow rates of 350 and 50 l h^{-1}, respectively. The capillary voltage was ±3.5 kV, the cone voltage ±50 V and the RF lens 1 energy ±20 V (±ESI). For collision-induced dissociation (CID) experiments, argon was used as collision gas at an indicated analyser pressure of 5×10^{-5} Torr and the collision energy was optimised for each parent ion (10 to 60 V). Lock mass correction, using appropriate cluster ions of an *ortho*-phosphoric acid solution (0.1% in H_2O/CH_3CN 50/50, v/v), was applied for accurate mass measurements. The mass range was typically 50–1000 Da and spectra were recorded at 1 s/scan in the profile mode at a resolution of 10,000 (FWMH). Data acquisition and processing were performed with MassLynx 4.0 software.

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