



## Identification by NMR and accumulation of a neolignan, the dehydrodiconiferyl alcohol-4- $\beta$ -D-glucoside, in *Linum usitatissimum* cell cultures

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### Abstract

Phenylpropanoids represent a broad range of secondary metabolites in plants, in which they are involved in defense mechanisms. This study deals with the NMR identification of a phenylpropanoid, which belongs to the class of neolignans, dehydrodiconiferyl alcohol-4- $\beta$ -D-glucoside (DCG), in *in vitro* cultures of *Linum usitatissimum*. The combination of 1- and 2-D NMR experiments such as COSY, HMBC and HMQC allowed the identification of this compound's structure unambiguously. In order to evaluate its implication in defense mechanism, the *L. usitatissimum* suspension cells were placed together with fungal extracts. Consequently, the DCG concentration decreased dramatically after 96 h of treatment. In correlation, the phenylcoumaran benzylic ether reductase (PCBER) expression increased rapidly and constantly immediately after elicitation until 96 h post elicitation, as shown by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). These two results are in agreement, since the aglycone form of DCG is one of the two substrates of PCBER, thus suggesting PCBER activation in plant defense mechanisms. **To cite this article:** J. Attoumbre *et al.*, *C. R. Chimie* 9 (2006).

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### Résumé

Les phénylpropanoïdes représentent une large classe de métabolites secondaires chez les végétaux chez lesquels ils sont souvent impliqués dans des mécanismes de défense. Cette étude présente l'identification par RMN d'un phénylpropanoïde

*Abbreviations:* BAP, benzylaminopurine; DCG, dehydrodiconiferyl alcohol-4- $\beta$ -D-glucoside; DW, dry weight; FW, fresh weight; HPLC, high pressure liquid chromatography; IFR, isoflavone reductase; NAA, naphthaleneacetic acid; NMR, nuclear magnetic resonance; PCBER, phenylcoumaran benzylic ether reductase; PLR, Pinoresinol lariciresinol reductase; RT-PCR, reverse transcriptase polymerase chain reaction.

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appartenant à la classe des néolignanes, l'alcool déhydrodiconiférylique-4- $\beta$ -D-glucoside (DCG) dans des cultures *in vitro* de *Linum usitatissimum*. La combinaison d'expériences mono- et bidimensionnelles, telles que COSY, HMBC et HSQC a permis d'identifier la structure de ce composé sans ambiguïté. Afin d'évaluer son implication dans les mécanismes de défense, les suspensions cellulaires de *L. usitatissimum* ont été mises en présence d'extraits fongiques. La concentration en DCG a alors chuté de manière importante, après 96 heures de traitement. Corrélativement, il a été remarqué par RT-PCR semi-quantitative que l'expression de la phénylcoumarane benzylique éther réductase (PCBER) avait augmenté rapidement dès les premières heures après l'élicitation et de manière stable jusqu'à 96 h post élicitation. Ces deux résultats sont en accord, puisque la forme aglycone du DCG est l'un des deux substrats de la PCBER. Ils suggèrent l'activation de la PCBER dans des situations de défense chez les végétaux. **Pour citer cet article :** J. Attoumbre et al., C. R. Chimie 9 (2006).

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**Keywords:** Cell suspension cultures; Dehydrodiconiferyl alcohol-4- $\beta$ -D-glucoside (DCG); Elicitation; HPLC; *Linum usitatissimum*; PCBER; RMN

**Mots-clés :** Alcool déhydrodiconiférylique-4- $\beta$ -D-glucoside (DCG) ; Cultures *in vitro* ; Élicitation ; CLHP ; *Linum usitatissimum* ; PCBER ; RMN

## 1. Introduction

In plants, the oxidative dimerization of two phenylpropanoid units leads to a great variety of secondary metabolites. When the oxidative process implies the 8-8' bonds, products are named lignans while the term neolignan is used to define all of the other type of linkage. Lignans and neolignans, found in a wide range of plant species, are responsible for their pharmacological activities and implied in their defense mechanisms [1,2]. Nevertheless, despite the importance of lignans in human health protection and in plant biology, systematic studies on the enzymes involved in their biosynthesis have only recently begun [3]. In this context it has been shown that phenylcoumaran benzylic ether reductases (PCBERs) that catalyze reductive process in 8-5' linked lignans are ubiquitous vascular plant enzymes, which could have a role in plant defense. Flaxseed is known to be a rich source of lignans and *Linum usitatissimum* *in vitro* cultures have been established as a very useful tool to study the biosynthesis and the accumulation of metabolites [4–6].

In these cultures, a 8-5' phenylpropanoid dimer (dehydrodiconiferyl alcohol-4- $\beta$ -D-glucoside (DCG)) has been isolated and identified by nuclear magnetic resonance (NMR) spectroscopy. To evaluate the role of this compound in the cell cultures defense response, elicitation experiments by two fungal pathogens have been carried out. In these cultures DCG accumulation was quantified as a function of time and the gene expression of *Lu PCBER* was monitored by semi-quantitative

reverse transcriptase polymerase chain reaction (RT-PCR).

## 2. Material and methods

### 2.1. Plant material

Cell suspension cultures of *L. usitatissimum* cv Barbara were established from hypocotyl-derived calli in MS-derived medium [7] supplemented with 3% sucrose, 8.88  $\mu$ M benzylaminopurine (BAP) and 2.68  $\mu$ M naphthaleneacetic acid (NAA). All suspension cultures were incubated on a gyratory shaker at 120 rpm in darkness at 25 °C and subcultured every 14 days.

### 2.2. DCG isolation and identification by NMR and mass spectrometry

A freeze-dried and ground sample of cells (5 g) cultured on MS-derived medium [7] was extracted with methanol/water (70/30, v/v) (200 ml) for 3 h at 60 °C. After filtration, the extract was concentrated to 15 ml. DCG was separated and purified by semi-preparative high pressure liquid chromatography (HPLC) using a  $\mu$ Bondapak C18 prepacked column [10  $\mu$ m, 25  $\times$  100 mm], eluted with acetonitrile/H<sub>2</sub>O 0.2% acetic acid (15/85, v/v), flow rate 8 ml min<sup>-1</sup>, UV detection 280 nm, retention time 25 min. DCG was identified by <sup>1</sup>H, <sup>13</sup>C NMR and ESI-MS.

The 1 D and 2 D NMR spectra were recorded at 300 K on a Bruker Avance 300 spectrometer operating

at 300.13 and 75.47 MHz, respectively, for  $^1\text{H}$  and  $^{13}\text{C}$  measurements. DMSO was used as the solvent and tetramethylsilane (TMS) as the internal standard. The chemical shifts are reported as parts per million (ppm) units relative to TMS. Mass spectral analysis was performed using a Finnigan SSQ 710 series mass spectrometer (LTE, UTC, France) in a solution of  $\text{MeOH}/\text{H}_2\text{O}/\text{CH}_3\text{COOH}$  (50:50:0.5 v/v).

### 2.3. Standard protocol for DCG quantitation

One gram, fresh weight (FW) of cells were separated from medium by filtration, rinsed with distilled water and freeze-dried. After extraction with methanol/water (70/30, v/v) (10 ml) for 3 h at 60 °C and after filtration, the extracts were made up to 20 ml with distilled water and analyzed in triplicate by HPLC using a kromasil C18 column (5  $\mu\text{m}$ , 250  $\times$  4 mm, Macherey Nagel, France) eluted isocratically with acetonitrile/0.2% acetic acid/THF (20/79/1, v/v/v) at 280 nm with a flow rate of 0.7 ml  $\text{min}^{-1}$ . In these conditions, the retention time was 15 min.

### 2.4. Elicitation of flax cell cultures

Fungal elicitors were prepared as described by Campbell and Ellis [8] with slight modifications. One of these fungal elicitors was obtained from mycelium extract of a specific pathogen of flax: *Fusarium oxysporum* F. sp. *Lini*, and the other from a mycelium extract of a broad-spectrum pathogen: *Botrytis cinerea*. Following transfer to fresh medium, flax cell cultures were inoculated at day 10 by fungal elicitors at a rate of 3% v/v. In the same way, controls were inoculated with sterile double distilled water and are used as reference during this study. Incubation continued on a gyratory shaker at 120 rpm in darkness at 25 °C. After treatment for a predetermined time, cells were collected for PCBER gene expression analyses.

### 2.5. Partial PCBER cDNA isolation and semi-quantitative reverse transcriptase-PCR

Total RNAs were purified from 150 mg of frozen tissues using the RNeasy Plant Kit (Qiagen). Degenerate PCR primers were designed to bind highly conserved sequences in plant PCBER genes. PCR product was resolved and purified from an agarose gel, sub-

cloned by TA-cloning into pGEM-Teasy vector (Promega), sequenced and analyzed for sequences homologies by BLASTn and ClustalW programs.

For semi-quantitative RT-PCR analysis first-strand cDNA was synthesized from 1  $\mu\text{g}$  of total RNA at 37 °C for 60 min, with 4 units of Omniscript Reverse Transcriptase (Qiagen). PCR was performed with specific primers designed from the partial cDNA sequence of *Lu PCBER* gene with PCBER-F sens primer (5'-TAIGTCTCCGCAAACCTGCTTTGC -3') and PCBER-R antisens primer (5'-TTCAACAGTGGTGTATTTAACCTC-3'). cDNA fragments were amplified during 20, 25 and 30 cycles. To normalize the amount of mRNA in each PCR reaction, a PCR product (632 bp) corresponding to the *Lu actin* gene was amplified with ACT-F sens primer (5'-TCTGGAGATGGTGTGAGCCACAC-3') and ACT-R antisens primer (5'-GGAAGGTACTGAGGGAGGCCAAG-3'). For this study 10  $\mu\text{l}$  of 25-cycle PCR products were loaded on 1% (w:v) agarose gel separated by electrophoresis and stained with BET.

## 3. Results and discussion

In order to evaluate their content in lignans, the flax cell suspension cultures were analyzed by a HPLC method, as previously described [9]. The analysis revealed that these suspensions contained little amounts of lignans (data not shown) in regard to another compound accumulated at a very high level. This compound was isolated by semi-preparative HPLC and its structure elucidated on the basis of its NMR and MS spectral data.

### 3.1. NMR identification of a neolignan

The MS spectrum exhibited a molecular ion at  $m/z$  538  $[\text{M} + \text{NH}_4]^+$  suggesting a molecular formula  $\text{C}_{26}\text{H}_{32}\text{O}_{11}$ . The  $^1\text{H}$  NMR spectrum showed five aromatic protons: three at  $\delta = 6.95$  ( $d$ ,  $J = 1.5$  Hz), 7.06 ( $d$ ,  $J = 8.0$  Hz), and 6.84 ( $dd$ ,  $J = 1.5, 8.0$  Hz) and two at  $\delta = 6.93$  implying the presence of two aromatic rings, one with a 1,3,4-trisubstitution pattern and the second with a 1,3,4,5-tetrasubstitution pattern. Moreover, the spectrum showed signals due to two aromatic methoxyl groups at  $\delta = 3.74$  and 3.80 and two trans olefinic protons at  $\delta = 6.21$  and 6.45 with a pattern correspond-

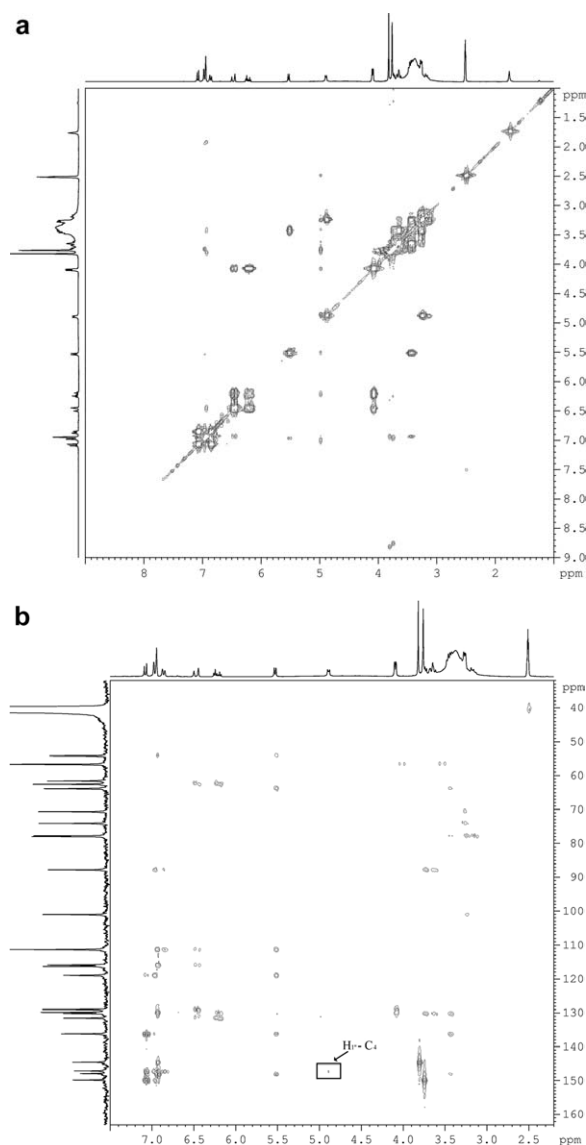


Fig. 1. DQF-COSY (a) and HMBC (b) spectra of a pure fraction of dehydrodiconiferyl alcohol-4- $\beta$ -D-glucoside (DCG).

ing to an allylic alcohol. In addition, a glucose anomeric proton was observed at  $\delta = 4.87$ .

The results of  $^1\text{H}$  and  $^{13}\text{C}$  NMR data and previous knowledge derived from lignans metabolites suggested that the isolated compound belonged to the neolignan class with a 8-5' bond and a 7-4' ether bond. The  $^{13}\text{C}$  NMR spectrum supported the presence of four oxygenated aromatic carbons at  $\delta = 144.6$ , 147.8, 149.8, and 147.1. Complete assignment of the proton spin sys-

tems was achieved by DQF-COSY (Fig. 1a), while carbons were assigned from HSQC and HMBC spectra (Fig. 1b). The evaluation of the HMBC spectrum permitted the identification of the aromatic hydroxyl and *O*-methyl positions. In particular, the HMBC spectrum showed a weak correlation between the anomeric protons at  $\delta = 4.87$  and a quaternary carbon at  $\delta = 147.1$  indicating that the sugar was located at C-4. All the chemical shifts are given in Table 1. These data allowed, to identify this compound as the dehydrodiconiferyl alcohol-4- $\beta$ -D-glucoside (DCG), a neolignan, which belongs to the phenylcoumaran family already described by Changzeng et al. [10]. In order to evaluate its involvement in plant defense mechanism, flax suspension cells were elicited with fungal pathogens (*F. oxysporum* F. sp. *Lini*, and *B. cinerea*).

### 3.2. Elicitation and RT-PCR semi-quantitative *LuPCBER* expression

After 96 h of treatment, the DCG content decreased by a factor of 13.1 and a factor of 2.1 when respectively treated with *F. oxysporum* F. sp. *Lini* and *B. cinerea*.

In order to monitor the effect of elicitation on phenylcoumaran lignans biosynthetic gene expression, degenerated primers were designed from global alignments of orthologous sequences of PCBER for PCR cloning. Indeed, PCBER catalyses the enantiospecific NADPH-dependent reduction of dehydrodiconiferyl alcohol and dihydrodehydrodiconiferyl alcohol (Fig. 2).

Based on cDNA sequences of conserved regions of plant PCBER, degenerated primers were designed and synthesized for amplification of a putative partial *LuPCBER* cDNA fragment. A putative *LuPCBER* partial cDNA of 625 bp was obtained, sequenced and analyzed for homology studies by local alignments (BLASTn and ClustalW) and submitted to GenBank under the accession number AY837829. The deduced amino acid sequence was determined and database retrieval with PSI-BLAST as well as multiple alignments revealed that *LuPCBER* is highly similar to many other PCBER, isoflavone reductase (IFR) and Pinoreosinol larciresinol reductase (PLR) from higher plants at the amino acid level, indicating that the *LuPCBER* belongs to the IFR superfamily (Table 2).

Table 1  
NMR spectral data of dehydroconiferyl alcohol-4-β-D-glucoside (DCG)

	<sup>1</sup> H	<sup>13</sup> C
1	–	136.2
2	6.95	111.3
3	–	149.8
4	–	147.1
5	7.06	116.2
6	6.84	118.9
7	5.51	87.7
8	3.43	54.2
9	3.63; 3.72	63.8
OC <sub>6</sub> H <sub>3</sub>	3.74	56.6
1'	–	131.5
2'	6.92	111.2
3'	–	144.6
4'	–	147.8
5'	–	130.2
6'	6.93	115.9
7'	6.45	129.8
8'	6.21	128.9
9'	4.07	62.5
OC <sub>6</sub> H <sub>3</sub>	3.80	56.5
1''	4.87	100.9
2''	3.23	74.1
3''	3.25	77.7
4''	3.14	70.5
5''	3.25	77.9
6''	3.65; 3.43	61.5

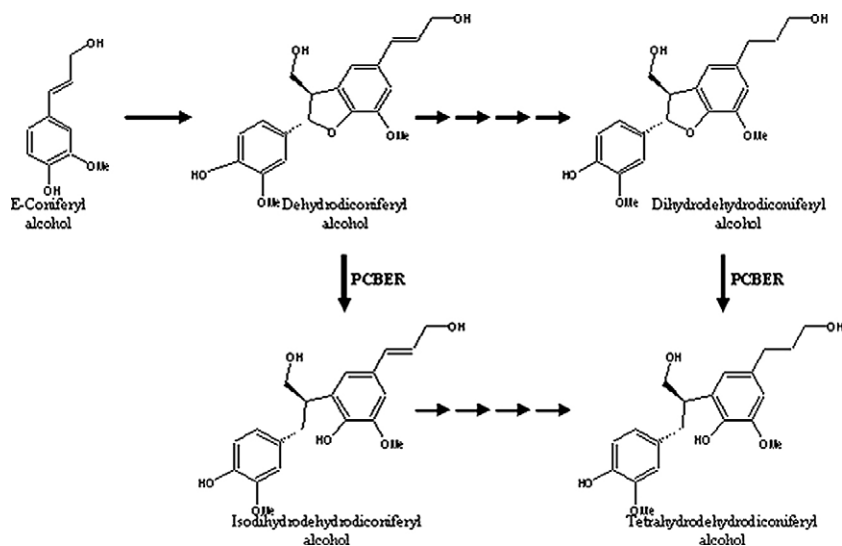
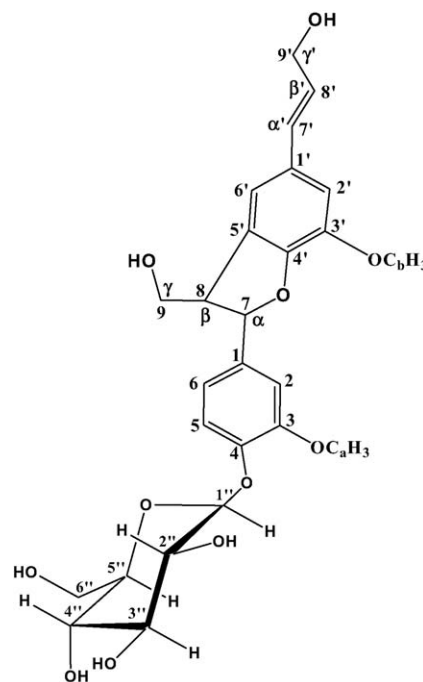


Fig. 2. Biosynthetic pathway to the 8-5'-linked lignan of *Pinus taeda* showing the reductions steps catalyzed by PCBER as described by Kwon et al. [11].

Table 2

BLASTp results showing identity and homology percentages of LuPCBER with other PCBER, IFR and PLR sequences (accession number from GenBank)

Organism	Enzyme	Accession number	Identity (%)	Homology (%)
<i>Tsuga heterophylla</i>	PCBER	AAF64178	71	80
<i>Forsythia x intermedia</i>	PCBER	AAF64174	70	83
<i>Glycine max</i>	IFR	AAF17578	70	83
<i>Populus trichocarpa</i>	PCBER	CAA06706	67	81
<i>Solanum tuberosum</i>	PCBER	CAA63056	67	80
<i>Pinus taeda</i>	PCBER	AAC32591	67	78
<i>Pinus pinaster</i>	PLR	AL751330	67	78
<i>Forsythia x intermedia</i>	PLR	AAC49608	49	62
<i>Thuja plicata</i>	PLR	AAF63310	47	63
<i>Tsuga heterophylla</i>	PLR	AAF64184	47	62

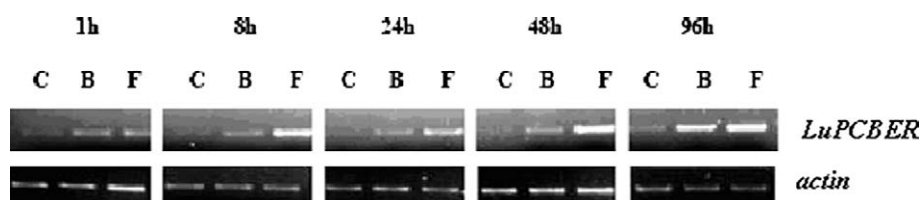


Fig. 3. RT-PCR analysis of *LuPCBER* gene expression in control and elicited cell suspension cultures. Total RNA isolated 1, 8, 24, 48, and 96 h post elicitation was subjected to RT-PCR semi-quantitative analysis using an *actin* gene as internal control. C, control cell suspension, B and F, cell suspensions treated with mycelium extract of *B. cinerea*, or *F. oxysporum*.

#### 4. Conclusion

The *LuPCBER* transcript accumulation in response to elicitation with fungal extracts of *B. cinerea* and *F. oxysporum* was monitored by semi-quantitative RT-PCR (Fig. 3). It revealed a marked elicitor effect on the steady state mRNA levels corresponding to the *LuPCBER* gene. A rapid and continuous induction of *LuPCBER* gene expression from 1 h to 96 h post elicitation was observed in both elicited cell cultures, this was particularly marked for *Fusarium* treated cultures. Given the reaction catalyzed by PCBER, the decrease of dehydroconiferyl alcohol accumulated as glucosides in flax cell suspension could be correlated with the increase in *LuPCBER* gene expression and would therefore suggest the activation of PCBER in plant defense mechanisms [3].

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