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Functional expression of cinnamate 4-hydroxylase from *Ammi majus* L.

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Total RNA was isolated from dark-grown cell suspension cultures of *Ammi majus* L. that had been induced with fungal elicitor or treated with water for control and used as template with cytochrome P450-specific primers for DD-RT-PCR amplifications. A cDNA clone was generated from the elicited transcripts and assigned to cinnamate 4-monooxygenase based on sequence alignments and functional expression in yeast cells. Comparison of the translated polypeptide with database accessions of heterologous cytochrome P450 monooxygenases revealed a high degree of similarity (99.6%) with 98.6% identity to cinnamic acid 4-hydroxylase from parsley, documenting the close evolutionary relationship within the Apiaceae family. Maximal activity of the *Ammi* hydroxylase in yeast microsomes was determined at 25 °C and in the pH range of 6.5–7.0 reaching 2.5 pkat/mg on average. An apparent K_m of 8.9 μM was determined for cinnamate. Preincubations with psoralen or 8-methoxypsoralen added up to 100 μM in the presence or absence of NADPH hardly affected the turnover rate. *A. majus* cell cultures accumulate sets of *O*-prenylated umbelliferones and linear furanocoumarins besides lignin-like compounds upon treatment with elicitor, and cinnamic acid 4-hydroxylase catalyzes the initial reaction leading from the general into the various phenylpropanoid branch pathways. Correspondingly, the hydroxylase transcript abundance was induced in the elicited cells.

Keywords: *Ammi majus*; Apiaceae; *trans*-Cinnamate 4-monooxygenase (EC 1.14.13.11); C4H; Furanocoumarin biosynthesis

1. Introduction

Cell cultures of the Apiaceae, in particular *Petroselinum crispum* (Tietjen et al., 1983; Wendorff and Matern, 1986; Hahlbrock and Scheel, 1989; Hahlbrock et al., 1995) and *A. majus* (Hamerski and Matern, 1988a,b), have been extensively used to study the elicitor induction of phytoalexins. Upon elicitation, both cultures produce linear furanocoumarins (psoralens) in addition to lignin-like compounds (Matern, 1991; Matern and Grimmig, 1994; Matern et al., 1995) or butylidenephthalides in case of parsley (Hagemeier et al., 1999). While the biosynthesis of psoralen from umbelliferone requires 6-*C*-prenylation to yield demethylsuberosin **1** (Fig. 1), *Ammi majus* concomitantly accumulates various 7-*O*-prenylated umbelliferones as the predominant coumarins (Hamerski et al., 1990a) (Fig. 1). Both the respective prenyltransferase activities were detected in vitro in the microsomal fraction of the elicitor-induced cells (Hamerski et al., 1990b). Furthermore, four cytochrome P450 monooxygenase activities were distinguished which sequentially convert demethylsuberosin **1** to (+)-marmesin, psoralen **2**, bergaptol and/or xanthotoxol. Higher activities were measured with microsomes from elicited *Ammi* cells as compared to *Petroselinum*, and these microsomes enabled the mechanistic investigation of the psoralen synthase reaction which releases acetone from (+)-marmesin (Matern et al., 1999; Stanjek et al., 1999). The number and the inducibility of these activities mark elicitor-treated *A. majus* cell cultures as an attractive system for the cloning of coumarin-related P450 monooxygenases.

Cinnamic acid 4-hydroxylase (C4H) catalyzes the hydroxylation of *trans*-cinnamate **3** to *trans*-4-coumarate

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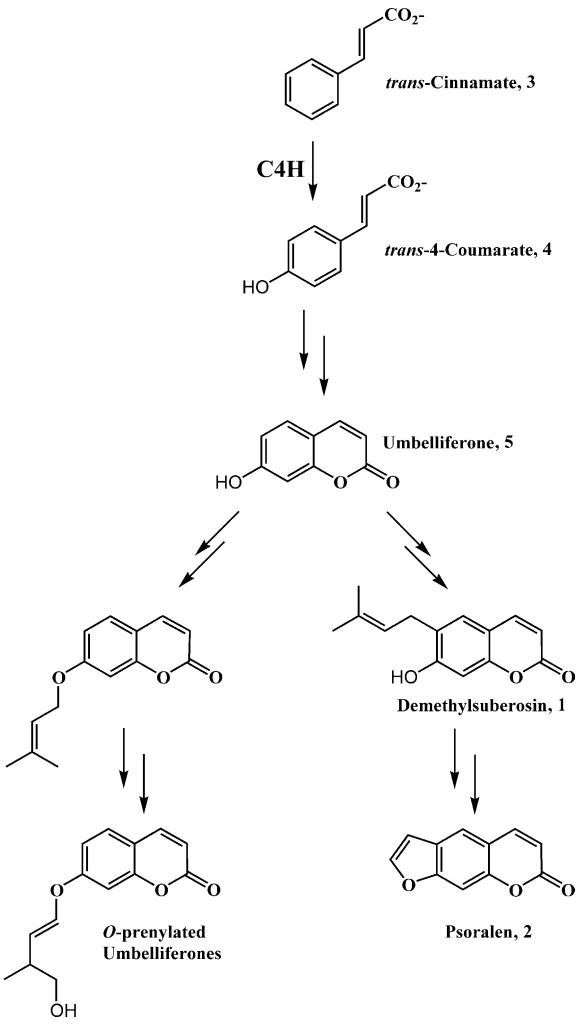

Fig. 1. Hydroxylation of *trans*-cinnamate **3** by C4H as a first step in the formation of coumarin derivatives (prenylated umbelliferones and psoralen **1**) in *Ammi majus*.

4 (Fig. 1), the first oxygenation step in phenylpropanoid biosynthesis, and the phenylpropanoid branch pathways lead to a wide array of secondary products including lignins, flavonoids, hydroxycinnamic esters and coumarins. These compounds are essential for UV-protection, differentiation of tissues and defence giving reason for the induction of C4H upon irradiation, wounding or infection of plants (Koopmann et al., 1999; Russell, 1971; Benveniste et al., 1978). Some plant genera are assumed to contain only one gene for C4H, such as *Pisum* (Frank et al., 1996), *Arabidopsis* (Bell-Lelong et al., 1997) and *Petroselinum* (Koopmann et al., 1999), while maize (Batard et al., 2000), alfalfa (Fahrendorf and Dixon, 1993) and periwinkle (Hotze et al., 1995) encoded small gene families. Following the addition of fungal elicitor, cultured *A. majus* cells reinforce their cell walls with ferulic esters (Matern, 1991) and produce various coumarins, such as umbelliferone **5**, (+)-marmesin and isopimpinellin besides umbelliferone methyl- and hydroxymethylbutenyl ethers, in a process mimicking the disease resistance response of the plant (Hamerski et al., 1990a,b). Both these classes of secondary metabolites are derived from the phenylpropanoid pathway (Murray et al., 1982; Matern et al., 1988), i.e. from 4-coumaric acid **4**, and the induction of C4H transcript abundance on elicitation of the cells was to be expected.

We report here the cloning and functional characterization of C4H from elicited *A. majus* cells. The enzyme sequence is very closely related to that of C4H from parsley, and kinetic investigations revealed a low sensitivity of the enzyme to the presence of psoralens.

2. Results

2.1. Cloning, functional expression and northern blot analysis

The accumulation of coumarins and other phenylpropanoids in *A. majus* cell suspensions had previously been triggered by the addition of crude cell wall fractions from *Phytophtora sojae* (formerly *P. megasperma* f. sp. *glycinea*, *Pmg*), and butenyl and hydroxybutenyl ethers of umbelliferone (Fig. 1) accumulated with a lag of 3–5 h in the culture fluid to maximal concentrations after about 35 h (Hamerski et al., 1990a). The activities of dimethylallyl-diphosphate:umbelliferone 6-*C*- and 7-*O*-dimethylallyltransferases increased concomitantly and reached a first maximum at about 12 h of elicitation (Hamerski et al., 1990b), suggesting maximal transcript abundances within the first 6 h. This time frame corresponds to the elicitor induction of C4H mRNA in pea which reportedly increased 3.78-fold with a lag of 1–3 h (Frank et al., 1996).

Total RNA was therefore isolated (Giuliano et al., 1993) from *A. majus* cells that had been treated with *Pmg* elicitor for 5 h and employed as a template for DD-RT-PCR amplification according to Fischer et al. (2001) with modifications. The cDNA from water-treated cells was used for control. The preferential amplification of fragments encoding cytochrome P450 monooxygenases is based on four and eight PCR primers, respectively, derived from the motifs EEF(R)PER and PFG highly conserved in these polypeptide sequences, and the procedure generated fragments of about 500 bp in length which were not amplified from control templates (Fig. 2). The fragments were cloned into the pCR2.1-TOPO vector, the inserts were sequenced, and individual full-length clones were generated by nested PCR, RACE and RLM-RACE techniques. Six cDNA clones were fully sequenced and classified by sequence alignments as encoding different P450 monooxygenases. One of these clones of 1623 bp was provisionally assigned to cinnamic acid 4-hydroxylase (C4H). The ORF of this clone was ligated into pYES2.1/V5-His-TOPO vector for

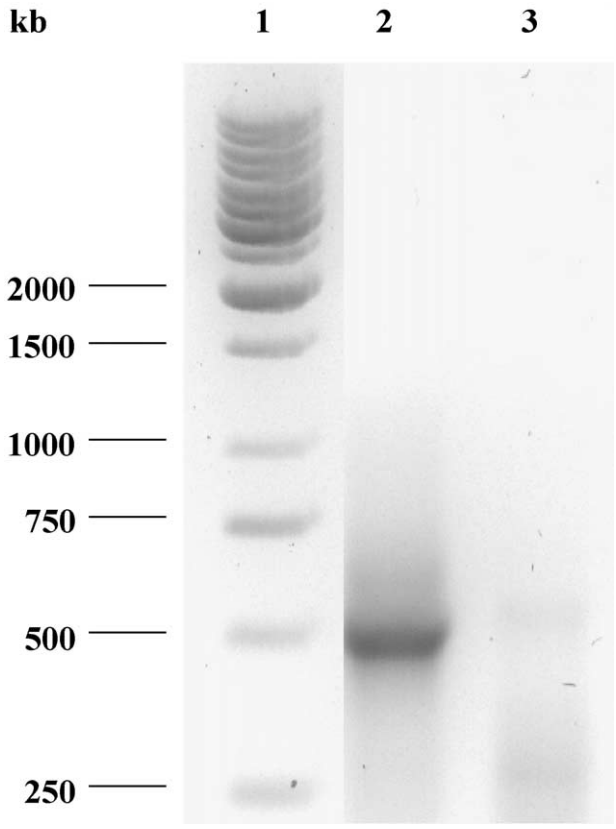

Fig. 2. Separation of amplified cytochrome P450 monooxygenase-related transcripts by DD-RT-PCR using total RNA from elicited (lane 2) or non-induced (lane 3) *Ammi majus* cells as template. Size markers are indicated in lane 1.

functional expression in *Saccharomyces cerevisiae* strain INVSc1. Microsomes were isolated from the transformants as described elsewhere (Pompon et al., 1996) and employed for C4H activity assays. The product of the reaction was identified as 4-coumaric acid **4** by radio-TLC on silica gel and comparison with an authentic sample. Yeast cells transformed with the antisense construct did not reveal C4H activity. The product was subjected further to a coupled assay employing 4-coumarate:CoA ligase (Endler et al., unpublished) and chalcone synthase (CHS) (Springob et al., 2000) from *Ruta graveolens* which, in the presence of [2-^{14}C]malonyl-CoA, converted the product to ^{14}C-labeled naringenin chalcone. The chalcone was isomerised to (2*S*/*R*)-naringenin (Springob et al., 2000), and the identity of this naringenin was corroborated by cochromatography with an authentic sample on cellulose thin-layer plates using 20% acetic acid (Springob et al., 2000), thus verifying the identity of the cloned C4H. The enzyme appears to possess narrow substrate specificity, because control incubations of the recombinant crude C4H with ^{14}C-labeled salicylic or 4-hydroxybenzoic acid did not yield any product.

Finally, the specific primers 5′-ACATGATGGACTTTGTTCTGTTAGAG (forward) and 5′-GGTATAATGGTATCAAGTCCACC (reverse), corresponding to nt 36–61 and nt 1601–1623, respectively, of the isolated C4H sequence, were employed for RT-PCR verification of the ORF using total RNA as template that had been extracted from leaf tissue of 6-day-old *A. majus* seedlings. The amplified ORF sequence fully matched the sequence determined from suspension cells and confirmed the identity of the *A. majus* cDNA clone which thus classified as CYP73A41 (GenBank accession number: AY219918). Furthermore, in suspension cells an ephemeral induction of C4H transcript abundance was apparent, since northern blot analysis showed an increase up to 3.5 h after elicitation followed by a moderate decrease of the specific mRNA level (data not shown).

2.2. Sequence alignments

The sequence of the translated C4H polypeptide (Fig. 3) revealed elements generally found in P450 monooxygenases, including subfamily CYP73A, such as the proline-rich region and the helical regions I, J and K, in addition to the PFG and (D)EFRPER-motifs (Fahrendorf and Dixon, 1993; Mizutani et al., 1997; Teutsch et al., 1993). The central part of the heme-binding loop contains a proline residue (Pro450) typically found in plant CYPs and shown by site-directed mutagenesis to be essential for heme-protein interactions (Schalk et al., 1999). Furthermore, the alanine dipeptide (307Ala-Ala) is present as in the I helix of all known CYP73A enzymes. The relative position of these two motifs varies slightly in the C4Hs of different plants from Pro449 and 307Ala-Ala in *Medicago sativa* and *Glycine max* over Pro448 and 306Ala-Ala in *Arabidopsis thaliana*, *Catharanthus roseus*, *Populus kitamasiensis*, *Pisum sativum*, *Glycyrrhiza echinata*, *Helianthus tuberosus* and *Zinnia elegans* to Pro447 and 305Ala-Ala in *Cicer arietinum*.

Most notable was the extraordinary degree of sequence identity of the *A. majus* enzyme with the C4H polypeptide from *Petroselinum crispum* (Koopmann et al., 1999) determined with a surprising 98.6% at 99.6% similarity (Fig. 3). Genes at a level of sequence identity exceeding 97% are considered as allelic variants according to Nelson et al. (1996). For comparison, the identity of the four C4H polypeptides identified so far from Fabaceae plants reached at most 91% as did the identity of the two constitutively expressed C4H polypeptides in *Lithospermum erythrorhizon* (Yamamura et al., 2001). *Citrus sinensis* Osbeck expresses a wound-inducible C4H1 and a constitutive C4H2, which share only 66% sequence identity at the polypeptide level (Betz et al., 2001). Overall, the unusually high degree of sequence conservation among the C4Hs from *Petroselinum* (CYP73A10) and *Ammi* (CYP73A41) suggests a short evolutionary distance between the Apiaceae.

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        1       10        20        30        40        50        60
C4HAm   MMDFVLLEKALLGLFIATIVAITISKLRGKKLKLPPGPIPVPVFGNWLQVGDDLNQRNLV
C4HPc   ------------------------------------------------------------

               70        80        90       100       110       120
C4HAm   EYAKKFGDLFLLRMGQRNLVVVSSPDLAKDVLHTQGVEFGSRTRNVVFDIFTGKGQDMVF
C4HPc   D---------M--------------E---------------------------------

              130       140       150       160       170       180
C4HAm   TVYSEHWRKMRRIMTVPFFTNKVVQQYRFGWEDEAARVVEDVKANPEAATNGIVLRNRLQ
C4HPc   ------------------------------------------------------------

              190       200       210       220       230       240
C4HAm   LLMYNNMYRIMFDRRFESVDDPLFLKLKALNGERSRLAQSFEYNFGDFIPILRPFLRGYL
C4HPc   ----------------------------------------H-----------------

              250       260       270       280       290       300
C4HAm   KLCQEIKDKRLKLFKDYFVDERKKLESIKSVGNNSLKCAIDHIIEAQEKGEINEDNVLYI
C4HPc   -------------------------------D---------------Q------------

              310       320       330       340       350       360
C4HAm   VENINVAAIETTLWSIEWGIAELVNNPEIQKKLRHELDTVLGAGVQICEPDVQKLPYLQA
C4HPc   ------------------------------------------------------------

              370       380       390       400       410       420
C4HAm   VIKETLRYRMAIPLLVPHMNLHEAKLAGYDIPAESKILVNAWWLANNPAHWNKPDEFRPE
C4HPc   ----------------------D-------------------------------------

              430       440       450       460       470       480
C4HAm   RFLEEESKVEANGNDFKYIPFGVGRRSCPGIILALPILGIVIGRLVQNFELLPPPGQSKI
C4HPc   ----------------------------------------------------------

              490              506
C4HAm   DTAEKGGQFSLQILKHSTIVCKPRSS
C4HPc   -------------------------
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Fig. 3. Alignment of the translated C4H polypeptides from *Ammi majus* L. (C4HAm) and *Petroselinum crispum* (C4HPc). Only those amino acids representing major changes in the overall charge (bold printed) in C4HPc as compared to C4HAm are highlighted. Sequence elements generally found in P450 monooxygenases are underlined.

2.3. Southern blot analysis

In order to determine the copy number of C4H genes in the genome of *A. majus*, genomic DNA was isolated and digested separately using various restriction enzymes. After agarose gel electrophoresis, the fragments were blotted to a nylon membrane and probed with the *A. majus* C4H-specific ^{32}P-labeled (random primed) cDNA fragment. Restriction of the DNA with *Eco*RV, *Bgl*II or *Pst*I resulted in two hybridizing bands, whereas only one band was apparent after treatment with *Eco*RI, *Xho*I or *Bam*HI (data not shown). These data suggest the presence of a single functional C4H gene in *A. majus* analogous to the results reported for parsley (Koopmann et al., 1999) and corresponding to the DD-RT-PCR amplification of only one such cDNA.

2.4. Kinetic characterization

Significant activity of the recombinant C4H was observed in a pH range from about 5.5 to 8.0 with maximal values in potassium phosphate and Tris–HCl buffer, respectively, of pH 6.5 or 7.0 (Fig. 4) and decreasing steeply above pH 7.5. This activity profile resembles that of parsley C4H (Koopmann et al., 1999) and is slightly lower than reported for other C4Hs (pH 7.5). The hydroxylase was active over a broad temperature range from 20 to 40° C with an optimum at 25 °C. Kinetic assays were conducted at 25 °C in 200 mM Tris–HCl buffer pH 7.0 employing 80 μg of microsomal protein per incubation and 1.2 mM NADPH in the presence of various concentration of [U-^{14}C]cinnamic acid (2.5–20 μM). The apparent K_m for cinnamic acid **3** was determined at 8.9 μM, which is in agreement with the values reported for C4Hs from other plant sources (Gabriac et al., 1991; Mizutani et al., 1997; Koopmann et al., 1999).

The *A. majus* C4H was expressed in yeast microsomes to only moderate levels of activity as compared to reports on other C4Hs (Koopmann et al., 1999; Batard et al., 2000; Fischer et al., 2001; Gravot, 2002) with provisional Vmax values of 2.5 pKat/mg on average.

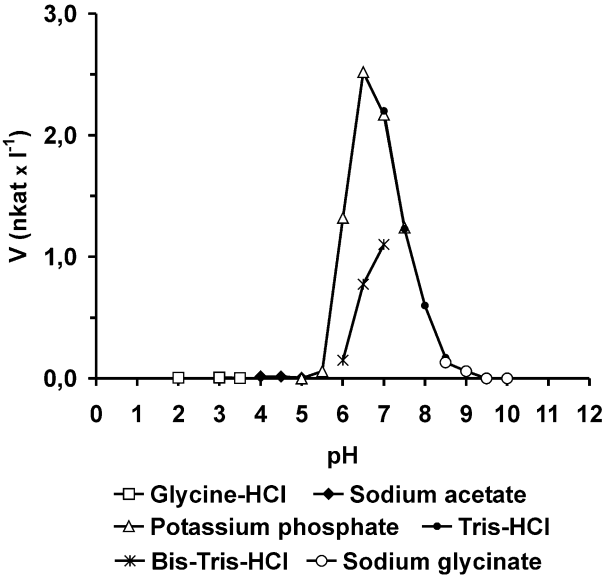

Fig. 4. Determination of the optimal pH range for the activity of *Ammi majus* cinnamic acid 4-hydroxylase expressed in yeast microsomes. The assays were conducted at 37 °C in 200 mM buffers (114 µl total volume) containing 1.2 mM NADPH and 80 µg of microsomal protein.

This is likely due in part to the yeast strain employed. The activity was nevertheless sufficient to examine the effect of psoralens which have been proposed to cause a mechanism-based inhibition of cytochrome P450 (CYP) monoxygenases (Koenigs et al., 1997; Koenigs and Trager, 1998). Psoralen-producing plants, e.g. species of the Apiaceae and Rutaceae, are believed to encode CYPs with enhanced psoralen **2** tolerance, and this has been documented recently for yeast-expressed C4H of *Ruta graveolens* in comparison with CYP73A1 (Gravot, 2002). In these experiments, an 85% inhibition of CYP73A1 activity was observed by 15 min preincubation of the enzyme in the presence of 50 µM psoralen or 8-methoxypsoralen and 1 mM NADPH. Preincubation of recombinant *A. majus* C4H for 20 min with psoralen **2** or 8-methoxypsoralen up to 100 µM inhibited the activity to only 28±1% or 34±4% in presence of NADPH and 29±4% or 31±2% in absence of NADPH.

3. Discussion

The approach to isolate elicitor-inducible Cyt P450 moonooxygenases from *A. majus* cells was taken, because these cultures effectively accumulate coumarin derivatives (Fig. 1) and yield active microsomal preparations which sequentially catalyze several steps of the biosynthesis. The cDNA described in this report is one of six elicitor-inducible transcripts putatively assigned to the Cyt P450 family. A polypeptide sequence identity of more than 80% with a functionally identified ortholog is usually accepted as a sufficient means of identification, although very few amino acid substitutions may already affect the substrate specificity (Lindberg and Negishi, 1989). The 98.6% identity of the *A. majus* polypeptide sequence with that of parsley C4H (Fig. 3) thus provides a reliable criterion for the assignment, and the amplification of the identical cDNA from leaf tissue RNA confirmed the *A. majus* indigeneous sequence. Nevertheless, the activity of the recombinant enzyme expressed in yeast cells was also assayed in vitro and found to *para*-hydroxylate cinnamate **3** to 4-coumarate **4**. This activity can be considered as the entry point reaction for the biosynthesis of coumarins and hydroxycinnamic esters, in particular since it is encoded by only one gene. The rapid induction of C4H transcript abundance upon elicitation with a maximum at about 3.5 h appears to support such a role for the enzyme in *A. majus* cells and sets a time scale for the induction of coumarin-specific transcripts.

Although a number of C4Hs have been cloned, biochemical data have remained scarce. The kinetic parameters and the optimal choice of temperature and pH conditions or buffer quality may be important for comparative analysis. Some coumarin derivatives, i.e. linear furanocoumarins, are known to affect the activity of Cyt P450 monooxygenases, a phenomenon that has been addressed as mechanism-based- or suicide-inhibition (Koenigs et al., 1997; Koenigs and Trager, 1998), and based on the C4Hs of parsley (CYP73A10) and the common rue (CYP73A32) reduced sensitivity was suggested for the enzymes involved in furanocoumarin biosynthesis (Gravot, 2002). The Km of 8.9 µM for cinnamate compared favourably with the data reported for other C4Hs (Koopmann et al., 1999). Furthermore, preincubation with psoralen **2** or 8-methoxypsoralen inhibited the activity only moderately at high concentrations (exceeding 50 µM), which is in contrast to the CYP73A1 activity from *Helianthus tuberosus* (Gravot, 2002). However, this effect was independent of NADPH and thus unlikely due to mechanism-based inhibition. The results suggest that *A. majus*, similar to the common rue and probably also parsley, encodes a form of C4H insensitive to the accumulation of psoralens. The particular differences in the polypeptides responsible for the enhanced psoralen **2** insensitivity remain to be established, but the amino acid substitutions H55Q, G123S, E198V and H491F(or Q) in the C-terminus have been noted for CYP73A10 and CYP73A32 (Gravot, 2002) and are recurring in the *A. majus* CYP73A41 sequence (Fig. 3).

4. Experimental

4.1. Cell cultures and induction

Cell suspension cultures of *A. majus* L. (40 ml in 250 ml flasks) were initiated and grown continuously in the

dark as described previously (Hamerski et al., 1988a,b). Crude *Pmg* elicitor in 1 ml water was added (5 mg/ml) to 5-day-old cultures, and the cells were harvested 5 h later and frozen in liquid nitrogen until use.

4.2. Materials

[2-^{14}C]Malonyl-CoA (55 mCi/mmol), [7-^{14}C]salicylic acid (56 mCi/mmol) and [carboxyl-^{14}C]hydroxybenzoic acid (55 mCi/mmol), L-[U-^{14}C]phenylalanine (396 mCi/mmol) were purchased from Hartmann Analytik (Braunschweig, Germany).

4.3. Cloning, sequence analysis and expression

Total RNA was isolated from the elicitor-induced and control cells that had received only water (1 ml) following the protocol of Giuliano et al. (1993). ESTs were generated by a cloning strategy developed by Fischer et al. (2001) for divergent plant cytochrome P450 genes. Briefly, four primers derived from the conserved EEF(R)PER motif were used in the first round of PCR amplifications. The individual PCR incubations were diluted 1:1000 and amplified in a second round of nested PCR with the decamer primers D1 through D8 as described by Schopfer and Ebel (1998). The cDNA fragments were cloned and sequenced, and full length clones were generated by RT-PCR and RNA ligase-mediated (RLM)-RACE with cDNA from induced cells. Bands of 200–600 bp separated by agarose gel electrophoresis were amplified in a third round of PCR using the same primers, and cloning of the PCR products was performed by TOPO TA Cloning. (Invitrogen). Sequence analysis was accomplished with advanced WU-Blast2 (EMBL) and alignments with ClustalW (EMBL-EBI). Expression of the ORF of the cDNA was achieved in the pYES2.1/V5-His-TOPO vector (Invitrogen) and using the yeast strain INVSc1 (Invitrogen) for transformation. Microsomes of the transformants were isolated as described elsewhere (Pompon et al., 1996).

4.4. Enzyme assays

CH4 activity was initially measured at 37 °C in 200 mM Tris–HCl-buffer pH 7.5 (total volume 114 µl) in the presence of NADPH (1.2–6.0 mM) and [U-^{14}C]cinnamic acid **3** as substrate (Urban et al., 1994). Labeled cinnamic acid **3** was from CEA France (46.3 mCi/mmol; 3.8 µM in the assay) or was generated from L-[U-^{14}C]phenylalanine (1.1×106 dpm) with *Rhodotorula glutinis* PAL (Sigma, Deisenhofen, Germany) (1 µM in the assay). The concentration of microsomal protein (17.25–138.0 µg/assay) and the time of incubation (10–50 min) were adjusted for linear conversion rates, and the incubations were stopped by the addition of 24% aq. trichloroacetic acid (20 ml). Cinnamic acids (**3** and **4**) were extracted subsequently from the incubation with ethyl acetate and analyzed by silica TLC in toluene–acetic acid 4:1 (solvent 1) or cyclohexane–ethyl acetate 2:1 (solvent 2). The developed plates were examined for radioactivity by a Bio Imager (FLA 2000 Fuji Photo Film, Tokyo, Japan) with software from Raytest (Straubenhardt, Germany), and the product was identified by comparison with authentic references (solvent 1: *trans*-cinnamic acid **3**, Rf 0.55; *trans*-4-coumaric acid **4**, Rf 0.39). No product other than 4-coumaric acid **4** was observed. For the determination of kinetic parameters, incubation conditions were modified to 25 °C in Tris–HCl buffer pH 7.0 and using 80 µg of microsomal protein. Protein was determined according to Lowry et al. (1951), and the data were extrapolated in double reciprocal Lineweaver–Burk plots.

4.5. Inhibition studies

The preincubation of yeast-expressed C4H from *A. majus* (400 µg protein) was conducted for 20 min at 25 °C in 200 mM Tris–HCl buffer pH 7.0 (100 µl total volume) containing 1.2 mM NADPH and various concentrations (from 2.5 to 100 µM) of psoralen **2** or 8-methoxypsoralen added in 2 µl ethyleneglycol monomethylether. Control preincubations were carried out in the absence of either NADPH or psoralen **2**, as well as in the absence of both psoralen **2** and NADPH. Aliquots of the preincubation (20 µl) were then added to 94 µl of the C4H assay mixture containing 3.8 µM L-[U-^{14}C] cinnamate **3** and 1.2 mM NADPH in 200 mM Tris–HCl buffer pH 7.0, and the incubation was continued for 5 min at 25 °C. The reaction was stopped by the addition of 24% aq. trichloroacetic acid (20 ml), and the cinnamic acids (**3**, **4**) were extracted with ethyl acetate and analysed as described above.

4.6. Blotting techniques

Genomic DNA was isolated from 3 g *A. majus* cell suspension cultures according to Dellaporta et al. (1983) and digested with the appropriate restriction enzymes. Genomic Southern blots with the *A. majus* C4H-specific ^{32}P-labeled (random primed) cDNA fragment were carried out following a standard protocol (Sambrook et al., 1989). For RNA dot blot (Northern blot) analysis, total RNA was isolated every 0.5 h and up to 6 h following the addition of elicitor to the cell suspensions. The RNA (4 µg) was denatured in 0.5×MOPS buffer pH 7.0, containing 2.2 M formaldehyde and 50% formamide, and transferred to a Hybond-N+ nylon membrane (Amersham Biosciences, Freiburg) in a I-SRc 96-Dot Blot Minifold (Schleicher and Schüll, Dassel) by standard techniques (Sambrook et al., 1989). Northern hybridization was carried out

overnight at 68 °C in 2×Denhardt's solution in the presence of 0.5% (w/v) SDS and 100 μg/ml salmon sperm DNA (Sigma, Deisenhofen).

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