Cytochrome P450 CYP79F1 from Arabidopsis Catalyzes the Conversion of Dihomomethionine and Trihomomethionine to the Corresponding Aldoximes in the Biosynthesis of Aliphatic Glucosinolates*

Received for publication, November 7, 2000, and in revised form, December 19, 2000
Published, JBC Papers in Press, December 22, 2000, DOI 10.1074/jbc.M010123200

Carsten Hörslev Hansen‡§, Ute Wittstock‡§, Carl Erik Olsen‡§, Alastair J. Hick‡, John A. Pickett‡, and Barbara Ann Halkier‡§***
From the ‡Plant Biochemistry Laboratory, Department of Plant Biology, ¶Department of Chemistry, and †Center for Molecular Plant Physiology (PlaCe), The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark and the †Integrated Approach to Crop Research-Rothamsted, Harpenden, Hertfordshire AL5 2JQ, United Kingdom

Glucosinolates are natural plant products that have received rising attention due to their role in interactions between pests and crop plants and as chemical protectors against cancer. Glucosinolates are derived from amino acids and have aldoximes as intermediates. We report that cytochrome P450 CYP79F1 catalyzes aldoxime formation in the biosynthesis of aliphatic glucosinolates in Arabidopsis thaliana. Using recombinant CYP79F1 functionally expressed in Escherichia coli, we show that both dihomomethionine and trihomomethionine are metabolized by CYP79F1 resulting in the formation of 5-methylthiopentanaldoxime and 6-methylthiohexanaldoxime, respectively. 5-methylthiopenta-naldoxime is the precursor of the major glucosinolates in leaves of A. thaliana, i.e. 4-methylthiobutylglucosinolate and 4-methylsulfinylbutylglucosinolate, and a variety of other glucosinolates in Brassica sp. Transgenic A. thaliana with cosuppression of CYP79F1 have a reduced content of aliphatic glucosinolates and a highly increased level of dihomomethionine and trihomomethionine. The transgenic plants have a morphological phenotype showing loss of apical dominance and formation of multiple axillary shoots. Our data provide the first evidence that a cytochrome P450 catalyzes the N-hydroxylation of chain-elongated methionine homologues to the corresponding aldoximes in the biosynthesis of aliphatic glucosinolates.

Glucosinolates are amino acid-derived secondary plant products containing a sulfate and a thioalcohol moiety. They are found in the order Capparales, which includes the economically important oilseed rape, Brassica vegetables, and the model plant Arabidopsis thaliana L. Upon tissue damage, glucosinolates are hydrolyzed by endogenous thiglucosidases (myrosinases) to produce a variety of degradation products, typically isothiocyanates, thiocyanates, and nitriles, which have a wide range of biological effects (1, 2). Generally, the glucosinolate/myrosinase system is believed to be involved in plant defense. In addition, it has been shown that glucosinolates or rather the isothiocyanates, particularly sulforaphane, the isothiocyanate of 4-methylsulfinylbutylglucosinolate, have anticarcinogenic properties (3, 4). There is a rising interest in being able to control the level of specific glucosinolates in crops to improve nutritional value and pest resistance.

Glucosinolates are grouped into aliphatic, aromatic, and indole glucosinolates, depending on whether they are derived from aliphatic amino acids, phenylalanine and tyrosine, or tryptophan (for review, see Ref. 5). The amino acid often undergoes a series of chain elongations prior to entering the biosynthetic pathway, and the glucosinolate product is often subject to secondary modifications such as hydroxylations, methylation, and oxidations giving rise to the structural diversity of glucosinolates. Biosynthetic intermediates common to all glucosinolates are aldoximes, thiohydroximates, and desulfglucosinolates. The glucosinolates found in A. thaliana ecotype Columbia are derived from tryptophan, several chain-elongated methionine homologues, chain-elongated phenylala-nine (6), and phenylalanine (7). However, the dihomomethionine-derived glucosinolates 4-methylthiobutylglucosinolate and 4-methylsulfinylglucosinolate account for more than 50% of the total glucosinolate content in the rosette leaves of A. thaliana (8).

A key step in the biosynthesis of glucosinolates is the N-hydroxylation of the precursor amino acids to the corresponding aldoximes. In the biosynthesis of cyanogenic glucosides, a group of natural plant products closely related to glucosinolates, cytochromes P450 belonging to the CYP79 family catalyze the conversion of amino acids to aldoximes (9–11). The nature of the enzymes catalyzing the formation of aldoximes in glucosinolate biosynthesis has been discussed controversially (12). Recently, evidence has been provided for the involvement of CYP79 homologues in the biosynthesis of aromatic (7) and indole glucosinolates (13, 14). Regarding the biosynthesis of aliphatic glucosinolates, extensive biochemical studies with preparations of Brassica sp. and chain-elongated methionine homologues as substrates have suggested that aldoxime formation from these amino acids is not catalyzed by cytochromes P450, but by flavin-dependent monoxygenases (15–17).

In the present paper, we report the identification of a cytochrome P450 of the CYP79 family, CYP79F1, which catalyzes the conversion of dihomomethionine and trihomomethionine to 5-methylthiopentanaldoxime and 6-methylthiohexanaldoxime, respectively. The reduced levels of aliphatic glucosinolates and
The PCR reaction was set up in a total volume of 50 µl. Primer 2 (Roche Molecular Biochemicals), 0.1 µM primer, was used for plant transformation by floral dip (25) using 0.005% Agrobacterium tumefaciens (L.) Moench as described earlier (21). In a typical enzyme assay, 5 µl of spheroplasts and 4 µl of NADPH:cytochrome P450 reductase (equivalent to 0.04 units defined as 1 µmol of cytochrome c/min) were incubated with substrate in buffer containing 30 mM KP, pH 7.5, 3 mM NADPH, 3 mM reduced glutathione, 0.042% Tween 80, 1 mg ml−1 BSA, 0.1 mM di- or tri-iodothyrine in 30 mMKP buffer. Reaction mixtures containing spheroplasts of E. coli C43(DE3) transformed with empty vector were used as controls in all assays. 3.3 µM L-14H14-phenylalanine (453 µCi/µmol; Amershram Pharmacia Biotech), 3.7 µM L-14H14-tyrosine (449 µCi/µmol; Amershram Pharmacia Biotech), 0.1 mM L-14H14-methionine (565 µCi/µmol; PerkinElmer Life Sciences) were tested as potential substrates. After incubation at 28 °C for 1 h, half of the reaction mixture was analyzed by TLC on Silica Gel 60 F254 sheets (Merck) using toluene/ethanol acetate 5:1 (v/v) as eluent. Radioisotopic bands were visualized and quantified by STORM 840 PhosphorImager (Amershram Pharmacia Biotech). For GC-MS analysis, 450 µl of reaction mixture containing 3.3 µM L-methionine (Sigma), 3.3 µM L-dihomomethionine or 3.3 µM L-trihomomethionine, respectively, were incubated for 4 h at 25 °C and extracted with a total volume of 600 µl CHCl3. The organic phase was collected and evaporated, and the residue was dissolved in 15 µl of CHCl3 and analyzed by GC-MS. GC-MS analysis was performed on an HPS5890 Series II gas chromatograph directly coupled to a JMS-Q 7100 mass spectrometer. An SE30 column (15 m; 0.75 mm inner diameter; 0.25-mm film thickness) was used (head pressure, 100-kPa, splitless injection). The program temperature was as follows: 80 °C for 3 min, 80 to 180 °C at 5 °C/min, 180 to 300 °C at 20 °C/min, 300 °C for 10 min. The ion source was run in EI mode (70 eV) at 200 °C. The retention times of the E- and Z-isomer of authentic 5-methylthiopentanaloxime were 14.3 and 14.8 min. The two isomers had identical fragmentation patterns with m/z 130, 129, 113, 82, 61, and 55 as the most prominent peaks. The retention times of the E- and Z-isomer of authentic 6-methylthiobialdoxime were 17.1 and 17.6 min. The two isomers had identical fragmentation patterns with m/z 143, 144, 98, 96, 69, 61, and 55 as the most prominent peaks. The di- and trihomomethionine, tri- and methylthiobialdoxime, 5-methylthiopentanaloxime, and 6-methylthiohexanoaloxime were synthesized as described previously (22) and authenticated by NMR spectroscopy.

For kinetic measurements of the conversion of L-dihomomethionine and L-trihomomethionine to the respective aldoximes by CYP79F1, enzymatic reactions were made essentially as described above. 5 nmol of phenylacetaldoxime was added to the reaction mixtures as internal standard. Product formation was determined after 0, 5, 10, 20, 40, 70, and 100 min of incubation and quantified based on authentic standards. The reaction mixtures were extracted with a total volume of 600 µl of CHCl3. The organic phase was collected and evaporated, and the residue was dissolved in 15 µl of 50% ethanol and analyzed by LC-MS. LC-MS was done on an HP1100 LC coupled to a Bruker Esquire-LC ion trap mass spectrometer. The LC was performed on a C18 column (Chrompack Inertsil 5 ODS-3 S15, 3 µm film thickness) was used (head pressure, 100-kPa, splitless injection). The program temperature was as follows: 30% B (2 min), linear gradient 30–70% B (28 min), then linear gradient 70–100% (5 min). The electropray ionization was done in positive ion mode. Each of the three aldoximes gave nicely separated peaks for the E- and Z-forms. The 210-nm UV trace was detected by the absorbance detector and used for quantitation with the genuine software.

Generation of Transgenic Plants—To construct plants, which express the CYP79F1 cDNA under control of the CaMV35S promoter (3SS: CYP79F1 plants), the CYP79F1 cDNA was PCR-amplified from the EST ATTS5112 (Arabidopsis Biological Resource Center, Columbus, OH), which contains the full-length sequence of CYP79F1. The C43(DE3) coding region was amplified from this EST by PCR with primer 1 (sense direction; 5′-CTCTAGATGACATATGCTACCTTTAAGGACGGAACTTTGGATAA) and primer 2 (antisense direction; 5′-CGGGATCCTTAATGGTGGTGAT-9′) with a PCR fragment was digested with BamHI restriction enzyme site upstream of the start codon. The PCR reaction was set up in a total volume of 50 µl in Pwo polymerase PCR buffer with 2 mM MgSO4, using 2.5 units of Pwo polymerase. The PCR reactions were run in 20 µl PCR cycles of 15 s at 94 °C, 30 s at 58 °C, and 2 min at 72 °C were run. The PCR fragment was digested with XbaI and BamHI and ligated into an XbaI/BamHI-digested pBluescript II SK (Strategene). The cDNA was sequenced to exclude PCR errors and transferred from pBluescript II SK to an NdeIBamHI-digested pSP119p10L expression vector (18).

DNA Sequencing and Computer Analysis—Sequencing was performed on an ALF-Express (Amershram Pharmacia Biotech) using a Thermo Sequence Fluorescent-labeled primer cycle sequencing kit (7-deaza-dGTP) (Amershram Pharmacia Biotech). Sequence computer analysis was done with programs of the GCG Wisconsin Sequence Analysis Package. The GAP program was used with a gap creation penalty of eight and a gap extension penalty of two to compare pairs of sequences.

Expression in E. coli—E. coli cells of strain JM119 (Strategene) and strain C43(DE3) (19) transformed with the expression construct were grown overnight in LB medium supplemented with 100 µg ml−1 ampicillin and used to inoculate 40 ml of modified TB medium containing 50 µg ml−1 ampicillin, 1 mM thiamine, 75 µg ml−1 3-aminolevulinic acid, 1 µg ml−1 chloramphenicol, and 1 mM isopropl-β-D-thigalactoside. The cultures were grown at 28 °C for 60 h at 125 rpm. The cells were pelleted and resuspended in buffer composed of 0.2 M Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl, and 0.02 M dithiothreitol. Lysosyme was added to a final concentration of 10 µg ml−1. After incubation at 30 min at 4 °C, Mg(OAc)2 was added to a final concentration of 10 mM. Spheroplasts were pelleted, resuspended in 3.2 ml of buffer composed of 10 M Tris-HCl, pH 7.5, 14 mM Mg(OAc)2, and 60 mM KOAc, pH 7.4, and homogenized in a Potter-Elvejem homogenizer. After DNase treatment, glycerol was added to a final concentration of 30%. Temperature-induced Triton X-114 phase partitioning resulted in the formation of a detergent-rich phase containing the majority of the cytochrome P450 and a detergent-poor phase (9). Functional expression of CYP79F1 was monitored by Fe2+ CO versus Fe2+ difference spectroscopy (20) performed on an Ahmed Aminco DW-2000 TM spectrophotometer (SLM Instruments, Urbana, IL) using 10 µl of Triton X-114-rich phase in 990 µl of buffer containing 50 mM KP, pH 7.5, 2 mM EDTA, 20% glycerol, 0.2% Triton X-100, and a few grains of sodium dithionate. The amount of functional CYP79F1 was estimated based on an absorption coefficient of 91 liters mmol−1 cm−1. Measurements of Enzyme Activities—The activity of CYP79F1 was measured in E. coli spheroplasts reconstituted with NADPH:cytochrome P450 oxidoreductase purified from Sorghum bicolor (L).

The abbreviations used are: EST, expressed sequence tag; RT-PCR, reverse transcriptase-polymerase-chain reaction; GC-MS, gas chromatography-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; CaMV35S, cauliflower mosaic virus 35S; HPLC, high pressure liquid chromatography.
CYP79F1 from Arabidopsis

and 0.9% agar. Transformants were selected after 2 weeks and transferred to soil. Seeds of these plants were harvested, and kanamycin-resistant T2 plants were selected on the same medium. The procedure was repeated to obtain T3 plants.

**HPLC Analysis of the Glucosinolate Content of Plant Extracts—**The analysis was performed with tissue harvested from 9-week-old primary transformants and 7-week-old T2 plants. Simultaneously grown wild-type plants of the same ages were used as controls. The tissue (two to three rosette leaves from each plant) was freeze-dried for 48 h. Glucosinolates were analyzed as desulfoglucosinolates as follows: 3.5 ml of a potassium phosphate buffer (Promega) supplemented with 500 μM p-hydroxybenzylglucosinolate; Bioraf, Denmark) were added, and the sample was incubated in a boiling water bath for 4 min. Plant material was pelleted, and the pellet was re-extracted with 3.5 ml of 70% (v/v) methanol and centrifuged. The supernatants were pooled and analyzed by HPLC after sulfatase treatment as described previously (7). The assignment of peaks was based on retention times, and UV spectra were compared with standard compounds. Glucosinolates were quantified in relation to the internal standard and by use of response factors (8, 26).

**Analysis of the Amino Acid Content of Plant Extracts—**Rosette leaves of 7-week-old plants (250 mg from each plant) were frozen in liquid nitrogen and homogenized using mortar and pestle. The tissue was extracted in 2.5 ml of 80% methanol. The plant material was pelleted (2500 g for 15 min), and the re-extracted supernatant was evaporated to dryness. The residue was dissolved in 100 μl of water. The individual protein amino acids in the sample were identified and quantified on an Ultraspec 8 Resin reverse phase HPLC column (200 × 4.6 mm) on a Biochrom 20 amino acid analyzer (Amerham Pharmacia Biotech) according to the manufacturer’s instructions. The elution program was modified to identify and quantify the chain-elongated homologues of methionine, dihomomethionine, and trihomomethionine. Each plant was analyzed in triplicate.

For quantification of dihomomethionine in the plant material, the sample was subjected to two elution programs. Program 1 was as follows: 53 °C for 7 min, buffer A; 50 °C for 35 min, buffer A; 95 °C for 34 min, buffer A. Program 2 was as follows: 53 °C for 7 min, buffer A; 58 °C for 12 min, buffer B; 95 °C for 25 min, buffer C. Buffer A was 0.2 M sodium citrate, pH 3–4.5; buffer B was 0.2 M sodium citrate, pH 4; buffer C was 1.2 M sodium citrate, pH 6.25. In program 1, phenylalanine and dihomomethionine coeluted at 63.6 min. In program 2, tyrosine and dihomomethionine coeluted at 25.3 min. Dihomomethionine was quantified as the difference between the peak area corresponding to tyrosine and dihomomethionine in program 2 and the peak area corresponding to tyrosine in program 1. The response factor for dihomomethionine was determined using an authentic standard.

For quantification of trihomomethionine in the plant material, the sample was subjected to program 3, which was as follows: 53 °C for 7 min, buffer A; 50 °C for 5 min, buffer B; 95 °C for 7 min, buffer B; 95 °C for 25 min, buffer C. Trihomomethionine eluted at 29.0 min and was quantified as the peak area using a response factor determined with an authentic standard.

**Synthesis of Control RNA—**RNA was synthesized from pBluescript II SK vector (Stratagene) linearized by digestion with ScaI. The synthesis of control RNA was used to check for inhibition of RT reactions by contaminating genomic DNA. The RNA was dissolved in diethylpyrocarbonate-treated water. The RNA was quantified spectrophotometrically and used to synthesize first-strand cDNA. To ensure linearity of the RT-PCR, first-strand cDNA synthesis was performed on 1, 0.3, and 0.1 μg of each pool of RNA. The cDNA was synthesized in First Strand buffer (Life Technologies, Inc.) supplemented with 0.5 mM dNTPs, 10 mM Tris-HCl, 50 mM KCl, 1 mM dithiothreitol, 200 mM random hexamers (Amerham Pharmacia Biotech), 3 μg of control RNA (internal standard), and 200 units of Superscript II reverse transcriptase (Life Technologies, Inc.) in a total volume of 20 μl. The reaction mixture was incubated at 27 °C for 10 min followed by incubation at 42 °C for 50 min and inactivation at 95 °C for 5 min. The RT reactions were purified by means of a PCR purification kit (Millipore) in solution with 50 μl of 1× Tris buffer, pH 8. The purified RT reactions was subjected to PCR. The PCR reactions were set up in a total volume of 50 μl in PCR buffer (Life Technologies, Inc.) supplemented with 200 μM dNTPs, 1.5 mM MgCl2, 50 pmol of sense primer, 50 pmol of antisense primer, and 2.5 units of Platinum Taq DNA polymerase (Life Technologies, Inc.). The PCR program was as follows: 2 min at 94 °C, 23–37 cycles (depending on transcript as specified below) of 30 s at 94 °C, 30 s at 53–57 °C (depending on transcript as specified below), 50 s at 72 °C. The following primers and specific conditions were used: for CYP79F1 primer 5 (sense direction; 5'-AAAGCTCAATGGTGTAAGATAGGG) and primer 6 (antisense direction; 5'-TTTTTAGACCACATTGTTTCTCCTTC) with 53 °C and 32 cycles for CYP79F2 primer 7 (sense direction; 5'-AAAGCTCAATGCAGGATTCTCA) and primer 8 (antisense direction; 5'-GACAGGAGGCAATGACA) with 53 °C and 37 cycles, for CYP79B2 primer 9 (sense direction; 5'-AGAGAAGAACGCAACACCA) and primer 10 (antisense direction; 5'-TCAATAAAATATACGGCGTCG) with 53 °C and 34 cycles, for CYP79B3 primer 11 (sense direction; 5'-GACAGAACGTGGGCAAGCAGCCT) and primer 12 (antisense direction; 5'-TCCTCGCCGACATCCGGC) with 55 °C and 32 cycles, for actin1 primer 13 (sense direction; 5'-GGAAACAGAATTACGTGTC) and primer 14 (antisense direction; 5'-TCTCAGAATGTGAAGACATACCG) with 57 °C and 23 cycles, for control RNA (internal standard) primer 15 (sense direction; 5'-TGATGAGGCGATTAGGCG) and primer 16 (antisense direction; 5'-CCAAAGAAATAGAAGGATAGGG) with 57 °C and 32 cycles. 10 μl of the PCR reactions were analyzed by gel electrophoresis on 1% agarose gels. Bands were visualized by ethidium bromide staining and quantified on a Gel Doc 2000 Transilluminator (Bio-Rad). Primers and PCR conditions were optimized to ensure amplification of the specific cDNAs. No PCR products from genomic DNA were obtained. PCR analysis of the internal standard showed that the RT reactions ran with the same efficiency in samples prepared with different amounts of RNA isolated from different plant tissues.

**RESULTS**

**Expression of CYP79F1 in E. coli and Identification of Substrates—**Cytochromes P450 of the CYP79 family have previously been shown to be involved in the biosynthesis of cyanogenic glucosides and glucosinolates. CYP79F1 is one of several CYP79 homologues identified in the genome of *A. thaliana*. The deduced amino acid sequence of CYP79F1 has 88% identity with the deduced amino acid sequence of CYP79F2 and 39–46% identity with other CYP79 homologues from glucosinolate and cyanogenic glucoside-containing species. A full-length EST of CYP79F1 (ATTSS5112) was identified by a data base search. The cDNA obtained from the EST clone was used for expression of CYP79F1 in *E. coli* using the vector pSP19g10L, which is optimized for expression of cytochromes P450 (18). The CYP79F1 expression construct was transformed into two different *E. coli* strains, C43(DE3) and JM109. A CO difference spectrum with the characteristic peak at 450 nm was obtained for CYP79F1 expressed in strain C43(DE3), indicating the presence of functional cytochrome P450 (Fig. 1). Based on the peak at 450 nm, the expression level of CYP79F1 in *E. coli* 543(DE3) was estimated to be 110 nmol of cytochrome P450 (liters of culture)−1. A peak at 418 nm was detected at all preparations independent of whether *E. coli* was transformed with a cytochrome P450 expression construct or the empty vector. The origin of the peak at 418 nm is unknown, but possibly could be derived from endogenous heme proteins (28). The absence of a peak at 450 nm in the CO difference spectrum obtained with a preparation of *E. coli* strain JM109 harboring...
the CYP79F1 expression construct indicates low expression level or failure of expression of functional protein. Recombinant CYP79F1 was therefore obtained by use of strain C43(DE3) for all further experiments.

To identify substrates of CYP79F1, activity measurements were carried out using spheroplasts of E. coli C43(DE3) reconstituted with NADPH:cytochrome P450 reductase from S. biocolor. When the reaction mixture containing CYP79F1 was incubated with dl-dihomomethionine, two compounds, which were not present in the control reactions, were detected by GC-MS (Fig. 2). The retention times and the mass spectral fragmentation patterns of these compounds were identical with those of the EZ-isomers of the authentic standard of 5-methylthiopentanaloxide. When dl-trihomomethionine was administered to the reaction mixture containing CYP79F1, two compounds with retention times and fragmentation pattern identical to those of the EZ-isomers of the authentic standard of 6-methylthiohexanaldoxime were detected by GC-MS. The formation of both 5-methylthiopentanaloxide and 6-methylthiohexanaldoxime was linear with time within 40 min. Product formation combined with cytochrome P450 quantification allowed the estimation of turnover number of 0.23 ± 0.01 min⁻¹ for dihomomethionine and 0.15 ± 0.01 min⁻¹ for trihomomethionine. No aldoximes were produced using boiled enzyme preparation. Administration of L-methionine, L-phenylalanine, L-tyrosine, and L-tryptophan to the reaction mixtures containing recombinant CYP79F1 did not result in the formation of detectable amounts of the corresponding aldoximes.

Analysis of 35S:CYP79F1 Plants—We have produced transgenic A. thaliana expressing the CYP79F1 cDNA under the control of the CaMV35S promoter to study the effect of altered expression levels of CYP79F1 on the content and composition of glucosinolates. Nine independent primary 35S:CYP79F1 transformants were investigated, and four were selected for analysis through the following two generations. The four primary transformants had dramatically reduced levels of short-chain aliphatic glucosinolates (plants S7 and S9) or slightly increased levels of these glucosinolates (plants S3 and S5)(Fig. 3). The four plants had a morphological phenotype characterized by reduced growth rates, reduced apical dominance, and production of multiple axillary shoots at the time of floral transition resulting in bushy plants. Analysis of plants of the T2 and T3 generations showed that the observed phenotype of changed glucosinolate profile and bushy appearance was not stable. However, T3 plants of the primary transformant S3 (S3.8.1–S3.8.4) had the characteristic bushy phenotype (Fig. 4) and a dramatically reduced content of aliphatic glucosinolates (Table I). The effect was very pronounced for the glucosinolates derived from short-chain methionine homologues, i.e. homomethionine and dihomomethionine. The levels of 3-methylsulfanylpropylglucosinolate (derived from homomethionine), 4-methylthiobutylglucosinolate and 4-methylsulfanylbutylglucosinolate (both derived from dihomomethionine) were reduced to 9–13% compared with wild-type. The level of glucosinolates derived from methionine elongated by three to six methylene groups was reduced to about 30 to 50% compared with wild-type. The total content of indole glucosinolates was increased to about the double of the level in wild-type plants. Plants S3.8.2 and S3.8.3 had the most pronounced phenotype and were selected for analysis of their content of the biochemically identified substrates of CYP79F1, dihomomethionine and trihomomethionine (Fig. 5). Both plants accumulated high amounts of the CYP79F1 substrates. Plant S3.8.2 contained as much as 50 times more dihomomethionine and ten times more trihomomethionine than wild-type plants. RT-PCR analysis of plant S3.8.4 showed that the levels of CYP79F1 and CYP79F2 transcripts were strongly reduced compared with the level in wild-type plants, suggesting that introduction of the transgene had led to cosuppression (Fig. 6). The transcript level of CYP79B2 was slightly increased compared with the level in wild-type plants (Fig. 6).

S3.8.1–S3.8.4 had normal growth rates, but the edges of the leaves were curling upwards (Fig. 4). Before floral transition became apparent, reduced apical dominance resulted in production of multiple axillary shoots, which later developed into lateral inflorescences. The plants had reduced fertility and produced only a few normal siliques and many short siliques with no or only few seeds.

CYP79F1 Expression Analysis—The level of CYP79F1 transcript was investigated in rosette leaves of plants of different developmental stages and in cauline leaves (Fig. 7). The transcript was detected in all tissues examined. The transcript level increased with maturation of the plants. The expression level was approximately four times higher in rosette leaves of 9-week-old flowering plants than in rosette leaves of 5-week-old plants. When the above-ground parts of 5-week-old plants were analyzed, less CYP79F1 transcript was detected than when only rosette leaves of the same plants were analyzed. This indicates that CYP79F1 is expressed at higher levels in rosette leaves than in petioles.

DISCUSSION

The CYP79 family comprises multifunctional cytochromes P450 that catalyze two consecutive N-hydroxylations of amino acids followed by dehydration and decarboxylation resulting in the formation of aldoximes (9). CYP79F1 is one of several CYP79 homologues identified in the genome of A. thaliana (available on the Web). In the present paper we report that CYP79F1 is an N-hydroxylase catalyzing the conversion of dihomomethionine and trihomomethionine to 5-methylthiopentanaloxide and 6-methylthiohexanaldoxime, respectively, a key step in the biosynthesis of aliphatic glucosinolates. CYP79F1 is the first aldoxime-forming enzyme in the biosynthesis of aliphatic glucosinolates to be heterologously expressed and characterized.

Using an EST clone containing the CYP79F1 cDNA we have expressed CYP79F1 in E. coli. The recombinant protein has the spectral characteristics of a cytochrome P450 enzyme. Both dihomomethionine and trihomomethionine are metabolized by CYP79F1 resulting in the formation of 5-methylthiopentanaloxide and 6-methylthiohexanaldoxime as proven by GC-MS analysis and comparison with authentic standards. Neither methionine nor the other protein amino acids tested are substrates of CYP79F1. Thus CYP79F1 seems to convert specifically chain-elongated methionine homologues to the corresponding aldoximes. Previously, aliphatic amino acids with different chain lengths have been shown to be substrates of the
same CYP79 as demonstrated for CYP79D1 and CYP79D2 from cassava (*Manihot esculenta* Crantz), which converts both valine and isoleucine to the corresponding aldoximes in the biosynthesis of the cyanogenic glucosides linamarin and lotaustralin (10). In contrast, CYP79A2 from *A. thaliana* has been shown to convert specifically phenylalanine, but not homophenylalanine, to the corresponding aldoxime (7).

Heterologous expression in *E. coli* of CYP79F1 was accomplished in the strain C43(DE3). Strain C43(DE3) is a mutant *E. coli* strain, which has been selected for its ability to accommodate high levels of heterologous membrane proteins (19). C43(DE3) has been used successfully for cytochrome P450 expression (14). In the present study, the use of strain C43(DE3) enabled sufficient expression levels for spectral characterization of CYP79F1, whereas this could not be accomplished in the strain JM109.

Two lines of evidence for the involvement of CYP79F1 in glucosinolate biosynthesis are provided by the analysis of transgenic *A. thaliana* containing the CYP79F1 cDNA under control of the CaMV35S promoter. First, several independent 35S:CYP79F1 transformants have either reduced or increased levels of aliphatic glucosinolates, and reduced CYP79F1 transcript levels in plants of the T3 generation are accompanied by a dramatically reduced content of aliphatic glucosinolates. Second, the substrates dihomomethionine and trihomomethionine of CYP79F1 accumulate in the plants with reduced content of aliphatic glucosinolates as would be expected upon down-regulation of CYP79F1. The accumulation of the chain-elongated methionines indicates that the enzymes catalyzing the chain elongation of methionine (29) are not subject to feed-back inhibition by the chain-elongated product. Furthermore, it suggests that the enzymes that catalyze additional chain elongation cycles are rate-limiting in the biosynthesis of longer chain methionine homologues.

Comparison of the biochemical data for recombinant CYP79F1 and the glucosinolate profiles of the 35S:CYP79F1 plants raises the question whether CYP79F1 metabolizes not only dihomono- and trihomomethionine, but also other chain-elongated methionine homologues produced in *A. thaliana*. Although the content of all aliphatic glucosinolates is reduced in the transgenic plants compared with wild-type, the effect is most pronounced for the glucosinolates derived from homo- and dihomomethionine. The decrease in the levels of other aliphatic glucosinolates than the dihomo- and trihomomethionine-derived ones might be explained by a broad substrate specificity of CYP79F1 for chain-elongated methionine homologues or by cosuppression not only of the CYP79F1 transcript but also of transcripts of other CYP79 homologues involved in the biosynthesis of aliphatic glucosinolates. As demonstrated by RT-PCR, introduction of the transgene resulted in down-regulation of both CYP79F1 and CYP79F2. CYP79F1 is 88% identical at the...
amino acid level to CYP79F2, and the two genes are located on the same chromosome, only separated by 1638 bp. This suggests that the two genes have been formed by gene duplication and that they might catalyze similar reactions. Because further investigations of the substrate specificity of CYP79F1 are limited by the unavailability of substrates, isolation of A. thaliana knock-out mutants of CYP79 homologues may facilitate such investigations. From the results of the RT-PCR, it appears that CYP79B2 is up-regulated approximately 2-fold in the 35S:CYP79F1 plants, resulting in an increased content of indole glucosinolates.

The transgenic A. thaliana plants with altered content of aliphatic glucosinolates possess a characteristic morphological phenotype characterized by production of multiple axillary shoots. A. thaliana has been reported to be able to tolerate overexpression of cytochromes P450 of the CYP79 family leading to a 2- to 5-fold increase in glucosinolate content (7, 30) without similar changes in the appearance of the plants. Therefore it seems unlikely that the morphological changes result from the presence or absence of specific glucosinolates. The accumulation of very high levels of chain-elongated methionine homologues in the transgenic plants suggests that the morphological phenotype may be a pleiotropic effect caused by disturbance of the plant’s sulfur metabolism, in which methionine plays a central role. Repression of cystathionine-γ-synthase, a

| Glucosinolate side chain | 35S:CYP79F1 | Wild-type |
|--------------------------|-------------|-----------|
| 3-Methylsulfinylpropyl-   | 0.171 (± 0.049) | 1.340 (± 0.082) |
| 4-Methylthiobutyl-        | 0.099 (± 0.026) | 1.120 (± 0.256) |
| 4-Methylsulfinylbutyl-    | 0.829 (± 0.214) | 7.728 (± 0.422) |
| 5-Methylthiopentyl-       | 0.070 (± 0.007) | 0.109 (± 0.014) |
| 6-Methylsulfinylhexyl-    | 0.090 (± 0.019) | 0.344 (± 0.022) |
| 7-Methylthioheptyl-       | 0.015 (± 0.007) | 0.048 (± 0.003) |
| 7-Methylsulfanylheptyl-   | 0.106 (± 0.010) | 0.117 (± 0.012) |
| 8-Methylthiooctyl-        | 0.036 (± 0.007) | 0.183 (± 0.009) |
| 8-Methylsulfinyloctyl-    | 0.072 (± 0.035) | 0.104 (± 0.019) |
| Indol-3-ylmethyl-         | 0.279 (± 0.067) | 0.561 (± 0.037) |
| 4-Hydroxyindol-3-ylmethyl-| 6.571 (± 1.463) | 2.449 (± 0.118) |
| 1-Methoxyindol-3-ylmethyl-| 0.067 (± 0.010) | 0.033 (± 0.004) |
| 4-Methoxyindol-3-ylmethyl-| 0.071 (± 0.011) | 0.042 (± 0.010) |
| 1-Methoxyindol-3-ylmethyl-| 1.142 (± 0.062) | 0.964 (± 0.101) |

Fig. 4. Morphological phenotype of 35S:CYP79F1 plants. A, 7-week-old 35S:CYP79F1 plant (S3.8.2) (right), wild-type plant of the same age (left). B, 11-week-old 35S:CYP79F1 plant (S3.8.3) (right), wild-type plant of the same age (left).

Fig. 5. Accumulation of chain-elongated methionine homologues in rosette leaves of 35S:CYP79F1 plants and wild-type plants. Extracts from two T3 plants derived from the primary transformant S3 and two wild-type plants were analyzed by HPLC. Each bar represents the mean (± S.E.) of four measurements on one individual plant.

Fig. 6. RT-PCR analysis of transcript levels of CYP79 homologues in rosette leaves of a 35S:CYP79F1 plant (S3.8.4). RT reactions were performed on 1, 0.3, and 0.1 μg of plant RNA. The experiment has been performed three times with similar results.
key enzyme in methionine biosynthesis, results in plants characterized by formation of a cluster of apical shoots at the time of floral transition and inability to produce flowers (31). 80% of the methionine synthesized by a plant is incorporated into S-adenosyl-methionine (32), which plays a central role in many biosynthetic processes, e.g. methylation reactions such as cytisine methyltransferase-catalyzed DNA methylation (33). One of the morphological changes seen in cytisine methyltransferase antisense plants is production of multiple axillary shoots (34). The onset of the morphological changes in CYP79F1-cosuppressed plants at the time of floral transition may be due to the requirement for methionine to support flower development. Alternatively, it coincides with an increase in the level of CYP79F1 expression in wild-type plants. The relation between CYP79F1 down-regulation and the morphological phenotype is the subject of future investigations.

Based on biochemical studies using microsomal enzyme preparations from species of the Brassicaceae, it has previously been proposed that the conversion of dihomomethionine, trihomomethionine, and tetrahomomethionine to their corresponding aldoximes is catalyzed by flavin-containing monoxygenases and not by cytochromes P450 (15–17). However, the conversion of chain-elongated methionine derivatives by flavin-containing monoxygenases was measured by indirect enzyme assays (15), in which the release of CO2 was used as a measure for enzyme activity. Despite high enzyme activity, the corresponding aldoximes have never been documented in these assays. Our study provides unequivocal evidence that a cytochrome P450 catalyzes the conversion of chain-elongated methionines (after having diverged away from the CYP79 homologues involved in the biosynthesis of cyanogenic glucosides). Degradation of 4-methylsulfinylbutylglucosinolate by myrosinase in broccoli sprouts leads to the formation of the isothiocyanate product sulforaphane. As demonstrated in rats, extracts of broccoli sprouts have a pronounced protective effect against breast cancer (4), and sulforaphane has been identified as the principle active agent (3). The identification of a gene involved in the biosynthesis of 4-methylsulfinylbutylglucosinolate is an important step in the development of functional foods that release elevated levels of sulforaphane. 5-Methylthiopenantaldoxime is not only the precursor of 4-methylthiobutylglucosinolate and 4-methylsulfinylbutylglucosinolate but also the precursor of a number of glucosinolates with secondary modifications of the side chain. Besides their occurrence in A. thaliana, such glucosinolates are important constituents of Brassica crops and vegetables. For example, the major glucosinolate in B. napus, the goitrogenic 2-hydroxy-3-butenylglucosinolate, is formed by side-chain modification of 4-methylthiobutylglucosinolate (37). The occurrence of 2-hydroxy-3-butenylglucosinolate in B. napus restricts the use of the protein-rich seed cake as animal feed. Thus availability of biosynthetic genes has great potential for the development of crops with reduced levels of such toxic glucosinolates while retaining glucosinolates with desirable effects, e.g. for pest resistance.

In conclusion, we have shown that CYP79F1 catalyzes the conversion of dihomomethionine and trihomomethionine to 5-methylthiopentanaldehyde and 6-methylthiohexanaldehyde, respectively, in the biosynthesis of aliphatic glucosinolates in A. thaliana. The identification of CYP79F1 provides an important tool for tissue-specific alterations of the level of aliphatic glucosinolates to improve the nutritional value of crop plants and vegetables as well as pest resistance. In addition, the availability of a biosynthetic gene for aliphatic glucosinolates is a valuable means for studying the physiological role of these glucosinolates in plants, e.g. in plant-insect interactions.

Acknowledgments—Dr. Bent L. Petersen is thanked for helpful advice and discussion regarding HPLC analysis, and Christina Mattson and Inga Olaen for technical assistance. Prof. Birger L. Møller is thanked for helpful discussions.

Note Added in Proof—Reintanz et al. (Reintanz, B., Lehnen, M., Reichelt, M., Gershenzon, J., Kowalczyk, M., Sandberg, G., Godde, M., Uhl, R., and Palme, K. (2001) Plant Cell 13, in press) have written an article on CYP79F1.

REFERENCES
1. Fenwick, G. R., Heaney, R. K., and Mullin, J. (1983) Crit. Rev. Food Sci. Nutr. 18, 123–201
2. Chew, F. S. (1988) in Biologically Active Natural Products (Cutler, H. G., ed)
Cytochrome P450 CYP79F1 from Arabidopsis Catalyzes the Conversion of Dihomomethionine and Trihomomethionine to the Corresponding Aldoximes in the Biosynthesis of Aliphatic Glucosinolates
Carsten Hørslev Hansen, Ute Wittstock, Carl Erik Olsen, Alastair J. Hick, John A. Pickett and Barbara Ann Halkier

J. Biol. Chem. 2001, 276:11078-11085. doi: 10.1074/jbc.M010123200 originally published online December 22, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M010123200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 33 references, 18 of which can be accessed free at http://www.jbc.org/content/276/14/11078.full.html#ref-list-1

Downloaded from http://www.jbc.org/ by guest on July 25, 2018