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Coproporphyrinogen III oxidase from barley and tobacco – sequence analysis and initial expression studies

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Abstract. Coproporphyrinogen III oxidase (coprogen oxidase; EC 1.3.3.3) is part of the pathway from 5-aminolevulinate to protoporphyrin IX which is common in all organisms and catalyses oxidative decarboxylation at two tetrapyrrole side chains. We cloned and sequenced full-length cDNAs encoding coprogen oxidase from barley (*Hordeum vulgare* L.) and tobacco (*Nicotiana tabacum* L.). They code for precursor peptides of 43.6 kDa and 44.9 kDa, respectively. Import into pea plastids resulted in a processed tobacco protein of approx. 39 kDa, which accumulated in the stroma fraction. Induction of synthesis of recombinant putative tobacco mature coprogen oxidase consisting of 338 amino-acid residues in *Escherichia coli* at 20°C result in a catalytically active protein of approx. 39 kDa, while induction of its formation at 37°C immediately terminated bacterial growth, possibly due to toxic effects on the metabolic balance of tetrapyrrole biosynthesis. The plant coprogen oxidase gene was expressed to different extents in all tissues investigated. This is most likely due to the differing requirements for tetrapyrroles in different organs. The steady-state level of mRNA did not significantly differ in etiolated and greening barley leaves. The content of coprogen oxidase RNA reached its maximum in developing cells and decreased drastically when cells were completely differentiated. Functioning of the two photosystems apparatus requires the synthesis of all pigment and protein components during plant development. It is speculated that the enzymes involved in tetrapyrrole synthesis are developmentally rather than light-dependently regulated. Regulation of these enzymes also guarantees a constant flux of metabolic intermediates and avoids photodynamic damage by accumulating porphyrins.

Key words: 5-Aminolevulinate synthesis – Chlorophyll synthesis – Chloroplast (development) – *Hordeum* – *Nicotiana* – Tetrapyrrole

Introduction

The biosynthesis of tetrapyrroles is of major importance because they are integral components of ubiquitous biological processes such as signal and energy transduction (for recent review, see Chadwick and Ackrill 1994; Beale and Weinstein 1990; Smith and Griffiths 1993). They are involved in oxidative and photo phosphorylation (cytochrome *c*), energy absorption (chlorophyll and biliverdin), light perception (phytochrome), nitrogen fixation (leghemoglobin), oxygen transport (haemoglobin) and storage (myoglobin), removal of oxidizing agents (catalases, peroxidases) and in detoxification reactions (cytochrome *P*450). The major precursor of tetrapyrroles is 5-amino-4-oxopentanoic acid, commonly known as aminolevulinic acid (ALA). Two biosynthetic pathways are involved in the synthesis of ALA. In animals, humans and yeast it is synthesized from glycine and succinyl-CoA in a condensation reaction catalyzed by ALA-synthase. In plants and many bacteria ALA is synthesized from glutamate in three enzymatic steps. Two molecules of ALA are involved in the synthesis of a pyrrole, phorphobilinogen, four molecular of which are subsequently combined in the formation of uroporphyrinogen, the initial cyclic tetrapyrrole. Subsequent modifications lead to protoporphyrin IX (proto IX) synthesis. The sequence of reactions from ALA to proto IX is common in all organisms. With insertion of one of several metal cations, the metabolic pathway branches to various metallo porphyrins, of which chlorophyll and heme are two of the important end products.

Little is known about regulation of plant tetrapyrrole metabolism and the enzymatic steps in which porphyrins

Accession number: The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X82830 (barley coprogen oxidase) and X82831 (tobacco coprogen oxidase).

Abbreviations: ALA = 5-aminolevulinic acid; copro = coproporphyrin III, coprogen = coproporphyrinogen III; IPTG = isopropyl β-D-thiogalactopyranoside; LHCP II = light-harvesting chlorophyll-binding protein of photosystem II; OD = optical density; proto IX = protoporphyrin IX: protogen IX = protoporphyrinogen IX

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are modified. Recent investigations indicate that enzymatic reactions leading to the synthesis of protoporphyrinogen IX (protogen IX) occur exclusively in plastids (Jacobs and Jacobs 1993; Smith et al. 1993). Subsequent modifications continue either in plastids (Mg- or Fe-containing tetrapyrroles), in mitochondria (mainly hemes) or in the cytoplasm (linear tetrapyrroles). Following the tetrapyrrole flux across various cellular compartments is experimentally very challenging.

In higher plants, light affects chlorophyll synthesis at two major steps. Protochlorophyllide is reduced to chlorophyllide when etiolated plants are illuminated, and ALA synthesis is stimulated by phytochrome induction. Repression of ALA formation prevents an increase in the protochlorophyllide pool in the dark (Kannangara and Gough 1978). Enzyme activities leading to protochlorophyllide synthesis are apparently not limiting in dark-grown seedlings; one might anticipate that regulation of individual genes involved in the expression of each enzymatic step ought to occur when plants are illuminated. Light-dependent regulation of ALA formation avoids photodynamic damage caused by accumulation of protochlorophyllide and other photosensitive intermediates.

Coproporphyrinogen III (coprogen) is one of the very photosensitive tetrapyrroles. Two propionate side chains of tetrapyrrole, rings A and B, are converted into vinyl groups by oxidative decarboxylation. Both of these propionate groups are synthesized by a single enzyme, coprogen oxidase, which has been purified to apparent homogeneity from bovine liver (Yoshinaga and Sano 1980a), yeast (Camadro et al. 1986) and mice (Bogard et al. 1989). The purified enzyme consists of two identical subunits of about 35–37 kDa. The genes for coprogen oxidase have been identified and sequenced from various organisms such as yeast (Zagorec et al. 1988), *Salmonella typhimurium* (Xu and Elliott 1993), mice (Bogard et al. 1989), humans (Martasek et al. 1994a; Taketani et al. 1994) and *Escherichia coli* (Troup et al. 1994). This enzyme has also been studied in the following plants: tobacco (Hsu and Miller 1970), pea and the spadices of cuckoo pint (Smith et al. 1993). Recently, the first plant gene encoding soybean coprogen oxidase was described (Madsen et al. 1993). Comparison of deduced soybean and yeast amino-acid sequences revealed greater than 50% homology, indicating that these coprogen oxidases are structurally related. In this communication, we describe sequence-analysis and preliminary expression studies of barley and tobacco coprogen oxidase.

Materials and methods

Plant material and growth conditions. Barley seedlings (*Hordeum vulgare* L. cv. Svalöf's Bonus, IPK, Gatersleben, Germany) were grown on moist vermiculite in a growth chamber at 25 °C under a 14 h/10 h light/dark cycle at a irradiance of 100 μmol photons $m^{-2} \cdot s^{-1}$ for 10 d. Etiolated seedlings were kept in complete darkness at 25 °C for 5 d and exposed to light (100 μmol photons $m^{-2} \cdot s^{-1}$) for the time indicated in the *Results*. Tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN; IPK, Gatersleben, Germany) were grown under greenhouse conditions or germinated for 3 d in light and subsequently kept in the dark for 8 d. Plant material used for RNA extraction was excised, immediately frozen in liquid nitrogen, and stored at − 80 °C. For plastid import experiments, pea seedlings (*Pisum sativum* L. cv. Rosa Krone; Heinje und Garvens, Hannover, Germany) were grown on vermiculite at 25 °C at 120 μmol photons $m^{-2} \cdot s^{-1}$ for 7 d in a 12-h light/12-h dark cycle and harvested 1–2 h after onset of illumination.

Isolation and analysis of cDNA clones for coprogen oxidase from tobacco and barley. Lambda ZAP II cDNA libraries from tobacco leaf (SR1 strain; Stratagene, La Jolla, Califa., USA) and greening barley seedlings were screened by standard procedures (Sambrook et al. 1989) using a ^{32}P-labelled cDNA-insert of soybean coprogen oxidase. The barley cDNA library was prepared and kindly made available by Sakari Kauppinen (Carlsberg Laboratory, Copenhagen, Denmark). The soybean coprogen oxidase cDNA was generously provided by Ole Madsen (Aarhus, Denmark). All cDNA fragments longer than 1.300 bp from positive clones were sequenced by the dideoxy-chain-termination method (Sanger et al. 1977) on both strands using internal primers. Results were analyzed with the PC/GENE programme (Intelli Genetics Inc., Mountain View, Calif., USA).

Posttranslational transport of radiolabelled coprogen oxidase precursor into intact pea chloroplast. Plasmid pTCPO containing the complete coding region for tobacco coprogen oxidase was linearized with *Eco* RV which catalyzes the hydrolysis of pBluescript polylinker downstream of the cDNA insert. In-vitro transcription with T3 RNA polymerase and translation in the wheat-germ system (Roberts et al. 1975) in the presence of [^{35}S]methionine were carried out as described by Kruse and Kloppstech (1992). Intact chloroplasts were isolated from 7-d-old pea seedlings grown as indicated above. In-vitro transport into chloroplasts was performed according to Grossman et al. (1982) with minor modifications (Grimm et al. 1989).

Extraction of RNA and Northern blot analysis. Total RNA from tobacco tissue was prepared as described by Chomczinsky and Sacchi (1987). Poly(A)-rich RNA from barley was isolated by oligo(dT) cellulose chromatography (Apel and Kloppstech 1978) with modifications as outlined previously (Pötter and Kloppstech 1993). The RNA was quantified spectrophotometrically. Equal amounts (15 μg of total RNA or 1 μg of poly(A)-rich RNA) were fractionated in formaldehyde-containing agarose gels and transferred to nylon membranes (Hybond N; Amersham, Braunschweig, Germany) by vacuum-blotting. Filters were probed with ^{32}P-labelled cDNA inserts under high-stringency conditions (Sambrock et al. 1989).

Construction of tobacco coprogen oxidase (CPO) expression vectors. The pUHE-expression system (Stüber et al. 1984) was used for overexpression of tobacco coprogen oxidase in *E. coli*. A cDNA-fragment containing the complete open reading frame, except for the sequence coding for a putative transit sequence, was amplified by poymerase chain raction using the following synthetic primers 5′-CGACGATGCTTGAGAAAGAAGTAGCTGTTTCC-3′ and 5′-CGTGGATCCGGGCGAATTAACATAAGACCAAC-3′. After digestion with *Sph* I and *Bam* HI the fragment was ligated into *Sph* I/*Bam* HI-digested pUHE 25-1 to obtain recombinant plasmid pCPOex. The ligation mixture was transformed into *E. coli* SG 13009 containing the pDMI 1 plasmid (obtained from D. Stüber, Hoffman-LaRoche, Basel, Switzerland). For the preparation of a second in-vivo expression construct, pTCPO was hydrolyzed with *Hind* III in the Bluescript polylinker site and in the coding sequence, resulting in a 1044-bp fragment with the complete 3′ end which codes for a truncated coprogen oxidase with the last 238 amino acids of the carboxy terminus. This fragment was inserted into pUHE 22-1. Recombinants were screened for correct orientation by restriction analysis of small-scale plasmid preparations.

Cells from overnight cultures containing recombinant plasmids were diluted tenfold in LB-medium isopropyl β-D-thiogalactopyranoside (IPTa) containing ampicillin and kanamycin

(100 μg·ml^{-1} and 25 μg ml^{-1} respectively) and grown at 37 °C. When the cell density had reached an optical density ($OD_{600\,nm}$) of 0.8–1, IPTG was added (to 1 mM) and bacteria were grown for 4 h under conditions specified in the results.

Measurement of coprogen oxidase activity in E. coli lysates. An aliquot of 100 ml *E. coli* cell culture was collected by centrifugation, resuspended in phosphate buffer (0.1 M potassium phosphate, pH 7.6; 1 mM EDTA) at a cell density of 40–50 $OD_{600\,nm}$ ml^{-1}. The suspension was incubated with lysozyme (final concentration of 0.1 mg·ml^{-1}) at room temperature for 20 min and subsequently sonicated. Aliquots of 50-μl this lysate (about 200 μg protein) were added to 550 μl assay mixtures containing 0.1 M potassium phosphate (pH 7.6), 1 mM EDTA, 1% dimethylsulfoxide, 2 mM dithiothreitol. Reactions were carried out for 20 min at 37 °C in the presence of 9 μl 200 μM coprogen [prepared from coproporphyrin III, by chemical reduction with sodium amalgam and generously provided by Dr. Klaus Gerbling, AgrEvo GmbH, Forschung Berlin, Germany]. Fluorescence emission spectra (λ_{ex}405 nm, λmax_{em}632 nm) were recorded on a Perkin-Elmer LS 50 B Luminescence Spectrophotometer (Perkin-Elmer, Norwalk, Conn., USA) before and after the addition of 10 μl 2-butanone peroxide and incubation in light for 1 h. The amount of product formed was calculated using calibration curves of proto IX fluorescence.

Miscellaneous. Escherichia coli total protein extracts were prepared by suspending the bacterial pellet in sample buffer (0.56 M $NaCO_3$, 0.56 M dithiothreitol, 12% sucrose, 2% SDS, 0.02% bromphenolblue) at a cell density of $OD_{600\,nm} = 10$ and boiling for 5 min. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard. The SDS-polyacrylamide gel electrophoresis was done according to Neville (1971) and fluorography according to Bonner and Laskey (1974).

The porphyrin content of *E. coli* cells was analyzed following centrifugation, extraction at a cell density of $OD_{600\,nm} = 150$ in methanol:0.1 N NH_4OH (v/v 9:1) and incubation on ice for 15 min. Cell debris and insoluble proteins were removed by centrifugation at 10000·*g* for 5 min. Extracted porphyrinogens were oxidized by addition of 50 μl of 1 M acetic acid and 50 μl of 2-butanone peroxide per ml extract and separated by HPLC on an RP 18 column (Novapak C18, 4 μm particle size, 4.6 × 150 mm; Waters Chromatography, Millipore, Eschborn, Germany) at a flow rate of 1 ml·min^{-1}. Porphyrins were eluted starting with 20% B (90% methanol in solvent A) in solvent A (0.1 M ammonium acetate, pH 5.2), and increasing concentration of B up to 100% during 10 min, then continuing with buffer B for at least 15 min. Column effluents were monitored fluorimetrically (λ_{ex} 405 nm and λ_{em} 625 nm), and porphyrins identified and quantified using authentic standards.

Results

Sequence analysis of tobacco and barley coprogen oxidase cDNAs. Barley coprogen-oxidase cDNA (Accession No. X82830) has an open reading frame of 1173 nucleotides, a 58-nucleotide 5′ non-translated leader, and 229 nucleotides of the 3′ non-coding region. Residues flanking the first ATG from position − 6 to + 5 match the putative consensus sequence proposed for the translation initiation site for monocotyledonous plants (Cavener and Ray 1991). The deduced amino-acid sequence consists of 391 residues yielding a molecular mass of 43.6 kDa. The tobacco sequence (Accession No. X82831) contains an open reading frame of 1191 nucleotides which corresponds to a protein of 397 amino acids with a predicted molecular mass of 44.9 kDa. The putative start codon is followed by a GC dinucleotide, and the upstream − 1 to − 10 region is enriched in A residues, characteristic of dicotyledonous -plant translation-initiation signals (Cavener and Ray 1991).

Barley and tobacco show about 63% sequence identity at the nucleotide level, and about 70% at the amino-acid level. Including the conservative amino-acid changes, increases the similarity to 76%. Homology is more pronounced in the C-terminal region.

Comparison of coprogen oxidase sequences from various organisms. Alignment of deduced amino-acid sequences of tobacco and barley coprogen oxidase with those from other organisms shows that there is a high degree of homology among these species (Fig. 1). The primary structures of the enzyme from different plant species are 70% homologous. Tobacco and soybean sequences are even more invariant (75% identity) compared with barley (70% identity), reflecting the phylogenetic origin of these species. Comparison of plant coprogen oxidase with those from other organisms yields 45–50% identity. Three highly conserved domains of coprogen oxidase from all phyla can be detected in the C-terminal region. The invariant residues are positioned between amino acids 220–237, 245–286 and 333–366 of the barley sequence (Fig. 1). These invariant residues are interrupted by stretches with a lower degree of conservation, in which mammals and yeast or prokaryotes show significant amino-acid sequence diversity.

Compared with coprogen oxidase of prokaryotes or yeast, plant enzymes have an amino-terminal extension of more than 60 amino acids. These amino-terminal extensions have features in common with known chloroplast targeting sequences (von Heinje et al. 1989, Gavel and von Heinje 1990): they are rich in alanine and hydroxylated amino acids, are nearly devoid of acidic amino acids and have an overall positive charge. We propose a processing-peptidase cleavage site at positions 57/58 of tobacco and 51/52 of barley, as indicated in Fig. 1. Consistent with the sequence data of Gavel and von Heinje (1990), negatively charged residues follow the cleavage site. The tobacco processing site partially matches the consensus motif V/I-X-A/CIA which is found in many chloroplast transit sequences. With this cleavage site, plant mature coprogen oxidase has an approximate molecular mass of 38.4 kDa. Significant homology with other nucleotide sequences or deduced peptide sequences was not observed.

Uptake of coprogen oxidase precursor by intact chloroplasts. To confirm the plastid localization of coprogen oxidase, the tobacco cDNA sequence was transcribed, mRNA translated and radiolabelled tobacco coprogen oxidase precursor was incubated with uptake-competent pea chloroplasts. As seen in the first lane of Fig. 2, the translational supernatant (TLS) of the in-vitro transcript from the tobacco cDNA clone in the wheat-germ system yielded one major protein band at 46 kDa and a minor band at 39 kDa. The bigger protein is consistent with the molecular mass predicted from the coding sequence analyzed; the smaller protein shows a electrophoretic mobility that corresponds to the molecular mass proposed for the mature protein, although identity of the protein was not proven. We include the possibility that this protein band

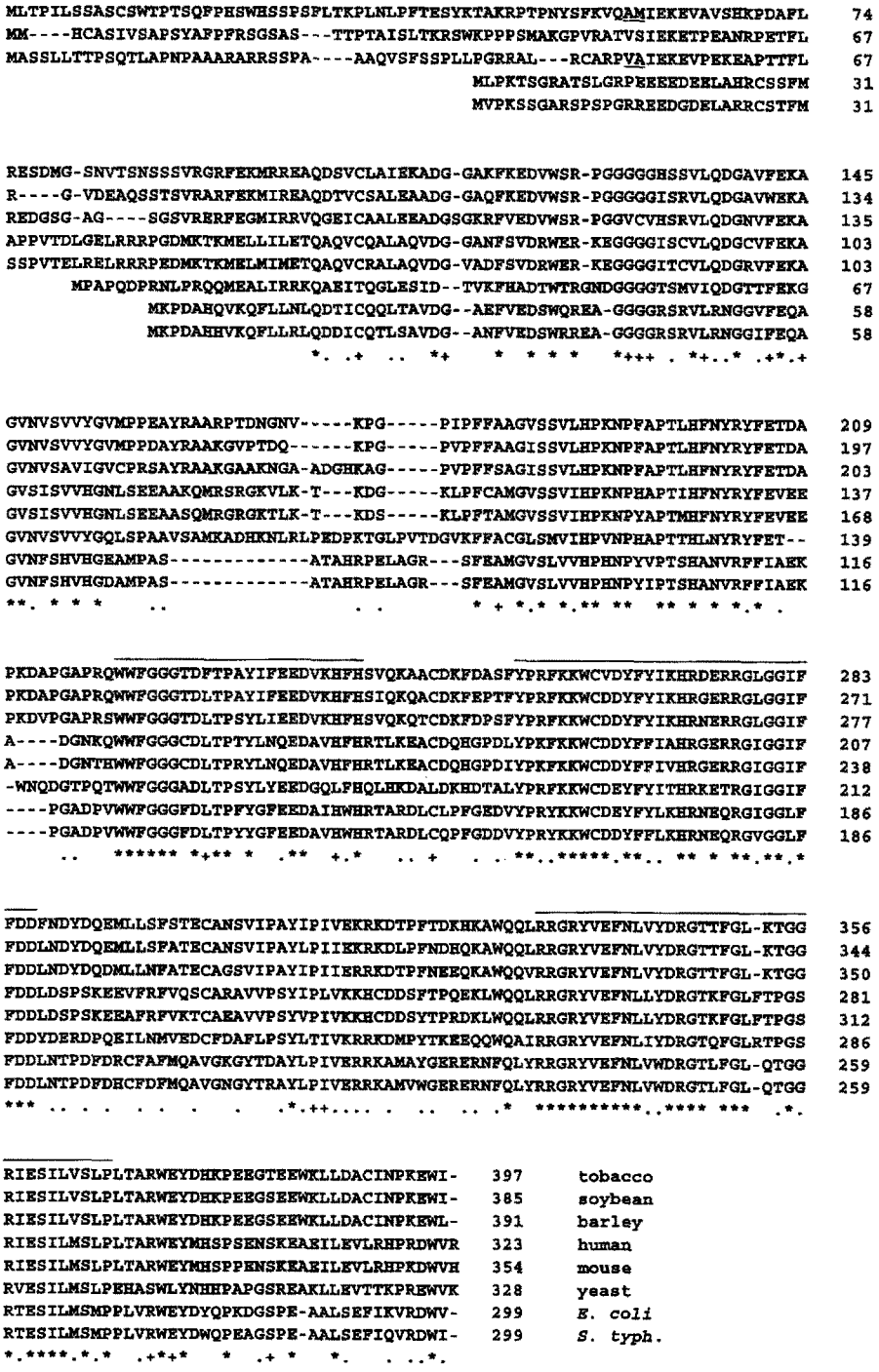

Fig. 1. Comparison of the primary structure of coprogen oxidase from various organisms. Deduced amino-acid sequences of tobacco and barley cDNA clones were aligned with those of soybean (Madsen et al. 1993), humans (Taketani et al. 1994, Martasek et al. 1994a), mice (Kohno et al. 1993), yeast (Zagorec et al. 1988), *E. coli* (Troup et al. 1994) and *Salmonella typhimurium* (Xu and Elliott 1993) using the PC/GENE CLUSTAL software program. *Asterisks* indicate identity and *dots* similarity of amino-acid residues. Residues that are identical in all but one protein are marked by *plus* signs. Highly homologous stretches are marked by *horizontal bars*. Proposed amino acids involved in transit-peptide cleavage are *underlined* in the tobacco and barley sequence

is due to processing activity present in the wheat-germ lysate (Pfisterer et al. 1982), or to translation initiation at the second methionine in the primary sequence, which is proposed to be the first residue in the mature peptide (Fig. 1). The latter has been observed during in-vitro translation (data not shown).

The 46- and 39-kDa proteins were associated with chloroplasts in the plastid-import assay. Plastids recovered after import assay were either analyzed directly (Fig. 2, lane PL) or treated with proteases, re-isolated and separated into membrane and stroma fractions (Fig. 2, lanes M and S). The mature protein of 39 kDa was exclusively found in the stroma fraction while proteins could not be seen in the membrane fraction. This experiment indicates import of the 46-kDa coprogen oxidase precursor into intact chloroplasts and processing to a stromal mature protein of apparent 39 kDa.

Overexpression of tobacco coprogen oxidase in E. coli. In order to promote further investigation of enzymic properties, the expression vector pCPOex for the overproduction of the proposed tobacco mature coprogen oxidase in

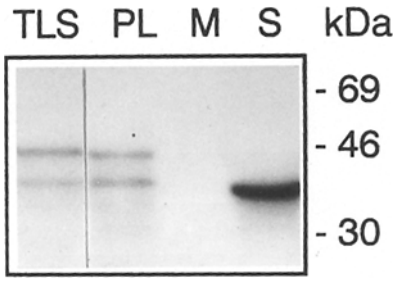

Fig. 2. Posttranslational uptake of tobacco coprogen oxidase by intact pea chloroplasts. Intact chloroplasts were incubated with radiolabelled precursor synthesized by in-vitro transcription/translation, treated with proteases, re-isolated by centrifugation through a 40% Percoll cushion, lysed osmotically, and separated into stroma and membrane fractions. Sampleswere analyzed by SDS-PAGE and fluorography. *TLS*, translational supernatant; *PL*, protein extract of plastids recovered from the import assay; *M*, membranes; *S*, stroma

E. coli was created. When bacteria carrying pCPOex were induced with IPTG at 37 °C, growth essentially terminated immediately, indicating toxicity of expressed recombinant protein. This putative toxic effect was less pronounced at growth temperature of 20 °C and an additional protein of 38 kDa was detected in cell extracts of *E. coli* after IPTG-induced expression of the recombinant coprogen oxidase gene (Fig. 3A, lanes 2 and 3). Bacteria carrying the non-recombinant plasmid, did not contain this protein (Fig. 3A, lane 1). Porphyrin analysis of exponentially growing *E. coli* cells indicated accumulation of coproporphyrin(ogen). In the presence of active plant coprogen oxidase, *E. coli* copro(gen) would be expected to decrease and protogen IX and/or heme increase. We therefore analyzed the porphyrin composition of IPTG-induced cells during exponential growth in the presence of pUHE or the recombinant plasmid pCPOex. As shown in Fig. 3B, the level of copro(gen) in control cells (with pUHE) was about tenfold higher than proto IX. In cells overexpressing the plant enzyme (with pCPOex8 and pCPOex10), reduced copro(gen) and increased proto IX contents were observed. This indicates that expression of plant coprogen oxidase affects the apparent levels of metabolic intermediates in the biosynthetic pathway of tetrapyrroles with the possible severe consequence for bacterial vitality.

A truncated coprogen oxidase peptide was overproduced in large amounts when bacterial cells containing pCPOΔex12 and 15 were grown at 37 °C. The IPTG-induced cells expressing the recombinant peptide did not show reduced growth compared to non-induced cells. The porphyrin pattern did not appreciably differ in induced and non-induced *E. coli* cells (data not shown).

Comparison of coprogen oxidase activity in *E. coli* cell extracts with and without recombinant plasmid revealed an approx. 15-fold increase in conversion of coprogen to proto IX in the presence of pCPOex8 or pCPOex10 (Fig. 3C). In the recombinant coprogen oxidase expression clones 11–15-nmoles of proto IX·(mg protein)$^{-1}$·h^{-1} were synthesized compared to control *E. coli* cells (0.8 nmol·mg^{-1}·h^{-1}). The specific activity of the plant recombinant coprogen oxidase is consistent with the

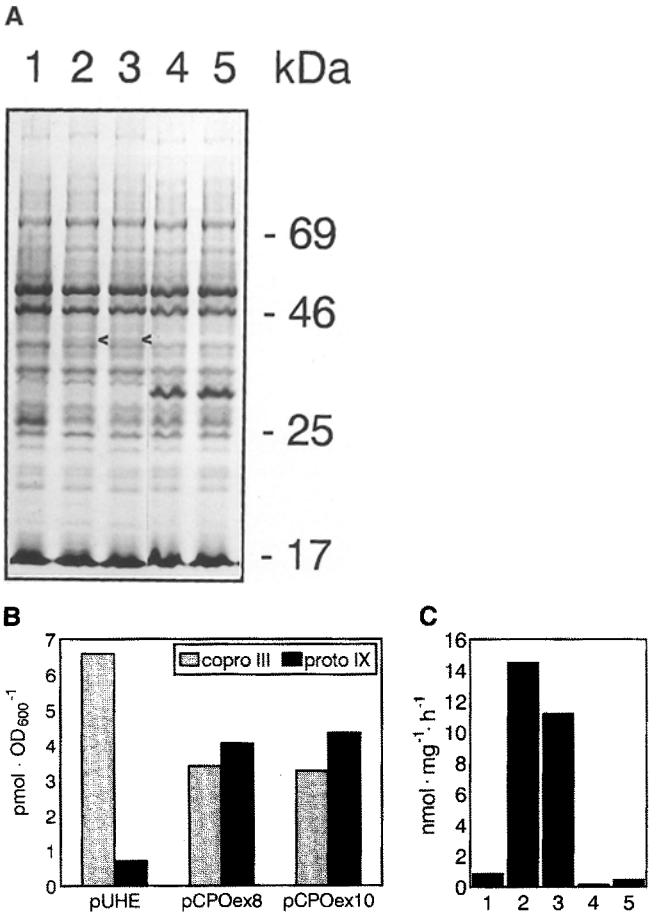

Fig. 3A–C. Overexpression of tobacco coprogen oxidase in *E. coli*. **A** Protein analysis. Cells carrying the pUHE-vector (*lane 1*) and independent recombinant clones (pCPOex8, pCPOex10, *lanes 2* and *3*) expressing the putative mature tobacco coprogen oxidase were grown at 20 °C to an $OD_{600\,nm}$ of 0.9, induced by IPTG and incubated for 3 h at 20 °C. In addition, two recombinant clones (pCPOΔex12 and 15, lanes *4* and *5*) expressing a truncated coprogen oxidase with 238 amino acids at the carboxy terminus were grown at 37 °C and treated as indicated above. Thereafter, cells were collected by centrifugation, and extracted proteins of lysed cells were separated by SDS-PAGE. The *arrowheads* in *lanes 2* and *3* mark the recombinant protein. **B** Porphyrin analysis. Cells carrying the non-recombinant vector (*pUHE*) or the vector with the gene for mature tobacco coprogen oxidase were grown and induced by IPTG as described in **A**. Cells were collected by centrifugation, and porphyrins were extracted and analyzed by HPLC. **C** Catalytic activity. Cell lysates were prepared as described in *Materials and methods* and assayed for coprogen oxidase activity. Activity is expressed as nmoles proto IX·mg^{-1} protein h^{-1}. *Samples 1–5* correspond to the cell extracts separated in lanes 1–5 of **A**

specific activity of the extract of *E. coli* expressing the human enzyme (Martasek et al. 1994a; Taketani et al. 1994). Like the control *E. coli* cells, extracts containing the truncated protein showed basal activity (0.4 nmol·mg^{-1}·h^{-1}).

Tissue-specific expression of coprogen oxidase mRNA in barley. Poly(A)-rich RNA was isolated from root, leaves and coleoptiles from 10-d-old light-grown barley plantlets and analyzed by Northern blot hybridization with barley

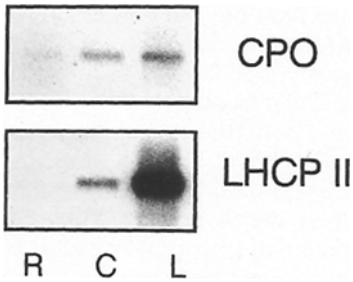

Fig. 4. Tissue-specific expression of coprogen oxidase in barley. Poly(A)-rich RNA was isolated from roots (*R*), coleoptiles (*C*) and primary leaves (*L*) of 10-d-old barley seedlings, subjected to Northern blot analysis using a radiolabelled cDNA insert for coprogen oxidase (*CPO*), and compared with the content of barley LHCP II mRNA

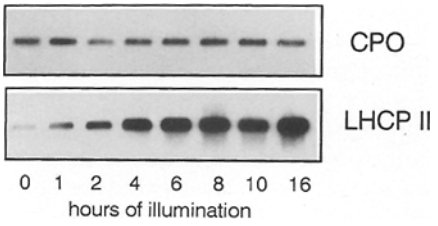

Fig. 5. Levels of coprogen oxidase transcripts (*CPO*) during greening of etiolated barley seedlings. Barley seedlings were grown in darkness for 5 d and then illuminated for the times indicated. Poly(A)-rich RNA was extracted and analyzed by Northern blot hybridization as described in Fig. 4. Determination of the level of LHCP II mRNA during greening was used as a light-induced control

cDNA probe. As shown in Fig. 4, coprogen oxidase transcripts could be detected in all tissues analyzed. Its steady-state RNA level in leaves was about twofold higher than in coleoptiles, and was considerably higher than in roots, presumably reflecting the metabolic requirements of tetrapyrroles needed in each of these tissues. In contrast, LHCP II mRNA, which encodes the light-harvesting chlorophyll-binding protem of photosystem II, one of the most-abundant chlorophyll-binding thylakoid membrane proteins, accumulated to very high levels in leaves, was poorly expressed in nearly non-green coleoptiles compared to leaves, and was not detectable in root tissue.

Effect of light on the accumulation of coprogen-oxidase transcripts. It was important to determine the influence of environmental stimuli on the expression activity of the coprogen oxidase gene from barley and tobacco. Such stimuli have been postulated to affect the expression of other enzymes in the tetrapyrrole biosynthetic pathway (Ilag et al. 1994). In order to determine the influence of light on the steady-state level of coprogen oxidase transcripts, analyses were carried out during greening of etiolated barley and tobacco plants. Relative to the low amount of chlorophyll, mRNA for coprogen oxidase was present in considerable amounts in etiolated barley seedlings and did not accumulate during greening (Fig. 5). No appreciable quantitative changes were observed for this mRNA in etiolated tobacco leaves, either, during a 10-h greening period (data not shown). In contrast, the mRNA level of the LHCP II gene family, which was used as an example of the expression of light-induced genes, increased drastically after onset of illumination.

Expression of coprogen oxidase during leaf development. A plant's developmental program is the primary regulatory factor for gene expression. Primary barley leaves, representing various developmental steps from the basal meristem (Fig. 6A, lane 1) to cells at the leaf tip with differentiated plastids (Fig. 6A, lane 6), were used to investigate the developmental expression of coprogen oxidase mRNA. In addition, leaves of different ages from a six-week-old tobacco plant were examined. Steady-state levels of coprogen oxidase mRNA within adjacent segments of primary barley leaves are shown in Fig. 6A.

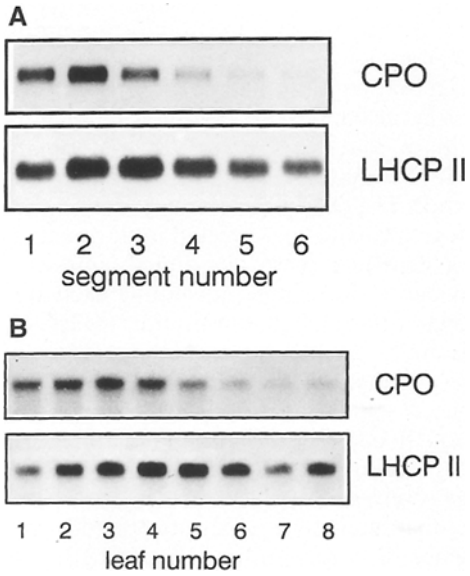

Fig. 6. Developmental expression of coprogen oxidase (*CPO*) gene in barley and tobacco. **A** Primary leaves of 10-d-old light-grown barley plantlets were carefully removed from the coleoptile, cut into six sections of equal length (about 2–2.5 cm each), processed for isolation of poly(A)-rich RNA and analyzed by Northern blot hybridization (*1* = basal, *6* = apical segment). **B** Northern blot hybridization assay of total RNA extracted from leaves of a 6-week-old tobacco plant. Analysis included successive leaves from the top to the base of the plant (*8*), beginning with the youngest fully expanded leaf (*1*)

Transcript levels were substantially higher in the lower half of the leaf and declined acropetally along the leaf axis to less than 5% in the oldest parts of the leaf (Fig. 6A, lanes 5 and 6). Maximal expression was found in the second segment, which is about 2–5 cm above the leaf base. A similar expression pattern was observed in tobacco leaves of different developmental stages (Fig. 6B). Starting from the top leaf (Fig. 6B, lane 1), mRNA content increased up to about the third leaf and then decreased to a more or less constant low level in fully expanded leaves. In both barley and tobacco, maximum levels of coprogen oxidase mRNA apparently preceeded increases in LHC II gene transcripts (Fig. 6A, B, lower panel) during plant development.

Discussion

This paper presents the characterization of the full-length cDNA sequence and initial expression studies for coprogen oxidase from a monocotyledonous plant, barley, and a dicotyledonous plant, tobacco. Compared with the sequence from soybean recently published (Madsen et al. 1993), the plant enzymes share 70% invariant residues. The primary structure of all known coprogen oxidases, including those of mammals and bacteria, show a remarkable overall identity of 30%. It is tempting to speculate that the conserved regions are functional domains for catalysis or tetrapyrrole binding. But structural analyses of the enzyme awaits large-scale enzyme isolation and purification. Definition of a porphyrin-binding motif requires the availability of more sequence data for porphyrin-synthesizing or heme-binding proteins. Particular amino acids have not been assigned to catalytic functions, except in the case of an active-site tyrosine which is putatively involved in initial oxidation steps of coprogen decarboxylation (Yoshinaga and Sano 1980b). Alignment of all coprogen oxidase sequences reveals five invariant tyrosine residues at positions 231, 257, 310, 337 and 373 of the tobacco sequence. The catalytic relevance of predicted residues awaits structural and site-directed mutation analyses. The human genetic disease hereditary coproporphyria is a consequence of a single nucleotide exchange resulting in arginine-to-tryptophan substitution (Martasek et al. 1994b). This mutation causes a partial deficiency of coprogen oxidase activity. The residue at position 231 (R276 in the tobacco sequence) is conserved in animals and plants but not in bacteria or yeast, indicating that arginine is not essential for catalysis or protein stability.

Saccharomyces cerevisae coprogen oxidase is a cytosolic enzyme. The mammalian peptides are found in the intermembrane space of mitochondria (Elder and Evans 1978; Grandchamp et al. 1978). These coprogen oxidase precursors contain a comparatively short amino-terminal transfer sequence. In contrast, the longer expendable amino-terminal end of plant coprogen oxidase implies a target site in plastids. The initial steps of chlorophyll synthesis are exclusively present in plastids and no alternative pathway in the cytosol or mitochondria has been detected in higher plants. We have provided evidence that the tobacco coprogen oxidase encoded in our cDNA sequence is targeted to plastids. In-vitro-translated coprogen oxidase can be imported into pea chloroplasts and is localized in the stroma. This is consistent with the putative transit sequence of the coprogen oxidase, which displays structural features similar to those of other plastid transit sequences. However, we are aware that this does not finally disprove the possibility that the destination of coprogen oxidase is the mitochondria. Consistent with our results is the fact that coprogen oxidase activity has only been found in plastids, and additional active isoforms in mitochondria have been ruled out (Smith et al. 1993). All indications suggest that tetrapyrrole synthesis is localized in plastids, at least up to coprogen oxidase, and that division of substrate flow into different cellular compartments occurs after protogen IX.

Chloroplast development in monocotyledonous species occurs during the conversion of meristematic cells of the leaf base into the mature mesophyll cells of the expanded leaf, as expressed along the developmental leaf gradient. Subsequent to an increase in the number of plastids and an amplification of plastid DNA, high nuclear and plastid transcription activities are observed in barley leaves (Baumgartner et al. 1989). This developmental stage corresponds to the maximum level of coprogen oxidase mRNA in segment 2, which is approximately 2–5 cm above the basal meristem of the primary leaf. It is predictable that this cellular state requires an increase in chlorophyll synthesis for the assembly of the photosynthetic apparatus. The mRNA of LHCP is most abundant in the middle region of barley primary leaves when photosynthetic competence reaches its maximum (Viro and Kloppstech 1980). The LHCP mRNA is stable in apical cells, while the coprogen oxidase mRNA is hardly detectable. The quantity of LHCP mRNA corresponds to the appearance of the protein (Viro and Kloppstech 1980). The content of coprogen oxidase protein in fully developed leaves needs to be shown by Western blot analysis or activity tests. The mRNAs for other enzymes involved in chlorophyll synthesis have shown the same occurrence in developing barley leaves as the coprogen oxidase mRNA. The amount of their translation products is stable up to the top of the primary barley leaf or in the fully differentiated leaf tissue (data not shown). We tentatively assume that the amount of coprogen oxidase remains constant once the protein has been synthezised. The plastid environment will stabilize the protein that guarantees a constant flux of porphyrins in the pathway and a constant supply of chlorophyll for the pigment-binding proteins and their assembly in the photosynthetic apparatus.

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