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Cloning and expression of the tobacco *CHLM* sequence encoding Mg protoporphyrin IX methyltransferase and its interaction with Mg chelatase

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Abstract

S-adenosyl-L-methionine:Mg-protoporphyrin IX methyltransferase (MgPMT) is an enzyme in the Mg branch of the tetrapyrrole biosynthetic pathway. The nucleotide sequence of tobacco (*Nicotiana tabacum*) *CHLM* was identified and the cDNA sequence was used to express the precursor, the mature and a truncated recombinant MgPMT for enzymatic activity tests and for the formation of polyclonal antibodies. Comparison of the mature and the truncated MgPMT revealed three critical amino acids at the N-terminus of MgPMT for the maintenance of enzyme activity. To assess the contribution of *CHLM* expression to the control of the metabolic flow in the tetrapyrrole pathway, *CHLM* transcripts and protein levels, the enzyme activity and the steady-state levels of Mg protoporphyrin and Mg protoporphyrin monomethylester were analysed during greening of seedlings and plant development as well as under day/night and continuous growth conditions. These expression studies revealed posttranslational activation of MgPMT during greening and light/dark-cycles. Using the yeast two-hybrid system physical interaction was demonstrated between MgPMT and the CHLH subunit of Mg chelatase. Activity of recombinant MgPMT expressed in yeast cells was stimulated in the presence of the recombinant CHLH subunit. Implications for posttranslational regulation of MgPMT are discussed for the enzymatic steps at the beginning of the Mg branch.

Abbreviations: ALA, 5-aminolevulinic acid; Chl, Chlorophyll; MgCh, Magnesium chelatase; MgPMT, Mg protoporphyrin IX methyltransferase; MgProto, Mg protoporphyrin IX; MgProtoMe, MgProtoporphyrin IX monomethylester; Proto, protoporphyrin IX; SAM, *S*-adenosyl-L-methionine

Introduction

S-Adenosyl-L-methionine:Mg protoporphyrin IX methyltransferase (MgPMT) is an enzyme of the Mg branch of tetrapyrrole biosynthesis and catalyzes the transfer of a methyl group to the 13-propionate side-chain of Mg protoporphyrin IX (MgProto) to yield Mg protoporphyrin IX monomethyl ester (MgProtoMe) (Gibson *et al.*, 1963). The enzymatic formation of MgProtoMe was first demonstrated in *Rhodobacter sphaeroides* (Gibson *et al.*, 1963) and chloroplasts of *Zea mays* (Radmer and Bogorad, 1967). MgPMT was purified from *Euglena gracilis* (Ebbon and Tait, 1969) and etiolated wheat seedlings (Hinchigeri *et al.*, 1981). It is a membrane-bound enzyme in *Arabidopsis*, barley, wheat and *Rhodobacter sphaeroides* (Shieh *et al.*, 1978; Hinchigeri *et al.*,

1981, 1997; Block *et al*., 2002). A ping-pong-type mechanism was demonstrated for the enzymatic reaction in wheat MgPMT (Hinchigeri *et al*., 1981). The gene encoding MgPMT was described first in R. *sphaeroides, R. capsulatus* and *Synechocystis* PCC 6803 and encodes a protein with a molecular mass of 25–27 kDa (Bollivar *et al*., 1994; Gibson and Hunter, 1994; Smith *et al*., 1996).

The homologous coding sequence for MgPMT was recently reported for *Arabidopsis thaliana* (Block *et al*., 2002). Interestingly, MgPMT of spinach and *Arabidopsis thaliana* is localised in both the envelope and the thylakoid membranes (Block *et al*., 2002). It has been deduced from previous studies of *Rhodobacter sphaeroides* that MgPMT and the preceding enzyme in the Mg branch, Mg chelatase (MgCh), are enzymatically coupled, because the metabolic intermediate MgProto could not be detected (Gorchein, 1972). MgCh consists of the subunits CHLH, CHLI, CHLD with different molecular masses (Walker and Willows, 1997). Consistent with the previous assumption, it was demonstrated that the MgCh H-subunit from *R. capsulatus* increased *in vitro* recombinant MgPMT activity up to seven fold (Hinchigeri *et al*., 1997) and that soluble extracts of *Synechocystis* PCC 6803 cultures co-expressing *CHLM*, and the three MgCh subunits rapidly converted protoporphyrin (Proto) to MgProtoMe (Jensen *et al*., 1999).

We identified the tobacco *CHLM* cDNA sequence encoding MgPMT and present expressions studies of this gene. The presumed physical protein–protein interaction between MgPMT and MgCh was finally proven to be a prerequisite for an efficient methylation reaction of MgPMT. Implications of the protein complex formation for the control of tetrapyrrole biosynthesis are discussed.

Materials and methods

Cloning of a CHLM cDNA sequence

Among EST sequences obtained from the moss *Physcomitrella patens* (Reski *et al*., 1998), a partial clone was discovered as being homologue to *Synechocystis chlM*. An AccI-500 bp fragment of this clone was used to screen 40×10^6 PFU of a λ-Zap-II cDNA library representing mRNA of 14-days-old leaf tissue from *Nicotiana tabacum* (var. SR1) (Stratagene, La Jolla, USA). Twelve putative positive phage clones were isolated and their cDNAs were sequenced with the dye-termination method on an automated DNA sequencer (Applied Biosystems, Darmstadt, Germany).

Growth of tobacco plants

Etiolated tobacco seedlings were grown in sterile liquid MS medium under constant shaking. Tobacco plants and barley seedlings were grown in soil under greenhouse conditions with average light intensity of 300 μmol photons m^{-2} s^{-1} or in growth chambers (Conviron, Manitoba, Canada) at 24 °C (tobacco) or 20 °C (barley) in a 12 h light/12 h dark period and 150 μmol photons m^{-2} s^{-1}. Plant material was harvested and immediately analysed or stored at −80 °C. Leaves were always counted from the top of the shoot.

RNA and DNA analysis

Genomic DNA extraction by the DNA easy-kit (Qiagen, Hilden, Germany) and Southern blotting were carried out according to standard procedures (Sambrock *et al.,* 1989). Total RNA was extracted by the guanidium thiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi (1987). Aliquots of 10–20 μg RNA and of 10 μg DNA were used for Northern and Southern analyses. Equal loading of RNA was visualised by ethidium bromide stained rRNA. Radioactively labelled DNA fragments were prepared by the random primer extension method using *E. coli* DNA polymerase I (AP-Biotech, Freiburg, Germany) and hybridised to the filter-bound nucleic acids. The hybridisation signal was monitored by a phosphoimager (Molecular Dynamics, Krefeld, Germany). Northern and Southern analyses based on samples of nucleic acids that were extracted from leaves of at least two different harvests.

Preparation of the tobacco CHLM coding sequence for the expression in E. coli

Three different cDNA fragments of the *CHLM* coding sequence with a length of 978, 852 and 714 bp, respectively, were amplified by PCR with

specific forward primers Exp-F1 (5′-GAT CCC ATG GGC TTT CTC TTC GCC ACT ATT C-3′), Exp-F2 (5′-GAT CCC ATG GGC AAC CGA CAT ATC GGC GGT CAC C-3′) , Exp-F3 (5′-TAT ACC ATG GGA GGT CGG CGG CGG CGA C-3′) and a reverse primer Exp-R1 5′-AAG CTT ATT GAG GCT GTC CCT GCG GAT CCA G-3′. The amplified DNA was inserted into the expression vector *pET 20b*$^{+}$-His6 (Novagen, Darmstadt, Germany) and introduced into the expression strain (BL21–*DE3*/*PlysS*) (Stratagene, La Jolla, USA). Expression of the recombinant genes was induced in *E. coli* by addition of 0.4 mM IPTG (isopropyl-*β*-D-thiogalactopyranoside).

Extraction of leaf and bacterial proteins and Western blot analysis

Leaves (100 mg) and *E. coli* cell pellets were ground under liquid nitrogen and re-suspended in solubilisation buffer (56 mM Na_2SO_4, 56 mM DTT, 2% SDS, 12% sucrose, 2 mM EDTA). After removal of non-solubilised cell debris, aliquots of the supernatant were subjected to SDS PAGE and subsequently to Western blot analysis. The antiserum was raised against recombinant tobacco MgPMT, which was purified by affinity chromatography using a Co-TALON-resin (Clontech, Heidelberg, Germany).

Chl and porphyrin content

Plant tissue (100 mg) was ground in liquid nitrogen and extracted in methanol/acetone/0.1 N NH_4OH (9:10:1 v/v). Aliquots of the supernatant were separated by HPLC (Agilent, Böblingen, Germany) on a RP 18 column (Novapak C18, 4 μm particle size, 3.9 × 150 mm, Waters Chromatography, Eschborn, Germany) at a flow rate 1 ml min^{-1} in a methanol/0.1 M ammonium acetate gradient (Papenbrock *et al*., 1999). Porphyrinogens were oxidised to porphyrins using 2-butanone peroxide in acetic acid. The eluted samples were monitored by a fluorescence detector at λ_{ex} 405 nm/ λ_{em} 620 nm for Proto and λ_{ex} 420 nm/ λ_{em} 595 nm for Mg porphyrins. The porphyrins and metalloporphyrins were identified and quantified by authentic standards purchased from Frontier Scientific (Logan, UT, USA). Chl was extracted from 100 mg leaf tissue in 80% alkaline acetone. Chl absorption was recorded at λ 664, 646 and 750 nm, respectively, and Chl content calculated according to Porra *et al*. (1989).

Enzyme assays

Ten gram of young leaves of 4-weeks-old tobacco plants and primary leaves of 7-days-old barley seedlings as well as cell pellets of 1l cultures of yeast (*Saccharomyces cerevisiae*) and *E. coli*, which were initially ground in liquid nitrogen, were homogenised in ice cold buffer (0.5 M sorbitol, 0.1 M Tris/HCl pH 7.5, 1 mM DTT, 0.1% BSA), filtered through two layers of Mira cloth and centrifuged (800 *g*, 4 °C, 2 min). The pellets were resuspended in assay buffer (500 mM Tris/HCl pH 7.8, 1 mM DTT, 5 mM EGTA). The crude plastid suspension and the yeast extracts were assayed at 33 °C with 1 mM SAM and 20 μm MgProto for 30 min. MgProtoMe produced was monitored by HPLC-fluorescence detection according to Papenbrock *et al*. (2000).

Yeast-two-hybrid-system

The coding sequence of the mature MgPMT was PCR amplified with the Exp-F2/Exp-R1-primers and subsequently cleaved with *Nco*I and *Bam*HI. The PCR fragments were subsequently inserted into the corresponding restriction sites of two vectors *pACT2* and *pAS2-1*. The *CHLD*, *CHLI* and *CHLH* coding sequences (Kruse *et al*., 1997; Papenbrock *et al*., 1997) for the mature proteins of the three MgCh subunits were amplified with the primer pairs: Mg-D-F (*Sma*I): 5′-TGA CCC GGG GGT AGT GGA ACC TGA AAA ACA ACC-3′ and Mg-D-R (*Sal*I): 5′-GCG GTC GAC TCA AGA TTC CTT TAA TGC AGA-3′; Mg-I-F (*Sma*I): 5′-CAG CCC GGG GGG TTC ACT ACT AGG-3′ and Mg-I-R (*Sal*I): 5′-CAG GTC GAC GCA CAG TAC AAA GCC-3′; Mg-H-F (*Eco*RV): 5′-GCT GAT ATC GGC TAT TGG CAA TGG TTT ATT CAC-3′ and Mg-H-R (*Sal*I): 5′-GC GTC GAC ATT TAT CGA TCG ATT CCC TCA A-3′. The amplified DNA fragments were cleaved with *Sal*I, *Sma*I, and *Eco*RV, respectively, and subsequently sub-cloned into the corresponding restriction sites of both vectors *pACT2* and *pAS2-1*. The different bait and prey vector-constructs were co-transformed into the YSF_{526} yeast strain (Klebe *et al*., 1983). Transformed yeast colonies were tested in filter and liquid

assays for galactosidase activity according to the manufacturers protocol (Breeden and Nasmyth, 1985; Munder and Fürst, 1992). Yeast extracts were subjected to enzyme assays in assay buffer as mentioned above.

Results

Heterologous hybridisation of a tobacco cDNA library with a partial *ChlM* clone from *Physcomitrella* revealed the cDNA clone MT-10/4 comprising the complete coding sequence (accession number AF 213968). The sequence contained 1134 nucleotides without the poly A-tail. The coding region between the nucleotide position 12 and a stop codon at position 987 codes for a protein containing 326 amino acids. A multiple sequence alignment of tobacco MgPMT with published MgPMT sequences from plants and photosynthetic bacteria revealed an overall identity of 77% to *A. thaliana* MgPMT (CAB 36750), of 63% to rice MgPMT (BAA 84812), of 66% to a complete *Physcomitrella* MgPMT, of 52% to *Synechocystis* CHLM and 23% to *Rhodobacter capsulatus* bchM.

The tobacco *CHLM* sequences possess an amino terminal extension with structural similarity to the expected plastid transit peptide. All MgPMTs contain three conserved domains for SAM-binding (Joshi and Chiang, 1998). As for other plant MgPMTs, a stretch of several hydrophobic amino acids at the N-terminus just behind the transit sequence is found in the mature tobacco MgPMT. This part of the enzyme is proposed to function as an anchor in plastid membranes (Block *et al.,* 2002).

Genetic analysis of CHLM *in tobacco*

Total genomic DNA was isolated from *N. tabacum* (var. SNN), *N. sylvestris* and *N. otophora* to determine the *CHLM* gene copy number. Latter two species are the ancestors of the amphidiploid *N. tabacum*. About 10 μg of *N. tabacum* genomic DNA was cleaved with various restriction enzymes and compared with *Eco*RI cleaved genomic DNA from the other two *Nicotiana* species. The *N. tabacum* genome always displayed two different hybridising bands with the *CHLM* cDNA probe. Only a single band was labelled in the respective genome of the two species *N. sylvestris* and *N. otophora*. Each band shows the same mobility as one of the two labelled DNA bands of the *Eco*RI-cleaved *N. tabacum* genome (Figure 1). This result indicates that the amphidiploid *N. tabacum* genome carries one *CHLM* gene from each ancestor.

Overexpression of CHLM *in* E. coli *and activity test*

By using the CHLOROP-program (Emanuelsson *et al.*, 2000) the putative transit peptide of tobacco MgPMT was predicted to consist of 42 amino acids resulting in a mature protein sequence with 284 amino acid and a molecular weight of 31.240 Da. Three different primer pairs were used to amplify by PCR three fragments of the *CHLM* sequence encoding: (i) the entire precursor protein (MgPMT I with 326 amino acids), (ii) the putative mature protein (MgPMT II with 284 amino acids) and (iii) a truncated peptide (MgPMT III with 238 amino acids) (Figure 2C). The truncated protein MgPMT II was equal in size to the MgPMT of *Synechocystis* PCC6803 (Smith *et al.*, 1996). Optimal expression of the three recombinant proteins was achieved with the *E. coli* strain BL21–*DE3*–*PlysS* that was induced with IPTG for 3 h at 30 °C under vigorous shaking in darkness. This procedure enabled the accumulation of 50% of the recombinant protein content in the soluble protein fraction of bacterial extracts. The soluble part of bacterial extracts was used for MgPMT purification and enzymatic activity tests. The purified recombinant MgPMT II was used to produce anti-MgPMT antibodies. Endogenous methyltransferase activity of the control *E. coli* strain harbouring only the empty pET vector was negligible. The *E. coli* protein extracts containing either the recombinant precursor protein, MgPMT I, or the mature protein, MgPMT II, displayed enzymic activity (Figure 2B), while the truncated MgPMT III peptide had no detectable methyltransferase activity.

The results of this activity test with the recombinant tobacco MgPMT were compared with results obtained from recombinant *Arabidopsis* MgPMT. A truncated *Arabidopsis* MgPMT sequence, lacking 79 N-terminal amino acid residues of the precursor protein, had a conserved leucine following the initial methionine. This leucine corresponds to the position 85 of tobacco MgPMT (Figure 2A; Block *et al.*, 2002).

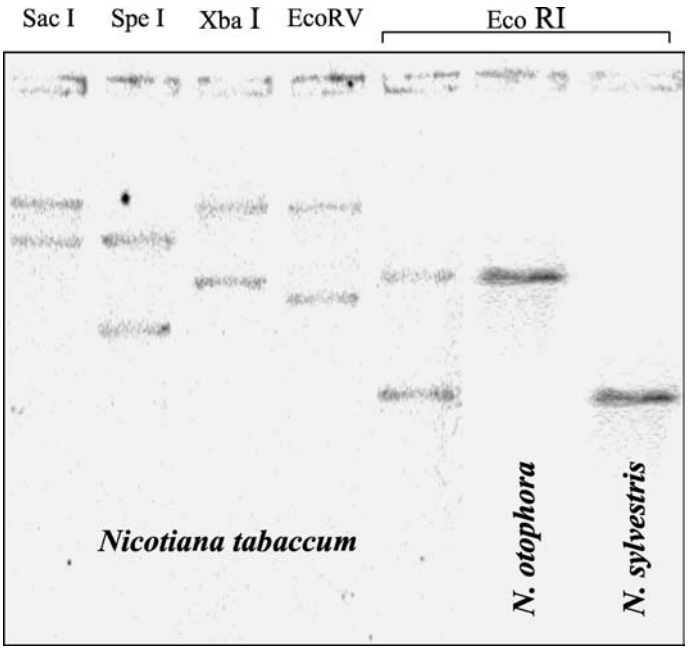

Figure 1. Southern blot analysis of *CHL M* in tobacco. Genomic DNA was isolated from *N. tabacum* and from *N. sylvestris* and *N. otophora*. Ten microgram DNA of *N. tabacum* was cleaved with *Eco*RI, *Eco*RV, *Xba*I, *Spe*I and *Sac*I (from right to left) 10 μg DNA of *N. sylvestris* and *N. otophora* DNA was cut with *Eco*RI. The digested DNA was subjected to Southern blot hybridisation with a ^{32}P-dCTP-labelled *CHLM* cDNA fragment. Amphidiploid DNA of *N. tabacum* displayed two hybridizing bands; whereas the *Eco*RI-cleaved DNA of the other two tobacco species contained only one labelled genomic DNA fragment each that corresponds to one of the hybridising bands of *N. tabacum Eco*RI-cleaved DNA.

Interestingly, this truncated protein possessed enzyme activity, while the truncated tobacco MgPMT III starting with glutamate at position 89 was expressed as an inactive protein. The four amino acids (leucine, glutamine, alanine and glutamate) at position 85–88 were conserved in plant MgPMT (Figure 2C). It is currently open, why the additional deletion of four amino acids caused an inactivation of the protein. This stretch of four amino acids is not present in bacterial MgPMT and may be a potential phosphorylation site (Block *et al*., 2002). Recombinant mutant MgPMT containing one or more of these conserved and essential amino acids are currently being generated to elucidate their function.

Expression of CHLM *during greening*

Chl synthesis is strongly influenced by environmental stimuli such as light intensity, day length and the daily changes between light/dark periods. Upon illumination, the Chl content rises in etiolated tobacco seedlings within 24 h from 0 to 147.6 nmol g^{-1} FW and in etiolated barley seedlings from 0 to 620 nmol g^{-1} FW and (data not shown). We addressed the question to what extent *CHLM* expression follows the light-induced Chl biosynthesis. Etiolated tobacco and barley seedlings were used for the analysis of the MgPMT expression because both species display a different morphogenetic development during etiolation. In contrast to the tiny etiolated tobacco seedlings, the full expanded etiolated barley leaf provided sufficient leaf material for MgPMT activity tests. Tobacco *CHLM* mRNA and protein were already present in etiolated tissue and their amount hardly increased during light exposure in the first 12 h of illumination (Figure 3A and B). Similar to tobacco seedlings, MgPMT was already present in etiolated barley seedlings and remained constant during a greening period of 24 h (Figure 4A).

The MgPMT activity of crude plastid extracts from seven-day-old primary barley leaves was low in etiolated tissue and increased more than 10-fold within 12 h after the onset of light. The activity

declined during the following 12 h in light (Figure 4B). The increase of enzyme activity did not correlate with the amount of available MgPMT protein. The steady-state levels of MgProto and MgProtoMe were analysed during greening of etiolated tobacco seedlings (Figure 3C). Both Mg porphyrin levels increased to a similar extent and reached a plateau 8–12 h after illumination and then decreased. The time-course of Mg porphyrin accumulation in tobacco seedlings resembled the MgPMT activity during greening of barley seedlings (Figure 3C). The synchronously increased pools of both Mg porphyrins are indicative (1) for a stimulated metabolic flow of chl precursors through the pathway most likely as result of light-induced increase of ALA-synthesis (Beator and Kloppstech, 1993), and (2) for a balanced activity of MgPMT and its neighbour enzymes up- and down-stream of the Mg branch.

Expression of CHLM *during leaf development*

Apart from the environmental factor light, the developmental program of plants is a determining regulatory factor for tetrapyrrole biosynthesis (He *et al.*, 1994). The development-dependent expression and activity of *CHLM* was examined in all leaves of 4-weeks-old tobacco plants,

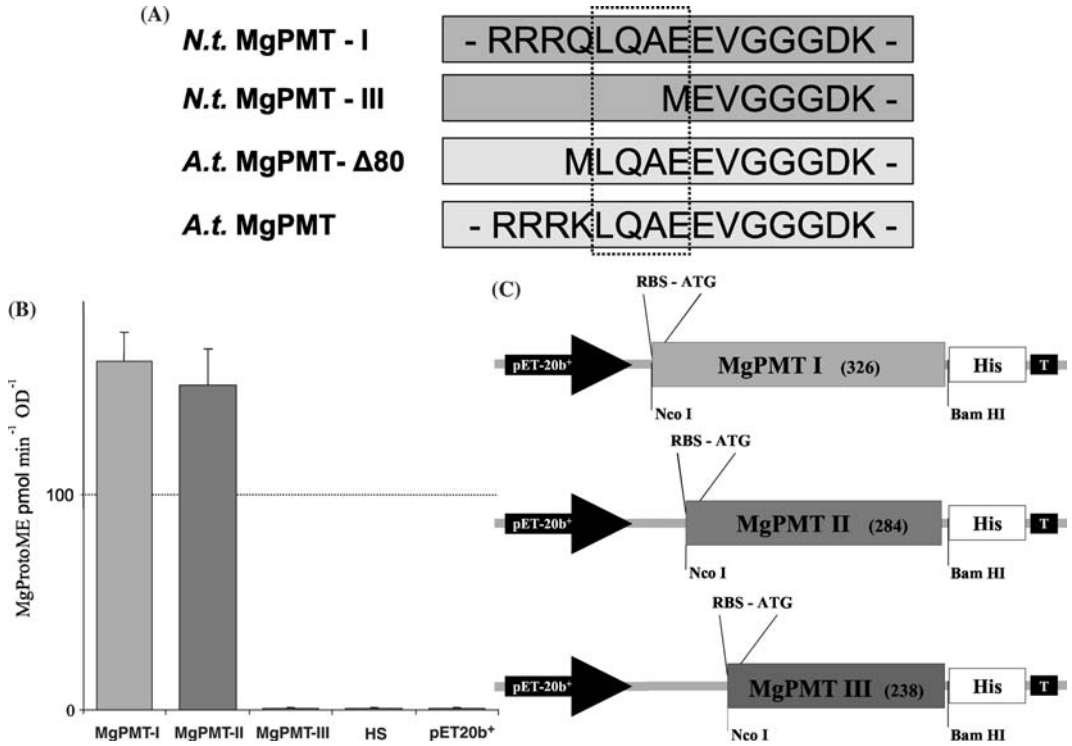

Figure 2. CHLM expression constructs and activity of the recombinant MgPMT. (A) Comparison of a stretch of amino acids in the N-terminal half of tobacco and *Arabidopsis* MgPMT. The sequence corresponds to amino acid No. 81–95 of the tobacco MgPMT. This part of the sequence highlights the N-terminal end of the recombinant MgPMT III of tobacco and the *A.t.* MgPMT-Δ80 that was deduced from the description in Block *et al.* (2002). The difference between inactive *N.t.* MgPMT III and *A.t.* MgPMT-Δ80 consists of four amino acids. (B) The recombinant MgPMT activity was determined from sonicated bacterial extracts containing the recombinant precursor, mature protein or the truncated peptide, respectively. Mg porphyrins were extracted from the assay mixture and analysed by HPLC. The MgPMT activities are presented after a 25 min enzyme reaction. Specific activity is indicated in pmol MgProtoMe min^{-1} OD^{-1}. A sample with heat shock denatured protein extract containing MgPMT II (HS) and a sample of extracts from a strain expressing the empty (*pET-20b*$^{+}$) vector were used as control and did not show MgPMT activity. (C) Restriction map of the pET20b expression vector with three different tobacco *CHLM* gene constructs for the expression of recombinant MgPMT in *E. coli*. T (black box) is the termination signal. The first gene construct (MgPMT I) encodes the precursor protein. The second gene construct encodes the putative mature protein (MgPMT II) and the third one encodes the truncated protein lacking 88 amino acid residues of the precursor protein (MgPMT III).

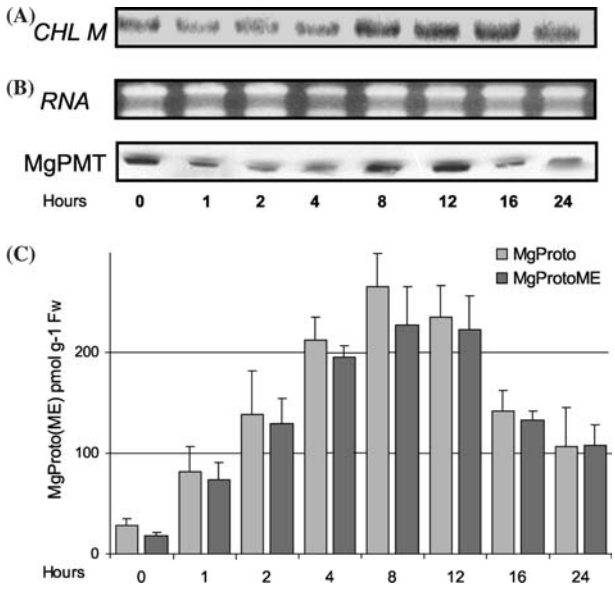

Figure 3. *CHLM* expression, MgPMT activity and steady-state levels of Mg porphyrins during greening in tobacco seedlings. Tobacco seedlings grown for 7 days in liquid MS medium with 100 rpm in darkness and, subsequently, in white light for the indicated times (150 μmol photons m^{-2} s^{-1}), were harvested and extracted. (A) Northern blot analysis of total tobacco RNA. The blot was subjected to radioactive hybridisation with a ^{32}P-labelled *CHLM* cDNA insert. Equal loading of the RNA was controlled with ethidium bromide-stain of total RNA. (B) Proteins were extracted from intact etiolated and greening seedlings of tobacco and applied to SDS-PAGE and Western blot analysis using anti-MgPMT antiserum. (C). Steady-state levels of MgProto and MgProtoMe in tobacco seedlings. The Mg porphyrins were extracted and analysed by HPLC.

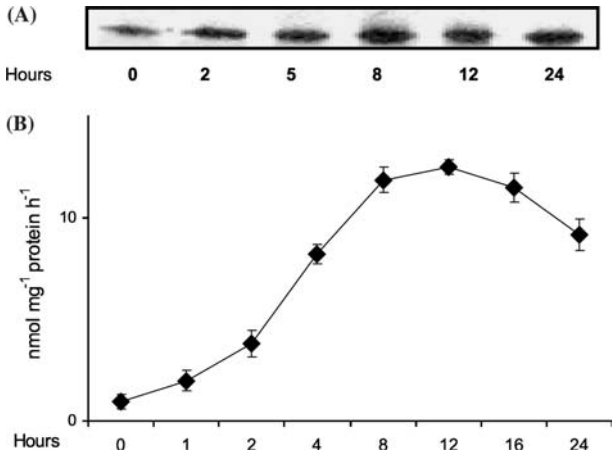

Figure 4. MgPMT accumulation and activity in greening barley seedlings. Etiolated 7-days-old barley primary leaves were exposed to light of 150 μmol photons m^{-2} s^{-1} for the indicated times and samples were harvested and processed. (A) Total proteins were extracted from etiolated and greening leaves and applied to SDS-PAGE and Western analysis using anti-MgPMT antiserum. (B) Analysis of MgPMT activity of plastid extracts from etiolated and greening barley seedlings. About 10 g of primary leaves were harvested to obtain crude chloroplast extracts, which were incubated for 30 min with MgProto and SAM. The enzyme activity is given in nmol of synthesised MgProtoMe mg^{-1} protein h^{-1}.

representing different developmental stages from the youngest premature leaf at the apex through a fully expanded and photosynthetically active leaf down to the senescent leaves at the base of the plant. *CHLM* RNA and protein content increased up to leaf No. 5 before the *CHLM* expression was attenuated in senescent leaves (Figure 5A and B).

For analysis of MgPMT activity, the premature leaves No. 1–4 were pooled to obtain sufficient leaf material. The MgPMT activity had a transient maximum in leaves No. 5/6 along the shoot axes before the activity dropped in older leaves (Figure 5C). The metabolic intermediates MgProto and MgProtoMe consistently accumulated to similar extent in each leaf analysed (Figure 5D) and their amounts correlated with the MgPMT activity rate. It is worth to emphasise that all kinetics of MgPMT activity and *CHLM* RNA and MgPMT contents show similarity during leaf development. The Mg porphyrin steady-state levels likely reflect the transiently enhanced metabolic activities of the Mg porphyrin branch during leaf development.

CHLM *expression during day/night growth condition*

The expression of some enzymes of the tetrapyrrole metabolism oscillates during a 12 h light/12 h dark period or in continuous dim light or darkness and contributes to the varying synthesis of Chl and heme in a 24 h period. (Papenbrock *et al*., 1999). We extended our knowledge on expression profiles in tetrapyrrole biosynthesis with the analysis of the *CHLM* expression under light/dark and constant growth condition. *CHLM* RNA levels did not vary during a 24 h period, neither under day/night conditions (Figure 6A and B) nor under continuous conditions (data not shown).

The contents of MgProto and MgProtoMe were determined during the daily dark/light transition. During darkness Mg porphyrin levels were extremely low and consistent with the general suppression of ALA synthesis and the inactivation of plant protochlorophyllide oxidoreductase in the dark (Reinbothe and Reinbothe, 1996; Beale, 1999). In contrast, a rapid rise of the steady-state levels of MgProto and MgProtoMe was observed in the first 4 h of the light phase (Figure 6C). It is proposed that steady-state levels of the Mg porphyrins account for the extent of synchronised metabolic activities in the Mg branch including the activity of MgPMT and its neighbouring enzymes. A light-dependent induction of the metabolic activities in the Mg branch could explain this burst of Mg porphyrins (Pöpperl *et al*., 1998).

It was previously shown that the rapid change from dark to light activates MgCh (Papenbrock *et al*., 1999). In the same experiment tobacco MgPMT displayed a maximum activity 5 h after illumination start and low activity rates during darkness. The activity of MgPMT was analysed in detail during the light period to interpret the steady-state levels of MgProto and MgProtoMe. During an 8 h period from the beginning of

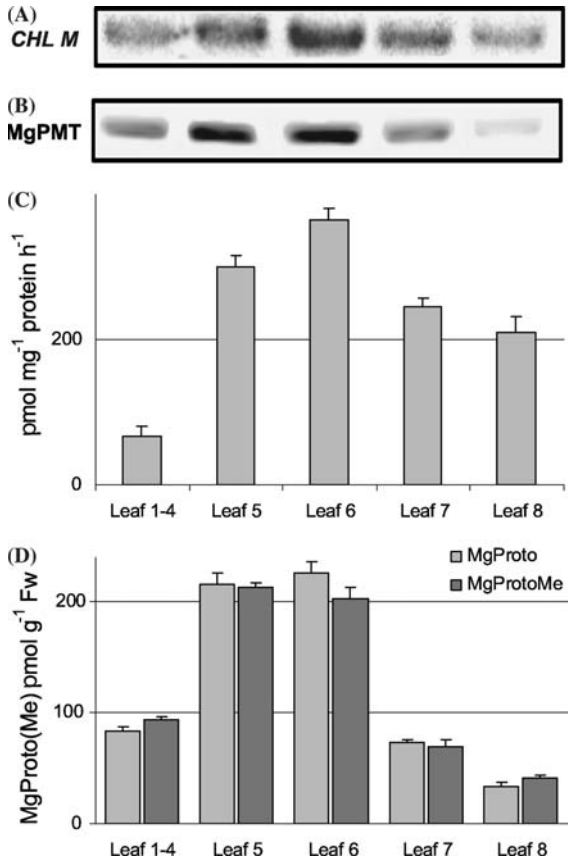

Figure 5. *CHLM* expression, MgPMT activity and steady-state levels of Mg porphyrins during plant development. Four-weeks-old wild type tobacco plants were grown in a growth chamber (12 h light/12 h dark; 300 μmol photons m^{-2} s^{-1}). Leaves of different age were harvested including the youngest leaves (No. 1–3) and the senescent fully expanded leaf (No. 8). (A) Total RNA was extracted and 10 μg RNA was subjected to Northern blot analysis using ^{32}P-labelled *CHL M* cDNA. (B) Total protein was extracted from leaf samples to determine accumulation of MgPMT during leaf development. About 20 μg samples were subjected to Western blot analysis using an antibody against MgPMT. (C) MgPMT activity in leaves of different ages: Crude chloroplast extracts of wild type (SNN) tobacco plants were prepared and incubated for 30 min with MgProto and SAM. (D) Steady-state levels of MgProto and MgProtoMe during tobacco leaf development were determined from three independent samples of corresponding leaves (No. 1–8) of three tobacco plants by HPLC.

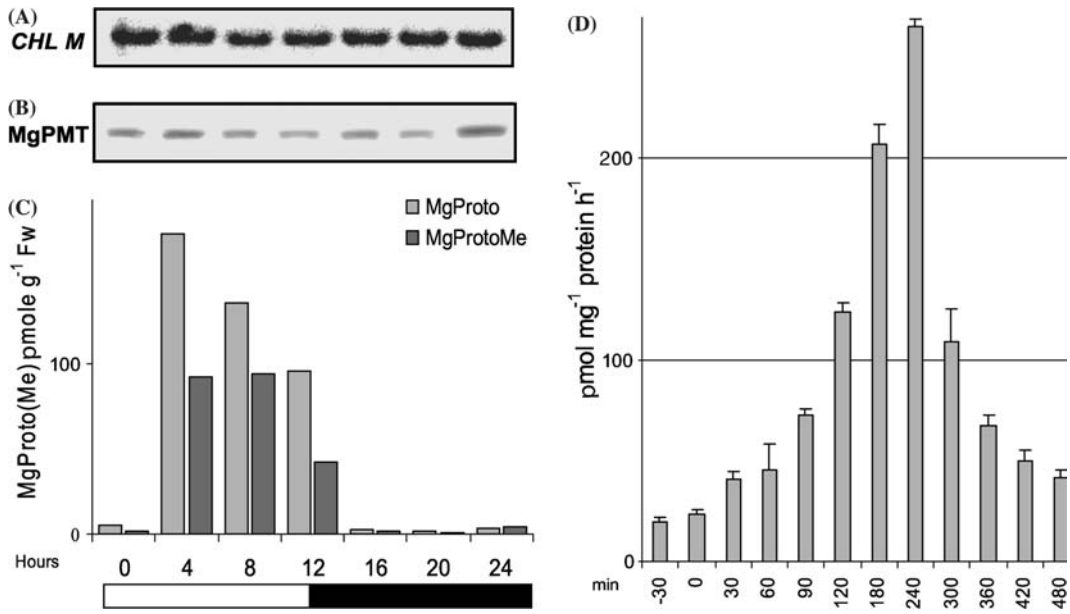

Figure 6. *CHLM* expression and accumulation of MgProto and MgProtoMe in tobacco leaves under diurnal growth conditions. Four-weeks-old tobacco plants were grown in 12 h light/12 h dark cycles. Material from leaf No. 4 and 5 was harvested in 4 h-intervals, starting after the transition from dark to light. (A) Total RNA was isolated and used for Northern blot analysis of the *CHLM* transcript. (B) Total protein content was separated by SDS-PAGE and subjected to Western blot analyses using anti-MgPMT antiserum. (C) MgProto and MgProtoMe levels were quantified by HPLC. (D) Leaves of a whole plant (leaf No. 1–8) was used for the isolation of a crude plastid extract. MgPMT activity of the crude plastid extract was determined to the indicated times. Assays were performed with extracts from two different harvests.

illumination, MgPMT showed a transient increase of activity after 4 h of illumination (Figure 6D). This activity peak resulted without any change in *CHLM* RNA and protein content and followed around 3 h after the MgCh activity peak. Referring to the Mg porphyrin levels in Figure 6C, it is suggested that the immediate rise of MgCh activity after transition form dark to light (Papenbrock *et al.,* 1999) has the potential to synthesise high amounts of accumulated MgProto at the beginning of the light period. During the following light exposure, MgProto is metabolised by the increasing capacity of MgPMT, before the activities of both enzymes, MgCh and MgPMT, are again attenuated in darkness. The light-induced burst in MgProto and MgProtoMe accumulation was not observed under constant dim light (10 μmol photons m^{-2} s^{-1}) (data not shown).

Interaction between Mg protoporphyrin IX methyltransferase and Mg chelatase

A tight MgCh–MgPMT complex was presumed without experimental evidence to explain the absence of detectable amounts of endogenous MgProto in *Rhodobacter* species and the accumulation of its methylester (Gorchein, 1972). Thus, these experiments (see also introduction) indicate a mutual interdependence of MgCh and MgPMT.

Physical interaction between both enzymes was tested by the yeast two-hybrid-system. The coding sequences of tobacco *CHLM, CHLH, CHLI* and *CHLD* were fused in frame behind the activation or DNA-binding domain of the *GAL4*-gene. MgPMT interaction with each MgCh subunit was qualitatively determined in filter and quantitatively in liquid assays (Figure 7A). The combination of fusion proteins containing MgPMT and CHLH caused the strongest protein interaction, while MgPMT with itself and the combination of MgPMT with CHLI or CHLD did not reveal significant interaction activity. The strong physical interaction between MgPMT and CHLH substantiates the previous observations (Gorchein *et al*., 1972) and is reasonable since CHLH binds Proto and could direct MgProto to the following enzyme, MgPMT. This example of substrate channelling lowers the potential risk of

photodynamic destruction by release of photoreactive MgProto.

Extracts of the yeast strains expressing different combinations of the two hybrid fusion proteins were tested for MgPMT activity (Figure 7B). The significance of the subunits of tobacco MgCh for the enzyme activity and the dissection of essential parts of CHLD have been successfully demonstrated with the yeast fusion protein system (Grafe *et al.*, 1999). Yeast extracts co-expressing recombinant MgPMT and either CHLI, CHLD or MgPMT, respectively, showed a significant activity. Control yeast extracts (without recombinant MgPMT) always have a low unspecific methyltransferase activity (Papenbrock *et al.*, 1997). The highest MgPMT activity rate was determined in yeast extracts from cells co-expressing *CHLM–CHLH*. Thus, the MgPMT–CHLH interaction apparently stabilises the protein complex or stimulates the methylation reaction (Figure 7B).

Discussion

The expression studies revealed that *CHLM* RNA and MgPMT levels did not consistently correlate with enzyme activities. During greening of dark-grown barley seedlings, the MgPMT activity increased more than 10-fold within the first 12 h of exposure to light, while the barley (Figure 4B) and the tobacco (Figure 3A and B) MgPMT content remained relatively constant during greening. The

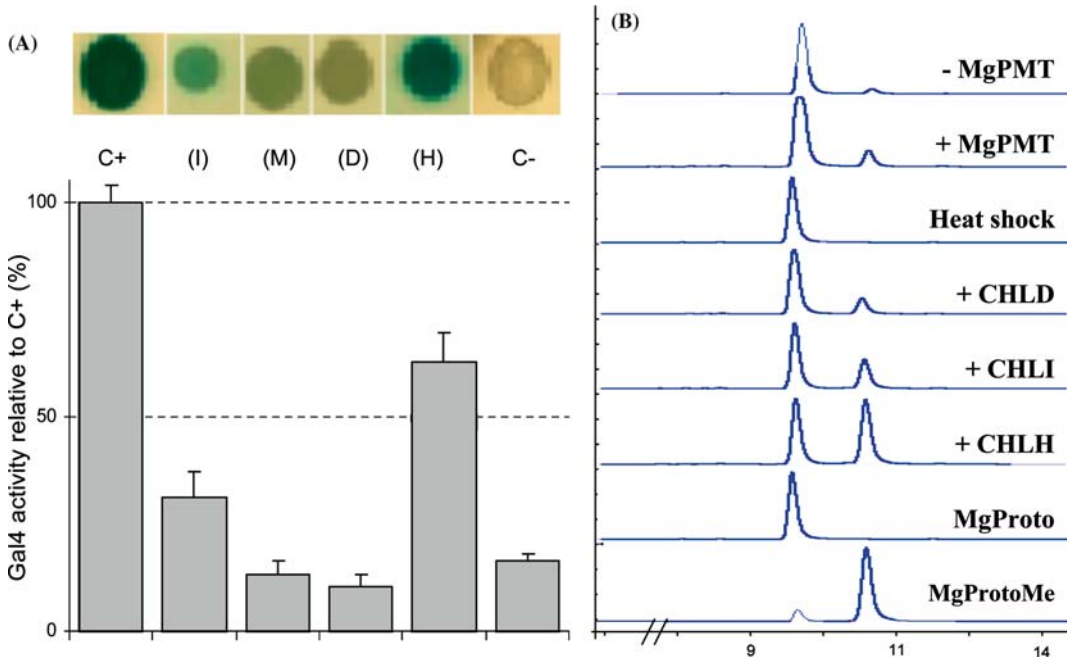

Figure 7. (A) Assay of MgPMT–MgCh interaction by using the yeast two-hybrid-system (*S. cerevisiae*). Yeast cells containing the reporter plasmid *pGAL4* were co-transformed with the prey plasmid *pACT2–CHLM* and different fusion constructs in the bait plasmid *pAS2-1*: with *CHLI* (I), *CHLM* (M), *CHLD* (D) and *CHLH* (H), respectively. C+ and C− stand for positive and negative control plasmids provided by the supplier. The transformants were resuspended in TE, dotted on X-gal-supplemented plates and incubated at 30 °C for 1–3 days. The upper panel shows the β-galactosidase filter assay, and the lower graph shows oNPG (*o*-nitrophenyl-β-D-galactopyranoside) liquid assay. The results of the quantitative β-galactosidase assay are the means of three independent assays. (B) MgPMT activity tests with extracts of the yeast strains expressing different combinations of the two hybrid–fusion protein with MgPMT and that with MgPMT and the three MgCh subunits CHLI, CHLD and CHLH, respectively. All extracts were incubated with SAM and MgProto. MgProto and MgProtoMe were extracted from the enzyme assays and subjected to HPLC. The chromatograms display the eluted Mg porphyrins monitored by HPLC fluorescence detection from the following assays (from the top to the bottom): −MgPMT: yeast extract without recombinant MgPMT; +MgPMT: yeast extract containing *pACT2–CHLM* fusion and co-expressed MgPMT; +HS: the same yeast extract after heat denaturation; +CHLD: yeast extract with *pACT2–CHLM* and co-expressed CHLD; +CHLI: – and co-expressed CHLI; +CHLH: – and with co-expressed CHLH; MgProto and MgProtoMe: authentic standards.

simultaneous accumulation of MgProto and MgProtoMe during greening corresponds to the MgPMT activity profile and is most likely explained with balanced activities of MgPMT and its neighbour enzymes, MgCh or MgProtoMe cyclase. Any alteration of their activity rates in relation to that of MgPMT would cause a change in the MgProto/MgProtoMe ratio.

In contrast to *CHLM* expression, the amount of the transcripts encoding the three MgCh subunits displays a rapid increase during greening in *Arabidopsis*, soybean, barley or tobacco (Gibson *et al*., 1996; Kruse *et al*., 1997; Papenbrock *et al*., 1997; Nakayama *et al*., 1998). Thus, additional posttranslational mechanisms are likely for the synchronisation of enzyme activities in the Mg branch and the increasing activity of MgPMT during greening. In addition, the conversion of etioplasts to thylakoids-containing chloroplasts and the association of enzymes to a multi-enzymatic complex for Mg porphyrin synthesis are proposed to positively affect MgPMT activity.

CHLM RNA, protein and activity levels correlated tightly during plant development (Figure 5). However, the *CHLM* RNA and protein contents as well as the Mg porphyrin levels remained constant during light/dark periods (Figure 6A–C), while the MgPMT activity increased up to eightfold in comparison to the measured activity in the dark (Figure 6D). We suggest that posttranslational activation accounts at least in part for the transient rise of MgPMT activity during the diurnal growth period.

The physical interaction of MgPMT and the substrate-binding subunit CHLH of MgCh (Figure 7A) stimulate MgPMT activity (Figure 7B, Hinchigeri *et al.,* 1997). It is reasonable, that direct channelling of MgProto from CHLH to MgPMT will enhance the methyltransferase reaction and lower the steady-state level of MgProto. During diurnal growth the MgCh activity peaks first after the immediate transition form dark to light before the MgPMT activity maximum follows a few hours later. In this interim MgProto levels are transiently higher than MgProtoMe (Figure 6C).

The tight interdependence of expression and activity of MgCh and MgPMT were also demonstrated in transgenic plants expressing antisense and sense RNA for MgPMT. The activity of MgCh and the *CHLH* transcript level increased in *CHLM* overexpressing plants and were lowered in *CHLM* antisense plants. This mutual activation and inactivation could occur in a bigger enzyme complex that stabilises proteins and channels Proto into the Chl synthesising pathway (Alawady and Grimm, 2005).

The MgProto/MgProtoMe ratio is also of particular interest, as both Mg porphyrins are suggested to be involved in reverse signalling from plastids to nucleus (Papenbrock *et al*., 2000; Mochizuki *et al.,* 2001; Rodermel, 2001; Surpin *et al*., 2002; Strand *et al.,* 2003). It is likely that the pool of Mg porphyrins or the ratio of MgProto/MgProtoMe are indicative of enzyme activities and the metabolic flow in the pathway (Papenbrock *et al.,* 2000).

In conclusion: MgPMT expression profiles are more characterised by constant *CHLM* and protein levels during greening and 24 h light/dark periods in comparison to that of the neighbour enzyme MgCh. The changes of MgPMT activities can be explained by posttranslational activation of the enzyme. The physical interaction between MgPMT and the MgCh subunit CHLH contributes to a fine-tuned balance of both enzyme activities. The constant ratio of MgProto/MgProtoMe analysed from different tissue and under different condition can account for a tight alliance of both enzymes. The participation of further enzymes in this complex cannot be excluded. The posttranslational control mechanisms are subject of our current investigations.

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