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ORIGINAL PAPER

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Characterization of *Chlamydomonas* mutants defective in the H subunit of Mg-chelatase

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Abstract Two chlorophyll-deficient mutants of *Chlamydomonas reinhardtii*, chl1 and brs-1, are light sensitive and, when grown heterotrophically in the dark, accumulate protoporphyrin IX and exhibit yellow/orange pigmentation. The lesions in both mutants were mapped to the gene (*CHLH*) for the plastid-localized H subunit of the heterotrimeric magnesium chelatase that catalyzes the insertion of magnesium into protoporphyrin IX. The genetic defects in the mutants could be assigned to +1 frameshift mutations in exon 9 (chl1) and exon 10 (brs-1) of the *CHLH* gene. In both mutants, the H subunit of magnesium chelatase was undetectable, but, as shown for chl1, the steady-state levels of the I and D subunits were unaltered in comparison to wild type. The *CHLH* gene exhibits marked light inducibility: levels of both the mRNA and the protein product are strongly increased when cultures are shifted from from the dark into the light, suggesting that this protein may play a crucial role in the light regulation of chlorophyll biosynthesis.

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Introduction

Porphyrins, complexed with iron and magnesium, are ubiquitous in photosynthetic organisms. The biosynthetic pathway for tetrapyrroles provides the macrocyclic ring structure of porphyrins, and metal ions are subsequently inserted. The whole pathway can be subdivided into three sections: the synthesis of protoporphyrin IX (PROTO) from glutamate, the conversion of PROTO into heme, and the synthesis of chlorophyll from PROTO (Beale and Weinstein 1990; von Wettstein et al. 1995; Rüdiger 1997). In plants and cyanobacteria, 5-amino levulinic acid is formed from glutamate in a tRNA-dependent pathway. Eight molecules of 5-amino levulinic acid are condensed to a linear and then to a cyclic tetrapyrrole. The product of the porphyrin-synthesizing pathway, PROTO, can be chelated with Mg^{2+} or Fe^{2+} cations to form Mg-protoporphyrin IX (MgPROTO) or protoheme. The latter is the precursor of various types of hemes which function as cofactors in proteins for electron and energy transfer, detoxification processes or signaling. The insertion of Fe^{2+} into PROTO is catalyzed by the monomeric protein ferrochelatase.

The Mg-porphyrin branch leads to the synthesis of chlorophyll and starts with Mg-chelatase, a heteromultimeric complex composed of subunits named D, H and I (reviewed in Walker and Willows 1997). Insertion of Mg^{2+} into PROTO may be subdivided into two steps, activation and Mg chelation. Activation can be achieved by incubating only subunits D and I with ATP, while subunit H was required for the chelation step. The H subunit is thought to bind PROTO and possibly Mg^{2+} (Gibson et al. 1995; Willows et al. 1996; Walker and Willows 1997).

Mg-chelatase has been proposed to play a crucial role in determining how much PROTO is directed into heme and chlorophyll biosynthesis (Gibson et al. 1996;

Papenbrock et al. 1999). Diurnal variations in enzyme activity and/or the concentration of subunit H may have an important role. The activity of Mg-chelatase in 8-day-old barley seedlings and young tobacco leaves increases threefold immediately after transfer from dark to light (Jensen et al. 1996; Papenbrock et al. 1999). The diurnal activity profile of Mg-chelatase does not entirely correspond to the expression pattern of the three genes that encode the Mg-chelatase subunits during the light/dark growth period. While only minor diurnal variations are observed in the levels of *CHLD* and *CHLI* mRNAs (encoding subunits D and I, respectively), amounts of *CHLH* mRNA oscillate drastically during a cyclic photoperiod in *Arabidopsis*, barley, soybean and tobacco. Transcript levels are very low during the dark period but show a substantial increase just prior to the start of the light period. The concentration of *CHLH* mRNA reaches a maximum in the first half of the light period, followed by a gradual decrease during the day (Gibson et al. 1996; Jensen et al. 1996; Nakayama et al. 1998; Papenbrock et al. 1999). These variations in *CHLH* expression led Gibson et al. (1996) to propose a role for the H subunit in regulating the flux through the tetrapyrrole biosynthesis pathways, primarily the chlorophyll branch. When abundant, the H subunit, by sequestering PROTO, may deplete the pool of substrate available for ferrochelatase. The consequent drop in heme levels is proposed to stimulate tetrapyrrole biosynthesis, since heme has been shown to repress the activity of glutamyl-tRNA reductase (Pontoppidan and Kannangara 1994), the first enzyme specific for porphyrin biosynthesis.

Pigment-deficient mutants have proven to be useful tools for the genetic and biochemical analysis of the metabolic pathways of chlorophyll synthesis (Granick 1948a, 1948b; von Wettstein et al. 1995; Suzuki et al. 1997). In *Chlamydomonas reinhardtii* several mutants have been identified which are defective in chlorophyll synthesis, and accumulate PROTO. The non-allelic yellowish-orange and light-sensitive nuclear mutants brs-1 and brs-2 were isolated and described by Wang and colleagues (Wang et al. 1974; Crawford et al. 1982). The mutant chl1 was also isolated as a chlorophyll-deficient strain (Stolbova 1971) and later it was shown that the mutation is recessive and affects a single nuclear gene (Chekunova and Kvitko 1986). It was suggested that the mutants have lesions at the branch point towards chlorophyll synthesis (Wang et al. 1974).

We have shown that mutant brs-1 is defective in the pathway that mediates the light-dependent induction of nuclear *HSP70* chaperone genes. In the mutant, feeding with MgPROTO and Mg-protoporphyrin IX dimethylester [used instead of the naturally occurring Mg-protoporphyrin IX monomethylester (MgPROTOMe)] in the dark resulted in induction of these genes, thus circumventing the mutational block (Kropat et al. 1997).

Here we extend the characterization of the genetic lesions in these mutants that are impaired in chlorophyll synthesis. We confirmed that brs-1 and chl1 are allelic and employed both mutants for biochemical analyses of the early steps in the Mg-porphyrin branch of chlorophyll synthesis. Western analyses revealed a lack of the *CHLH* product, but subunits D and I are present in normal amounts. The +1 frameshift mutations defined in the *CHLH* genes of both mutants, however, do not result in an absence of *CHLH* mRNA. Genetic studies with these mutants should help to define the role of the CHLH protein in regulatory signaling.

Materials and methods

Strains and cell culture conditions

The wild-type stocks of *C. reinhardtii* used in this work were derived from strains 137C *mt+* and CC-124 *mt-*. The chl1 mutant was isolated following treatment of cells of strain CC-124 with N-nitroso-N-methyl urea, and selected from among chlorophyll-deficient mutants (Stolbova 1971). The brs-1 mutant was generated by UV irradiation of strain 137 C *mt+*, and described by Wang et al. (1974, 1975), and was kindly provided to us by Dr. Wang (University of Iowa, Iowa City, Iowa). The wild-type strains and the arginine-requiring mutants CC-50 (*arg7,mt+*) and CC-49 (*arg2,mt-*) were obtained from the Chlamydomonas Genetics Center (Duke University, Durham, N.C.).

Chlamydomonas cells were grown in TRIS-acetate-phosphate (TAP) medium (Harris 1989). Cell cultures were grown photomixotrophically on agar plates or in flasks on a rotary shaker at 24°C under continuous irradiation with white light (30 μM photons per m^2/s) from fluorescent tubes (Osram L36W/25). Light-sensitive mutants were grown heterotrophically in the dark. For arginine-requiring mutants the medium was supplemented with arginine (final concentration 100 μg/ml).

Genetic analysis of mutants

Crosses, maturation of zygotes and analysis of progeny were performed by standard techniques, with some modifications necessitated by the light-sensitive nature of the mutants (Harris 1989). For the mutants dim light was used for gametogenesis and germination of zygotes.

Complementation tests were performed as described by Ebersold (1967). Diploids were identified on the basis of three characteristics: arginine prototrophy, larger cell volume in comparison to haploid cells and mating type (-), since this mating type is dominant.

For recombination tests the zygotes were germinated and incubated for several days in the dark before scoring for the presence or absence of green sectors within yellow/orange colonies. When mutant strains were crossed, the colonies produced by the meiotic products of the zygotes that exhibited green sectors were identified as recombinant clones, while those showing only yellow/orange colors were classified as non-recombinant clones.

Analysis of pigments

Pigment content was determined in cells grown in liquid cultures. Cells were harvested by centrifugation and sonicated. Porphyrins were extracted with acetone/methanol/0.1 N NH_3 (10/9/1, v/v). HPLC analyses were performed on a RP 18 reverse-phase column as described by Kruse et al. (1995) with a few modifications. The column eluate was monitored using a fluorescence detection system

(λ_{ex} /λ_{em} = 405 nm/625 nm and 420/595 nm). Porphyrins were identified and quantified using authentic standards obtained from Fluka or Porphyrin Products. The chlorophyll content was determined in alkaline acetone extracts according to Porra et al. (1989). The extraction of heme was perfomed as described previously (Schneegurt and Beale 1986). Pigments were first extracted from *Chlamydomonas* cells by washing (four times) with cold acetone (80%, v/v) containing 5% (v/v) concentrated HCl. Extracted heme was transferred to chloroform:butanol (2/1; v/v), concentrated and washed on a DEAE-Sepharose column. Absorbance was monitored at 398 nm ($E = 144\ mM^{-1}\ cm^{-1}$). The concentration of protoheme and heme standard solutions was determined spectrophotometrically (Weinstein and Beale 1983).

Determination of 5-amino levulinic acid-synthesizing capacity

The 5-amino levulinic acid-synthesizing capacity was determined according to Miller et al. (1979) with some modification. Cell suspensions (100 ml) were centrifuged and resuspended in 10 ml of fresh TAP medium containing 20 mM levulinate, and incubated for 24 h in the dark. Then cells were centrifuged and resuspended in 0.7 ml of TCA (7.5%), sonicated for 30 s, kept on ice for 30 min and centrifuged for 20 min at 10,000×*g*. The supernatants were adjusted to pH 4.6 by adding 3 M sodium acetate. Three drops of acetylacetone and 0.1 ml of acetate buffer was added to 500-µl aliquots of these samples, which were then boiled for 10 min. The samples were cooled on ice for 5 min, mixed with 1 vol of Ehrlich's reagent and, after a 15-min incubation at room temperature, the 5-amino levulinic acid content was determined spectrophotometrically at 555 nm.

Determination of Mg-chelatase and Mg-protoporphyrin IX methyl transferase activities

For determination of Mg-chelatase and Mg-protoporphyrin IX metyltransferase activities, the *Chlamydomonas* cells were grown in 500 ml of liquid TAP medium to a density of 2–3×10^6 cells/ml, harvested by centrifugation and resuspended in homogenization buffer (0.5 M sorbitol, 0.1 M TRIS-HCl, 1 mM DTT and 0.1% BSA, pH 7.5). The cells were disrupted by two passages through an Amicon French press. The cell extracts were centrifuged at 15,000×*g* for 30 min and the supernatants were used for enzyme assays. Mg-chelatase was assayed as described by Lee et al. (1992) with a few modifications. The assay mixture contained 40 mM $MgCl_2$ and 20 mM ATP. The reaction, performed at 28°C in individual tubes, was started by adding PROTO dissolved in DMSO to a final concentration of 100 µM, and stopped after 15, 30, 45 and 60 min by freezing the tubes in liquid nitrogen.

Mg-protoporphyrin IX methyl transferase activity was measured according to Gibson and Hunter (1994). The enzyme activity was assayed in protein extracts in the presence of 500 µM S-adenosylmethionine and 20 µM MgPROTO for 0, 10 and 20 min at 28°C.

Samples from each enzyme assay were extracted twice with acetone/methanol/0.1 N NH_3 (10/9/1; v/v) and quantified by HPLC using authentic porphyrin standards.

Protein extraction and immunoblot analysis

Total *Chlamydomonas* proteins were extracted as described by Höfgen et al. (1994). Protein concentrations were determined according to Bradford (1976). The SDS-PAGE and immunoblotting procedures were performed as described by Sambrook et al (1989). Alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse serum was used to detect the primary antibodies. The primary antibodies recognizing the CHLI and the CHLH proteins were raised against the recombinant *N. tabacum* proteins, respectively, as described previously (Papenbrock et al. 2000b).

Extraction of RNA and Northern hybridization analysis

Total RNA was isolated and processed for RNA blot analysis as described by von Gromoff et al. (1989). Samples of RNA (10 µg) were fractionated in formaldehyde-containing agarose gels, blotted and hybridized following published protocols (von Gromoff et al. 1989).

DNA extraction and Southern hybridization

Total genomic DNA was isolated from *Chlamydomonas* strains as described by von Gromoff et al. (1989). Aliquots (about 1 µg) of total genomic DNA were digested with various restriction enzymes, fractionated by electrophoresis on 0.8% agarose gels and transferred onto nylon membranes, which were used for hybridization with a ^{32}P-labeled fragment of the *CHLH* gene. Hybridization was performed as previously described (von Gromoff et al. 1989).

Cloning and sequencing of the *CHLH* gene

Two conserved regions in the protein sequences (FGYEGDPM and LEFMPGKQ) deduced for the H subunits of Mg-chelatase from barley (*Xantha-f*), *Antirrhinum majus* (*Olive*), *Synechocystis* PCC6803 (*chlH*) and *Rhodobacter capsulatus* (*bchH*) were selected for the design of degenerate primers. The upstream primer 5′-T T(CT)GG(ACTG)TA(CT)GAGGG(ATGC)GA(CT)CC(AGCT) ATG-3′ and downstream primer 5′-CTGCTT(ACGT)CC(ACGT) GGCAT(AG)AA(CT)TC-3′ were used in a PCR with total DNA from a *C. reinhardtii* wild-type strain as template. The reaction was carried out for 30 cycles of 1 min at 94°C, 2 min at 57°C and 2 min at 72°C. A 162-bp DNA fragment was obtained, cloned into the TA-cloning vector PCRII (Invitrogen) and sequenced. This PCR fragment showed homology to part of the *Xantha-f* gene, and was used as a probe to screen a cDNA library constructed in λNM1149 that was generated from mRNA isolated from vegetative *C. reinhardtii* cells (kindly provided by J.-D. Rochaix, University of Geneva, Switzerland). Two clones with inserts of 1.4 kb and 3.1 kb were identified. The cDNA fragments were amplified by PCR and subcloned into the pGEM-T vector (Promega, Madison, Wis.) for sequencing. The sequence of the 3.1-kb insert exhibited a short polyA tail and, in close proximity to it, the polyadenylation signal TGTAA. About 800 bp upstream of this signal, an ORF with distinct homology to that of other *CHLH* genes was detected. Sequencing of the 1.4-kb insert revealed a 200-bp overlap with the 3.1-kb fragment (the region of the PCR product) and 1.2 kb of upstream sequences. However, approximately 1 kb of coding sequence was still missing. Since rescreening of the cDNA library did not yield clones harboring the front end of the gene, we used the 5′RACE protocol (Gibco BRL). Using two specific primers corresponding to the front end of the 1.4-kb fragment (5′-CTCCAGCTTGTCCGAGTTACC-3′ and 5′-AG-CAGGTTCTC CAGGTTGTCC-3′ and the anchor primer 5′-GGCCACGCG CCGTCGACTAGTACGGGIIGGGIIGG-GIIG-3′), a 605-bp fragment of upstream coding region was amplified, subcloned into the pBluescript II SK + (Stratagene) vector, and sequenced.

In order to test whether the 5′ end of this fragment represented the beginning of the gene, we screened a *Chlamydomonas* genomic DNA library constructed in λEMBL3 (Goldschmidt-Clermont 1986) using the 605-bp 5′RACE product as a probe. Phage DNA from three positive clones was digested with either *Sal*I/*Pst*I or *Sal*I/*Sma*I, transferred onto a nylon membrane and hybridized with the 5′RACE fragment. After stripping, the membranes were rehybridized with a probe derived from the 3′ end of the 3.1-kb cDNA fragment. One DNA fragment (1.75 kb) which hybridized only with the 5′RACE product was eluted from the agarose gel using the JETsorb kit (Genomed) and subcloned into pBluescript II SK + (Stratagene) for sequencing. The sequence of this fragment revealed ORFs with homology to the CHL H subunit, interrupted by multiple introns. Exons and introns in this fragment were

assigned by comparison with a 445-bp EST fragment from an EST database (Asamizu et al. 1999; Accession No. AV394213) which overlapped with the 5′RACE fragment. Based on the EST fragment the beginning of the coding region as well as the potential start site of transcription could be localized. The combined sequence data could be arranged to form a full-length cDNA of the *CHLH* gene (5054 bp). The sequence of the 3′ end is identical to that deposited in GenBank by Willows and Beale (Accession No. AF069768). The genomic sequence of the *Chlamydomonas CHLH* gene was amplified in fragments from genomic DNA (strain CC-124) by PCR with a thermostable proofreading DNA polymerase (PfuTurbo DNA polymerase, Stratagene) using the reaction conditions: 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 2 min at 65°C, and 3 min at 72°C, with a final extension step for 10 min at 72°C. The primer pairs used to obtain six overlapping fragments constituting of the entire *CHLH* gene were designed on the basis of the *CHLH* cDNA sequence. The upstream (A) and downstream (B) primers were: 1A ($^{-23}$AACTAGGGAGGGGCA ACA^{-6}) and 1B ($^{+465}$ATGAAGATGTTGGCAGAGGCC $^{+485}$); 2A ($^{+454}$GCCAACATCT TCATCGGCTCGC^{+475}) and 2B ($^{+1135}$TGTTGACGGGGTCGGAGAA^{+1153}); 3A ($^{+1133}$TCCG ACCCCGTCAACAAGT^{+1151}) and 3B ($^{+1893}$GGCTGCACGCC GATGAAGACG^{+1913}); 4A ($^{+1893}$CGTCTTCATCGGCGTGCA GCC^{+1913}) and 4B ($^{+3039}$GC GGCCTGAGTGCCAATC $^{+3056}$); 5A ($^{+3020}$ACGGACGCCCTG GACCCTCAGT^{+3037}) and 5B ($^{+4226}$CAACCACAACCCGCCAACT^{+4244}); and 6A ($^{+4170}$CGT GGAGGACAAGATTG^{+4186}) and 6B ($^{+4951}$TCCGAGGGA GCCGTGAG^{+4967}). The numbers refer to the sequence of the *CHLH* cDNA with +1 representing the start of the ORF. Fragments generated with these primers were subcloned into the pGEM-T vector and sequenced. In this manner, the sequence of the entire *CHLH* gene was obtained.

The genomic sequence was used to define the intron/exon structure and, later, for the identification of the chl1 and brs-1 mutations.

Screening of libraries by plaque hybridization, isolation of positive clones, cloning and subcloning procedures were performed according to standard protocols (Sambrook et al. 1989). Sequencing was carried out by the dideoxynucleotide chain-termination method (Sanger et al. 1977) using the ALF DNA analyzing system (Pharmacia Biotech), and the sequences presented were deposited in the EMBL database under the Accession Nos. AJ307054 (*CHLH* cDNA) and AJ307055 (*CHLH* gene).

The analysis of DNA sequences and the analysis of putative ORFs and restriction sites were performed with the programs BCM Search Launcher and ExPASy Tools.

Transformation

Nuclear transformation of the *Chlamydomonas* mutants was done using the method described by Kindle (1990). The light-sensitive mutants chl1 and brs-1 were transformed with a genomic DNA clone (approximately 20 kb long) which includes the entire *CHLH* gene. Transformants were either selected by their ability to grow in the light or the genomic DNA was co-transformed with a selection plasmid containing the paromomycin resistance gene *aphVIII* (kindly provided by Dr. I. Sizova and Dr. P. Hegemann, University of Regensburg, Germany). Paromomycin-resistant transformants were selected in the dark on plates of TAP medium containing paromomycin sulfate (Sigma) at 12 μg/ml. These transformants were screened for colonies that exhibited a green colour, indicating successful complementation.

Statistical evaluation

The biochemical analyses of enzyme activities and determinations of pigments were performed at least in triplicate on each independent sample. The results from different measurements were combined.

Results

Genetic analysis of the mutants

The two mutants brs-1 and chl1, which are apparently defective in chlorophyll synthesis, can only be grown in the dark or at very low fluence rates (< 125 nmol per m^2/s) (Stolbova 1971; Wang et al. 1974). At moderate or high fluence rates the cells were killed within 24 h. The mutants exhibit a yellow/orange color, their phenotypes in the dark and in light are documented in Fig. 1.

Prior to molecular characterization of these phenotypically similar mutants, we tested whether they were affected in the same locus. To assay for their ability to complement each other we generated gametes from haploid mutant strains of opposite mating types that carried additional selective markers (*arg7.8* in the chl1

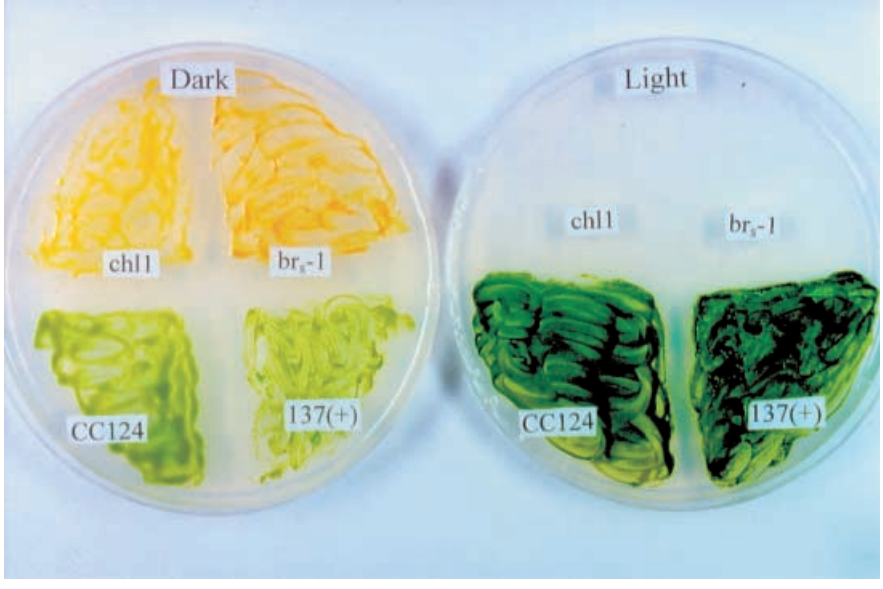


Fig. 1 Growth phenotypes and pigmentation of the mutant strains brs-1 and chl1 after incubation in the dark and in the light. Strains were incubated on TAP-containing plates for 4 days. The photosensitivity of the mutants prevents growth in the light

mutant and *arg2* in the brs-1 mutant). Diploid vegetative cells were subsequently selected for their ability to grow in the absence of arginine (Ebersold 1967). All 50 diploid clones tested exhibited the mutant phenotype and did not produce chlorophyll (data not shown). This lack of complementation suggests that the mutations are allelic. In the recombination test, zygotes were generated from a cross between the chl1 and brs-1 mutants to confirm and extend this result. After zygote germination, the spores released were assayed for their phenotype. Among more than 10,000 clones assayed none exhibited a green color, indicating that the defect was not repaired by recombination and that the mutations must be closely linked.

Biochemical characterization of mutants

To elucidate the physiological and genetic lesions in these mutants their intracellular levels of chlorophyll, heme, and of three intermediates of chlorophyll biosynthesis were compared with those in wild-type cells. Only residual amounts of chlorophyll (< 1% of the wild-type content) were found in the mutants. Levels of the chlorophyll precursors MgPROTO and MgPROTOMe were drastically reduced (Table 1). However, both mutants showed a huge increase in the steady-state levels of PROTO. This accumulation of PROTO is the reason for the yellow/orange pigmentation observed (Fig. 1). The heme concentration in these mutants was elevated by about 5-fold, relative to that in wild-type cells grown in the dark. These results suggested an enzymatic impairment of chlorophyll biosynthesis in these mutants and preferential direction of tetrapyrrolic metabolites into heme synthesis.

We next assayed the activity of Mg-chelatase and MgPROTO methyl transferase, the first two enzymes in the chlorophyll-specific branch of the pathway (Table 2). In mutant chl1, the activity of Mg-chelatase was reduced to about one-third of the wild type level, while the methyl transferase activities were similar in mutant and control. The mutant also exhibited an unaltered capacity to synthesize 5-amino levulinic acid, indicating that the initial and rate-limiting steps of tetrapyrrole biosynthesis were not affected. The combined results from the porphyrin measurements and the enzyme assays suggested that both mutants are perturbed in the first step of the chlorophyll biosynthetic branch.

Characterization of the mutants by Western analysis

The reduction in Mg-chelatase activity observed in the mutant chl1 could most easily be explained by an impairment in the synthesis of this enzyme or by a defect in the assembly of the active enzyme complex. Mg-chelatase in photosynthetic bacteria and higher plants is composed of three non-identical subunits termed CHL D, H and I for plants and BchD, H and I for bacteria (Gibson et al. 1995; Walker and Willows 1997). The mutant chl1 was assayed for the presence of the three subunits using antibodies raised against the corresponding polypeptides from tobacco. Antibodies directed against the I subunit, which also crossreact with the D subunit, detected the corresponding proteins in wild-type *C. reinhardtii* and in the chl1 mutant extracts (Fig. 2). The antibody raised against the H subunit recognized a protein of about 154 kDa in wild-type *C. reinhardtii* cells. No specific immune reaction was detectable in either of the mutant strains (Fig. 2). These results suggested that the CHL H subunit is either missing or present at non-detectable levels in the mutants chl1 and brs-1.

Cloning and sequence analysis of the gene for the H subunit

Combining sequence data from cDNA clones, 5′RACE experiments and an EST library (Asamizu et al. 1999),

Table 1 Levels of tetrapyrrole pools in wild-type and mutants

Strains and assay conditions	Levels of pigments (nmol/10^9 cells)[a]				
	PROTO	MgPROTO	MgPROTOMe	Heme	Chlorophyll
CC-124 (light)	0.31	0.17	0.14	23	3800
CC-124 (dark)	0.17	0.09	0.09	50	2400
chl1 (dark)	30	n.d.	n.d.	270	15
brs-1 (dark)	50	n.d.	n.d.	210	20

[a]n.d., not detectable

Table 2 Activity of various enzymes involved in chlorophyll synthesis in the wild type and mutants

Strains and conditions tested	Enzymes assayed		Relative levels of 5-amino levulinic acid
	Mg-chelatase (pkat/g protein)	Mg-proto-methyl transferase (μkat/g protein)	
CC-124 (light)	144	3.5	100
CC-124 (dark)	90	2.7	56
chl1 (dark)	27	2.7	63

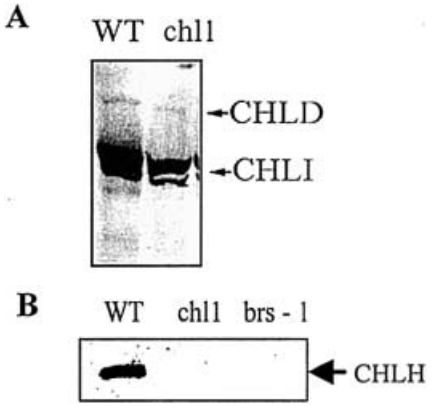


Fig. 2A, B Western analysis of whole cell extracts from the wild type (WT) and the mutants chl1 and brs-1. **A** Aliquots (10 μg) of total protein from wild type (WT) and the chl1 mutant, grown in the dark, were fractionated by PAGE and analyzed with a polyclonal antiserum raised in mouse against the recombinant CHL I protein of Mg-chelatase from *Nicotiana tabacum*. This antibody also crossreacts with the D subunit of Mg-chelatase. **B** Cells of the wild type and both mutant strains were grown in the dark for 3 days and then transferred into the light for 2 h. Total protein extracts were analyzed by the Western blot technique using a polyclonal antiserum raised in rabbit against the recombinant CHL H protein of Mg-chelatase from *N. tabacum*

we obtained the complete cDNA for *CHLH*, which comprises 5050 bp. This cDNA had a short polyA tail, and nearby is the polyadenylation signal TGTAA, which is typical for *C. reinhardtii* protein-encoding genes. This cDNA contains an ORF that encodes a protein of 1399 amino acids. At the N-terminal end a potential chloroplast targeting sequence of 36 amino acids was predicted by the chloro P program (Emanuelson et al. 1999). The sequence surrounding the postulated ATG start codon (AGAAATGCAG) closely resembles the *Chlamydomonas* consensus sequence which is (A/C)A(A/C)(A/C)ATG(G/C) C(G/C) (Silflow 1998). The structure of the genomic DNA was elucidated using PCR techniques. A schematic diagram of the *CHLH* gene of *C. reinhardtii*, with 11 introns and 12 exons, is presented in Fig. 3. It exhibits a rather short 5′UTR of 25 bp and a rather long 3′UTR of 829 bp. The GC content of the coding region is 60.4%, typical for *C. reinhardtii* genes.

Fig. 3 Structure of the *CHLH* gene. The *filled boxes* represent the exons, *open boxes* the introns. The 5′UTR and 3′UTR are shown as *narrow open boxes*. The start of the protein coding sequence (ATG) as well the translational stop signal (TAA) and the polyadenylation signal typical for *C. reinhardtii* (TGTAA) are indicated. The positions of the +1 frameshift mutations in the two mutants chl1 and brs-1, as well as the bases inserted, are also indicated

The H subunit is encoded by a single-copy gene

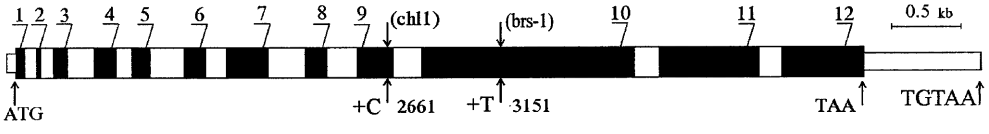
Southern analysis was employed to determine how many copies of the gene encoding the H subunit of Mg-chelatase are present in the *C. reinhardtii* genome. Hybridization of genomic DNA, digested individually with four different restriction enzymes that either do not cut within the gene or only at its very ends revealed one unique fragment in each case (data not shown). This result argues that *CHLH* is a single-copy gene.

Complementation of the mutant phenotypes

The mutations brs-1 and chl1 have been shown to affect the synthesis of the H subunit of Mg-chelatase. The mutations could lie within the *CHLH* sequence or affect a gene that controls the expression of *CHLH*. To distinguish between these possibilities, we performed complementation tests. For the complementation analyses genomic DNA containing the complete *CHLH* gene was introduced by transformation into both mutants. We first screened for colonies in which chlorophyll synthesis was restored and which could grow in the light. In a second approach we used cotransformation of the genomic DNA together with the *aphVIII* gene and selected for paromomycin-resistant clones in the dark. Since spontaneous revertants of the mutants were never detected, all green colonies that turned up were assumed to be the result of successful complementation. From both mutants transformants were recovered that had the ability to grow in the light. Their chlorophyll content corresponded to that of the wild-type strain (data not shown). In the cotransformation experiment 2% of the paromomycin-resistant transformants recovered in the dark were green in color and contained normal levels of chlorophyll. We conclude that the genomic clone complements both mutants; providing evidence that in mutants chl1 and brs-1 the *CHLH* gene is affected.

Identification of the alteration in *CHLH* in the mutants brs-1 and chl1

A PCR-based strategy was chosen for the identification of the mutational defects in the *CHLH* genes of both mutants. Six pairs of primers were designed from regions of the cDNA sequence that are unlikely to represent exon/intron borders. Amplified fragments ranged in size from 0.7 kb to 1.6 kb and covered the complete coding region of *CHLH*. Comparison of the genomic sequence with that of the cDNA allowed the identification of 11 introns (Fig. 3). The absence of an intron within the 3′UTR was also confirmed by PCR.



Analysis of the *CHLH* sequence from the mutant chl1 revealed a 1-bp insertion (C) at position 2661 within exon 9 (Fig. 3). This frameshift mutation leads to a stop codon after 22 triplets. The mutant would code for a truncated protein of approximately 52 kDa. The absence of a detectable polypeptide of this size on Western blots probed with the H-subunit-specific antibodies (Fig. 2) may be explained by the instability of the truncated protein.

Since no recombinants were observed among the progeny of crosses between the chl1 and brs-1 mutants (see above) we initially speculated that the two mutations must be closely linked. Partial sequencing of the genomic DNA of the brs-1 mutant indeed revealed a mutation in exon 10 (position 3151), at a site 490 bp downstream from the chl1 mutation. Again a frameshift mutation, resulting from the insertion of one nucleotide (T) was discovered (Fig. 3). The altered reading frame in this mutant ends with a stop codon after 164 triplets.

Besides the two frameshift mutations that prevent synthesis of the complete full-length H subunit, we discovered seven polymorphisms within exons 10, 11 and 12 of *CHLH* when cDNA and genomic DNA sequences were compared (data not shown). In five cases, these polymorphisms do not alter the amino acid sequence of the predicted gene product. However, in two cases, different amino acids appear to be present in the H subunit proteins. Thus, a change from T to C at position 1050 of the cDNA sequence leads to the incorporation of proline instead of leucine and a change from G to C at position 1226 results in a change from serine to threonine. Since the strain from which the cDNA library (137c *mt*+) was derived and the parental strain of chl1 (CC-124) do not exhibit a lack of chlorophyll or a light-sensitive phenotype we conclude that these alterations in the amino acid sequence do not crucially affect the function of the H subunit.

Regulation of Mg-chelatase expression by light

The level of *CHLH* mRNA was very low in dark-grown wild-type cells but distinctly higher when cells were grown under continuous illumination (Fig. 4). *CHLH* mRNA accumulation, assayed in wild-type cells during a shift from dark to light, begins within the first hour of illumination, has increased by 10-fold after 3 h and remains at elevated levels thereafter (Fig. 4).

Northern analysis revealed that *CHLH* mRNA was present in both mutant strains (Fig. 4). Accumulation of *CHLH* mRNA in the light was also observed in the mutants. The size of the mRNA species appeared to be unaltered in comparison to that in the wild type RNA. This result indicates that the frameshift mutations and subsequent stops in translation appear to have no pronounced effect on the expression of *CHLH* at the RNA level, nor on the stability of the mRNA.

We also assayed the response of *CHLH* to light at the protein level. A nine-fold increase in the amount of the H subunit was evident after 2 h of exposure to light

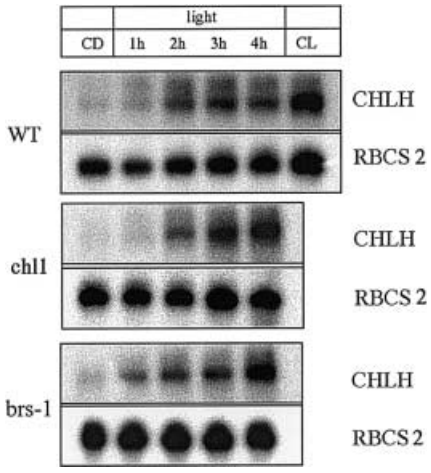


Fig. 4 Expression of *CHLH* at the RNA level. Aliquots (10 μg) of total RNA from wild type (WT) and the chl1 and brs-1 mutants were assayed by Northern analysis using the 1.4-kb and 3.1-kb CHLH cDNA fragments as probes. Transcript levels were determined from cells grown either in continuous light (CL), or first continuous darkness (CD) and then transferred to light for 1, 2, 3 and 4 h. As a loading control, the membranes were hybridized with an *RBCS2* probe

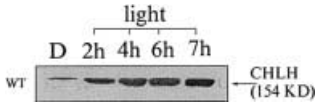


Fig. 5 Expression of *CHLH* at the protein level. Aliquots (20 μg) of total protein isolated from wild-type *Chlamydomonas* cells grown in the dark (D) and after transfer into the light for 2, 4, 6 and 7 h were assayed by Western analysis using antisera raised against the Mg-chelatase H subunit from *N. tabacum*

(Fig. 5). Mg-chelatase activity also increased by 60% in a cell culture grown in the light in comparison to the activity of a dark-grown culture (Table 2). In parallel, a significantly enhanced rate of synthesis of chlorophyll and its precursors has to be postulated in the light, since the chlorophyll content of light-grown cells is about 35% higher than that of cells grown in the dark (Table 1). A higher rate of chlorophyll biosynthesis in light-grown cells is certainly required because they grow faster by a factor of about 1.4 in comparison to cells that grow heterotrophically in the dark (data not shown).

Discussion

At the branchpoint of tetrapyrrole biosynthesis towards chlorophyll and heme, Mg-chelatase catalyzes the insertion of Mg^{2+} into the tetrapyrrole ring and it is likely that a finely tuned mechanism controls the relative amounts of PROTO assigned to Mg-chelatase and ferrochelatase. Here we report on the molecular and genetic characterization of two allelic mutants (brs-1 and chl1) that are defective in the H subunit of Mg-chelatase. In both mutants the chlorophyll levels were below 1% of wild-type levels, and MgPROTO, as well

as MgPROTOMe, was undetectable. In contrast, the concentration of PROTO in the mutants was elevated by a factor of more than 100 (Table 1). Therefore, continuous growth of the mutants in the light is not expected, since the accumulation of PROTO is extremely deleterious to light-grown *Chlamydomonas* cells, leading to photodynamic damage and cell death (Fig. 1). In contrast to the chlorophyll deficiency, heme levels were higher in the mutants than in wild type, reflecting a redirection of the excess PROTO that would normally be assigned to chlorophyll synthesis towards protoheme biosynthesis.

It was demonstrated in previous work that the PROTO-accumulating *Chlamydomonas* mutant brs-1, transgenic tobacco plants with Mg-chelatase deficiency and the *Arabidopsis* mutant *gun5*, which has a mutation in the *CHLH* gene, showed modified expression of a subset of nucleus-encoded genes (Kropat et al. 1997; Papenbrock et al. 2000a; Mochizuki et al. 2001). The newly identified mutant locus in the *Chlamydomonas* mutant brs-1 codes for CHLH. The genetic lesion in brs-1 is due to a +1 frameshift mutation in exon 10 of *CHLH*, and results in a defect in the light induction of the chaperone genes *HSP70A* and *HSP70B* (von Gromoff et al. 1989; Kropat et al. 1997, 1999). Feeding of MgPROTO to this mutant and to wild-type cells in the dark was shown to substitute for the light signal in activating the *HSP70* genes (Kropat et al. 1997). These results implicated the chlorophyll intermediate MgPROTO, which is not detectable in the brs-1 strain (Table 1) as a plastid molecule involved in the regulation of a specific set of nuclear genes. We suggested in this context a role for the porphyrin-binding H subunit of Mg-chelatase in making MgPROTO accessible to downstream signaling components in cytoplasm/nucleus (Kropat et al. 2000). The brs-1 mutant was also shown to be affected in the light regulation of an *LHC* gene (Johanningmeier and Howell 1984; Johanningmeier 1988).

A regulatory role for the active Mg-chelatase complex or single subunits thereof was deduced from an analysis of transgenic tobacco plants expressing antisense RNA for *CHLH*. In the transformants, Mg-chelatase activity was reduced to various extents, leading to lower steady-state levels of MgPROTO, MgPROTOMe, and chlorophyll, Surprisingly, however, they did not accumulate the Mg-chelatase substrate PROTO (Papenbrock et al. 2000a, 2000b). This lack of PROTO accumulation was attributable to reduced levels of the enzyme activities that catalyze the early steps in tetrapyrrole synthesis. As shown by mRNA analyses, the drop in the concentration of the H subunit resulted in reduced expression of the nuclear genes encoding glutamyl-tRNA, 5-amino levulinic acid dehydratase and LHCP (Papenbrock et al. 2000a). This contrasts with the situation in *Chlamydomonas* where we observed an accumulation of PROTO in the Mg-chelatase mutants but no reduction in 5-amino levulinic acid-synthezising capacity (Table 2).

These differences between plants and algae must reflect differences in the mechanism that regulates tetrapyrrole biosynthesis. Accumulation of PROTO as a result of Mg-chelatase deficiency indicates that the green alga *Chlamydomonas* did not evolve a feedback control system similar to that of angiosperms that prevents the accumulation of PROTO. We speculate that the lack of such a control system may be a consequence of the ability of these algal cells to synthesize chlorophyll in the dark. In angiosperms, dark incubation interrupts the light-dependent reduction of protochlorophyllide to chlorophyllide by protochlorophyllide oxidoreductase. This results in an accumulation of protochlorophyllide in dark-grown plants, which in turn may cause inhibition of the synthesis of early non-photosensitizing metabolites by a feedback mechanism (Dörnemann et al. 1989). In *Chlamydomonas,* chlorophyll synthesis may continue in the dark due to the presence of an enzymatic machinery that converts protochlorophyllide to chlorophyllide in the dark. The evolution of regulatory mechanisms that prevent an accumulation of intermediates like PROTO that are highly toxic upon irradiation may provide a selective advantage for plants that in a diurnal manner periodically turn off chlorophyll synthesis.

An involvement of the H subunit in plastid-to-nucleus signaling has been strongly suggested based on an analysis of *Arabidopsis* mutants with point mutations in the *CHLH* gene (*gun5* and *cch*). In these mutants, the expression of a subset of nuclear genes that encode chloroplast-localized proteins was found to be uncoupled from the physiological state of the plastid (Susek et al. 1993). Surprisingly, in the *gun5* mutant, the chlorophyll content was reduced by only 30% relative to wild-type levels and the MgPROTO pools in this mutant were not significantly reduced, while the PROTO concentration was somewhat less than in wild-type (Mochizuki et al. 2001). These minor influences of the *gun5* mutation on the synthesis of chlorophyll and its clear-cut effect on nuclear gene expression suggested a special role for the H subunit in signaling from chloroplast to nucleus. This special role of the H subunit was corroborated by the observation that mutants with defects in a different Mg-chelatase subunit (CHLI) were unaffected in the regulation of these nuclear genes (Mochizuki et al. 2001).

Although in all of the cases presented above the nature of the regulatory function of Mg-chelatase, and in particular that of subunit H, are still open, there is now increasing evidence that at least one regulatory circuit that links nuclear gene expression to the physiological state of the chloroplast involves the enzyme and/or its substrate/product that make up the first step specific for chlorophyll synthesis (Beck 2001).

During growth in the light, *Chlamydomonas* cells have a higher demand for chlorophyll than in the dark. Light treatment of dark-adapted cells indeed causes a pronounced increase in *CHLH* mRNA within 2 h (Fig. 4). This increase in mRNA is followed by an

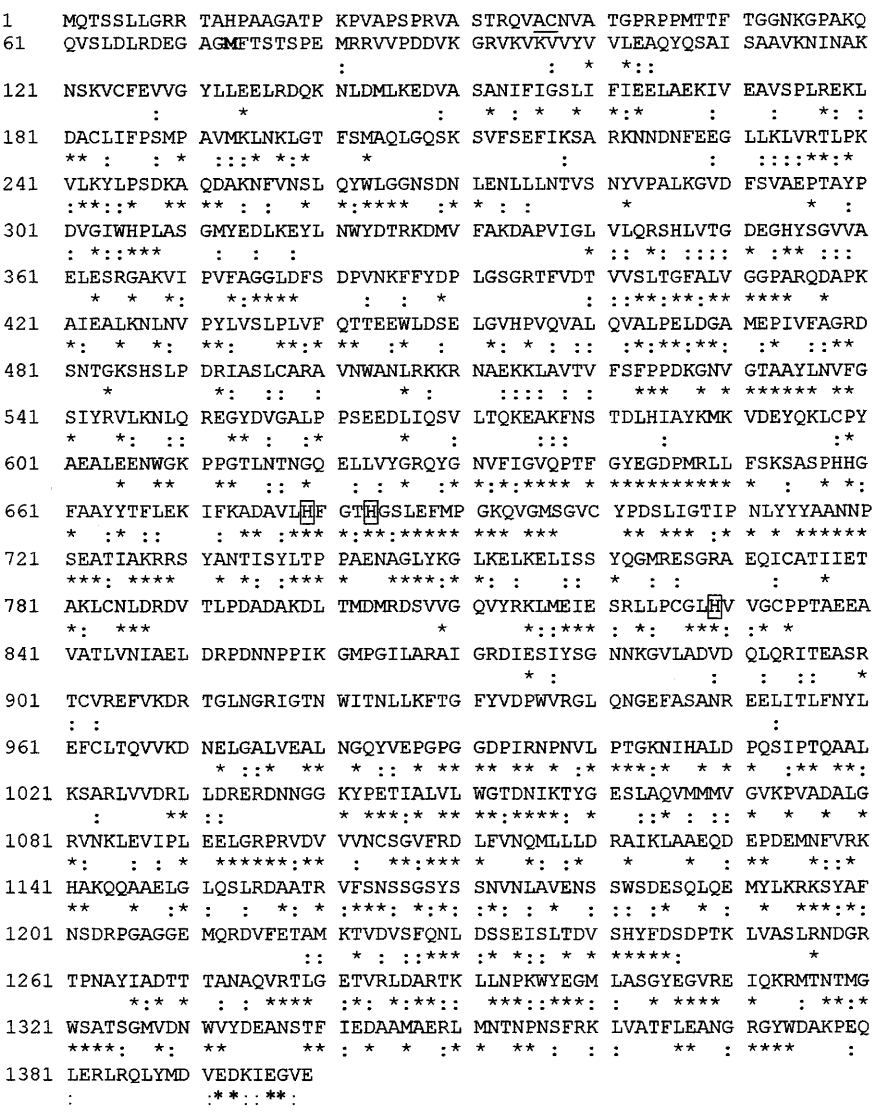


Fig. 6 Amino acid sequence of the *C. reinhardtii* Mg-chelatase H subunit, deduced from the gene sequence. Those amino acids that are conserved in Mg-chelatase H subunits from eight species are indicated. The amino acid sequences of the H subunits compared were those from *C. reinhardtii*, *Synechocystis* PCC6803 (*chlH*; Accession No. S75000), *Rhodobacter sphaeroides* (*bchH*; CAM38723), *Antirrhinum majus* (*Olive*; S37310), *Arabidopsis thaliana* (*chlH*; S71288), *Nicotiana tabacum* (*ChlH*; TO1789), *Hordeum vulgare* (*Xantha-f*; S64721), and *Glycine max* (*chlH*; TO7126). Sequence identities are indicated by the *asterisks*, conserved residues by *colons*. The methionine residue that is the first amino acid of the *Synechocystis* H subunit is indicated in *bold*. The predicted site of the proteolytic cleavage that removes the transit peptide lies *between* the *underlined* amino acids. The three conserved histidine residues mentioned in the text are *boxed*. The diagram was created with the aid of CLUSTALW multiple alignment program

accumulation of subunit H within 4 h after transfer from dark to light (Fig. 5). However, Mg-chelatase activity in light-grown cultures was only 60% higher than that in cells grown in the dark (Table 2). This discrepancy between the modest increase in enzyme activity and the marked accumulation of H subunit protein suggests either a modified stoichiometry of the H subunit in the active Mg-chelatase complex of light-grown cultures in comparison to the enzyme complex of dark-incubated cells or the availability of free H subunits that may engage in the regulatory functions discussed above. It should, however, be stressed that more work is needed to elucidate the role of the H subunit in regulation.

Comparison of the *Chlamydomonas* H subunit with seven H subunits of Mg-chelatases from bacteria and higher plants revealed extensive homologies (Fig. 6). Since the function of the H subunit is thought to involve both the binding of PROTO (Gibson et al. 1995; Willows et al. 1996) and the binding of Mg^{2+} used for chelation, some of the conserved regions must be involved in porphyrin and Mg^{2+} binding and possibly in catalysis (Walker and Willows 1997). It has been suggested that the biological insertion of Mg^{2+} into PROTO requires the formation of a nitrogenous base-Mg^{2+}-porphyrin complex. The most likely candidate for the nitrogenous base is a histidine residue. Candidates for partners in this reaction are three conserved histidine residues (H^{679}, H^{683}, and H^{829}; Fig. 6). The degree of sequence identity between the *C. reinhardtii* protein and the others ranges from 37% (for the *Rhodobacter capsulatus* protein) to 65% for the homologs from *Antirrhinum majus*, *Arabidopsis thaliana*, and *Glycine max*. The overall sequence of the *C. reinhardtii* subunit H is thus closely related to those of its homologs from higher plants.

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References

Asamizu E, Nakamura Y, Sato S, Fukuzawa H, Tabata S (1999) A large scale structural analysis of cDNAs in a unicellular green alga, *Chlamydomonas reinhardtii*. I. Generation of 3433 non-redundant expressed sequence tags. DNA Res 6:369–373

Beale SI, Weinstein JD (1990) Tetrapyrrole metabolism in photosynthetic organisms. In: Dailey HA (ed) Biosynthesis of heme and chlorophylls. McGraw-Hill, New York, pp 287–391

Beck CF (2001) Signaling pathways in chloroplast-to-nucleus communication. Protist 152:175–182

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chekunova EM, Kvitko KV (1986) Genetic study of *Chlamydomonas* mutants which accumulate protoporphyrin IX (in Russian). Issledovaniya po Genetike 10:104–112

Crawford MS, Wang W-Y, Jensen KG (1982) Identification of the primary lesion in a protoporphyrin-accumulating mutant of *Chlamydomonas reinhardtii*. Mol Gen Genet 188:1–6

Dörnemann D, Kotzabasis K, Richter P, Breu V, Senger H (1989) The regulation of chlorophyll biosynthesis by the action of protochlorophyllide on ^{glu}t-RNA-ligase. Bot Acta 102:112–115

Ebersold WT (1967) *Chlamydomonas reinhardtii*: heterozygous diploid strains. Science 157:447–449

Emanuelsson O, Nielsen H, von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci 978–984

Gibson LCD, Hunter CN (1994) The bacteriochlorophyll biosynthesis gene, *bchM* of *Rhodobacter shaeroides* encodes S-adenosyl-L-methionine:Mg-protoporphyrin IX methyltransferase. FEBS Lett 352:127–130

Gibson LCD, Willows RD, Kannangara CG, von Wettstein D, Hunter CN (1995) Magnesium-protoporphyrin chelatase of *Rhodobacter sphaeroides*: reconstitution of activity by combining the products of the *bchH*, *-I* and *-D* genes expressed in *Escherichia coli*. Proc Natl Acad Sci USA 92:1941–1944

Gibson LCD, Marrison JL, Leech RM, Jensen PE, Bassham DC, Gibson M, Hunter CN (1996) A putative Mg-chelatase subunit from *Arabidopsis thaliana* cv. C24. Plant Physiol 111:61–71

Goldschmidt-Clermont M (1986) The two genes for the small subunit of RuBP carboxylase/oxygenase are closely linked in *Chlamydomonas reinhardtii*. Plant Mol Biol 6:13–21

Granick S (1948a) Protoporphyrin IX as a precursor of chlorophyll. J Biol Chem 172:717–727

Granick S (1948b) Mg-protoporphyrin IX as a precursor of chlorophyll. J Biol Chem 175:333–342

Harris EH (1989) The *Chlamydomonas* source book: a comprehensive guide to biology and laboratory use. Academic Press, San Diego

Höfgen R, Axelsen KB, Kannangara CG, Schüttke I, Pohlenz HD, Willmitzer L, Grimm B, von Wettstein D (1994) A visible marker for antisense mRNA expression in plants: inhibition of chlorophyll synthesis with a glutamate 1-semialdehyde aminotransferase antisense gene. Proc Natl Acad Sci USA 91:1726–1730

Jensen PE, Willows RD, Petersen BL, Vothknecht UC, Stummann BM, Kannangara CG, von Wettstein D, Henningsen KW (1996) Structural genes for Mg-chelatase subunits in barley: *Xantha-f, -g* and *–h*. Mol Gen Genet 250:383–394

Johanningmeier U (1988) Possible control of transcript levels by chlorophyll precursors in *Chlamydomonas*. Eur J Biochem 177:417–424

Johanningmeier U, Howell SH (1984) Regulation of light-harvesting chlorophyll-binding protein mRNA accumulation in *Chlamydomonas reinhardtii*. J Biol Chem 259:13541–13549

Kindle KL (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Proc Nat Acad Sci USA 87:1228–1232

Kropat J, Oster U, Rüdiger W, Beck CF (1997) Chlorophyll precursors are signals of chloroplast origin involved in light induction of nuclear heat-shock genes. Proc Natl Sci USA 94:14168–14172

Kropat J, Oster U, Pöpperl G, Rüdiger W, Beck CF (1999) Identification of Mg-protoporphyrin IX as a chloroplast signal that mediates the expression of nuclear genes. In: Wagner E, Normann J, Greppin H, Hackstein JHP, Herrmann RG, Kowallik KV, Schenk HEA, Seckbach J (eds) From symbiosis to eukaryotism – Endocytobiology VII. Geneva University Press, Geneva, pp 341–348

Kropat J, Oster U, Rüdiger W, Beck CF (2000) Chloroplast signalling in the light induction of nuclear HSP70 genes requires the accumulation of chlorophyll precursors and their accessibility to cytoplasm/nucleus. Plant J 24:523–531

Kruse E, Mock HP, Grimm B (1995) Reduction of coproporphyrinogen oxidase level by antisense RNA synthesis leads to deregulated gene expression of plastid proteins and effects of oxidative defense system. EMBO J 14:3712–3720

Lee HJ, Ball MD, Parham R, Rebeiz CA (1992) Chloroplast biogenesis. 65. Enzymic conversion of protoporphyrin IX to Mg-protoporphyrin IX in a subplastidic membrane fraction of cucumber etiochloroplasts. Plant Physiol 99:1134–1140

Miller GW, Denney A, Wood JK, Welkie GW (1979) Light-induced delta-aminolevulinic acid in dark-grown barley seedlings. Plant Cell Physiol 20:131–143

Mochizuki N, Brusslan JA, Larkin R, Nagatani A, Chory J (2001) Arabidopsis *genomes uncoupled 5* (*gun5*) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction. Proc Natl Sci USA 98:2053–2058

Nakayama M, Masuda T, Bando T, Yamagata H, Ohta H, Takamiya K-I (1998) Cloning and expression of the soybean *chlH* gene encoding a subunit of Mg-chelatase and localization of the Mg^{2+} concentration-dependent ChlH protein within the chloroplast. Plant Cell Physiol 39:275–284

Papenbrock J, Mock HP, Kruse E, Grimm B (1999) Expression studies in tetrapyrrole biosynthesis: inverse maxima of magnesium chelatase and ferrochelatase activity during cyclic photoperiods. Planta 208:264–273

Papenbrock J, Mock H-P, Tanaka R, Kruse E, Grimm B (2000a) Role of magnesium chelatase activity in the early steps of the tetrapyrrole biosynthetic pathway. Plant Physiol 122:1161–1169

Papenbrock J, Pfündel E, Mock H-P, Grimm B (2000b) Decreased and increased expression of the subunit CHLI diminishes Mg chelatase activity and reduces chlorophyll synthesis in transgenic tobacco plants. Plant J 22:155–164

Pontoppidan B, Kannangara CG (1994) Purification and partial characterization of barley glutamyl-tRNA^{Glu} reductase, the enzyme that directs glutamate to chlorophyll biosynthesis. Eur J Biochem 225:529–537

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim Biophys Acta 975:384–394

Rüdiger W (1997) Chlorophyll metabolism: from outer space down to the molecular level. Phytochemistry 46:1151–1167

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2nd edn). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sanger F, Nicklen S, Coulson, AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schneegurt MA, Beale SI (1986) Biosynthesis of protoheme and heme *a* from glutamate in maize. Plant Physiol 81:965–971

Silflow CD (1998) Organization of the nuclear genome. In: Rochaix J-D, Goldschmidt-Clermont M, Merchant S (eds) Molecular biology of *Chlamydomonas*: chloroplasts and mitochondria. Kluwer Academic, Dordrecht, pp 25–40

Stolbova AV (1971) Genetic analysis of light-sensitive mutants of *Chlamydomonas reinhardtii*. I. Identification of the main

pigments and description of collection of pigment forms (in Russian). Genetika 7:90–94

Susek RE, Ausubel FM, Chory J (1993) Signal transduction mutants of Arabidopsis uncouple nuclear *CAB* and *RBCS* gene expression from chloroplast development. Cell 74:787–799

Suzuki JY, Bollivar DW, Bauer CE (1997) Genetic analysis of chlorophyll biosynthesis. Annu Rev Genet 31:61–89

Von Gromoff ED, Treier U, Beck, CF (1989) Three light-inducible heat shock genes of *Chlamydomonas reinhardtii*. Mol Cell Biol 9:3911–3918

Von Wettstein D, Gough S, Kannangara CG (1995) Chlorophyll biosynthesis. Plant Cell 7:1039–1057

Walker CJ, Willows RD (1997) Mechanism and regulation of Mg-chelatase. Biochem J 327:321–333

Wang WY, Wang WL, Boynton JE, Gillham NW (1974) Genetic control of chlorophyll biosynthesis in *Chlamydomonas*: analysis of mutants at two loci mediating the conversion of protoporphyrin IX to magnesium protoporphyrin. J Cell Biol 63:806–823

Wang WY, Boynton JE, Gillham NW (1975) Genetic control of chlorophyll biosynthesis in *Chlamydomonas*: analysis of a mutant affecting synthesis of δ-aminolevulinic acid. Cell 6:75–84

Weinstein JD, Beale SI (1983) Separate physiological roles and subcellular compartments for two tetrapyrrole biosynthetic pathways in *Euglena gracilis*. J Biol Chem 258:6799–6807

Willows RD, Gibson LCD, Kannangara CG, Hunter CN, von Wettstein D (1996) Three separate proteins constitute the magnesium chelatase of *Rhodobacter sphaeroides*. Eur J Biochem 235:438–443