Running head: Arabidopsis CHLI2 can substitute for CHLI1

To whom correspondence should be addressed:

**Hsou-min Li**
Institute of Molecular Biology, Academia Sinica, Nankang, Taipei 11529, Taiwan
Tel: +886-2-27899220
Fax: +886-2-27826085
E-mail: mbhmli@gate.sinica.edu.tw

Category: Bioenergetics and Photosynthesis
Associate Editor: Susanne von Caemmerer
Arabidopsis CHLI2 can substitute for CHLI1

Yi-Shiuan Huang¹,² and Hsou-min Li²,*

¹Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 114, Taiwan
²Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan
Footnotes

1. This work was supported by the National Science Council (grant no. NSC-97-2321-B-001-001 to HmL) and the Academia Sinica of Taiwan.

*Corresponding author; e-mail mbhml@gate.sinica.edu.tw; fax +886-2-27826085.
ABSTRACT

The I subunit of Mg-chelatase is encoded by two genes in Arabidopsis, CHLI1 and CHLI2. Conflicting results have been reported concerning the functions of the two proteins. We show here that the chli1/chli1 chli2/chli2 double-knockout mutant was albino. Comparison with the pale-green phenotype of a chli1/chli1 single-knockout mutant indicates that CHLI2 could support some chlorophyll biosynthesis in the complete absence of CHLI1. Real-time quantitative RT-PCR showed that CHLI2 was expressed at a much lower level than CHLI1. The chli1/chli1 chli2/chli2 double mutant could be fully rescued by expressing a transgene of CHLI2 driven by the CHLI1 promoter. These results suggest that differences between CHLI1 and CHLI2 lie mostly in their expression levels. Furthermore, both the chli1/chli1 and the chli2/chli2 single-knockout mutants had lower survival rates during de-etiolation than the wild type, suggesting that both genes are required for optimal growth during de-etiolation. In addition, we show that a semi-dominant chli1 mutant allele and the chli1/chli1 chli2/chli2 double mutant accumulated Lhcb1 transcripts when treated with the herbicide norflurazon, indicating that knocking out the CHLI activity causes the genome-uncoupled (gun) phenotype.
INTRODUCTION

Magnesium (Mg) chelatase catalyzes the first committed step toward chlorophyll synthesis in the tetrapyrrole biosynthesis pathway. The enzyme inserts Mg\(^{2+}\) into protoporphyrin IX (Proto IX) and produces Mg-protoporphyrin IX (Mg-proto). The enzyme is composed of three subunits: CHLH, CHLD and CHLI (corresponding to BchH, BchD and BchI in \textit{Rodobacter} and XAN-F, XAN-G, XAN-H in barley, respectively). Both in vitro and in vivo evidence has shown that all three subunits are essential for the Mg chelatase activity (Gibson et al., 1995; Willows et al., 1996; Kannangara et al., 1997). The H subunit binds Proto IX and may be the catalytic subunit for the metallation reaction. The D and I subunits form an activation complex in an ATP- and Mg\(^{2+}\)-dependent manner. In Arabidopsis, both the D and H subunits are encoded by single genes.

CHLI belongs to the AAA\(^{+}\)-(ATPases associated with various cellular activities) family of ATPases. It forms a ring-shaped homo-hexamer. Many semidominant and recessive alleles of \textit{chli} mutants have been isolated from various species (Nguyen, 1995; Kjemtrup et al., 1998; Hansson et al., 2002; Soldatova et al., 2005). The semidominant alleles are all mis-sense mutations that still produce full-length proteins. The mutant proteins can assemble with the wild-type proteins into the hexameric ring, hindering the ATPase activity and therefore resulting in the semidominant phenotype (Hansson et al., 2002).

CHLI isoforms in Arabidopsis are encoded by two genes: \textit{CHLI1} (At4g18480) and \textit{CHLI2} (At5g45930). CHLI1 seems to be the major functional form since chlorophyll levels in \textit{chli1}-null mutants are reduced to 10 to 17\% of the wild-type level (Rissler et al., 2002). Some reports have shown that \textit{CHLI1} and \textit{CHLI2} RNA are expressed at a similar level (Rissler et al., 2002; Apchelimov et al., 2007). Because CHLI2 protein was not detected even in isolated \textit{chli1} mutant plastids, it has been proposed that CHLI2 is subjected to rapid post-translational turnover and does not accumulate in vivo (Rissler et al., 2002). Alternatively, it has also been suggested that CHLI2 cannot assemble into the hexameric-ring structure due to changes in residues in its C terminus (Apchelimov et al., 2007). However, from a miniarray experiment examining all genes involved in
tetrapyrrole biosynthesis (Matsumoto et al., 2004) and also from the Genevestigator (https://www.genevestigator.ethz.ch/, Zimmermann et al., 2004) and MPSS (http://mpss.udel.edu/at/, Brenner et al., 2000) databases, the expression level of CHLI2 is shown to be much lower than that of CHLI1. It is possible, therefore, that CHLI2 function could not be observed due to its very low expression level. Indeed, it has been shown that recombinant CHLI2 has ATPase activity although with a lower $V_{\text{max}}$ and $K_{\text{mATP}}$ than CHLI1 (Kobayashi et al., 2008). A double mutant of a partially functional light-green allele of chli1, cs (or named ch42-2), with a chli2-knockout mutant, is albino. This result indicates that at least in the presence of a partially functional CHLI1, CHLI2 can contribute to some chlorophyll biosynthesis (Kobayashi et al., 2008).

The tetrapyrrole biosynthesis pathway also seems to be important for retrograde signaling from plastids to the nucleus. One frequently used method for studying the signaling pathway is to treat plants with the herbicide norflurazon (Nf), which inhibits carotenoid biosynthesis and causes photo-oxidative damage to plastids. This treatment results in repressed expression of nucleus-encoded photosynthetic genes like Lhcb1, in response to signals sent by the damaged plastids. Arabidopsis mutants that still express Lhcb1 in the presence of Nf have been identified and are named gun (genome uncoupled) mutants (Susek et al., 1993; Cottage et al., 2008). Noticeably, four of the original five gun mutants are defective in tetrapyrrole biosynthesis. The gun2 and gun3 mutants are defective in heme oxygenase and phytochromobilin synthase respectively. The gun5 mutant has a mis-sense mutation in CHLH, and GUN4 encodes a regulator of Mg chelatase (Mochizuki et al., 2001; Larkin et al., 2003). A knockout mutation in CHLD also results in the gun phenotype (Strand et al., 2003). Therefore it seems that perturbation of Mg chelatase activity would result in the gun phenotype. However, two alleles of the chli1 mutants, ch42 (ch42-1) and cs (ch42-2), do not accumulate Lhcb1 transcripts when treated with Nf and are therefore not gun mutants (Mochizuki et al., 2001). It has been proposed that the presence of CHLI2 in the chli1 mutants may be sufficient to allow functioning of the retrograde signaling pathway (Nott et al., 2006).

During our search for Arabidopsis mutants defective in protein import into chloroplasts, several pale-green or albino mutants were collected from various sources. One of the mutants from the Arabidopsis Biological Resource Center (ABRC), cs215,
although not defective in protein import (data not shown), showed an interesting semi-
dominant phenotype. Homozygous cs215 mutants were albino while heterozygous plants
were pale green. Positional cloning of the cs215 locus revealed that cs215 is a new allele
of chli1 mutants. To compare cs215 with other chli mutants, we obtained T-DNA- or Ds-
insertion alleles of chli1 and chli2 mutants and generated various double mutants. Our
results indicated that the low functionality of CHLI2 was mostly due to its low expression
level compared to that of CHLI1. We further tested the homozygous cs215 mutant and
the chli1/chli1 chli2/chli2 double mutant for the gun phenotype. When treated with Nf,
the chli1/chli1 chli2/chli2 double mutant accumulated Lhcb1 to a level similar to that
observed in a chld-knockout mutant, and the cs215 homozygous mutant accumulated an
even higher level of Lhcb1, similar to that observed in a chlh-knockout mutant,
suggesting that knocking out the CHLI activity also caused the gun phenotype.

RESULTS

Identification of the cs215 locus

Homozygous cs215 (cs215/cs215) mutants were albino while heterozygous (cs215+)/
plants were pale green (Fig. 1). To identify the cs215 locus, we crossed the cs215/+mutant with Columbia (Col) and Lansberg (Ler) wild-type plants because the cs215
mutant is in the ecotype Enkheim (Enk). We identified PCR-based polymorphic markers
that distinguish between the Enk and Col/Ler ecotypes (see Methods). Initial mapping
placed the cs215 locus between markers AG and SGCSNP43 on chromosome IV (Fig.
2A). Data from three recombinant plants delimited the cs215 locus to the region
encompassed by BACs F28J12 and F28A21. One of the genes in this region, At4g18480
encoding CHLI1, has been shown to have mutant alleles with semidominant pale-green
phenotypes (Nguyen, 1995; Kjemtrup et al., 1998; Hansson et al., 2002; Soldatova et al.,
2005). We therefore sequenced the region of At4g1848 from cs215 and found that cs215
indeed had a C to T mutation at nucleotide 584 that converted a threonine at residue 195
to an isoleucine (Fig. 2B). Pale green plants were heterozygous at this position.
Threonine 195 is located between the Walker A and Walker B motifs of the ATPase domain of CHLI and is only 10 residues downstream from the mutations in the barley Xan-h\textsuperscript{clo161} and maize Oyl-N1989 mutants (Fig. 2B), both of which are semi-dominant alleles of the chli mutants. When a cs215/+ plant was crossed with a chli1-knockout mutant (see below), the F\textsubscript{1} seedlings had the same phenotype as cs215/cs215 (cs215/chli1, Fig. 1A), further supporting that cs215 was allelic to chli1. We have also sequenced the CHLI2 gene from the Enk ecotype and the result indicated that CHLI2 from Enk has an identical deduced amino acid sequence to that of CHLI2 from Col (data not shown). Therefore it is unlikely that the severe phenotype of cs215 was caused by a synthetic effect with natural variations in the CHLI2 gene of the Enk ecotype.

**CHLI2 supports some chlorophyll biosynthesis in the absence of CHLI1**

To compare the phenotype of c215 with other chli1 mutants, a mutant line with a T-DNA insertion in the third exon of the CHLI1 gene (SAIL_230_D11, Fig. 3A) was obtained from ABRC. This mutant produced a truncated CHLI1 mRNA but no full-length transcripts or transcripts behind the T-DNA insertion site (Fig. 3B, lanes 4 to 6). It was yellow in appearance (Fig. 1A) and had less than 10% of the wild-type level of chlorophylls (Fig. 1B), similar to the levels reported for other chli1 null mutants (Rissler et al., 2002). These data suggested that SAIL_230_D11 is most likely also a chli1 null mutant. SAIL_230_D11 will be referred to as the chli1/chli1 mutant herein.

Comparison of chli1/chli1 with cs215/cs215 revealed that chli1/chli1 had more chlorophylls than cs215/cs215 (Fig. 1B). Arabidopsis has two genes encoding CHLI: CHLI1 and CHLI2. It is possible that in the chli1/chli1 mutant, CHLI2 can still support some chlorophyll biosynthesis. In the cs215/cs215 mutant, the presence of the cs215 mutant protein might prevent CHLI2 from functioning, resulting in the phenotype difference between cs215/cs215 and chli1/chli1. To verify this, we obtained two mutant lines, GT13937 (Kobayashi et al., 2008) and GT18178, with Ds transposon insertion in the CHLI2 gene (Fig. 3A). RT-PCR analyses indicated that neither mutant produced any full-length CHLI2 mRNA or any CHLI2 mRNA 3’ to the Ds insertion site (Fig. 3B, lanes 8 and 9 and inset). Both mutants were wild-type in appearance (Fig. 1A) and in
chlorophyll levels (Fig. 1B). GT13937 and GT1817 are referred to herein as chli2-I and chli2-2, respectively.

We crossed cs215/+ and CHLI1/chli1 with chli2-I/chli2-I and chli2-2/chli2-2 to generate various double mutants. Results from crosses to chli2-I/chli2-I and to chli2-2/chli2-2 were identical and data from chli2-I are presented unless specified. Double mutants of chli1/chli1 chli2/chli2 were albino (Fig. 1A) with no detectable chlorophylls (Fig. 1B). Compared with the chli1/chli1 single mutant, the albino phenotype of the double mutant indicated that CHLI2 protein must be stable enough to provide some Mg-chelatase activity even in the absence of CHLI1. Furthermore, double mutants of cs215/cs215 chli2/chli2 were indistinguishable from the cs215 single (cs215/cs215 CHLI2/CHLI2) mutant (Fig. 1A and 1B). It is therefore likely that the presence of the cs215 mutant protein had prevented CHLI2 from functioning. This may be because the expression level of CHLI1 is much higher than that of CHLI2, as shown previously by Matsumoto et al (2004). In the cs215 mutant, the high amount of cs215 mutant protein may out-compete the low amount of CHLI2 in assembly with CHLD. It is also possible that the expression levels of CHLI1 and CHLI2 were similar (Rissler et al., 2002; Apchelimov et al., 2007) and the presence of the cs215 mutant protein inhibited the expression of CHLI2 as previously suggested (Soldatova et al., 2005).

The expression level of CHLI2 is much lower than CHLI1 and is not affected by mutations in CHLI1

To clarify the role of CHLI2, we compared the expression levels of CHLI1 and CHLI2 using real-time quantitative RT-PCR. Amounts of CHLI1 and CHLI2 RNA were determined by comparing to standard curves generated from known quantities of CHLI1 and CHLI2 plasmid DNA. The results showed that, in 14-day-old seedlings, the ratio of CHLI1 to CHLI2 RNA was 5.86 ± 1.31 (n=5). This result agrees with the reported miniarray data (Matsumoto et al., 2004) and is also supported by data found in Genevestigator (expression value 9764 vs. 2121 in rosette leaves) and MPSS (928 vs. 132 transcripts per million in 21-day leaves) databases. The level of CHLI2 was not changed in the chli1/chli1 or cs215/cs215 mutants (Fig. 4, lower panel), indicating that mutations
in CHLI1 did not affect the expression of CHLI2. The level of CHLI1 was also not changed in the chli2/chli2 or cs215/cs215 mutants (Fig. 4, upper panel).

CHLI2 driven by CHLI1 promoter can rescue the chli1/chli1 chli2/chli2 double mutant

It has been suggested that CHLI2 is defective in hexameric-ring assembly due to changes in its C terminus (Apchelimov et al., 2007). However, it has also been shown that CHLI2 is an active ATPase (Kobayashi et al., 2008). To test the function of CHLI2 in vivo, we fused the CHLI2 gene behind a 2.1-kb CHLI1 promoter fragment and used this transgene (referred to herein as pCHLI1::CHLI2) to complement the chli1/chli1 chli2/chli2 double mutant. Transgenic plants with one copy of the pCHLI1::CHLI2 transgene were referred to as the pCHLI1::CHLI2 (chli1/chli1 chli2/chli2) plants. For fair comparison, these plants were compared to plants with only one endogenous copy of CHLI1 (genotype CHLI1/chli1 chli2/chli2 and referred to herein accordingly). Nonetheless, CHLI1/chli1 chli2/chli2 plants were similar to the wild type in both appearance (Fig. 1A) and chlorophyll levels (Fig. 1B).

The pCHLI1::CHLI2 (chli1/chli1 chli2/chli2) transgenic plants had phenotypes that ranged from fully green to slightly yellow in younger leaves (Fig. 5A, “Green” and “Yellow” respectively). The “Green” transgenic plants had a chlorophyll level similar to the CHLI1/chli1 chli2/chli2 plants (Fig. 5B). This result indicated that CHLI2 could substitute for CHLI1 if expressed at a sufficient level. The “Yellow” transgenic plants had chlorophyll and CHLI2-transcript levels about half of those in the “Green” transgenic plants (Fig. 5B and 5C). This result further demonstrated that the expression level of CHLI2 was correlated with the level of chlorophyll-biosynthesis activity observed.

The chli mutants have lower survival rates during de-etiolation

To investigate if CHLI2 contributes to plant fitness, we compared the chli mutants with the wild type in de-etiolation experiments. Seeds were imbibed at 4°C in the dark for 3 days, exposed to light at 24°C for one day and then moved to the dark to germinate at 24°C for 7 days. Etiolated seedlings were then transferred to a regular 16 h light/8 h dark
cycle and seedling survival rates were scored 6 days later. Seedlings that failed to expand their cotyledons, i.e. the two cotyledons had a combined length less than 0.25 cm, were scored as dead (see Fig. 6A for examples). As shown in Figure 6B, the chli1/chli1 mutant had a 14% survival rate and the two alleles of chli2/chli2 mutants had a 40% survival rate. Both rates were significantly lower than that of their corresponding wild type. This result suggested that both genes contributed to increase the seedling survival rate upon de-etiolation. We then analyzed the CHLI1 and CHLI2 RNA levels during de-etiolation, comparing with the CHLI1 and CHLI2 RNA levels in seedlings that were grown under a regular 16 h light/8 h dark cycle after cold stratification (Fig. 6C). The results indicated that the expression of both genes was induced by light upon de-etiolation. When the RNA levels of the two genes were directly compared, CHLI1 was still several folds higher than CHLI2, agreeing with the result that the survival rate of the chli1 mutant was more severely affected than the chli2 mutants. However, CHLI2 contributed to about 27% of total CHLI RNA in the dark and only about 16% of total CHLI after de-etiolation (Fig. 6D), suggesting that CHLI2 might have slightly more contribution in the dark. This might be the reason that chli2 mutants had no clear phenotype when directly grown under the light but had a lower survival rate in transitions from dark to light.

**The chli1/chli1 chli2/chli2 double mutant and the cs215/cs215 mutant are gun mutants**

Mutants in the H and D subunits of Mg chelatase are gun mutants (Strand et al., 2003) but two alleles of the chli1 mutants are not (Mochizuki et al., 2001). To investigate if the chli1/chli1 chli2/chli2 double mutant or the cs215/cs215 mutant would show the gun phenotype, we first obtained mutants with T-DNA insertion in the CHLD and CHLH genes for use as controls. The chld mutant allele was identical to the allele reported previously and has been shown to be a gun mutant (SALK_150219; Strand et al., 2003; Ankele et al., 2007). The chlh mutant has a T-DNA insertion in the first exon of the CHLH gene (SALK_062726, Fig. 7A). No transcripts across the T-DNA insertion site or 3’ to the T-DNA insertion site could be detected in either of the mutant (Fig. 7B) and both mutants had an albino phenotype similar to that of the chli1/chli1 chli2/chli2 double...
mutant (Fig. 7C). We then measured *Lhcb1* transcript levels in these mutants after treatment with Nf. As shown in Figure 7D, the *chli1/chli1 chli2/chli2* double mutant accumulated a higher level of *Lhcb1* transcripts than the wild type, similar to the level found in the *chld/chld* mutant. Interestingly, *cs215/cs215* and *chlh/chlh* mutants accumulated an even higher level of *Lhcb1* transcripts. These results indicated that the *chli1/chli1 chli2/chli2* double mutant and the *cs215/cs215* mutant were *gun* mutants.

**Absence of CHLI caused instability of CHLD**

It has been shown that changes in the level of one Mg-chelatase subunit causes instability in other subunits (Hansson et al., 1999; Petersen et al., 1999). We therefore investigated whether the level of CHLD or CHLH proteins was affected in the various *chli* mutants. We used immunoblots to detect the levels of the three Mg-chelatase subunits. As shown in Figure 8, in the *chli1/chli1* and *chli1/chli1 chli2/chli2* mutants CHLI was reduced or absent, and the amount of CHLD protein was also greatly reduced (lanes 4 and 6). In comparison, the *cs215/cs215* mutant still contained a normal amount of CHLI protein (lane 7) and its amount of CHLD protein was also normal. On the other hand, the amount of CHLI was not affected by the *chld* mutation (lane 8). These results agreed with results from barley showing that *chli* null mutants also lacked CHLD, but *chld* mutations did not affect the amount of CHLI (Petersen et al., 1999). Furthermore, the normal amount of CHLD in the *cs215* mutant suggested that the *cs215* mutant protein could still complex with CHLD, agreeing with previous observations in barley that semi-dominant mutant *chli* protein could still complex with CHLD (Hansson et al., 1999). In addition, the presence of CHLD and CHLH in the *cs215/cs215* mutant also indicated that the *gun* phenotype of the *cs215/cs215* mutant was not caused by the absence of CHLD or CHLH. Interestingly, loss of CHLI or CHLD activity in the *chli1/chli1, chli1/chli1 chli2/chli2, cs215/cs215* and *chld/chld* mutants seemed to have resulted in an increase of the CHLH protein level (lanes 4 and 6 to 8).
DISCUSSION

The expression level of CHLI2 was much lower than that of CHLI1. When driven by the CHLI1 promoter, expression of CHLI2 alone rescued the chli1/chli1 chli2/chli2 double mutant. These data indicate that CHLI2 can be functionally equivalent to CHLI1 if expressed at a sufficient level. This result also agrees with results from Kobayashi et al. (2008) showing that CHLI2 is a functional ATPase. Previous proposals of CHLI2 rapid turnover and inability in hexameric-ring assembly were based on data suggesting that CHLI1 and CHLI2 had similar expression levels (Rissler et al., 2002; Apchelimov et al., 2007), which might have resulted from probe or primer cross-reactions.

The chli2/chli2 mutants had no clear phenotype when grown in the light but had a lower survival rate during de-etiolation, suggesting that the presence of a second copy of CHLI may contribute to plant fitness under certain growth conditions. Although CHLI1 was the major isoform in both light and dark, CHLI2 contributed to about 27% of total CHLI RNA in the dark and only about 16% of total CHLI after de-etiolation. These results agreed with the miniarray data showing that CHLI1 RNA was almost three folds of CHLI2 in 3-week-old light-grown seedling but only 2 folds of CHLI2 in 3-d-old etiolated seedlings (Matsumoto et al., 2004). These results suggest that the presence of CHLI2 may be more important in the dark or in a sudden dark to light transition as in the de-etiolation experiments. We searched several sequenced plant genomes and found that two copies of CHLI are present in Chlamydomonas reinhardtii, Physcomitrella patens, and Populus trichocarpa, but only one copy is present in rice (Oryza sativa) and Sorghum bicolor. It is not clear why monocots seem to have lost the second copy of CHLI. It is possible that changes in the physiology in monocots have rendered the second copy unnecessary.

It has been suggested that, because the N terminus of CHLD has some sequence similarity to CHLI, CHLD may interact with CHLH in the absence of CHLI and provide a low level of Mg-chelatase activity (Rissler et al., 2002). However our data showed that both the chli1/chli1 chli2/chli2 double mutant and the cs215/cs215 mutant had no detectable chlorophylls and were indistinguishable from the chld/chld and chlh/chlh knockout mutants. In fact, similar to barley, the stability of CHLD seems to rely on the presence of CHLI. These results suggest that CHLD and CHLH support no significant
chlorophyll biosynthesis in the absence of CHLI, agreeing with results from Rodobacter and barley that all three subunits are required for the Mg-chelatase activity (Gibson et al., 1995; Willows et al., 1996; Kannangara et al., 1997).

We showed that the chli1/chli1 chli2/chli2 double mutant and the cs215/cs215 mutant showed the gun phenotype. Our data help remove doubts that knocking out the Mg-chelatase activity causes the gun phenotype. Indeed mutants in all three subunits in barley have been shown to be gun mutants (Gadjieva et al., 2005). It has been shown recently that the immediate product of Mg-chelatase, Mg-proto, does not accumulate under Nf treatment and therefore is unlikely to be the determinant for retrograde signaling (Mochizuki et al., 2008; Moulin et al., 2008). It therefore remains to be elucidated why perturbation of the tetrapyrrole biosynthesis pathway often causes the gun phenotype. It is also interesting that the cs215/cs215 and the chlh-knockout mutants showed a higher level of Lhcb1 accumulation than the chli1/chli1 chli2/chli2 double mutant and the chld/chld mutant, even though they are indistinguishable in appearance. These mutants may be useful materials to facilitate identification of compounds whose accumulation levels correlate with the Lhcb1 transcript levels and who may therefore be candidates for the signaling molecules.

MATERIALS AND METHODS

Positional cloning of the cs215 locus

The cs215 mutant is in the Enk ecotype. Heterozygous cs215 plants were crossed with wild-type Col and Ler plants. DNA from F2 seedlings with the cs215/cs215 phenotype was isolated for mapping. SSLP and CAPS markers between the Col and Ler ecotypes (http://www.arabidopsis.org) were tested on Enk DNA to identify markers that could distinguish Enk. vs. Col. or Enk. vs. Ler. ecotypes.
Plant materials, growth conditions, and treatments.

The cs215 (Enk ecotype), chli1, chld and chlh mutants (Col ecotype) were obtained from ABRC (http://www.arabidopsis.org/abrc/). The chli2 mutants GT13937 and GT18178 (Ler ecotype) were obtained from Cold Spring Harbor Laboratory (http://genetrap.cshl.org). The T-DNA or Ds insertion positions in the mutants were confirmed by PCR and direct sequencing of the PCR products (See Table SI for primer sequences). Mutants were backcrossed to their corresponding wild type and lines with single T-DNA or Ds insertion were selected based on the kanamycin- or BASTA-resistance segregation ratio.

Sterilized seeds were plated on MS media containing 0.3% Gelrite, 1x Murashige and Skoog salts with Gamborg’s B5 vitamin and 2% sucrose. After a 3-d cold stratification, seeds were grown in growth chambers under a 16-h photoperiod with a light intensity approximately 60 µmol m⁻² s⁻¹ at 22°C. Total chlorophyll and carotenoid contents were determined as described (Lichtenthaler, 1987). Kanamycin selection of T-DNA and Ds transposon-containing seedlings were performed on media supplemented with kanamycin (Sigma-Aldrich, St. Louis, MO) at 50 mg L⁻¹. The pCHLI1::CHLI2 construct was obtained by two-step PCR (primers used were KpnI-CHLI1F and KpnI-CHLI2R for the first step; P1 and P2 for the second step), cloned into the transformation vector pPZP221 (Hajdukiewicz et al., 1994), transformed into Agrobacterium GV3101 and introduced into the CHLI1/chli1 chli2/chli2 mutant using the floral spray method (Chung et al., 2000). Transgenic plants were screened on MS media containing 100 mg L⁻¹ G418.

For the chli1/chli1 de-etiolation experiment, because the chli1/chli1 mutant was sterile and could only be sowed from seeds of CHLI1/chli1 heterozygous plants, seeds from CHLI1/chli1 heterozygous plants were first grown in the light to confirm the segregation ratio of the chli1/chli1 mutant, which was almost always around ¼. The same batch of seeds was then used for the de-etiolation experiments. When calculating survival rate, the theoretical number of chli1/chli1 seedlings was deduced using the segregation ratio from light-grown seedlings. The number of survived chli1/chli1 seedlings was then counted using the yellow cotyledons of the chli1/chli1 mutant as an indication.
For Nf-treatment experiments, seeds (from heterozygous plants of the albino and pale-green mutant lines and from homozygous plants of others) were sterilized and plated on MS media. After a 3-d cold stratification, plates were moved to a growth chamber for 5 days. Albino (cs215/cs215, chli1/chli1 chli2/chli2, chld/chld and chlh/chlh) and pale-green (chli1/chli1) mutants were selected, and together with chli2-1/chli2-1 and wild-type seedlings, were transferred to new MS media containing 5 µM Nf or the same volume of DMSO, and grown for another 5.5 days under strong continuous light (100 µmol m⁻² s⁻¹).

**RNA Analysis**

Total RNA was isolated from Arabidopsis shoots with TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with RQ1 RNase-Free DNase (Promega, Madison, WI). Template cDNA was prepared using 1 µg of total RNA and the MMLV reverse transcription system (Promega). Real-time quantitative RT-PCR was performed using the LightCycler system (Roche Applied Science, Indianapolis, IN) and the Lightcycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). Each PCR reaction contained 10-50 ng of cDNA and 0.5 µM of each of the primer pairs. The initial denaturing step of 10 min was followed by 40 PCR cycles of 95°C for 10 sec, 60°C for 5 sec and 72°C for 1 sec per 25 bp of the expected product. After the PCR reaction, the melting temperature was tested. Quantification was performed using LightCycler Relative Quantification software version 1.0. Normalization was done using the transcript level of H3G and also confirmed by the transcript level of ubiquitin 10.

**Immunoblots**

Total proteins from leaves of 14-d-old plants were extracted with SDS sample buffer (300 mM Tris-HCl, pH 8.5, 1 mM EDTA, pH 8.0, 8% SDS and 1 mM PMSF). 40 µg of total proteins were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA). Immunostaining was performed with anti-sera to soybean CHLI and CHLH and Plectonema boryanum CHLD at a 1:1000 dilution followed by secondary staining with an alkaline phosphatase-conjugated goat-anti-rabbit serum at a 1:1000 dilution. Colorimetric development with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium was used to visualize protein bands.
Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

SUPPLEMENTAL MATERIAL

Table SI: Primers used in this study.

ACKNOWLEDGEMENTS

We thank Wei-Ning Hwang, Ming-Da Yang and Dr. Chi-Chou Chiu for initial mapping of the cs215 locus. We thank ABRC for the cs215, chld and chlh mutants, and Cold Spring Harbor for the chli2 mutants GT13937 and GT18178. We thank Dr. Tatsuru Masuda for providing the CHLI, CHLD and CHLH antibodies. We thank Dr. Harry Wilson of Academia Sinica for English editing.
REFERENCES

Ankele E, Kindgren P, Pesquet E, Strand A (2007) In vivo visualization of Mg-
protoporphyrin IX, a coordinator of photosynthetic gene expression in the nucleus and the chloroplast. Plant Cell 19: 1964-1979

Aphelimonov AA, Soldatova OP, Ezhova TA, Grimm B, Shestakov SV (2007) The
analysis of the Chl 1 and Chl 2 genes using acifluorfen-resistant mutant of
Arabidopsis thaliana. Planta 225: 935-943

Brenner S, Johnson M, Bridgham J, Golda G, Lloyd D, Johnson D, Luo S,
McCurdy S, Foy M, Ewan M, Roth R, George D, Eletr S, Albrecht G,
Vermaas E, Williams SR, Moon K, Burcham T, Pallas M, DuBridge RB,
Kirchner J, Fearon K, Mao J, Corcoran K (2000) Gene expression analysis by
massively parallel signature sequencing (MPSS) on microbead arrays. Nature
Biotech. 18: 630-634

Chung MH, Chen MK, Pan SM (2000) Floral spray transformation can efficiently
generate Arabidopsis transgenic plants. Transgenic Res 9: 471-476

Cottage AJ, Mott EK, Wang J-H, Sullivan JA, MacLean D, Tran L, Choy M-K,
Newell C, Kavanagh TA, Aspinal S, Gray JC (2008) GUN1 (GENOMES
UNCOUPLED1) encodes a pentatricopeptide repeat (PPR) protein involved in
plastid protein synthesis-responsive retrograde signaling to the nucleus. In J
Allen, E Gantt, J Golbeck, B Osmond, eds, Photosynthesis. Energy from the Sun:
14th International Congress on Photosynthesis, pp 1201-1205

Gadjieva R, Axelsson E, Olsson U, Hansson M (2005) Analysis of gun phenotype in
barley magnesium chelatase and Mg-protoporphyrin IX monomethyl ester cyclase
mutants. Plant Physiol Biochem 43: 901-908

Gibson LC, 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

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of
Agrobacterium binary vectors for plant transformation. Plant Mol Biol 25: 989-994

Hansson A, Kannangara CG, von Wettstein D, Hansson M (1999) Molecular basis for
semidominance of missense mutations in the XANTHA-H (42-kDa) subunit of
magnesium chelatase. Proc Natl Acad Sci USA 96: 1744-1749

Hansson A, Willows RD, Roberts TH, Hansson M (2002) Three semidominant barley
mutants with single amino acid substitutions in the smallest magnesium chelatase
subunit form defective AAA+ hexamers. Proc Natl Acad Sci USA 99: 13944-13949

Kannangara CG, Vothknecht UC, Hansson M, von Wettstein D (1997) Magnesium chelatase: association with ribosomes and mutant complementation studies identify barley subunit Xantha-G as a functional counterpart of Rhodobacter subunit BchD. Mol Gen Genet 254: 85-92

Kjemtrup S, Sampson KS, Peele CG, Nguyen LV, Conkling MA, Thompson WF, Robertson D (1998) Gene silencing from plant DNA carried by a Geminivirus. Plant J 14: 91-100

Kobayashi K, Mochizuki N, Yoshimura N, Motohashi K, Hisabori T, Masuda T (2008) Functional analysis of Arabidopsis thaliana isoforms of the Mg-chelatase CHLI subunit. Photochem Photobiol Sci 7: 1188-1195

Larkin R, Alonso J, Ecker J, Chory J (2003) GUN4, a regulator of chlorophyll synthesis and intracellular signaling. Science 299: 902-906

Lichtenthaler H (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol 148 350–382

Matsumoto F, Obayashi T, Sasaki-Sekimoto Y, Ohta H, Takamiya K, Masuda T (2004) Gene expression profiling of the tetrapyrrole metabolic pathway in Arabidopsis with a mini-array system. Plant Physiol 135: 2379-2391

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 Acad Sci USA 98: 2053-2058

Mochizuki N, Tanaka R, Tanaka A, Masuda T, Nagatani A (2008) The steady-state level of Mg-protoporphyrin IX is not a determinant of plastid-to-nucleus signaling in Arabidopsis. Proc Natl Acad Sci USA 105: 15184-15189

Moulin M, McCormac A, Terry M, Smith A (2008) Tetrapyrrole profiling in Arabidopsis seedlings reveals that retrograde plastid nuclear signaling is not due to Mg-protoporphyrin IX accumulation. Proc Natl Acad Sci USA 105: 15178–15183

Nguyen LV (1995) Transposon Tagging and Isolation of the SULFUR Gene in Tobacco (Nicotiana tabacum). North Carolina State University

Nott A, Jung HS, Koussevitzky S, Chory J (2006) Plastid-to-nucleus retrograde signaling. Annu Rev Plant Biol 57: 739-759
Petersen BL, Møller MG, Jensen PE and Henningsen KW (1999) Identification of the Xan-g Gene and Expression of the Mg-chelatase Encoding Genes Xan-f, -g and -h in Mutant and Wild Type Barley (Hordeum Vulgare L.). Hereditas 131: 165-170

Rissler HM, Collakova E, DellaPenna D, Whelan J, Pogson BJ (2002) Chlorophyll biosynthesis. Expression of a second chl I gene of magnesium chelatase in Arabidopsis supports only limited chlorophyll synthesis. Plant Physiol 128: 770-779

Soldatova O, Apchelimov A, Radukina N, Ezhova T, Shestakov S, Ziemann V, Hedtke B, Grimm B (2005) An Arabidopsis mutant that is resistant to the protoporphyrinogen oxidase inhibitor acifluorfen shows regulatory changes in tetrapyrrole biosynthesis. Mol Genet Genomics 273: 311-318

Strand A, Asami T, Alonso J, Ecker JR, Chory J (2003) Chloroplast to nucleus communication triggered by accumulation of Mg-protoporphyrinIX. Nature 421: 79-83

Susek R, Ausubel F, Chory J (1993) Signal transduction mutants of Arabidopsis uncouple nuclear CAB and RBCS gene expression from chloroplast development. Cell 10: 787-799

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

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol. 136: 2621-2632
FIGURE LEGENDS

**Figure 1.** Phenotypes of various mutants and double mutants. A, Plants of the indicated genotypes were grown on MS media for 12 days. B, Chlorophyll and carotenoid contents in 12-d-old plants as shown in A. Data shown are means ± SD from 5 independent samples per genotype, each sample containing 5 to 8 plants.

**Figure 2.** Identification of the cs215 locus. A, Summary of cs215 mapping. Vertical lines indicate the positions of PCR-based markers. Values beneath the lines indicate the number of recombinant plants. The direction of transcription of the At4g18480 ORF is indicated (arrow). B, Alignment of Arabidopsis CHLI1 and related proteins. Alignment of Arabidopsis CHLI1 (AtCHLI1) and CHLI2 (AtCHLI2), Sulfur from *Nicotiana tabaccum* (tobacco, NtCHLI), and CHLI from *Hordeum vulgare* (barley, HvCHLI) and *Zea mays* (maize, ZmCHLI). Walker A (Wlk A) and Walker B (Wlk B) motifs of the ATP-binding fold are marked above the sequence. Mutation positions of the chli1 (SAIL_230_D11), cs215 and the maize Oyl-N1989/barley Xan-h<sup>clo161</sup> mutants are also indicated.

**Figure 3.** Transcript analyses of the chli1/chli1 and chli2/chli2 mutants. A, Schematic representation of the structure of the CHLI genes and the location of each insertion site. Exons are represented by open boxes and introns are represented by lines between boxes. Location and direction of primers used for RT-PCR are also indicated (arrows). B, Analyses of CHLI transcripts in chli1/chli1 and chli2/chli2 mutants. Total RNA extracted from wild-type and mutant plants was analyzed by RT-PCR. Histone 3G (H3G, At4g40040) transcripts were analyzed as a control.

**Figure 4.** CHLI1 and CHLI2 mRNA levels in the wild type and chli mutants. Total RNA was isolated from 12-day-old seedlings. CHLI and CHLI2 mRNA levels were determined by real-time quantitative RT-PCR as described in Materials and Methods. Data shown are means ± SD from 4 independent samples per genotype and each sample contained 5 plants.

**Figure 5.** CHLI2 driven by the CHLI1 promoter rescued the chli1/chli1 chli2/chli2 double mutant. A, Plants of the indicated genotypes were grown on MS media for 12 days and then photographed. The pCHLI1::CHLI2 (chli1/chli1 chli2/chli2) transgenic plants had phenotypes that ranged from fully green (Green) to slightly yellow in younger leaves (Yellow). Representative transgenic lines were photographed and used for analyses shown in B and C. B, Chlorophyll and carotenoid contents of 12-d-old plants as shown in A. Data shown are means ± SD derived from 5 independent samples per genotype, each sample containing 5 to 8 plants. C, Comparison of CHLI2 mRNA levels in the wild type and chli mutants. Seedlings were grown as described in A. mRNA levels were determined by real-time quantitative RT-PCR as described in Materials and Methods. Data shown are means ± SD derived from 5 independent samples per genotype, each one containing 5 to 8 plants. CHLI1 transcript levels were also determined as controls.
Figure 6. The chli mutants had lower survival rates during de-etiolation. Seeds were grown on MS media and imbibed at 4°C in the dark for 3 days, exposed to light at 22°C for 1 day and then move to the dark to germinate at 22°C for 7 days. Etiolated seedlings were then transferred to 16 h light/8 h dark cycle for another 6 days (de-etiolation) and then photographed. A, Examples of seedlings being scored as “live” or “dead”. B, Seedling survival rates of Col, Ler, chli1/chli1, chli2-1/chli2-1 and chli2-2/chli2-2 after de-etiolation. Data shown are means ± SD of 6 independent experiments, each experiment containing 100 to 120 seedlings per genotype. C, Comparison of CHLI1 and CHLI2 mRNA levels in Ler during de-etiolation (filled triangles and solid lines). CHLI1 and CHLI2 mRNA levels were determined by real-time quantitative RT-PCR as described in Materials and Methods. D, Percent of CHLI2 in total CHLI (CHLI1+CHLI2) during de-etiolation. Amounts of CHLI1 and CHLI2 mRNA were determined by real-time quantitative RT-PCR and deduced from standard curves produced from known quantities of CHLI1 and CHLI2 plasmid DNA. For both C and D, time in the x-axis indicates hours after transferring to the 16 h light/8 h dark cycle after etiolation. Seedlings were harvested 3 hours after light came on each day. Seedlings of the same age but were grown under a 16 h light/8 h dark cycle since stratification were analyzed as a control (open squares and dotted lines). Data shown are means ± SD of 4 independent samples, each sample containing 50 to 60 plants.

Figure 7. Lhcb1 mRNA levels in Nf-treated wild type (Col, Ler, Enk), various chli mutants, and chld/chld and chlh/chlh mutants. A, Schematic representation of the CHLD and CHLH genes. Exons are represented by open boxes and introns are represented by lines between the boxes. The locations of T-DNA insertion sites and RT-PCR primers were indicated. B, No transcript across the T-DNA insertion site or 3’ to the insertion site could be detected in the chld/chld or chlh/chlh mutants. Total RNA extracted from wild-type and mutant plants was analyzed by RT-PCR using primers indicated in A. H3G transcripts were analyzed as a control. C, Phenotypes of the chli1/chli1 chli2/chli2, chld/chld and chlh/chlh mutants. Plants were grown on MS media for 12 days. D, Comparison of Lhcb1 mRNA levels in the wild type and mutants. Seedlings were grown on MS media for 5 days. Homozygous pale-green or albino mutants (chli1/chli1, cs215/cs215, chli1/chli1 chli2/chli2, chld/chld and chlh/chlh) were selected and, together with the chli2/chli2 and wild-type seedlings, were transferred to MS media containing either 5 µM norflurazon or the same volume of DMSO. Seedlings were grown for another 5.5 days under strong continuous light (100 µmol m⁻² s⁻¹) and then used for total RNA isolation. Lhcb1 mRNA levels were determined by real-time quantitative RT-PCR as described in Materials and Methods. Data shown are means ± SD derived from 4 independent samples per genotype, each sample containing 10 to 15 plants.

Figure 8. Immunoblot analyses of the three Mg-chelatase subunits in various mutants and corresponding wild type. Total proteins extracted from leaves of 14-d-old plants were analyzed by SDS-PAGE and immunoblots with antibodies against CHLI, CHLD, and CHLH. RBCL from the same set of samples as revealed by Coomassie blue staining was
analyzed as a control.
Figure 1. Phenotypes of various mutants and double mutants. A, Plants of the indicated genotypes were grown on MS media for 12 days. B, Chlorophyll and carotenoid contents in 12-d-old plants as shown in A. Data shown are means ± SD from 5 independent samples per genotype, each sample containing 5 to 8 plants.
Figure 2. Identification of the cs215 locus. A, Summary of cs215 mapping. Vertical lines indicate the positions of PCR-based markers. Values beneath the lines indicate the number of recombinant plants. The direction of transcription of the At4g18480 ORF is indicated (arrow). B, Alignment of Arabidopsis CHLI1 and related proteins. Alignment of Arabidopsis CHLI1 (AtCHLI1) and CHLI2 (AtCHLI2), Sulfur from Nicotiana tabacum (tobacco, NtCHLI), and CHLI from Hordeum vulgare (barley, HvCHLI) and Zea mays (maize, ZmCHLI). Walker A (Wlk A) and Walker B (Wlk B) motifs of the ATP-binding fold are marked above the sequence. Mutation positions of the chli1 (SAIL_230_D11), cs215 and the maize OyI-N1989/barley Xan-h^{clo161} mutants are also indicated.
Figure 3

A

**CHLI1** (At4g18480)

SAIL_230_D11

start

1F

1-1

1-2

1R

stop

**CHLI2** (At5g45930)

chl2-2

(chl2-2 (GT13937))

chl2-1

(chl2-1 (GT13937))

start

2F

2R

stop

B

Col

SAIL_230_D11

Ler

chl2-2/chl2-2

chl2-1/chl2-1

1F+1R

2F+2R

1F+1-1

2F+2R

1F+1-2

2F+2R

1F+1R

2F+2R

Histone 3G (**H3G**, At4g40040)

Figure 3. Transcript analyses of the **chl1**/**chl1** and **chl2**/**chl2** mutants. A, Schematic representation of the structure of the **CHLI** genes and the location of each insertion site. Exons are represented by open boxes and introns are represented by lines between boxes. Location and direction of primers used for RT-PCR are also indicated (arrows). B, Analyses of **CHLI** transcripts in **chl1**/**chl1** and **chl2**/**chl2** mutants. Total RNA extracted from wild-type and mutant plants was analyzed by RT-PCR. **Histone 3G** (**H3G**, At4g40040) transcripts were analyzed as a control.
Figure 4. *CHLI1* and *CHLI2* mRNA levels in the wild type and *chli* mutants. Total RNA was isolated from 12-day-old seedlings. *CHLI1* and *CHLI2* mRNA levels were determined by real-time quantitative RT-PCR as described in Materials and Methods. Data shown are means ± SD from 4 independent samples per genotype and each sample contained 5 plants.
Figure 5. CHLI2 driven by the CHLI1 promoter rescued the chli1/chli1 chli2/chli2 double mutant. A, Plants of the indicated genotypes were grown on MS media for 12 days and then photographed. The pCHLI1::CHLI2 (chli1/chli1 chli2/chli2) transgenic plants had phenotypes that ranged from fully green (Green) to slightly yellow in younger leaves (Yellow). Representative transgenic lines were photographed and used for analyses shown in B and C. B, Chlorophyll and carotenoid contents of 12-d-old plants as shown in A. Data shown are means ± SD derived from 5 independent samples per genotype, each sample containing 5 to 8 plants. C, Comparison of CHLI2 mRNA levels in the wild type and chli mutants. Seedlings were grown as described in A. mRNA levels were determined by real-time quantitative RT-PCR as described in Materials and Methods. Data shown are means ± SD derived from 5 independent samples per genotype, each one containing 5 to 8 plants. CHLI1 transcript levels were also determined as controls.
Figure 6. The *chli* mutants had lower survival rates during de-etiolation. Seeds were grown on MS media and imbibed at 4°C in the dark for 3 days, exposed to light at 22°C for 1 day and then move to the dark to germinate at 22°C for 7 days. Etiolated seedlings were then transferred to 16 h light/8 h dark cycle for another 6 days (de-etiolation) and then photographed. A, Examples of seedlings being scored as “live” or “dead”. B, Seedling survival rates of *Col, Ler, chli1/chli1, chli2-1/chli2-1* and *chli2-2/chli2-2* after de-etiolation. Data shown are means ± SD of 6 independent experiments, each experiment containing 100 to 120 seedlings per genotype. C, Comparison of *CHLI1* and *CHLI2* mRNA levels in *Ler* during de-etiolation (filled triangles and solid lines). *CHLI1* and *CHLI2* mRNA levels were determined by real-time quantitative RT-PCR as described in Materials and Methods. D, Percent of *CHLI2* in total *CHLI* (*CHLI1+CHLI2*) during de-etiolation. Amounts of *CHLI1* and *CHLI2* mRNA were were determined by real-time quantitative RT-PCR and deduced from standard curves produced from known quantities of *CHLI1* and *CHLI2* plasmid DNA. For both C and D, time in the x-axis indicates hours after transferring to the 16 h light/8 h dark cycle after etiolation. Seedlings were harvested 3 hours after light came on each day. Seedlings of the same age but were grown under a 16 h light/8 h dark cycle since stratification were analyzed as a control (open squares and dotted lines). Data shown are means ± SD of 4 independent samples, each sample containing 50 to 60 plants.
Figure 7

A, Schematic representation of the CHLD and CHLH genes. Exons are represented by open boxes and introns are represented by lines between the boxes. The locations of T-DNA insertion sites and RT-PCR primers were indicated. B, No transcript across the T-DNA insertion site or 3’ to the insertion site could be detected in the chld/chld or chlh/chlh mutants. Total RNA extracted from wild-type and mutant plants was analyzed by RT-PCR using primers indicated in A. H3G transcripts were analyzed as a control. C, Phenotypes of the chli1/chli1 chli2/chli2, chld/chld and chlh/chlh mutants. Plants were grown on MS media for 12 days. D, Comparison of Lhcb1 mRNA levels in the wild type and mutants. Seedlings were grown on MS media for 5 days. Homozygous pale-green or albino mutants (chli1/chli1, cs215/cs215, chli1/chli1 chli2/chli2, chld/chld and chlh/chlh) were selected and, together with the chli2/chli2 and wild-type seedlings, were transferred to MS media containing either 5 µM norflurazon or the same volume of DMSO. Seedlings were grown for another 5.5 days under strong continuous light (100 µmol m⁻² s⁻¹) and then used for total RNA isolation. Lhcb1 mRNA levels were determined by real-time quantitative RT-PCR as described in Materials and Methods. Data shown are means ± SD derived from 4 independent samples per genotype, each sample containing 10 to 15 plants.
Figure 8. Immunoblot analyses of the three Mg-chelatase subunits in various mutants and corresponding wild type. Total proteins extracted from leaves of 14-d-old plants were analyzed by SDS-PAGE and immunoblots with antibodies against CHLI, CHLD, and CHLH. RBCL from the same set of samples as revealed by Coomassie blue staining was analyzed as a control.