Arabidopsis genomes uncoupled 5 (*GUN5*) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction

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A plastid-derived signal plays an important role in the coordinated expression of both nuclear- and chloroplast-localized genes that encode photosynthesis-related proteins. *Arabidopsis GUN* (*ge*nomes *un*coupled) loci have been identified as components of plastid-to-nucleus signal transduction. Unlike wild-type plants, *gun* mutants have nuclear *Lhcb1* expression in the absence of chloroplast development. We observed a synergistic phenotype in some *gun* double-mutant combinations, suggesting there are at least two independent pathways in plastid-to-nucleus signal transduction. There is a reduction of chlorophyll accumulation in *gun4* and *gun5* mutant plants, and a *gun4gun5* double mutant shows an albino phenotype. We cloned the *GUN5* gene, which encodes the ChlH subunit of Mg-chelatase. We also show that *gun2* and *gun3* are alleles of the known photomorphogenic mutants, *hy1* and *hy2*, which are required for phytochromobilin synthesis from heme. These findings suggest that certain perturbations of the tetrapyrrole biosynthetic pathway generate a signal from chloroplasts that causes transcriptional repression of nuclear genes encoding plastid-localized proteins. The comparison of mutant phenotypes of *gun5* and another Mg-chelatase subunit (ChlI) mutant suggests a specific function for ChlH protein in the plastid-signaling pathway.

A number of components required for plastid structure and development are encoded by the nuclear genome. There is a considerable body of evidence that suggests that the proper and timely expression of these genes depends in part on the functional state of the chloroplast. For example, nuclear mutations that result in developmentally arrested chloroplasts also result in the reduced expression of nuclear-localized photosynthetic genes (1). These results tie the functional state of the chloroplast to nuclear function and suggest that the chloroplast signals the nucleus in a retrograde fashion (2).

Such retrograde signaling between chloroplast and nucleus has been studied primarily by using carotenoid-deficient plants induced either by mutations or by carotenoid biosynthesis inhibitors such as Norflurazon (Nf; refs. 3 and 4). Carotenoids prevent the production of reactive oxygen species by excited triplet states of chlorophyll. Carotenoid-deficient plants thus suffer a rapid photooxidation of most chloroplast components under intense light. Although most nuclear-encoded cytoplasmic enzymes are present at normal levels in photooxidatively damaged plants, a small set of nuclear-encoded chloroplast enzymes is absent (3, 4). Accordingly, it was hypothesized that plastids send an unknown signal(s) to the nucleus that regulates the expression of a small subset of nuclear-localized photosynthetic genes (2, 5).

The molecular nature of the plastid signal(s) and the mechanism by which it is relayed to the nucleus remain ambiguous. Although both plastid transcription and translation are necessary for the production of the plastid signal (4, 6), the plastid signal is not a direct translational product of a plastid gene. Light does not seem necessary for the activity of the plastid signal. Dark-grown pea *lip1* and *Arabidopsis cop1-4* mutants contain plastids that do not arrest as etioplasts but continue to differentiate into prechloroplasts. Nuclear *Lhcb1* is derepressed in these dark-grown mutants, implying that the plastid signal does not depend on photosynthesis (7). The plastid signal observed in these mutants grown in the dark still depends on early plastid translation, because *Lhcb1* expression is sensitive to lincomycin (7).

Early studies using chlorophyll-deficient mutants led to the proposal that the plastid signal does not depend on chlorophyll biosynthesis (8). However, Johanningmeier and Howell (9) reported an inhibition of light-induced *Lhcb* mRNA accumulation in *Chlamydomonas* treated with 2, 2′-dipyridyl (DP), which is presumed to cause Mg-protoporphyrin IX (MgProto) and Mg-protoporphyrin IX monomethyl ester (MgProtoME) accumulation [MgProto(ME)]. They proposed that chlorophyll precursors are negative regulators of *Lhcb* mRNA accumulation. Kropat *et al.* (10, 49) recently reported that the addition of MgProto or Mg-protoporphyrin IX dimethyl ester ($MgProtoME_2$) in the dark can substitute for light signals in the induction of nuclear *HSP70* genes in *Chlamydomonas*. Together, this evidence suggests that chlorophyll precursors can act as plastid-derived signals that influence nuclear gene expression. However, the mechanism by which these putative signals are relayed to the nucleus is still unclear.

We have taken a genetic approach to understanding the mechanisms of the plastid-to-nucleus signal transduction pathway(s). We have reported previously the isolation of *Arabidopsis* mutants, referred to as *gun* (*g*enomes *un*coupled) mutants, that express nuclear-encoded *Lhcb* and *RbcS* transcripts in the absence of chloroplast development (11). We described three nonallelic loci (*GUN1*, *GUN2*, and an unspecified locus) that are involved in the plastid-dependent regulation of the *Lhcb1* promoter.

In this paper, we report the identification of previously unidentified *GUN* loci (*GUN3*, *GUN4*, and *GUN5*) and the genetic interactions between *GUN1*, *GUN4*, and *GUN5* genes. We cloned the *GUN5* gene and show that it encodes the ChlH subunit of Mg-chelatase. A comparison of Mg-chelatase activity and the *gun* phenotypes in *gun5* and the *cs/ch42* mutants, which have defects in the ChlI subunit of Mg-chelatase, shows that the ChlH subunit may have a distinct function in plastid signaling in addition to acting as a subunit of the Mg-chelatase enzyme.

Materials and Methods

Strains and Media. The transgenic line, pOCA107–2 (12), contains a pOCA18-based Lhcb1*2-hpt/Lhcb1*2-β-glucuronidase

Abbreviations: Nf, Norflurazon; DP, 2, 2′-dipyridyl; MgProto, Mg-protoporphyrin IX; MgProtoME: Mg-protoporphyrin IX monomethyl ester; MgProto(ME), MgProto and MgProtoME; $MgProtoME_2$, Mg-protoporphyrin IX dimethyl ester; GUS, β-glucuronidase; ALA, 5-aminolevulinate; PΦB, phytochromobilin.

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(GUS) transgene integrated near GPA1 and was used as the wild type in all the experiments described here. *gun* mutants were isolated by their elevated expression of Lhcb1*2-GUS in the presence of Nf, as described (11). Because a *gun3* allele was not specified in the previous study, we assigned the 11th isolate of 12 *gun* mutants as *gun3-1*. *gun4-1* and *gun5-1* were designated originally as *gun1-4* and *gun0-6*, respectively (11). Unless specified, *gun1*, *gun2*, *gun3*, *gun4*, and *gun5* represent *gun1-1*, *gun2-1*, *gun3-1*, *gun4-1*, and *gun5-1*, respectively. *cch* (*c*onditional *ch*lorina) was isolated from ethyl-methanesulfonate-treated *Columbia* for its light-sensitive reduction of chlorophyll accumulation (ref. 13 and J.A.B., unpublished data). *ch42* and *cs* are Mg-chelatase *chl I* mutants isolated by x-ray irradiation (14) and transferred-DNA insertion (15), respectively. Plants were grown on soil or on Murashige–Skoog (Wako-Jyunyaku, Japan) agar medium containing 2% (wt/vol) sucrose. Seedlings were photobleached by including 5 μM Nf (provided by Sandoz Pharmaceutical) in the growth media and exposing the seedlings to continuous white light (100 μmol/m^2/sec).

GUS Assay. GUS activity was measured according to the methods of Jefferson (16). For quantitative assays, plant extracts were prepared from 5- to 6-day-old seedlings, and protein concentrations were determined by Protein Assay reagent (Bio-Rad). One unit of GUS activity was defined as the amount of activity that could produce 1 pmol of 4-methylumbelliferone/minute/mg protein. For a semiquantitative assay, a piece of cotyledon was excised from a 5- to 6-day-old plant and placed into 100 μl of GUS-assay mixture (16). After a 12-h incubation at 37°C, the reaction was terminated by adding 100 μl of 1 M sodium bicarbonate. The fluorescence (excitation wavelength, 365 nm; emission wavelength, 455 nm) was measured with a Perkin–Elmer Luminescence Spectrophotometer LS50B. For histochemical staining, harvested tissues were incubated for 12 h at 37°C in 2 mg/ml 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) in 10 mM sodium phosphate, pH 7.0 (16).

Chlorophyll Measurements. Chlorophyll was extracted from approximately 0.1 g of fresh 1-week-old seedlings with *N*,*N*′-dimethylformamide for 12 h at 4°C in complete darkness. The extract was subjected to spectrophotometric measurements at 603, 647, and 664 nm. Specific chlorophyll content was calculated by using the equations of Moran (17) and normalized to the total fresh weight for each sample.

Porphyrin Measurements. Porphyrins were extracted on the basis of the method of Rebeiz *et al.* (18). Approximately 0.1 g of tissue was homogenized with a microtube pestle in ammonium-acetone (0.1 M NH_4OH/acetone 1:9) and centrifuged for 15 min at 0°C. The supernatant was extracted with hexane three times. The excitation (Ex) and emission (Em) wavelength for each porphyrin was: Proto, Ex 400 nm and Em 632 nm; MgProto(ME), Ex 420 nm and Em 595 nm (19).

5-Aminolevulinate (ALA)–DP Feeding. ALA–DP feeding experiments were performed on the basis of the methods of Falbel and Staehelin (20). Seedlings were grown on Murashige–Skoog agar plates containing 50 μM gibberellin A_3 in the dark for 4 days at 24°C. Then, 3 ml of an ALA–DP feeding solution (10 mM ALA/10 mM DP/5 mM $MgCl_2$/10 mM KPO_4, pH 7.0) was added to each plate. After a 12-h incubation at 24°C in the dark, porphyrin levels were determined as described above. Fluorescence intensity of the extract was normalized to the total fresh weight for each sample.

Northern Analysis. Total RNA was purified by using the TRIzol reagent (GIBCO/BRL). Then, 3 μg of total RNA was separated on a denaturing agarose gel and blotted onto a nylon membrane. Hybridization was carried out as described (21). *Lhcb1* DNA probes were generated from a plasmid containing the *Lhcb1*2* genomic DNA (22), and an 18S rRNA genomic sequence was cloned by PCR and used as a probe.

DNA Markers and Clones for *GUN5* Cloning. Molecular markers (23–25) were used to map the *GUN5* locus. Yeast-artificial chromosome DNA (CIC7F6, CIC5H3) and a full-length cDNA clone of *CHLH* from the *Columbia* ecotype were provided generously by M. Seki and K. Shinozaki (The Institute of Physical and Chemical Research, Tsukuba, Japan). P1 clones were from S. Tabata (Kazusa DNA Research Institute, Chiba, Japan). The plant transformation vector, pPZP221 (26), was from P. Maliga (Rutgers University, Piscataway, NJ). We noticed that the published cDNA sequences of *CHLH* from *C24* ecotype (27) have an insertion and a deletion of a T-residue in the third exon, which results in three amino acid differences from 987–989, compared with the amino acid sequences predicted from the cDNA sequence of *Columbia CHLH*. We sequenced the corresponding region of genomic DNA from C24, *Columbia*, and *Landsberg erecta*. All sequences were identical to the *Columbia CHLH* in this region. In addition, the *Columbia* amino acid sequence is conserved perfectly between known *BchH*/*CHLH* genes. Therefore, the *Columbia* sequence is used as the wild-type *CHLH* sequence in this paper.

Results

Genetic Characterization of *gun* Mutants. In a previous study, we reported the isolation and characterization of 12 *gun*-mutant candidates (11). To understand the genetic relationships between the mutants better, they were backcrossed to the wild-type parent (pOCA107–2), and complementation tests were done. The progeny were assayed for the mutant phenotype in the F_1 and F_2 generations and a total of five *GUN* loci (*GUN1-GUN5*) were identified (data not shown). *gun2* and *gun3* have long hypocotyl phenotypes under white light, and they were found to be alleles of *hy1* and *hy2*, respectively (data not shown). We examined *hy1-1* and *hy2-1* (28) for the *gun* phenotype and found that they had elevated *Lhcb* expression in the presence of Nf (N.M., unpublished data). We roughly mapped *gun5* to approximately 29.5 centimorgans on chromosome 5, and we noticed that *cch*, which affects chlorophyll accumulation, was located near *GUN5* (J.A.B., unpublished data). Complementation analysis and a comparison of *Lhcb1* mRNA levels in photobleached seedlings indicated that *cch* and *gun5* are allelic (N.M., unpublished data, and Fig. 6*A*). Although *cch* is paler than *gun5* (Figs. 2 and 3), *Lhcb1* mRNA levels were equivalent in photobleached seedlings (Fig. 6*A*).

Genetic Interactions Between *gun* Mutants. During complementation analysis, we noticed that approximately 1/16 of the F_2 individuals from the *gun1* × *gun4* and the *gun1* × *gun5* crosses expressed extremely high levels of *Lhcb1*2-GUS* in the presence of Nf (data not shown). Thus, we hypothesized that they were *gun1gun4* and *gun1gun5* double mutants. We backcrossed self-fertilized F_3 progeny of the putative double mutants with pOCA107–2. As expected for a double mutant, the phenotype segregated in an approximately 9:7 (wild-type/mutant) ratio (data not shown). *Lhcb1*2-GUS* expression in the *gun1gun4* and *gun1gun5* double mutants was 4.8- and 6.3-fold higher than the single mutants, respectively, and was about 11–17% of that observed in nonphotobleached wild-type plants (Fig. 1*A*). The endogenous *Lhcb1* mRNA levels were also derepressed synergistically in these double mutants (Fig. 1*B*).

Individuals with extremely high levels of *Lhcb1*2-GUS* expression were not observed among *gun4* × *gun5* F_2 progeny, but we found approximately 1/16 of *gun4* × *gun5* F_2 progeny were extremely pale under normal growth conditions (Fig. 2). We also

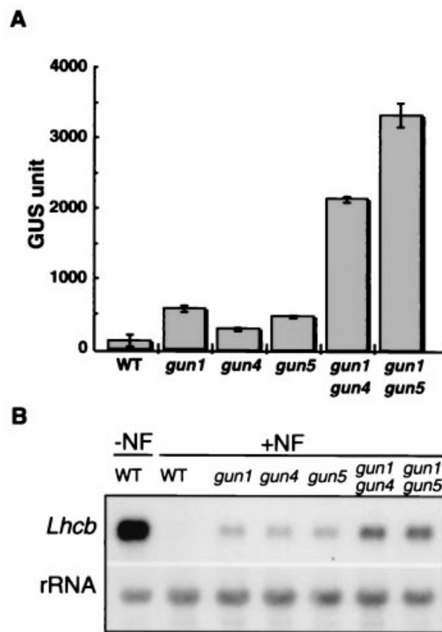

Fig. 1. *Lhcb1*2-GUS* and *Lhcb1* mRNA accumulation in wild-type and mutant seedlings. (*A*) *Lhcb1*2-GUS* activity in photobleached wild-type (WT), *gun* single, and *gun* double mutants. Seedlings were photobleached for 5 days, and GUS was extracted and assayed as described in *Materials and Methods*. In the absence of Nf, wild type expressed 20,120 ± 2,197 GUS units. (*B*) *Lhcb1* mRNA accumulation in photobleached wild-type, *gun* single, and *gun* double mutants. Total RNA was analyzed as described in *Materials and Methods* from seedlings that were grown as described in *A*.

crossed *gun1gun4* with *gun1gun5* to obtain a *gun1gun4gun5* triple mutant, and albino seedlings appeared in a ratio of approximately 1/16 in the F_2 generation (data not shown). We were unable to backcross the putative *gun4gun5* double mutants and the putative triple mutants because of low fertility. *gun4* and *gun5* have lower chlorophyll levels than wild type, but *gun1* mutants accumulate normal levels of chlorophyll (Figs. 2 and 3). Additionally, *gun1gun4* and *gun1gun5* accumulate the same levels of chlorophyll as *gun4* and *gun5* single mutants, respectively (Figs. 2 and 3). Thus, it is most likely that the extreme chlorophyll phenotype of the *gun4gun5* double mutant is because of the additive effect of these two mutations. Although we were able to extract low levels of chlorophyll "a" from the putative *gun4gun5* double mutants, we could not extract measurable levels of chlorophyll from the putative triple mutants (N.M., unpublished results). It is not surprising to see a more severe chlorophyll phenotype in the triple mutant, because the *gun1*

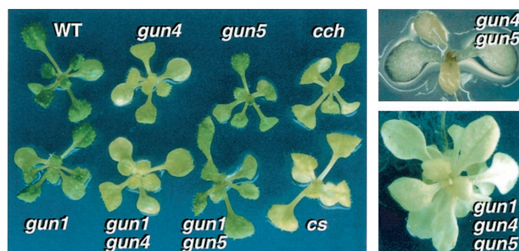

Fig. 2. Comparison of the leaf color of wild-type, *gun*, *cch*, and *cs* plants. Two-week-old wild-type and mutant plants (*Left*), a 1-week-old putative *gun4gun5* double mutant (*Upper Right*), and a 1-month-old putative *gun1gun4gun5* triple mutant (*Bottom Right*). Mutants were grown in long-day conditions at fluence rates of 50 μmol/m^2/sec.

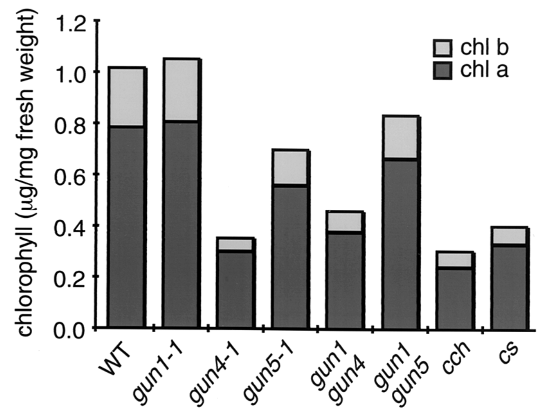

Fig. 3. Comparison of chlorophyll levels in wild-type, *gun*, *cch*, and *cs* mutants. Average chlorophyll content of wild-type and mutant plants. Chlorophyll "a" is presented as a black bar and Chlorophyll "b" as a gray bar. $n = 4$.

mutation can impair chloroplast development under some conditions (29). These results suggest that there are at least two separate but partially redundant pathways for plastid-to-nucleus signal transduction and that *GUN1* affects a separate pathway from *GUN4* and *GUN5*. Also, the *GUN4*/*GUN5* pathway is required for proper chlorophyll accumulation.

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gun5-1 and cch Are Defective in Mg-Protoporphyrin IX Synthesis. Because *gun5* and *cch* have pale phenotypes, we decided to search for defects in the chlorophyll biosynthetic pathway. Potential bottleneck steps in chlorophyll biosynthesis were determined by measuring chlorophyll precursor levels after feeding dark-grown seedlings with ALA and DP (ref. 20 and Fig. 4*A*). ALA is an early precursor, and DP inhibits ferrochelatase isocyclic ring formation and causes MgProto(ME) accumulation in dark-grown plants (30). The *cs* mutant, which has a lesion in the ChlI subunit of Mg-chelatase, produced approximately 74% of the MgProto(ME) levels as wild type (cf. ref. 20 and Fig. 4*B*). *cch* made only approximately 28% of the MgProto(ME) that was synthesized by wild type, and *cch* accumulated more Proto than wild type, which suggests that there is a defect in Mg-chelatase in this mutant. MgProto(ME) levels were not reduced significantly in *gun5*, and both *gun5* and *cs* produced less Proto than wild type (Fig. 4*B*). *gun5* has a weak chlorophyll phenotype (Figs. 2 and 3), which may explain why a significant difference in MgProto(ME) levels was not detected between *gun5* and wild type with this assay.

Cloning of GUN5/CCH. We crossed *gun5* to *L. erecta* to create an F_2 mapping population. F_2 plants were grown on Nf under continuous bright light, and *gun5* mutants were selected by the semiquantitative GUS assay. Then *gun5* F_2 recombinants were subjected to genetic-linkage analysis by using PCR-based markers as described in *Materials and Methods*. In the initial mapping effort, *GUN5* was localized to the interval between mi174 and CHS (<0.1 centimorgans) on chromosome 5. Previous work by Falbel and Staehelin had suggested that mutants in several crop species with phenotypes similar to *cch* might have reduced Mg-chelatase activity (20). On the basis of this work, we decided to focus our cloning efforts on the genes that encode the subunits of Mg-chelatase. Mg-chelatase is composed of three subunits, which are commonly referred to as ChlD, ChlH, and ChlI. Because the sequence information was available only for *CHLI* and *CHLH* when we mapped *GUN5*, and because *CHLI* was mapped on chromosome 4, we tested chromosome 5 yeast-artificial chromosomes and P1 clones from the interval for the

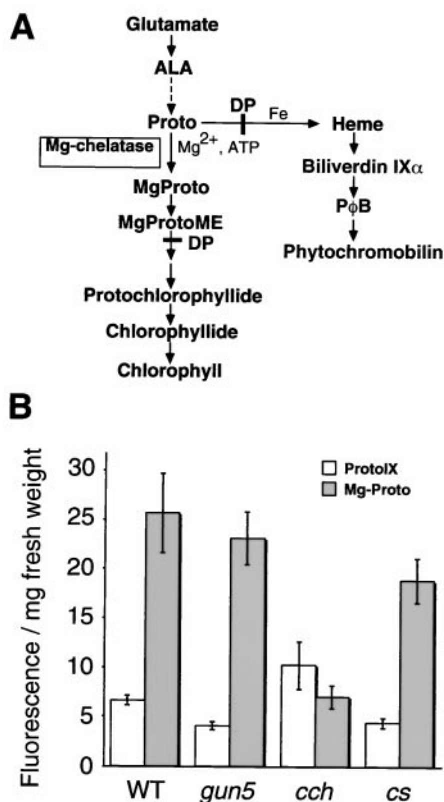

Fig. 4. Proto and MgProto levels in ALA–DP-fed plants. (*A*) Schematic diagram of chlorophyll biosynthetic pathway. Dashed arrows represent multiple steps, and the steps that are inhibited by DP are indicated by solid lines across the arrows. (*B*) Proto and MgProto accumulation in seedlings fed with ALA–DP. Etiolated seedlings (4 days old) were fed with ALA and DP, and porphyrin levels were measured as described in *Materials and Methods*.

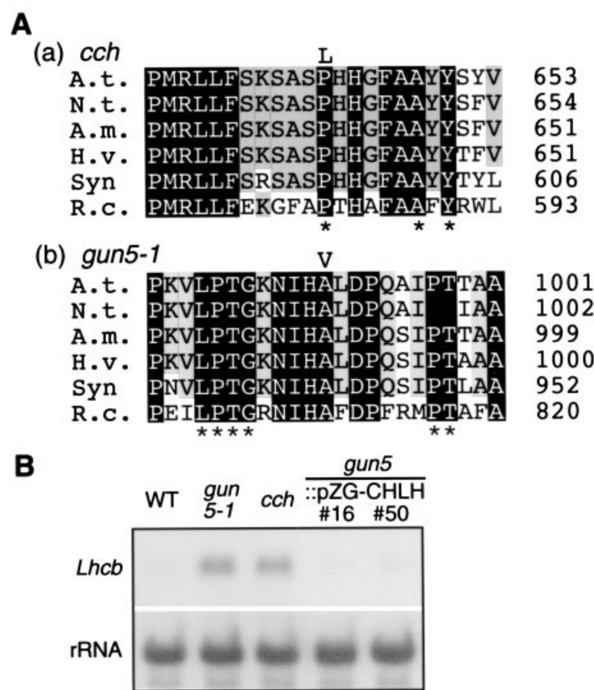

Fig. 5. Cloning of *GUN5/CCH*. (*A*) Partial amino acid sequence alignment of ChlH around the mutation site of (*a*) *cch* and (*b*) *gun5*. The substituted residues in the mutants are indicated above the aligned sequences. The derived amino acid sequences of *Arabidopsis thaliana* ChlH, *Nicotiana tabacum* ChlH, *Antirrhinum majus* ChlH, *Hordeum vulgare* ChlH, *Synechocystis* sp. PCC6803 ChlH, and *Rhodobacter capsulatus* BchH are shown. Conserved residues in six ChlH/BchH and in the ChlH counterparts of Co-chelatase CobN from *Paracoccus denitrificans*, putative Co-chelatase from *Methanococcus jannaschii*, and Ni-chelatase from *Synechocystis* PCC 6803 are marked by asterisks. (*B*) The wild-type *CHLH* gene rescues *gun5*. *Lhcb* mRNA levels were measured as described in Fig. 1*B* from two representative *gun5* lines (#16 and #50) that were transformed with pZG-CHLH.

presence of the *CHLH* gene by using PCR. We obtained a specific amplification of the *CHLH* sequence when CIC7F6, CIC5H3 and MSH12 were used as PCR templates (data not shown). We then amplified the *CHLH* region (8.5 kb) containing 5′-upstream sequence (2 kb), all the exons and introns, and 3′-sequence (1.9 kb) from *Columbia*, *gun5*, and *cch* and sequenced the products. Both *gun5* and the *cch* mutants have nucleotide substitutions in the third exon. In *gun5*, the change is from a C to a T, resulting in an A990V mutation. In *cch*, another C to T substitution results in a P642L mutation (Fig. 5*A*). Both amino acid substitutions reside in the region conserved among all of the reported ChlH subunits, and P642 is also conserved in Co-chelatase CobN from *P. denitrificans* (Fig. 5*A*).

The *gun5* phenotype was rescued by introducing the wild-type *CHLH* gene into the *gun5* mutant, as described below. We subcloned a 9.1-kb *Bam*HI–*Xba*I fragment from MSH12 that contains the genomic sequence of *CHLH* into pPZP221. The resulting construct, pZG–*CHLH*, was introduced into *gun5-1* by *Agrobacterium*-mediated vacuum infiltration (31). Gentamicin-resistant plants (T1 generation) were self-fertilized, and their progeny (T2 generation) were tested for the zygosity of transferred-DNA (T-DNA) insertion. T2 and T3 generations that were homozygous for T-DNA insertion (five independent lines) were subsequently tested for the *gun* phenotype. These plant lines showed reduced (i.e., wild-type) levels of *Lhcb1* mRNA accumulation in the presence of Nf (Fig. 5*B*). Therefore, we conclude that the *gun5/cch* phenotype is caused by defects in the *CHLH* gene.

chlI Subunit Mutants Are Not *gun* Mutants. Our analysis of *gun2*, *-3*, *-4*, and *-5* indicates that perturbations in tetrapyrrole metabolism uncouple Lhcb expression from chloroplast development and suggest that Proto and/or MgProto(ME) levels might be responsible for the *gun* phenotype. To test this hypothesis, we measured *Lhcb1* mRNA levels in photobleached *cs* and *ch42* mutants. *cs* is an insertional mutant in which the last four residues of ChlI are replaced with 11 new residues (15), and the chlorophyll phenotype of *cs* is comparable to *cch* (Figs. 2 and 3). *ch42* is an x-ray generated allele of *chlI* that is completely albino (14). Because *ch42* homozygotes were unable to grow without sucrose, we used albino progeny derived from *CH42/ch42* heterozygous parents for this analysis. Unlike *cch* and the *gun* mutants, *cs* and *ch42* *Lhcb1* mRNA levels were comparable to wild type in the presence of Nf (Fig. 6 *A* and *B*). Thus, plastid-to-nucleus signaling is fully functional in *cs* and *ch42*, even though *cs* has less Mg-chelatase activity than *gun5* (Fig. 4*B*), and the chlorophyll phenotype of *ch42* is severe. These data strongly suggest that modulation of Proto and/or MgProto(ME) levels is not sufficient to derepress *Lhcb1* genes in photobleached seedlings and that ChlH is an interorganelle signaling molecule.

Discussion

Here we show that the *GUN5/CCH* gene encodes the ChlH subunit of Mg-chelatase, which is a key enzyme in the chlorophyll branch of the tetrapyrrole biosynthetic pathway. We also found that ChlI subunit of Mg-chelatase is not necessary for plastid signal transduction, because the *chlI* (*cs* and *ch42*)

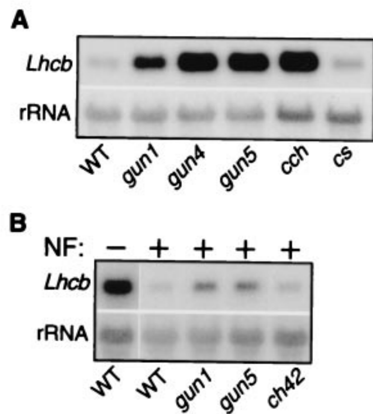

Fig. 6. Lhcb1 mRNA accumulation in photobleached pOCA107–2 (WT), *gun*, *cch*, *cs*, and *ch42* mutants. (*A*) *cs* is not a *gun* mutant. Wild-type and mutant plants were photobleached for 6 days as described in *Materials and Methods*, and *Lhcb1* mRNA levels were determined as described in Fig. 1*B*. (*B*) *ch42* is not a *gun* mutant. Seedlings were grown on Murashige–Skoog media for 3 days. After 3 days, albino *ch42* homozygotes, wild-type, and *gun* mutant seedlings were transferred to media containing Nf and grown for 4 days under strong continuous light. *Lhcb1* mRNA levels were determined as described in *Materials and Methods*.

mutants do not exhibit a *gun* phenotype. Thus, our results provide strong genetic evidence in support of earlier studies that suggest that chlorophyll precursors may function as signal molecules in the plastid-to-nucleus signal transduction pathway; however, they also point to a specific role for the ChlH protein in this signaling pathway.

Multiple Signaling Pathways from Plastids to the Nucleus. At present, we have identified five loci that uncouple *Lhcb1* transcription from chloroplast development. Double-mutant studies suggest that *GUN1* might function in a pathway that is separate from but partially redundant with the *GUN4/GUN5* pathway. Strong enhancements of the *gun* phenotype were observed in *gun1gun4* and *gun1gun5* double mutants. The putative *gun4gun5* double mutant did not exhibit such an enhanced *gun* phenotype despite a drastic reduction in chlorophyll accumulation. There is another difference between the mutants: *gun1* is defective in greening after a prolonged period of dark growth, whereas neither *gun4* nor *gun5* has such a defect (refs. 11 and 29, and N.M., unpublished data). Therefore, it is likely that there are at least two independent *GUN* pathways, one that is composed of *GUN1* and the other composed of *GUN4* and *GUN5*. However, it is possible that *GUN1* and *GUN2*, *-3*, *-4*, and *-5* function in the same pathway as our results, which might be due to the synthetic enhancement of leaky mutant combinations (32). Lastly, it should be noted that chloroplasts also regulate *Lhcb* genes with a light-intensity signal that is mediated by the redox status of plastoquinone (33), thereby suggesting yet a third pathway of signaling from plastids to the nucleus.

Tetrapyrroles as Signals from the Plastid. We have found that *gun2* and *gun3* are alleles of *hy1* and *hy2*, respectively, and that *hy1-1* and *hy2-1* (28) are *gun* mutants. *hy1-6.2*, a null allele (34), is also a *gun* mutant (48). *HY1* and *HY2* are required for the synthesis of phytochromobilin (PΦB) from heme. *HY1* encodes a heme oxygenase that converts heme to biliverdin IXα (BV; refs. 35 and 36), and *HY2* is thought to encode a PΦB synthase that converts BV to 3(Z)-PΦB (37). Chlorophyll synthesis is repressed in *gun2* (R.L., unpublished results), *hy1*, *hy2*, the corresponding mutants of tomato and pea (ref. 38 and M. Terry, personal communication), and in transgenic plants that attenuate PΦB synthesis by targeting mammalian biliverdin IXα reductase to the plastids (39). Heme, PΦB, and chlorophyll are derived from a group of common precursors in the chloroplast. Repression of chlorophyll synthesis in these mutants is thought to be mediated by repressive effects of heme (ref. 38 and M. Terry, personal communication) or other linear tetrapyrroles (39) on the synthesis of early tetrapyrrole precursors. *hy1gun4* and *hy1gun5* double mutants have albino phenotypes that resemble the *gun4gun5* double mutant (48). Because *gun2* is an allele of *hy1*, and because *gun2* and *gun3* affect chlorophyll accumulation like *gun4* and *gun5*, it is likely that *gun2–gun5* mutations affect the same plastid-to-nucleus signaling pathway by perturbing tetrapyrrole synthesis.

One technical concern is that *gun2–gun5* are not completely photobleached by Nf. Nf inhibits chloroplast development by blocking carotenoid synthesis. Carotenoids quench excited triple states of chlorophyll, which can interact with molecular oxygen and produce reactive oxygen species. It is possible that there is less photooxidative damage in the photobleached plastids of chlorophyll-deficient mutants, but two observations indicate that inefficient photobleaching is not the cause of the *gun* phenotype in *gun2–gun5*. First, *Lhcb1* expression is derepressed in these mutants when chloroplast development is blocked with chloramphenicol (N.M., unpublished results), which inhibits chloroplast translation. Second, the *Arabidopsis* chlorophyll-deficient mutants *cs*, *ch42*, *ch1-2*, and *ch5-1* are not *gun* mutants (Fig. 6 and N.M., unpublished results).

The ChlH Subunit in Chlorophyll Biosynthesis and Plastid Signaling. We found that the *GUN5/CCH* gene encodes the BchH/ChlH subunit of Mg-chelatase. The subunit varies in size (123–154 kDa among different species), binds to Proto, and is thought to be largely responsible for catalysis. The two smaller subunits of Mg-chelatase, ChlI and ChlD, form an ATP-dependent complex that associates with ChlH–Proto and stimulates this reaction. After Mg insertion, the complex is thought to dissociate into ChlH–MgProto and ChlI-ChlD–MgADP (40). The residues that are altered in *gun5* and *cch* (A990 and P642, respectively) are in highly conserved regions, and P642 is even conserved in Co-chelatase and Ni-chelatase (Fig. 5*A* and ref. 40). Although these substitutions have dramatically different effects on chlorophyll synthesis, the *gun* phenotypes of these alleles are very similar. The functional topography of ChlH has not been determined, but our results indicate that catalytic and signaling functions of ChlH can be uncoupled.

Plastid Signal Transduction: A Model. Feeding and inhibitor experiments have implicated intermediates in the chlorophyll biosynthetic pathway, especially Proto(ME), in plastid-mediated repression of *Lhcb* genes in *Chlamydomonas* and cress seedlings (9, 41, 42), and Proto(ME) feeding activates nuclear heat-shock genes through a light-responsive promoter element in dark-grown *Chlamydomonas* cultures (10, 49). MgProto(ME) levels should be reduced in *gun2–gun5* and *cch*, which is consistent with MgProto(ME) acting as a plastid signal in *Arabidopsis*. However, MgProto(ME) levels are reduced also in the ChlI subunit mutants *cs* and *ch42*, which are not *gun* mutants. Also, Proto and MgProto(ME) are below the limits of detection in *Arabidopsis* seedlings that have been treated with Nf for 6 days (N.M., unpublished results). Also, over- or underexpressing the ChlH and the ChlI subunit in tobacco has pleiotropic effects on tetrapyrrole metabolism, including inhibition of Mg-chelatase activity and suppression of early tetrapyrrole biosynthetic enzyme levels (43, 44). However, ChlH subunit levels were not affected by altered ChlI levels in these studies, and therefore we expect that *cs* and *ch42* mutants might contain wild-type levels of the ChlH subunit.

Together, these results suggest a model (Fig. 7). We propose that ChlH measures the flux at the beginning of the chlorophyll branch of the plastid tetrapyrrole biosynthetic pathway and sends information about the rate of chlorophyll synthesis to the nucleus. ChlH might exist in free, Proto-bound, or MgProto-

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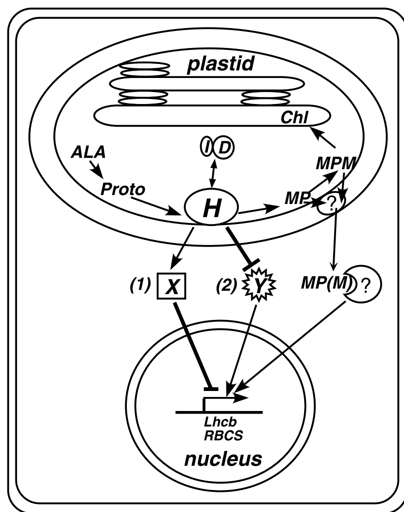

Fig. 7. Model for plastid signaling mediated by the ChlH subunit of Mg-chelatase. The details of the model are explained in the text. "X" and "Y" represent a putative repressor and an activator of *Lhcb1* transcription, respectively. MP, MgProto; MPM, MgProtoMe.

bound states, and ChlI and ChlD might interact with each of these ChlH forms. Each of these ligands might affect the signaling activity of ChlH. *gun2*, *-3*, and *-4* might affect the signaling activity of ChlH indirectly by modulating the levels of one or more of these ligands or by altering ChlH levels. In contrast, *cs* and *ch42* mutations may not reduce the levels of MgProto(ME), ChlD, or ChlH below a threshold that is required to block the signaling activity of ChlH and uncouple *Lhcb1* expression from chloroplast development.

Mg-chelatase has been found in the inner envelope of chloroplasts in the presence of 5 μM Mg^{2+}, which is within the estimated range of chloroplast Mg^{2+} concentrations (45), although the enzyme is also localized in the stroma at lower Mg^{2+} concentrations (27, 46). Some steps in chlorophyll biosynthesis including MgProto(ME) production are presumed to occur on the inner envelope. Thus, the chloroplast inner envelope seems like a reasonable location for a receptor or a sensor of chlorophyll precursors. ChlH might monitor porphyrin levels by binding excess Proto and/or MgProto and (*i*) send a negative signal or (*ii*) inhibit a positive signal to the nucleus via a hypothetical downstream factor(s) (Fig. 7). Proto and/or MgProto(ME) synthesis might be out of balance with the plastid's needs when the plastid is damaged by reactive oxygen species, as described in these experiments, or when the levels of chlorophyll precursors fluctuate in response to internal and external stimuli (47). If MgProto(ME) is exported from the chloroplast as proposed by Kropat *et al.* (10, 49), another factor(s) probably mediates the transduction of this porphyrin signal from the chloroplast inner envelope to the cytosol (Fig. 7). It is possible also that very small amounts of ChlH are transported across the chloroplast envelope and interact with porphyrins that are released from the chloroplast.

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