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Light-Independent Chlorophyll Biosynthesis: Involvement of the Chloroplast Gene *chlL* (*frxC*)

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The *Chlamydomonas reinhardtii* chloroplast gene *chlL* (*frxC*) is shown to be involved in the light-independent conversion of protochlorophyllide to chlorophyllide. The polypeptide encoded by *chlL* contains a striking 53% amino acid sequence identity with the bacteriochlorophyll (bch) biosynthesis *bchL* gene product in the photosynthetic bacterium *Rhodobacter capsulatus*. In a previous analysis, we demonstrated that *bchL* was involved in light-independent protochlorophyllide reduction, thereby implicating *chlL* in light-independent protochlorophyllide reduction in photosynthetic eukaryotes. To perform a functional/mutational analysis of *chlL*, we utilized particle gun-mediated transformation to disrupt the structural sequence of *chlL* at its endogenous locus in the chloroplast genome of Chlamydomonas. Transformants for which the multicopy chloroplast genome was homoplasmic for the disrupted *chlL* allele exhibit a "yellow-in-the-dark" phenotype that we demonstrated to be a result of the dark accumulation of protochlorophyllide. The presence of a *chlL* homolog in distantly related bacteria and nonflowering land plants, which are thought to be capable of synthesizing chlorophyll in the dark, was also demonstrated by cross-hybridization analysis. In contrast, we observed no cross-hybridization of a probe of *chlL* to DNA samples from representative angiosperms that require light for chlorophyll synthesis, in support of our conclusion that *chlL* is involved in light-independent chlorophyll biosynthesis. The role of *chlL* in protochlorophyllide reduction as well as recent evidence that both light-independent and light-dependent protochlorophyllide reductases may be of bacterial origin are discussed.

INTRODUCTION

In angiosperms, a key regulatory step for chlorophyll biosynthesis is the *trans*-reduction of ring D in protochlorophyllide to form chlorophyllide (Rüdiger and Schoch, 1988). This reaction has been extensively studied in barley, oat, and wheat, among others, where it has been shown to involve the nuclear-encoded chloroplast enzyme protochlorophyllide oxidoreductase (PCR). PCR is known to require light energy for catalysis; hence, the dependence on light for protochlorophyllide reduction is thought to be a major contributing factor in the requirement for light by angiosperms for chlorophyll biosynthesis (Griffiths, 1978; Apel et al., 1980; reviewed in Castelfranco and Beale, 1983; Schulz et al., 1989). In contrast, it has long been recognized that a number of photosynthetic eukaryotes such as gymnosperms and algae, as well as photosynthetic prokaryotes, synthesize an enzyme that reduces protochlorophyllide irrespective of light, thereby providing these organisms the ability to synthesize chlorophyll in the dark (Bogorad, 1950; Lascelles, 1960; Nikolič and Bogdanovič, 1972; Oku et al., 1974; Laudi and Manzini, 1975; Michel-Wolwertz, 1977; Dring, 1988). However, only a few studies on light-independent protochlorophyllide reductase activity have been published (Peschek et al., 1989a, 1989b).

Even though it is generally recognized that gymnosperms and algae have the ability to synthesize chlorophyll in the dark, there is accumulating evidence that many of these "dark-greening" eukaryotes synthesize both light-independent and light-dependent versions of protochlorophyllide reductase. For example, studies with pine have shown the existence of a protein immunologically related to the light-dependent PCR. This protein is found in dark-grown cotyledons, where it forms phototransformable complexes with protochlorophyllide, thereby suggesting that it functions in a manner analogous to the light-dependent PCR from angiosperms (Seltsam et al., 1987; Ou et al., 1990). In addition, genetic analysis with *Chlamydomonas reinhardtii* (Wang, 1979; Ford and Wang, 1980a, 1980b; Ford et al., 1981, 1983) as well as with *Scenedesmus obliquus* and a number of other algae (Senger and Brinkmann, 1986) have demonstrated the existence of both light-independent and light-dependent forms of protochlorophyllide reductase. Thus, it appears that many of these dark-greening organisms have both light-dependent and light-independent routes for chlorophyll biosynthesis.

In contrast to the limited information that exists on genes involved in chlorophyll biosynthesis of plants and algae, most of the genes involved in photopigment biosynthesis in the photosynthetic prokaryote *Rhodobacter capsulatus* have been genetically identified and sequenced (Taylor et al., 1983; Youvan

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et al., 1984; Zsebo and Hearst, 1984; Yang and Bauer, 1990). This bacterium synthesizes a tetrapyrrole known as bacteriochlorophyll *a* that is structurally and functionally related to chlorophyll *a* (reviewed in Beale and Weinstein, 1991). Indeed, genetic and biochemical analyses have demonstrated that chlorophyll *a* and bacteriochlorophyll *a* biosynthetic pathways both involve similar intermediates (Griffiths, 1975; Beale and Weinstein, 1991). Because it appears that these pathways are related, we felt that there was a strong possibility that genes involved in photopigment biosynthesis would be evolutionally conserved among prokaryotic and eukaryotic organisms. Indeed, we demonstrated in this study that the chloroplast of Chlamydomonas contains a gene (*chlL*) that exhibits significant amino acid sequence identity to gene products of *bchL* of *R. capsulatus* and *frxC* of the filamentous cyanobacterium *Plectonema boryanum*, genes that have been shown in previous studies to be involved in light-independent conversion of protochlorophyllide to chlorophyllide (Yang and Bauer, 1990; Fujita et al., 1992). To verify that the chloroplast-encoded *bchL* homolog has a similar function, we constructed a site-directed mutational disruption of *chlL* in Chlamydomonas using particle gun–mediated transformation. The resulting strain exhibits a "yellow-in-the-dark" phenotype that we demonstrated to be a result of the mutant's inability to reduce protochlorophyllide in the dark. We also demonstrated by cross-hybridization analysis the presence of *chlL*-like sequences in numerous dark-greening bacteria and nonflowering land plants and the absence of cross-hybridizing fragments in a number of angiosperms, a group of plants that require light for greening under normal growth conditions.

RESULTS

Identification and Characterization of *chlL*: A Chlamydomonas Chloroplast Gene Homolog of *bchL*

Previous studies have provided mutational evidence that bacterial homologs of the chloroplast *frxC* gene, originally described from liverwort (Kohchi et al., 1988; Ohyama et al., 1988b; Fujita et al., 1989), are involved in light-independent protochlorophyllide reduction (Yang and Bauer, 1990; Fujita et al., 1992). The goal of this study was therefore to perform a molecular genetic analysis to determine whether the chloroplast-encoded *frxC* was also involved in light-independent protochlorophyllide reduction. Toward this goal, we attempted to discern whether the transformable alga Chlamydomonas (Boynton et al., 1988; Newman et al., 1990; Kindle et al., 1991) contained *frxC* within its chloroplast genome and, if so, to create a chloroplast gene disruption in this species. The results

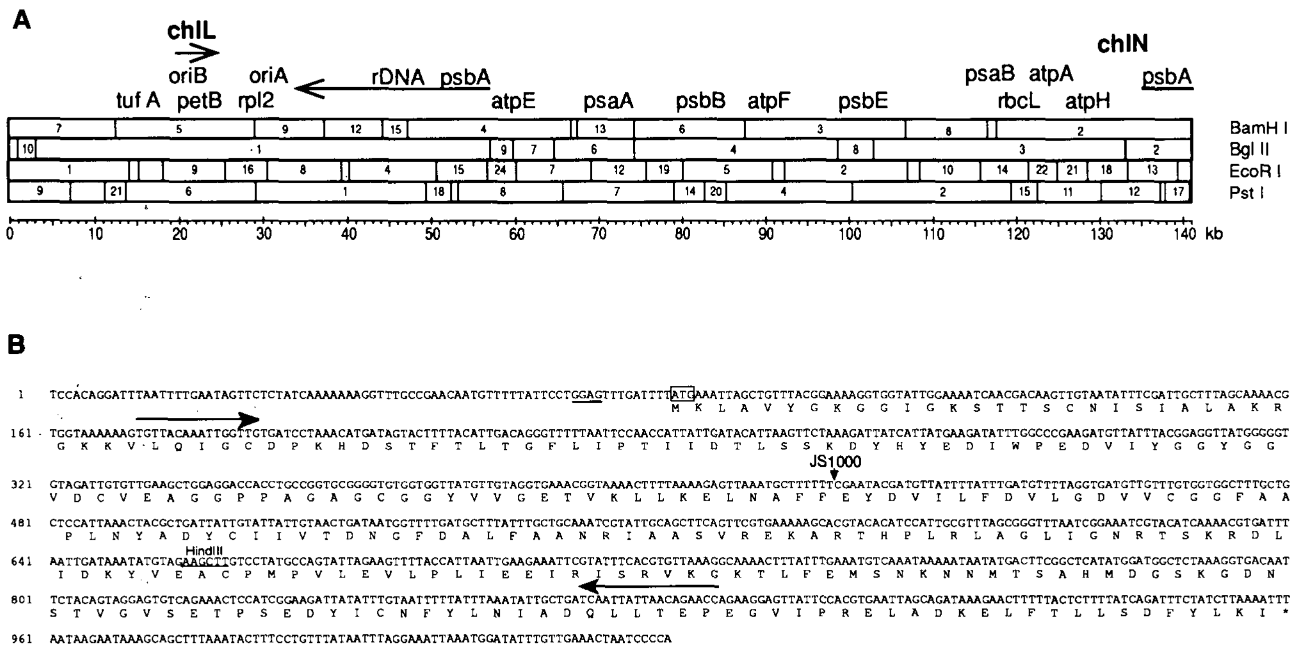

Figure 1. Location and Sequence Analysis of *chlL*.

(A) Position and orientation of *chlL*, homolog of the liverwort gene *frxC*, on a partial chloroplast restriction map of Chlamydomonas. *chlL* is located entirely within the EcoRI-9 fragment. The map was redrawn from Harris (1990).

(B) Primary nucleotide and deduced amino acid sequence of *chlL*, including flanking regions. The putative initiation codon is boxed, and a candidate ribosome binding site is underlined. The position marked JS1000 denotes the site in *chlL* that was disrupted in this study. Arrows mark the positions of polymerase chain reaction primers (see Methods). The *chlL* sequence has been submitted to EMBL as accession number X60490.

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                                                                 ATP Binding
ChlL  Chlamydomonas                                            MKLAVYGKGGIGKS
FrxC  Liverwort                                                MKIAVYGKGGIGKS
FrxC  Lodgepole Pine                                           VKIAVYGKGGIGKS

FrxC  P. boryanum                                              MKLAVYGKGGIGKS
BchL  R. capsulatus  MSPRDDIPDLKGFDGDGEGSVQVHDSEDIGLDVGGARVFSVYGKGGIGKS
                                                                **********

TTSCNISIALAKRGKKVLQIG C DPKHDSTFTLTGFLIPTIIDTLSSKDYHYEDIWPEDVIYG
TTSCNISIALARRGKKVLQIG C DPKHDSTFTLTGFLIPTIIDTLQSKDYHYEDVWPEDVIYK
TTSCNISVALARRGQKVLQIG C DPKHDSTFTLTGFLIPTIIDTLQSKDYHYEDIWPEDVIHK

TTSCNISVALAKRGKKVLQIG C DPKHDSTFTLTGFLIPTIIDTLQEKDYHYEDVWAEDVIYK
TTSSNLSAAFSLLGKRVLQIG C DPKHDSTFTLTGRLQETVIDILKQVNFHPEELRPEDYVTE
*** * * *     *  ***** * ************ *  * ** *     * *    **

GYGGVD C VEAGGPPAGAG C GGYVVGETVKLLKELNAFFEYDVILFDVLGDVV C GGFAA
GYGRCD C VEAGGPPAGAG C GGYVVGETVKLLKELNAFYEYDIILFDVLGDVV C GGFAA
GYGGVD C VEAGGPPAGAG C GGYVVGETVKLLKELNAFYEYDIILFDVLGDVV C GGFAA

GYGGVD C VEAGGPPAGAG C GGYVVGETVKLLKELNAFDEYDVILFDVLGDVV C GGFAA
GFNGVM C VEAGGPPAGTG C GGYVVGQTVKLLKQHHLLEDTDVVVFDVLGDVV C GGFAA
*      * ********* * • ****** ******          *   ******** * *****

PLNYADYCIIVTDNGFDALFAANRIAASVREKARTHPLRLAGLIGNRTSKRDLIDKYVEACPMP
PLNYADYCIIITDNGFDALFAANRIAASVREKARTHPLRLAGLVGNRTSKRDLIDKYVEACPMP
PLNYADYCVIITDNGFDALFAANRITASIREKARTHPLRLAGLVGNRTSRRDLINKYVEACPMP

PLNYADYCMIVTDNGFDALFAANRIAASVREKARTHPLRLAGLIGNRTAKRDLIEKYVDAVPMP
PLQHADRALIVTANDFDSIYAMNRIIAAVQAKSVNYKVRLAGCVANRSRETNEVDRYCEAANFK
**  **   * * * **   * *** *   *      ****   **            *  *

VLEVLPLIEEIRISRVKGKTLFEMSNKNNMTSAHMDGSKGDNSTVGVSETPSEDYICNFYLNIA
VLEVLPLIEDIRVSRVKGKTLFEM.....................VELQPSLKYVCDFYLNIA
VIEVLPIIEDIRVSRVKGKTLFEM.....................VGSEPSLNYVCNYYLGIA

ILEVLPLIEDIRVSRVKGKTLFEM.....................AESDPSLNYVCDYYLNIA
RIAHMPDLDSIRRSRLKKRTLFEM.....................DDAEDVVMARA.EYIRLA
     *    ** ** *  *****                                  *   *

DQLLTEPEGVIPRELA..DKELFTLLSDFYLKI*
DQILSKPEGIIPKEVP...RELFSLLSDFYLNPVNTVNEKNKPNLIDFMII*
DQILSQPEGIVPKEIP..DRELFSLLSDLYLNPIGGGGQKKNKKEILLGFTRI*

DQILANPEGVVPKDAA..DRDLFSLLSDFYLNPQQPKTAEEELDLMMV*
ETLWRSTGEPGLTPEPLTDRHIFELLGFD*
                          * **
```

Figure 2. Sequence Similarity of ChL to Bacterial and Plant Homologs.

The deduced amino acid sequence of ChlL from Chlamydomonas is aligned with FrxC from liverwort, lodgepole pine, and *P. boryanum* and BchL of *R. capsulatus*. Identical residues shared among the five protein sequences are indicated with asterisks. Regions containing additions or deletions are indicated by periods. The ChlL homologs share a conserved ATP binding motif (GXXXXGKS) as well as cysteine residues (boxes) found in the same relative positions as in NifH (dinitrogenase reductase), a subunit of the prokaryotic nitrogen-fixing enzyme nitrogenase (Table 1). Conserved cysteine residues that are functional for Fe-S binding in NifH are indicated by shaded boxes.

of a DNA gel blot of the Chlamydomonas chloroplast genome using the *frxC* gene from liverwort as a probe demonstrated that a region of the *C. reinhardtii* genome exists that exhibits strong cross-hybridization with *frxC* (J. Y. Suzuki, unpublished results). By comparing the size pattern of various cross-hybridizing restriction fragments to restriction maps available for the Chlamydomonas chloroplast genome, we were able to localize the area of cross-hybridization to the central region of the EcoRI-9 (Harris, 1990) fragment, as shown in Figure 1A. Sequence analysis of a subclone of the EcoRI-9 fragment revealed that it contained an open reading frame (ORF) that encodes a polypeptide of 293 residues (Figure 1B), which, as shown in Figure 2 and Table 1, exhibits 89% identity to FrxC of liverwort, 85% identity to FrxC of lodgepole pine (Lidholm and Gustafsson, 1991), 86% identity to FrxC of *P. boryanum*, and 53% identity to BchL of *R. capsulatus* (Youvan et al., 1984; Hearst et al., 1985; Yang and Bauer, 1990). (The Chlamydomonas *frxC* homolog is termed *chlL*, based on sequence and functional similarity to the bacteriochlorophyll biosynthesis gene *bchL*. Note also that a similar sequence was recently reported during the preparation of this manuscript [Huang and Liu, 1992].) Although ChlL exhibits a unique sequence near the carboxyl terminus causing a gap in alignment, this sequence results in overall size conservation as compared to its homologs. Conserved structural features among ChlL, FrxC, and BchL (Figure 2) that suggest that this polypeptide represents a subunit of the protochlorophyllide reductase enzyme will be covered more fully in the Discussion.

Site-Directed Disruption of *chlL* via Particle Gun Transformation

A plasmid-borne insertion mutation of *chlL* (pChlL::KanH) was constructed by ligating a kanamycin resistance (Km^r) and bleomycin resistance (Ble^r) gene cassette into the coding region of *chlL*, as shown in Figure 3. The Km^r, Ble^r gene cassette used in this experiment does not function as an effective selectable marker for chloroplast transformation; thus, a cotransformation procedure was performed to facilitate screening for recombinants. The gene disruption plasmid

Table 1. Percent Sequence Identity and Similarity among ChlL Homologs and NifH

	R. caps-BchL	*P. bory*-FrxC	*C. rein*-ChlL	*M. poly*-FrxC	*P. cont*-FrxC
P. bory-FrxC	51/70				
C. rein-ChlL	53/73	86/94			
M. poly-FrxC	51/71	84/92	89/95		
P. cont-FrxC	51/71	82/91	85/96	87/94	
R. caps-NifH	38/58	36/55	32/52	33/52	35/55

Numbers for the sequence comparison were calculated using the Bestfit program from the University of Wisconsin GCG sequence analysis software package. *R. caps*, *R. capsulatus* (R-caps-BchL, Yang and Bauer, 1990; R-caps-NifH, Jones and Haselkorn, 1988); P. bory, *P. boryanum* (Fujita et al., 1991); *C. rein*, Chlamydomonas (Figure 1B); *M. poly*, liverwort (Kohchi et al., 1988); *P. cont*, lodgepole pine (Lidholm and Gustafsson, 1991).

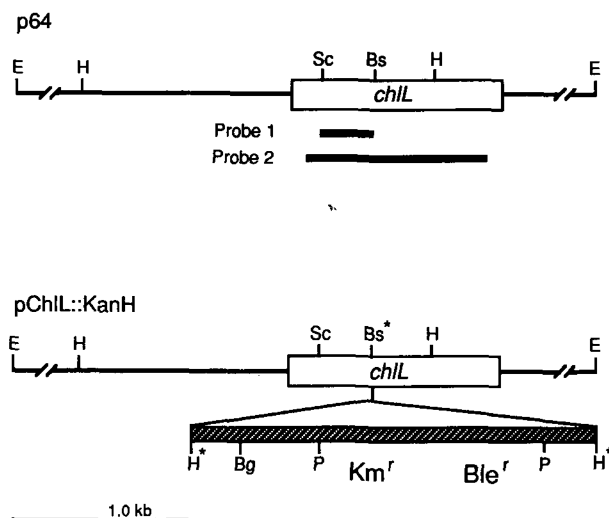

Figure 3. Diagram of the Wild-Type *chlL* Clone and Mutant *chlL* Construct.

Plasmid p64 is the 7.6-kb EcoRI-9 fragment of the Chlamydomonas chloroplast genome containing *chlL* cloned into the EcoRI site of pUC8. Plasmid pChlL::KanH is a derivative of p64 that was used for constructing the Km^r, Ble^r disruption of *chlL*. Relevant restriction sites are abbreviated as Bs, BstBI; Bg, BglII; E, EcoRI; H, HindIII; P, PstI; Sc, ScaI, with an asterisk denoting loss of a site as a result of cloning.

pChlL::KanH and the transformation selection vector p183, which contains a copy of the Chlamydomonas 16S and 23S chloroplast ribosomal DNA (rDNA) that confers spectinomycin resistance (Sp^r) and streptomycin resistance (Sm^r), were annealed to tungsten particles and subsequently bombarded onto wild-type Chlamydomonas strain 2137 as described by Newman et al. (1990). Transformants that were both Sp^r and Sm^r arose at a frequency of 3.3×10^{-6} using a 1-to-1 weight ratio of p183 to pChlL::KanH DNA, whereas a second bombardment using a 1-to-5 ratio resulted in a transformation frequency of 7.6×10^{-7}. Transformants were subsequently tested for cotransformation with the Km^r, Ble^r–disrupted copy of *chlL* by assaying for restriction fragment length polymorphism (RFLP) of the chloroplast genome. As diagrammed in Figure 3, wild-type Chlamydomonas has a 1.4-kb HindIII fragment that encodes the amino-terminal region of ChlL; in contrast, *chlL*::Km^r-Ble^r transformants should have an additional 1.6 kb of DNA inserted into this HindIII fragment.

Figure 4A shows an RFLP analysis of the Sp^r/Sm^r transformants using a restriction fragment containing *chlL* as a probe. The results demonstrate that 73 colonies (94%) from the first bombardment (Figure 4A, lanes 1 to 78) and 14 colonies (93%) from the second bombardment (Figure 4A, lanes 79 to 93) exhibited the presence of the predicted 3.0-kb HindIII RFLP corresponding to the mutated copy of *chlL* (an identical DNA gel blot with the Km^r, Ble^r cassette as a probe also hybridized to the 3.0-kb band; J. Y. Suzuki, unpublished results). Further inspection of the autoradiograph in Figure 4A, as well as a greatly overexposed copy (J. Y. Suzuki, unpublished results), shows that 59% (43) of the cotransformed cell lines from the first bombardment and 79% (11) of the cotransformed lines from the second bombardment were homoplasmic for *chlL*::Km^r-Ble^r, whereas the remaining cell lines had both wild-type and mutated copies of *chlL*. It is probable that the cell lines described in this experiment as heteroplasmic are in fact mixed colonies of segregants that are homoplasmic for either the wild-type or mutant gene. In any case, it is apparent that for the majority of samples, homoplasmicity to the mutant gene probably occurred early, by mechanisms previously described (Newman et al., 1991), because the analyzed DNAs were extracted from cell populations of the original drug-resistant colony and were not from cell lines restreaked for individual isolates.

An extensive analysis of one transformant (JS1000) from the first bombardment was undertaken by analyzing BamHI, BglII, PstI, EcoRI, and HindIII RFLP patterns at the *chlL* locus (Figure 4B). *chlL* hybridizing fragments from JS1000 contained the predicted 1.6-kb increase as compared with the wild-type using BamHI (19.5 to 21.2 kb), EcoRI (~8.0 to 9.7 kb), and HindIII digests (1.4 to 3.0 kb). In samples digested with BglII and PstI, a decrease in the hybridizing band size was observed in JS1000 compared with the wild-type from approximately 52 to 21.1 kb and 18.1 to 9.8 kb, respectively. This latter result is also consistent with the presence of the 1.6-kb Km^r, Ble^r gene cassette that, as diagrammed in Figure 3, introduces a BglII site and PstI restriction sites at the endogenous *chlL* locus in the chloroplast genome. These results indicate no apparent rearrangement of sequences flanking the endogenous disrupted *chlL* locus. In addition, JS1000 appears to be homoplasmic for the mutant *chlL* based on the absence of wild-type restriction fragments and based on polymerase chain reaction amplification products expected of mutant and not wild type–sized templates (J. Y. Suzuki, unpublished results). The results of this experiment demonstrated that site-directed disruption of a gene to the multivalent chloroplast genome by homologous recombination can occur at high frequencies and without direct selection. This allowed us to screen for the mutant phenotype of a chloroplast gene, *chlL* (*frxC*), of previously unknown function.

chlL Mutants Are Deficient in Light-Independent Chlorophyll Biosynthesis

Each of the Sp^r/Sm^r transformants from two bombardments were transferred onto Tris-acetate phosphate agar (TAP; Harris, 1989) plates and grown mixotrophically in the light or heterotrophically in the dark. In confirmation of the RFLP data, dark-grown cell lines that had the wild-type RFLP for the *chlL* locus were deep green, those cell lines that exhibited both wild-type and mutant RFLP patterns of *chlL* were light green, and yellow colonies were observed from dark-grown cell lines that exhibited only a mutant RFLP pattern for the *chlL* locus (J. Y. Suzuki, unpublished results).

As shown in Figure 5, JS1000, a strain that is homoplasmic for the *chlL* disruption, exhibits a pigment defect specifically under dark growth conditions; this phenotype is qualitatively identical to the yellow-in-the-dark phenotype that has been described for Y-5, a strain containing a nuclear mutation that disrupts light-independent protochlorophyllide reduction (Ford and Wang, 1980a). To further characterize the step in chlorophyll biosynthesis that was affected, pigments from wild-type, JS1000, and Y-5 cells were extracted into diethyl ether and scanned for fluorescence emission (Bednarik and Hoober, 1985; Roitgrund and Mets, 1990).

As shown in Figure 6, a wild-type strain transformed only with the transformation vector p183 exhibited a characteristic chlorophyll *a* and *b* emission spectrum with maxima at 666

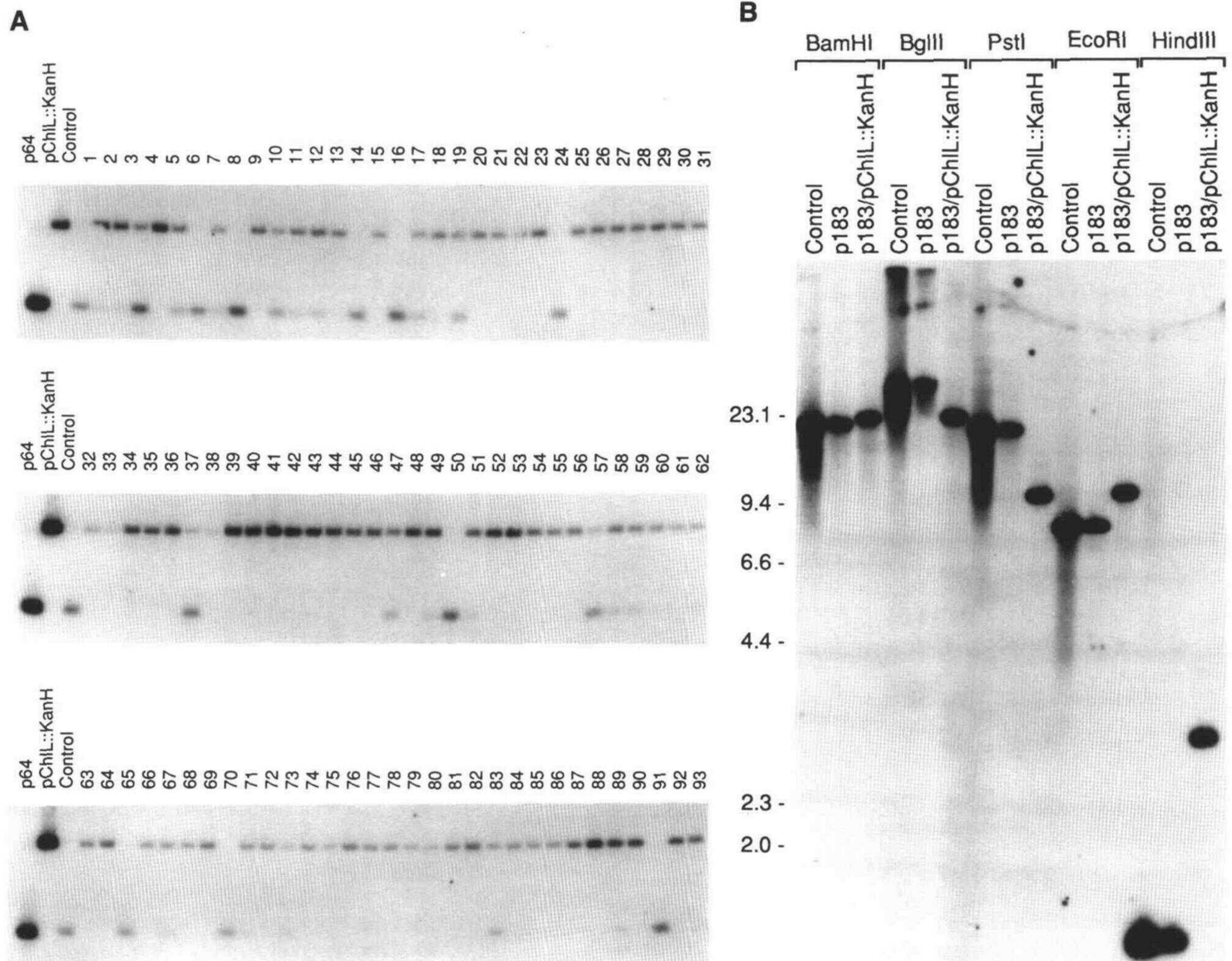

Figure 4. DNA Gel Blot RFLP Analysis of Transformants.

(A) DNA gel blot RFLP analysis of transformed (Sp^r/Sm^r) cell lines of Chlamydomonas strain 2137 obtained after bombardment with transformation vector p183 and pChlL::KanH. Purified plasmid DNA of wild-type and mutant *chlL* constructs (lanes marked p64 and pChlL::KanH, respectively) was run as RFLP markers along with total DNA from a cell line that was transformed with p183 only (lanes marked control). Total DNA from individual cell lines that were obtained from a single bombardment using equal weight ratios of transforming DNA p183 and pChlL::KanH is labeled 1 to 78, whereas a second bombardment using a 1:5 weight ratio of p183 to pChlL::KanH DNA resulted in samples in lanes 79 to 93. All DNA samples were digested with HindIII and fractionated on a 0.7% agarose gel. ^{32}P-labeled probe 1 (Figure 3) was used to analyze the size of the amino-terminal HindIII fragment of *chlL* in the transformants.

(B) Extensive RFLP analysis of DNA from JS1000, a cell line that is homoplasmic for the chloroplast *chlL*::Km^r-Ble^r disruption hybridized with ^{32}P-labeled probe 1 (Figure 3). Control denotes purified Chlamydomonas chloroplast DNA from wild-type cells. Lane p183 is total DNA from a colony that grew under spectinomycin and streptomycin selection after particle bombardment with the p183 vector only, whereas lane p183/pChlL::KanH denotes total DNA from cell line JS1000. Molecular size markers at left are given in kilobase pairs.

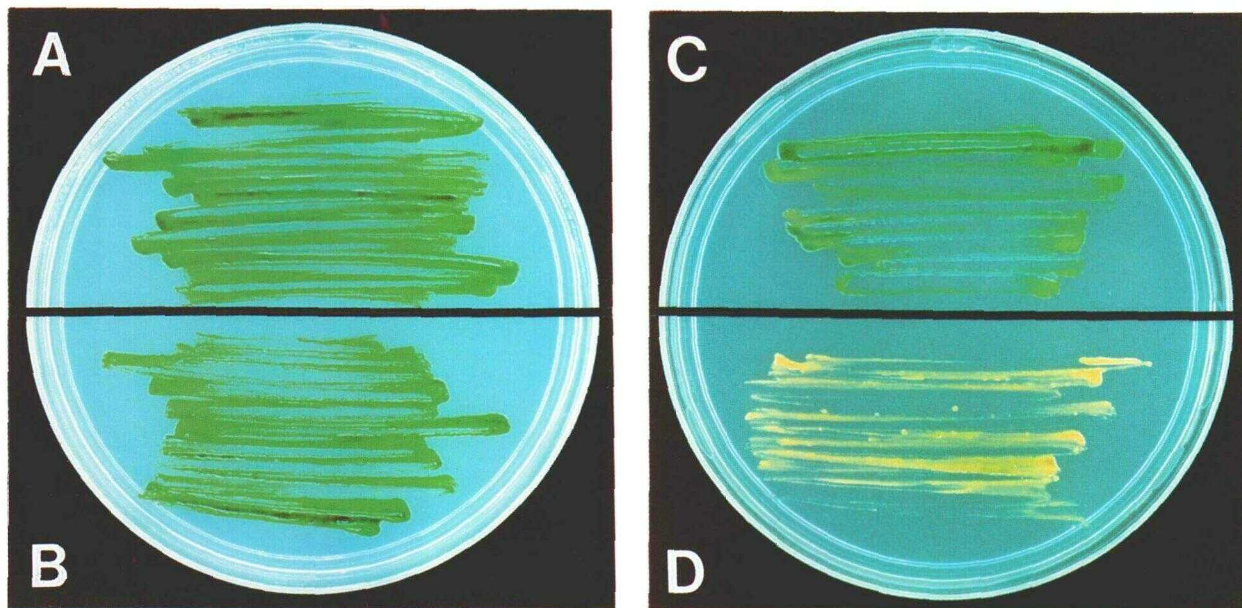

Figure 5. Phenotype of Transformants under Light and Dark Growth Conditions.

(A) Wild type grown in light.
(B) Cell line JS1000 grown in light.
(C) Wild type grown in dark.
(D) Cell line JS1000 grown in dark.
The wild-type strain shown is strain 2137 that was transformed with p183 only. Cultures were streaked from TAP agar plates to HSHA agar media and cultured for 9 days in the light (2000 to 3000 lux) or in dim light (0.5 lux).

and 648 nm, respectively (Rüdiger and Schoch, 1988), when grown either in the dark or light. In contrast, the *chlL*::Km^r-Ble^r cell line JS1000 accumulated a pigment that had a major fluorescence emission peak at 627 nm when grown in the dark; this emission peak is characteristic of protochlorophyllide (Bednarik and Hoober, 1985) and is identical to the major peak observed with dark-grown control strain Y-5. These results show that mutations in the chloroplast gene *chlL* result in a defect in the light-independent reduction of protochlorophyllide.

chlL Is Broadly Distributed among Photosynthetic Organisms

An attempt was made to correlate the ability of various organisms to undergo light-independent greening with the presence or absence of *chlL*. For this analysis, a DNA gel blot of total DNA from various organisms was hybridized with a *chlL* probe from Chlamydomonas. As shown in Figure 7, *chlL* hybridized to DNA preparations from the cyanobacteria *Anacystis nidulans* and *Synechococcus* sp (*Synechococcus* R2, PCC 7942), which, like other photosynthetic prokaryotes, do not require light for greening (Stanier and Cohen-Bazire, 1977). With one exception, cross-hybridizing bands were observed with DNA prepared from all nonflowering vascular plants tested, including the horsetail *Equisetum arvense* (field horsetail), the lycopsid *Selaginella willdenovii* (J. Y. Suzuki, unpublished results), the ferns *Pellaea glabella missouriensis* (Missouri cliff-brake), *Polystichum acrostichoides* (Christmas fern), *Cystopteris fragilis* (fragile fern), *Athyrium filix-femina* (lady fern), and the gymnosperms *Pseudotsuga menziesii* (Douglas fir), *Ginkgo biloba* (ginkgo), *Taxus* × *media* (yew), *Juniperus virginiana* (red cedar), and *Araucaria* sp. The cross-hybridizing sequences observed in the plant samples are believed to represent chloroplast sequences based on the amount of plant DNA loaded in each lane, an amount that was normalized for similar levels of chloroplast DNA. (Note that in most photosynthetic eukaryotes the chloroplast genomes are represented at levels that are typically several thousandfold that of the respective nuclear genomes; see Methods.) The surprising exception is the absence of cross-hybridization to *Psilotum nudum* (whisk fern), morphologically the simplest extant vascular land plant (Bold et al., 1980). An overexposure of the blot shown in Figure 7 (approximately three- to fourfold exposure) yielded qualitatively the same results (J. Y. Suzuki, unpublished results). Although we cannot rule out the possibility of major sequence divergence or transfer of *chlL* to the nucleus of Psilotum in this study, this organism also appears to lack the ability to green in the dark (Whittier, 1988), an observation that supports our cross-hybridization analysis.

We also failed to observe *chlL* hybridization with representative angiosperms maize, tobacco, Arabidopsis, and *Bougain-*

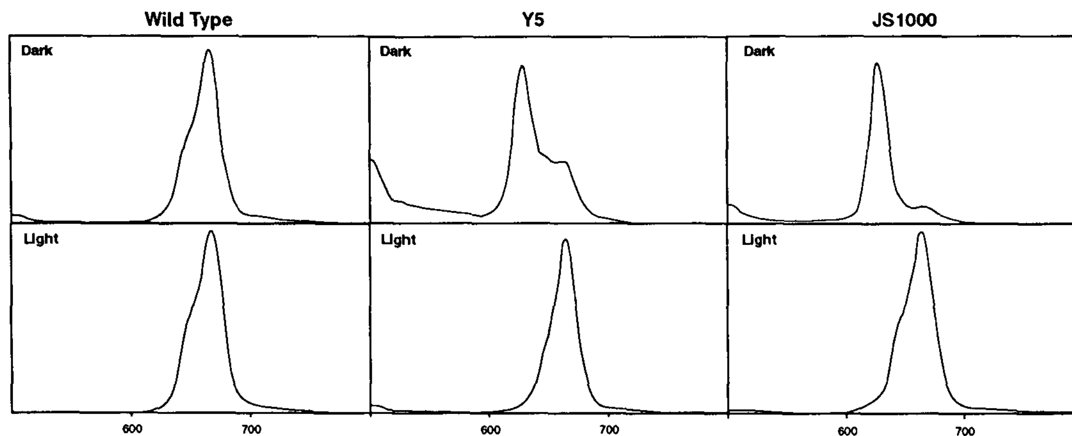

Figure 6. Spectral Analysis of Photopigments Produced by Transformants.

Fluorescence emission spectra of pigments extracted into diethyl ether from wild-type and mutant cell lines grown in total darkness (top panels) and in high light (bottom panels). The wild-type control cell line (strain 2137 transformed with the Sp^r/Sm^r plasmid p183) exhibits qualitatively identical fluorescence emission maxima at 666 and 648 nm under both light and dark growth conditions. Y-5, a yellow-in-the-dark nuclear mutant control strain, accumulates a pigment intermediate with an emission maximum at 627 nm corresponding to protochlorophyllide in the dark and fluorescing species at 666 and 648 nm in the light. Cell line JS1000 that harbors the Km^r, Ble^r gene disruption of *chlL* exhibits an identical phenotype, i.e., dark-specific accumulation of a pigment fluorescing at 627 nm as described for the mutant control strain Y5.

villea glabra. In general, angiosperms are thought to require light for greening with the exception of several studies that have reported dark chlorophyll biosynthesis only under preillumination conditions (Adamson et al., 1985). Our negative cross-hybridization results with these species are also consistent with the observation that this gene is not present in the completely sequenced chloroplast genomes of the angiosperms tobacco and rice (Shinozaki et al., 1986; Hiratsuka et al., 1989; Shimada and Sugiura, 1991). In addition, proteins that are immunologically cross-reactive to antibodies against liverwort FrxC have not been detected in the angiosperms spinach and tobacco (Fujita et al., 1989). Collectively, these results suggest that, with perhaps the exception of Psilotum, a gene involved in light-independent protochlorophyllide reduction is conserved in photosynthetic prokaryotes as well as in the chloroplast genomes of an alga and nonflowering land plants.

DISCUSSION

A Role for ChlL in Light-Independent Protochlorophyllide Reduction

Our results demonstrated that chloroplast genomes from dark-greening organisms contain a gene involved in light-independent chlorophyll biosynthesis. The striking amino acid sequence identity that BchL has with its chloroplast-encoded homologs coupled with the similar phenotype observed upon disruption of *bchL* from *R. capsulatus*, *frxC* from *P. boryanum*, and *chlL* from Chlamydomonas clearly suggest that these gene products have a similar function in photopigment biosynthesis. It should be noted that there is as yet no direct evidence for enzymatic function for the polypeptide encoded by *bchL* or its homologs. However, several lines of evidence suggest that these gene products represent one subunit of a multisubunit complex involved in catalyzing protochlorophyllide reduction. A strong argument for an enzymatic role is based on the previously noted amino acid sequence similarity that BchL and its homologs have to dinitrogenase reductase (NifH), which is the Fe-S subunit of the nitrogen-fixing enzyme nitrogenase (Figure 2 and Table 1; Hearst et al., 1985; Kohchi, et al., 1988; Ohyama et al., 1988b; Fujita et al., 1991). Sequence similarity between these proteins includes conservation in overall length as well as conservation of functional domains such as the ATP binding motif and cysteine residues known to be important for Fe-S binding (Figure 2; Howard et al., 1989; Georgiadis et al., 1990; Fujita et al., 1991). The NifH-like feature of BchL and its homologs is also supported by biochemical studies of the FrxC polypeptide from liverwort (Fujita et al., 1989). This analysis has demonstrated that FrxC, like NifH, is a soluble protein that exists as a dimer in solution and that FrxC has a functional ATP binding domain. In addition to the nitrogenase-like feature of the BchL homologs, we and others have recently sequenced and genetically disrupted two additional genes involved in light-independent protochlorophyllide reduction in the bacterium *R. capsulatus* (J. Y. Suzuki, J. M. Dobrowolsky, and C. E. Bauer, manuscript in preparation). It is interesting that one of these additional genes codes for a polypeptide that has significant sequence identity to the β subunit of dinitrogenase, thereby suggesting the tantalizing conclusion that enzyme complexes for light-independent protochlorophyllide reduction may be evolutionally related to the

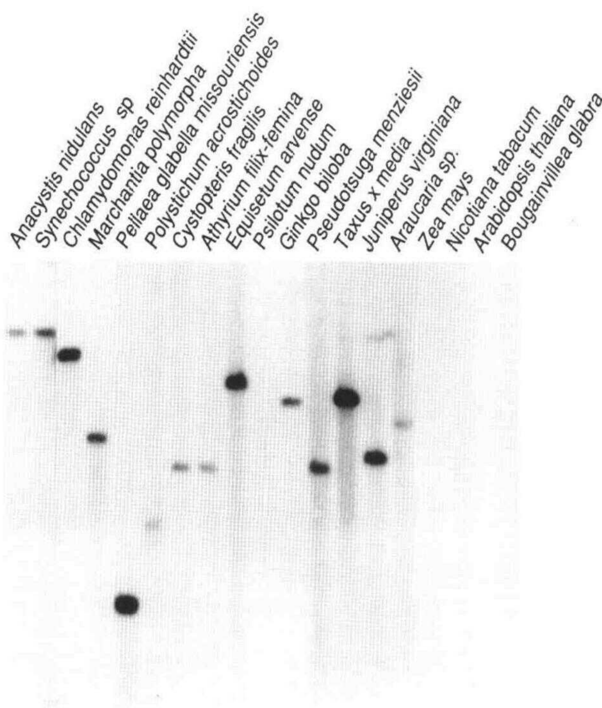

Figure 7. DNA Gel Blot Analysis for the Presence or Absence of *chlL* from Cyanobacteria, an Alga, and Major Groups of Land Plants.

Total or chloroplast-enriched DNAs were isolated from cyanobacteria, an alga, or land plants, digested with EcoRI and fractionated on a 0.7% agarose gel, and treated as described in Methods.

nitrogenase holoenzyme. Additional biochemical analysis of this enzyme is clearly warranted to address these speculations.

There is also a compelling evolutionary argument based on the genetic and physiological disparity between prokaryotic and eukaryotic organisms that would indicate a structural rather than a regulatory role for BchL/ChlL in (bacterio)chlorophyll biosynthesis. One strong argument against a regulatory role is based on the observation that *R. capsulatus* does not appear to regulate chlorophyll biosynthesis at the step of protochlorophyllide reduction. Photosynthetic bacteria also do not compartmentalize gene expression or its photosynthetic apparatus into organelles; therefore, it seems unlikely that ChlL would be involved in transport of an essential component into the chloroplast or in any aspect of nuclear–organellar interaction that may be involved in protochlorophyllide reduction. It seems more likely that the high degree of sequence conservation among BchL/ChlL and NifH reflects a conserved structural rather than regulatory role for these proteins.

The Involvement of Additional Chloroplast and Nuclear Loci in Light-Independent Protochlorophyllide Reduction

Several studies have identified additional loci in Chlamydomonas that are involved in protochlorophyllide reduction. Recently, a deletion in a 4.0-kb region of the Chlamydomonas chloroplast genome was reported to have a similar yellow-in-the-dark phenotype as described for *chlL* (Roitgrund and Mets, 1990; Goldschmidt-Clermont et al., 1991). Sequence analysis of this region, which the authors have termed *chlN*, demonstrates that the deletion disrupts an open reading frame homologous to the chloroplast ORF465 from liverwort (Choquet et al., 1992). A specific function for *chlN* in protochlorophyllide reduction has not yet been ascribed. However, *chlN* exhibits significant amino acid sequence similarity to *bchN* (D. H. Burke, M. Alberti, and J. E. Hearst, unpublished sequence data, GenBank accession number Z11165), an additional gene from *R. capsulatus* that we have shown by mutational analysis to be involved in protochlorophyllide reduction (J. Y. Suzuki, J. M. Dobrowolski, and C. E. Bauer, manuscript in preparation). Thus, by analogy it would appear that *chlN* may encode for an additional subunit of the enzyme complex. Furthermore, although *chlN* and *chlL* are distantly located in the Chlamydomonas chloroplast genome (Figure 1A), in liverwort, lodgepole pine, and *R. capsulatus*, homologs of *chlN* and *chlL* (*frxC*) are physically linked in what appears to be an operon (Kohchi et al., 1988; Yang and Bauer, 1990; Lidholm and Gustaffson, 1991). This arrangement is typical for genes involved in a similar function. Finally, *chlN* and *chlL* homologs are not present in the completely sequenced chloroplast genomes of the angiosperms rice and tobacco (Shinozaki et al., 1986; Hiratsuka et al., 1989; Shimada and Sugiura, 1991), which is consistent with the characteristic light-dependent greening of this group of organisms as well as with the results of our *chlL* gene cross-hybridization analysis.

In addition to *chlN* and *chlL*, previous genetic studies by Wang and others have identified at least six nuclear loci that are involved in light-independent protochlorophyllide reduction in Chlamydomonas (Ford and Wang, 1980a, 1980b). These yellow-in-the-dark or "Y" mutants have the same phenotype, i.e., dark accumulation of protochlorophyllide, as do chloroplast mutations in *chlN* and *chlL*. The involvement of these nuclear loci in protochlorophyllide reduction has not yet been elucidated. However, because protochlorophyllide reductase from *R. capsulatus* appears to involve three gene products and only two chloroplast genes have been identified as homologs, it leaves open the possibility that a third subunit is nuclear encoded. An additional possibility is that the nuclear loci could be involved in aspects controlling *chlL* and/or *chlN* expression. We are currently testing this possibility at the transcriptional and translational level. Finally, it is also likely that one or more of the nuclear loci are not directly involved in protochlorophyllide reduction but are instead affecting a step in chloroplast development or physiology that in turn has a pleiotropic effect on light-independent protochlorophyllide reductase expression.

Have Angiosperms Lost a Light-Independent Protochlorophyllide Reductase?

Our evidence that *chlL* hybridizes to representative gymnosperms, ferns, and photosynthetic bacteria, but not to

angiosperms, correlates well with physiological and biochemical evidence that angiosperms as a group require light for greening. Although we cannot definitively exclude the possibility that absence of *chlL* cross-hybridization is due to gene sequence divergence or gene transfer to the nucleus, our results indicate that structural genes involved in light-independent protochlorophyllide reduction may have simply been lost in the angiosperm lineage. A question remaining, however, is the evolutionary nature of the nuclear-encoded, light-dependent PCR enzyme of angiosperms. A number of studies indicate that light-dependent PCR is not unique to angiosperms. This argument is based on the overwhelming genetic evidence for a light-dependent protochlorophyllide reductase activity in algae such as Chlamydomonas and Scenedesmus (Wang, 1979; Ford and Wang, 1980a, 1980b; Ford et al., 1981, 1983; Senger and Brinkmann, 1986). There is also immunological and sequence information for the existence of a light-dependent PCR from the gymnosperm pine (Ou et al., 1990; Spano et al., 1991). In addition, Fujita et al. (1992) recently reported the inactivation of a *bchL* homolog present in the cyanobacterium *P. boryanum*, which resulted in a strain that failed to reduce protochlorophyllide in the dark, but surprisingly retained chlorophyll biosynthetic capability in the light. This result indicates that *P. boryanum* harbors a light-dependent PCR activity and therefore suggests that both the light-independent and the light-dependent protochlorophyllide reductases of plants are of prokaryotic origin.

METHODS

Cell Lines and Growth Conditions

The stable "green-in-the-dark" wild-type strain 2137 (mt+) of *Chlamydomonas reinhardtii* was used as the recipient for particle gun transformation and was obtained from R. Togasaki, Indiana University. Mutant strain CC-1169 (Y-5, mt+) was used as a control for analysis of protochlorophyllide accumulation in the dark and was obtained from the *Chlamydomonas* Genetics Center, Duke University (Durham, NC). Cell lines were routinely grown in Tris-acetate phosphate media (TAP) or TAP agar plates (Harris, 1989) at 25°C under dark, dim (0.5 lux), or high illumination (2000 to 10,000 lux) growth conditions.

chlL Isolation and Sequence Analysis

A Chlamydomonas homolog of the *Marchantia polymorpha* (liverwort) *frxC* gene was identified by cross-hybridization of Chlamydomonas chloroplast DNA with a liverwort *frxC* probe. For this analysis, a 2.1-kb BglII-HincII fragment containing the *frxC* chloroplast gene of liverwort was subcloned from plasmid pMP323. pMP323 contains a 6.9-kb BglII fragment (Bg6) of the liverwort chloroplast genome (Ohyama et al., 1988a). The subclone of *frxC* was random-primer labeled (Random Primed DNA Labeling Kit; US Biochemical Corp.) with ^{32}P-dATP (Amersham) and hybridized against a DNA gel blot of purified Chlamydomonas chloroplast DNA that was digested with various restriction enzymes. A cross-hybridization pattern was observed which placed the putative *frxC* gene within the 7.6-kb EcoRI-9 chloroplast restriction fragment (Figure 1A) that was subsequently confirmed by cross-hybridization to the insert fragment of plasmid p64, a clone containing the EcoRI-9 region of the Chlamydomonas chloroplast genome (a gift of J. Palmer, Indiana University). *chlL* was further shown to span a HindIII restriction site located within the EcoRI-9 fragment and was subsequently subcloned as 1.4-kb HindIII and 3.7-kb EcoRI-HindIII restriction fragments into the respective sites of M13. Sequence analysis of both strands of *chlL* was then performed on the M13 subclones by dideoxy nucleotide sequencing (Sequenase Kit; US Biochemical Corp.) using synthetic primers synthesized with an oligonucleotide synthesizer (Model No. 394; Applied Biosystems, Foster City, CA).

Plasmid-Encoded *chlL* Disruption

A *chlL* disruption was constructed from plasmid p64 by subcloning a 1.6-kb HindIII restriction fragment containing the kanamycin resistance (Km^r) and bleomycin resistance (Ble^r) structural genes from pUC4Kixx (Pharmacia) into a unique BstBI site in plasmid p64; this was accomplished by blunt ending the restriction fragments with the Klenow fragment of DNA polymerase I prior to ligation. The resulting construct pChlL::KanH contains a 1.6-kb insertion at codon 114 of *chlL*.

Particle Gun Transformation

Particle gun–mediated transformation of the Chlamydomonas chloroplast genome followed the protocol of Newman et al. (1990). Transformation vector p183 was a generous gift of E. Harris and B. Randolph-Anderson, Duke University. Two separate bombardments were performed; one utilized a 1:1 (2.5 to 2.5 μg) ratio, and the second transformation utilized a 1:5 (2.5 to 12.5 μg) ratio of the drug resistance marker plasmid p183 to the *chlL* disruption plasmid pChlL::KanH, respectively. Recipient cell strain 2137 (mt+) was grown in 0.5 mM FrUrd as described by Newman et al. (1990). Transforming DNA was annealed to tungsten particles and then bombarded into 9.66×10^7 cells using a particle accelerator gun (prototype; Nippon Zeon Co., Tokyo, Japan) containing a low-acceleration charge. After bombardment, cells were replated onto HSHA agar media (Harris, 1989) containing 100 μg/mL each of spectinomycin and streptomycin and 50 μg/mL ampicillin. Drug-resistant colonies, which appeared after 5 days of incubation at 25°C under high light (6000 lux), were allowed to enlarge for an additional 9 days, after which individual colonies were restreaked as whole cell populations for two rounds on TAP agar drug selection plates. Subsequent rounds of restreaking were performed on drug-free TAP agar media.

Restriction Fragment Length Polymorphism Analysis

For restriction fragment length polymorphism (RFLP) analysis, individual transformed cell lines were grown mixotrophically under high light (10,000 lux) with shaking (250 rpm) for 5 days in 2.5 mL of TAP media without drugs. Total DNA was then isolated using a previously described DNA miniprep procedure (Newman et al., 1990) that was modified by use of 1.5 mL of liquid culture as the source of cell material. DNA samples were then digested with various restriction enzymes, fractionated on a 0.7% agarose gel, blotted to a Nytran membrane (Schleicher & Schuell), and hybridized with ^{32}P-labeled probe 1 (Figures 4A and 4B). Probe 1, a 217-bp BstBI-ScaI restriction fragment of *chlL*, was gel purified from low-melting temperature agarose (NuSieve GTG; FMC Corp., Rockland, ME) and used as a template for random-primer labeling.

Pigment Determination

Cells were inoculated from TAP agar plates into 50 mL of TAP medium and shaken in an Erlenmeyer flask at 200 rpm under high light (10,000 lux). After 4 to 5 days of growth (to saturation), the cells were then subcultured at 1/500 dilution into 50 mL of TAP media and grown for an additional 4 days under high light (10,000 lux) or total darkness. Pigments were then extracted from light- or dark-grown cells as follows: 20 mL of cell culture was pelleted by centrifugation at 1100*g* for 5 min, resuspended with 2.0 mL of fresh Tris-acetate phosphate media, and then lysed by sonication. Unbroken cell debris were then removed by centrifugation at 1100*g* for 5 min, and the resulting supernatant was then extracted of pigments by emulsifying with 2.0 mL of diethyl ether. The pigment-containing diethyl ether phase was separated from the aqueous phase by centrifugation at 1100*g* for 5 min and analyzed for room temperature fluorescence emission spectra using a Hitachi F-2000 fluorescence spectrophotometer with an excitation wavelength set at 438 nm (Bednarik and Hoober, 1985; Roitgrund and Mets, 1990). Fluorescence peaks at 666 and 648 nm corresponding to chlorophyll *a* and *b*, respectively, were qualitatively similar using either excitation wavelength of 438 nm or the excitation wavelengths for maximum fluorescence. The minor fluorescence emission peaks observed in the pigment spectra from dark-grown Y-5 (644 and 662 nm) and JS1000 (664 nm) are minor components that vary in levels from extraction to extraction.

DNA Gel Blot Analysis

Total or chloroplast-enriched DNAs from various photosynthetic organisms were isolated as previously described (Palmer, 1986; Doyle and Doyle, 1987; Wagner et al., 1987; Wilson, 1990) and subjected to one round of $CsCl_2$ gradient purification. DNA samples were digested with EcoRI and fractionated on a 0.7% agarose gel. DNA gel blots (Southern, 1975) were prepared in duplicate and hybridized against ^{32}P-labeled probe 2 (Figure 3) under low-stringency conditions in 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% Blotto, 1.0% Nonidet P-40 at 42°C for 12 hr. The filters were subsequently subjected to two 20-min washes at room temperature in 2 × SSC, 0.1% SDS and 1 × SSC, 0.1% SDS, an additional room temperature wash in 0.1 × SSC, 0.1% SDS, and a final 20-min wash at 42°C in 0.1 × SSC, 0.1% SDS. Probe 2 is a 716-bp polymerase chain reaction–amplified product of a region internal to the structural sequence of the Chlamydomonas chloroplast gene *chlL*. This probe was obtained using the following primers: N terminus, 5'-GTGTTACAAATTGGTTG-3'; C terminus, 5'-GGTTCTGTTAATAATTG-3'. Preliminary DNA gel blot analysis was performed using as a probe the 16S and 23S ribosomal DNA (rDNA) from the Chlamydomonas chloroplast transformation vector p183 (J. Y. Suzuki, unpublished results). We used the resulting rDNA hybridization signals as a guide to adjust for similar levels of chloroplast DNA in the subsequent blot shown in Figure 7. Similar levels of chloroplast DNA were confirmed by examining the relative hybridization signals in a blot identical to that in Figure 7 using as a probe a radiolabeled 970-bp EcoRI fragment from p183 containing the 16S rDNA (J. Y. Suzuki, unpublished results).

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