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The *chlL* (*frxC*)* gene: phylogenetic distribution in vascular plants and DNA sequence from *Polystichum acrostichoides* (*Pteridophyta*) and *Synechococcus* sp. 7002 (*Cyanobacteria*)

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Abstract: We examined *chlL* (*frxC*) gene evolution using several approaches. Sequences from the chloroplast genome of the fern *Polystichum acrostichoides* and from the cyanobacterium *Synechococcus* sp. 7002 were determined and found to be highly conserved. A complete physical map of the fern chloroplast genome and partial maps of other vascular plant taxa show that *chlL* is located primarily in the small single copy region as in *Marchantia polymorpha.* A survey of a wide variety of non-angiospermous vascular plant DNAs shows that *chlL* is widely distributed but has been lost in the pteridophyte *Psilotum* and (presumably independently) within the Gnetalean gymnosperms.

Most green, chlorophyll-containing organisms have both a light-dependent and a light-independent pathway to chlorophyll (Chl) synthesis, due to the presence of two distinct enzymes for protochlorophyllide (PChlide) reduction. The broad spectrum of photosynthetic organisms capable of dark PChlide reduction includes the

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* The name *frxC* was originally used to denote a gene encoding a product with probable Fe : S cluster binding activity. This activity was postulated due to the amino acid sequence similarity between this product and the Fe : S-binding nitrogenase iron protein *nifH*. Fe : S-binding is a property shared by ferredoxins, which are denoted by the prefix "*frx*". However, this gene does not encode a ferredoxin. It is much larger than any known ferredoxin, it binds its Fe : S cluster between two halves of a homodimer (FUJITA & al. 1989, BURKE & al. 1993 a, c) instead of within a single subunit, and it lacks the pattern of clustered cysteines present in all ferredoxins (MEYER 1988). Therefore, we use the name *chlL* to recognize the sequence and functional similarities to the bacterial PChlide reductase subunit, *bchL*. Similar usage has been adopted for this (SUZUKI & BAUER 1992) and other (CHOQUET & al. 1992, BURKE & al. 1993 b) PChlide reductase subunits.

green, gliding bacterium, *Chloroflexus aurantiacus* (T. SULLIVAN and DHB, unpubl. obs.), the purple bacterium, *Rhodobacter capsulatus* (MADIGAN & GUEST 1978, YEN & MARRS 1977), the cyanobacterium, *Plectonema boryanum* (FUJITA & al. 1992), the green alga, *Chlamydomonas reinhardtii* (CHOQUET & al. 1992, ROITGRUND & METS 1990), and the conifer, *Pinus nigra* (BOGDANOVIĆ 1973), among others (CASTELFRANCO & BEALE 1983). Flowering plants are thought to lack the ability to reduce PChlide in the dark and instead rely entirely on the light-dependent pathway to chlorophyll synthesis (GRIFFITHS 1991), although there is tenuous evidence that some angiosperms may be capable of dark Chl synthesis under preillumination conditions (ADAMSON & PACKER 1984, CASTELFRANCO & BEALE 1983).

The enzyme system for dark PChlide reduction has been shown to be distinct from that of the light-dependent reduction (ROITGRUND & METS 1990, CHOQUET & al. 1992, SUZUKI & BAUER 1992, BURKE & al. 1993 a). The dark reductase is composed of three subunits (BURKE & al. 1993 b), the light-dependent enzyme of only one (SCHULTZ & al. 1989, DARRAH & al. 1990). In *Chlamydomonas reinhardtii*, there are a number of uncharacterized nuclear mutations (the *yellow* mutants) that block dark Chl synthesis without interfering with normal greening in the light (FORD & WANG 1980 a, b). In addition, two *C. reinhardtii* chloroplast genes for the dark PChlide reductase have been characterized genetically: *chlN* (*gidA*) and *chlL* (*frxC*^{I}) (ROITGRUND & METZ 1990, CHOQUET & al. 1992, SUZUKI & BAUER 1992). Similar genetic studies have been done on the *bchL* gene from *Plectonema boryanum* (FUJITA & al. 1992). All of these chlorophyll-synthesis mutants do green in the light because of the light-dependent PChlide reductase. Mutations in the postulated third chloroplast subunit, *chlB* (ORF 513 in the chloroplast genome of the bryophyte *Marchantia polymorpha*), have yet to be described.

Nucleotide sequences of dark PChlide reductase subunits (*chlL, chlN*, and *chlB*) from several organisms have recently been determined. Sequence conservation is strongest among the various *chlL* genes. The amino acid sequences of *chlL* are 80 to 95% identical among two cyanobacteria (FUJITA & al. 1992, OGURA & al. 1992), the cyanelle of the flagellar eukaryote *Cyanophora paradoxa* (V. L. STIREWALT & D. BRYANT, pers. comm.), and the chloroplasts of *Chlamydomonas reinhardtii* (SUZUKI & BAUER 1992), *Marchantia polymorpha* (OHYAMA & al. 1986), and *Pinus nigra* (LIDHOLM & GUSTAFSSON 1991). Each is roughly 50% identical with the *bchL* gene of *Rhodobacter capsulatus* (BURKE & al. 1993 a). The *chlN* gene has been sequenced from the chloroplast genomes of *C. reinhardtii* (CHOQUET & al. 1992) and *P. nigra* (LIDHOLM & GUSTAFSSON 1991), from the *C. paradoxa* cyanelle (V. L. STIREWALT & D. BRYANT, pers. comm.), and from the cyanobacterium *Synechocystis* PCC 6803 (OGURA & al. 1992). Each of these *chlN* genes shares strong amino acid identities (70%) with ORF 465 from the *M. polymorpha* chloroplast, moderate similarity (36%) with the *bchN* gene product of *R. capsulatus* (BURKE & al. 1993 b), as well as similarities (19%) with the *nifK* gene product (SUZUKI & BAUER 1992, OGURA & al. 1992, BURKE & al. 1993 c). The amino acid sequence of the *bchB* product from *R. capsulatus* is 34% identical with ORF 513 from *M. polymorpha*.

None of the genes for the enzymes in the dark Chl synthesis pathway is found in the completely sequenced chloroplast genomes of tobacco and rice (SHINOZAKI

& al. 1986 and HIRATSUKA & al. 1989, respectively), whereas all three genes are present in the chloroplast genome of *Marchantia* (OHYAMA & al. 1986). Studies to determine the distribution of these genes among land plants have been undertaken only recently. The *chlL* and *chlN* genes have been sequenced from the conifer, *Pinus nigra* (LIDHOLM & GUSTAFSSON 1991) and Southern hybridizations have been used to test some pteridophytes and gymnosperms for the presence of *chlL* (SUZUKI & BAUER 1992, YAMADA & al. 1992). Of the DNAs tested only the pteridophyte *Psilotum* failed to demonstrate presence of the gene. We initiated an investigation of the evolution of the *chlL* gene in non-angiospermous vascular plants as part of earlier studies (RAUBESON 1991, BURKE-AQÜERO 1992, BURKE & al. 1991). Here we report sequence information from the fern, *Polystichum acrostichoides* (plus the cyanobacterium, *Synechococcus* sp. 7002) as well as more extensive surveys examining all major extant groups of non-flowering vascular plants to determine the phylogenetic distribution of the *chlL* gene.

Material and methods

Heterologous PCR with either purified bacterial (1–10 ng) or fern chloroplast (0.1–1 ng) DNA was carried out using 100 pmoles of each primer: Fe-1, TA(TC)GGIAA(AG)GGIGGIAT(TCA)GGNA>; Fe-4, GTCGACITT(TC)ACI(TC)TIACNGG>; Fe-8, GGITG(TC)GGIGGITA(TC)GTIGTNGG>; Fe-9, <CCIACIAC(AG)TAICCICC(GA)CANCC; Fe-13, <GC(AG)AAICCICC(AG)CAIACIAC(AG)TC. Parenthesis enclose degenerate sets of nucleotides used at that position, and arrowheads indicate the relative orientation of each primer within the gene. A master mix containing 2.5 U Taq DNA polymerase and 17 nmoles of each dNTP in 1x reaction buffer [1x = 10 mM Tris-HCl (pH 8.3) 50 mM KCl, 2.5 mM $MgCl_2$, 0.02% gelatin] was prepared and 10 µl added at 90 °C to 90 µl containing freshly boiled primer and template in 1x reaction buffer. A two-step amplification (COMPTON 1990) was used that included 5 cycles of (94 °C, 60 sec; 37 °C, 60 sec; a 150 second ramp up to 72 °C; 72 °C, 60 sec) followed by 25 cycles of the same profile without the slow ramp. PCR products were assayed by running 20 µl of the reaction mix on a 1.4% agarose gel. The remainder was extracted, desalted on a Sephadex G-50 spin column (Pharmacia), precipitated, and cloned into Sma I-cut M 13 for dideoxy chain-terminating sequencing (SANGER & al. 1977). Oligonucleotide synthesis was performed as described previously (BURKE & al. 1993 a). Cyanobacterial DNA was isolated by JOHNNY CHANG from *Synechococcus* sp. 7002, which was a gift of Prof. KENNETH SAUER. Protein and nucleic acid databanks were searched with the fast-db program (BRUTLAG & al. 1990) using the Intelligenetics Software release 5.37. The DNA sequences reported here will be deposited in GenBank.

We generated a physical map of the chloroplast genome of *Polystichum acrostichoides*. The procedures are described in detail elsewhere (STEIN 1993). Briefly, a total DNA preparation was digested with the enzymes Pst I, Stu I, Pvu II, and Bam HI singly and in paired combinations. The resulting fragments were separated by gel electrophoresis in 0.7% agarose and transferred to Zetabind (Cuno, CT) nylon membrane. The order of the *P. acrostichoides* restriction fragments was determined by sequentially hybridizing DNA fragments used as overlapping probes. These probes were labelled with ^{32}P by nick translation (SAMBROOK & al. 1989) and included cloned chloroplast DNA of lettuce (provided by R. JANSEN), *Petunia* (provided by J. PALMER), and *Adiantum capillus-veneris* (provided by M. HASEBE). In addition, we cloned Pst I-digested cpDNA from *P. acrostichoides* into pUC 8 and used these clones to complete the mapping. All the Pst I fragments 1 kb or larger except the 16.3 and the 2.9 kb fragments were successfully cloned.

For the phylogenetic survey, total genomic DNA was extracted from each plant, digested singly with Bam HI, Hind III, and in combination. Gel electrophoresis, transfer to nylon membranes and probe labelling were as described above. Hybridization was for 40 h at 55 °C (in 4 × SSC, 0.5% SDS, 1% powdered milk) and the filters were washed in 2 × SSC at 55 °C. Maps were prepared via overlap hybridization (PALMER 1986) using cloned tobacco cpDNA fragments (OLMSTEAD & PALMER 1992). Gene positions within mapped chloroplast genomes were determined by hybridization with a 532 bp fragment internal to the *Marchantia chlL* gene (or the 363 bp PCR product from *P. acrostichoides*), a cloned fragment of tobacco cpDNA containing the 3′ 1680 bp of the 23S rRNA gene, and a gel-excised fragment of tobacco cpDNA containing the *ndhF* gene. Cloned tobacco fragments were kindly provided by J. PALMER.

Results

We first used the polymerase chain reaction to detect the presence of *chlL* genes. Five fully degenerate PCR primers were synthesized by reverse translating amino acid sequences conserved between *Rhodobacter capsulatus bchL* and *Marchantia polymorpha chlL*. Inosine was used in positions that called for the inclusion of all four nucleotides to reduce the chemical complexity of each primer. Most of these conserved regions are also shared with nitrogenase Fe protein, *nifH*, where they are known to be involved in binding MgATP and a (4Fe4S) cluster (BURKE & al. 1993 c, FUJITA & al. 1989, HEARST & al. 1985), and with the chlorin reductase subunit *bchX* (BURKE & al. 1993 a). As our initial template, we examined *Synechococcus* sp. 7002 because this cyanobacterium cannot grow under nitrogen-limiting conditions and fails to hybridize to probes carrying the *Klebsiella pneumoniae nifHDK* genes (D. BRYANT, pers. comm.). It was therefore considered unlikely to give false amplification signals arising from a *nifH* gene. PCR reactions using this template produced bands of the appropriate sizes in all five combinations of primers, and when the larger fragments were cut from a gel and reamplified in nested or heminested reactions, they, too, gave bands of the appropriate size. The 363 bp fragment arising from primers 1 and 13 was cloned and sequenced. The nucleotide and deduced amino acid sequences (Fig. 1) of the amplified product are 75% and 91% identical, respectively, to the corresponding region in the *Marchantia* chloroplast *chlL*. Purified chloroplast DNA from the fern *Polystichum acrostichoides* (family *Dryopteridaceae*) then was used as template for PCR reactions as above, and again the 363 bp product was cloned and sequenced.

```
     S  T  T  S  C  N  I  S  V  A  L  A  K  R  G  K  K  V  L  Q  I  G  C  D  P  K  H  D
>>>>>ATCTACCACCAGTTGTAATATTTCCGTCGCCCTCGCCAAACGCGGTAAAAAGGTTCTCCAAATTGGCTGTGACCCCAAGCATGAC
Fe-1 TAGATGGTGGTCAACATTATAAAGGCAGCGGGAGCGGTTTGCGCCATTTTTCCAAGAGGTTTAACCGACACTGGGGTTCGTACTG

S  T  F  T  L  T  G  F  L  I  P  T  I  I  D  T  L  Q  E  K  D  F  H  Y  E  D  I  W  P  E
AGCACCTTTACCTTGACGGGCTTTTTGATCCCGACGATCATCGATACTCTCCAGGAAAAAGATTTTCACTACGAAGACATTTGGCCGGAG
TCGTGGAAATGGAACTGCCCGAAAAACTAGGGCTGCTAGTAGCTATGAGAGGTCCTTTTTCTAAAAGTGATGCTTCTGTAAACCGGCCTC

D  V  I  Y  K  G  Y  G  G  V  D  C  V  E  A  G  G  P  P  A  G  A  G  C  G  G  Y  V  V  G
GATGTTATTTACAAAGGCTATGGCGGCGTTGATTGCGTGGAAGCAGGCGGGCCGCCGGCTGGGGCTGGCTGCGGTGGTTATGTGGTCGGG
CTACAATAAATGTTTCCGATACCGCCGCAACTAACGCACCTTCGTCCGCCCGGCGGCCGACCCCGACCGACGCCACCAATACACCAGCCC

E  T  V  K  L  L  K  E  L  N  A  F  D  E  Y  D  V  I  L  F  D  V  L  G...
GAAACGGTCAAACTCCTCAAAGAACTCAATGCCTTGCATGAATACGATGTAATTTTGTTTGATGTGTTGGG         Fe-13
CTTTGCCAGTTTGAGGAGTTTCTTGAGTTACGGAACGTACTTATGCTACATTAAAACAAACTACACAACCC<<<<<<<<<<<<<<<<<<
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Fig. 1. Nucleotide and amino acid sequences of *Synechococcus* amplicon. Arrows indicate the position of the primers Fe-1 and Fe-13 used in the amplification

A physical map of the *P. acrostichoides* chloroplast genome was generated for four restriction enzymes (Fig. 2). Summation of the fragments resulting from these digests gives an approximate genome size of 149 kb. The Pst I fragments that flank the boundary of the inverted repeats and the large single copy region (2.9 and 10.3 kb) cross-hybridize with each other, as do those on the side of the small single copy region (5.4 and 8.3 kb). The maximum and minimum sizes of the inverted repeats are therefore 17.8 and 27.7 (but see below).

Amplified cyanobacterial *chlL* DNA (363 bp product between primers 1 and 13) was used as a hybridization probe to map the location of the *chlL* gene. The probe hybridizes only to a 5.4 kb Pst I fragment, and not to the 8.3 kb Pst I fragment on the other side of the small single copy region (Fig. 3). This would seem to locate *chlL* to the small single copy DNA adjacent to IR_A. (The boundary between the small single copy region and IR_A lies 6 bp downstream of the start of *chlL* in the *Marchantia* chloroplast genome.) However, hybridization to *P. acrostichoides* PCR products (the 363 bp product between primers 1 and 13 and a 261 bp product between primers 4 and 13) suggest that almost 200 bp of the 5′ end of the gene has been copied from the small single copy region into IR_B. Evidentally, when using the *Synechococcus* probe, base pair mismatch between the DNAs was high enough that the duplicated region (about 120 base pairs of the 363 bp probe) was not detected. From the homologous hybridizations, it appears that about 5.1 kb of the 5.4 kb PstI fragment is repeated and the total size of the inverted repeat is 22.9 to 25.8 kb, depending on how much of the 2.9 kb fragment, at the IR-large single copy boundary, is included. In contrast to this small growth of the IR boundary into the small single copy region, a major expansion of the inverted repeats into the large single copy region of *P. acrostichoides* and several other fern species has been demonstrated previously (Stein & al. 1992).

The 5.4 kb Pst I fragment containing *P. acrostichoides chlL* was subcloned into M 13 phage for single-strand sequencing. More than one kb of sequence was determined (A + T content 56.5%), which included most of the *chlL* gene and > 500 bp of DNA upstream from *chlL* (Fig. 4). This clone did not contain the 3′ end of the gene. The *Marchantia chlL* sequence continues for an additional 407 nucleotides beyond the site that aligns with the end of the *P. acrostichoides chlL* clone.

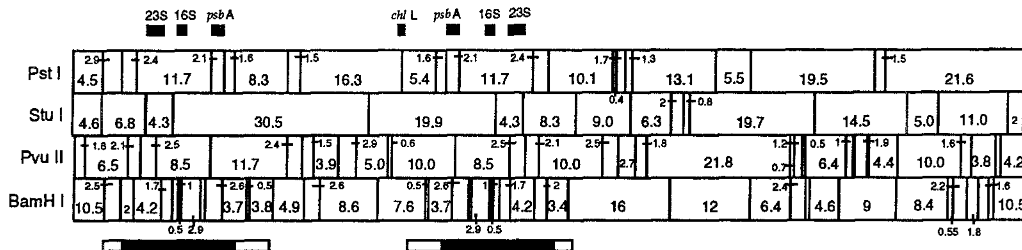

Fig. 2. Map of the *Polystichum acrostichoides* chloroplast genome, showing positions of restriction sites for four enzymes. The locations of four genes, including *chlL*, are shown as thick bars above the restriction map. The thick bars below the map are the approximate locations of the two inverted repeats, with their maximum sizes shown in white and minimum sizes in black. Fragment sizes are given in kilobases

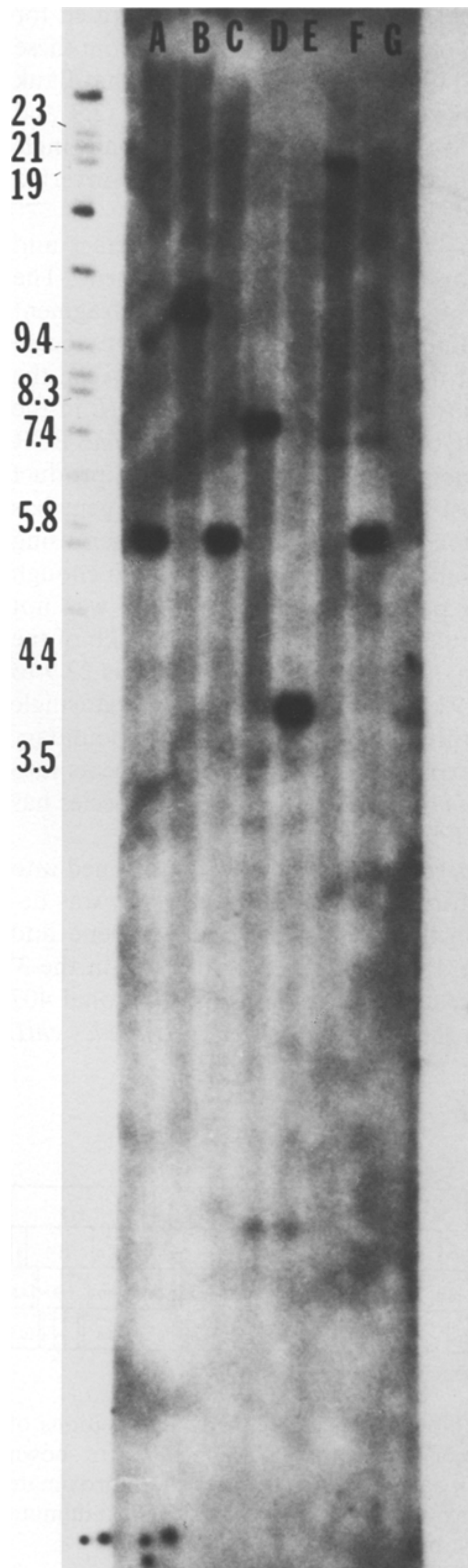

Fig. 3. Southern hybridization of the *Synechoccus* amplicon to *Polystichum acrostichoides* DNA. The *chlL* gene probe (363 bp) hybridizes only to the 5.4 kb Pst I (A), 10.0 kb Pvu II (B), 7.6 kb Bam HI (D), and 19.9 kb Stu I (F) demonstrating that the location of the *chlL* gene is in the small single copy region. Lanes C, E, and G are double digests of the enzymes above (B, D, and F, respectively) together with Pst I (A)

```
        10        20        30        40        50        60        70        80        90
GCACAAATTTGTTTCTCTCCAATTGTTAGAGCTGGAAATAAGCACGATAGTGAATCATTCACTGGTTGGTGGATCATGGTCCAACACAAT
CGTGTTTAAACAAAGAGAGGTTAACAATCTCGACCTTTATTCGTGCTATCACTTAGTAAGTGACCAACCACCTAGTACCAGGTTGTGTTA

       100       110       120       130       140       150       160       170       180
TTGATTTGGCATATGTGGGAAACACAACTACCCAATTAGTAAGAGTTGTTGGGGTAGATTCGAACGAATCTCCCCGGGTAGGATTCGAAC
AACTAAACCGTATACACCCTTTGTGTTGATGGGTTAATCATTCTCAACAACCCCATCTAAGCTTGCTTAGAGGGGCCCATCCTAAGCTTG
                                                                      3'-GAGGaGuCCAUCCUAAaCUUG

       190       200       210       220       230       240       250       260       270
CTACGACCAATCGGTTAACAGCCGACCGCTCTACCGCTGAGCTACCGAGGAAAGTGGTAGGGGATTCGGTCTCATACACCCTCAACTTTG
GATGCTGGTTAGCCAATTGTCGGCTGGCGAGATGGCGACTCGATGGCTCCTTTCACCATCCCCTAAGCCAGAGTATGTGGGAGTTGAAAC
GAUGCUGGUUAGCCAAUUGUCGGCUGGCGAGAUGGuGACUCGAUGauUCCU-5'  tRNA Asn(GUU)

       280       290       300       310       320       330       340       350       360
TTCTTTCGTTCTCTGAATCGCTTCTAAATCTGTAAGACGCTAAGCTTTCTCCGACATTTTGTGAAGTAGACTTCGCGTACACTCTAACTC
AAGAAAGCAAGAGACTTAGCGAAGATTTAGACATTCTGCGATTCGAAAGAGGCTGTAAAACTCTTCATCTGAAGCGCATGTGAGATTGAG

       370       380       390       400       410       420       430       440       450
CCATTATAGGAGGAGGCAGCGGCGATAGCAATCCCATTTGAATGGAAGGGTACCCAAATCGTGGGAGAGGTGGGGGGGGGGGGGGGGCAC
GGTAATATCCTCCTCCGTCGCCGCTATCGTTAGGGTAAACTTACCTTCCCATGGGTTTAGCACCCTCTCCACCCCCCCCCCCCCCCCGTG
  -10                  -35
       460       470       480       490       500       510       520       530       540
CGATCGAGGTCGAGATCACCCCATAAGCCCCCCACGATCTGTATCGATCGGGTCACCAATTGGGTACTTCCTATTCCCCCCCCCCCCAAG
GCTAGCTCCAGCTCTAGTGGGGTATTCGGGGGGTGCTAGACATAGCTAGCCCAGTGGTTAACCCATGAAGGATAAGGGGGGGGGGGGTTC

       550       560       -35       580       -10       600       610       620       630
AAGCATAGTAGAATAGCCGCAGGATTCTCCTACCTCATAGAAAGAAGTAATATCTTTGAGAAATAGAAGTAGCAAAGAGAAGAGAGATAG
TTCGTATCATCTTATCGGCGTCCTAAGAGGATGGAGTATCTTTCTTCATTATAGAAACTCTTTATCTTCATCGTTTCTCTTCTCTCTATC

       640       650       660       670       680       690       700       710       720
                                    chlL =(M) E  L  R  E  T  K  V  A  V  Y  G  K  G  G  I
AGATGGGGAATATGAACAATAGTCGGGATAAATGAACGCGAATTGGAGCTTCGTGAAACGAAAGTAGCAGTTTACGGCAAGGGCGGAATT
TCTACCCCTTATACTTGTTATCAGCCCTATTTACTTGCGCTTAACCTCGAAGCACTTTGCTTTCTACGTCAAATGCCGTTCCCGCCTTAA

       730       740       750       760       770       780       790       800       810
G  K  S  T  T  S  C  N  T  S  I  A  L  A  R  R  G  R  R  I  L  Q  I  G  C  D  P  K  H  D
GGGAAATCAACAACTAGCTGCAACACATCGATAGCTTTAGCTAGACGAGGGAGACGGATATTACAAATTGGGTGCGATCCCAAACATGAT
CCCTTTAGTTGTTGATCGACGTTGTGTAGCTAGCGAAATCGATCTGCTCCCTCTGCCTATAATGTTTAACCCACGCTAGGGTTTGTACTA

       820       830       840       850       860       870       880       890       900
S  T  F  T  L  T  G  F  S  I  P  T  I  I  D  T  S  Q  S  K  D  Y  H  Y  E  D  V  W  P  E
AGTACTTTCACTCCCACAGGATTCTCAATACCTACAATTATAGATACTTCACAATCGAAAGATTATCATTATGAAGATGTGTGGCCTGAA
TCATGAAAGTGAGGGTGTCCTAAGAGTTATGGATGTTAATATCTATGAAGTGTTAGCTTTCTAATAGTAATACTTCTACACACCGGACTT

       910       920       930       940       950       960       970       980       990
D  V  I  H  R  G  Y  G  G  V  D  C  V  E  A  G  G  P  P  A  G  A  G  C  G  G  Y  V  V  G
GATATGATTCACAGAGGTTACGGTGGAGTAGACTGCGTCGAAGCTGGCGGACCCCCTGCGGGGGCGGGCTGTGGGGGATATGTCGTGGGA
CTATACTAAGTGTCTCCAATGCCACCTCATCTGACGCAGCTTCGACCGCCTGGGGGACGCCCCCGCCCGACACCCCCTATACAGCACCCT

      1000      1010      1020      1030      1040      1050      1060      1070      1080
E  T  V  K  P  L  K  E  S  N  A  F  Y  E  Y  D  I  I  L  F  D  V  L  G  D  V  V  C  G  G
GAAACGGTGAAACCATTAAAAGAATCAAATGCCTTTTATGAATACGACATTATCTTATTCGACGTCTTGGGAGCAGATGTTTGTGGGGGC
CTTTGCCACTTTGGTAATTTTCTTAGTTTACGGAAAATACTTATGCTGTAATAGAATAAGCTGCAGAACCCTCGTCTACAAACACCCCCG

      1090      1100      1110      1120      1130      1140      1150
F  A  A  P  L  N  Y  A  D  Y  C  I  I  I  T  D  N  G  F  D  A  L  S  A...
TTCGCTGCCCCACTGAATTACGCAGATTACTGCATCATTATAACTGACAATGGATTTGATGCTCTTTCTGCAG
AAGCGACGGGGTGACTTAATGCGTCTAATGACGTAGTAATATTGACTGTTACCTAAACTACGAGAAAGACGTC
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Fig. 4. *Polystichum acrostichoides chlL* and upstream sequence from plasmid pFePac 1. Small letters (given under the sequence in reverse orientation) in the sequence of tRNA Asn(GUU) indicate non-identity between *P. acrostichoides* and *Marchantia polymorpha* tRNA sequences. Ribosome binding sites and the -35 and -10 elements of potential promoters are underlined

The nucleotide and amino acid sequences of *P. acrostichoides chlL* (Fig. 5) are very similar to the *Marchantia chlL* gene (75% nucleotide and 90% amino acid identity). There is a potential promoter, TTCTCC(N_{17})TAATAT, approximately 100 nucleotides upstream from the first codon to align with the other *chlL* sequences.

```
bchL     (insert)RVFSVYGKGGIGKSTTSSNLSAAFSLLGKRVLQIGCDPKHDSTFTLTGRLQ
                    .|||||||||||||.|.|.|..  ||.|||||||||||||||||| | 
Marc             MKIAVYGKGGIGKSTTSCNISIALARRGKKVLQIGCDPKHDSTFTLTGFLI
                  |.|||||||||||||||| ||||||||...|||||||||||||||||| |
Pac         MELRETKVAVYGKGGIGKSTTSCNTSIALARRGRRILQIGCDPKHDSTFTLTGFSI
                              |||||| |.|||.||...|||||||||||||||||| |
Syn                   >>>>>>>>STTSCNISVALAKRGKKVLQIGCDPKHDSTFTLTGFLI


bchL     ETVIDILKQVNFHPEELRPEDYVTEGFNGVMCVEAGGPPAGTGCGGYVVGQTVKLLKQHH
          |.|| |   ..|.|.. |||  .  |.    ||||||||||.||||||||.||||||.
Marc     PTIIDTLQSKDYHYEDVWPEDVIYKGYGRCDCVEAGGPPAGAGCGGYVVGETVKLLKELN
         |||||| ||||||||||||||||..|||  ||||||||||||||||||||||||.||| |
Pac      PTIIDTSQSKDYHYEDVWPEDVIHRGYGGVDCVEAGGPPAGAGCGGYVVGETVKPLKESN
         |||||| | ||.||||.|||||||||||||||||||||||||||||||||||||.||| |
Syn      PTIIDTLQEKDFHYEDIWPEDVIYKGYGGVDCVEAGGPPAGAGCGGYVVGETVKLLKELN


bchL     LLEDTDVVVFDVLGDVVCGGFAAPLQHADRALIVTANDFDSIYA + 113 amino acids
         .. . |...|||||||||||||||| .||  .|.| | ||...|
Marc     AFYEYDIILFDVLGDVVCGGFAAPLNYADYCIIITDNGFDALFA + 134 amino acids
         |||||||||||||||||||||||||||||||||||||||||| |
Pac      AFYEYDIILFDVLGDVVCGGFAAPLNYADYCIIITDNGFDALSA
         || |||.||||||
Syn      AFDEYDVILFDVL<<<<<<<<<
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Fig. 5. Amino acid sequence alignment of the chlorophyll iron protein subunits of various protochlorophyllide reductases with the nitrogenase reductase iron protein subunit encoded by nif H. Amino acid sequences used in designing PCR primers are underlined. Identical amino acids are indicated by |. *bchL* from *Rhodobacter capsulatus* (YOUVAN & al. 1984, YANG & BAUER 1990); Marc, *Marchantia polymorpha* (OHYAMA & al. 1986) and Pac, *Polystichum acrostichoides* chloroplasts; Syn, *Synechococcus* sp. 7002; *chlL* (*frxC*). The Syn sequence is from PCR products (Fig. 3), while the Pac sequence is from pFePacl (Fig. 4) "Insert" in the *bchL* sequence refers to an N-terminal 36 amino acid extension that is present in *bchL* and *bchX*, but not in any of the *chlL* or *nifH* sequences

Translation initiation in *P. acrostichoides* likely begins at the TTG resulting in a protein five amino acids longer than that of *Marchantia* at the N-terminus. An in-frame TGA stop codon ten codons upstream of this TTG eliminates the possibility of initiation further upstream. There is also a potential ribosome binding site (AAAtGAA) located seven nucleotides upstream from the TTG. TTG starts are less common than GTG or ATG occurring with a frequency of 1 : 10 : 89 in *E. coli* (GOLD & STORMO 1987).

Nearly 460 nucleotides upstream from *chlL* and in the opposite orientation lies the *trnN*(GUU) gene for tRNA^{Asn}. A candidate promoter sequence [TTGCTA(N_{17})TATAAT] occurs 158 nucleotides prior to the *trnN* start (towards *chlL*). The sequence of *trnN*(GUU) from *P. acrostichoides*, in 67 of 72 positions, is identical to *trnN*(GUU) from pine. Between *chlL* and *trnN*(GUU) in the *P. acrostichoides* chloroplast genome, there is a curious region of unknown function which contains both a poly-G tract and a poly-C tract, with a short spacer between the two. Since this region is between divergently transcribed genes, it is unlikely

to form a hairpin in mRNA or to serve as a transcriptional terminator. No function can presently be ascribed to these sequences; however, in an AT-rich genome, it seems unlikely that they are without significance.

We also gathered data from Southern hybridizations to survey the distribution of the *chlL* gene in the major extant lineages of non-angiospermous vascular plants (Table 1). All but two of the taxa examined retain *chlL* (Fig. 6). (In addition to the figured taxa, *Lycopodium, Selaginella, Botrychium,* and *Angiopteris* were examined and results indicate that the gene is present in the chloroplast genomes of these pteridophytes.) In taxa where hybridization signals were observed, the region detected with various *chlL* probes mapped to the expected location in the small single-copy region adjacent to IR_A (Fig. 7). However, the pteridophyte *Psilotum* and the gnetalean gymnosperm *Welwitschia* gave no detectable signal when hybridized to any of the *chlL* probes, even in duplicate experiments with additional filters, although these DNAs gave strong signal when hybridized to other gene specific probes such as 23S rDNA or *ndhF* (data not shown).

Table 1. Land plant taxa examined for presence of *chlL*

Taxon	Source[a]	*chlL*	Data[b]
Lycopodium obscurum L.	F	present	1, 2
Selaginella spec.	UCONN	present	1
Isoetes melanopoda Guy & Durieu	UCONN	present	1, 2
Equisetum scirpoides Michx.	UCONN	present	1
Equisetum arvense L.	F	present	1, 2
Botrychium virginianum (L.) Sw.	F	present	1, 2
Psilotum nudum (L.) Beauvois	UCONN	ABSENT	1
Osmunda cinnamomea L.	F	present	1, 2
Lygodium palmatum (Bernh.) Sw.	UCONN	present	1
Adiantum spec.	UCONN	present	1
Polystichum acrostichoides (Michx.) Schott	F	present	1, 2, 3
Marattia spec.	NYBG	present	1, 2
Angiopteris spec.	UCONN	present	1
Podocarpus macrophyllus (Thunb.) D. Don	UCONN	present	1, 2
Ginkgo biloba L.	F	present	1, 2
Cycas revoluta Thunb.	UCONN	present	1, 2
Encephalartos spec.	UCONN	present	1, 2
Gnetum spec.	UCB	present	1, 2
Ephedra spec.	UCB	present	1, 2
Welwitschia mirabilis Hook. f.	OSU	ABSENT	1

[a] Material from field collections (F) or from living collections of Ecology and Evolutionary Biology Greenhouses, Univ. of Connecticut, Storrs (UCONN); University of California (Berkeley) Botanical Garden (UCB); New York Botanical Garden (NYBG); and Department of Botany Greenhouses, Ohio State Univ. (OSU).

[b] Presence or absence of *chlL* in the cpDNA of taxon inferred from/determined by *1* signal in southern hybridization; *2* location mapped in cpDNA; *3* nucleotide sequence

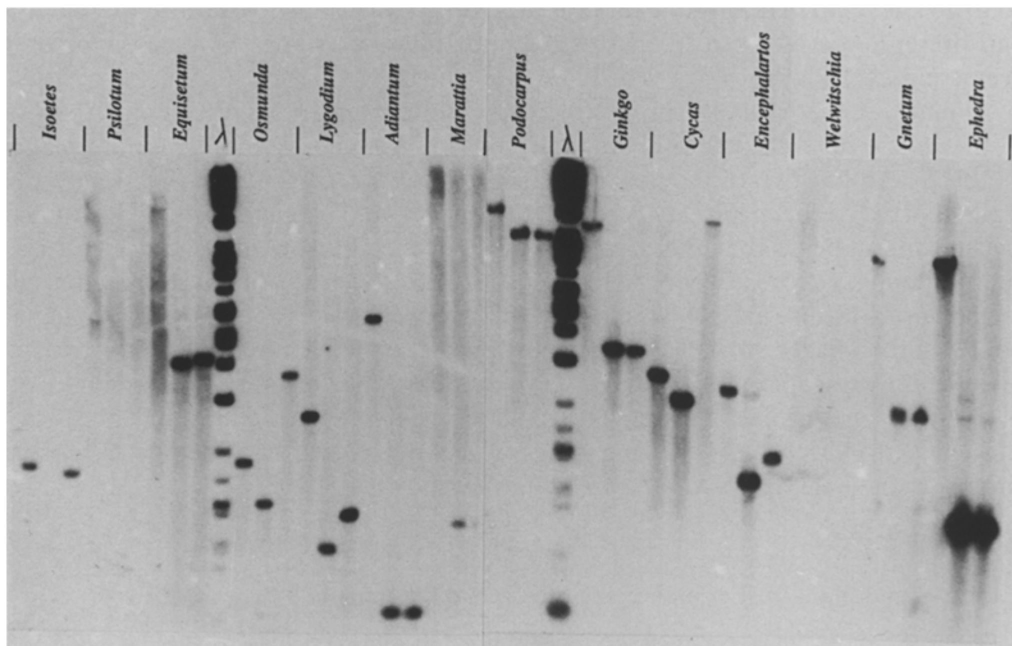

Fig. 6. Southern hybridization of *chlL* to vascular plant DNAs. The taxa represented (in order, left to right) are the lycopsid *Isoetes, Psilotum* (whisk fern), *Equisetum* (horsetail), the ferns *Osmunda, Lygodium*, and *Adiantum*, the eusporangiate fern *Marattia*, the conifer *Podocarpus*, the cycads *Cycas* and *Encephalartos*, and the three gnetalean gymnosperms *Welwitschia, Gnetum*, and *Ephedra*. A fragment of 532 bp internal to the *chlL* gene gel-excised from the chloroplast DNA of *Marchantia polymorpha* was used to probe total genomic DNA to produce the autoradiograph shown. *Synechococcus* and *Polystichum acrostichoides* PCR products were used as probes in additional experiments with the same results. Each taxon is represented by three lanes – the first is a Bam HI digest, the third a Hind III digest, and the middle lane the double digest Bam HI/Hind III. Note the lack of hybridization in *Psilotum* and *Welwitschia*

Discussion

We report here the partial sequences of the PChlide reductase Fe protein subunit *chlL* from a cyanobacterium and from the chloroplast of the fern *Polystichum acrostichoides*. These sequences were efficiently amplified from purified DNA using degenerate, inosine-containing primers. So far, *chlL/bchL*-like sequences have been reported from two of the five photosynthetic bacterial divisions (cyanobacteria/chloroplasts and protobacteria). A similar PCR-based strategy may prove fruitful in detecting homologous sequences in the three photosynthetic other bacterial groups (*Heliobacteria, Chloroflexaceae*, and *Chlorobiaceae*).

We have mapped the location of the *chlL* gene mostly to the small single copy region adjacent to IR_A in the chloroplast genomes of *Equisetum, Polystichum, Osmunda, Ginkgo*, and *Cycas*. This location is the same as the position of *chlL* in *Marchantia polymorpha* (OHYAMA & al. 1986). Furthermore, the location and orientation of *trnN* with respect to *chlL* in *P. acrostichoides* are identical to those in *Pinus nigra* (LIDHOLM & GUSTAFSSON 1991) and *M. polymorpha* (OHYAMA &

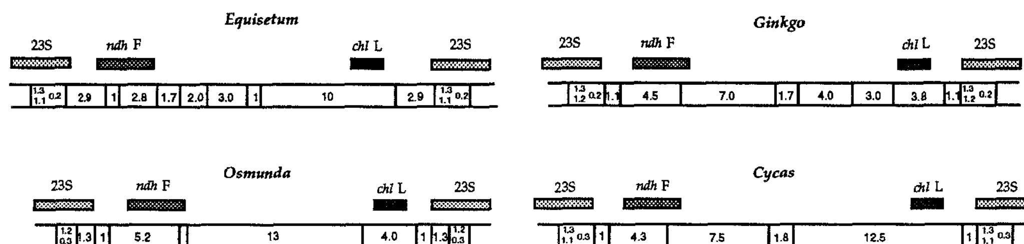

Fig. 7. Physical maps showing location of *chlL* in the small single copy region of the chloroplast genome. Maps are shown for the enzyme HindIII for four taxa – two pteridophytes (*Equisetum* and *Osmunda*) and two gymnosperms (*Ginkgo* and *Cycas*). The 23S rDNA is within the inverted repeat. The *chlL* gene maps to one end of the small single copy (as in *Marchantia* and *Polystichum*) and at the opposite end of the small single copy from the *ndhF* gene. Positions of *chlL* within large fragments is determined from double digest information

al. 1986) chloroplasts. In both *Pinus* and *Marchantia*, as well as in the cyanobacterium *Synechocystis* PCC 6803, the next gene downstream from *chlL* is *chlN*. It would therefore not be surprising to find a *chlN* gene downstream from *chlL* in the fern chloroplast as well, although our sequence does not answer this question.

Given the level of conservation among known amino acid and nucleotide sequences of *chlL* genes, including those presented here, the lack of hybridization signal from *Psilotum* and *Welwitschia* suggests a loss of the *chlL* gene from the chloroplast genomes in these taxa. Lack of hybridization signal from *Psilotum* was reported by SUZUKI & BAUER (1992) during the course of the preparation of this manuscript. They also reported positive hybridization of a *chlL*-specific probe to *P. acrostichoides* DNA. These investigators examined additional taxa, but they did not assay for the presence of *chlL* in gnetalean gymnosperms, cycads, or eusporangiate ferns.

All bacterial and organellar genomes that have been thoroughly investigated (*Rhodobacter capsulatus, Marchantia polymorpha, Cyanophora paradoxa*, rice, and tobacco) either contain all three subunits of the light-independent PChlide reductase (*chlL/bchL, chlN/bchN*, and *chlB/bchB*) or none of them. In each case, the presence or absence of the genes correlates with the ability or inability, respectively, to green in the dark. Thus, detection of any one of these genes suggests both the presence of the other genes and the ability to reduce PChlide in the dark. It will therefore be of interest to determine whether the taxa examined here that retain *chlL* also retain dark Chl synthesis ability, and whether those that show no *chlL* signal retain dark PChlide reductase activity. Dark PChlide reductase activity may have been retained through the transfer of some or all of the genes for this enzyme from the chloroplast to the nucleus. We did not test for the presence of these genes in the nucleus.

The distribution we observe for *chlL* implies that the gene has been lost from the chloroplast genome on more than one occasion, as it seems unlikely that *Psilotum, Welwitschia*, and the angiosperms share a unique common ancestor, one not shared by other vascular plants. At a minimum, the apparent loss in *Psilotum* is likely independent, since seed plants are widely viewed as monophyletic. It is

possible that a single (shared) loss event is responsible for the lack of *chlL* hybridization signal in *Welwitschia* and all angiosperms examined to date. If that is the case, the *chlL* character would be in conflict with current ideas that consider the *Gnetales* to be monophyletic (CRANE 1985, DOYLE & DONOGHUE 1986, HAMBY & ZIMMER 1991).

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