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Structure of a Co-Transcribed Gene Cluster, ndh1-frxB3-ndh6-ndh4L, Cloned from the Filamentous Cyanobacterium Plectonema boryanum4

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The frxB gene found in chloroplast genomes encodes a bacterial ferredoxin-like (Fd-like) Fe-S protein, whose function has not been established. Using an frxB gene probe from liverwort, we have isolated and determined the nucleotide sequence of a 4.6-kb fragment of DNA that contains the frxB gene from the filamentous cyanobacterium Plectonema boryanum. The frxB gene from Plectonema encodes a protein of 194 amino acids that shows 66% sequence identity to that encoded by liverwort chloroplasts. The sequence contains two clusters of cysteine residues (C-X-X-C-X-X-C-X-X-X-C-P), typical of bacterial ferredoxins that chelate two [4Fe-4S] clusters. Such sequences are found in highly conserved regions in the seven available sequences of frxB-related proteins. In addition, we identified three ORFs homologous to the chloroplast ndh1, ndh6 and ndh4L genes that encode the components of a putative NADH dehydrogenase. They show 60%, 50\% and 65\% amino acid sequence identity to the respective gene products from liverwort chloroplasts, but they show a lower degree of similarity to the mitochondrial homologues. The ndh1, frxB, ndh6 and ndh4L genes, occurring as single genomic copies, are tightly linked to one another, and they are co-transcribed as a 2.8-kb mRNA which was identified by Northern blot analysis with gene-specific probes. These four genes occur in the same order in the chloroplast genomes of liverwort, tobacco and rice as they do in *Plectonema*. Therefore, the organization of ndh1-frxB-ndh6-ndh4L may represent a case of clustering and co-transcription of functionally related genes in both cyanobacteria and chloroplasts. These findings lend a support to the hypothesis that cyanobacteria and chloroplasts contain an enzyme complex that is closely related to the mitochondrial complex I, although such a highly organized enzyme complex has not been identified to date in an extensive survey of cyanobacterial NAD(P)H dehydrogenases.

Key words: Chloroplast DNA — Complex I — Cyanobacterial DNA — Iron-sulfur protein — NAD(P)H dehydrogenase — *Plectonema boryanum*.

The complete nucleotide sequences are known of the chloroplast genomes of liverwort (Marchantia poly-

morpha; Ohyama et al. 1986), tobacco (Nicotiana tabacum; Shinozaki et al. 1986), and rice (Oryza sativa; Hiratsuka et al. 1989). Genes encoding bacterial Fd-like proteins (frxA and frxB) have been found in these genomes (Ohyama et al. 1988, Kohchi et al. 1988). These Fe-S proteins are not related to the soluble chloroplast-type Fd which functions in photosynthetic electron transport. frxA encodes a 9-kDa protein of the photosystem I complex which carries two [4Fe-4S] clusters (centers A and B; Oh-oka et al. 1987, 1988), while the function of the frxB protein has not been established. The product of expression of the frxB gene has been identified at the protein level in chloroplasts of Chlamydomonas reinhardtii (Wu et al. 1989), tobacco and spinach (Lin and Wu 1990). The prod-

Abbreviations: Fd, ferredoxin; ORF, open reading frame. ³ For renaming "frxB" two proposals have been made: "ndhK", by dePamphilis and Palmer (1990); and "ndhI", by Dupuis et al. (1991).

⁴ The sequence data will appear in the DDBJ/EMBL/GenBank Nucleotide Sequence Databases under the accession number D01014.

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uct appears to be a membrane protein that is bound to the peripheral portion of thylakoids since it can be extracted by high-salt washing. This conclusion is consistent with our recent finding that the *frxB* protein can be solubilized by detergent from the thylakoid membranes and is recovered in a fraction that contains unidentified protein complexes (Shonai et al. unpublished results).

In chloroplast genomes, seven ORFs have been identified as the homologues of the mitochondrion-encoded components of complex I (NADH:ubiquinone reductase). The ORFs of liverwort chloroplasts are named ndh1, 2, 3, 4, 4L, 5 and 6 (Ohyama et al. 1986, 1988), and the corresponding genes (ndhA, B, C, D, E, F and G) are also found in chloroplasts of tobacco and rice (Matsubayashi et al. 1987, Hiratuka et al. 1989). The frxB gene is located in a gene cluster that encodes four homologues of components of mitochondrial complex I: ORF392(or 393)-ndh1(A)frxB-ndh6(G)-ndh4L(E)-frxA(psaC)-ndh4(D). It has recently been demonstrated that some of the nucleus-encoded subunits of mitochondrial complex I have also homologues encoded by ORFs in chloroplast genomes. They are the 49-, 30- and 23-kDa subunits of bovine complex I, which are related to product of liverwort ORF392, ORF169 and frxB, respectively (Fearnly et al. 1989, Pilkington et al. 1991a, Dupuis et al. 1991). These findings have led to the suggestion that the putative ndh proteins, together with the product of frxB are components of an NAD(P)H:plastoquinone reductase that is responsible for the respiratory electron-transport activity in Chlamydomonas chloroplasts (Godde 1982, Maione and Gibbs 1986). Another possibility is exemplified by the results of Wu and co-workers (1989) who showed that the salt-extracted frxB protein has an affinity for the origin of replication of ctDNA. However, both these piece of evidence are indirect and fail to disclose the functional role of this protein in chloroplasts.

We have studied structure-function and evolutionary relationships among various iron-sulfur proteins and we have initiated a molecular genetic study using cyanobacteria which is aimed at elucidating the functional role of the frxB protein. Here we report the cloning, nucleotide sequence and co-transcription of the ndh1, frxB, ndh6 and ndh4L genes of a filamentous cyanobacterium, Plectonema boryanum. These results are discussed in terms of the putative function of the frxB protein.

Materials and Methods

Preparation of genomic DNA and hybridization—A filamentous, non-heterocystous cyanobacterium, Plectonema boryanum strain M101, was kindly provided by Dr. T. Kato (Kyoto University). Cells were grown in BG-11 medium (Rippka et al. 1979) at 30°C, in an illuminated incubator. They were harvested by centrifugation at the

stationary phase of growth, and DNA was prepared as described by Fujita et al. (1991). Fractionated digests of DNA were transferred from agarose gels to nitrocellulose as described by Southern (1975). A 363-bp Bcl I-Alu I fragment, containing the truncated frxB gene from liverwort chloroplasts, was radioactively labeled with $[a^{-32}P]dCTP$ by the Multiprime DNA labeling system (Amersham) and used as a hybridization probe. This probe contained nucleotides 99916 to 100278 of the liverwort ctDNA (Kohchi et al. 1988). Hybridization was carried out with the radiolabeled probe for 18 h at 45°C. The filters were washed twice in a mixture of $6 \times SSC$ and 0.5% SDS at room temperature, twice again in the same solution at 45°C, twice at 55°C, and twice more at 60°C. For other details, including hybridization conditions, see Fujita et al. (1991).

Screening of the genomic DNA library—Approximately 2×10^4 recombinants of a *Plectonema* genomic DNA library in pBR322 (Fujita et al. 1991) were screened by the colony hybridization method of Hanahan and Meselson (1983). The probe and conditions used were as described above. Two recombinants gave a strong hybridization signal with the liverwort frxB probe. Both of the positive recombinants carried inserted DNA fragments whose restriction maps were indistinguishable from one another. One, named pB-45, was selected for further study.

Subcloning and DNA sequencing—A 3.1-kb Pst I fragment, which hybridized to the probe for frxB, and a 1.5-kb Pst I-Hind III fragment (see Fig. 1) were subcloned into M13mp18 and -19 vectors in both orientations. Their DNA sequences were determined by the modified dideoxy method (Sanger et al. 1977, Biggin et al. 1983) after the construction of deleted subclones by means of the ExoIII/ mung bean nuclease deletion technique (Henikoff 1984). The complete sequences of the 3.1-kb and 1.5-kb fragments were determined for the entire inserts in both orientations with the aid of one internal primer. A 1.7-kb Ssp I-Hind III fragment (see Fig. 1) was also subcloned into M13mp19 which had previously been cut with Sma I and Hind III. One-sided sequencing, starting from the Ssp I site, gave an overlapping sequence across the Pst I site. Sequence comparisons were made using the GENETYX (Software Development Co., Ltd., Tokyo, Japan) programs. Predicted amino acid sequences of more than 33 amino acids in length were compared with the sequences in the databases of NBRF-PDB (National Biochemical Research Foundation) and SWISS-PROT (European Molecular Biology Laboratory).

Northern blot analysis—Total RNA was isolated from Plectonema cells that had been grown mixotrophically in BG-11 medium supplemented with 5 mm glucose (Fujita et al. 1991). The RNA was fractionated by electrophoresis on 1.0% agarose/formaldehyde gels and transferred to an Immobilon-N membrane (Millipore). Single-strand hybridization probes were synthesized (Sambrook et al. 1989)

from the inserts of deletion clones used for sequence analysis: for ndh1, nucleotides 1556 to 1083 of the DNA sequence shown in Figure 2 (probe 1); for frxB, nucleotides 2225 to 1807 (probe 2); for ndh6, nucleotides 2758 to 2345 (probe 3); for ndh4L, nucleotides 3103 to 2905 (probe 4); for the 3' non-coding strand, nucleotides 3951 to 3624 (probe 5); for ORF105, nucleotides 1496 to 1786 (probe 6); and for ORF295, nucleotides 3526 to 4011 (probe 7). Hybridization was performed in $6 \times SSC$, $5 \times Denhardt's$ solution, 0.5% SDS and $100 \mu g ml^{-1}$ salmon sperm DNA at $65^{\circ}C$ for 16 h. The washings were performed in $0.2 \times SSC$ and 0.1% SDS at $65^{\circ}C$.

Results and Discussion

Cloning and DNA sequence—The radiolabeled probe derived from the frxB gene from liverwort chloroplasts hybridized to specific fragments in restriction digests of DNA from P. boryanum, namely, the 5.6-kb Hind III fragment, the 4.0-kb EcoR I fragment, and the 2.7-kb

Hind III-EcoR I fragment (data not shown). With the same gene probe, two positively hybridizing recombinants, pB-05 and pB-45, were identified in a library of Plectonema DNA which had been constructed with the Hind III digest of the genomic DNA. These two positive recombinants carried a 5.6-kb Hind III fragment (Fig. 1) and were indistinguishable from one another with respect to the sizes of various restriction fragments. Subsequent Southern hybridization showed that the length of the hybridizing fragment in the Pst I digest of the recombinants was 3.1 kb. The DNA sequence of the 3.1-kb Pst I fragment from pB-45 corresponds to nucleotides 1 to 3108, shown in Figure 2. As described below, the sequence did not contain the 3' portion of the ndh4L homologue. Therefore, the sequence was extended in the 3' direction by the use of a 1.5-kb Pst I-Hind III fragment (nucleotides 3103 to 4616) derived from pB-45. The complete sequence presented in Figure 2 is 4616 bp long. Each nucleotide was identified at least once on each strand of the DNA.

Identification of potential genes—The DNA sequence

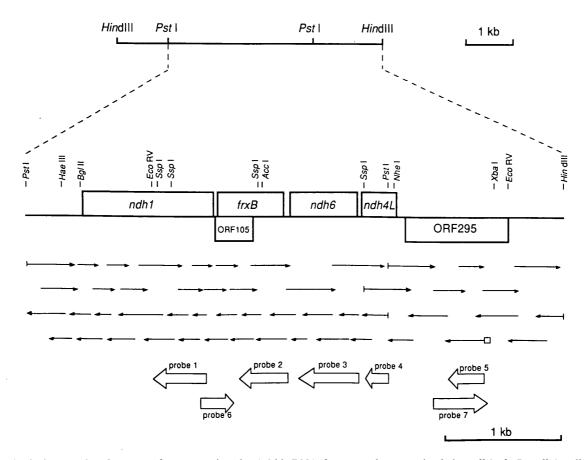


Fig. 1 Physical map of and strategy for sequencing the 4.6-kb DNA fragment that contained the ndh1, frxB, ndh6, ndh4L gene cluster, cloned from the filamentous cyanobacterium P. boryanum. The four genes and two unidentified reading frames (ORF105 and ORF295) are shown as open boxes on the map. The direction and extent of analyses of sequences of individual restriction fragments or deletion clones are indicated by the arrows below the map. An open box attached to an arrow indicates that an internal synthetic oligonucleotide primer was used in the sequence analysis. Open arrows at the bottom of the Figure correspond to single-strand DNAs used as gene-specific probes for the Northern blotting analysis in Fig. 3. For other details see Materials and Methods.

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10 20 CTGCAGGCTGGTTAGCGCATTGGA	30 40 AAGAGCAGCTTGCAGAAAAC	50 60 CGGATCTTCCGTCCGACG	70 80		
130 140 GATTGCGTAATTTCGATGATGGGT	150 160	170 180	190 200) 210 22	20 230 240
250 260 CTTTTATTTTTACAAATTCTGACT	270 280	290 300	310 320) 330 34	10 350 360
370 380	390 400	410 420	430 440) 450 4 <i>6</i>	io 470 480
AAATCGGCAGAAATTCAGTAGTTCC	510 520	530 540	550 560	570 58	590 600
TTCTTACTGCGTTG <u>GGA</u> ACCATGAI ndh1> M N					CCCTCTGGATGCCATTGCCGA
610 620 TGCTGATCATGCTCCTTGCAGCGAC	630 640 CGGTCAGCGTTCTGGTTGTGG	650 660 GTGTGGCTAGAACGGAAA	670 680 ATCTCAGCCGCCGCGCAA	CAGCGGATTGGTCCTGAAT	TCATCGGACCGCTCGGCGTTC
LIM LLAAT 730 740	750 760	770 780	790 800	810 82	0 830 840
TCGCCCCGCTTGCGGATGGCTTAAA A P L A D G L K	LVLKED V	GTGGTTCCGGCGAAAGCA V V P A K A	GATAAGCTGCTCTTTACC D K L L F T	CTAGGTCCCGCGATCGTGG L G P A I V V	TTATTCCCGTCTTTTTGTCCT I P V F L S Y
850 860 ACTTGATTCTGCCGTTTGGACAAAA L I L P F G Q N	870 880 ACCTTCAAATCACCGATGTCC L Q I T D V C	890 900 GGGCTGGGAATCTTCCTT G L G I F L	910 920 TGGATTGCCTTATCGAGC W I A L S S	GTTGTGCCGATCGGGCTTT	TGATGTCGGGCTATGCCTCGA
970 980 ATAACAAATACTCGCTGCTGGGTGC	990 1000	1010 1020	1030 1040	1050 106	0 1070 1080
NKYSLLGG	LRAAAQS	SISYEL	PLALSV	LAVVMMS	N S L S T V D
1090 1100 ATATCGTCAATCAGCAAGCGGGCTA I V N Q Q A G Y	1110 1120 ACGGAATTCTGGGCTGGAATA G I L G W N 1	1130 1140 ATTTGGCGACAACCGGTT I W R Q P V	1150 1160 GGATTTATTATTTCTGG G F I I F W	ATTGCGGCTCTGGCAGAAT	GTGAGCGGATTCCCTTTGACT
1210 1220 TACCAGAAGCGGAAGAAGAACTCGT	1230 1240 TTGCAGGGTATCAGACTGAAT	1250 1260 TATTCAGGCATGAAGTTT	1270 1280 GCCCTGTTCTATCTCGGG	TCTTATGTGAACTTGACGC	TCTCAGCCTTGCTGTTTGCTG
P E A E E E L V	A G Y Q T E Y	Y S G M K F .	A L F Y L G 1390 1400		
TCCTTTACCTGGGTGGTTGGGAATT L Y L G G W E F	PISLS V	ATCTCCGGACTGATTGGC ISGLIG	GTGCCCGAATCGACTCCT V P E S T P	TGGTTGCAGTTGATTTTCG	CCACAATCGGCATTGGAATGA
1450 1460 CGCTCTTGAAGGCTTATTCCTTGAT	1470 1480 FCTTCCTGGCGATTTTGATGG	1490 1500 CGTTGGACTGTGCCCCGT	1510 1520 GTGCGGATTGACCAACTG	CTCGACCTCGGCTGGAAGT	TTCTACTTCCCGTCTCGCTTG
L L K A Y F L I	1590 1600	1610 1620	1630 1640	1650 166	0 1670 1680
TCAACTTGCTGATCACCGCAGGTTT N L L I T A G L					CAAACAAGTCGGTGATTACAC K Q V G D Y T
1690 1700 GAAAGAGGCGATCCAAGCAGGCAAG		1730 1740 CTGTGACCTTTGACCACA	1750 1760 TGCGCCGTCGCCCTATCA		
K E A I Q A G K	Y I G Q G L S	V T F D H M	RRRPIT	V Q Y P Y E	K L I L S E R
CTTCCGGGGACGGATTCACTTTGAA F R G R I H F E	ATTTGACAAGTGTATTGCTTG	GCGAAGTCTGTGTGCGGG	PTTGCCCGATCAACTTGC	CTGTCGTAGATTGGGAATT	CAACAAAGAAACCAAGAAAAA
1930 1940 GAAACTCAATCATTACAGTATTGAT	1950 1960	1970 1980	1990 2000		
KLNHYSID	F G V C I F C	GNCVEY	CPTNCL	S M T E E Y	
2050 2060 ACATGAACTGAACTACGACAATGTG H E L N Y D N V	2070 2080 GCACTGGGTCGTCTGCCGTA A L G R L P Y	2090 2100 ACAAAGTGACGAATGATC K V T N D P	2110 2120 CGATGGTGACTCCGTTGC M V T P L R	GTGAGTTTGCATACTTACO	GAAGGGTGCGATCGATCCACA
2170 2180	2190 2200	2210 2220	2230 2240	2250 226	0 2270 2280
TGATCTACCTGCTGGTTCGCGTCGG D L P A G S R R				GGGCGTGACTGCCCCACTT	rdh6> M
2290 2300 TGAATTTAGCGGAAGGTGTTCAAAT N L A E G V Q I				TTACTGGAAAATGTGGTTT	ATTCTGCCTTTCTTTTAGGCG
2410 2420 GCGTTTTCATTAGCATTGCGGGATT	2430 2440 PATATTTGCTGCTGAATGCAG	2450 2460 GACTTCGTGGCAGCGGCG	2470 2480 CAAGTTTTGATTTACGTC	2490 250 GGGGCAGTCAACGTCTTGA	0 2510 2520
V F I S I A G L	Y L L L N A D	2570 2580	2590 2600	3 A V N V L I 2610 262	L F A I M L V 0 2630 2640
TGAACAAGCGTGAGGCATTTCAGCC N K R E A F Q P					

Cyanobacterial ndh1-frxB-ndh6-ndh4L gene cluster

CGATTTCAACAGCGGTTCCGATCC	2670 2680 GAGAGTTCGATTATTACGATCC	2690 2700 GGCTGCATTTCTTCACCGA	2710 2720	2730 2740	2750 2760
ISTAVPI	SSIITIO	L H F F T D	F L L P F E	L A S I L L L	M A L V G A
2770 2780 CGATCGTGCTGGCACGTCGTGAGT	2790 2800 TTCTGCCGGATGAGGATGAGG	2810 2820 GCGGATACCGCTTTAACTTT	2830 2840	2850 2860	2870 2880 CAACCCTGAAAACTAAG
IVLARREE					
2890 2900 <u>GA</u> CTTTGAACGATGCAACTTCAAT	2910 2920	2930 2940	2950 2960	2970 2980	2990 3000
				R N A V R V L	
3010 3020	3030 3040	3050 3060	3070 3080	3090 3100	3110 3120
TGCTGAACGCTGTGAATTTGAATT LNAVNLNL	TGATGGCGTTTTCTAACTATC MAFSNYL	TTGATCCGCAAGAGATCAA DPQEIK	AGGTCAGATGTTTACGAT	TTTCGTGATTACGATCGCGGCT F V I T I A A	TGCAGAAGCGGCAGTTG A E A A V G
3130 3140	3150 3160	3170 3180	3190 3200	3210 3220	3230 3240
GTTTAGCGATCGTGCTAGCGATCT L A I V L A I Y	ATCGGAACCGCGATACGGTCG	SATATGGAACAATTCAATTT) M E Q F N L	ACTCAAGTGGTAAATTGT: L K W *		GGTGTAGTGAGTCTGCA
3250 3260	3270 3280	3290 3300	3310 3320	3330 3340	3350 3360
CCTCTTTTTTGTTTGAGGATAGCA				CACTTCAGCAATGAGTTCCATA V E A I L E M	
3370 3380	3390 3400	3410 3420	3430 3440	3450 3460	3470 3480
GCATATTGAGAGCAATCGCCTGCT L M N L A I A Q	TTTCTTCCAATTTTGCTCTCC	GCTCTAGTGATGACACATA	CTTCATCCGTCTAGCCTC	CTCAAAAGTTTCTAACTCAGC	CTGTAACTGGCGCTCTA
3490 3500	3510 3520	3530 3540	3550 3560	3570 3580	3590 3600
ACTCTTCAGGCAAATTCATCAGCC	AGTCTAAAAAGTTGTGTAGCT	CTAGTATATCTTGCTCACT	ATAGCCACGATCGTAAAG	CATTGTCGTCAGATGATACCGC	CCAGCGCTTGCGCTCAA
3610 3620	3630 3640	3650 3660	3670 3680	3690 3700	3710 3720
GTGGCTGATTGTGCGTTTCCTTGG	TCTTTAGATGAGCCATCGCGA	CGACAGCAAAAGGATTTTG.	AATGGCTTCTAATTCTGTG	CATTGAGATTGATAGTCTGAT	TAGCTTCACGATTGGAA
LPQNHTEK	TKLHAMA	CGACAGCAAAAGGATTTTG V V A F P N Q	AATGGCTTCTAATTCTGTC I A E L E T	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S	TAGCTTCACGATTGGAA L K V I P
L P Q N H T E K 3730 3740 ACTCAAAATGAGTGGCACAGCCCC	T K L H A M A 3750 3760 ACAATTCGTCATAAAACCTGC	CGACAGCAAAAGGATTTG V V A F P N Q 3770 3780 TGGGTCGCCAATTCGGGCG	AATGGCTTCTAATTCTGTC I A E L E T 3790 3800 GTCATCACCCAGAATGGCT	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S 3810 3820 FAAGCTGACAACAGGGCAATTA	TAGCTTCACGATTGGAA L K V I P 3830 3840 ATAGCGATCGCGCAATC
L P Q N H T E K 3730 3740 ACTCAAAATGAGTGGCACAGCCCC F E F H T A C G	T K L H A M A 3750 3760 ACAATTCGTCATAAAACCTGC W L E D Y F R	CGACAGCAAAAGGATTTTG. V V A F P N Q 3770 3780 TGGGTCGCCAATTCGGCGG S P R W N P R	AATGGCTTCTAATTCTGTC I A E L E T 3790 3800 GTCATCACCCAGAATGGCT D D G L I A	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S 3810 3820 TAAGCTGACAACAGGCAATTA L S V V P C N	TAGCTTCACGATTGGAA L K V I P 3830 3840 ATAGCGATCGCGCAATC Y R D R L
L P Q N H T E K 3730 3740 ACTCAAAATGAGTGGCACAGCCCC F E F H T A C G 3850 3860 GATAGTTGTAGCTGTACATCCTGG	T K L H A M A 3750 3760 ACAATTCGTCATAAAACCTGC W L E D Y F R 3870 3880 CGACGAAATCGCGCTCCTCTT	CGACAGCAAAAGGATTTTG. V V A F P N Q 3770	AATGGCTTCTAATTCTGTC I A E L E T 3790 3800 GTCATCACCCAGAATGGCT D D G L I A 3910 3920 ACAAATCACTAAGGTTCTC	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S 3810 3820 PAAGCTGACAACAGGGCAATTA L S V V P C N 3930 3940 CTCACCCGCAGACGGTGAACT	TAGCTTCACGATTGGAA L K V I P 3830 3840 ATAGCGATCGCGCAATC Y R D R L 3950 3960 TTCGACTAATTTATCGG
L P Q N H T E K 3730 3740 ACTCAAAATGAGTGGCACAGCCCC F E F H T A C G 3850 3860 GATAGTTGTAGCTGTACATCCTGG R Y N Y S Y M R	T K L H A M A 3750 3760 ACAATTCGTCATAAAACCTGC W L E D Y F R 3870 3880 CGACGAAATCGCGCTCCTCTT A V F D R E E	CGACAGCAAAAAGGATTTTG. V V A F P N Q 3770 3780 TGGGTCGCCAATTCGGCGC S P R W N P R 3890 3900 GACTTTGCACTTCAATGTG. Q S Q V E I H	AATGGCTTCTAATTCTGTC I A E L E T 3790 3800 GTCATCACCCAGAATGGCT D D G L I A 3910 3920 ACAAATCACTAAGGTTCTC C I V L T R	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S 3810 3820 PAAGCTGACAACAGGGCAATTA L S V V P C N 3930 3940 CTCACCCGCAGACGGTGAACT	TAGCTTCACGATTGGAA L K V I P 3830 3840 ATAGCGATCGCGCAATC Y R D R L 3950 3960 TTCGACTAATTTATCGG
The bound of the color of the	T K L H A M A 3750 3760 ACAATTCGTCATAAAACCTGC W L E D Y F R 3870 3880 CGACGAAATCGCGCTCCTCTT A V F D R E E C 3990 4000 CTCTGACGATTTTCTGTAATT	CGACAGCAAAAGGATTTTG. V V A F P N Q 3770	AATGGCTTCTAATTCTGTC I A E L E T 3790 3800 GTCATCACCCAGAATGGCT D D G L I A 3910 3920 ACAAATCACTAAGGTTCTC C I V L T R 4030 4040	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S 3810 3820 PAAGCTGACAACAGGGCAATTA L S V V P C N 3930 3940 CTCACCCCGCAGACGGTGAACT E G R L R H V 4050 4060	TAGCTTCACGATTGGAA L K V I P 3830 3840 ATAGCGATCGCGCAATC Y R D R L 3950 3960 TTCGACTAATTTATCGG E V L K D 4070 4080 AAAGAAGGCTAGAAACT
L P Q N H T E K 3730 3740 ACTCAAAATGAGTGGCACAGCCCC F E F H T A C G 3850 3860 GATAGTTCTAGCTGTACATCCTGG R Y N Y S Y M R 3970 3980 CATAGCGTTTGGGAATCTCTGCAT A Y R K P I E A	T K L H A M A 3750 3760 ACAATTCGTCATAAAACCTGC W L E D Y F R 3870 3880 CCACCAAAATCGCGCTCCTCTTT A V F D R E E 3990 4000 CTCTGACGATTTTCTGTAATT D R V I K Q L	CGACAGCAAAAGGATTTTG. V V A F P N Q 3770 3780 TGGGTCGCCAATTCGGGCG S P R W N P R 3890 3900 GACTTTGCACTTCAATGTG. Q S Q V E I H 4010 4020 CTTTATCTAGAAAGCGAACG	AATGGCTTCTAATTCTGTC I A E L E T 3790 3800 GTCATCACCCAGAATGGCT D D G L I A 3910 3920 ACAAATCACTAAGGTTCTC C I V L T R 4030 4040 CGGCTTTGACCAGCAAAAA P K S W D V	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S 3810 3820 PAAGCTGACAACAGGGCAATTA L S V V P C N 3930 3940 CTCACCCCGCAGACGGTGAACT E G R L R H V 4050 4060	TAGCTTCACGATTGGAA L K V I P 3830 3840 ATAGCGATCGCGCAATC Y R D R L 3950 3960 TTCGACTAATTTATCGG E V L K D 4070 4080 AAAGAAGGCTAGAAACT
3730 3740	T K L H A M A 3750 3760 ACAATTCGTCATAAAACCTGC W L E D Y F R 3870 3880 CGACGAAATCGCGCTCCTCTT A V F D R E E C 3990 4000 CTCTGACGATTTTCGTAATT D R V I K Q L 4110 4120	CGACAGCAAAAAGGATTTTG. V V A F P N Q 3770	AATGGCTTCTAATTCTGTC I A E L E T 3790 3800 GTCATCACCCAGAATGGCT D D G L I A 3910 3920 ACAAATCACTAAGGTTCTC C I V L T R 4030 4040 CGGCTTTGACCAGCAACA P K S W D V 4150 4160	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S 3810 3820 PAAGCTGACAACAGGGCAATTA L S V V P C N 3930 3940 CTCACCCCCCAGACGGTGAACT E G R L R H V 4050 4060 VCTCGCCTCAATCGTTGGGAAA D A E I T P F	TAGCTTCACGATTGGAA L K V I P 3830 3840 ATAGCGATCGCGCAATC Y R D R L 3950 3960 TTCGACTAATTTATCGG E V L K D 4070 4080 AAAGAAGGCTAGAAACT F F A L F
3730 3740	T K L H A M A 3750 3760	CGACAGCAAAAGGATTTTG. V V A F P N Q 3770	AATGGCTTCTAATTCTGTC I A E L E T 3790 3800 GTCATCACCCAGAATGGCT D D G L I A 3910 3920 ACAAATCACTAAGGTTCTC C I V L T R 4030 4040 CGGCTTTGACCAGTCAACA P K S W D V 4150 4160 ITGGGTCATTACCCAAAAA Q T M <0RF295	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S 3810 3820 PAAGCTGACAACAGGGCAATTA L S V V P C N 3930 3940 CTCACCCCCCAGACGGTGAACT E G R L R H V 4050 4060 VCTCGCCTCAATCGTTGGGAAA D A E I T P F	TAGCTTCACGATTGGAA L K V I P 3830 3840 ATAGCGATCGCGCAATC Y R D R L 3950 3960 TTCGACTAATTTATCGG E V L K D 4070 4080 AAAGAAGGCTAGAAACT F F A L F
3730 3740	T K L H A M A	CGACAGCAAAAAGGATTTTG. V V A F P N Q 3770	AATGGCTTCTAATTCTGTC I A E L E T 3790 3800 GTCATCACCCAGAATGGCT D D G L I A 3910 3920 ACAAATCACTTAAGGTTCTC C I V L T R 4030 4040 CGGCTTTGACCAGTCAACA P K S W D V 4150 4160 TTGCGTCATTACGCCAAAA Q T M <0RF295 4270 4280	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S 3810 3820 PAAGCTGACAACAGGGCAATTA L S V V P C N 3930 3940 CTCACCCCCCAGACGGTGAACT E G R L R H V 4050 4060 ACTGGCCTCAATCGTTGGGAAA D A E I T P F 4170 4180 ATTAGTTGATAGTTCTATATC	TAGCTTCACGATTGGAA L K V I P 3830 3840 ATAGCGATCGCGCAATC Y R D R L 3950 3960 TTCGACTAATTTATCGG E V L K D 4070 4080 AAAGAAGGCTAGAACT F F A L F 4190 4200 CCTATCATTGCATGATT 4310 4320
The control of the	T K L H A M A	CGACAGCAAAAGGATTTTG. V V A F P N Q 3770	AATGGCTTCTAATTCTGTC I A E L E T 3790 3800 GTCATCACCCAGAATGGCT D D G L I A 3910 3920 ACAAATCACTAAGGTTCTC C I V L T R 4030 4040 CGGCTTTGACCAGTCAACA P K S W D V 4150 4160 TTGCGTCATTACCCCAAAA Q T M <0RF295 4270 4280 ATTATGAGTGAGTGGCTCGCTCA4390 4400	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S 3810 3820 PAAGCTGACAACAGGGCAATTA L S V V P C N 3930 3940 CTCACCCCGCAGACGGTGAACT E G R L R H V 4050 4060 ATCTGCCTCAATCGTTGGAAA D A E I T P F 4170 4180 ATTAGTTGATTATTGTTCTATATC 4290 4300 GAAACTGTTCGAGAAATTTGGAAATTTGGAAACTGTTTCGAGAAATTTGGA	TAGCTTCACGATTGGAA L K V I P 3830 3840 ATAGCGATCGCGCAATC
3730	T K L H A M A	CGACAGCAAAAAGGATTTTG. V V A F P N Q 3770	AATGGCTTCTAATTCTGTC I A E L E T 3790 3800 GTCATCACCCAGAATGGCT D D G L I A 3910 3920 ACAAATCACTAAGGTTCTC C I V L T R 4030 4040 CGGCTTTGACCAGTCAACA P K S W D V 4150 4160 TTGCGTCATTACCCCAAAA Q T M <0RF295 4270 4280 ATTATGAGTGAGTGGCTCGCTCA4390 4400	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S 3810 3820 PAAGCTGACAACAGGGCAATTA L S V V P C N 3930 3940 CTCACCCCGCAGACGGTGAACT E G R L R H V 4050 4060 ATCTGCCTCAATCGTTGGAAA D A E I T P F 4170 4180 ATTAGTTGATTATTGTTCTATATC 4290 4300 GAAACTGTTCGAGAAATTTGGAAATTTGGAAACTGTTTCGAGAAATTTGGA	TAGCTTCACGATTGGAA L K V I P 3830 3840 ATAGCGATCGCGCAATC
The control of the	T K L H A M A	CGACAGCAAAAGGATTTTG. V V A F P N Q 3770	AATGGCTTCTAATTCTGTC I A E L E T 3790 3800 GTCATCACCCAGAATGGCT D D G L I A 3910 3920 ACAAATCACTAAGGTTCTC C I V L T R 4030 4040 CGGCTTTGACCAGTCAACA P K S W D V 4150 4160 TTGGGTCATTACCCAAAA Q T M <0RF295 4270 4280 ATTATGAGTGAGAGAAGAAC 4510 4520	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S 3810 3820 PAAGCTGACAACAGGGCAATTA L S V V P C N 3930 3940 CTCACCCCGCAGACGGTGAACT E G R L R H V 4050 4060 ATCTGCCTCAATCGTTGGAAA D A E I T P F 4170 4180 ATTAGTTGATATGTTCTATATC 4290 4300 SAAACTGTTCGAGAAATTTGGAAATTTGGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAACTGTTCGAGAAATTTGGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAAATTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTGGAAATTGAAATTGGAAATTGGAAATTGGAAATTGGAAATTGGAAATTTGGAAATTGAAAATTGAAATTGAAAATTGAAATTGAAATTGAAAATTGAAAATTGAAATTGAAATTGAAATTGAAATTGAAATTGAAATTGAAATTGAAAATTGAAATTGAAAATTGAAA	TAGCTTCACGATTGGAA L K V I P 3830 3840 ATAGCGATCGCGCAATC
The color of th	T K L H A M A 3750 3760 ACAATTCGTCATAAAACCTGC W L E D Y F R 3870 3880 CGACGAAATCGCGCTCCTCTT A V F D R E E C 3990 4000 CTCTGACGATTTTCTGTAATT D R V I K Q L 4110 4120 TTTTCCAAGGATTATCGTAATT T K W P N D Y 4230 4240 CTGTAAACGATATCCTATCTT 4350 4360 CGCACAGTCTTTTCAGGCGAAC 4470 4480 CGCAGATTGGGGATCGGTCACC 4590 4600	CGACAGCAAAAGGATTTTG. V V A F P N Q 3770	AATGGCTTCTAATTCTGTC I A E L E T 3790 3800 GTCATCACCCAGAATGGCT D D G L I A 3910 3920 ACAAATCACTAAGGTTCTC C I V L T R 4030 4040 CGGCTTTGACCAGTCAACA P K S W D V 4150 4160 TTGGGTCATTACCCAAAA Q T M <0RF295 4270 4280 ATTATGAGTGAGAGAAGAAC 4510 4520	CCATTGAGATTGATAGTCTGAT W Q S Q Y D S 3810 3820 PAAGCTGACAACAGGGCAATTA L S V V P C N 3930 3940 CTCACCCCGCAGACGGTGAACT E G R L R H V 4050 4060 ATCTGCCTCAATCGTTGGAAA D A E I T P F 4170 4180 ATTAGTTGATATGTTCTATATC 4290 4300 SAAACTGTTCGAGAAATTTGGAAATTTGGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAACTGTTCGAGAAATTTGGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAGAAATTTGGAAATTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTTGGAAATTGGAAATTGAAATTGGAAATTGGAAATTGGAAATTGGAAATTGGAAATTTGGAAATTGAAAATTGAAATTGAAAATTGAAATTGAAATTGAAAATTGAAAATTGAAATTGAAATTGAAATTGAAATTGAAATTGAAATTGAAATTGAAAATTGAAATTGAAAATTGAAA	TAGCTTCACGATTGGAA L K V I P 3830 3840 ATAGCGATCGCGCAATC

Fig. 2 Nucleotide sequence of the ndh1, frxB, ndh6, ndh4L gene cluster from P. boryanum. Nucleotide sequences are shown in the direction of transcription except in the case of ORF295. Deduced amino acid sequences are shown below the nucleotide sequence in the one-letter code. Genes with an arrowhead (> or < indicating the direction of transcription) are named at the start of their coding sequences. Putative promoter sequences (-35 and -10 sequences) and SD sequences are indicated by underlining and double underlining, respectively. Broken arrows indicate inverted repeat sequence.

was translated into amino acid sequences in all six possible reading frames and the predicted sequences were compared with known sequences contained in the databases of NBRF-PDB and SWISS-PROT. In this way, homologues of the *ndh1*, *frxB*, *ndh6* and *ndh4L* proteins of liverwort chloroplasts were identified in the same strand of DNA. In the case of *ndh6*, GTG might act as an initiation codon,

as in the frxC gene of P. boryanum (Fujita et al. 1991). Possible ribosome-binding sites (Shine-Dalgarno sequences) were found preceding the initiation codons for these genes. In addition to the possible genes described above, eight ORFs encoding proteins of more than 33 amino acids in length were detected on the same and on the complementary strands, respectively. No significant ho-

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(a) frxB
                                                   MMKFLKQVGDYTKEAIQAGKYI-----GQGLSVTFDHMRRRPITVQYPYEKLILSERFRG-----RIHFEFDKCIACEVCVRVCPINLPVVDWEFNK
ſ₽ì
                                                      [L]
                                                      (T)
                                                                 \texttt{M} \cdot \texttt{PMVT} \cdot \texttt{FMGQQT} \cdot R \cdot \texttt{AR} \cdot \dots - \dots \cdot \texttt{SFII} \cdot \texttt{LS} \cdot \texttt{TN} \cdot \texttt{L} \cdot \dots \cdot \texttt{IH} \cdot \dots \cdot \texttt{S} \cdot \texttt{T} \cdot \dots \dots - \dots \cdot \texttt{D} \cdot \texttt{L} \cdot \dots \cdot \texttt{R} \cdot \texttt{E} \cdot \texttt{E} \cdot \texttt{C} \cdot 
[R]
                                    \texttt{MN} \cdot \texttt{FPMVTGFMS} \cdot \texttt{GQQT} \cdot \texttt{R} \cdot \texttt{TR} \cdot ---- \cdot \texttt{SFIT} \cdot \texttt{LS} \cdot \texttt{TN} \cdot \texttt{L} \cdot \cdot \cdot \texttt{IH} \cdot \cdots \cdot \texttt{S} \cdot \texttt{TP} \cdot \cdots ---- \cdot \texttt{G} \cdot \cdots \cdot \texttt{D} \cdot \cdots \cdot \texttt{R} \cdot \texttt{E} \cdot \texttt{S} \cdot \texttt{TM} \cdot \texttt{G} \cdot \texttt{G
[W]
                              \texttt{TYKYVNLREPSM} \cdot \texttt{MKSVTDR} \cdot \texttt{AQTLLWTELIR} \cdot \cdot \texttt{GM} \cdot \texttt{LSYLF} \cdot \texttt{E} \cdot \texttt{A} \cdot \texttt{IN} \cdot \cdot \texttt{F} \cdot \cdot \texttt{GP} \cdot \cdot \texttt{P} \cdot \cdots \\ \texttt{EHALR} \cdot \texttt{YPSGEER} \cdot \cdots \cdot \texttt{KL} \cdot \texttt{EA} \cdot \cdots \cdot \texttt{AQAITIEA} \cdot \texttt{PRA} \cdot \texttt{PRA
[B]
                                                                                                                                                                                                                                                          \texttt{MFTFIKKVIKTGTA} \cdot \texttt{SS} \cdot \cdot \texttt{L} \cdot \texttt{PIAVDKN} \cdot \cdot \cdot -----\texttt{KPEQNPQQ} \cdot \cdot \texttt{G} \cdot \texttt{AA} \cdot \cdot \texttt{NA} \cdot \cdot \texttt{S} \cdot \texttt{ALT} \cdot \texttt{ETDLAT}
(E)
                            ETKKKKI.NHYSTDFGVCTFCGNCVEYCPTNCI.SMTEEYEI.STYDRHEI.NYDNVAI.GRLPYKVTNDPMVTPLREFAYLPKGAI.DPHDLPAGSRRAGI.RPEE
[P]
[T] DIR.R.LN....I.....NQI.....MS.ID.YTIRTISNLPQIKNE (167)
[W] DI...Q.LN......S......NQI..S...ISIMG.YTIQTI.NSSESKINKEKSSNS (176)
[B] DGSR-RTTR.D..MTK..Y..F.Q.A..VDAIVEGPNF.F..ETHE..L.NKEK.LNNGD.WEAEIAANIQADYL.R (176)
 \texttt{[E]} \quad \texttt{GE-----LAWEFNL} \cdot \texttt{H} \cdot \cdots \cdot \texttt{R} \cdot \texttt{E} \cdot \texttt{V} \cdot \cdots \texttt{AAIKLSQ} \cdot \cdots \cdot \texttt{AVW-KK} \cdot \texttt{DFLQQSRFALCNCR} \cdot \texttt{C} \cdot \texttt{R} \cdot \texttt{FAV-QK} \cdot \texttt{ID} \cdot \texttt{AIALLKHNG} \cdot \texttt{SR} \cdot \texttt{ENH} \cdot \texttt{ESFETCP} 
[P] TVEOSOO (194)
[E] ECKROKCLVPSDRIELTRHMKEAI (180)
(b) ndh1
[P] MNPGIDLOGTFIETVOSLGIPAGAAKALWMPLPMLIMLLAATVSVLVVVWLERKISAAAQORIGPEFIGPLGVLAPLADGLKLVLKEDVVPAKADKLLFT
[L] ·ISN·N·EDK·FSFFFT·FSKEFFNF·IIFSI·LM·GV·IG··L·············YA···IIOA···I·F····I··OG·VW·N
                                 MIIDTTEIET.NSFSK.ESLKEVYGII..LF.I.TLV.GI.IG...I....E...GI......YA....I.QA....T..L...NLI.STG.TR..S
[T]
                                MIIDRV.VEA.NSFSN.ELLKEVYGLI.I-..I.TL..GI.IE...I.....E...SI.......YA....L.QAI...T..LF...IL.SRG.IP..S
[R]
                                                                                                                                                                                                                                \texttt{MIVASI} \cdot \cdot \texttt{LIVPVLL} \cdot \cdot \texttt{AMFTLA} \cdot \cdot \texttt{TVM} \cdot \texttt{SM} \cdot \texttt{R} \cdot \texttt{F} \cdot \cdot \texttt{QVS} \cdot \texttt{IS} \cdot \texttt{L} \cdot \texttt{Q} \cdot \texttt{FW} \cdot \cdot \cdot \cdot \cdot \texttt{GV} \cdot \texttt{P} \cdot \texttt{L} \cdot \texttt{DSSSAGA} \cdot \texttt{A}
(C)
                                                                                                                                                                                                                                \texttt{MFMINI} \cdot \texttt{MLIIPILLA} \cdot \texttt{AFLTLV} \cdot \cdot \cdot \vee \texttt{LGYM} \cdot \texttt{L} \cdot \texttt{K} \cdot \cdot \texttt{NVV} \cdot \cdot \texttt{Y} \cdot \texttt{L} \cdot \texttt{Q} \cdot \texttt{I} \cdot \cdot \texttt{AI} \cdot \cdot \texttt{FI} \cdot \cdot \texttt{PLR} \cdot \cdot \texttt{TSSASM} \cdot \texttt{I}
ſBl
(E)
                                                                                                                                                                                                                                \texttt{MSV} \cdot \texttt{YP} \cdot \cdot \texttt{QA} \cdot \texttt{VLFAVAPLLSGIT} \cdot \texttt{VAR} \cdot \texttt{RLHN} \cdot \texttt{R} \cdot \cdot ----- \cdot \texttt{V} \cdot \texttt{QEYR} \cdot \texttt{II} \cdot \cdot \texttt{LGRQS} \cdot \texttt{G} \cdot \texttt{DAS} - \texttt{GWV} \cdot \texttt{R}
[P] LGPAIVVIPVFLSYLILPFGQNLQITDVGLGIFLWIALSSVVPIGLLMSGYASNNKYSLLGGLRAAAQSISYELPLALSVLAVVMMSNSLSTVDIVNQQA
[L] \quad I \cdot IL \cdot L \cdot \dots \cdot VI \cdot EY \cdot VILANFSI \cdot V \cdot F \cdot \dots \cdot L \cdot \dots \cdot A \cdot G \cdot \dots \cdot F \cdot \dots \cdot \dots \cdot I \cdot \dots \cdot SIALL \cdot \dots \cdot EA \cdot S
 (R) \quad I \cdot . S \cdot A \cdot . S I L \cdot F \cdot V I \cdot L \cdot Y F F V L A \cdot L S I \cdot V \cdot \cdots I \cdot I A \cdot \cdots A \cdot S \cdot \cdots \cdot F S \cdot \cdots \cdot I \cdot T F C \cdot \cdots I S L L \cdot C \cdot S \cdot \cdots \cdot E A \cdot S \cdot \cdots A \cdot S \cdot C \cdot A \cdot S \cdot C \cdot A \cdot S \cdot C \cdot A \cdot A \cdot S \cdot C \cdot S \cdot A \cdot S \cdot S \cdot C \cdot A \cdot S \cdot C \cdot A \cdot S \cdot A \cdot S \cdot C \cdot S \cdot A \cdot S \cdot S \cdot C \cdot S \cdot S \cdot A \cdot S \cdot S \cdot S \cdot S \cdot S \cdot S \cdot
 [C] \quad \text{AS-M-SFVLSQVAWVGIC------S-ASFQGLVIM-I--LAVY-VMLA-W---S--AF--C--SV-LMV-----S-GAAL-SIGLFVTDGTGMKCL-FAE} 
                                [B]
                              \cdot \texttt{T} \cdot \texttt{YVM} \cdot \texttt{GVMLTIATA} \cdot \cdot \texttt{VVTVGSPLPQLGDLITLLY} \cdot \texttt{FAIARFFFAI} \cdot \cdot \texttt{LDTGSPFTAI} \cdot \texttt{AS} \cdot \texttt{E} \cdot \texttt{MLGVLV} \cdot \texttt{PM} \cdot \texttt{L} \cdot \texttt{GLWVAAQVAG} \cdot \texttt{TN} \cdot \texttt{SNITDTVY}
[E]
[P] GYGILGWNIWRQPVGFIIFWIAALAECERIPFDLPEAEEELVA-GYQTEYSGMKFALFYLGSYVNLTLSALLFAVLYLGGWEFPISL-SVISGLIGVPES
  (L) \quad \text{$K \cdot F \cdot S \cdot L \cdot \dots I \cdot \dots V \cdot F \cdot S \cdot \dots \cdot L} \\ \text{$K \cdot F \cdot S \cdot L \cdot \dots I \cdot \dots V \cdot F \cdot S \cdot FVTI \cdot \dots \cdot H \cdot S \cdot PFF \cdot LFKNFEWNLM \cdot \dots \cdot F \cdot 
[T] K.-FW......I...V.L.SS.....L....RSRRRISSRVSNRIFRYQIWFD.TVA..L..LV.S.FVT.....NLS.PY----FVPELFGIN
(C) ---MPTTPOYAMLPLCL..LVCI...TK.D.......NV...SLG....FIAE.A.MAVMSAIASIYF...FS-AL------
[B] OMWLI----LPAWPLAMMWF·ST···TN·A····T·G·S···S-·FNV··AAGP····FMAE·A·IIMMNIFT·I·F··TSHN·HMP-------
[E] HWPLSOS--IPLVLALCACAF.TFI.MGKL....A...Q..QE-.PLS....SG.GVMKW.ISLKQLVVLQM.VGVFI-P.GQMETF------
[P] TPWLQLIFATIGIGMTLLKAYFLIFLAILMRWTVPRVRIDQLLDLGWKFLLPVSLVNLLITAGLKLAFPVAFGG (372)
[L] NGISEV.SII...VI..V.S.LFL.IS.MT...L..I...N....IA.G...L.TSFQ.FLL (368)
 \{T\} \quad \text{K-RGKVFGTL} \cdots \text{FI} \cdot \text{A} \cdot \text{T} \cdot \text{LFL} \cdot \text{IP} \cdot \text{AT} \cdots \text{L} \cdot \text{L} \cdot \text{M} \cdot \cdots \cdot \text{N} \cdot \cdots \cdot \text{I} \cdot \text{G} \cdots \text{L} \cdot \text{TSSQ} \cdot \text{LSL} \  \, (364) 
[R] K-MVGILEMTMS·FI··T···LFL·IS·TI···L··M·M····N·····I··G···L·TSSQ·VSL (362)
[C] -----ITA·F·AFVWT·G·L··Y·Y··FMR····AF··LT·AFFALH·SVAI (292)
 [B] -----ELYTINFTI.SLL.TMSFLWI.ASY..F.Y...MH.L..NF..LT.ALCMWHVS.PILTSGIPPQT (318)
 [E] ----TAGGLLLALVIAIV.LVVGVLVIA.FENSMA.L.L.ITPRIT.AGFGFAF.AFVSLL.A (307)
```

mology was found between any of the predicted proteins and the sequences in the databases. The proposed arrangement of genes, including the two large ORFs (ORF105 and

ORF295), is summarized in Figure 1.

The frxB gene—The frxB gene extends from nucleotides 1646 to 2227, as shown in Figure 2, and could encode

Cyanobacterial ndh1-frxB-ndh6-ndh4L gene cluster

(C) ndh6

(d) ndh4L

```
[P] MQLQYFLLIAAALFCIGVYGLVTSRN-AVRVLMSIELMLNAVNLNLMAFSNYLDPQEIKGQMFTIFVITIAAAEAAVGLAIVLAIYRNRDTVDMEQFNLLKW (101)
[L] M.EHI.TLS.F.....F.I....-M.A.CL.IF...I.V...F.SSQ...EI.S.I.A....TI......KSTRID..... (100)
[T] ·I.EHV.VLS.Y.S.I...I...-M.A.CL.I...I.FVT.DFF.NRQL.DI.S...A....I...SS...KSTRIN.S..NN (101)
[R] ·MFEHV.FLSVY.S.I...I...-M.A.ICL.I.SI...VT.DLF.SRQL.DI.A...AL...I.S.LSS.H..KSTRIN.S.F.NN (101)
[M] ·MFERV.FLSVY.S.I...I...-M.A.ICL.I.SI...VT.DLF.SRQL.DI.A...AL...I.S.LSS.H..KSTRIN.S.F.NN (101)
[B] MSMVYMN.MM.-.TVSLV.LLMY.SHLMSS.LCL.G.M.-LS.FV.ALTI.NSHFTLAS.MP.ILLVF.C...L.SLLVMVSNTYG.DYVQNL...QC (98)
```

Fig. 3 Comparison of the amino acid sequences deduced from the frxB (a), ndh1 (b), ndh6 (c) and ndh4L (d) genes of Plectonema boryanum with those of related proteins. [P], P. boryanum (present study); [L], liverwort chloroplasts (Kohchi et al. 1988); [T], tobacco chloroplasts (Matsubayashi et al. 1987); [R], rice chloroplasts (Hiratsuka et al. 1989); [W], wheat chloroplasts (Dunn and Gray 1988); [M], maize chloroplasts (Schantz and Bogorad 1988); [B], bovine mitochondria (Anderson et al. 1982, Dupuis et al. 1991); [C], Chlamydomonas mitochondria (Boer and Gray 1988, 1989); [E], E. coli (Böhm et al. 1990). Amino acid residues identical to the corresponding residues in the proteins from Plectonema are denoted by dots. Bacterial Fd-like arrangements of cysteine residues found in frxB sequences are indicated by asterisks.

a protein of 194 amino acids with a mol wt of 22,528. It lies between the *ndh1* and *ndh6* genes; the ATG initiation codon of the *frxB* gene (at position 1646) is located 27 bases downstream from the TAA stop codon of *ndh1* gene (at position 1619), and the TAG stop codon of the *frxB* gene (at position 2230) is 50 bases upstream from the GTG initiation codon of the *ndh6* gene (at position 2280). A possible ribosome-binding site, GGAG, lies 11 bases

upstream from the initiation codon ATG.

Figure 3(a) shows an alignment of the sequence of the frxB protein with other sequences that are currently available. The protein from Plectonema has 60-66% homology with each of the chloroplast proteins (Table 1). In the case of these five sequences shown, no deletions or insertions were necessary for proper alignment. The 23-kDa subunit of bovine mitochondrial complex I, probably carry-

Table 1 Percentage identities between products of *Plectonema boryanum* genes and homologous proteins from chloroplasts, mitochondria and *E. coli*

	Chloroplasts		Mitochondria		Eli	
	Liverwort	Tobacco	Rice	Chlamydomonas	Bovine	E. coli
frxB	66	64	60	_	29	25
ndh1	60	51	55	34	32	20
ndh6	50	_	43	18	12	_
ndh4L	65	57	50	_	24	_

Similarities in amino acid sequences were calculated on the basis of the sequence alignments shown in Figure 3(a-d).

ing the iron-sulfur cluster(s) known as N-2 (Dupuis et al. 1991), is also related to the cyanobacterial and chloroplast frxB proteins but to a lesser extent. In addition, the product of ORF6 encoded in the hyc operon of $Escherichia\ coli$ (Böhm et al. 1990) was found to show a significant degree of sequence identity to the frxB protein. Hydropathy profiles of these proteins (not shown) also show striking similarities between them. The data suggest that these proteins are hydrophilic and contain no large hydrophobic segments that span the membrane. These profiles are consistent with the experimental observation that the frxB proteins from tobacco and spinach chloroplasts can be extracted from thylakoid membranes by high-salt washing (Lin and Wu 1990).

The frxB protein is unusual among the soluble bacterial-type Fds. A typical bacterial Fd, as exemplified by Fd from Clostridium pasteurianum, has two [4Fe-4S] clusters carried by a polypeptide of about 6 kDa. In these clusters, each of the eight iron atoms is chelated by cysteine residues, which are arranged in two -C-X-X-C-X-C-X-X-X-C-P- sequences (reviewed by Bruschi and Guerlewquin 1988). Similar arrangements of cysteine residues are also found in two highly conserved regions of the frxB proteins. Presumably, the conserved regions are indispensable for the operation of the Fe-S protein in electron-transfer reactions. However, the frxB protein is much larger than the bacterial-type Fds, with extensions mainly in the N- and C-terminal regions of about fifty residues (Kohchi et al. 1988). Obviously, these proteins must have a common ancestor and must have diverged during evolution, one to a group of proteins, such as soluble bacterial Fds, and the other to a component of a membrane-bound enzyme complex. The N- and C-terminal regions of the frxBprotein may be involved in interactions with thylakoid membranes or with other components of the membranebound complex.

ndh genes—The coding region of the ndh1 gene extends from nucleotides 501 to 1616, shown in Figure 2, and encodes a protein of 372 amino acids with a mol wt of 40,239. It terminates 27 bases upstream from the frxB gene. The ndh6 gene (nucleotides 2280 to 2876; Fig. 2) follows the frxB gene. It encodes a protein of 199 amino acids with a mol wt of 21,267. The ndh4L gene extends from nucleotides 2892 to 3194. It lies 13 bases downstream from the TAA stop codon of the ndh6 gene (at position 2879) and encodes a protein of 101 amino acids with a mol wt of 11,335. Potential binding sites for 16 S ribosomal RNA were located for each of the three ndh genes.

Alignments of the sequences of the products of the *ndh* genes with those of analogous proteins from chloroplasts, mitochondria and *E. coli* are shown in Figure 3(b-d), and their homologies are summarized in Table 1. The deduced amino acid sequences of products of the *ndh1*,

ndh6 and ndh4L genes from Plectonema show significant similarities to the corresponding gene products from chloroplasts. Although these sequences are not obviously homologous to the mitochondrial sequences, in particular in the case of the ndh6 protein, comparisons of their hydropathy profiles reveal striking similarities (data not shown). The ndh1, ndh6 and ndh4L proteins contain seven, three and three hydrophobic segments of more than 20 amino acids each, respectively, and these segments are consistent with the presence of transmembranous α -helical segments in these proteins.

Unidentified potential genes—ORF295 extends from nucleotides 4153 to 3269 on the opposite strand of the DNA to the frxB and ndh genes (Fig. 1 and 2). It could encode a hydrophilic protein of 295 amino acids, with a mol wt of 35,541. It terminates 74 bp downstream from the ndh4L gene. ORF105 extends from nucleotides 1933 to 1619, encoding a protein of 105 amino acids with a mol wt of 12,187. It lies on the complementary strand of the frxB coding region and the transcription of ORF105 is less probable (see below).

Co-transcription of the genes in the ndh1-frxB-ndh6ndh4L gene cluster-A prokaryotic promoter-like sequence can be found upstream from the ndh1 gene at bases 322 to 329 (AATTTACT, -35 BOX) and 347 to 352 (TACTTT, -10 BOX). Nucleotides that can form a stemloop structure, followed by a run of T residues, occur downstream from the ndh4L gene (nucleotides 3218 to 3248), which may act as a signal for ρ -independent termination of transcription. Therefore, it seems likely that the gene cluster ndh1-frxB-ndh6-ndh4L constitutes an operon. Further support for this proposal is provided by the presence of very short intergenic non-coding regions: 29 bp between ndh1 and frxB; 52 bp between frxB and ndh6; and 15 bp between ndh6 and ndh4L. In order to ascertain the significance of these transcriptional signals, Northern blot analysis was carried out. Total RNA isolated from mixotrophically grown cultures of Plectonema was hybridized with single-strand DNA probes for each of the coding strands, synthesized on recombinant M13 DNAs as templates. The ndh1-, frxB-, ndh6- and ndh4L-specific probes (probes 1 to 4) each recognized a transcript of about 2.8 kb, which is sufficient in terms of length to cover these genes (Fig. 4). In addition to the 2.8-kb transcript, both the ndh1 and frxB probes recognized mRNA species of about 1.5 kb. Transcripts of 1.4 kb and 1.1 kb hybridized with the ndh6 and ndh4L probes with different relative intensities. No signals were observed when a probe for the 3' region (probe 5), downstream from the terminator-like sequence, was tested with the same preparation of RNA and, therefore, it appears that the clustered ndh1-frxB-ndh6ndh4L genes are co-transcribed as a single mRNA. The length of the largest transcript (about 2.8 kb) is consistent with the length of the entire operon, from close to the

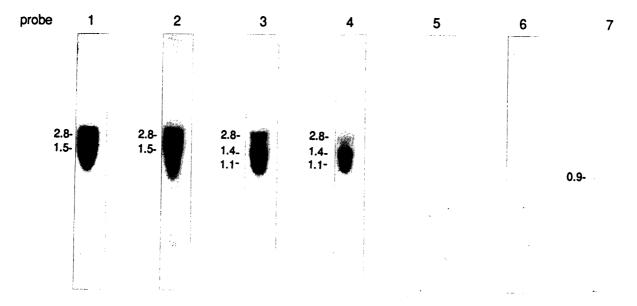


Fig. 4 Northern blot analysis of the ndh1, frxB, ndh6, ndh4L and ORF295 genes in P. boryanum. Total RNA isolated from a batch culture of mixotrophically grown Plectonema was tested with the gene-specific single-strand DNA probes shown in Fig. 1. The coding strand of each probe was labeled with $[a^{-32}P]dCTP$ as described in Materials and Methods. The sizes (in kb) of hybridizing species are shown to the left of respective lanes.

"-10"-like sequence to the possible termination sequence shown in Figure 2. The presence of smaller mRNA species suggests that there may be other sites of initiation of transcription, or that the 2.8-kb transcript may be specifically processed or degraded. Essentially the same results were obtained with preparations of RNA from mixotrophically grown and photoautotrophically grown cultures of Plectonema, suggesting that the expression of this gene cluster is not regulated by glucose or its metabolites. Two additional hybridization probes, (probes 6 and 7; Fig. 4), were used to examine the expression of ORF105 and ORF295, respectively, both of which are found on the opposite strand of the DNA from the frxB and ndh genes. The ORF295 probe recognized a weak and diffuse band at around 0.9 kb. The mRNA is of sufficient length to cover this gene (885 bp), and it is likely to be a monocistronic transcript. Under the same conditions as described above, no transcript of ORF105 could be detected. Since we used equally labeled probes, each of almost the same radioactive intensity and specific activity, the expression of the ORF105 gene seems less likely when compared with that of other genes.

Organization of the operon—Each of the ndh1-, frxB-, ndh6- and ndh4L-specific probes (the same probes as used for the Northern blot analysis) hybridized to a single 5.6-kb Hind III and a single 3.2-kb EcoR V fragment in digests of Plectonema genomic DNA (data not shown). These results indicate that these genes occur as single copies in the genome. Arrangement of the genes in Plectonema is shown in Figure 5 and is compared to the corresponding regions in the chloroplast genomes. The genes equivalent

to frxB in the ctDNAs of tobacco and rice are represented by ORF167 and ORF178, respectively. In tobacco chloroplasts, a transcript of about 4 kb has been detected with ndhA- and ndhE-specific probes, and it is long enough to include genes ndhA to ndhE, if the intron is excluded (Matsubayashi et al. 1987). Since the ORF393- or ndhDspecific probes did not recognize the 4-kb transcript, the gene cluster of ndhA-ORF167(frxB)-ndhG-ndhE appears to constitute a transcription unit. The same is true for these genes in Plectonema. The conserved amino acid sequences, the order of genes and the co-transcription of the ndh1, frxB, ndh6 and ndh4L genes in cyanobacteria and chloroplasts support an endosymbiotic origin for plastids. However, the intron contained in the plastid *ndh1* gene is not found in the *Plectonema* sequence, and the lengths and the sequences of the intergenic non-coding regions show no significant similarities. Therefore, the conserved order of genes may reflect some aspect of transcriptional or translational regulation, for example, it may be involved in the production of programmed amounts of functionally related proteins. Alternatively, the polycistronic mRNA could allow for the immediate assembly of the products of translation into an enzyme complex. The structure of the ndhC-psbG-ORF157/159(ndhJ) operon and the corresponding deduced amino acid sequences are also conserved in both the cyanobacterium Synechocystis PCC6803 and chloroplasts (Steinmüller et al. 1989) and, therefore, it is clear that cyanobacteria and chloroplasts contain a highly conserved enzyme complex.

Phylogenetic implications—Chloroplast genomes con-

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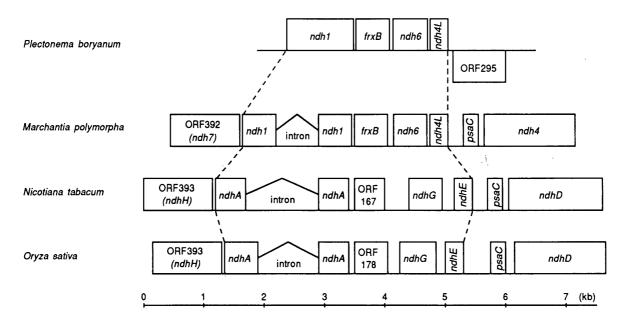


Fig. 5 Arrangement of cyanobacterial and chloroplast genes in the vicinity of frxB. The diagram is based on the sequence data published by Kohch et al. (1988), Shinozaki et al. (1986) and Hiratsuka et al. (1989). frxB homologues in the ctDNAs of tobacco and rice are denoted by ORF167 and ORF178, respectively. ORF392(393) is homologous to the nuclear-encoded 49-kDa subunit of bovine mitochondrial complex I (Fearnley et al. 1989). psaC (formerly frxA) encodes a component of photosystem I which carries two [4Fe-4S] clusters. The arrangement of genes that is conserved among the four species is enclosed by dotted lines.

tain ten genes that encode homologues to the components of mitochondrial complex I. They are ndh1 (A), 2 (B), 3 (C), 4 (D), 4L (E), 5 (F), 6 (G), 7 (H), 9 (J), and frxB(Ohyama et al. 1988, Matsubayashi et al. 1987, Hiratsuka et al. 1989, Fearnly et al. 1989, Pilkington et al. 1991a, Dupuis et al. 1991). In addition, it has been suggested, from indirect evidence, that the psbG gene is a candidate for an ndh gene but its product is not considered to be a component of photosystem II (Nixon et al. 1989, Mayes et al. 1990). In cyanobacterial genomes, eight related genes have been identified; ndh2(B), 3(C), 9(J) and psbG from Synechocystis (Steinmüller et al. 1989, Mayes et al. 1990, Ogawa 1991), and ndh1 (A), 4L (E), 6 (G) and frxB from Plectonema (this study). These findings lend support to the hypothesis that chloroplasts and cyanobacteria contain an enzyme that is related to mitochondrial complex I. Consequently, an NADPH:plastoquinone oxidoreductase has been proposed as the most probable candidate (Fearnly et al. 1989, Pilkington et al. 1991a, Dupuis et al. 1991). However, it seems premature to draw a firm conclusion because of the lack of biochemical evidence. Furthermore, the following sets of observations are in disagreement with the putative assignation of NADPH:plastoquinone oxidoreductase in both cyanobacteria and chloroplasts.

First, the amino acid sequences of the mitochondrial components are poorly conserved in the cyanobacterial and chloroplast homologues. As shown in Table 1, the extent of sequence identities between the components of *Plec*-

tonema and bovine mitochondria is about 30% or less: not sufficiently high to allow us to postulate an identical function in these organisms. On the other hand, sequence similarities have been reported between components of formate hydrogenlyase in E. coli and the products of the ndh genes of chloroplasts and mitochondria (Böhm et al. 1990). The enzyme from E. coli, releasing hydrogen via the reduction of protons that is coupled to the oxidation of formate, is used in the non-energy-conserving system to eliminate excess formate. Four of the eight ORFs identified in a hyc operon of E. coli, namely, ORF3, 4, 5 and 7, exhibit homologies with products of the ndh4 (D), ndh1 (A), ndh7 (H) and psbG genes, respectively (Böhm et al. 1990). In addition, we found sequence similarities between the product of ORF6 from E. coli and the products of frxB from Plectonema and chloroplasts (Fig. 3, Table 1). The similarity to ORF6 is not restricted to the Fd-like arrangement of cysteine residues but also extends to the other parts of the amino acid sequence. These observations highlight the difficulty in assigning a functional role from the limited homologies.

Second, not all of the components of mitochondrial complex I have been correlated with the products of chloroplast or cyanobacterial genes. Mitochondrial complex I is an enzyme composed of at least 25 subunits (Hatefi 1985). With the limited sequence data available to date, at least six components of bovine mitochondrial complex I appears to have no homologues encoded in ctDNAs (Pilkington et al.

1991b, Masui et al. 1991). One of the subuhnits is the functionally well-defined 51-kDa subunit which binds the substrate NAD(H) and contains an FMN (Pilkington et al. 1991b). We can speculate that some of these genes have been transferred to the plant nucleus after endosymbiosis, although none of the additional genes has been identified so far, even in the *ndh* operons of cyanobacteria. Weiss and co-workers (1991) have proposed the hypothesis that the mitochondrial complex I was constructed originally by the combination of at least two preexisting enzyme complexes. If so, it is not surprising that the chloroplast and cyanobacterial enzymes contain some but not all of the mitochondrial components.

Third, no enzyme resembling mitochondrial complex I has been identified so far in an extensive survey of cyanobacterial NAD(P)H dehydrogenases. Because the thylakoid membranes of cyanobacteria are known to participate in respiration (Scherer et al. 1988a, Scherer 1990), there have been several attempts to identify the cyanobacterial NAD(P)H dehydrogenases that provide reducing equivalents for the respiratory transport of electrons (Sandmann and Malkin 1983, Scherer et al. 1988b, Alpes et al. 1989). Such an NADH dehydrogenase has recently been purified from Anabaena variabilis and it consists of 17-kDa polypeptide(s). Compared to mitochondrial complex I, the enzyme from Anabaena contains FAD but not FMN and is only weakly inhibited by rotenone (Alpes et al. 1989, Scherer 1990). As for the cyanobacterial NADPH dehydrogenase, evidence has been obtained to suggest that the Fd-NADP+ oxidoreductase is responsible for the respiratory oxidation of NADPH in Anabaena cells (Scherer et al. 1988b).

The limited data available today suggest the presence of functionally divergent enzyme complexes in cyanobacteria (chloroplasts) and mitochondria and, therefore, the possibility can not be ignored that the products of the *ndh* genes, together with the *frxB* protein, may not be involved in the respiratory electron-transport systems. It has recently been demonstrated that a mutant of *Synechocystis*, lacking the *ndhB* gene, is defective in the transport of CO₂ (Ogawa 1991). Further genetic studies of cyanobacteria will assist in the biochemical characterization of the enzyme complex.

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