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Abstract

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Reference

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Sequence homology between the 32K dalton and the D2 chloroplast membrane polypeptides of *Chlamydomonas reinhardii*

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Summary

The region of the chloroplast genome of *Chlamydomonas reinhardii* containing the gene of the thylakoid polypeptide D2 (psbD) has been sequenced. A unique open reading frame of 350 codons exists in this region. Because the first ATG is followed 11 codons downstream by a second one, the D2 polypeptide consists of either 339 or 350 amino acids. Comparison of the sequences of D2 and the 32K dalton polypeptides, both of which are associated with photosystem II, reveals partial homology. Although, the overall homology of these two polypeptides is only 27%, they contain several related regions and their hydropathic profiles are strikingly similar. These data suggest that the two polypeptides may have related functions and/or that their genes may have originated from a common ancestor. Alternatively, convergent evolution of these polypeptides may be due to structural constraints in the thylakoid membrane. Limited sequence homology is also observed between the D2 polypeptide and some of the subunits of the reaction centers of photosynthetic bacteria.

Introduction

Chloroplast membranes are highly specialized structures capable of harvesting and of converting light energy into useful chemical energy. These membranes consist of numerous polypeptides; over 50 have been detected in *Chlamydomonas reinhardii* (1, 2). Recent studies reveal that as many as 40 thylakoid polypeptides are synthesized on chloroplast ribosomes in this organism (2). It is very likely that they are all encoded by the chloroplast genome. Thylakoid polypeptides synthesized within chloroplasts are made on membrane bound ribosomes (3, 4). These polypeptides can be divided into two classes based on their turnover. The first class includes stable polypeptides such as the ATP synthase subunits α and β, while the polypeptides of the second class are unstable. In *C. reinhardii* two polypeptides of similar size, D1 and D2, are associated with photosystem II and are prominent among the latter group (1, 5). The D1 polypeptide is the product of the psbA gene and corresponds to the 32K dalton polypeptide of higher plant chloroplasts, since its sequence is 90% homologous to the corresponding sequence in spinach and tobacco (6).

In order to gain insights into the structure, and possibly into the function and the regulation of expression of the D2 polypeptide, the sequence of its gene and of the surrounding chloroplast DNA regions have been determined in *C. reinhardii.* An intriguing finding is the partial sequence homology observed between the D1 and D2 polypeptides and the striking resemblance of their hydrophatic (hydrophilic/hydrophobic) profiles.

Materials and methods

Enzymes. Restriction endonucleases were purchased from Bethesda Research Laboratories and Genofit (Geneva) and used as recommended by the manufacturer.

DNA and RNA. Chloroplast DNA (7), plasmid DNA (8) and RNA from *C. reinhardii* (9) were prepared as described. Mapping of restriction endonuclease sites was performed according to Smith & Birnstiel (10). DNA hybridizations were done in 6 × SSC, 1 mM EDTA at 65 °C for 12 to 16 h (1 × SSC: 0.15 M NaCl, 0.015 M Na citrate, pH 7.0). Filters were washed 3 times in 0.1 × SSC, 0.1% SDS at 55 °C. Northern hybridizations were performed as described by Alwine *et al.* (11). S1 nuclease mapping was as described (12).

Cloning. Most chloroplast DNA fragments used were cloned in pBR322 (13). The HindIII fragment H3 was cloned in λ 1149 (constructed by N. Murray).

Sequencing. 5′ and 3′ end-labelling of DNA fragments and sequencing were performed by the method of Maxam & Gilbert (14) DNA sequences were analyzed with a HP3645 computer.

Protein analysis. The hydrophatic (hydrophilic/hydrophobic) character of the polypeptides was established according to Kyte & Doolittle (15). The hydrophatic profile was obtained by adding the hydropathy index of each amino acid in a 9 amino acid stretch and by plotting the sum, as a point, over the central residue of that stretch.

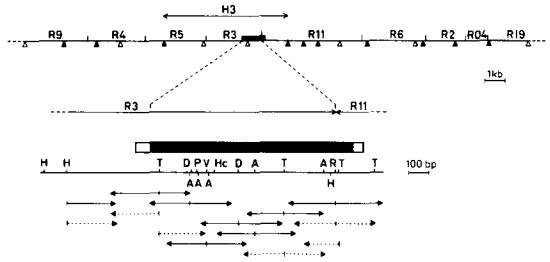


Fig. 1. Restriction map of the region of the chloroplast genome of *C. reinhardii* containing the psbD gene. The gene is drawn on the top line. Transcription proceeds from the left to the right. Restriction endonuclease sites are indicated in the upper part of the figure by ▲, HindIII, and △, PvuII. In the lower part the sites are indicated by H, HinfI; T, Taq I; A, AluI; D, DdeI; P, PvuII, V, AvaII; Hc, HincII; R, EcoRI. The lower part shows an enlargement of the psbD region and the sequencing strategy used. Coding and untranslated regions are represented by black and white bars, respectively. 5′- and 3′-end labelled fragments are marked by ⟶ and ⇢, respectively.

Results

The gene of the thylakoid polypeptide D2, psbD, had previously been mapped on the chloroplast EcoRI fragment R3 (16) which is located close to one of the inverted repeats of the chloroplast genome of *C. reinhardii.* However, its precise location within a set of four chloroplast fragments R11, R6a, R5 and R3 had not yet been determined. The order of these fragments was established by hybridizing each of them with chloroplast DNA HindIII and PvuII digests and by determining the HindIII and PvuII sites of the four EcoRI fragments. Figure 1 shows the arrangement of these fragments on the chloroplast DNA as well as the position of the D2 gene.

The sequence of a region of 1.6 kb between a HinfI site on the left and beyond an EcoRI site on the right was determined according to the strategy shown in Fig. 1. While most of the coding region is contained within the R3 fragment, the 3′ terminal portion of the gene is located on fragment R11. The sequence of the left end of R11 revealed a Taq I site located 12 bases from the EcoRI site (Fig. 1). In order to obtain an overlap between the R3 and R11 fragments, the HindIII fragment H3 was cloned, isolated and cut with Taq I. The 270 bp Taq I fragment spanning the EcoRI site was sequenced (Fig. 1).

There is a unique open reading frame of 350 codons within this region from position 389 to 1438 (Fig. 2). At position 422 there is a second ATG in phase with the first one. If the second ATG were used as an initiation codon, the polypeptide would consist of 339 amino acids. We favor this alternative because in contrast to the first ATG, the second is preceded by 5′GGAG, a sequence which is complementary to the 3′ end of the 16S rRNA of *C. reinhardii* (17) and which could act as a ribosome binding site. An unambiguous proof will have to rely on protein sequence data.

In order to determine the location of the 5′ end of the mRNA of D2, the R3 fragment was cut with Taq I, 5′ end-labelled and redigested with HinfI. The 470 bp Taq I-HinfI fragment, spanning the 5′ end of the gene was denatured and annealed with non-polyadenylated RNA, treated with S1 nuclease and electrophoresed together with an A + G sequencing tract. The results are shown in Fig. 3 (lanes b, c, d); two protected fragments can be seen,

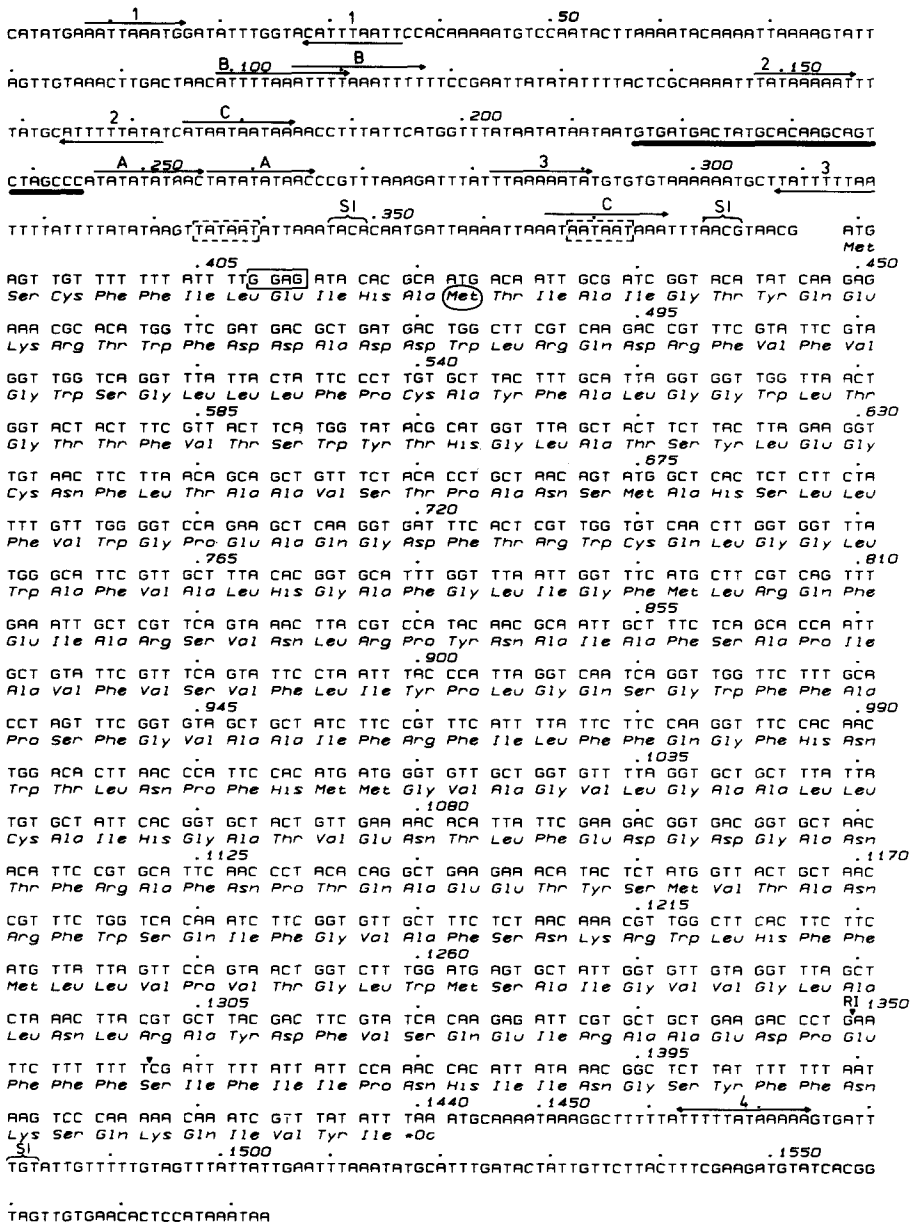


Fig. 2. Sequence of the psbD gene and of its surrounding regions. Only the non-coding strand is drawn. Direct repeats of 12 bp (B) and 10 bp (A, C) and inverted repeats of 9 bp (1, 2, 3) and 12 bp (4) are indicated by arrows. The underlined region represents the GC rich island. The positions of the ends of the transcripts determined by S1 nuclease mapping are marked by S1. Regions related to the bacterial-10 box (18) – and to the Shine-Dalgarno sequence (17, 32) – are framed. The second met residue is circled and has been chosen as reference site for numbering the amino acid residues of D2 (on the left of the figure). Nucleotides have been numbered arbitrarily from the start of the sequence.

one around position 347, the other, which is considerably stronger, around position 380 of Fig. 2. It is not yet clear whether there are two transcription initiation sites or whether the second signal is due to processing of the primary transcript. If one assumes that the second ATG is used as initiation codon, the 5′ untranslated region would be 75 and 42 nucleotides long, respectively. Both of the mapped sites for initiation of transcription are preceded by elements that are related to the bacterial Pribnow box

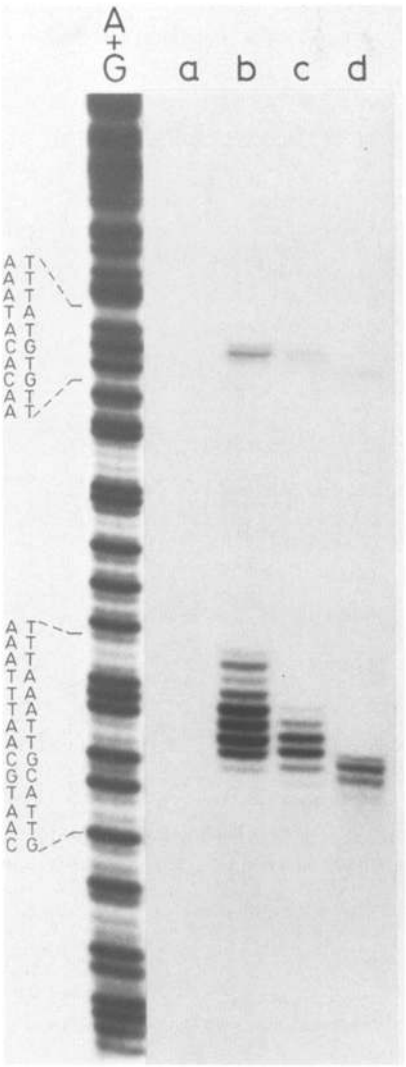


Fig. 3. Mapping of the 5′-end of the psbD transcript.

The 470 bp HinfI-Taq I fragment (position I to 464 in Fig. 2) was 5′ end labelled at its Taq I site, hybridized with *C. reinhardii* RNA, digested with S1 nuclease and co-electrophoresed on an 8% polyacrylamide-7 M urea gel with the A + G cleavage products of the same labelled fragment. Lane a shows a control with yeast RNA. Lanes b, c and d correspond to increasing concentrations of S1 nuclease (1 000, 2 500 and 5 000 units, respectively).

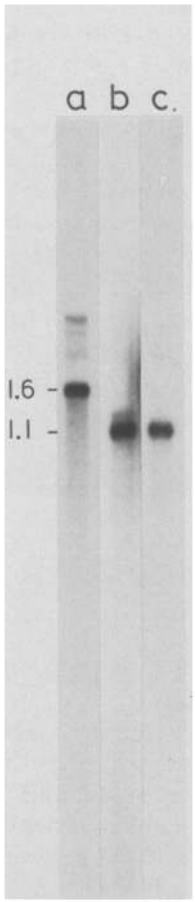


Fig. 4. Comparison of the sizes and steady state levels of the transcripts of rbcL (a), psbD (b) and psbA (c). Non-polyadenylated RNA from *C. reinhardii* was fractionated by electrophoresis on a denaturing 1.5% agarose gel containing 5 mM methylmercuric hydroxide (19), transferred to DBM (diazobenzyloxymethyl) paper (11) and hybridized with the cloned labelled chloroplast EcoRI fragments R15 (6a), R3 (b) and R14 (c) which contain the genes of interest (16). The sizes of the mRNAs of the large subunit of ribulose-1,5-bisphosphate carboxylase (a) and of the 32K dalton polypeptide (c) are from sequence and mapping data (12, Erickson & Rochaix, unpublished results).

at position −10 (18). The 5′ upstream region of the D2 gene is characterized by a high AT content (82%). It contains a short relatively GC rich island of 29 bp (51.7% GC) which precedes two tandemly arranged elements of 10 bp (indicated by A in Fig. 2). Other features include the presence of two partially overlapping 12 and 11 bp direct repeats (B and C in Fig. 2) and of three 9 bp inverted repeats (indicated by 1, 2, 3 in Fig. 2). With an AT content of 60%, the D2 gene coding region is significantly richer in GC than its flanking regions.

The 3′ end of the D2 gene transcript was located around position 1482 (Fig. 2) by S1 nuclease mapping using the 1.3 kb EcoRI-HindIII fragment of H3 (Fig. 1) with the EcoRI site labelled at its 3′ end as hybridization probe (data not shown). The ter-

mination site shortly follows a 12 bp palindromic sequence (indicated by 4 in Fig. 2). The 3′ untranslated region contains 45 bases and the size of the mRNA of D2 can be estimated at 1.1 kb which agrees with the value obtained by measuring this mRNA by agarose gel electrophoretic fractionation followed by hybridization with labelled R3 fragment (Fig. 4b). The steady state level of this mRNA is comparable to those of the transcripts from the genes of the large subunit of ribulose 1,5 biphosphate carboxylase (lane a) and of the 32K dalton polypeptide (lane c). The probes used in these hybridizations had comparable specific activities.

Discussion

Structural features of the D2 polypeptide of C. reinhardii

The sequence of the D2 coding region has revealed a single large open reading frame which encodes a polypeptide of 339, possibly of 350 amino acids. The corresponding molecular weights can be calculated to be 38 062 and 39 355 respectively. These estimates are considerably higher than the value obtained by SDS-polyacrylamide gel electrophoresis (1). Similar discrepancies between molecular weight values obtained by these two methods have been reported for other hydrophobic proteins such as the 32K dalton membrane polypeptide of spinach (20) and for the M subunit of the reaction center from *Rhodopseudomonas sphaeroides* (24). Furthermore, the relative mobilities of D2 and the 32K dalton polypeptide are reversed when SDS-urea gels are used (2).

Relationship between D2 and other polypeptides

The D2 polypeptide has been shown to be an intrinsic thylakoid polypeptide (5). The hydrophatic profile of this polypeptide shown in the middle part of Fig. 5 clearly demonstrates the hydrophobic character of this polypeptide. Rao *et al.* (21) proposed a model for the 32K dalton polypeptide with seven membrane-spanning helical regions of 20 to 28 amino acids, each containing mostly hydrophobic residues, which are connected by exposed turn segments. These spans are indicated on the hydrophatic profile of the 32K dalton membrane polypeptide of *C. reinhardii* (Fig. 5, upper part) except that the location of spans V and VI has been changed (Trebst, pers. comm.). Comparison of the hydrophatic profiles of D2 and of the 32K dalton membrane polypeptide reveals an intriguing resemblance (Fig. 5) which suggests that these two polypeptides may be folded in a similar fashion in the thylakoid membrane. A recent analysis of the distribution of the hydrophobic residues in cytochrome b of the mitochondrial complex III and of the chloroplast b6/f complex has also revealed similarities in the membrane folding pattern of these polypeptides (22). While the overall homology between the two polypeptides 32K and D2, is only 27%, there are several domains (A to G in Fig. 5)

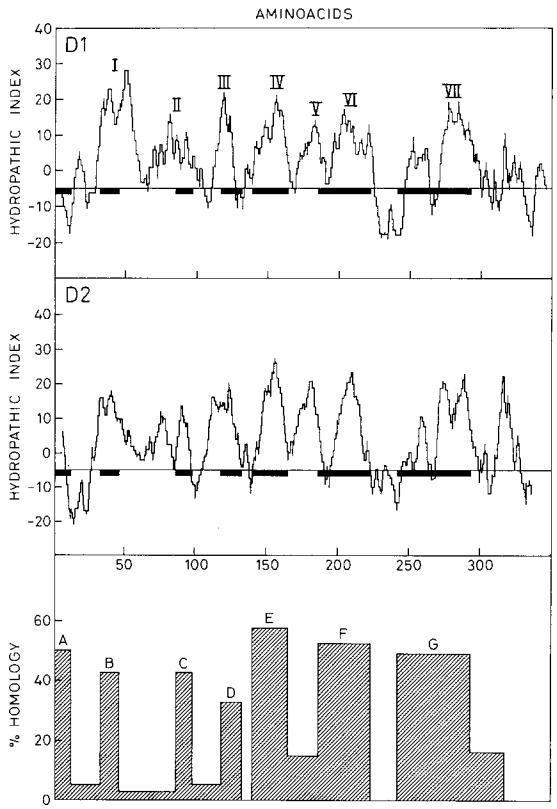
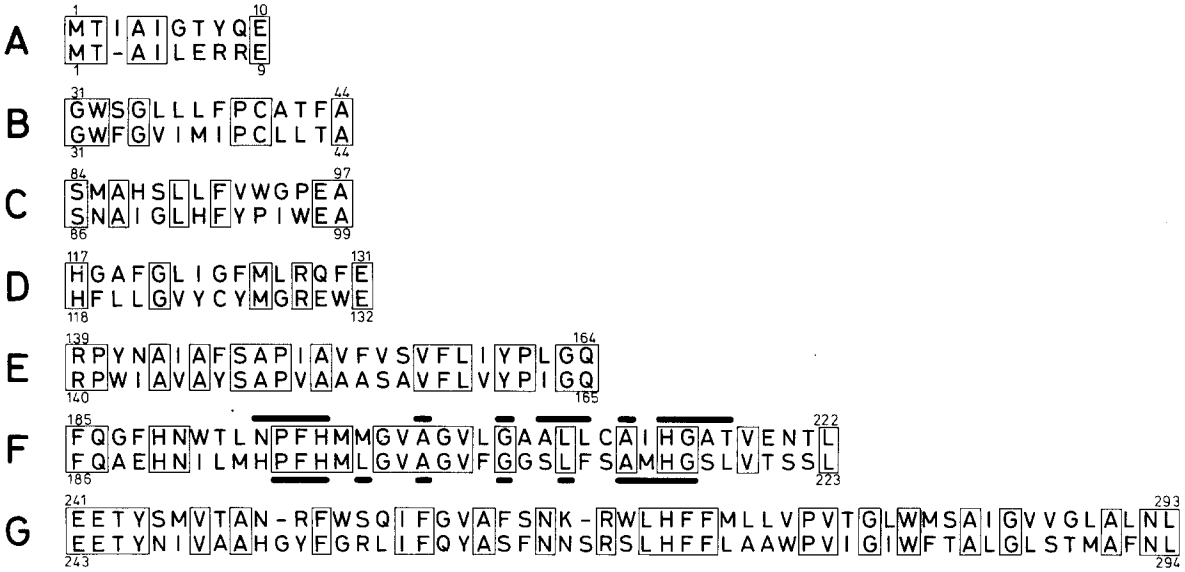


Fig. 5. Comparison of the hydrophatic profiles of the D1 (32K dalton) and D2 polypeptides of *C. reinhardii* (upper and middle parts of figure, respectively). Regions of homology are represented by thickened black lines and the degree of homology is indicated in the lower part of the figure. Numbers on the ordinate indicate the percentage homology. Hydrophatic profiles were determined according to Kyte & Doolittle (15). The sequence of the 32K dalton polypeptide of *C. reinhardii* was determined by Erickson *et al.* (6).

Table 1. Comparison of the sequences of the conserved regions of the D2 and 32K dalton polypeptides of *C. reinhardii.*



The regions indicated correspond to those shown in Fig. 5. Numbering of the amino acid residues of D2 (upper line) follows that of Fig. 2. The corresponding sequences of the 32K dalton polypeptides are shown in the lower line (6). Conserved amino acids are framed. The amino acids of the M subunit of the reaction center of *R. sphaeroides* (residues 199–222; ref. 24) that are conserved in D2 and in the 32K dalton polypeptide are overlined and underlined, respectively, in domain F between residues 194 and 217 of D2.

which are related with a homology ranging between 33% in D to 58% in E (Table 1). Most of these conserved regions are located within the spans (Fig. 5). It is noteworthy that the amino termini of the two polypeptides are related whereas the carboxy termini differ from each other considerably (Fig. 5, Table 1). The D2 polypeptide contains 4 lys residues in contrast to the 32K dalton polypeptide which lacks lysine.

Recently the sequences of the highly hydrophobic L and M subunits of the reaction centers from the photosynthetic bacteria *Rhodopseudomonas capsulata* (23) and *R. sphaeroides* (24) have been determined. It is interesting to note that the conserved amino acid stretches observed between the 32K dalton polypeptide of higher plants (20) and the L subunit (pro_{196}, phe, his, met; 23) and the M subunit (ala_{213}, met, his, gly; 24) are nearly fully conserved in the D2 polypeptide at similar positions, except for the met residue of the last peptide which is replaced by ile in D2 (Table 1). The only region of significant homology between D2 (positions 194 to 217) and the M subunit (positions 199 to 222) is comprised within domain F in Fig. 5 (cf. Table 1). In this region the M subunit is more related to D2 (58% homology) than to the 32K dalton polypeptide (46% homology).

Studies on the reaction center subunits of *R sphaeroides* based on azido-anthroquinone binding and on inhibition of electron transfer by antibodies directed against the individual subunits strongly suggest that the primary and secondary quinone binding sites are on, or very close to, the M subunit (25, 26). On the other hand, the L subunit of the reaction center of *R. sphaeroides* has been shown to bind azido atrazine (27), a property that is shared by the 32K dalton polypeptide (28). A sequence analysis of the psbA gene of an atrazine resistant biotype of *Amaranthus hybridus* (29) and of an atrazine and diuron resistant mutant of *C. reinhardii* (6) has shown that in both cases the 32K dalton polypeptide from the resistant and sensitive species differ by a single amino acid at position 264, which is serine in wild type. Although this residue belongs to the conserved domain G (Fig. 5), it has not been conserved in the D2 sequence (cf. Table 1).

These observations point towards a parallel relationship between the L and M subunits on one side and the 32K dalton and D2 polypeptides, on the other. In particular, the possibility that the D2 poly-

Table 2. Codon usage in chloroplast genes of *C. reinhardii.*

U			C			A			G			
Phe	12	(20)	Ser	6	(30)	Tyr	4	(8)	Cys	5	(23)	U
Phe	30	(87)	Ser	1	(1)	Tyr	6	(48)	Cys	0	(1)	C
Leu	21	(85)	Ser	8	(37)	Ochre	0	(4)	Opal	0	(0)	A
Leu	1	(2)	Ser	1	(1)	Amber	0	(0)	Trp	13	(46)	G
Leu	7	(42)	Pro	5	(20)	His	1	(2)	Arg	12	(67)	U
Leu	0	(0)	Pro	0	(0)	His	8	(37)	Arg	1	(1)	C
Leu	4	(19)	Pro	7	(31)	Gln	10	(32)	Arg	0	(0)	A
Leu	0	(0)	Pro	0	(3)	Gln	2	(4)	Arg	0	(0)	G
Ile	16	(52)	Thr	9	(52)	Asn	1	(2)	Ser	4	(5)	U
Ile	4	(37)	Thr	0	(0)	Asn	14	(65)	Ser	0	(5)	C
Ile	2	(2)	Thr	10	(30)	Lys	3	(37)	Arg	0	(1)	A
Met	9	(41)	Thr	1	(1)	Lys	1	(1)	Arg	0	(0)	G
Val	13	(42)	Ala	27	(106)	Asp	3	(20)	Gly	29	(140)	U
Val	0	(0)	Ala	0	(1)	Asp	7	(44)	Gly	1	(6)	C
Val	9	(57)	Ala	9	(31)	Glu	9	(65)	Gly	0	(1)	A
Val	0	(0)	Ala	1	(31)	Glu	3	(8)	Gly	0	(0)	G

The number of times each codon appears in psbD is indicated. Numbers of parentheses refer to the codon used in rbcL (19), psbA (6), psbD and in a portion of two unidentified open reading frames X and Y (19).

peptide may be involved in quinone binding has to be considered.

Little is known about the function of the D2 polypeptide. It is synthesized on membrane bound chloroplast ribosomes and its synthesis appears to be light induced (3). Studies on transcription of the chloroplast genome of *C. reinhardii* during synchronous cell growth and at various stages of the greening process in a yellow mutant show that transcription of the psbD gene is strongly stimulated by light (30).

The partial sequence homology between D2 and the 32K dalton polypeptide raises the possibility that these two polypeptides have evolved from a common ancestor. An alternative is that there has been convergent evolution either because these two membrane polypeptides perform similar photosynthetic functions or because of structural constraints in the thylakoids. There may also be a link between the relatedness of these two polypeptides and their susceptibility to degradation.

Codon usage in the psbD gene

Analysis of the codon usage in the psbD gene reveals that only 45 codons are used (Table 2). The restricted codon usage was already noted for the rbcL gene of *C. reinhardii* (12). The data obtained from three chloroplast genes of this alga (rbcL, psbA, psbD and partial sequences of two genes X and Y, ref. 12) are summarized in Table 2. Out of 1505 codons examined only 51 different codons are utilized. The 10 codons which have not been found are CUC, CUG (leu), GUC, GUG (val), CCC (pro), ACC (thr), CGA, CGG, AGG (arg), GGG (gly). Among the three termination codons only UAA has been found. Since a tRNA specific for CGA and CGG may be missing in *C. reinhardii,* a minimum number of 32 distinct tRNAs would be required for proper translation assuming normal wobble pairing (31). Codons terminating with A are highly favored over those terminating with G.

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