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Resistance to the photosystem II herbicide diuron is dominant to sensitivity in the cyanobacterium *Synechococcus* sp. PCC7942

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The transformable cyanobacterium, *Synechococcus* sp. PCC7942, was used to study the genetics of resistance to the herbicide diuron. In wild-type cells, diuron binds to one of the core proteins, called D1, of photosystem II reaction centres. This binding prevents the transfer of electrons from Q_A, the primary quinone acceptor, to Q_B, which is necessary to create the charge separation that drives ATP synthesis. A single amino acid substitution in the D1 protein reduces diuron binding and confers herbicide resistance to reaction centres containing the substituted D1 protein. In *Synechococcus* 7942, the D1 protein is encoded by three functional genes called *psbAI, psbAII* and *psbAIII*. By selectively altering one member at a time of the three-member *psbA* gene family, we have demonstrated that diuron-resistant alleles are dominant to diuron-sensitive alleles. The relative abundance of the different *psbA* gene transcripts is correlated with the fraction of diuron-resistant reaction centres and with the degree of diuron resistance. Growth in sublethal diuron selectively increases the steady-state levels of transcripts of genes (*psbA* and *psbD*) encoding the core proteins of photosystem II. We have also found that turnover of the D1 protein can be uncoupled from electron transport through photosystem II.

Key words: cyanobacteria/diuron/D1 turnover/herbicide resistance/photosystem II

Introduction

Photosynthesis in plants, algae and cyanobacteria requires two photosystems which convert light energy into chemical energy. Photosystem I (PSI) and photosystem II (PSII) receive light energy at 700 nm and 680 nm, respectively, and use this energy to excite an electron. In PSII the excited electron is transferred from a special chlorophyll to different chromophores before it is stabilized on two different quinones. All of these reactions occur in a thylakoid membrane-bound pigment–protein complex termed the reaction centre, which is located in the chloroplasts of higher plants and algae or in the intracellular membranes of cyanobacteria (reviewed in Barber, 1987). The PSII reaction centre is thought to be similar in structure and organization to the reaction centre of photosynthetic bacteria (Deisenhofer *et al.*, 1985; Trebst, 1986). Two PSII reaction centre proteins, D1 and D2, similar in sequence to the L and M polypeptides of photosynthetic bacteria, house the chromophores and the quinones (Q_A and Q_B) essential to electron transport. In plants, the D1 and D2 proteins are encoded by the *psbA* and *psbD* genes, located in the chloroplast.

Many herbicides stop PSII electron transport by blocking electron flow from $Q_A \rightarrow Q_B$. Diuron, 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea, has been shown to compete with Q_B for the Q_B-binding pocket located on the D1 protein (reviewed in Kyle, 1985). Mutations in the gene encoding the D1 protein alter the Q_B-binding pocket in a way that permits Q_B binding, but prevents binding by diuron (reviewed in Brusslan and Haselkorn, 1988). One of these mutations is a Ser → Ala change at amino acid 264 of the D1 polypeptide. This mutation also slows electron transport from $Q_A \rightarrow Q_B$ (Hirschberg and McIntosh, 1983; Golden and Sherman, 1984b).

Attempts to engineer resistance to herbicides which affect PSII have so far been unsuccessful in higher plants and algae. One problem is that the gene which encodes the D1 protein, *psbA*, is located on the chloroplast genome which is difficult to manipulate. Recently, transformation of the chloroplast genome has been reported (Boynton *et al.*, 1988), and thus new attempts to introduce a herbicide-resistant D1 protein may succeed. Another approach has been to transform the nuclear genome of a plant with a *psbA* gene containing a plastid transport leader sequence (Cheung *et al.*, 1988). This attempt had minimal success; the most resistant plant survived 3 weeks in herbicide-containing culture, and then bleached and died.

Another problem in engineering herbicide resistance is the rapid turnover of the D1 protein. Turnover of the D1 protein has been studied extensively in the aquatic angiosperm, *Spirodela oligorrhiza*. When whole plants are grown for a short time with [^{35}S]methionine, the D1 protein is strongly labelled. As the label is chased in the light, the radioactive D1 protein disappears much more rapidly than other labelled proteins (Weinbaum *et al.*, 1979). A D1-specific membrane-bound protease is believed to be responsible for D1 turnover (Greenberg *et al.*, 1987). The rapid degradation of the D1 protein does not occur in the dark or in the presence of diuron (Mattoo *et al.*, 1984), but the D1 protein from a diuron-resistant strain of the alga *Chlamydomonas reinhardtii* (that does not bind diuron) continues to turn over when the herbicide is present. Thus, diuron appears to protect the D1 protein from degradation concomitant with its action in blocking electron transport (Kyle *et al.*, 1984). An empty Q_B-binding pocket, which occurs as the Q_B shuttles to and from the PSII complex, may be a signal for protein degradation (Trebst *et al.*, 1988). It has also been predicted that a mixed population of D^r (diuron-resistant) and D^s (diuron-sensitive) D1 proteins will not support photosynthetic growth in the presence of diuron because the D^s proteins will not be able to turn over. Indeed, D^r has been found to be recessive to D^s in 'cytohets' of *C.reinhardtii* (Mets *et al.*, 1985) and in certain strains of *Synechococcus* sp. PCC7942 (Pecker *et al.*, 1987).

In the present study of the relationship of diuron-resistant and sensitive alleles in the cyanobacterium *Synechococcus* sp. PCC7942 (referred to as *Synechococcus* in this text) we have exploited the three-member *psbA* gene family (Golden *et al.*, 1986). By altering each *psbA* gene, alone, at Ser-264 → Ala, we find that D^r is dominant to D^s in strains with two or three copies of the *psbA* gene. Different transcriptional levels of each gene result in correlated degrees of diuron resistance. These results indicate that engineering diuron resistance in plants may require no more than sufficient expression of the gene encoding the D^r allele.

We have also found that in *Synechococcus*, concentrations of diuron, which completely inhibit electron transport and presumably keep the Q_B-binding pocket of the D1 protein occupied, do not block turnover of the D1 protein. This result is not consistent with the current model for D1 turnover outlined above.

Results

Strain construction

Synechococcus has an efficient homologous recombination system. Transformation with a plasmid that does not replicate in *Synechococcus*, e.g. any colE1 derivative, will require integration if the plasmid is to be stably maintained. If a mutant gene is cloned into a non-replicating plasmid, it can replace the endogenous wild-type (WT) gene by homologous recombination involving a double cross-over. This event can be selected by screening for the mutant phenotype. If a cloned gene is interrupted by a selectable marker, then transformation with this interrupted gene on a non-replicating vector can result in replacement and thus inactivation of the endogenous gene. We used mutant D^r *psbA* genes and interrupted *psbA* genes to construct and analyse strains of *Synechococcus* which contain a D^r *psbA* allele in any of the three gene copies leaving D^s *psbA* alleles in the other two copies. These strains have a mixed population of D^r and D^s D1 proteins. Growth of the altered strains in media containing diuron was then measured to assess dominance of D^r alleles. The strains constructed for these analyses are listed in Table I.

In previous work we showed that any one of the three *psbA* genes in *Synechococcus* is sufficient to sustain the WT photosynthetic growth rate (Golden *et al.*, 1986). We also found that the relative steady-state level of transcripts of the three genes in WT (equivalent to WT_f, below) was as follows: *psbAI*, 94%; *psbAII*, 1.5%; *psbAIII*, 4.5%. In the course of the experiments reported below we found that the steady-state level of transcripts of the *psbAII* gene, in our WT and derivative strains that had been subcultured in the laboratory for several years, had increased ~100-fold. In this paper we distinguish these strains as WT_f (frozen) having the low level expression of *psbAII* and WT_c (cultured) having the high level expression of *psbAII*.

At the nucleotide sequence level, the *psbAI* gene is 91% similar to *psbAII* and *psbAIII*; the latter two differ by only 1.4% within the coding region. Outside the coding region the sequences are completely divergent. The unique flanking sequences make it possible to use antibiotic resistance cassettes, inserted into each *psbA* gene, for allele-specific inactivation by transformation (Golden *et al.*, 1986).

Strain construction for the analysis of dominance is outlined in Figure 1. We began with a 700-bp *Taq*I DNA fragment cloned from a diuron-resistant (D^r) mutant isolated following chemical mutagenesis. This fragment is within the *psbAI* gene, differing from WT by a single nucleotide that changes Ser-264 to Ala. Transformation of WT with this fragment, selecting for diuron resistance, gave the strain called TAQ1 (Table I, Figure 1). The left column of Figure 1 shows the double cross-over event required to replace the chromosomal *psbAI* Ser-264 with Ala. The genotype of this strain was shown to be as given in Table I by selective inactivation of each allele. Inactivation of *psbAI* (Figure 2, strain TAQ1::Tn*5*) returned the phenotype to D^s, while inactivation of *psbAII* or *psbAIII* left the phenotype D^r (data not shown). Thus, only the *psbAI* allele is altered in the TAQ1 strain. Even though selection was based on diuron resistance, no additional mutations resulting in resistance occurred.

Table I. *Synechococcus* sp. PCC7942 strains

Strain/designation	Amino acid 264			Phenotype
	psbAI	*II*	*III*	
WT	Ser	Ser	Ser	D^s
TAQ1	Ala	Ser	Ser	D^r
KΔ1	deleted	Ser	Ser	D^s
KΔ1TAQ3	deleted	Ser	Ala	D^r
KΔ1TAQ3C3	deleted	Ser	interrupted	D^s
TAQ3	Ser	Ser	Ala	D^r
TAQ3C3	Ser	Ser	interrupted	D^s
KΔ1C3	deleted	Ser	interrupted	D^s
KΔ1C3TAQ2	deleted	Ala	interrupted	D^r
Stp2	deleted	Ala	interrupted	D^r
KΔ1TAQ2	deleted	Ala	Ser	D^r
KΔ1TAQ2C2	deleted	deleted	Ser	D^s
TAQ2	Ser	Ala	Ser	D^r
TAQ2C2	Ser	deleted	Ser	D^s

The strain designations have the following meanings (see Figure 1 for the constructions): TAQ1 is a diuron-resistant transformant of wild-type produced by transformation with a 700-bp *Taq*I fragment of DNA isolated from a diuron-resistant mutant. KΔ1 has had the *psbAI* gene replaced by a Kan^r cassette. TAQ3, TAQ2 have the Ser → Ala replacement in the *psbAIII* and *psbAII* genes, respectively. C2, C3 refer to the chloramphenicol resistance cassette used to inactivate *psbAII* and *psbAIII*, respectively (Golden *et al.*, 1985). D^r and D^s mean resistant and sensitive, respectively, to 1 μM diuron.

In order to move the Ser → Ala mutation to the other alleles, we first deleted the *psbAI* gene entirely. This was done by transforming WT with a cloned *psbAI* gene in which ~2 kb of coding and downstream sequence are replaced by a kanamycin resistance cassette (see Materials and methods). The resulting strain, KΔ1, was then transformed with the 700-bp *Taq*I fragment of *psbAI* containing the Ser → Ala mutation, selecting for diuron resistance. In principle the strains selected could have integrated the *Taq*I fragment at either *psbAII* or *psbAIII*. In fact, 19 of 20 tested by inactivation with allele-specific cassettes (Figure 2, strain KΔ1TAQ3C3) showed that the D^r gene had entered at *psbAIII* (strain KΔ1TAQ3, Figure 1, Table I). The 20th D^r clone is not shown in Table I or Figure 1 because it was unstable.

The two strains, TAQ1 and KΔ1TAQ3, have the D^r allele in their most abundantly transcribed *psbA* gene. Figure 1 shows, in the right hand column, the construction of a TAQ3 strain in which the *psbAI* wild-type allele is restored. Total chromosomal DNA from the KΔ1TAQ3 strain was

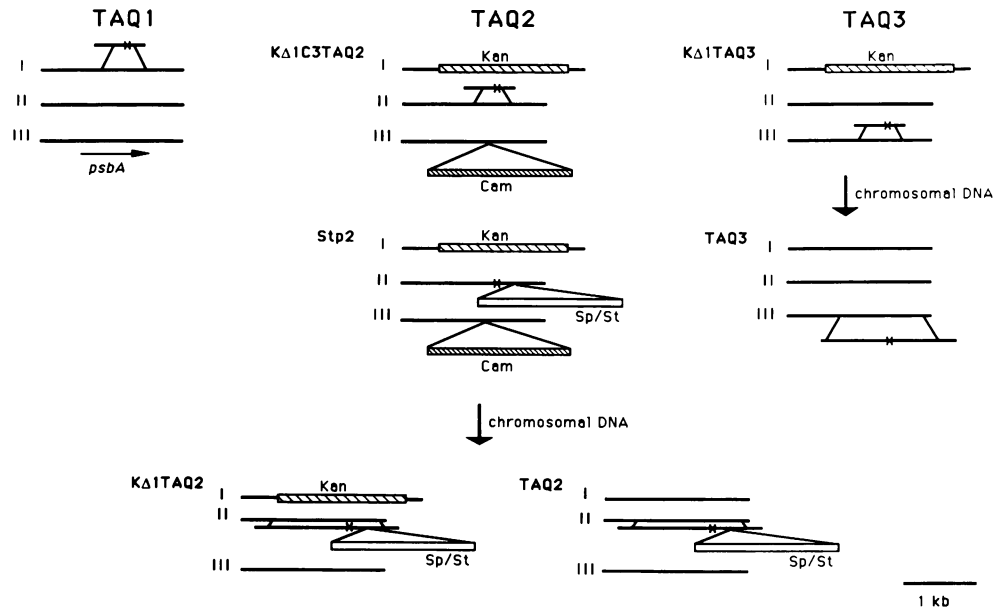

Fig. 1. Procedures for construction of the TAQ series of strains of *Synechococcus*. The three chromosomal *psbA* genes are indicated by I, II, III to the left of each column. The extent of the *psbA* coding region is shown by the horizontal arrow under TAQ1. x indicates the location of the Ser-264 → Ala mutation. The bar labelled Kan replaces (deletes) the *psbAI* gene. The bar labelled Cam is an insert that interrupts the *psbAIII* reading frame in the TAQ2 construction intermediates. The bar labelled Sp/St is an insert conferring resistance to spectinomycin, near, but not within the transcribed region of the *psbAII* gene. See Table I for properties of the final strains and the intermediates used in their construction; see the text for details of the constructions.

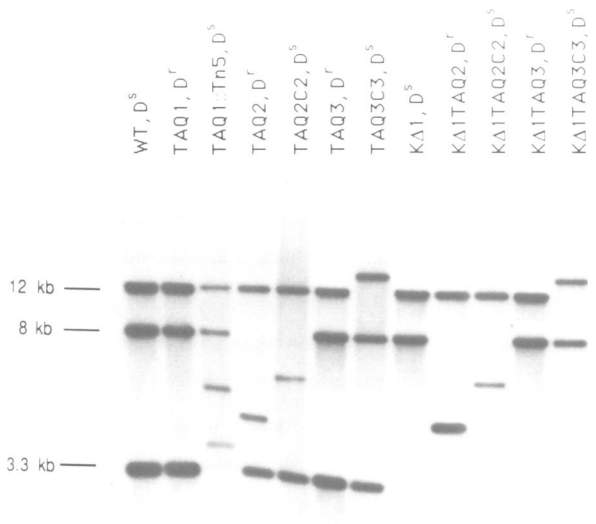

Fig. 2. Southern blot showing *psbA* genes of TAQ series strains and cognate inactivated strains. Chromosomal DNA was digested with *Bam*HI, blotted to GeneScreen+, and hybridized with a non-specific *psbA* gene probe: a 1.5-kb *Sal*I fragment from *psbAI* which contains most of the gene plus 500 bp downstream sequence. In WT, *psbAIII* is located on a 12-kb *Bam*HI fragment, *psbAII* on an 8-kb fragment and *psbAI* is found on a 3.3-kb fragment. Inactivation of *psbAI* with pTn5K1 results in a new *Bam*HI site located on a fragment the size of the *psbAI* fragment plus 5.7 kb of Tn5. In TAQ2 a new *Bam*HI site is located downstream of the Spc^r−Str^r gene resulting in a 4.6-kb *psbAII* fragment. This fragment increases to 6.6 kb when *psbAII* is inactivated with pCAM2. The 12-kb *psbAIII*-containing fragment is increased by 2 kb as a result of inactivation with pCAM3.

used to transform WT, selecting for diuron resistance. The donor strain has no *psbAI* gene and the Ser → Ala mutation is in the *psbAIII* gene. This it was expected that homology of flanking regions would direct the mutation to *psbAIII*. One D^r transformant, TAQ3, was selected (Table I, Figure 2). Inactivation of *psbAIII* in this strain (TAQ3C3) restored the D^s phenotype, but when *psbAI* or *psbAII* was inactivated the D^r phenotype was retained (data not shown). Only the *psbAIII* gene contains the Ser → Ala substitution in the TAQ3 strain indicating again that selection on diuron did not result in the isolation of secondary mutations. These results mean that the protein encoded by the *psbAIII* gene alone can confer the D^r phenotype, even when the gene's transcripts are in the minority (see below).

Since a stable TAQ2 strain was not obtained in the construction just described, an alternate route was planned that did not require selection for diuron resistance in the final step. First, the strain KΔ1C3 was made; in this strain *psbAI* is deleted and *psbAIII* is inactivated (Figure 1, centre column). Transformation with the 700-bp *Taq*I fragment, selecting for diuron resistance, gave a strain with the Ser → Ala mutation in *psbAII* (Figure 1). This strain was transformed with DNA containing a Spc^r−Str^r cassette inserted 100 bp downstream of the *psbAII* stop codon. The transforming DNA fragment contained 200 bp of the carboxy-terminal of the *psbAII* coding region plus 5.7 kb downstream. Since the Ser → Ala mutation is 83 bp upstream of this fragment, transformation to Spc^r−Str^r should not affect the D^r mutation. Indeed, the Spc^r−Str^r transformants retained the D^r phenotype of the parent KΔ1C3TAQ2 strain. One such transformant, Stp2, was then used to transform both KΔ1 and WT, selecting again for Spc^r−Str^r. Linkage of the Spc^r−Str^r cassette to the *psbAII* gene would allow co-transformation; the diuron resistance phenotype could then be tested on a number of Spc^r−Str^r transformants. Extremely tight linkage was observed: 19 of

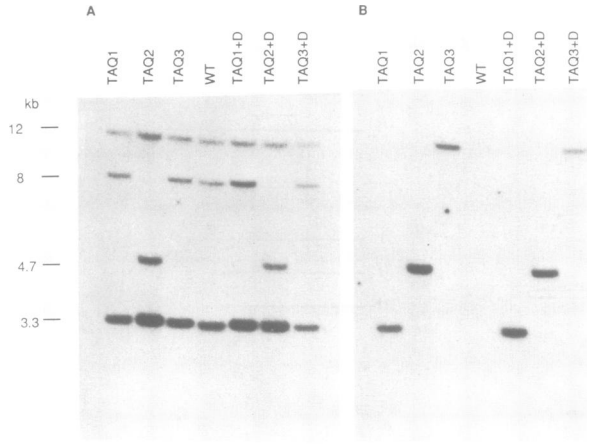

Fig. 3. Southern blots of the TAQ series probed with (**A**) a non-specific *psbA* gene probe or (**B**) a D^r oligonucleotide. Cells were grown without or with (+D) 10 μM diuron (TAQ1 and TAQ2 or 1 μM diuron (TAQ3) to late exponential stage before DNA was extracted. DNA was digested with *Bam*HI as in Figure 2. The WT strain does not contain any D^r alleles, and as expected is not labelled by the D^r oligonucleotide.

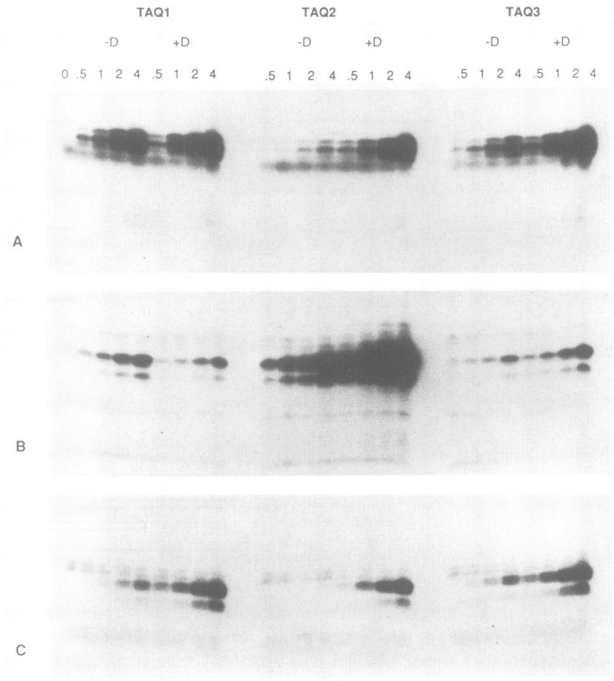

Fig. 4. Quantitative RNase protection anaslysis of *psbA* gene transcripts in the TAQ series strains grown without (−D) or with (+D) diuron. Cells were grown as in Figure 3 and total RNA was extracted. 0.5, 1, 2 and 4 μg of each RNA, as indicated at the top of the figure, was hybridized to a (**A**) *psbAI*-specific probe, (**B**) *psbAII*-specific probe or (**C**) *psbAIII*-specific probe, and then digested with RNase as described in Materials and methods. For each probe a lane which did not contain any *Synechococcus* RNA (0) was included to assess background. Following autoradiography the protected material at the centre of each lane was excised and counted.

20 Spc^r-Str^r derivatives of KΔ1 and 18 of 19 derivatives of WT were diuron resistant (Figure 1, Table I). A number of these derivatives were tested further by allele-specific inactivation (Figure 2), which confirmed that only the *psbAII* gene contained the Ser → Ala mutation.

The TAQ1, TAQ2 and TAQ3 strains were unstable under long term cultivation in the presence of diuron due to intrachromosomal gene conversion leading to D^r alleles at loci that were originally D^s (J.Brusslan, B.Gaster and R. Haselkorn, in preparation). To demonstrate that the strains we analysed below contained only one D^r gene, a 19mer oligonucleotide which recognizes only a D^r *psbA* gene was used to probe chromosomal DNA from TAQ1, TAQ2 and TAQ3 (Figure 3b). By comparison with the same DNA probed with a non-specific *psbA* probe (Figure 3a), it can be seen that each strain contains only one D^r *psbA* allele. Even after the cells have been grown in diuron, there is still only one D^r *psbA* gene. Therefore, the D^r phenotype can be manifest from any position within the *psbA* gene family.

Phenotypes of D^r strains

The TAQ3 strain was more sensitive to diuron than TAQ1 or TAQ2. The growth of TAQ3 was 60% inhibited at 1 μM diuron while growth of both TAQ1 and TAQ2 was inhibited by only 35% at 10 μM diuron (J.Brusslan, Ph.D. dissertation, University of Chicago). These values can be compared to WT which was 60% growth inhibited at 50 nM diuron. All these TAQ derivatives grow at the same rate as WT in the absence of diuron.

Transcription of the psbA genes in the D^r strains

A quantitative assay using RNase A and RNase T1 protection was employed to measure steady-state transcript levels from each of the three *psbA* genes in WT and the TAQ strains. In all cases total RNA was isolated from cells harvested in late exponential phase, $OD_{750} = 0.1$, to avoid any shading effects. (After a lag, cyanobacteria enter a brief stage of logarithmic growth. When the cells reach a density at which they shade one another, arithmetic growth ensues. Arithmetic growth continues until the cells reach stationary phase.) Growth in media containing a concentration of diuron which reduced the growth rate by 60%, increased the transcript levels of all three *psbA* genes in all strains but one, yielding a doubling in total *psbA* mRNA (see Figures 4 and 5).

In all strains, the increase in the *psbAI* mRNA level varied from 2- to 5-fold, while the increase in the *psbAIII* mRNA level varied from 3- to 14-fold. These increases in mRNA abundance were independent of the position of the D^r gene. On the other hand, the *psbAII* transcript level was strain dependent. The steady-state level of the *psbAII* transcript was increased 40-fold in TAQ2 compared to WT_f even when grown without diuron. Our WT_c strain, which had been sub-cultured for 5 years, also had acquired a high steady-state *psbAII* transcript level, unlike the WT_f strain which had been kept in 20% glycerol at −80°C during this time (Figure 5). The high steady-state level of the *psbAII* transcript in our sub-cultured strain might explain why it eventually became possible to construct a *psbAII* D^r strain. The cause of this increase is not yet known, but it is distinct from the induction by diuron because a further increase in the *psbAII* mRNA level was observed when these *psbAII* transcript-elevated strains were grown with diuron (Figure 4b). Growth in diuron had a different effect on the steady-state level of *psbAII* mRNA in different strains. In the TAQ1 strain there was no *psbAII* mRNA induction by diuron, the TAQ3 strain showed a 4-fold increase, while WT_f showed a 30-fold increase. Note that TAQ1 and TAQ3 are derived from WT_f; TAQ2 was derived from WT_c (Figure 5).

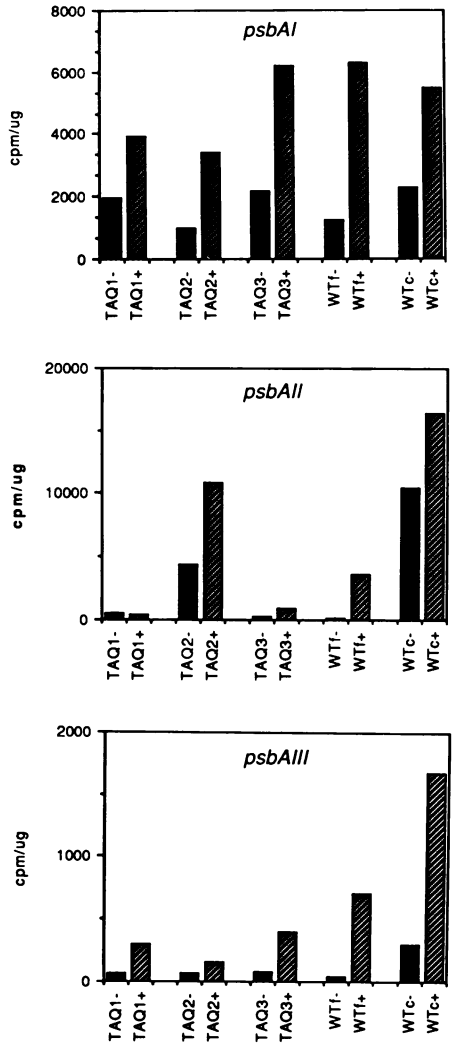

Fig. 5. *psbAI*, *psbAII* and *psbAIII* steady-state mRNA levels. Data from the experiments shown in Figure 4 and other similar experiments were quantitated and normalized to the number of radioactive U residues in each copy-specific probe. Results are presented for each strain grown without (−) and with (+) diuron. Note that the scale of c.p.m. protected/μg RNA changes for each *psbA* gene. For the TAQ series the average of two separate experiments is shown, while the WT frozen (WT_f) and WT cultured (WT_c) strains are data from single experiments. The diuron concentration was 50 nM for WT, 1 μM for TAQ3 and 10 μM for TAQ1 and TAQ2.

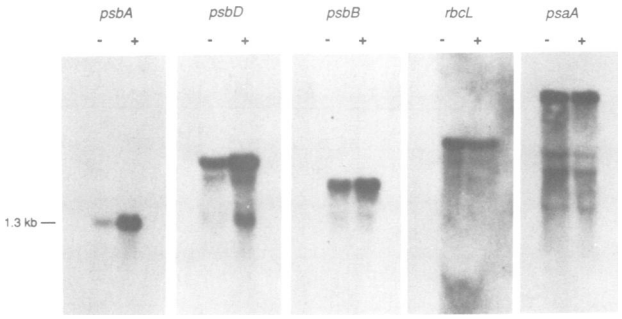

Fig. 6. Northern blot analysis of the effect of diuron on the level of specific transcripts. RNA was extracted from late exponential stage cells of the WT frozen strain grown without (−) and with (+) 50 nM diuron, denatured with glyoxal, electrophoresed, blotted to GeneScreen+ and probed with various cloned genes as indicated. The *psbA* probe identifies all three *psbA* transcripts. The *psbD* gene encodes the D2 protein of PSII reaction centres. There are two such genes; the larger transcript includes the *psbC* gene as well. The *psbB* gene encodes the 47-kd chlorophyll-binding protein of PSII. The *rbcL* gene encodes the large subunit of RuBP carboxylase (Rubisco). The *psaA* gene encodes a large polypeptide component of PSI reaction centres.

Table II. Correlation of *psbA* gene transcript levels with the proportion of diuron-resistant PSII reaction centres

Strain	Proportion of *psbA* mRNA from D^r allele	Proportion of D^r PSII reaction centres
TAQ1	0.77	0.75
TAQ2	0.74	0.63
TAQ3	0.037	0.17

RNA data are taken from Figures 4 and 5. The proportion of D^r reaction centres was determined by Howard Robinson (University of Illinois) as described in Robinson *et al.* (1987), by measuring the inhibition of PSII fluorescence decay from whole cells as a function of diuron concentration.

Response of other PSII gene transcripts to diuron stress

In the preceding section, we showed that stressing cells with sublethal concentrations of diuron, sufficient to reduce the growth rate by 60%, resulted in elevated levels of *psbA* mRNA. To see if diuron induction of *psbA* mRNA was due simply to slowed growth of the cells, Northern blots of total RNA from WT cells grown in the absence or presence of 50 nM diuron were probed with various clones. Slow growth results in fewer ribosomes, and thus possibly a greater proportion of mRNA in total RNA (Gausing, 1977). It was found that the transcript levels of *rbcL* (encoding the large subunit of RuBP carboxylase) and *psaA/B* (encoding the apoproteins of photosystem I) were not altered by growth in diuron, but that the mRNA levels of *psbB* and *psbD/C* (both encoding essential components of PSII), as well as *psbA* were increased (Figure 6). The fact that different transcripts are individually affected rules out a general effect of slow growth. The 2.0-kb transcript of *psbB* and the 2.5-kb transcript of *psbDI/C* were increased to the same extent, but the level of the 1.3-kb *psbDII* transcript and the 1.3-kb *psbA* transcript were increased much more. Thus it appears that the *psbA* and *psbD* transcripts which encode the PSII core proteins, D1 and D2, were increased most by addition of diuron.

Correlation of transcript levels with reaction centre populations

The quantitative measurements of *psbA* transcript levels described in the preceding sections make it possible to calculate the fraction of *psbA* transcript corresponding to the D^r alleles in each of the TAQ strains, with or without the addition of diuron. The results of these calculations, for cells grown without diuron, are listed in Table II.

The relationship between transcript level and finished, assembled, functional reaction centres depends on the efficiency with which each mRNA is translated, the selection of each D1 protein for assembly, and the stability of each D1 protein. While each of these steps might be measured individually, it is possible to bypass them and determine directly the fraction of PSII reaction centres by biophysical methods. This was done for us by Dr Howard Robinson at the University of Illinois, who measured the variable

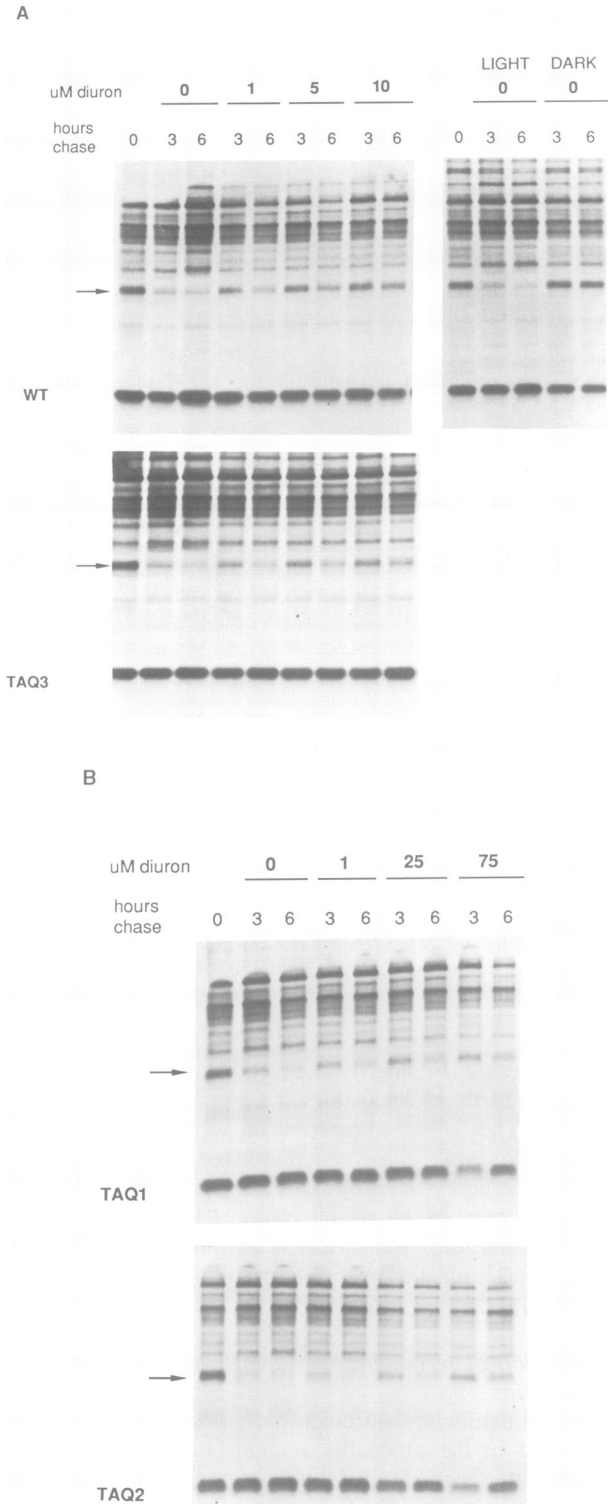

Fig. 7. Turnover of ^{35}S-labelled proteins in the (**A**) WT and TAQ3 strains and (**B**) TAQ1 and TAQ2 strains of *Synechococcus*. Cells were labelled for 90 min, and then chased for 3 or 6 h in the presence of unlabelled methionine with varying concentrations of diuron, as indicated. The D1 protein is indicated by the arrow. For the WT strain, a separate experiment comparing turnover in the light and dark without diuron is included. Following autoradiography the labelled D1 bands were excised and counted.

fluorescence from PSII reaction centres as a function of herbicide concentration, using whole cells grown without diuron (Robinson *et al.*, 1987). His results are included in Table II. They show a remarkably good correlation between the fraction of *psbA* mRNA due to D^r alleles and the fraction of PSII reaction centres that are functionally resistant to diuron.

Turnover of the D1 protein in D^r strains

The unusual metabolism of the D1 protein has been studied for many years. The protein is rapidly and abundantly synthesized in chloroplasts; it is also rapidly degraded. Among the many rationalizations of this result, a prominent idea has been that the D1 protein is sacrificed to oxygen radicals in order to spare the remainder of the reaction centre. Blockade of D1 turnover by darkness or by herbicide have been interpreted to be consistent with this idea. However, in studies to determine whether the D1 protein of cyanobacteria also turns over (Goloubinoff *et al.*, 1988) we noted that diuron had an incomplete inhibitory effect on turnover. Those experiments were therefore extended to examine in detail the effect of diuron on D1 turnover in the TAQ strains.

The proteins of *Synechococcus* were pulse-labelled with $H_2{}^{35}SO_4$, and then chased in the light in the presence of excess cold methionine. The D1 protein was strongly labelled, and turned over rapidly, as has been reported for the D1 protein of *S.oligorrhiza*. The identity of the D1 protein was confirmed by immunoblotting (M.Shaefer and S.Golden, personal communication). Turnover in the WT and TAQ3 strains was compared at 0, 1, 5 and 10 μM diuron (Figure 7a). It can be seen that turnover is identical in both strains, being inhibited to the same extent with diuron. Eighty per cent of the labelled D1 protein was degraded in 6 h without diuron, and this turnover was reduced 20% in the presence of 1 μM diuron and 45% at 10 μM diuron. The TAQ3 strain showed an increase in cell density at 1 μM diuron during the 6-h experiment (ΔOD_{750} = 0.025) while the WT strain showed no increase at this diuron concentration.

Turnover of the D1 protein in the TAQ1 and TAQ2 strains was not slowed at 1 μM diuron, showed 10% inhibition at 25 μM, and was reduced 45% at 75 μM (Figure 7b). The turnover of the D^r proteins was partially resistant to diuron; the concentration of diuron required for 10% inhibition of turnover reduces electron transport by 90% (Robinson *et al.*, 1987). Thus the D^r strains, like WT, showed that diuron inhibits both electron transport and D1 turnover, but the two processes can be uncoupled.

Discussion

Diuron resistance is dominant to diuron sensitivity

Strains of *Synechococcus* sp. PCC7942 containing both D^r and D^s D1 proteins were constructed by exploiting the *psbA* multigene family. The phenotype of these strains was D^r. Thus, in strains expressing both D^r and D^s alleles of the *psbA* gene, containing both sensitive and resistant PSII reaction centres, resistance is dominant to sensitivity.

The degree of resistance depends on the relative expression of each gene (Table II). The TAQ1 strain contains 75% D^r reaction centres as determined by fluorescence, and the *psbAI* gene contributes 77% of the total transcript in TAQ1 grown

without diuron. In TAQ2, the *psbAII* transcript is 74% of total *psbA* mRNA, and there are 63% D^r reaction centres. Finally, in TAQ3 there are 17% D^r reaction centres, while the *psbAIII* transcript accounts for 3.7% of the total *psbA* message. The TAQ3 strain must be close to the threshold of the number of D^r reaction centres needed to sustain growth in diuron. At 1 μM diuron almost all D^r reaction centres in this strain are functioning (Robinson *et al.*, 1987), and the strain grows at 60% of the WT rate.

When diuron is absent all the strains grew at the same rate, probably because the D^s reaction centres, which are more efficient in $Q_A \rightarrow Q_B$ electron transfer (Gressel, 1985) can function. Although TAQ1 contains 25% D^s reaction centres, TAQ2 contains 37% D^s reaction centres, and TAQ3 contains 83% D^s reaction centres, all these strains grew at the same rate.

Previously it was reported that a *psbAI* duplication in *Synechococcus* containing one D^s *psbAI* gene adjacent to a D^r *psbAI* gene is D^s (Pecker *et al.*, 1987). The duplication strain was constructed by integration, at *psbAI*, of the entire plasmid carrying the D^r *psbAI* gene. It seems possible that the integrated plasmid has an effect on transcription of the D^r *psbAI* allele, but transcript levels have not been measured in these strains.

Gingrich *et al.* (1988) report that a herbicide-resistant *psbA* gene located on a replicating plasmid is dominant to the three herbicide-sensitive *psbA* genes located on the chromosome of *Synechococcus* sp. PCC7002. At present these results cannot be interpreted in terms of gene dosage because the copy number of the plasmid was not measured.

D1 protein turnover

It has been assumed that a heterozygote containing both D^r and D^s D1 proteins would be D^s due to inhibition of D^s D1 turnover by diuron. In *Synechococcus*, D1 turned over at 80% of its maximal rate at a concentration of diuron where no or very little (5%) electron transport occurs (Figure 7, Table II). If diuron does not prevent turnover, then a heterozygous strain should function in the presence of diuron. In another system, *S.oligorrhiza*, turnover was completely inhibited at 10 μM diuron (Mattoo *et al.*, 1984), but data concerning D1 turnover at 1 μM diuron was not presented. This experiment was performed on intact fronds, and perhaps higher concentrations of herbicide are required due to problems with uptake. More compelling evidence that turnover and electron transport are not strictly correlated is the observation that 10 μM diuron did not completely stabilize the D1 protein in isolated pea thylakoids (Ohad *et al.*, 1985). Here electron transport was inhibited by 50% at 0.1 μM diuron, by 95% at 1 μM diuron and completely stopped at 10 μM diuron (Wraight, 1985). Clearly the Q_B-binding pocket of the D1 protein should be protected at 10 μM diuron, yet the D1 protein still turned over.

Turnover of the D1 protein was completely inhibited in the dark without herbicide addition (Goloubinoff *et al.*, 1988 and Figure 7a). Perhaps D1 protein degradation requires light but not electron transport for partial activity, full activity being restored by electron transport. This is consistent with the observation of D1 degradation occurring in far red light, which can activate only PSI (Gaba *et al.*, 1987). Also, in the isolated pea thylakoid system, turnover was completely blocked in the dark (Ohad *et al.*, 1985). As in higher plants (Mattoo *et al.*, 1984), ATP synthesis is not required for D1 turnover in *Synechococcus*. This was demonstrated by the observation of complete turnover of the D1 protein in $CaCl_2$-permeabilized cells in the presence of the uncoupler, gramicidin (J.Brusslan, Ph.D. dissertation, University of Chicago).

If D1 turnover is not related to PSII function, what role does it play, and why is turnover observed in all plants, algae and cyanobacteria where it has been studied? The rapidly-labelled D1 protein could be a pool of excess D1 which is present to prevent PSII shutdown in case of emergency.

Transcript levels of psbA and other PSII genes

In the D^r TAQ strains, all three *psbA* genes are expressed in the presence or absence of diuron, indicating that dominance is not due to inactivation of D^s gene transcription. Cells whose growth is slowed by diuron exhibited a general stress response which includes the shade-type appearance, high phycocyanin:chlorophyll ratios (Koenig, 1987), as well as our observation of an increase in the steady-state levels of PSII transcripts. Preliminary data, using the transcription inhibitor rifampicin, suggest that the stability of the *psbA* mRNA is not affected by growth in diuron (J.Brusslan, Ph.D. dissertation, University of Chicago). If this is the case, then the *psbA* genes must be transcribed more often during PSII stress. It appears that different members of the *psbA* and the *psbD* gene family respond in different degrees to this stress.

The steady-state transcript level of *psbAII* changed in WT cells that had been subcultured for several years. This increase could be due to greater transcription or to a change in transcript stability. Such an increase in *psbAII* transcript level appears to acount for our ability eventually to isolate a strain containing a D^r *psbAII* gene which expressed a D^r phenotype (TAQ2). Transformation of the WT_f, which has a low level of *psbAII* mRNA, with DNA from Stp2 resulted in Spc^r-Str^r clones which were also D^r and had a high steady-state level of *psbAII* mRNA. This result suggests that the mutation which causes high *psbAII* expression is linked to the Spc^r-Str^r cassette. A likely candidate would be the promoter region of the *psbAII* gene, which is being examined at present.

Engineering herbicide resistance

Mutation of the single chloroplast-encoded *psbA* gene in plants, converting Ser-264 to Gly or Ala, results in herbicide resistance (Hirschberg and McIntosh, 1983; Gressel, 1985). Chloroplast membranes from the resulting homozygous plants transfer electrons from $Q_A \rightarrow Q_B$ more slowly than WT. The herbicide-resistant plants grow more slowly and suffer significantly in yield, so the *psbA* mutants were believed to be agronomically insignificant.

Herbicide-resistant mutants which are not altered in electron transport have been isolated in *Chlamydomonas* (Erickson *et al.*, 1985), but these are substituted at different positions in the D1 protein and they are not as resistant to herbicides as the Ser-264 → Ala mutation.

At present it appears that the only limit to engineering diuron resistance in higher plants is the problem of gene dosage; clearly a heterozygote can be herbicide resistant. Considering that there are 100 chloroplasts/cell and 60 genomes/chloroplast in a mature leaf (Lamppa *et al.*, 1980),

Table III. Plasmids used for transformation and allele-specific inactivation

Plasmid	Vector	*Synechococcus* fragment	Insert	Reference
pTaq700	pBR328	700-bp *Taq*I carrying Ser → Ala mutation, internal to *psbAI* reading frame		Golden and Haselkorn (1985)
pTn5K1	pBR328	3.5-kb *Xho*I fragment containing *psbAI* gene	Tn*5* at position 846 of *psbAI* coding region	Golden *et al.* (1986)
pCam2	pBR328	2.7-kb *Bam*HI−*Hind*III fragment containing *psbAII* gene	1.7-kb Cam^r *Bam*HI fragment from pSKS114 replaces 600-bp *Bst*EII internal fragment	Golden *et al.* (1986); this work
pCam3	pBR322	1.0-kb *Sal*I/*Bam*HI fragment containing most of *psbAIII* gene	1.7-kb Cam^r *Bam*HI fragment from pSKS114 inserted at internal *Bcl*I site	Golden *et al.* (1986)
pKanΔ1	pBR328	3.5-kb *Xho*I fragment containing *psbAI* gene	2-kb Kan^r *Sal*I fragment from pSKS101 replaces *Sal*I fragment containing most of *psbAI*	This work

this problem may be difficult to overcome. Yet the *rbcS* genes, a nuclear gene family which encode the small subunit of Rubisco, are able to produce as much protein as *rbcL*, a plastid gene encoding the large subunit of Rubisco, and so this problem should not be insurmountable.

Materials and methods

Strains and plasmids

Synechococcus sp. PCC7942 (*Anacystis nidulans* R2) was grown in BG-11 medium (Allen, 1968) at 31°C at 110 μE/m^2 fluorescent light. Diuron was obtained from Serva, and was used at 1 μM except when otherwise indicated. The transformation procedure was that reported in Golden and Sherman (1984a). *psbA* clones as well as inactivation plasmids were mainly described in Golden *et al.* (1986), but see Table III. pCam2 is identical to pStp2 (Golden *et al.*, 1986) except that a cassette encoding chloramphenicol resistance replaces a 600-bp internal *Bst*EII fragment of *psbAII*. pTaq700 was described in Golden and Haselkorn (1985). The strain KΔ1 was constructed using pKanΔ1, a derivative of pDH140 (Golden and Haselkorn, 1985) in which a cassette encoding Kan^r was inserted into *Sal*I sites located 120 bp inside of the amino-terminal of *psbAI* and 1.2 kb downstream of *psbAI*, replacing most of the gene. Kan^r transformants were confirmed by Southern analysis (Figure 2, KΔ1) to show that a replacement recombination occurred. Selection for the Spc^r−Str^r cassette was done at 40 μg/ml spectinomycin and 5 μg/ml streptomycin.

Preparation of chromosomal DNA

5 ml of liquid culture or a thick streak of cells from BG-11 agar was washed twice with 0.5 M NaCl, then resuspended in 800 μl of 10 mg/ml lysozyme (Sigma) in 10 mM Tris−HCl pH 8.0, 10 mM EDTA. Cells were incubated at 37°C for 1 h, and then brought to 1.6% Sarkosyl and 1 mg/ml proteinase K, and incubated at 55°C for 1 h. Lysed cells were phenol extracted and then precipitated with 1/9 vol. 3 M NaOAc and 0.6 vol. ethanol. The resulting pellet was resuspended in 10 mM Tris−HCl pH 8.0, 1 mM EDTA, phenol extracted, reprecipitated and washed with 70% ethanol.

D^r oligonucleotide hybridization

A 19mer 5′TGTTGAACGCTGCGTATTG3′ was end-labelled, and a Sephadex G-25 spin column was used to remove unincorporated label. DNA was blotted to GeneScreen + (DuPont), hybridized in standard hybridization buffer (Maniatis *et al.*, 1982) at 58°C, and washed in 5 × SSPE, 1% SDS at 58°C. The G indicates a nucleotide which differs in *psbAIII*, and the C is the D^r mutation.

Quantitation of steady-state transcripts

Copy-specific probes were constructed in BRL T7/T3 plasmids. U1 (upstream *psbAI*) is a 203-bp *Sau*3A fragment beginning 4 bp upstream from the start codon. U2 (upstream *psbAII*) is a 117-bp *Hin*fI−*Pvu*I fragment beginning 8 bp upstream from the start codon. U3 (upstream *psbAIII*) is a 118-bp *Hin*fI−*Pvu*II fragment also beginning 8 bp upstream from the start codon (Golden *et al.*, 1986). Run-off anti-sense riboprobes were made using T7 RNA polymerase according to BRL. RQ1 DNase from Promega was used to digest the DNA templates, and reaction products were ethanol-precipitated twice with NH_4OAc. 60 fmol of labelled RNA were used in each reaction. Total *Synechococcus* RNA was isolated according to Golden *et al.* (1987), and increasing amounts of RNA: 0.5, 1, 2 and 4 μg, were used in RNase A plus RNase T1 protection assays. These assays were done according to the Promega Gemini System technical manual with the following changes: 15 μg tRNA was used in all reactions, hybridization was performed at 41°C, nuclease digestion was at 14°C and 330 units/ml of RNase T1 was used. A final 70% ethanol wash was found to be unnecessary. Samples were electrophoresed on 8% acrylamide sequencing gels and, after autoradiography, radioactive protected fragments were cut out of the gel and counted using a Beckman scintillation counter in the Cerenkov mode. Data were normalized to the number of U residues in each protected fragment, subjected to linear regression analysis, and samples with correlation coefficients below 0.95 were discarded.

Northern analysis

RNA was extracted from WT cells grown to OD_{750} = 0.1 either with or without 50 nM diuron, and subjected to Northern analysis as described in Thomas (1983). The *rbcL* gene was isolated from *Anabaena* 7120 (Nierzwicki-Bauer *et al.*, 1984). *psbB* is also from *Anabaena* 7120 (J.D.Lang, unpublished results). *psbD* was isolated from *Synechococcus* sp. PCC7942 (Golden and Stearns, 1988), and *psaA* was also cloned from *Synechococcus* sp. PCC7942 (J.Brusslan, unpublished) using a *psaA* cloned gene from spinach as a probe (Westhoff *et al.*, 1983). Homologous hybridization was done at 65°C while heterologous hybridization was done at 60°C.

Labelling, turnover and membrane solubilization

Labelling and turnover were performed similar to Goloubinoff *et al.* (1988) with the following changes: cells were grown at 110 μE/m^2 sec and 40 μg/ml cold methionine was added to the chase medium. For membrane solubilization, the procedure of Guikema and Sherman (1980) was used with the following alterations: 1.5 ml of OD_{750} = 0.25 cells were digested with 10 mg/ml lysozyme in buffer A (Guikema and Sherman, 1980) with 2 μM aminocaproic acid, 2 μM benzamidine and 10 mM EDTA for 45 min at 37°C. After osmotic shock, membranes were resuspended in 27 μl of 10 mM Tricine pH 7.8, 6.5% sucrose, 5 mM $MgSO_4$, 0.2% NP-40 and 1 unit of DNase I, and incubated for 5 min at 37°C. 0.6 μl of 250 mM EDTA, 5 μl of 20% lithium dodecyl sulphate (LDS) and 3 μl of 1 M dithiothreitol were added, mixed and centrifuged briefly. 20 μl of the solubilized membrane preparation containing 800 000 c.p.m. were loaded onto a pre-chilled 12% polyacrylamide gel, and subjected to electrophoresis in the presence of LDS overnight at 4°C according to Delepelaire and Chua (1979). Gels were treated with En^{3}Hance (NEN) and autoradiographed. The D1 protein was excised and counted using Amersham scintillation fluid.

For immunoblot analysis, a gel containing proteins from a 6-h experiment was electroblotted to nitrocellulose and probed with D1 antibody (M. Schaefer, unpublished). One 35-kd band was detected; no bands were seen at 66 kd or 23.5 kd, the positions of D1−D2 aggregates or the major D1 degradation product, respectively.

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References

Allen,M.M. (1968) *J. Phycol.*, **4**, 1−4.
Barber,J. (1987) *Trends Biochem.*, **12**, 321−326.

Boynton,J.E., Gillham,N.W., Harris,E.H., Hosler,J.P., Johnson,A.M., Jones,A.R., Randolph-Anderson,B.L., Robertson,D., Klein,T.M., Shark,K.B. and Stanford,J.C. (1988) *Science*, **240**, 1534–1538.
Brusslan,J. and Haselkorn,R. (1988) *Photosynthesis Res.*, **17**, 115–124.
Cheung,A.Y., Bogorad,L., Van Montagu,M. and Schell,J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 391–395.
Deisenhofer,J., Epp,O., Miki,K., Huber,R. and Michel,H. (1985) *Nature*, **318**, 618–624.
Delepelaire,P. and Chua,N.-H. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 111–115.
Erickson,J.M., Rahire,M., Rochaix,J.-D. and Mets,L. (1985) *Science*, **228**, 204–207.
Gaba,V., Marder,J.B., Greenberg,B.M., Mattoo,A.K. and Edelman,M. (1987) *Plant Physiol.*, **84**, 348–352.
Gausing,K. (1977) *J. Mol. Biol.*, **115**, 335–354.
Gingrich,J.C., Buzby,J.S., Stirewalt,V.L. and Bryant,D.A. (1988) *Photosynthesis Res.*, **16**, 83–99.
Golden,S.S. and Sherman,LA.. (1984a) *J. Bacteriol.*, **158**, 36–42.
Golden,S.S. and Sherman,L.A. (1984b) *Biochim. Biophys. Acta*, **764**, 239–246.
Golden,S.S. and Haselkorn,R. (1985) *Science*, **229**, 1104–1107.
Golden,S.S. and Stearns,G.W. (1988) *Gene*, **67**, 85–96.
Golden,S.S., Brusslan,J. and Haselkorn,R. (1986) *EMBO J.*, **5**, 2789–2798.
Golden,S.S., Brusslan,J. and Haselkorn,R. (1987) *Methods Enzymol.*, **153**, 215–231.
Greenberg,B.M., Gaba,V., Mattoo,A.K. and Edelman,M. (1987) *EMBO J.*, **6**, 2865–2869.
Goloubinoff,P., Brusslan,J., Golden,S.S., Haselkorn,R. and Edelman,M. (1988) *Plant Mol. Biol.*, **11**, 441–447.
Gressel,J. (1985) In Miflin,B. (ed.), *Oxford Surveys of Plant Molecular and Cell Biology*. Oxford University Press, Oxford, Vol. 2, pp. 321–328.
Guikema,J.A. and Sherman,L.A. (1980) *Biochim. Biophys. Acta*, **536**, 189–201.
Hirschberg,J. and McIntosh,L. (1983) *Science*, **222**, 1346–1348.
Kyle,D.J. (1985) *Photochem. Photobiol.*, **41**, 107–116.
Kyle,D.J., Ohad,I. and Arntzen,C.J. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4070–4074.
Koenig,F. (1987) *Z. Naturforsch.*, **42c**, 727–732.
Lamppa,G.K., Elliot,L.V. and Bendich,A.J. (1980) *Planta*, **148**, 437–443.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mattoo,A.K., Hoffman-Falk,H., Marder,J.B. and Edelman,M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1380–1384.
Mets,L., Galloway,R.E. and Erickson,J.E. (1985) In Zaitlin,M., Day,P. and Hollaender,A. (eds), *Biotechnology in Plant Science*, Academic Press, NY, pp. 301–312.
Nierzwicki-Bauer,S.A., Curtis,S.E. and Haselkorn,R. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5961–5965.
Ohad,I., Kyle,D.J. and Hirschberg,J. (1985) *EMBO J.*, **4**, 1655–1659.
Pecker,I., Ohad,I. and Hirschberg,J. (1987) In Biggins,J. (ed.), *Progress in Photosynthetic Research*. Martinus Nijhoff Pub., Dordrecht, Vol. III, pp. 811–814.
Robinson,H., Golden,S.S., Brusslan,J. and Haselkorn,R. (1987) In Biggins, J. (ed.), *Progress in Photosynthetic Research*. Martinus Nijhoff Pub., Dordrecht, Vol. IV, pp. 825–828.
Thomas,P.S. (1983) *Methods Enzymol.*, **100**, 255–266.
Trebst,A. (1986) *Z. Naturforsch.*, **41c**, 240–245.
Trebst,A., Depka,B., Kraft,B. and Johanningmeier,U. (1988) *Photosynthesis Res.*, **18**, 163–177.
Weinbaum,S.A., Gressel,J., Reisfeld,A. and Edelman,M. (1979) *Plant Physiol.*, **64**, 828–832.
Westhoff,P., Alt,J., Nelson,N., Bottomley,W., Bunemann,H. and Herrmann,R.G. (1983) *Plant Mol. Biol.*, **2**, 95–107.
Wraight,C.A. (1985) *Biochim. Biophys. Acta*, **809**, 320–330.

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