A Study of the Molecular Biology of the Small Subunits of Photosystem Two

A thesis submitted for the degree of
Doctor of Philosophy
By

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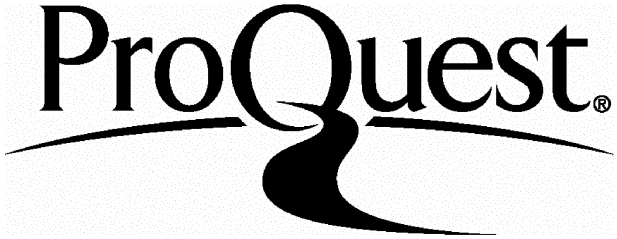

Abstract

Photosystem two is a multi subunit protein complex which carries out a photochemical reaction producing reduced plastoquinone and molecular oxygen as the products. The proteins which form this centre are encoded for by both nuclear and chloroplast genes and while the function of some has been determined, many play an unknown role. This thesis will discuss mutants created in the subunits PSII-H and PSII-N, the isolation and sequencing of the *psb*W gene in *C.reinhardtii* and the characterisation of the conserved gene *ycf*9.

The first chapter discusses the creation of a series of mutants in genes of the *psb*B operon. The *psb*H deletion mutants gave a PSII minus phenotype and were unable to grow photoautotrophically. In comparison PSII-N minus cells were capable of photosynthetic growth but appeared to be more susceptible to high light damage. The role of the phosphorylatable threonine of PSII-H was also addressed by the creation of a site directed mutation at this position. The resulting data from the analysis of this mutant did not explain the reason for this characteristic.

The next chapter begins with the screening of a series of nuclear PSII mutants, with the aim of discovering new nuclear factors controlling the expression of chloroplast PSII genes. This leads into the creation of deletion mutants for the conserved gene *ycf*9, introduced into both *C. reinhardtii* and *Synechocystis* sp PCC 6803. Analysis of these mutants does not lead to a simple hypothesis of the role of this protein as the characteristics from the two organisms are not identical. Further analysis is planned to elucidate the function of the *ycf*9 protein.

The final chapter discusses the nuclear encoded subunit of the PSII core, PSII-W. We report the isolation and partial sequencing of the gene for this protein in *C. reinhardtii*. Further analysis showed this to be a single copy gene containing two introns, which is expressed as a 1kb transcript. The mature protein sequence showed high similarity to other species over the N-terminal region, with a greater degree of variability in the C-terminal portion.

To Paul, Mum and Dad,

With Love

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Abbreviations

AMV	avian myeloblastosis virus
bp	base pair
BSA	bovine serum albumin
C-terminal	carboxy-terminal
Chl	chlorophyl
CIAP	calf intestinal alkaline phosphatase
Cyt	cytochrome
Da	daltons
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone
DCMU	3-(3,4-dichloro-phenyl)-1,1-dimethylurea
DEPC	diethylpyrocardonate
DMBQ	2,5-dimethyl-*p*-benzoquinone
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNP-INT	2,4-dinitrophenyl ether of iodonitrotymol
dNTP	2`deoxynucleoside 5`-triphosphate
E. coli	*Escherichia coli*
EDTA	diaminoethanetetra-acetic acid
EPR	electron paramagnetic resonance
EST	expressed sequence tag
Fe-S_A, Fe-S_B, Fe-S_X,	low potential iron sulphur centres in PSI
FSBA	5`-*p*-fluorosulphonylbenzoyladenosine
HSM	high salt minimal medium
HQNO	2-(*n*-heptyl)-4-hydroxyquinoline *N*-oxide
IPTG	isopropylthio-β-galactoside
kb	kilobase
LB	Luria-Bertani medium
LHC	light harvesting complex
LHC-P	phosphorylated light harvesting complex
mRNA	messenger ribonucleic acid
MOPS	3-[N-morpholino] propanesulphonic acid

N-terminal	amino terminal
$NAD(P)^+$	β-nicotinamide adenine dinucleotide phosphate
NAD(P)H	reduced β-nicotinamide adenine dinucleotide phosphate
P680	primary electron donor of PSII
P700	primary electron donor of PSI
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PNK	polynucleotide kinase
PQ	plastoquinone
PSI	photosystem one
PSII	photosystem two
PVP	poly vinyl pyrrolidone
Qa	primary quinone electron acceptor of PSII
Qb	secondary quinone electron acceptor of PSII
RACE	rapid amplification of cDNA ends
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
Sp	spectinomycin
TAP	tris acetate phosphate medium
TAE	tris acetate EDTA buffer
TE	tris EDTA buffer
TEMED	*N,N,N`,N`*-tetramethylethylenediamine
TES	N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid
T_M	melting temperature
Tricine	*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
Tris	2-amino-2-hydroxy-methylpropane-1,3-diol
WOC	water oxidising complex
X-gal	5-bromo-4-chloro-3-indolyl-*b*-D-galactoside

Chapter 1

Introduction

1.1 Photosynthesis

The process of photosynthesis has made possible the development of advanced life as we know it. Photosynthesis, was and is, responsible for the conversion of atmospheric carbon, in the form of the unusable gas, carbon dioxide, into an organic form which is more accessible. Oxygen, as a by-product from this process, was released into the atmosphere. Over millions of years it formed the ozone layer which protects life on earth from UV irradiation and also provides the terminal oxidant for the process of respiration.

The process of photosynthesis does not necessarily involve the evolution of oxygen and the early photosynthetic organisms did not carry out this part of the reaction. The basic characteristics of photosynthesis are the absorption of light energy by pigment molecules which pass the excitation on to a pair of chlorophyll (or bacteriochlorophyll) molecules. The reaction centre pigments enter an excited state which is a very strong reductant and charge separation occurs with an electron being passed to a primary electron acceptor. This step converts light energy into redox chemical energy. In order to prevent charge recombination, which results in energy being lost as heat, the electron is passed onto secondary acceptors. This causes spatial separation of the charge components and stabilises the system energetically. At this point in the process the primary donor is oxidised and the final acceptor is reduced. Before a further charge separation event can occur the redox components need to be returned to their initial state. There are two methods by which this can be achieved. A cyclic method e.g. used by purple sulphur bacteria, uses the reduced acceptor to donate an electron back to the donor via other components. This causes the formation of an electrochemical gradient across the membrane which is used to convert the energy of the system into a chemical bond of ATP. Another method of reducing the primary donor uses independent substrates as oxidant and reductant e.g. in higher plants water is oxidised by P680 leaving oxygen as a by product. The terminal electron acceptor is carbon dioxide but an intermediate acceptor NADPH stores the reducing power until it is used in the Calvin cycle. This non cyclic form of photosynthesis also creates an electrochemical gradient used to drive ATP synthesis. Under certain conditions plants also perform cyclic photophosphorylation which involves only PSI and does not lead to the production of NADPH .

The reaction centres which carry out this photochemistry all have a basic similarity. They are all formed by a dimer of proteins which co-ordinate the electron donors and acceptors in favourable orientations and environments required to make the photochemistry efficient. The ancestral organisms of today's photosynthetic cells contained a homodimer of identical proteins whereas most modern organisms utilise a heterodimer of two related proteins. During evolutionary time the gene for the reaction centre protein must have been duplicated and the two copies have subsequently diverged as mutations in each gene increased the efficiency of the process. Both proteins of the dimer co-ordinate redox components but electrons pass solely along one pathway through the reaction centre. In modern organisms two sets of redox components are still present but the divergence of the two genes has inactivated one branch of the pathway while increasing the efficiency of the other.

1.2 Types of Reaction Centre

All reaction centre complexes, with the exception of PSII, have a special pair of chlorophyll molecules as the electron donor, a chlorophyll based molecule as the primary acceptor and a quinone molecule as the secondary acceptor. The identity and characteristics of these, however, vary enormously between organisms. There are two basic examples of reaction centre, known as type I and type II, both of which are present in higher plants, algae and cyanobacteria but only one type has ever been found in a single anoxygenic photosynthetic bacterium. The better characterised example is the type II reaction centre e.g. found in purple sulphur bacteria and PSII (Blankenship 1992). The cofactors which carry out the photochemistry, in this type of reaction centre, are a special pair of chlorophyll molecules (bacteriochlorophyll in the bacteria) which donate an electron to a pheophytin molecule (a chlorophyll molecule without the Mg cofactor). This is followed by the next electron acceptors which are a pair of quinone molecules Qa and Qb. A non heme iron molecule is present between these two quinone molecules on an axis of symmetry through the reaction centre. The redox components are co-ordinated by a heterodimer composed of subunits L and M in purple sulphur bacteria and D1 and D2 in PSII. The protein sequences of these polypeptides are available but the similarity at sequence level between bacterial and plant protein is now low. Despite this, examination of

predicted structure shows that they all have five transmembrane helices and some residues from bacterial species are conserved in their plant counterparts.

The similarity seen between purple bacterial and plant PSII reaction centres has led many people to propose that D1 is descended from L and D2 from M, based on Qa association with D2 and M and Qb with D1 and L. However phylogenetic trees based on sequence data show that D1 and D2 are more closely related to each other than to L and M (Ruffle et al. 1992). A further analysis by Lockhart et al. (1996) points out that the compilation of alignments assumes that the open reading frame does not vary. They highlight one example of a frame shift mutation which has changed the sequence of D1 compared to D2. In light of this, it is very difficult to predict whether the gene duplication giving rise to a heterodimer occurred before or after divergence of the plant and bacterial reaction centres.

While the purple bacteria contain reaction centres which are related to PSII, they are unable to oxidise water. There are two addition features of PSII which are required for this activity. Firstly the midpoint potential of P680, which at more than 1V is almost double that of P870, is high enough to oxidise water and the complex has evolved a system for charge accumulation needed for the four-electron chemistry of water splitting. These two characteristics cannot conceivably have evolved simultaneously but are likely to have arisen as a result of a series of transitional forms. A scheme which could explain how the modern situation has developed is proposed by Blankenship & Hartman (1998). They suggest that hydrogen peroxide may have been a transitional electron donor which has since been replaced by water and that the Mn containing WOC may have evolved from a catalase enzyme. The oxidation of hydrogen peroxide has a low redox potential which is within the capabilities of anoxygenic photosynthetic bacteria. Mn catalase enzymes are known to contain a binuclear metal centre which is similar in structure to half of the Mn centre of the WOC. In summary the authors propose that a pre-existing catalase enzyme was able to donate electrons to the reaction centre and over time evolved to become the four manganese structure which is capable of extracting electrons from water.

The other characteristic required for water oxidation is the high redox potential of the special pair. The same authors propose a plausible scenario to explain how this may also have occurred. The difference in redox potential

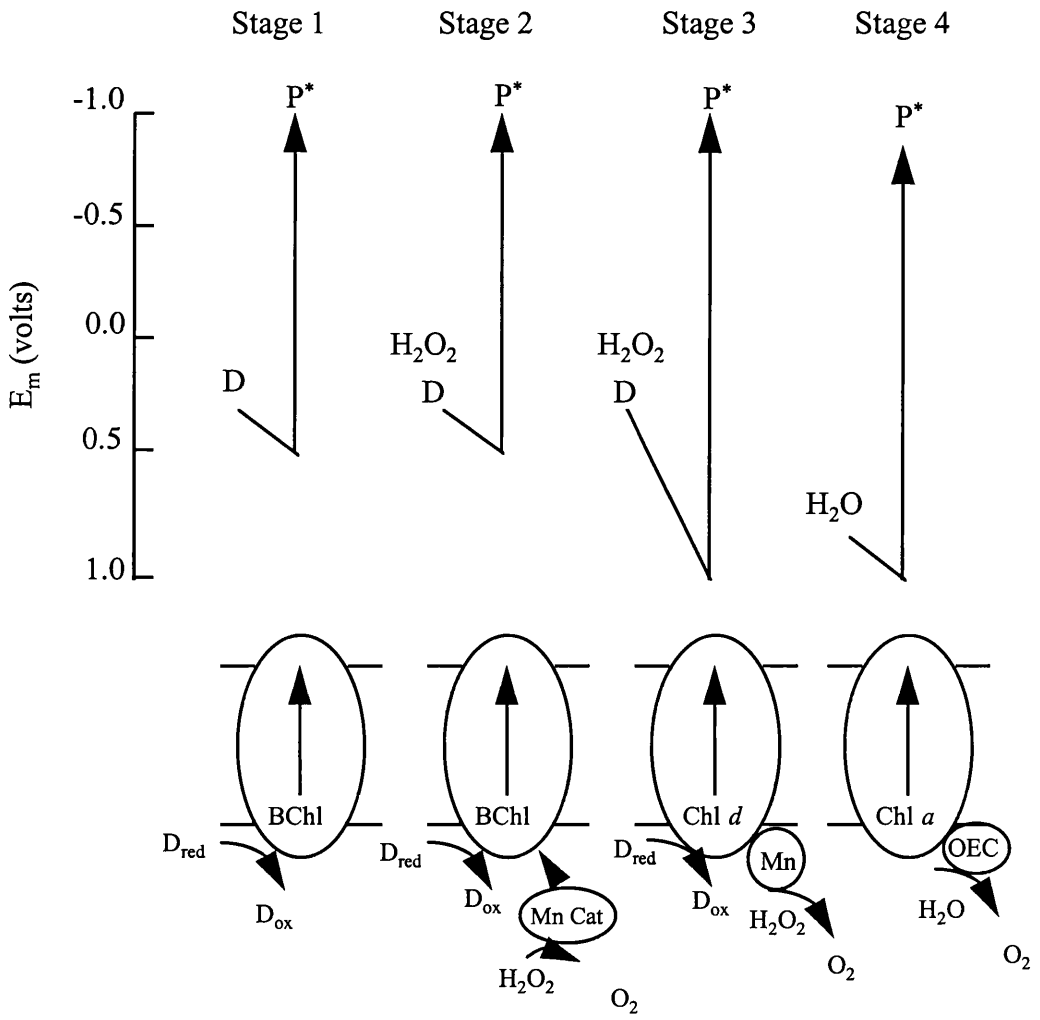
between PSII and purple bacterial centres lies in the fact that they contain different pigments. The bacterial organisms utilise bacteriochlorophylls as reaction centre pigments and these absorb longer wavelength light with less energy than the chlorophyll *a* found in PSII. This results in lower excited state energy and hence a smaller redox potential span in the bacteriochlorophyll containing organisms. The authors propose that an intermediate between chlorophyll *a* and the bacteriochlorophylls could have been chlorophyll *d*. This has the oxidised ring characteristic of chlorophyll *a* but also an acetyl side chain present in bacteriochlorophylls. Chlorophyll *d* has a slightly longer wavelength than chlorophyll *a* (716nm as opposed to 680nm) but has a redox potential capable of oxidising water. Figure 1.1 shows the scheme that is proposed which links all these steps together and forms a workable scheme for the evolution of a functioning water oxidising system from an anoxygenic complex.

The second type of reaction centre, type I, is typified by PSI and contains iron sulphur centres (Nitschke & Rutherford 1991, Golbeck 1993). The similarity of PSI to a bacterial counterpart was not confirmed as early as the relationship between the PSII and purple sulphur bacteria reaction centres. It is now recognised that green sulphur bacteria of the genus *Chlorobiaceae* and the gram positive heliobacterium both contain iron sulphur centres.

The redox cofactors of this type of reaction centre follow the ancestral characteristics with a special pair of chlorophylls followed by the primary acceptor A_o, a chlorophyll *a* molecule and the secondary acceptor, A_1, a quinone. After A_1 the electron passes to the first of the iron sulphur centres Fe-S_x. This is a rare example of a (4Fe-4S) cluster which is co-ordinated jointly by two polypeptides. This cofactor lies on a similar plane of symmetry to the PSII non heme iron but participates directly in the photochemistry by passing an electron to the other iron sulphur centres Fe-S_A and Fe-S_B.

The PSI cofactors are co-ordinated by a heterodimer of PSI-A and PSI-B which are 82 and 83kDa respectively. These are larger than the PSII proteins (39-40kDa) and have eleven transmembrane spanning helices. The *psa*A and *psa*B sequences are 45% identical, 55% if conservative replacements are considered which is consistent with the genes arising by duplication and subsequent divergence from an ancestral gene. The sequence of a green sulphur bacterial

Figure 1.1 Evolutionary stages of oxygen evolution capacity



This figure shows four possible stages of the evolution of the OEC but intermediates undoubtedly also existed. For each stage the upper diagram shows an energetic picture, and the lower diagram a schematic of the reaction centre protein. Stage 1 is equivalent to a purple bacterial system. The pigment is bacteriochlorophyll *a* and an external donor (D) reduces the special pair pigment (P). Stage 2 is equivalent to stage 1 but a Mn catalase is present and can donate electrons to P. In stage 3 the pigment has altered to chlorophyll *d* and the catalase enzyme has become associated with the reaction centre. Stage 4 shows a PSII centre containing chlorophyll *a* and a fully incorporated OEC. The figure has been reproduced from Blankenship and Hartman (1998).

reaction centre gene also gave a protein of predicted molecular mass of 82kDa with eleven transmembrane helices. There is however only one gene for a reaction centre protein so the cofactors in this organism must be co-ordinated by a homodimer. This fact provokes questions about secondary cofactors may be active in a homodimeric complex.

Higher plants, algae and cyanobacteria have both types of reaction centre present in a single cell and linked in series via the cytochrome b_6f complex. The simplest explanation for this situation is explained by Blankenship (1992). It is proposed that two organisms, each containing a single type of reaction centre, were involved in a fusion event giving rise to a cell with both photosystems present which were initially independent of each other. At a subsequent point in evolution the transfer chain must have altered to link the two systems.

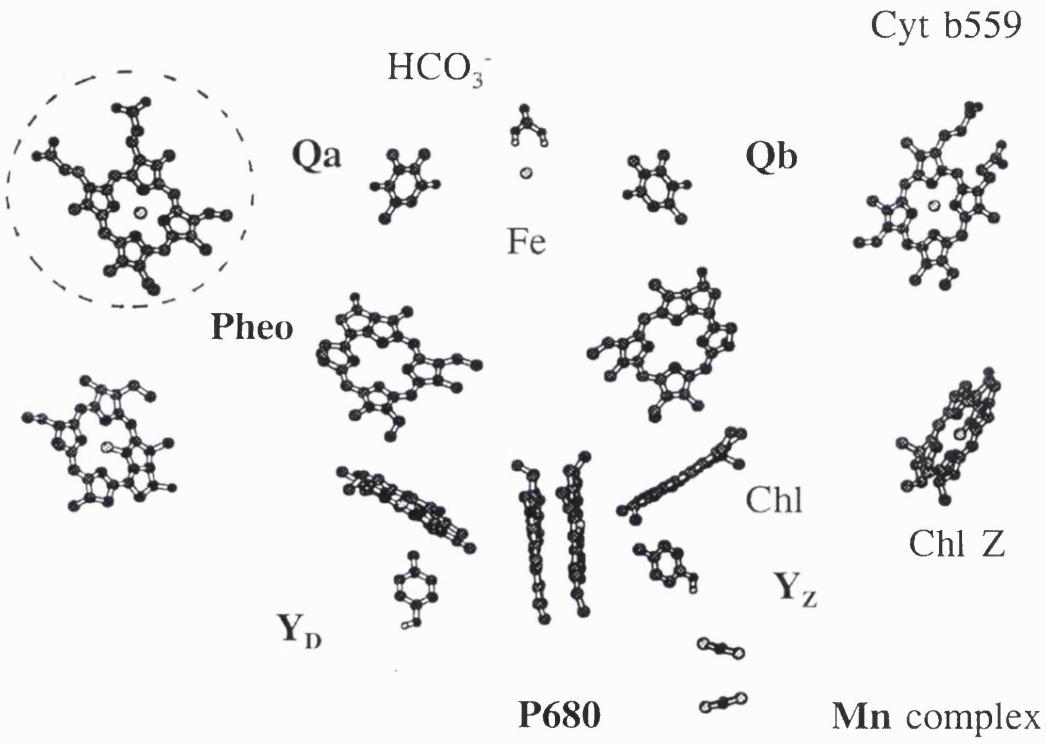
1.3 Photosystem Two

Higher plants, algae and cyanobacteria employ non cyclic photophosphorylation as a method of producing energy which is stored as ATP molecules and reducing power in the form of NADPH.This is achieved by light energy causing excitation of a chlorophyll dimer, P680, to a higher state, P680*, which is a strong reductant. Charge separation then occurs and an electron is rapidly passed through a series of electron acceptors, from pheophytin to Qa and Qb. Once Qb is doubly reduced and protonated it dissociates from PSII and is replaced by a plastoquinone molecule from the plastoquinone pool in the membrane. The other main feature of PSII is its ability to oxidise water and release O_2.

The donor side reactions (so called because they donate electrons to P680) occur at the water oxidising complex and involve an amino acid on the D1 backbone. The electrons required by $P680^+$ are extracted from water by the Mn containing water oxidising complex. Reduced $Y_Z^{tyrosine}$ acts as the direct donor to oxidised P680. The relative positions of the cofactors are shown in Figure 1.2. This figure was complied using available data and the molecular modelling of D1 and D2 (Ruffle et al. 1992).

The photosystem, which carries out the photochemistry is a large multi-subunit protein-chromophore complex composed of at least twenty different

Figure 1.2 Diagrammatic view of PSII cofactors



The view shown is in the plane of the membrane. The presumed P680 dimer is shown edge towards the viewer and it is assumed that a pair of accessory chlorophylls may be present either side of the dimer. Above these are two plastoquinones, Qa and Qb, either side of a ball representing the non-hem iron. Bicarbonate is shown bound at or near the non heme iron. Near the P680 dimer are the redox active tyrosine residues Y_D and Y_Z, with two Mn dimers close to Y_Z. Two hemes are shown for either one or two (circled) cytochrome b_{559}.
This figure was reproduced from Nugent (1997).

proteins and the five redox components. The electron carriers are all co-ordinated by a heterodimer of the two related 32kDa proteins, D1 and D2. The other protein subunits present include two antenna chlorophyll binding proteins, CP43 and CP47 and many smaller polypeptides the function of many of which is as yet unknown. Table 1.1 shows a summary of all the known PSII subunits and some basic information about each. Biochemical preparations have been devised which purify the complexes from thylakoid membranes to varying degrees. The smallest core complex capable of charge separation (Nanba & Satoh 1987) contains the D1 and D2 proteins, the α and β subunits of cytochrome b_{559} and two small polypeptides, one encoded for by the chloroplast genome PSII-I

1.4 The photochemistry of Photosystem II

1.4.1 P680

The primary donor of PSII, P680, is the first redox component of the light reactions of photosynthesis. Light energy from the antenna pigments causes P680 to enter an excited state which leads to the primary charge separation event within a few picoseconds. The precise nature of P680 is as yet unclear*. One important characteristic of P680 is its very high midpoint potential of approximately 1.1V. This is 370mV higher than the oxidation potential of chlorophyll *a in vitro* and provides the driving force required for the sequential oxidation of water. It is not known what factors are responsible for the high E_m value of $P680^+$/P680 couple. The number of chlorophyll molecules present in P680 is also a point of much debate. The chlorophyll special pair in the purple bacterial reaction centre is known to be a dimer. PSII shares many characteristics with the bacterial reaction centre including the similarity of D1/D2 and L/M heterodimers so it is possible that the special pair shares the same arrangement. Experimental evidence is available which reports the properties of P680 to be best described by either a chlorophyll monomer or a chlorophyll dimer (reviewed by van Gorkom & Schelvis 1993) but the general consensus on this matter appears to favour P680 as a weakly interacting chlorophyll dimer. Site directed mutagenesis has been used to try and address the issue of the high midpoint potential of $P680^+$/P680 and to provide insights into its organisation. Two residues, histidine 198 of both D1 and

* but could be described as a chlorophyll monomer, a weakly coupled dimer or a monomer

Table 1.1

Summary of the PSII genes and corresponding proteins

Gene	Location of gene	Protein	Mass (kDa)	Proposed Function
***psb*A**	C	D1	38.0 (S)	Binds redox cofactors
***psb*B**	C	CP47	56.3 (S)	Chl *a* binding inner antenna
***psb*C**	C	CP43	50.0 (S)	Chl *a* binding inner antenna
***psb*D**	C	D2	39.4 (S)	Binds redox cofactors
***psb*E**	C	α-subunit of cyt *b*559	9.3 (S)	Binds heme, photo protection?
***psb*F**	C	β-subunit of cyt *b*559	4.4 (S)	Binds heme, photo protection?
***psb*H**	C	PSII-H	7.7 (S)	phosphoprotein, PSII stability ?
***psb*I**	C	PSII-I	4.2 (S)	PSII stability ?
***psb*J**	C	PSII-J	4.1 (P)	unknown function
***psb*K**	C	PSII-K	4.3 (S)	PSII stability ?
***psb*L**	C	PSII-L	4.4 (S)	regulation of electron transfer
***psb*M**	C	PSII-M	3.8 (P)	unknown function
***psb*N**	C	PSII-N	4.7 (T)	unknown function
***psb*O**	N	33kDa extrinsic protein	26.5 (S)	Stabilisation of water oxidation
***psb*P**	N*	23kDa extrinsic protein	20.2 (S)	Stabilisation of water oxidation
***psb*Q**	N*	17kDa extrinsic protein	16.5 (S)	Stabilisation of water oxidation
***psb*S**	N	PSII-S	21.7 (S)	Chl *a/b* binding
***psb*T**	C	Ycf8 protein	3.8 (S)	optimal PSII activity
***psb*T$_n$**	N*	5-kDa protein	3.3 (S)	unknown function
***psb*U**	**	U-protein	~10 (Sy)	Stabilisation of water oxidation
***psb*V**	**	Cyt *c*550	15.1 (Sy)	Stabilisation of water oxidation
***psb*W**	N*	PSII-W	5.9 (S)	unknown function
***psb*X**	N	PSII-X	4.2 (S)	unknown function

Location of the gene.

C chloroplast encoded, N nuclear encoded, * found only in plants, ** found only in cyanobacteria

Molecular mass values given relate to :

S	Spinach
P	Pea
T	Tobacco
Sy	*Synechococcus* sp.

D2, (residue 197 in *C. reinhardtii*) provide ligands to P680. Mutagenesis of the D2 ligand to a tyrosine in *Synechocystis* sp PCC 6803 resulted in loss of ability to evolve oxygen and altered fluorescence characteristics (Vermaas et al. 1987). In contrast, the equivalent substitution in the purple bacterial system did not cause destabilisation of the complex but bacteriopheophytin replaced bacteriochlorophyll in the special pair.

1.4.2 Pheophytin

The primary electron acceptor of PSII is one of two pheophytin molecules bound to the heterodimer. This cofactor is reduced by the excited state of P680. Of the two pheophytin molecules present only the one associated with D1 (PheoI) participates in the electron transfer reactions. The position of the pheophytin molecule has been predicted by modelling experiments of Svensson et al. (1990) and Ruffle et al. (1992) to be homologous to that seen in the bacterial reaction centre.

1.4.3 Electron acceptors Qa and Qb

Both quinone acceptors Qa and Qb are molecules of plastoquinone-9 which are associated with the D1/D2 heterodimer. Qa is reduced by Pheo I^- 200-400ps after the initial charge separation event. Qa^- then passes the electron to the second quinone Qb. Although both quinones are identical molecules they act differently. In the course of normal electron transfer Qa accepts only a single electron while Qb becomes doubly reduced and protonated. In this state QbH_2 becomes dissociated from PSII and is replaced by a plastoquinone molecule. Under high light conditions Qa can also become doubly reduced. This is just one signal of photoinhibitory light conditions and initiates the changes in PSII which are associated with this state (Keren et al. 1995).

The region of D1 which binds Qb is known to be the site of action of a number of herbicides which inhibit electron transfer out of PSII. There have been many reports of herbicide resistant mutants, either spontaneous or chemically induced, which have been characterised (reviewed by Erickson & Rochaix 1992). The analysis of these mutants has shown a wide variety of effects. The alteration of residues in the 211-275 region of D1 can affect the Qa^- to Qb, Qa^- to Qb^- electron

transfer, neither or both. This implies that specific residues participate in different stages of Qb reduction.

Between the two quinone binding sites lies the non heme iron. This is not thought to participate directly in electron transfer through PSII. In purple bacteria the non heme iron can be removed, or replaced with other divalent cations and electron transfer between the two quinones can still be detected, although at a reduced rate (Debus et al. 1986). The ligands for non heme iron in purple bacteria include four histidines and one glutamate residue. The amino acid sequence of the PSII reaction centre proteins shows that the four histidines are conserved but the glutamate residue is missing. It has been proposed that the final ligand is provided by a bicarbonate ion in PSII (Nugent et al. 1988). Depletion of bicarbonate has been shown to slow electron transfer between the two quinones and is thought to be involved in the protonation of Qb^{-}. In addition to the effect bicarbonate has on electron transfer it is also important in photoinhibition and phosphorylation of PSII proteins.

1.4.4 Tyrosine residues D and Z

In addition to the co-ordinated cofactors of PSII, amino acid residues from proteins D1 and D2 are involved in the process of electron transfer. $P680^{+}$ is reduced by the intermediate donor Y_Z which is in turn reduced by the water oxidising complex. The speed of the oxidation of Y_Z by $P680^{+}$, which occurs on a nanosecond time scale, stabilises the acceptor side of PSII. If the reduction of $P680^{+}$ were slower, recombination events would be more likely to occur inhibiting forward electron transfer.

The radical Z can be detected by EPR and its spectrum is known as signal II_{fast}. This indicates the rapid decay of the signal seen in the dark. An alternative signal which is spectrally similar but dark stable, signal II_{slow} is formed by tyrosine D (Babcock et al. 1989). The identity of these electron donors was first supported by the work of Barry & Babcock in 1987 who showed that deuteration of tyrosines in cyanobacteria had a significant effect on the line shape of signal II_{slow}. Site directed mutagenesis in cyanobacteria then altered the tyrosine residues, which had been proposed as candidates, leading to the identification of D2- Y160 as Y_D

(Debus et al. 1988a, Vermaas et al. 1988) and D1- Y161 as Y_Z (Debus et al. 1988b, Metz et al. 1989).

While Y_D can be oxidised by $P680^+$ it does not participate directly in electron flow. This residue is thought to be involved in bringing the WOC to a stable state in the dark, see Section 1.4.5 below.

1.4.5 The water oxidising complex of PSII

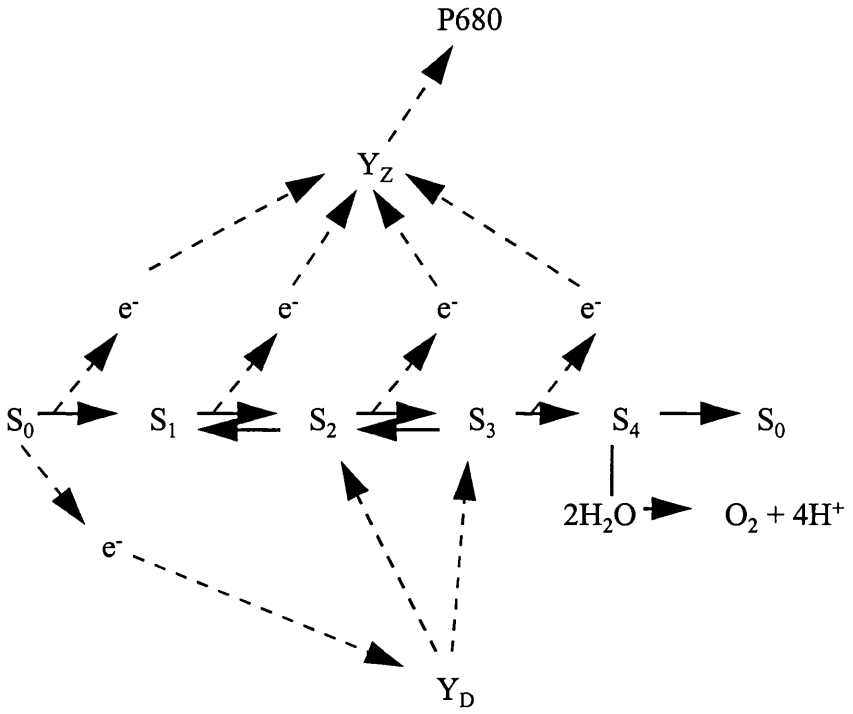
The oxidation of water, which results in the release of molecular oxygen and the reduction of Y_Z, involves a three subunit protein complex on the lumenal side of PSII. The proteins of this part of the PSII complex are discussed further in Section 1.7. They are involved in binding both Cl^- and Ca^{2+}, which are required for optimal rates of oxygen evolution, and in protecting the manganese atoms from the environment.

It is known that molecular oxygen is evolved in a regular fashion after every four turnovers of the PSII complex (Joliot & Joliot 1968). The water oxidation complex is able to store the four oxidising equivalents generated by PSII and use them to oxidise the two water molecules required for the evolution of one O_2 molecule. A model explaining a mechanism for this action was proposed by Kok et al. in 1970 and is shown in Figure 1.3. The S-states which Kok propose each store an additional oxidising equivalent produced by each turnover of the reaction centre until S_4 is reached. This state spontaneously decays to S_0 in less about 1ms with the concomitant release of oxygen. In the dark, S states S_2 and S_3 decay back to S_1 within minutes. The S_0 state is converted slowly to S_1 via oxidation by $Y_D^{\cdot}$. There are known to be four Mn atoms associated with the WOC and these are involved in storing the oxidising equivalents and the reaction with water.

1.5 PSII Subunits of the Reaction Centre Complex

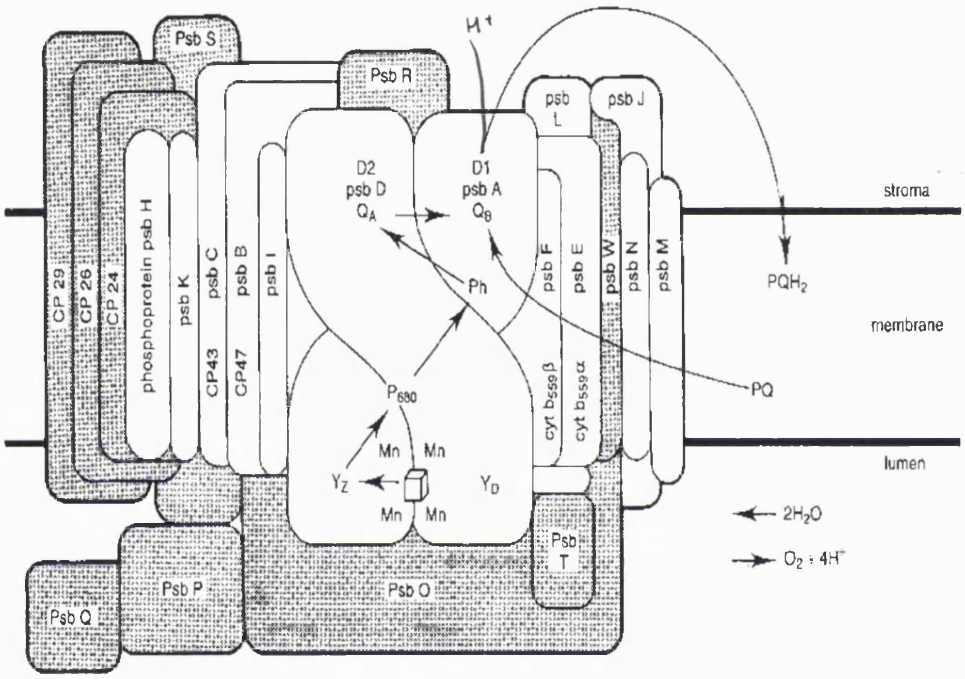
There are over twenty known subunits in the PSII complex. The proteins are located on genes in both the nucleus and the chloroplast and the function of many is as yet unknown. Figure 1.4 shows all the proteins known to be present in PSII in a schematic diagram. The chloroplast encoded subunits are shown in white

Figure 1.3 The S-states of the water oxidising complex



Electrons are removed sequentially by $P680^+$ via Y_Z. The S-state number indicates the number of oxidising equivalents stored. On reaching S_4, oxygen is released and the cycle reset. The dark stable state is S_1. This is reached by slow oxidation of S_0 by Y_D and reduction of Y_D by states S_2 and S_3. This figure is reproduced from Nugent (1997).

Figure 1.4 Subunit composition of PSII



The subunits encoded by chloroplast genes are shown in white while the nuclear encoded subunits are shaded. The precise location of the Mn complex with respect to the D1 and D2 subunits has yet to be established. The arrows indicate the direction of electron flow.
Figure reproduced from Bowyer and Leegood (1997).

while the nuclear proteins are shaded. This diagram shows the presence of the PSII-R protein which is not discussed here (Barber et al 1997).

1.5.1 D1 and D2

The two proteins which form the heterodimer which lies at the heart of the PSII complex are D1 and D2. The identification of D1 as a PSII component was made by comparison of polypeptide profiles between wild type and PSII minus thylakoids from *C. reinhardtii* (Chua & Bennoun 1975). Both proteins form a diffuse band of approximately 32kDa on an SDS-PAGE gel hence the names D1 (diffuse band 1) and D2. Further evidence linking D1 to PSII came from herbicide studies using diuron and atrazine which were shown to bind to this protein (Mattoo et al. 1981, Pfister et al. 1981). The gene encoding the D1 protein (*psb*A) was first isolated by Zurawski et al. (1982) from spinach and tobacco. The *C. reinhardtii* sequence was determined two years later by Erickson et al. (1984) and in addition to being one amino acid residue shorter than the higher plant protein the gene was shown to contain four introns. The sequence for the D2 gene, *psb*D, determined in *C. reinhardtii* by Rochaix et al. (1984) shows that the predicted protein sequences of D1 and D2 show 25% identity. Comparison of the sequences from a wide range of oxygenic organisms shows that the genes are highly conserved, 65% of the residues in D1 and 75% in D2 are the same (Svensson et al. 1991). In addition to their conservation and relationship to each other, it has been reported that D1 and D2 show a high degree of similarity to the purple bacterial reaction centre proteins L and M (Williams et al. 1984). Models of the PSII proteins (Svensson et al. 1990 & 1996, Ruffle et al. 1992) have been created and were based on the X-ray structures of the L and M purple bacterial reaction centre proteins. All four proteins share the same basic structure with five transmembrane spanning alpha helices and specific residues, which are believed to co-ordinate some of the co-factors, being conserved. The first evidence of D1 and D2 co-ordinating the cofactors involved in the primary charge separation event came when Nanba & Satoh (1987) isolated the reaction centre complex. They found this consisted of only D1, D2 cytochrome b_{559} and PSII-I and could demonstrate light dependent oxidation of P680 and reduction of pheophytin from this minimal complex. The

P680 chlorophyll *a* molecules which make up the special pair are assumed to be ligated by His 198 of D1 and D2 by analogy to the purple bacterial reaction centre.

The primary electron acceptor, pheophytin, appears to follow the bacterial model and is believed to be bound via a hydrogen bond to the D1 residue E130 (Moënne-Loccoz et al. 1989). The non heme iron molecule which is located between the quinone acceptors is bound by four histidine residues (H215 and H 272 of D1 and H215 and H269 of D2) with the fifth ligand being provided by a bicarbonate molecule instead of the glutamate residue which is involved in purple bacteria (Diner & Petrouleas 1990, Petrouleas et al. 1994). The quinone molecules bind on the loop region between these residues Qa associating with D2 and Qb with D1.

The donor side cofactors are also strongly associated with D1 and D2. The tyrosine residue Y_Z which donates electrons directly to $P680^+$ has been identified by site directed mutagenesis as Y161 of D1 and the corresponding residue on D2 Y_D has also been shown to display redox activity. The D1/D2 heterodimer is also expected to supply at least some of the ligands to the Mn cluster in the oxygen evolving complex. Site directed mutagenesis has identified residues E69 of D2 (Vermaas et al. 1990) and D170 of D1 (Boerner et al. 1992, Nixon & Diner 1992) as possible Mn ligands. The C-terminal portion of D1 is also expected to play a role in Mn ligation. It contains a number of conserved amino acid residues which could potentially provide ligands and correct C-terminal processing is required for Mn complex assembly.

Both D1 and D2 proteins undergo post translational modifications which produce the mature form of the protein. The N-terminal methionine is cleaved in both cases exposing a threonine at position two which becomes acetylated and can be reversibly phosphorylated (Michel et al. 1988). The phosphorylation of D1 has been linked to the process of photoinhibition of the PSII complex, see Section 1.11. Further internal modifications have been reported to occur via oxidation of specific residues of D1 and D2 (Sharma et al. 1997) particularly those which are positioned close to the electron carriers.

The D1 protein is also known to undergo modification of its C-terminal domain. As the precursor protein is translocated across the thylakoid membrane nine residues are removed from the C-terminus by a lumenal peptidase (Reisfeld et

al. 1982). The inability of certain mutants to carry out this modification results in the failure to assemble a functional water oxidising complex (Metz et al. 1980). The nuclear gene for the carboxyl-terminal processing protease of D1 has been cloned and sequenced and characterisation of the enzyme suggests that it does not belong to any known class of protease (Oelmüller et al. 1996).

1.5.2 Cytochrome b_{559}

Cytochrome b_{559} is present in reaction centre preparations together with D1, D2, PSII-I and PSII-W. There are two protein subunits which together with a co-ordinated heme make up the cytochrome. The α-subunit is encoded for by the *psb*E gene and the β-subunit by *psb*F. In all higher plants and *Synechocystis* both genes are transcribed together in an operon with *psb*L and *psb*J. In *C. reinhardtii* however the arrangement of the genes is altered with *psb*E and *psb*F separated by three other genes and transcribed in opposite orientations (Alizadeh et al. 1994, Mor et al. 1995). The products of these genes show a high degree of similarity to related sequences from other species. The *psb*E gene product in *C. reinhardtii* is predicted to contain 82 amino acids and the mature protein, formed by removal of the N-terminal methionine has a molecular mass of 9.2kDa. The PSII-E protein sequence contains one conserved histidine residue thought to be involved in the ligation of the heme group.

The *psb*F gene product is smaller with only 44 amino acids in *C. reinhardtii* although this is five more than seen in higher plants. The original sequence for the Chlamydomonas *psb*F gene (Fong & Surzycki 1992) predicted no conserved histidine to be present, although one had been seen in higher plants. However further sequence analysis by Alizadeh et al. (1994) and Mor et al. (1995) differed slightly from the original and showed a histidine to be present in the equivalent position to that seen in plants. The co-ordination of the heme has been investigated and it was shown to be ligated by two histidines (Babcock et al. 1985). To account for the physical data this group collected and sequence data published elsewhere they proposed that the heme crosslinks the two protein subunits of the cytochrome complex.

The *psb*E gene has been disrupted in *Synechocystis* sp PCC 6803 (Shukla et al. 1992) and mutants recovered showed no PSII activity. The proteins CP47,

CP43, 33kDa and D1 are present but at reduced amounts while no significant amount of D2 was seen. The authors propose that the presence of cytochrome b_{559} stabilises D2 in the membranes. Another mutational study of cytochrome b_{559} in *Synechocystis* sp PCC 6803 altered the two histidine residues from *psb*E and *psb*F to leucines (Pakrasi et al. 1991). The presence of single and double mutations resulted in the complete loss of PSII activity. The other subunits of the PSII core failed to accumulate and in the cells carrying the single mutation the *psb*E product appeared truncated. This effect may occur when a protease recognises the α-subunit to be in an incorrect conformation caused by lack of ligation to the heme group. A study which deleted the *psb*F gene also resulted in lack of autotrophic growth and failure to accumulate the core proteins (Pakrasi et al. 1990).

There are a number of issues surrounding cytochrome b_{559} about which there remains some doubt. There are conflicting reports relating to the stoichiometry of cytochrome b_{559} per reaction centre with researchers favouring either one or two per centre. The make up of the cytochrome complex also has not been resolved; if two cytochromes are present there could be two heterodimers (αβ) or two homodimers (αα and ββ). A study by McNamara et al. (1997) has created a fusion protein with the N-terminus of the β-subunit fused to the C-terminus of the α-protein. Cells carrying the αβ fusion protein were capable of photoautotrophic growth, heme was bound to the protein and it was incorporated into PSII. This data supports a model which has each heme co-ordinated by a homodimer of either α_2 or β_2. In addition the role of cytochrome b_{559} has never been positively confirmed. The main proposals all centre on this subunit being involved in protecting the reaction centre complex from photoinhibition, the process whereby reaction centres can be inhibited by light.

1.5.3 PSII-I

The PSII-I protein has been identified as being present in reaction centre preparations (Nanba & Satoh 1987) which means that it is located at the heart of the complex. Crosslinking studies have shown that this protein is in close proximity to the D2 protein and the α subunit of cytochrome b_{559} both of which are also present in the reaction centre (Tomo et al. 1993).

The PSII-I protein is predicted to have 37 amino acids which corresponds to 4.8kDa and is encoded for on a chloroplast located gene. The function of this protein is unknown but it is not believed to be associated with any cofactors. Deletion mutants in *C. reinhardtii* and *Synechocystis* sp PCC 6803 have been created to try and elucidate the role of PSII-I. In *C. reinhardtii* a region of DNA carrying the *psb*I gene was replaced by the *aad*A spectinomycin resistance cassette as a selection marker (Künstner et al. 1995). Analysis of these mutants showed that in the absence of PSII-I the PSII complex is partially inactivated. Oxygen evolution levels of between 10-20% of wild type rates were observed and fluorescence analysis showed a significantly altered pattern. This effect was due to low levels of D1 accumulation, 10-20% of that seen in a wild type sample. The mutant cells were capable of photoautotrophic growth but displayed increased photosensitivity and were unable to grow at 600μE m^{-2} s^{-1} on either TAP or HSM media. The absence of the I protein from the core of the PSII complex caused an increase in the instability of PSII but did not lead to its complete inactivation.

The cyanobacterial PSII-I deletion mutants created in *Synechocystis* sp PCC 6803 also showed a decrease in PSII activity but to a lesser extent than that seen in Chlamydomonas (Ikeuchi et al. 1995). They detected no reduction of accumulation in any other PSII polypeptide and saw a 25-30% loss in oxygen evolving activity. The increased photosensitivity seen in *C. reinhardtii* was also observed in this prokaryotic study and Ikeuchi and coworkers conclude that PSII-I is not essential for PSII function but optimises its activity.

1.5.4 PSII-W

The PSII-W subunit is a small (6.1kDa) hydrophobic protein which has been found to be present in reaction centre preparations (Irrgang et al. 1995). It was not initially identified as being a component of the reaction centre in the publication by Nanba & Satoh (1987) probably because it stains very weakly on SDS PAGE gels. It is thought to be the only nuclear encoded subunit located in the core of the complex.

The nuclear gene encodes a 137 amino acid precursor protein in spinach, (Lorkovic et al. 1995) 83 residues of which make up the transit peptide which endows the ability to transverse the chloroplast envelope and the thylakoid

membranes. The amino acid sequence of the transit peptide resembles that of a lumenal protein rather than one with a membrane location such as PSII-W. The purified protein shows no evidence of attachment of a chromophore (Irrgang et al. 1995). An examination of light induced proteolysis (Hagman et al. 1997) concluded that the PSII-W protein is degraded at a rate equivalent to that of the D1 protein but that this is not due to direct photodamage rather as a response to the general destabilisation of the photosystem. The exact role of this protein is unknown but it has been proposed that this nuclear encoded subunit could play a regulatory role in the assembly of PSII or provide docking domains for other central subunits.

1.6 Other Chloroplast Encoded Subunits of PSII

1.6.1 CP43 and CP47, the antenna chlorophyll-protein complex of PSII

CP43 and CP47 are chlorophyll binding proteins found in oxygen evolving PSII preparations but absent in reaction centre complexes. They are proposed to transfer excitation energy to the reaction centre primary donor chlorophyll P680. These two proteins are structurally related to each other, each having six membrane spanning regions and a large hydrophilic loop between helices 5 and 6 (Bricker 1990). The overall protein sequence homology between these proteins is small (< 20%) although the position of a number of histidine residues is maintained in both (Sayre & Wrobel-Boerner 1994). Histidines are candidates for providing ligands for the chlorophyll molecules bound by these two proteins. The conserved histidine residues are all located near the membrane surface on both the lumenal and stromal faces. The precise number of chlorophylls bound by each protein has not been determined but de Vitry et al. (1984) demonstrated that both CP43 and CP 47 bind between 20 and 25 chlorophylls per protein. Neither of these proteins contain sufficient histidine residues for this number of chlorophylls to be exclusively histidine liganded so either fewer pigment molecules are present or alternative residues also act as ligands. There are other examples of other residues acting as ligands e.g. LHCII.

In addition to playing a role in excitation energy distribution these two proteins have been linked to oxygen evolution. They are present in all oxygen evolving preparations. In particular, evidence exists which suggests that the 33kDa

protein of the oxygen evolving complex (or PSII-O) interacts with the large hydrophilic loop of CP47 e.g. an antibody raised to a region of this extrinsic domain fails to bind in the presence of the 33kDa WOC protein but after alkaline Tris washing, which removes this protein, the antibody could bind to its epitope (Frankel & Bricker 1989). The two proteins CP47 and PSII-O can be chemically cross linked (Odom & Bricker 1992) which implies that they associate closely.

Site directed mutagenesis studies in *Synechocystis* sp PCC 6803 have produced mutants with small deletions of 3-8 amino acid residues within the large hydrophilic loop of CP47 (Haag et al. 1993, Gleiter et al. 1994). The phenotypes of these mutants were varied. They discovered that the residues close to the hydrophobic membrane spanning regions were required for normal assembly of PSII. This could be due to either the deletion of specific residues or a more general structural effect. A particular region of this loop domain, E364-D440 has been inferred to be involved in the binding to the 33kDa protein (Odom & Bricker 1992). Mutations in this region did not result in a PSII-O minus phenotype but some mutants shared an increase in susceptibility to photoinhibition linked to the PSII-O deletion mutant. The two studies conclude that Mn and PSII-O are still bound to PSII in the mutants but that normal function is impaired. An additional *Synechocystis* sp PCC 6803 CP47 site directed mutant (Putnam-Evans & Bricker 1994) with the site directed change R448G exhibits an altered requirement for Cl^- ions and affects PSII assembly or stability in a chloride limiting environment.

The CP43 protein had been linked to a role in the stabilisation of Qa because biochemical extraction of this protein always caused a loss of Qa activity. Mutants which lack the *psb*C gene product have been shown to have reduced levels of CP47, D1 and D2 (Rochaix et al. 1989) and accumulated no PSII reaction centres. In other publications trace amounts of reaction centre have been reported in CP43 deletion mutants. One such study by Rögner et al. (1991) constructed and analysed CP43 site directed deletion mutants and found approximately 10% of the wild type level of PSII. They were able to show that the few complexes formed in the absence of CP43 were capable of light driven electron transfer to Qa with a quantum yield similar to a wild type sample.

1.6.2 PSII-H

The gene encoding the PSII-H protein is located in the *psb*B operon which in higher plants contains the genes *psb*B, *psb*T, *psb*H, *pet*B and *pet*D. In *C. reinhardtii* the cytochrome b_6f subunit genes are located elsewhere (Johnson & Schmidt (1993). The protein contains 88 amino acids in *C. reinhardtii* (only 73 in the higher plant protein) with a single predicted transmembrane span and is phosphorylated on a threonine at position two of the mature protein (Dedner et al. 1988).

Deletion mutants have been created to try and determine the function of this phosphoprotein within the PSII complex. In *C. reinhardtii,* deletion mutants are unable to grow photoautotrophically, do not evolve oxygen and fail to accumulate PSII core complex proteins (Summer et al. 1997, Ruffle et al. 1995 and this thesis). This implies that PSII-H is required as a structural component of the PSII complex. The role of the phosphorylatable threonine will be discussed in this thesis. Cyanobacterial deletion mutants of PSII-H, created in *Synechocystis* sp PCC 6803 (Mayes et al. 1993), show cells which are capable of autotrophic growth but have impaired Qa to Qb electron transfer. In cyanobacterial PSII it appears that the *psb*H gene product is not required for the assembly of a functional complex. The gene for *psb*H in *Synechocystis* does however lack part of the N-terminal sequence seen in higher plants including the phosphorylatable threonine. The possibility of a second residue becoming phosphorylated has been proposed (Race & Gounaris 1993) but this has never been confirmed or disproved.

1.6.3 PSII-J

The sequence for the *psb*J gene has been reported in liverwort (Ohyama et al. 1986), tobacco (Shinozaki et al. (1986), rice (Hiratsuka et al. 1989) and *Synechocystis* sp PCC 6803 (Kaneko et al. 1996). The gene has been sequenced in *C.reinhardtii* in our laboratory and the data has been entered onto the database (Accession no. AFO25877). In Synechocystis and higher plants the genes for the two subunits of the cytochrome b_{559} complex, *psb*E and *psb*F, are followed by two further open reading frames, coding for *psb*L and *psb*J (Cushman et al. 1988, Cantrell & Bryant 1988). The naming of the *psb*J gene was tentative because the protein had yet to be shown to be associated with PSII. In order to verify this, Lind & coworkers

(1993) created a PSII-J deletion mutant in *Synechocystis* sp PCC 6803 by introducing a premature stop codon into the coding sequence. Mutant cells were able to grow under autotrophic conditions but with a growth rate of only 45% that seen in wild type cells. Electron transfer rates through PSI were unaffected in this mutant but PSII mediated electron transfer was only 46% of that in a wild type sample. This confirmed that the gene named *psb*J encodes a subunit of PSII but does not give us enough information to determine a specific function for this subunit.

1.6.4 PSII-K

The mature PSII-K protein has 37 amino acid residues (approximately 4kDa) and has a high level of similarity, 80%, in the species sequenced to date. The first residue of the post translationally modified polypeptide appears to be a lysine in all situations and analysis of the protein sequence predicts a single transmembrane spanning domain. The presence of a cleavable presequence is conserved among species but the identity of this is varied. In higher plants the 24 amino acids prior to the lysine are cleaved but in *C. reinhardtii* and *Synechocystis* sp PCC 6803 there are less than half this number removed (Silk et al. 1990, Ikeuchi et al. 1991).

In higher plants the *psb*K gene is found between the tRNA gene *trn*Q and PSII gene *psb*I (Sexton et al. 1990). *Psb*K and *psb*I have been shown to be cotranscribed then the precursor RNA is cleaved to give the gene specific transcripts (Neuhaus & Link 1990). In *C. reinhardtii* the *psb*K gene, which is located far away from the *psb*I gene, is followed by ORF 46 but is transcribed on a monocistronic transcript (Takahashi et al. 1994).

The *psb*K gene has been disrupted in *C. reinhardtii* by insertion of the *aad*A cassette within the coding sequence (Takahashi et al. 1994). The mutant cells were unable to grow photoautotrophically and showed complete loss of PSII activity with respect to oxygen evolution and chlorophyll fluorescence measurements. The lack of active PSII centres was determined to be caused by instability of the complex rather than reduced synthesis of the major subunits.

A *psb*K disruption mutant has also been created in *Synechocystis* sp PCC 6803 (Ikeuchi et al. 1991) but analysis of this gave contrasting results to those seen

in *C. reinhardtii.* The mutant cells were capable of autotrophic growth although at a reduced rate and further analysis showed no significant alteration in PSII function including susceptibility to photoinhibition (Zhang et al. 1993). In Synechocystis the PSII-K polypeptide is not essential for PSII activity but is required for optimum function. Ikeuchi et al. (1991)

1.6.5 PSII-L

The gene for *psb*L is found in higher plants in an operon with *psb*E, F and J. In *C. reinhardtii* it is in the vicinity of similar genes but the arrangement differs. *Psb*E is in the opposite orientation to *psb*F and separated by three other genes and *psb*L follows the *psb*F sequence (Fong & Surzycki 1992). The PSII-L protein is 38 amino acids long in higher plants and consists of 46 amino acid residues in *C. reinhardtii.* It has a single predicted membrane spanning region and all species show a high degree of similarity (74-80%).

A deletion mutant has been created in cyanobacteria (Anbudurai & Pakrasi 1993) and the lack of the PSII-L protein caused an inability to grow autotrophically. It was concluded that the PSII-L protein was required for stable assembly of the PSII complex. Reconstitution experiments have been performed where purified reaction centre complexes were added to purified PSII-L and led to a restoration of Qa activity (Kitamura et al. 1994). An extension of this study (Ozawa et al. 1997) repeated the experiment with PSII-L, over expressed in *E. coli,* then purified and determined that PQ9 was able to rebind to the reaction centre complex in the presence or absence of PSII-L but its activity increased from 11 to 37% when the L protein was included in the reconstitution. This suggests that PSII-L acts in a way other than stabilising the quinone at the Qa site. Studies with mutated copies of PSII-L determined that the residues at the C-terminus were essential for the recovery of this activity. Assuming that PSII-L is inserted into the membrane in the typical manner i.e. with the N-terminus in the stroma, this places the highlighted residues nearer to the donor side of PSII. Additional work by the same group (Hoshida et al. 1997) led to the proposal that the *psb*L gene product is involved in the oxidation of Y_Z by $P680^+$.

1.6.6 PSII-M

The PSII-M protein has been shown to consist of 37 amino acid residues and the *C. reinhardtii* (Higgs et al. 1996) and tobacco sequences (Shinozaki et al. 1986) show 74% identity. There have been no reports of any deletion mutants made for this gene in either *C. reinhardtii* or *Synechocystis* sp PCC 6803, in which the gene has also been identified. No conclusion can be drawn as to what the role of this protein may be.

1.6.7 PSII-N

The gene for the PSII-N protein is located in the *psb*B gene cluster along with the genes for *psb*T and *psb*H. The *psb*N gene lies between *psb*T and *psb*H but on the opposite strand of DNA. The gene arrangement is conserved in higher plants and *C. reinhardtii* (Johnson & Schmidt 1993) but in the alga there are much larger intergenic spaces. The coding sequence for the gene predicts a 43 residue protein with a putative membrane spanning region. A disruption mutant created in *C. reinhardtii* will be discussed in Sections 3.1.5 and 3.4. *

1.6.8 PSII-T

The gene for this subunit is also located in the *psb*B gene cluster as mention above. It was previously known as *ycf*8 until Monod & coworkers determined its presence within PSII. In *C. reinhardtii* this 31 amino acid polypeptide has been sequenced by Monod et al. (1992) and Johnson & Schmidt (1993).

In order to determine the role this protein plays Monod et al. (1994) replaced the coding sequence with the *aad*A cassette. Analysis of this mutant strain revealed that under high light or high levels of the antibiotic spectinomycin, cell growth and PSII function were impaired. Under normal growth parameters the protein appears to be dispensable but is required for optimal PSII function under adverse conditions.

1.7 The Proteins of the Oxygen Evolving Complex

A range of nomenclature is seen for the three extrinsic polypeptides known to play a role in oxygen evolution. In many references they are known by their

* Mayes et al. (1993) created a *Synechocystis* PCC 6803 mutant which showed that a functional *psb*N gene is not essential for PSII activity.

spinach molecular weights of 33, 23 and 16 kDa although the exact figures given can vary. In *C. reinhardtii* they are known as oxygen evolving enhancer proteins (OEE) 1, 2 and 3, with 1 being the largest and 3 the smallest. The genes for these proteins have been named *psb*O, *psb*P and *psb*Q so the polypeptides can also be named PSII-O etc. In this section they will be referred to as the 33, 23 and 16 kDa polypeptides.

The genes for all three of the oxygen evolving complex proteins (reviewed Seidler 1996) are located in the nucleus. The proteins are translated on cytosolic ribosomes complete with a transit peptide composed of two domains. The first, the chloroplast targeting domain, ensures that the protein crosses the chloroplast envelope to the stroma while the second targets the protein across the thylakoid membrane. Examination of the second stage of this targeting procedure reveals a range of methods by which this can be brought about. The largest WOC protein, 33kDa, is imported via a mechanism which resembles the bacterial Sec-dependent pathway and has an absolute requirement for ATP and a stromal factor. The two smaller proteins enter the thylakoid system using a process dependent only on a proton gradient.

1.7.1 The 33kDa Oxygen Evolving Complex Protein

The mature 33kDa protein contains 241-247 residues, depending on species, and is highly conserved. It is targeted to the lumen of the thylakoids and a mutant in *C. reinhardtii* has shown that it is essential for oxygen evolution in eukaryotes (Mayfield et al. 1987a). A nuclear mutant with a large insertion in the 5` region of the gene has been characterised and in the absence of the protein complete deficiency in oxygen evolution is seen. Absence of the 33kDa protein does not affect accumulation of the other nuclear encoded WOC proteins but it has not been determined whether they are able to associate with the membranes. In contrast PSII core polypeptide accumulation is decreased to 10-15% of wild type levels. This effect was determined to be due to protein turnover rather than lack of expression.

The situation in *Synechocystis* sp PCC 6803 is different to that seen in Chlamydomonas and higher plants. This cyanobacterium has only the largest WOC protein, 33kDa, neither the 23 nor the 16kDa proteins are present. The

deletion of the *psb*O gene did not cause the loss of ability to grow autotrophically, although growth rates were slower, and the cells were still capable of evolving 60-70% of the wild type level of oxygen (Burnap & Sherman 1991). An additional study of *psb*O deletion mutants (Komenda & Barber 1995) showed that under high light conditions these cells were more susceptible to photoinhibition than wild type cells.

Another difference between plant and prokaryotic systems is the presence of a low potential cytochrome c_{550} and an additional 12kDa polypeptide in *Synechocystis* PCC 6803. These proteins are able to bind to the PSII complex in the absence of the 33kDa protein. This contrasts with the situation in the eukaryotic system which loses binding of all three WOC proteins when the 33kDa protein is absent (Mayfield et al. 1987a).

The extrinsic polypeptides were initially considered to be directly involved in water splitting since removal of the 23 and 16kDa proteins led to a partial inactivation of oxygen evolution (Åkelund et al. 1982) and removal of all three by alkaline tris-washing led to a total loss of activity and Mn release (Kuwabara & Murata 1982). The partial loss of activity on removal of the two smaller polypeptides was later determined to be due to loss of Ca^{2+} (Ghanotakis et al. 1984) and Cl^- ions (Andersson et al. 1984). In addition a method was developed which allowed the removal of the 33kDa protein without the loss of Mn (Miyao & Murata 1983). The activity seen in this preparation was much lower than that seen for intact PSII but its activity could be restored on rebinding of the 33kDa protein. This and other similar reports conclude that the 33kDa protein cannot provide a direct binding site for the Mn cluster. The absence does however allow greater access to the Mn cluster for external reductants so this protein plays a protective role.

Site directed mutagenesis studies in *Synechocystis* sp PCC 6803 have been carried out to determine the significance of some highly conserved residues (Burnap et al. 1994) which were proposed to influence the binding of the protein to PSII. The first residue altered, Asp9, lies in an N-terminal portion of the protein determined by Eaton-Rye & Murata (1989) to be important in binding to the reaction centre. The alteration of this residue to a lysine had no dramatic effect on

oxygen evolution causing Burnap and co-workers to assume that either this residue does not form a part of the linkage to the reaction centre or that it is not critical.

A second target for mutagenesis was one of a conserved pair of cysteine residues proposed to form an intramolecular disulphide bridge. The phenotype of this site directed mutant was very similar to the mutant lacking the 33kDa protein suggesting that the disulphide bond is required to hold the protein in a specific conformation which allows binding to the complex. The last residue altered was D159, a strictly conserved aspartate residue in a highly charged region. It has been considered to potentially be involved in binding to the reaction centre or Ca^{2+}. The change to D159N at this location lead to the accumulation of normal amounts of protein but negatively influences the rate and stability of oxygen evolution.

1.7.2 23kDa Subunit of the Oxygen Evolving Complex

The 23kDa protein which is part of the oxygen evolving complex is encoded for by the nuclear gene *psb*P. The mature protein (188 amino acids in *C. reinhardtii*) is found in the lumen after its transit across the chloroplast envelope and the thylakoid membrane. A naturally occurring mutant in *C. reinhardtii* was determined to lack the 23kDa protein (Mayfield et al. 1987b). These cells are still capable of photoautotrophic growth and oxygen evolution but with only rates equivalent to 5% of that seen in wild type cells. The loss of the 23kDa protein does not affect the accumulation or association of the other PSII polypeptides. In the absence of this 23kDa protein efficient rates of oxygen evolution are possible when exogenous chloride ions are included in the medium (Rova et al. 1994). Under conditions of low chloride ion concentration the mutant was highly susceptible to irreversible photoinhibition targeted mainly to the donor side of PSII.

Another effect caused by the lack of the 23kDa WOC protein was reported by Rova et al. (1996). Photoactivation is the process by which the Mn cluster is added to the pre-formed PSII complex when the cells are transferred from darkness into the light. The process was found to be impaired being both slower and less efficient than the process in the wild type sample. In addition to the level of activation, the mutant was highly dependent on the light intensity. The authors propose a scheme for photoactivation which has a requirement for Cl^- ions for an

efficient reaction to occur. The mutant is known to have a lower affinity for chloride and hence the activation is impaired.

1.7.3 16kDa Subunit of the Oxygen Evolving Complex

The third and smallest protein of the oxygen evolving complex is encoded for by the nuclear gene *psb*Q. Like the other two nuclear encoded subunits of this complex this protein is expressed with a bipartite transit sequence to ensure its import into the thylakoid lumen. The gene has been sequenced in *C. reinhardtii* by Mayfield et al. (1989) and the mature protein was shown to share only 28% similarity with higher plant sequences.

This subunit of the water oxidation complex is known to bind to PSII only when both the 33kDa and 23kDa proteins are already in position (Miyao & Murata 1989). It is known from reconstitution experiments to be required together with the other two subunits of the WOC for maximal rates of oxygen evolution in the absence of Cl^- and Ca^{2+} ions (Åkerlund et al. 1982).

1.7.4 Cytochrome c_{550} or PSII-V

The protein encoded for by the *psb*V gene was found to be involved in the oxygen evolution mechanism in cyanobacteria in 1994 when Nishiyama & coworkers washed thylakoid membranes with 0.1% Triton X-100 and destabilised the oxygen evolving machinery. They went on to purify a protein which could restore the activity in reconstitution experiments and were able to clone the gene. The mature protein has 136 amino acid residues and a cleaved transit peptide which enables it to cross into the thylakoid lumen. The protein shows characteristics of a *c*-type cytochrome and has been identified as a low potential cytochrome c_{550}.

The function of a protein is often determined by deleting the gene encoding it and this has been done for cytochrome c_{550} in *Synechocystis* sp PCC 6803 (Shen et al. 1995a). The resulting *psb*V deletion mutants were capable of photoautotrophic growth at a reduced rate and showed a 40% drop in the rate of oxygen evolution. An extension of this deletion study created a mutant lacking both the *psb*O and *psb*V gene products (Shen et al. 1995b). The double deletion mutant was unable to grow on unsupplemented media and showed oxygen

evolution of less than 10% while both single deletion mutants were capable of photosynthetic growth and a higher rate of oxygen evolution. The conclusion from this work was that both PSII-O and PSII-V are capable of supporting oxygen evolution in the absence of the other but that both are required for optimal activity.

1.7.5 PSII-U

The PSII-U protein is approximately 12kDa, as estimated from SDS PAGE gels, and the sequence is mainly hydrophilic. The DNA sequence of the cloned genes from *Synechocystis* sp PCC 6803 (Shen et al. 1997) and *Synechococcus* sp PCC 7002 (Nishiyama et al. 1997) show the protein is expressed with a transit sequence which is cleaved as the protein crosses the thylakoid membrane to give a mature size of 95 and 96 residues respectively.

This protein is known to act to protect the oxygen evolving machinery, which corresponds to its lumenal location. Reconstitution experiments with thylakoids depleted of cytochrome c_{550} and PSII-U (Nishiyama et al. 1997) show that both subunits act independently to stabilise the complex against heat but both are required for optimal thermal stability. PSII-U deletion mutants in *Synechocystis* sp PCC 6803 (Shen et al. 1997) show a small decrease in oxygen evolution and are capable of autotrophic growth. In the absence of Ca^{2+} or Cl^- however the mutants performed less well implicating the 12kDa protein in the role of maintaining the optimum Ca^{2+}/Cl^- environment.

1.8 Other Nuclear Encoded Subunits of Photosystem II

1.8.1 PSII-S

The 22kDa protein encoded by the *psb*S gene has been found to be associated with PSII in all plants examined . It has yet to be reported in *C. reinhardtii*. The protein, being from a nuclear encoded gene, is synthesised as a 274 amino acid precursor in spinach (Kim et al. 1992, Wedel et al. 1992) which is cleaved after import to give a mature protein of 200 residues.

Sequence comparison has revealed that the PSII-S protein shows distant similarity to the chlorophyll binding proteins CP27 and CP29 (Wedel et al. 1992). In addition to this, the four transmembrane helix protein shows internal similarity

indicating that at some point in the past the sequence for the first two helices has been duplicated. It has been demonstrated (Funk et al. 1995) that the PSII-S protein binds 4-5 chlorophyll molecules, as might be predicted from its similarity to light harvesting proteins, but is stable in their absence. Each PSII reaction centre appears to be associated with two PSII-S proteins.

Further investigation into *psb*S expression in spinach seedlings (Adamska et al. 1996) has shown that the level of *psb*S mRNA increases with progressing etiolation and is controlled by a number of mechanisms. Initially transcriptional activity regulates the amount of mRNA present and an extended period of darkness resulted in post transcriptional control. The exposure of the seedlings to light also increases the level of the *psb*S transcript as gene expression is positively regulated by phytochrome. The observations of Adamska and coworkers (1996) lead to the hypothesis that PSII-S binds chlorophyll molecules before they are assembled into the active photosynthetic complexes to avoid any negative effects of unbound chlorophyll molecules.

1.8.2 PSII-T

A second gene has been named *psb*T but this one is nuclear rather than chloroplast encoded. This gene has been cloned and sequenced from cotton (Kapazoglou et al. 1995) and the predicted sequence is homologous to that of a PSII-T protein partially sequenced in spinach. The cotton gene encodes a 105 amino acid protein with a molecular mass of 11kDa and examination of the sequence reveals potential stroma and thylakoid targeting domains. Import studies with radiolabelled PSII-T precursor show that this results in a 3kDa lumenal protein in cotton (Kapazoglou et al. 1995).

1.8.3 PSII-X

The nuclear gene for the PSII-X protein has been recently cloned and sequenced in *Arabidopsis thaliana* (Kim et al. 1996). The gene for this small, 4.1kDa, highly hydrophobic protein has also been sequenced in the eukaryotic alga *Odontella sinensis* (Kowallik et al. 1995) but it was in this case found to be present in the chloroplast genome. This suggests that the relocation to the nuclear genome is a recent event in evolutionary terms.

One consequence of the movement of the gene to the nucleus is the requirement for a chloroplast targeting sequence. DNA sequence of the arabidopsis cDNA has located a presequence typical of a lumenal protein although hydrophobicity analysis strongly predicts PSII-X to have a membrane location. Studies of the import of the PSII-X precursor (Kim et al. 1996) have found that this process is not affected by sodium azide or nigericin, inhibitors of the Sec related and ΔpH related pathways respectively. This protein must therefore be imported by an independent mechanism.

1.9 Photoinhibition

Light is one of the main substrates of PSII but it is also the cause of constant damage to the complex. The presence of oxygen and highly reactive radicals results in the continuous damage in particular to the D1 protein. D1 has been shown to be rapidly turned over within PSII and under low light conditions the rate of damage is exceeded by the rate of repair allowing the complex to function at full capacity. The complex is partially disassembled and the damaged protein is degraded while the other subunits are recycled when the complex is reassembled. As light intensity increases the rate of damage reaches a point where it exceeds the rate of repair and inhibition of PSII activity results. This process of damage induced loss of activity is known as photoinhibition (reviewed Anderson et al. 1997).

Damage to the photosystem is proposed to occur via two different mechanisms. One consequence of increased light stimulation can be the over reduction of the quinone acceptors. The first quinone, Qa, may become doubly reduced and protonated and then dissociate from its binding site (Styring et al. 1990, Vass et al. 1992). This increases the likelihood of a back reaction occurring between pheophytin and P680 which results in the formation of the P680 triplet state. This is proposed to interact with oxygen generating the highly reactive singlet oxygen (Telfer et al. 1990) which causes damage to the complex.

An alternative consequence of high levels of light stimulation occurs via the donor side of PSII. The chloroplast lumen becomes acidified as the rate of electron transfer results in increased proton translocation. This causes Ca^{2+} to be released from the oxygen evolving complex, which in turn down regulates PSII

activity and results in the formation of Yz^+ and $P680^+$ radicals which cause oxidative damage. Any situation which causes a prolonged formation of these radicals can lead to damage, it is not limited to high light conditions (Keren et al. 1997)

The D1 product has a specific role in the turnover of the PSII complex which is required to rectify the damage caused by light. This protein is phosphorylated in a light dependent manner (Callahan et al. 1990) and it was hypothesised that phosphorylation may mark damaged D1 for degradation. More recently it has become apparent that phosphorylation of D1 is not induced by damage of D1 and that the phosphorylated form of this protein is more stable (Ebbert & Godde 1994). Rintamäki et al. (1995) postulated that phosphorylation of this protein retards the degradation of damaged D1 until replacement is possible hence preventing the complex from entering total disassembly.

Many deletion and site directed mutants of subunits of PSII have phenotypes which show increased susceptibility to photoinhibition. This does not mean that they are all involved with the light induced loss of PSII activity as described above. Rather some of these mutants contain altered PSII complexes which are not as competent as wild type centres so the chances of photoinhibitory damage occurring is increased.

1.10 State Transitions

The two photosystems (PSI and PSII) are optimally activated at differing wavelengths of light. When conditions provide light favouring one photosystem over the other an adaptation occurs resulting in the redistribution of absorbed excitation energy to the light limited system. Therefore when PSII is over stimulated more absorbed energy is directed to PSI and vice versa. The adaptation to PSI light is termed state 1 and adaptation to PSII light is referred to as state 2 hence the change from one to the other is known as a state transition. This process occurs over a time scale of minutes and leads to an increase in the yield of oxygen evolution and a change in fluorescence emission.

A long term adaptation is also known to occur in which the relative quantities of PSI and PSII are altered to compensate for unequal utilisation of light energy. This affects the levels of gene expression within the cell and leads to the

production of more of the limited photosystem. Using the long term stoichiometric alterations and the finely tuned short term state transitions the distribution of excitation energy can be balanced between the two photosystems and rapidly altered as conditions change to keep the system working most effectively.

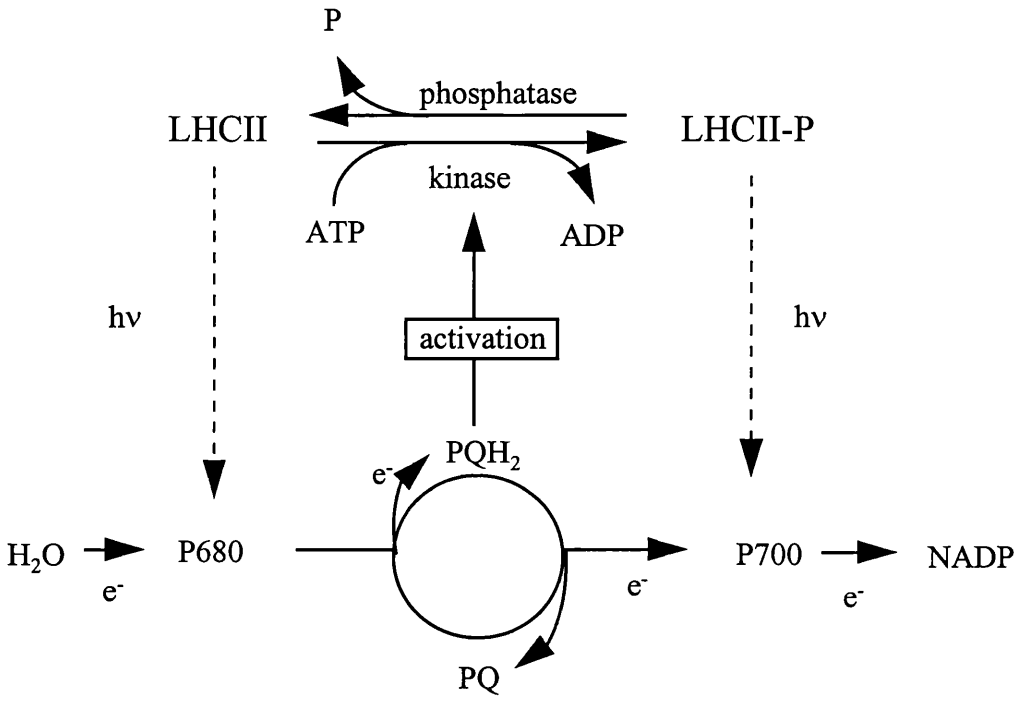
An imbalance in the stimulation of the photosystems leads to the intermediate between them, PQ, becoming relatively reduced or oxidised depending on the conditions. The PQ pool is said to act as the redox sensor for the system. In PSII activating light the PQ pool becomes reduced which causes the activation of a kinase responsible for phosphorylating LHCII, Figure 1.5.

Original work by Bennett (1979) showed that the kinase which phosphorylates LHCII is light dependent and membrane bound. Initial hypotheses assumed that light was required for the conversion of ^{32}P orthophosphate into labelled ATP. However further studies (Bennett 1979), showed that the addition of [^{32}P]-ATP did not diminish the light requirement and that protein phosphorylation could be blocked by DCMU, an electron transfer inhibitor (Bennett 1979). This suggests that electron transfer was required to activate the kinase rather than a mechanism involving direct excitation by light. Further support for this theory came when it was discovered that reducing agents e.g. dithionite (Allen et al. 1981) and reduced ferredoxin (Bennett 1979a) could activate the kinase.

The plastoquinone pool was suggested as the redox sensor of the kinase. DCMU acts at the Qb site of PSII preventing its reduction and both dithionite and reduced ferredoxin are known to reduce plastoquinone. In addition redox titrations of kinase activation (Horton et al. 1981) most closely match the redox titrations of the plastoquinone pool and the n value of approximately 2 requires a double electron carrier like plastoquinone. An inhibitor, DBMIB, which blocks electron flow from reduced plastoquinone to the cytochrome b_6f complex does not inhibit phosphorylation (Allen et al. 1981, Coughlan 1988) which tells us that the reduction of electron carriers after plastoquinone is not required for activation.

The activation of the LHC kinase by the reduced plastoquinone pool is known to involve the cytochrome b_6f complex. Evidence is available from mutants deficient in cytochrome b_6f, which are unable to phosphorylate LHCII, (Wollman & Lemaire 1988, Gal et al. 1987) and from inhibitor studies. DNP-INT blocks electron flow into the cytochrome complex and inhibits LHC

Figure 1.5 A scheme for the control of the LHCII protein kinase by the redox state of plastoquinone



An imbalance of excitation between the two photosystems in favour of PSII results in increased reduction of PQ which leads to the activation of the LHCII kinase. Phospho-LHCII decouples from PSII and transfers energy to PSI. This will tend to oxidise PQH_2, inactivate the kinase and allow the LHCII phosphatase to predominate.
Reproduced from Allen (1992).

phosphorylation (Bennett et al. 1988). HQNO stops electron flow leaving cytochrome b_6f but does not affect LHC phosphorylation.

Activation and deactivation rates of the LHC kinase are not compatible with direct interaction with the changing redox states of the PQ pool. Reduction of the pool occurs within seconds and activation of the kinase has been reported to take 2-5 minutes while the re-oxidation of the pool takes 1-2 minutes in the dark. The intermediary stage has been proposed to be the plastoquinol mediated interaction between cytochrome b_6f and the kinase (Gal et al. 1990, Frid et al. 1992). Frid et al. (1992) propose that the rate limiting step of activation occurs while the reduced b_6f complex has to associate with the kinase. Deactivation is proposed to require the oxidation of the kinase or the occupation of a quinone binding site causing kinase deactivation then separation. A recent report by Vener et al. (1997) provides additional evidence for the requirement of cytochrome b_6f in the activation of the kinase. They proposed that when the cytochrome complex has its high potential path components completely reduced and a plastoquinol molecule in the Q_o site, it forms an activator state. This occurs when the plastoquinone pool is reduced by PSII faster than it can be oxidised by cytochrome *f*. Under conditions which prevent re-reduction of the PQ pool, a single turnover flash can cause the withdrawal of a single electron from the plastoquinol at Q_o which inactivates the kinase.

1.11 Photosystem Two Kinases

The first kinase purified with the ability to phosphorylate LHCII was reported by Coughlan & Hind (1986). This protein has an apparent molecular mass of 64kDa and the N terminal sequence was published in 1992 (Gal et al. 1992). More recently, using the technique of liquid phase isoelectric focusing, this 64kDa protein has been separated from the kinase activity (Race et al. 1995). The gene for the 64 kDa protein has been cloned and sequenced. Its deduced protein sequence shows similarity to a group of plant polyphenol oxidases. In the same paper two additional proteins capable of phosphorylation are identified. In 1996 Race & Hind described a 58kDa protein kinase which co-purifies with the core complex of PSII. This kinase is capable of phosphorylating both PSII and LHCII. The report does not rule out the possible existence of other kinase proteins.

There has been much debate over whether there is a single or multiple kinases phosphorylating LHCII and the PSII phosphoproteins. Evidence supporting a single kinase includes the inhibition of both LHCII and PSII protein phosphorylation by DBMIB (Larsson et al. 1983) and antibodies raised against the 64 kDa protein also inhibit both (Coughlan & Hind 1987). However if all kinases involved in the process, or one early in a cascade of events, conforms to these facts then this evidence does not preclude the presence of more than one kinase. The adenosine affinity inhibitor 5`-p-fluoro-sulfonylbenzoyladenosine inhibits 95% of LHCII phosphorylation but only 35% inhibition of 10kDa phosphorylation is seen (Farchaus et al. 1985). The phosphorylation site of LHCII differs from the other substrates i.e. LHCII is phosphorylated on threonine 6 or threonine/serine 4 with basic residues on either side while the PSII polypeptides are phosphorylated on a threonine residue number two with a neutral residue at position one. Also the phosphorylation of LHCII requires an active cytochrome b_6f complex, unlike PSII proteins. Evidence to support this has been gained from inhibitor studies and a maize cytochrome b_6f deficient mutant (Bennett et al. 1988).

1.12 Thylakoid Protein Phosphatases

Historically there has been less interest in the LHCII and PSII phosphatases than kinases. The initial demonstration of thylakoid LHCII phosphatase activity was reported by Bennett (1979b). The rates of dephosphorylation vary widely between the known phosphoproteins but fall broadly into a fast category (LHCII, 27kDa and 25kDa subunits and an unidentified 12kDa protein) and a much slower group (Carlberg & Andersson 1996, Cheng et al. 1995). The half times of dephosphorylation start at seven minutes for phosphorylated LHCII and rise to over 180 minutes for PSII-H (Cheng et al. 1994).

Initial experiments carried out on thylakoid membranes found the phosphatase activity to be independent of light (Bennett 1980) and redox activity Silverstein et al. 1993). *In vivo* experiments using *Spirodela* plants (Elich et al. 1993) report that phosphatase activity is stimulated by light. All evidence agrees that the phosphatase activity is not affected by the presence of okadaic acid, a phosphatase inhibitor, but is inhibited by 20mM NaF.

No phosphatase proteins have yet been purified but there is evidence that there are at least two enzymes involved. Phosphatase activity has been located in both a stromal fraction and within the thylakoid membranes (Hammer et al. 1995). Both activities were inhibited by NaF and EDTA but differed in their response to molybdate ions and pH optima. The report of Carlberg & Andersson (1996) proposes that different enzymes catalyse the dephosphorylation of the fast group including LHCII and the slower class of PSII proteins. The two groups also show different sensitivities to light intensity with the LHCII and 12kDa protein reaching a maximum level of phosphorylation at 125μE/m^2/s then a decrease in phosphorylation until at 2500μE/m^2/s it is only 25% of its maximum. When NaF is added to the experiment the level of phosphorylation falls to only 40% of its maximum showing that dephosphorylation is stimulated at higher light intensity. In comparison the PSII family of phosphoproteins are maximally phosphorylated at 500μE/m^2/s and the level does not decline as light intensity increases. Addition of NaF does not have the same effect as just discussed (Carlberg & Andersson 1996).

There must also be similarities between the two proposed phosphatases because one synthetic phospho-polypeptide, which is an analogue of the N terminal segment of phosphorylated LHCII, inhibits the dephosphorylation of all phosphoproteins. This means that the features determining recognition of the substrate by the enzyme must have some common characteristics (Cheng et al. 1994). A factor shown to stimulate the dephosphorylation of D1 and other PSII polypeptides was far red (720nm) light. This is absorbed preferentially by PSI and the authors (Elich et al. 1993) propose that PSI excitation can regulate PSII core protein dephosphorylation.

1.13 *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii is a unicellular, eukaryotic, biflagellate green alga isolated from freshwater. It is used as a model system for studying many areas including photosynthesis, flagellar function and biosynthesis, phototaxis, metabolism of carbon, sulphur and nitrogen and cell wall synthesis. It has many characteristics which make it particularly amenable for genetic research in these areas which will be highlighted in the following section.

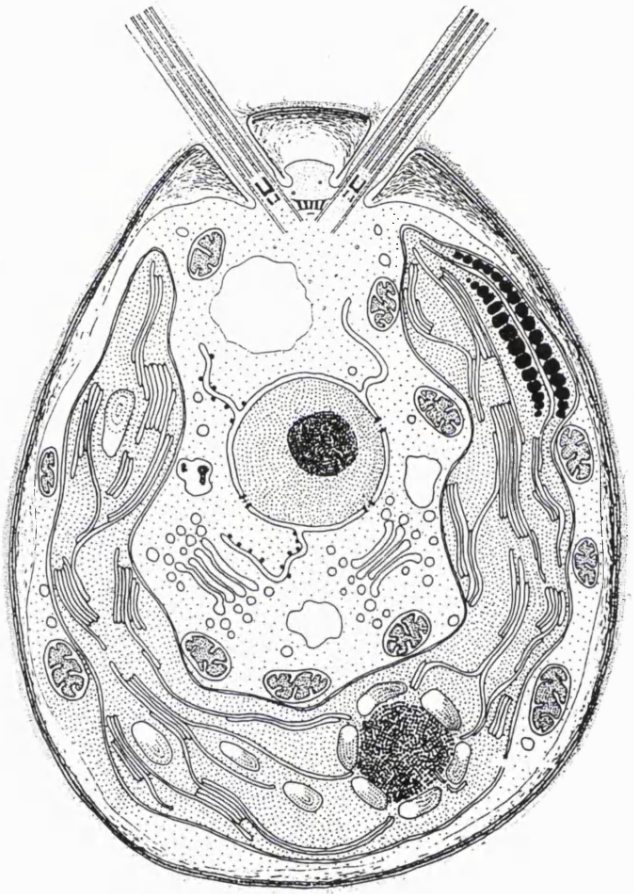
C. reinhardtii cells are typically 10μm in length and 3μm in width and have two flagella at their anterior pole. Figure 1.6 shows a diagram of a single *C. reinhardtii* cell. They possess a single large cup shaped chloroplast which occupies approximately 40% of the cell volume. Along with the typical eukaryotic organelles *C. reinhardtii* also possess an eye spot which consists of stacks of carotenoid-containing lipid granules which focus the incoming light onto the photoreceptor located in the plasma membrane. The ability to perceive light results in phototaxis towards an optimum light intensity and in response to a sudden increase in intensity *Chlamydomonas* cells move backwards away from the light.

One important feature for photosynthetic studies in *C. reinhardtii* is the flexibility it shows in relation to its growth conditions. This cell is able to grow photoautotrophically in the light using CO_2 as a sole carbon source, mixotrophically in the light with acetate present or heterotrophically in the dark using acetate as a sole carbon source. The ability to dispense with photosynthesis allows the creation and propagation of mutants with defective photosynthetic apparatus.

C. reinhardtii can exist as haploid cells which are designated either mating type + or mating type - and are propagated vegetatively. A cycle of sexual reproduction can be initiated by placing the cells under nitrogen starvation which promotes the formation of gametes. When opposing mating type gametes are bought together a series of reactions are triggered which results in zygote formation. Induction of meiosis in the zygote leads to the production of a tetrad of haploid progeny, two mating type + and two mating type -. This cycle of sexual reproduction is often exploited to determine which genome a particular mutation is located in or to study linkage between genes.

C. reinhardtii has three autonomous genetic systems located in the nucleus, the chloroplast and the mitochondria. A mutation in the chloroplast genome can be distinguished from a nuclear based lesion because the genetic systems are inherited in a different manner. Inheritance of the nuclear genome is based on Mendelian rules whereas the organellar genetic material is inherited uniparentally, chloroplast genomes are transmitted through the mating type + parent and mitochondrial DNA is received from the mating type - parent.

Figure 1.6 ***Chlamydomonas reinhardtii***



The figure shows a *C.reinhardtii* cell with its two bipolar flagella. The cell has a single, large cup shaped chloroplast which contains all the thylakoid membranes. The nucleus is the large organelle shown in the centre of the cell.

The chloroplast genome in *C. reinhardtii* has yet to be completely sequenced although a large proportion of this work has been done. There are approximately 80 copies of the 196kb circular genome in the single chloroplast. The genes which have been located in this organelle can be characterised into one of three groupings. Over 40 are involved in plastid gene expression including subunits for RNA polymerase, transfer RNAs and ribosomal RNA and protein. Many more encode proteins which are subunits of the photosynthetic complexes and finally there are conserved open reading frames whose function is largely unknown. Although the genes for the core subunits of PSII are chloroplast encoded, with the exception of PSII-W, many more are located in the nuclear genome.

The nuclear genome has been estimated to contain 7-10 x 10^5 kbp. One major difference between the chloroplast and nuclear DNA is the codons used to signify the amino acids which make up the proteins. In the nucleus the third position of a given codon is most likely to be a guanine or a cytosine which reflects the generally higher GC content of nuclear DNA. This trend is particularly apparent in highly expressed genes. Chloroplast DNA is generally more AT rich and as a consequence is less dense.

To become a useful model organism it must be possible to transform the genetic material. The last ten years have seen the introduction of reliable, consistent techniques for both chloroplast and nuclear transformation systems. The first obstacle to be overcome is the delivery of the exogenous DNA into the organelle to be transformed. A number of techniques have been successfully used to introduce DNA into *C. reinhardtii* including microparticle bombardment (Boynton et al. 1988), electroporation (Brown et al. 1991) and agitation with glass beads (Kindle 1990) or silicon carbide whiskers (Dunahay 1993). The most frequently chosen delivery mechanism for nuclear transformation uses the glass bead method. This is only suitable for cell wall deficient strains or after removal of the cell wall but results in a high transformation rate of approaching 1 in $1x10^4$ cells treated (Kindle 1990, Purton & Rochaix 1995). An alternative strategy, microparticle bombardment, is employed for the introduction of exogenous DNA into the chloroplast. The cells are bombarded with DNA coated tungsten or gold particles using a particle gun with either a gun powder charge or a helium driven system. Once inside the chloroplast the incoming DNA recombines homologously

with the equivalent wild type sequence. In 1993 Randolph-Anderson and coworkers demonstrated transformation of the mitochondrial genome but this has yet to be exploited.

Once DNA has been delivered into the cell and it has combined with the native DNA either homologously e.g. in the chloroplast or in a non homologous fashion in the nucleus there needs to be a technique for selection of transformant cells. Several markers are available for the direct selection of chloroplast transformants including the bacterial *aad*A gene (aminoglycoside adenyl transferase) which confers resistance to spectinomycin and streptomycin (Goldschmidt Clermont 1991) and more recently a kanamycin resistance cassette (Hu et al. 1997). The *aad*A coding sequence is flanked by chloroplast regulatory sequences and has been widely used for both specific gene disruption and site directed mutagenesis.

The first successful nuclear transformations used strains with defective arginosuccinate lyase, nitrate reductase or a non functioning photosynthetic gene. Foreign DNA was introduced with a functioning copy of the defective *arg*7, *nit*1 or photosynthetic gene and transformants were selected for on media lacking arginine (Debuchy et al. 1989), ammonium (Kindle 1989) or acetate (Mayfield & Kindle 1990).

The selectable marker used for chloroplast transformation, *aad*A, has been used to generate nuclear transformants (Cerruti et al. 1997) but it was found that a proportion of the mutants displayed unstable expression in the absence of antibiotic selection. The gene does not show the same codon bias as nuclear DNA but the fact that some transformants were obtained shows that this alone does not stop expression. A further bacterial resistance gene successfully used as a nuclear marker came from *Streptoalloteichus hindustanus* and encodes resistance to phleomycin and zeomycin (Stevens et al. 1996). The initial low transformation rates were significantly improved by the introduction of an RBCS2 intron into the coding sequence (Lumbreras et al. 1997).

1.14 *Synechocystis* sp PCC 6803

This cyanobacteria (or blue green alga) is an aquatic prokaryote which is capable of oxygenic photosynthesis. Purple or green sulphur bacteria are also

photosynthetic but unlike cyanobacteria they do not utilise water as an electron donor. It is generally believed that eukaryotic photosynthetic cells arose from an endosymbiotic event involving an ancient ancestor of modern cyanobacteria. Both higher plants and blue green algae show many common characteristics in both the structure and mechanism of the photosynthetic apparatus. In light of these similarities *Synechocystis* sp PCC 6803 has been used as a model organism for studying all aspects of photosynthesis.

Synechocystis sp PCC 6803 has a number of features which make it particularly suitable as a model organism. The first of these is the fact that it can be cultured under a range of conditions from full photoautotrophy to heterotrophic growth. Photoautotrophic growth can be achieved on a minimal media with no added carbon source in cells with active PSI and PSII complexes. In the presence of an added carbon source e.g. glucose *Synechocystis* sp PCC 6803 cells are able to grow in the absence of PSII. The growth rate of such cells on supplemented media is not significantly different from that of photoautotrophic cells. Cyclic electron flow around PSI is sufficient to maintain efficient levels of growth. Cells which lack PSI grow at a much slower rate independent of the absence or presence of PSII. PSI minus mutants can be cultured but are extremely light sensitive because PSII becomes over reduced leading to damage to the photosystem.

The problem of this light sensitivity can be avoided if the mutants are generated in a strain with a deletion in the *apc*E gene (Shen et al. 1993). The protein encoded by this gene is responsible for anchoring the phycobilisomes (the light harvesting apparatus of cyanobacteria) to the photosystems. *Synechocystis* sp PCC 6803 cells can also be grown in photomixotrophic culture with added glucose or by light activated heterotrophic growth (Anderson & McIntosh 1991). This form of heterotrophic growth requires the cells to be exposed to five minutes of light per day. The wavelength of light which stimulates this growth, in the range of 400-500nm, precludes any involvement of electron transport involving either photosystem. The authors conclude that the blue light is required as an environmental signal which regulates some aspect of metabolism or cell division.

There are differences in both the antenna system and the ratio of the two photosystems between cyanobacteria and algae and higher plants. Cyanobacteria, like red algae, use a phycobilisome system to capture photons and present them to

the photosystems. These are large multi-subunit complexes which are located on the stromal side of the membrane. The light harvesting portions of this structure are the phycobiliproteins which are a family of soluble proteins with covalently bound open-chain tetrapyrroles, known as phycobilins. The precise structure of the phycobilisomes is able to vary as a method of adaptation to the environmental conditions.

Another variation between eukaryotic cells and *Synechocystis* sp PCC 6803 is the ratio of PSI to PSII. In cyanobacteria I have already discussed the differing effects of disrupting PSI and PSII and this is extended when the relative amount of each photosystem is examined. *Synechocystis* cells have approximately six times as many PSI centres as they do PSII. In consequence PSI minus mutants tend to appear more blue in colour in comparison to PSII minus cells which do not differ significantly in appearance to wild type cells. These two features imply that for *Synechocystis* sp PCC 6803 cyclic electron flow around PSI is a physiologically important phenomenon.

There are two further features which make *Synechocystis* sp PCC 6803 a particularly attractive model organism. Firstly the cells are naturally transformable (reviewed Thiel 1994). The mechanism of uptake of DNA has not been discovered. Once inside the cells the transforming DNA undergoes homologous double recombination which replaces the wild type region with the introduced section. A gene conferring antibiotic resistance is usually included on the transforming DNA to provide a method of selection for transformants. Resistance genes for chloramphenicol, erythromycin, kanamycin and spectinomycin have all been successfully used. An additional selectable marker which can be employed is the *sac*B gene (Reid & Collmer 1987, Cai & Wolk 1990). This encodes a levan sucrase and expression in the presence of sucrose is lethal. If used in combination with a resistance marker, transformants can be selected for then the markers removed. This is achieved by a second transformation with a construct carrying DNA which flanks the original insertion site. Second round transformants (lacking both markers) are selected for in the presence of sucrose. Spontaneous transformation of *Synechocystis* sp PCC 6803 occurs in the range of 1 in 10^{3}-10^{5} and depends on the state of the cells and the length of the incoming DNA. A final relevant factor in the use of *Synechocystis* sp PCC 6803 as a model organism is the

availability of the entire 3,573,470bp sequence (Kaneko et al. 1996). The presence of this data, available on a Web page based in Japan, facilitates the construction of mutants. 3,168 potential protein coding genes have been assigned (Kaneko & Tabata 1997). Of these a function has been deduced by comparison to other known sequences for 1416 but 55% remain unclassified.

Chapter 2

Materials and Methods

2.1 Chemicals

All chemicals used were of the highest analytical grade available.

2.2 Growth and storage of bacterial strains

Bacterial strains used were DH5α (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1) and JM109 (*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*Δ(*lac-proAB*).

Luria-Bertani media (LB) (Sambrook et al. 1989) was used as the growth media, unless an enriched media was required then 2YT was chosen. Media was used as either a liquid culture or solidified with 2% w/v bactoagar (Difco). Additional antibiotics were added, where appropriate, at the concentrations suggested in Sambrook et al. (1989). The cultures were grown at 37°C and stored, for up to four weeks, at 4°C. Long term glycerol stocks were made by adding 0.15ml of sterile glycerol to 0.85ml of bacterial culture. They were then frozen in liquid nitrogen before being stored at -70°C.

2.3 Growth and storage of *Chlamydomonas reinhardtii* strains

The strains of *C. reinhardtii* used are listed in Table 2.1 along with the source of this material. Cultures were maintained on TAP media solidified with 2% bactoagar and incubated at 18°C. The petri dishes were placed under 8μE/m^2/s illumination and the cells transferred to fresh plates every four weeks. When photosynthetic growth conditions were required, HSM media was used and the cells were grown with 50μE/m^2/s illumination.

Table 2.1

C. reinhardtii strains

Strain	Characteristics	Source
CC1021	Wild type with cell wall	E. Harris
Mlu1	*aad*A downstream of *psb*H	H.E. O'Connor
T3A	*psb*H site directed mutant	H.E. O'Connor
H Null -Same	*psb*H minus, *aad*A same orientation	H.E. O'Connor
H Null -Opp	*psb*H minus, *aad*A opposite orientation	H.E. O'Connor
PB5	*psb*N minus, *aad*A opposite orientation	S. Ruffle
PS6	*aad*A before N, same orientation as H	S. Ruffle
PS10	*aad*A before N, opp. orientation to H	S. Ruffle
B4	PSI minus	R. Sayre
FuD7	PSII minus	Culture collection
A4d	PSII minus, LHCII minus	Culture collection
CW10	Cell wall deficient wild type	Culture collection
Mϕ14,16,20,21,25,27,38	Nuclear mutants with defective PSII	N. Gumpel
PM10 and PM26	Nuclear mutants with defective PSII	M.Turner
Cycf	*aad*A inserted between *psb*M and *ycf*9	This thesis
Δycf	*ycf*9 null mutant	This thesis

Cells for molecular and biochemical examination were streaked out weekly on solid media. A loopful of cells was used as the inoculum for 25ml of liquid TAP or HSM. The cultures were grown under the light conditions listed above until they reached stationary phase then either harvested by centrifugation or an aliquot used as an inoculum for a larger culture.

The recipes for making the components of this media can be found in Chapter 10 of Plant Molecular Biology (Rochaix et al. 1988). Table 2.2 shows the quantities of stock solutions used for each type of media. The amounts shown are diluted with water to give a final volume of one litre.

Table 2.2

Recipes for *C. reinhardtii* media

For one litre	Tris Acetate Phosphate (TAP)	High Salt Minimal (HSM)
Tris	2.42g	-
4x Beijerinck salts	25ml	25ml
1M (K) PO_4 pH 7	1ml	-
2x PO_4 for HSM	-	50ml
Trace Elements	1ml	1ml
Glacial Acetic Acid	approx. 1ml	-

2.4 Counting *Chlamydomonas* cells using a haemocytometer

The cell solution to be counted was first diluted, if necessary; for example when using a stationary phase culture a dilution factor of 1 in 10 would be appropriate. A 10ml aliquot was killed using 10µl iodine solution (25mg/ml in ethanol). A drop of this solution was placed on the grid of a haemocytometer (Weber Scientific International Ltd.) and the number of cells visible through a light microscope in both grids was counted and an average taken. The average count was multiplied by 10^4 (after taking into account any dilutions made) to give the number of cells per ml.

2.5 Growth and storage of cyanobacterial strains

A glucose tolerant strain (Williams 1988) of the cyanobacterium *Synechocystis* PCC 6803 was provided by Dr C. Mullineaux (UCL). This organism was grown heterotrophically in BG11 media (Castenholz 1988) supplemented with glucose, TES (N-tris [Hydroxymethyl]methyl-2-aminoethanesulfonic acid) and sodium thiosulphate. Autotrophic growth occurs when glucose is omitted from the medium. When solid media was required, for pouring of agar plates, 2% w/v agar was added prior to autoclaving.

Liquid cultures were inoculated, with a loop of cells or an aliquot from a stationary phase culture, then incubated at 30°C with 25 $\mu E/m^2/s$ of light and the shaker set to 150rpm. Cultures on agar plates were also incubated at 30°C but with a light intensity of $7\mu E/m^2/s$.

2.6 Transformation techniques

2.6.1 Bacterial transformation

Ligation reactions, carried out as detailed in Section 2.7.6, were transformed into competent cells using a method which is based on the observations of Mandel & Higa (1970). The competent cells used were either high efficiency JM109 cells purchased from Promega or DH5α cells made according to the directions of Sambrook et al. (1989). When competent cells were made they were used the same day or frozen in liquid nitrogen and stored at -70°C. To initiate transformation, 1µl of a ligation reaction was added to a 100µl aliquot of competent cells and then incubated on ice for thirty minutes to allow DNA to adhere to the cells. They were then heat shocked at 42°C for 45-60 seconds and incubated on ice for two minutes. After this 1ml of 2YT was added and the cells were left to incubate at 37°C for an hour. A 100µl aliquot was spread on media containing an appropriate antibiotic and X-gal (40µg per ml)/ IPTG (0.1mM), if blue white selection was required. The plates were incubated overnight at 37°C.

2.6.2 Biolistic chloroplast transformation of *C. reinhardtii*

The chloroplast transformation of *C. reinhardtii* can be achieved by bombarding a lawn of algal cells with particles of tungsten or gold coated with the transforming DNA (Boynton et al. 1988).The system we used for this work was the PDS1000/ Helium driven system (Bio-Rad) with gold microcarriers.

The wild type *C. reinhardtii* cells to be transformed were streaked onto fresh TAP plates every two days to encourage healthy growth then used to inoculate a liquid culture. 25ml of log phase cells were required per plate to be transformed. The cells were collected by centrifugation in 25ml aliquots then resuspended in 5ml TAP agar (0.5%) and poured onto TAP plates containing 100µg/ml spectinomycin.

6mg of 1.0µm gold particles were weighed out and placed in a microfuge tube with 0.5ml 70% ethanol. This was vortexed for two minutes, incubated for 15 minutes then pelleted by a 5 second centrifugation step before the ethanol was removed. The particles were then washed in 0.5ml sterile double deionised water, vortexed, allowed to settle and the wash liquid removed. This was carried out three

times then sterile 50% glycerol was added to give a final concentration of gold particles of 60mg/ml.

All equipment to be used for the transformation was placed in a laminar flow hood and washed with 70% ethanol prior to use. The gold particles were resuspended by vortexing then a 25µl (1.5mg) aliquot was removed. To this 4µl of transforming plasmid (1mg/ml), 25µl of 2.5M $CaCl_2$ and 10µl of 0.1M spermidine were added and the tube was vortexed vigorously for three minutes. The microcarriers were pelleted by centrifugation and the supernatant discarded. 70µl of 70% ethanol was added then removed without disturbing the gold pellet and this was repeated with 100% ethanol. The particles were finally resuspended in 24µl of 100% ethanol and 6µl aliquots were spread over the central portion of the macrocarriers and left to dry in the laminar flow hood.

The helium gun was assembled according to the manufacturers instruction guide with one agar plate inside and the chamber pressurised to 27,000psi. The helium flow was then switched on and when the pressure behind the rupture disc reached 11,000psi the microparticles were propelled towards the algal lawn. The chamber was vented and the plate removed and placed for incubation at 20°C under 8µE/m^2/s.

2.6.3 Segregation of the chloroplast genome to obtain homoplastic colonies

The plates were incubated at 18°C under a sheet of filter paper to ensure low levels of light intensity and the colonies first became visible after approximately two weeks. Once they had reached the size of about a millimetre in diameter they were transferred with a sterile cocktail stick to a fresh TAP spectinomycin (100µg/ml) plate. When there was sufficient cell mass, a loopful of the transformant cells was used to inoculate a 20ml TAP spectinomycin (100µg/ml) flask. This was incubated under the conditions listed above until the cells had grown to stationary phase. A one in thousand dilution was made then 100µl was spread onto another TAP spectinomycin plate which was incubated until single colonies were obtained. This process of selection was repeated twice more to ensure all wild type copies of the genome were removed by selection

pressure. Southern blot analysis was used to ensure the cells were homoplasmic for the mutant copy of the gene.

2.6.4 Transformation of *Synechocystis* sp PCC 6803

This method of transformation is based on the work of Williams (1988) and Ermakova et al. (1993). A liquid culture of wild type *Synechocystis* sp PCC 6803 was grown until it reached an A_{750} of 0.5, diluted with an equal volume of BG11 then left to grow in the shaking incubator overnight. The following day the absorbance at 750 nm was again measured and the number of cells per ml calculated using the formula :-

$$\text{cells /ml} = A_{750} \times 1.15 \times 10^8$$

The volume of culture which would contain 4×10^8 cells was then harvested by centrifugation in an MSE Mistral 1000 at 3500rpm for 5 minutes and washed in fresh medium before being resuspended in 1ml of BG11. An aliquot of 200µl of this cell suspension was used per transformation and to it 5µg DNA in 10µl TE was added. After mixing, this was placed in a shaking incubator for four hours at 30°C and a light intensity of 25µE/m^2/s. At the end of this time 100µl aliquots were spread onto solid agar plates with and without the glucose supplement and left in the incubator of three days.

Antibiotic selection for transformants was then imposed by pouring 3ml of 0.6% agar with added kanamycin to bring the final concentration on the plates to 50µg/ml. The plates were then incubated for approximately two weeks until colonies could be seen.

Once the transformant colonies were visible they were moved using a sterile cocktail stick to a fresh BG11 kanamycin plate to allow further growth. Three or more further rounds of subculture were required to allow for full segregation.

2.7 DNA Techniques

2.7.1 Plasmid DNA Vectors

1. pBluescript SK (Stratagene)
2. p207 (*Chlamydomonas* Culture Centre) subclone of *Bam*HI fragment 4 containing the 5` end of *rps*7, *psb*M and *ycf*9.
3. pSKaadA. (Saul Purton, UCL). Contains the *aad*A cassette between two *Bam*HI sites in a pSK background.
4. pBSSK. (Mark Ashby, UCL) Contains the kanamycin cassette cloned into pSK.
5. pCycfSp. (This thesis) p207 with the *aad*A cassette cloned into a *Bgl*II site between *ycf*9 and *psb*M.
6. pΔycf. (This thesis) pCycfSp without the *Pst*I fragment carrying *ycf*9 and part of *aad*A.
7. pSycf. (This thesis) PCR product carrying *Synechocystis ycf*9 gene cloned into pUC19.
8. pKn2 & 5. (This thesis) pSycf after *Bsm*FI digest and introduction of kanamycin cassette.
9. pPsbW. (This thesis) PCR product amplified from λZAP and cloned into pUC19
10. pHW. (This thesis) *psb*W RACE product cloned into pSK
11. pGW. (This thesis) *psb*W cosmid clones
12. pGWP. (This thesis) *Pvu*II subclone of pGW

2.7.2 Restriction analysis of DNA

DNA digests using restriction enzymes were set up using an appropriate restriction buffer, approximately 500ng DNA, an excess of the restriction enzyme and distilled water to make up to the desired volume. The digests were incubated at 37°C for at least an hour. Digests of *Chlamydomonas* genomic DNA were incubated overnight. After an appropriate period to allow complete digestion the samples were run on a 1% agarose gel to ensure that the reaction was successful.

2.7.3 Agarose gel electrophoresis

Gels were made by melting 1% agarose in TAE buffer (40mM Tris acetate, 10mM EDTA.Na_2, pH 8) unless otherwise stated (Sambrook et al. 1989). Known molecular weight standards were obtained by digesting λ DNA with *Ava*I and *Bgl*II and a 250ng aliquot was applied to each gel. Sample loading buffer (0.1M EDTA.Na pH 8, 40% glycerol, 0.01% SDS and 0.01% bromophenol blue) was added to all samples. The electrophoresis was carried out in TAE buffer at 75V for one and a half to two hours. The gel was then soaked in 0.01% ethidium bromide for twenty minutes so that the DNA bands could be visualised on a UV transilluminator. A record of each gel was kept using either a polaroid instant camera or a UVP gel documentation system.

2.7.4 Recovering DNA from agarose gels

A gel slice, containing the required DNA fragment, was removed from an ethidium bromide stained agarose gel using a scalpel. The DNA was recovered using the Qiaquick gel purification system (Qiagen) following the manufacturers instructions. The DNA was eluted from the column in 30 or 50 µl of 10mM Tris HCl pH 8.0.

2.7.5 Filling in recessed 5` ends of double stranded DNA

The 5` overhang, left after digestion at 65°C with the enzyme *Bsm*F1 (New England Biolabs.), was filled in to give a blunt ended molecule using the method found in Sambrook et al. (1989). After incubation with the restriction enzyme the desired fragment was gel purified (Section 2.7.4), then incubated with nick translation buffer (10x buffer, 0.5M Tris.Cl pH 7.7, 0.1M $MgSO_4$, 1mM dithiothreitol, 500µg/ml bovine serum albumin), 1µl of a 2mM solution of each dNTP and one unit of Klenow at 37°C for one hour. The reaction was terminated by adding 1µl of 0.5M EDTA.

2.7.6 Construction of recombinant DNA plasmids

For a successful ligation a purified linearised plasmid is required which has compatible ends to the DNA insert. The DNA to be inserted could be a product of a restriction digest, with an enzyme to give compatible ends to those of the vector, or a PCR product which would be a blunt ended molecule. The vector was digested with an appropriate enzyme. If both vector and insert had 5` phosphate groups present they were removed from the vector by treatment with 10 units of CIAP (Sambrook et al. 1989). This was then phenol chloroform purified to remove any proteins and redissolved in TE pH 8.0. Ligations were performed as detailed in the Promega Protocols and Applications Guide (Third edition). The amounts of vector and insert required for each ligation were calculated using the equation :

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

An insert : vector ratio of 3:1 was used in all cases unless otherwise stated and the ligations were incubated at 16°C overnight or at 4°C for 48 hours.

2.7.7 Plasmid DNA isolation

Small scale preparations of plasmids were made from 1-3ml of overnight culture using either the plasmid mini preparation method of Sambrook modified from the original methods of Birnboim & Doly (1979) and Ish-Horowicz & Burke (1981) or the Wizard Plus miniprep kit (Promega). Larger quantities of plasmid DNA were prepared using Qiagen maxi columns and following the manufacturers instructions. DNA from both methods was redissolved in TE then analysed by restriction enzyme digest.

2.7.8 Small preparations of *C. reinhardtii* total DNA

Two methods of preparation of total *C. reinhardtii* DNA were used. The first was taken from Plant Molecular Biology by Rochaix et al. (1988) and the second involved using the QIAamp tissue kit from Qiagen. The tissue kit has not been optimised by Qiagen for use with plant material but gives satisfactory amounts of DNA using the method outlined here. A 25ml stationary phase culture was pelleted by centrifugation at 3500rpm for 3 minutes then the cells were

resuspended in 360µl of buffer ATL (supplied with the kit). Pronase was added to a final concentration of 2mg/ml and the suspension mixed by vortexing. This was then incubated at 55°C for three hours with occasional shaking. After this time the tubes were placed in a centrifuge for 5 minutes at 13000rpm and the supernatant was removed. An addition of 400µl of buffer AL (supplied by Qiagen) and 20µl of RNase (10µg/ml) was made to each sample, the tubes were again mixed using a vortex then incubated at 70°C for ten minutes. This resulting solution was loaded onto a QIAamp spin column by centrifugation at 8000rpm and the column was washed with two 500µl aliquots of Qiagen buffer AW. To elute the DNA, 200µl of preheated 10mM Tris-HCl pH 9.0 was added to each column and they were then placed in a 70°C water bath for 5 minutes. The DNA containing buffer was removed from the column by a 1 minute centrifugation step at 8000rpm. A second aliquot of heated Tris was then used to repeat this elution step. The resulting DNA solution was ethanol precipitated using two volumes of absolute ethanol and a tenth volume of 3M sodium acetate pH 5.2. After a five minute room temperature incubation the DNA was collected by centrifugation at 13000rpm for 10 minutes. The pellet was washed with 70% ethanol, the centrifugation repeated, then the pellet was air dried before being resuspended in 50µl of 10mM Tris-HCl pH 9.0.

2.7.9 Preparation of genomic DNA from *Synechocystis* sp PCC 6803

A flask containing 25ml of BG11 media was inoculated either from an existing liquid culture or from a plate and the cells were grown to stationary phase. The cells were harvested by a ten minutes centrifugation step at 4500rpm in the bench top centrifuge. Most of the supernatant was removed from the cells then they were resuspended in the remaining liquid and transferred to an eppendorf tube. This was placed in a microfuge for two minutes at 13,000rpm before the remaining culture media was separated from the cell sample. The tube was quickly frozen in liquid nitrogen. DNA was prepared using the PhytoPure kit (Nucleon). The manufacturers instructions were followed with the exception of first steps. The frozen cells samples were removed from liquid nitrogen and allowed to thaw. 600µl of Solution 1 was added to the tube during the thawing step and the sample was thoroughly mixed. After this point the instructions were followed through the cell lysis and DNA precipitation steps.

2.7.10 Polymerase Chain Reaction (PCR)

PCR was used to amplify DNA from both plasmids and genomic DNA. Template DNA was purified as detailed in Section 2.7.8 and approximately 10ng was used per reaction. Primers were designed to be complementary to the sequences flanking the region of interest and ordered from Perkin Elmer. A final concentration of 1μM of each primer was used in each reaction. The enzyme of choice for all PCR experiments was Vent polymerase (New England Biolabs.) and 2 units were added to each 50μl reaction. The reaction buffer supplied with the enzyme was diluted as appropriate and supplemented with $MgSO_4$, to give a final concentration of 4μM, and with all four dNTPs, to give 0.2mM of each in the reaction tube.

A programmable thermal cycler (Techne Progene) was used for the amplification process. An initial denaturation step of three minutes at 94°C was followed by 25 cycles of 1 minute denaturation at 94°C, 1 minute annealing at a temperature below the T_M of the primers, and 2 minutes extension at 72°C. A final extension time of 5 minutes at 72°C completed the reaction. One fifth of the reaction volume was then run on a 1% agarose gel to analyse the products.

The primers used for PCR experiments are listed in Table 2.3. A suitable annealing temperature was calculated for each pair of primers based on the melting temperature calculated from the following equation.

$T_M = 2$ (no. A + T bases) + 4 (no. G + C bases)

During design of primers the T_M would be calculated to ensure that the pair of primers gave similar values. The initial annealing temperature used was 5°C below the average T_M.

Table 2.3

Primers used for PCR experiments

Name	Sequence (5`-3')	Location
atpA down	ggcaatgcgtactccagaagaactt	At 5` end of the aadA cassette
psbhnest	gggactgcctactgcggtcttagg	Between psbH and psbN genes
T3	cgaaattaaccctcactaaaggg	Flanking MCS of pSK
T7	gtaatacgactcactatagggc	Flanking MCS of pSK
Sycf9 5`	gccagggaaattgatcgttggagtg	300bp upstream of ycf9
Sycf9 3'	cacatcaatgccatgacgtttgaggc	200bp downstreanm of ycf9
psbW	ctggt(g/c)gacgagcgcatgaacgg	5` end of *psb*W

2.7.11 DNA sequencing reactions

All DNA sequence was obtained via automated sequencing using an ABI Prism 310 Genetic Analyser (Perkin Elmer) and carried out by Laura Winskill at UCL. The reactions were prepared by cycle sequencing using 500ng DNA, 0.5µl of 10µM stock of primer, AmpliTaq FS and its supplied buffer (Perkin Elmer), dNTPs and BigDye labelled terminator dNTPs. After 25 cycles of amplication the reactions were ethanol precipitated, resuspended in loading buffer and denatured for 1 minute before being loaded onto the sequencer. GC rich DNA was sequenced by adding 2µl of 5% DMSO to the reaction and increasing the amount of terminator ready reaction mix from 4µl to 16µl. The primers used for sequencing reactions are shown in Table 2.4.

2.8 RNA techniques

2.8.1 Extraction of total cellular RNA

RNA was extracted from *C. reinhardtii* cells using the RNeasy Plant Mini Kit (Qiagen). The cells were harvested during mid log phase (2-4 x10^6 cells per ml) by centrifugation at 3500rpm for 5 minutes. The supernatant was removed and the cells resuspended in 450µl buffer RLT (provided by Qiagen) and 4.5µl of β-

Table 2.4

Primers used for sequencing

Primer	Sequence	Target
T3	cgaaattaaccctcactaaaggg	MCS of pSK
T7	gtaatacgactcactatagggc	MCS of pSK
pk3	agacacaacgtggc	Kanamycin cassette
pk7	acttgacgggacggcggc	Kanamycin cassette
Wseq1	ttgtcagcagctctgcggtg	Internal *psb*W primer
Wseq2	caccgcagagctgctgacaa	Internal *psb*W primer

mercaptoethanol was added. The cells were subjected to 3 cycles of freezing in liquid nitrogen and thawing at room temperature, then the manufacturers instructions were followed. The RNA was eluted from the final column in 50µl DEPC treated distilled water and the yield and purity was determined by absorbance measurements at 260nm and 280nm (Sambrook et al. 1989).

2.8.2 Electrophoresis of RNA

Electrophoresis of RNA samples was carried out on 1% agarose gels made with MOPS buffer (0.2M MOPS, 80mM Na acetate, and 15mM EDTA pH 7 with 2M NaOH) and containing 1% formaldehyde. The loading buffer (12µl ethidium bromide, 300µl 10x MOPS buffer, 80µl formaldehyde and 300µl formamide) was mixed with the RNA in the ratio of 6µl loading buffer to 8µl RNA. The sample was then heated to 95°C for two minutes and cooled on ice just prior to loading. When RNA markers (Promega) were required 3µg of RNA was loaded. Electrophoresis was carried out at 75V for three hours.

2.8.3 Rapid Amplification of cDNA Ends (RACE)

The first stage of the RACE protocol (Frohman et al. 1988) involves using the enzyme, reverse transcriptase, to synthesise cDNA molecules from mRNA. The mRNA sample was prepared from wild type *C. reinhardtii* cells using Ambion Poly(A) Pure kit. The cDNA was then synthesised using an oligonucleotide

(DT58) which, would hybridise to the poly A tail found on mRNA molecules, and AMV reverse transcriptase. The reaction was incubated at 55°C for 60 minutes then the enzyme was denatured by elevating the temperature to 65°C for 5 minutes.

The second stage of the RACE protocol uses two primers, one designed against the end of the primer DT58 and the other specific to the required sequence. The sequence of all the primers used in the RACE protocol is listed in Table 2.5. The PCR reaction was composed of 0.5µl cDNA, 0.2µM each primer, 0.2mM each dNTP, 1µl HiFi Expand Taq polymerase (Boehringer Mannheim), 10µl of supplied buffer No.2 and sterile distilled water to bring the final volume to 100µl.

Using the Perkin Elmer Gene Amp PCR System 2000 the reactions were denatured for 2 minutes then taken through 30 cycles of 30 seconds at 94°C to denature the DNA, 30 seconds at 47°C for the primers to anneal and 72 seconds at 72°C to allow polymerisation to occur. This was followed by a 7 minute incubation at 72°C to ensure full polymerisation of all molecules. To ensure amplification of the correct product a second round of PCR was carried out using 2 internal primers and 1µl of reaction 1 product as the template. The products of both reactions were examined on a 2% agarose gel using a 100bp ladder (MBI Fermentas).

2.9 Filter hybridisation of nucleic acids

2.9.1 Southern analysis

Southern analysis was carried out using the method of Southern (1975) and described in Sambrook et al. (1989). DNA was transferred to Hybond N (Amersham International) nylon filters using the capillary blot technique described in the Amersham International protocol but with 10xSSC as the transfer buffer. After 16 hours the blotting apparatus was dismantled and the blot baked in an 80°C oven for 2 hours.

Table 2.5

Primers used for RACE.

Name	Sequence
DT-58	aaggatccgtcgacatcgataatacgactcactataagggatttttttttttttttt
Race 1	aaggatccgtcgacatcgataat
Race 2	gataatacgactcactataaggga
W1	tsgtsgaygagcgbatgaa
W2	atgaayggngayggnacngg

Redundancy codes

s = g or c

y = c or t

b = c,g or t

n = a,c,g or t

2.9.2 Northern analysis

Formaldehyde gels were photographed using the gel documentation system then the RNA was transferred to either a Hybond N+ (Amersham International) nylon filter or to a nitro-cellulose filter (Schleicher & Schuell). Both types of filter were soaked in 2x SSC before use. Method used for transfer of RNA to the filter is described by Sambrook et al. (1989). After overnight transfer the gel was placed on a UV light box to ensure the transfer had been completed successfully and the filter was baked in an 80°C oven for 2 hours.

2.9.3 Screening a genomic cosmid library

The cosmid library used was prepared in the vector pARG7-8cos by Dr Saul Purton of UCL. This was plated out at a density of 20-50,000 colonies per 13cm petri dish on ten different plates. Duplicate filters were then prepared for each using the method outlined in Sambrook et al. (1989). After overnight incubation of the cultures at 37°C the plates were placed at 4°C for 30 minutes. Dry nylon filters were placed onto the surface of each plate until they were completely wet. The filter was then marked at three asymmetrical locations using a needle then

the filter was removed. The colonies which adhered to each disc were lysed and attached to the filters using a method from Sambrook et al. (1989) which is closely based on the original work of Grunstein & Hogness (1975).

Filter paper was placed into the lids of four large square petri dishes then soaked in one of four solutions. The first was 10% SDS then denaturing solution (0.5N NaOH, 1.5M NaCl) followed by neutralising solution (1.5M NaCl, 0.5M Tris.Cl pH7.4) and finally 2xSSC. Excess liquid was removed from each tray. The filters were placed colony side up onto each of the soaked filter papers. Contact with the SDS tray was limited to 3 minutes while the following incubations all lasted 5 minutes. After the 2xSSC tray the filters were placed on 3MM paper to dry then baked at 80°C for 2 hours to fix the colonies. The filters were then ready for use in a hybridisation reaction.

2.10 Radiolabelling DNA probes

2.10.1 Random labelling of DNA

Random labelling was achieved using the Prime It Random Labelling kit from Stratagene and α-^{32}P dCTP* from Amersham International. 25ng of DNA from a plasmid or a PCR product, listed in Table 2.6, was purified and labelled according to the manufacturers protocol. The enzyme was inactivated and the DNA denatured by boiling for 2 minutes before addition to the prehybridisation solution.

2.10.2 End labelling oligonucleotide probes

10ng of oligonucleotide (see Table 2.7), 1µl of 10x PNK buffer and enough distilled water to give a final volume of 10µl were placed in an Eppendorf tube. Then 50µCi of γ^{32}P-ATP* and 2 units of bacteriophage T_4 polynucleotide kinase (PNK) were added. The tube was incubated at 37°C for 45 minutes before being heated to 65°C for a further 10 minutes to inactivate the enzyme. This was then added to the hybridisation solution in the tubes.

* 3000 Ci/mmol

Table 2.6

DNA used as random labelled probes

Probe	Source	Reference
***psb*A**	1kb *EcoR*1/*Hind*III fragment of pSHC	Johanningermeirer & Heiss (1993)
***psb*B**	1kb *EcoR*1/*Pst*1 fragment of pUC8-Eco19	H.E. O'Connor (Thesis, UCL)
***psb*C**	930bp *Hind*III fragment of pDC-R9	Rochaix et al (1989)
***psb*D**	650bp *EcoR*1/*Xba*1 fragment of pH10	Chlamydomonas Centre
***psb*H**	700bp *EcoR*1/*Hind*III fragment of pUC9-psbH	H.E. O'Connor (Thesis, UCL)
***aad*A**	*Pst*1/*Nco*1 digest of *aad*A cassette	Goldschmidt-Clermont (1991)
***psb*W**	RACE11 product	This Thesis
S *ycf*9	PCR product using primers Sycf9 5` and 3'	This Thesis
C *ycf*9	1kb *Pst*I/*Spe*I fragment of p207	This Thesis

Table 2.7

Oligonucleotides used as end labelled probes.

Name	Sequence	Reference
***psb*E**	cttgttctaaagcattgaaacggtcag	Alizadeh et al. (1994)
***psb*F**	ggtactgcaataccgtggatagctaacc	Alizadeh et al. (1994)
***psb*I**	cctgggttacgagcagggtcattag	Leu et al. (1992)
***psb*J**	ccaatagctaaagtaccaacaacagtacc	AFO25877
***psb*K**	aggtgcaaatggtgcgtatgcttcagg	Silk et al. (1990)
***psb*L**	ctcattaactaatgtctataac	Fong & Surzycki (1992)
***psb*M**	gcagtagctgttaatccgt	Higgs et al. (1996)
***psb*N**	gcactaggaaaacttcttgtgcttc	Johnson & Schmidt (1993)
***psb*T**	catacgtggaggatctctgaag	Monod et al. (1992)
***ycf*9**	ctacaggtacacctactac	Higgs et al. (1996)

2.11 Hybridisation of probes

2.11.1 Southern blots

A baked DNA filter was soaked in 2xSSC (20x SSC, 3M NaCl, 0.3M $Na_3C_6H_5O_7$) at room temperature for five minutes. A prehybridization solution of 5xSSC, 5x Denhardts solution (50x Denhardts : 5g Ficoll, 5g BSA, 5g PVP, in 500ml water) and 0.5% SDS were prepared. Then 0.5ml of a 1mg/ml solution of salmon sperm DNA was denatured by boiling for 5 minutes and added to it. This was then placed in the hybridisation bottle with the filter and incubated at 65°C in a hybridisation oven for four hours.

After this time the denatured probe was added to the prehybridisation solution and the filter was incubated with it overnight at 65°C. The following day the filter was washed to removed non-specifically bound probe, following the directions in the Amersham protocol before being wrapped in Saranwrap and placed with a film for autoradiography.

2.11.2 Northern blots

RNA blots probed with a random labelled piece of DNA were treated as detailed above but the hybridisation and wash steps were carried out at 42°C unless otherwise stated. When end labelled oligonucleotide probes were used the incubation temperature was influenced by the T_M. The incubation was carried out 5°C below the calculated T_M. All other stages of the hybridisation process were carried out as detailed above in Section 2.11.1.

2.12 SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

Gels and buffers were prepared according to the methods in Plant Molecular Biology (Rochiax et al. 1988). All PSII-H samples were analysed on 18% slab or 10-20% gradient gels and the quantity of sample used was equivalent to 25µg of chlorophyll for a large (20 cm) gel. The samples were subject to acetone extraction to remove chlorophyll because its presence interferes with the migration and visualisation of low molecular weight proteins like PSII-H. The 25µg samples were pelleted in a microfuge at 13000rpm for two minutes and the pellet was resuspended in 200µl of 90% acetone and left for five minutes. After this time the samples are again spun in the same manner and the acetone removed. This was

repeated if the pellet was still green in colour then the tubes were air dried for 10 minutes to ensure all the acetone had evaporated.

To solubilise the proteins, 40µl of sample buffer (2.5% SDS, 2.5% β-mercaptoethanol, 0.2M Tris.HCl pH 8.3, 0.1M sucrose, 4M urea, 0.1% bromophenol blue) was added then left at room temperature until needed. Immediately prior to loading, the sample was heated to 70°C for five minutes. Pre stained colour markers (Sigma) were loaded onto every gel according to the manufacturers instructions.

Large gels were electrophoresed overnight at 4°C at a constant current of 8mA. Gels used for analysis of radiolabelled proteins were dried before use for autoradiography. These gels were put into a solution of 50% methanol and 2% glycerol for two hours. They were then placed in a vacuum drier between sheets of cellophane (Biorad), soaked in the same solution, and dried for two hours at 60 °C. The dried gel was exposed to autoradiography film.

2.13 *In vitro* phosphorylation assay

In vitro phosphorylation was examined in both broken cells and thylakoid membrane preparations from *C. reinhardtii* using an adaptation of the method of Wollman & Delepelaire (1984). Each assay was carried out on samples containing 25µg of chlorophyll and the entire sample was analysed by SDS PAGE on either an 18% slab gel or a 10-20% gradient gel.

Two Eppendorfs, each containing 25µg aliquots of the cell type to be examined, were washed in reaction buffer (25mM Tricine, 0.3M sorbitol, 5mM $MgCl_2$, 15mM KCl, pH 7.8) and resuspended a volume of the same buffer so that the final volume after all subsequent additions would be 100µl. One of the tubes was then wrapped in foil for dark adaptation and the other was placed in front of a 300 W light, with a water filter to absorb heat from the light source. The samples were then incubated for 30 minutes to poise the membranes with the electron acceptors in a photo reduced or oxidised state.

After this time NaF, known to inhibit the phosphatase reaction, and ATP were added to a final concentrations of 1mM each. A final addition of 4µCi of ^{32}P-γATP* was made and then the samples were returned to their dark or light incubation conditions for another thirty minutes. After this time 50µl of 50mM

* 3000 Ci/mmol

EDTA was added to stop the phosphorylation reaction. The samples were spun in a microfuge for two minutes and the supernatant removed. The pellet was resuspended in 200µl of reaction buffer and the spin repeated; this removed any unincorporated radionucleotide. The samples were then prepared for electrophoresis as described.

2.14 Fluorescence analysis

Fluorescence characteristics were analysed using the Hansatech Plant Efficiency Analyser. The samples were streaked evenly onto TAP plates and left to grow in dim light for 48 hours. Prior to analysis the samples were dark adapted for at least 30 minutes. The cells were then analysed with a light intensity of 50% and an illumination time of fifteen seconds.

2.15 *Chlamydomonas* thylakoid membrane preparation

The method used to prepare thylakoid membranes from *C. reinhardtii* is an adaptation of the methods of Diner & Wollman (1980) and Shim et al. (1990).
The 30 litres of culture required for this preparation was grown at 20°C in three ten litre vessels, each inoculated with a one litre stationary phase culture. They were incubated with constant stirring and aeration through Millipore 0.22µM filters and an illumination level suitable for the cell type as indicated in Section 2.3.

The large volume of culture was condensed to approximately one litre using a Millipore pump with a 0.22µm membrane. This culture was then pelleted for 5 minutes at 5000g and 4°C. The supernatant was discarded and the pellet resuspended in a small volume of HSM buffer (20mM Hepes, 0.35M sucrose, 2mM $MgCl_2$, pH 7.5). The concentration of chlorophyll was assayed and then adjusted with more HSM to 1mg/ml. Lysis of the cells was achieved by one passage through a French press at 4000lb per $inch^2$.

The broken cells were pelleted at 50,000g for thirty minutes at 4°C. Following this the pellet was resuspended in 180ml of a dense sucrose solution (2.2M sucrose, 10mM EDTA, 5mM Hepes pH 7.5). This solution was evenly distributed between four centrifuge pots, overlaid with 15ml of a second sucrose solution (0.5M sucrose, 5mM Hepes, pH 7.5) and spun at 100,000g for 2 hours at

4°C. The thylakoid membranes float at the interface between the two buffers and were carefully collected. These were washed in thylakoid buffer A (0.4M sucrose, 5mM $MgCl_2$, 20mM Hepes, pH 7.5) then finally resuspended in a small volume of the same buffer. The membrane samples were frozen in liquid nitrogen for long term storage.

2.16 Broken cell membrane preparations

This type of preparation was used for *in vitro* phosphorylation assays and protein gels. The method bursts the cells using sonication then a centrifugation step separates the suspension into crude membrane and soluble fractions. This process was carried out exactly according to the instructions in Plant Molecular Biology (Rochaix et al. 1988) with only one exception. The resuspension buffer A used for the *in vitro* phosphorylation was prepared leaving out the reducing agent β-mercaptoethanol.

2.17 Oxygen evolution

Oxygen evolution analysis was performed using a Clark type electrode and a volume of cells or membranes containing 50μg of chlorophyll, made up to a total volume of 3ml with resuspending medium (20mM MES, 15mM NaCl, 5mM $MgCl_2$, pH 6.3). Two electron acceptors were used, DMBQ and ferricyanide, each to a final concentration of 1mM. Oxygen levels were measured for a minute without illumination and for between one and two minutes under saturating light conditions.

2.18 EPR Analysis

Samples for EPR analysis consisted of either intact cells or membrane preparations. Membrane samples were obtained in a concentrated form then 0.3ml was added to an EPR tube. Whole cell samples were concentrated by centrifugation then the cells were resuspended in buffer containing 20mM Tris, 5mM EDTA pH8.0. The centrifugation was repeated then the sample was resuspended in as small a volume as possible. The sample was then placed in an EPR tube. Both types of sample were dark adapted, on ice, for at least one hour, then frozen in liquid nitrogen.

EPR analysis was performed using a Jeol REIX spectrometer with an Oxford Instrument liquid helium cryostat.

2.19 Chlorophyll Assays

Chlorophyll assays were carried out on both cell suspensions and membrane preparations according to the methods of Arnon (1949) and Porra et al. (1989).

Chapter 3

The characterisation of PSII-H and PSII-N mutants in *C. reinhardtii*

3.1 Introduction

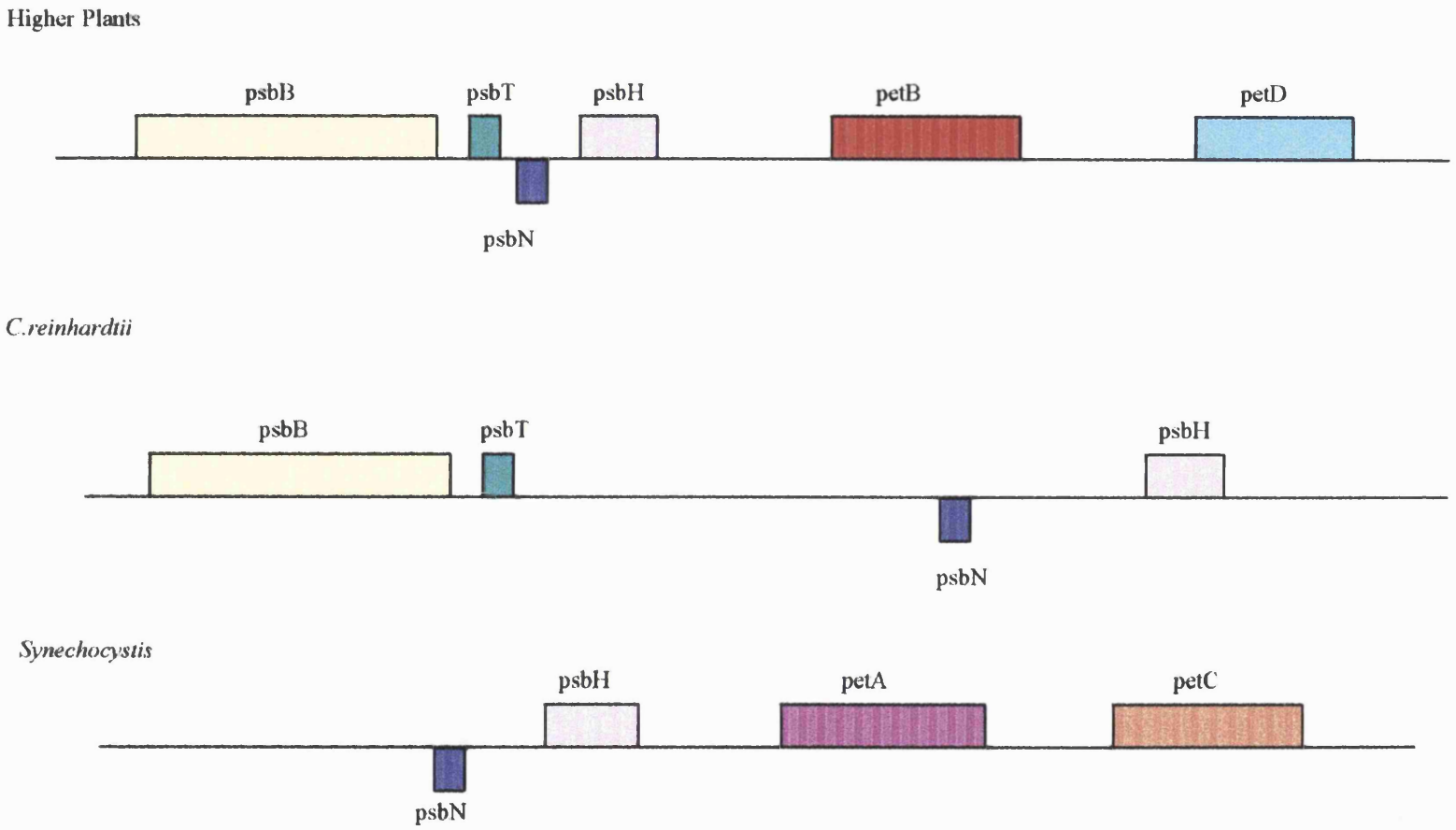
3.1.1 PSII-H

The 10kDa phosphoprotein, as the PSII-H subunit is also known is found on the chloroplast genome in the *psb*B operon. In higher plants the genes are arranged in the order *psb*B, *psb*T, *psb*N, *psb*H, *pet*B and *pet*D with the *psb*N open reading frame found on the complementary strand. The operon in *C. reinhardtii* (Johnson & Schmidt 1993) consists only of the first four genes; *pet*B and *pet*D are known to be located elsewhere on the chloroplast genome and the intergenic spaces are much larger in *C. reinhardtii* as can be seen in Figure 3.1.

The *psb*H gene has been sequenced in many species including spinach (Westhoff et al. 1986), wheat (Hird et al. 1986), pea (Allen & Findlay 1986), *Synechocystis* PCC 6803 (Abdel-Mawgood & Dilley 1990, Mayes & Barber 1990) and *C. reinhardtii* (Johnson & Schmidt 1993, O'Connor et al. 1998). The sequence data raises some noteworthy points. The amino residue, a methionine, is in all cases removed post translationally. The protein has one membrane spanning helix which contains a large number of conserved hydrophobic residues. A feature, conserved in all sequences, with the exception of the cyanobacterial gene, is the presence of a phosphorylatable threonine at position two of the mature polypeptide. This was first shown to be phosphorylated in spinach by Michel & Bennett (1987) but the presence of an additional phosphorylation site has never been conclusively ruled out. Sequence from *Synechocystis* PCC 6803 *psb*H gene shows that the protein is truncated at the amino terminus and is consequently missing the confirmed phosphorylation site. Work by Race & Gounaris (1993) has suggested that another unspecified residue may be phosphorylated in the cyanobacteria.

The phosphorylation characteristics of the PSII-H protein have been examined. It is the second most heavily phosphorylated protein in PSII after the LHCII proteins and its phosphorylation like, as LHCII, is under redox control. The kinetics of the phosphorylation of LHCII and PSII-H differ quite significantly, as shown in the *in vitro* incorporation of ^{32}P into thylakoids of Stys et al. (1995). The LHCII proteins become heavily phosphorylated within fifteen minutes of the start of a light incubation and then slowly decline. In contrast PSII-H becomes

Figure 3.1 A schematic comparison of the genes surrounding *psb*H in higher plants, *C.reinhardtii* and *Synechocystis* PCC 6803



phosphorylated over a time course of two hours. The dephosphorylation reactions of LHCII and PSII-H also proceed with different kinetics (Bennett 1980). The dephosphorylation of LHCII is relatively fast with a half time of approximately 8-10 minutes while PSII-H has the phosphate group removed over a much longer time scale with a half time greater than 180 minutes (Cheng et al. 1994).

3.1.2 The function of PSII-H within PSII

There is as yet no confirmed function for PSII-H. The fact that its phosphorylation is under redox control suggests that it has an active role and is not just a structural component of the complex. With a view to confirming the function of this protein, a *psb*H deletion mutant has been created by Mayes et al. (1993) in *Synechocystis* PCC 6803. This PSII-H null mutant is still photosynthetically competent at low light intensity but has a reduced growth rate in comparison to wild type cells. Experimental evidence leads to the conclusion that in the absence of PSII-H the Qb site is adversely affected leading to an impaired electron flow from Qa to Qb. Their later studies (Komenda & Barber 1995) discovered that the changes occurring in the vicinity of the Qb site affect the turnover of the D1 protein causing a defective repair/replacement process after D1 has been damaged. They conclude that it is this double effect which renders the null mutant more susceptible to photoinhibition. The cyanobacterial PSII-H protein lacks the conserved phosphorylation site of eukaryotic organisms and has not conclusively been shown to be phosphorylated elsewhere, so it is possible that there may be an additional role for PSII-H in higher plant cells.

An early suggestion as to a possible role for PSII-H was proposed by Packham (1988) who observed that in phosphorylated membranes Qa^- had a higher degree of stability. In his experiments using FSBA which inhibits LHC phosphorylation (Packham 1987), he concluded that this effect could not be caused by LHCP because the degree of inhibition of electron flow was unaltered He attributed the effect to phosphorylated PSII-H binding at the Qb pocket. These findings are supported by Harrison & Allen (1991). In purple bacteria there is a subunit of the reaction centre called the H subunit, which shows no sequence homology to the PSII-H protein, and which binds at the Qb pocket to ensure complete reduction of the second quinone. Packham proposed that PSII-H could

have a similar role and in its phosphorylated form cause a decrease in affinity of PSII for plastoquinone and hence an increase in the stability of Qa^-.

This effect links in with theories which connect PSII-H with protection from photoinhibition. It has been suggested (Thompson & Brudvig 1988) that electron flow could be switched from its normal pathway out of PSII and be diverted from Qa to cytochrome b_{559}. The cytochrome is proposed to rereduce chlorophyll photooxidised by P680. The presence of phosphorylated PSII-H is suggested to inhibit electron flow to Qb and therefore promote the theoretical reduction of cytochrome b_{559} by Qa^-.

The phosphorylation induced reduction in electron transfer through PSII could occur as a response to photoinhibition. The research of Sundby et al. (1989) also supports a role for phosphorylation in photoinhibition. Her results show that thylakoid protein phosphorylation is influenced by the concentration of bicarbonate ions. In the presence of 20mM bicarbonate the phosphorylation of the 25kDa protein of LHCII is highly stimulated. It is this LHC protein which has been shown to be capable of migration away from the PSII reaction centre (Larsson et al. 1987, Spangfort et al. 1987). In contrast to this the phosphorylation of PSII-H appears to be inhibited by increasing bicarbonate concentration.

Bicarbonate concentration is known to affect the rate of electron transfer from Qa to Qb and Sundby et al. (1989) suggests that the cluster of basic amino acids at the amino terminus of PSII-H may provide a binding site for this ion. This is consistent with the positioning of PSII-H, which is known to span the membrane once with the N terminus on the quinone binding side of PSII, and would account for the competition between phosphorylation and bicarbonate binding. In summary, in the presence of bicarbonate electron transfer between Qa and Qb is efficient and PSII-H is unphosphorylated, in its absence PSII-H is phosphorylated and Qa to Qb electron transfer is inhibited.

Another theory which provides a role for phosphorylated PSII-H in the protection against photoinhibition is given by Stys et al. (1995). Their research studied the variation in phosphorylation levels of the major PSII phosphoproteins over a time course of two hours. They found that the level of LHCP reached its maximum within fifteen minutes and then declined over the remaining time period. For the theory of LHCII fluorescence quenching to be accomplished by its

migration away from PSII a continuous level of phosphorylation would be expected over the longer term, rather than the observed decline. The authors propose that a quenching complex is formed involving another of the thylakoid phosphoproteins. They continue to propose that this other component maybe the *psb*H gene product as this is the only other component to achieve an equivalent level of phosphorylation to that of LHCII. The participation of PSII-H in such a complex may help to explain its very slow dephosphorylation rate if the complex were to protect its phosphorylation site from the phosphatase.

It is known that under high light conditions LHCII dissociates from PSII reducing its light harvesting abilities. An initial proposal for the mechanism of this involved the electrostatic repulsion between phosphorylated LHCII and phosphate groups attached to PSII phosphoproteins like PSII-H (Allen & Findlay 1986). However the paper by Harrison & Allen (1991) showed that the effect is still observed even when only LHCII can be phosphorylated which means that PSII-H cannot be involved in this mechanism

The dissociation of LHCII from PSII is a short term response to changes in light quality and quantity. A longer term adaptation occurs at the level of gene expression which can alter the ratio of PSI to PSII and vary the antenna size. This is proposed to be brought about by a redox sensor being able to interact, as the conditions change, with a second factor, the response regulator. This has a direct influence on transcription of certain genes. PSII-H, as a redox regulated phosphoprotein, could be a potential redox sensor in a system of this nature (Allen 1992).

3.1.3 PSII-N

There are at the latest count over 20 proteins associated with PSII many of which have no known function. One of these is the *psb*N gene product. In *C. reinhardtii* this gene is located within the *psb*B operon between *psb*T and *psb*H but in the opposite orientation to them (Johnson & Schmidt 1993) as shown in Figure 3.1. This configuration is highly conserved among eukaryotic species which would suggest that this arrangement may have some significance.

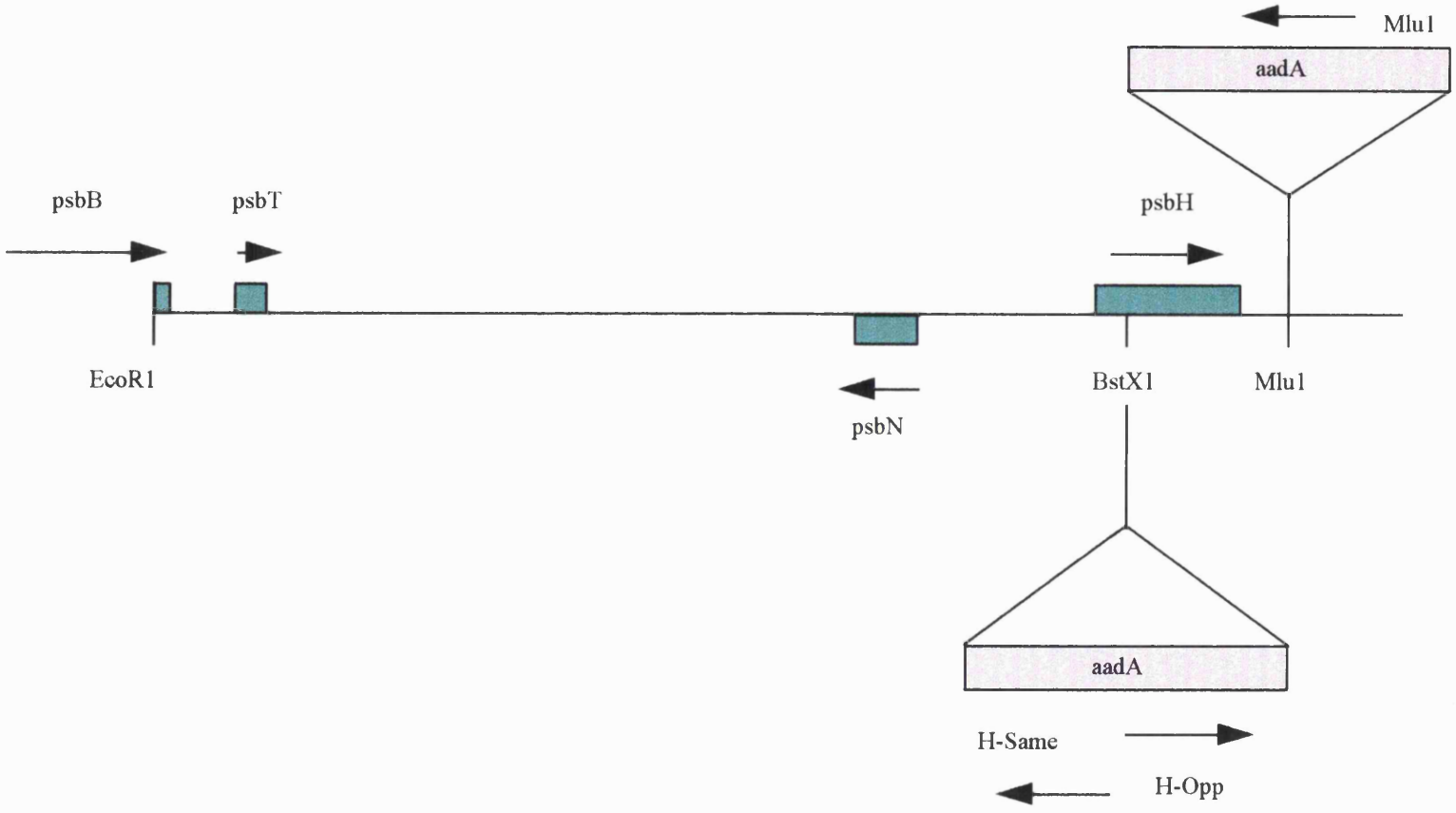
It has been confirmed that the *psb*N gene is transcribed in liverwort (Kohchi et al. 1988), pea and *C. reinhardtii* (Johnson & Schmidt 1993) and the

PSII-N protein has been purified from a cyanobacterial reaction centre preparation. The protein, in *C. reinhardtii*, is predicted to be 5210Da which is similar to the size found in higher plants. The high percentage of hydrophobic residues implies that it is an integral membrane protein but there is no known function for this protein.

3.1.4 Creation of *psb*H mutants

In order to investigate the roles of both PSII-H and PSII-N in *C. reinhardtii* a series of mutants have been generated. The *psb*H mutants were created by Helen O'Connor in this laboratory (O'Connor et al. 1998). The four different cell types created all have the *aad*A cassette introduced into the area around the *psb*H gene as shown in Figure 3.2. Two classes of PSII-H null mutant were created by inserting the *aad*A sequence into a unique *Bst*XI site, within the gene coding region, in both the same and opposite orientation to *psb*H. The null mutants will be referred to as H-same and H-opp to differentiate between them. A site directed change was also introduced which altered the phosphorylatable threonine residue at position 2 to an alanine. This was achieved by placing the *aad*A cassette into an *Mlu*I site immediately downstream of *psb*H then using site-directed PCR mutagenesis to alter the coding sequence from ACA (a threonine codon) to GCA (an alanine codon). In doing this a novel *Pst*I site was introduced in the mutants lacking the phosphorylatable threonine which have been named T3A. In the mature protein the phosphorylatable threonine is at position 2 but the N-terminal methionine is cleaved from the precursor. The fourth mutant which will be discussed had the *aad*A sequence cloned into the *Mlu*I site but no further change made. This acted as a control to ensure that any changes noted in the site directed mutants were not due to the *aad*A cassette being present downstream from the gene.

Figure 3.2 Schematic diagram showing the positions of the *aad*A cassette in the *psb*H mutants



3.1.5 Creation of *psb*N mutants

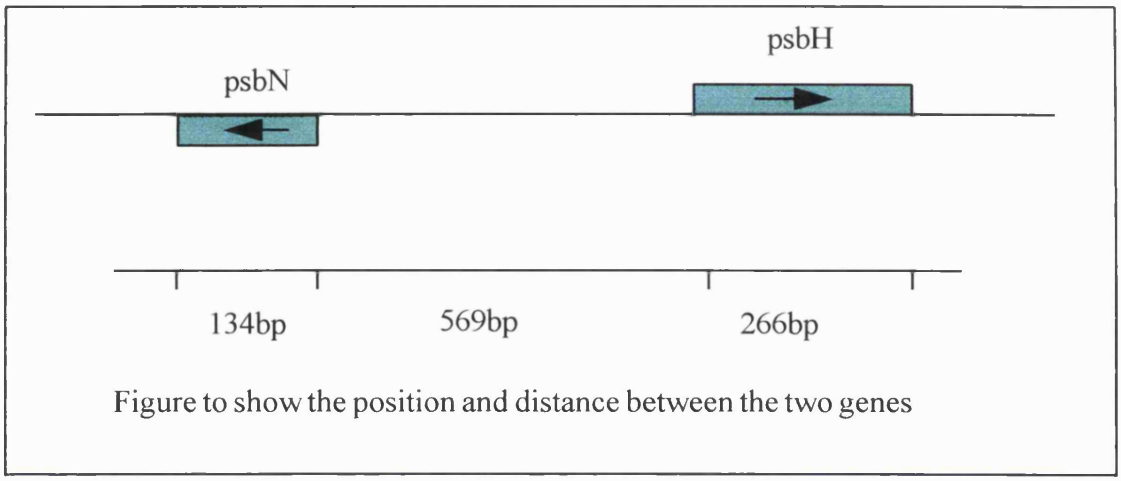
The *psb*N gene lies upstream from *psb*H but on the opposite strand of DNA and in the opposite orientation. To determine the function of this PSII protein a number of mutants, shown in Figure 3.3, were created by Stuart Ruffle in this laboratory. The *psb*N gene was disrupted by cloning the *aad*A cassette into a *Bsa*AI site within the coding sequence. Plasmids were selected which had the *aadA* cassette in both the same orientation as the gene, PB5, and the opposite orientation, PB2. The spectinomycin cassette was also introduced into a *Sca*1 site 15bp downstream of the *psb*N gene, in mutants PS6 and PS10, to determine whether the presence of this exogenous DNA was affecting the transcript of *psb*N and the other genes of the *psb*B operon.

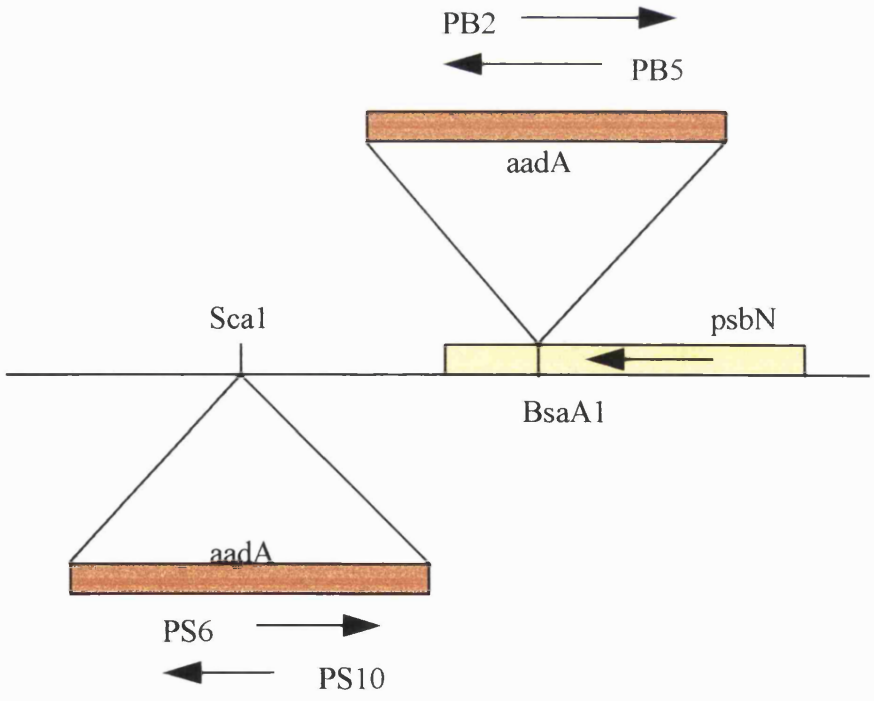
The plasmids carrying the changes were introduced into *C. reinhardtii* by particle bombardment and the transforming DNA entered the genome by homologous recombination. Mutant colonies were selected for on spectinomycin containing media then taken through three rounds of selection to encourage the appearance of homoplasmic colonies. A southern analysis was performed on DNA from PB5, PS6 and PS10 cells which confirmed that all three cell lines no longer carried the wild type copy of the gene.

The PB2 cells which were isolated on the spectinomycin selection plates grew very poorly and despite a second attempt at trying to introduce this change into *C. reinhardtii,* the transformants all died before they could be analysed. It is possible that in PB2 the 3' *rbc*L region of the *aad*A cassette, which contains a transcription terminator disrupts the expression of the region of DNA downstream from *psb*N.

In higher plants it is well established that *psb*B is expressed in an operon with the genes *psb*T, *psb*H, *pet*B and *pet*D. In *C. reinhardtii* the two *pet* genes are located elsewhere on the chloroplast genome and the intergenic regions are much larger. Nuclear mutants have been described (Sieburth et al. 1991, Monod et al. 1992, Johnson & Schmidt 1993) which have impaired accumulation of not only the *psb*B transcript but also the *psb*H and *psb*T transcripts. This suggests that in *C. reinhardtii* a single transcript may encompass the whole operon. Attempts to use *in vitro* capping have however failed to identify a primary

Figure 3.3 Schematic diagram to show the creation of the PSII-N mutants





The *aad*A cassette is introduced both within and after the *psb*N gene.

transcript for any of the *psb*B gene cluster (Monod et al. 1992, Johnson & Schmidt 1993).

The recently published results of Summer et al. (1997) describe a series of mutations made in the region of the *psb*B operon in *C. reinhardtii*. They inserted the *aad*A cassette into the regions between *psb*T and *psb*N (sense), between *psb*N and *psb*H (antisense) and between *psb*H and *trn*E; in addition they replaced the *psb*H coding sequence with the *aad*A cassette. They conclude from northern analyses that inserting *aad*A upstream of *psb*H, in the sense orientation does not affect the accumulation of the *psb*H transcript. They propose that the absence of the H transcript in the nuclear mutants 222E and GE2.10 results from the defective nuclear protein failing to bind to similar motifs on a transcript for *psb*H, which has its own promoter upstream of the gene, and that *psb*T and *psb*N transcript accumulation is independent to that of *psb*H.

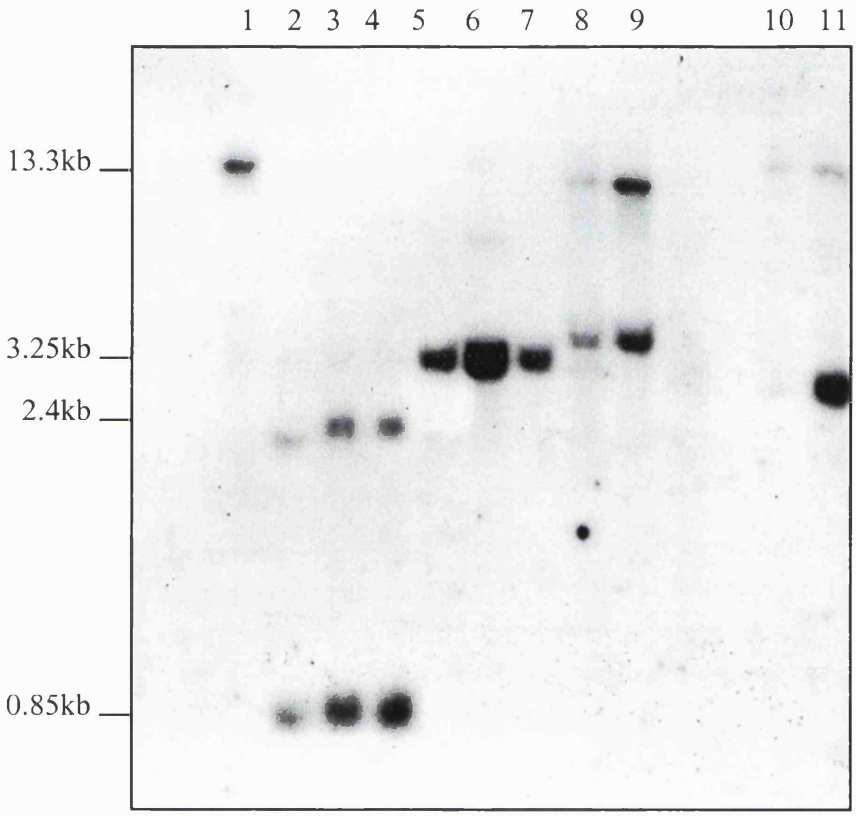
Results

3.2 Confirmation of PSII-H mutant phenotypes

Before any further analysis of these mutants was performed I decided to confirm that the cell lines were still homoplasmic for the mutations by performing a southern blot. Genomic DNA was prepared from all the mutants and wild type CC1021 cells by the method of Rochaix et al. (1988), then digested with the enzyme *Pst*I, separated by electrophoresis and transferred to a nylon membrane. This filter was then hybridised with a random labelled probe made from a 700bp fragment containing the *psb*H coding sequence and the results can be seen in Figure 3.4.

The wild type copy of the *psb*H gene lies on a 13.3kb *Pst*I fragment. The *aad*A cassette, present in all the mutants contains an internal *Pst*I site which means that all the mutants will have the probe hybridised to a smaller fragment. The *psb*H insertional mutants were created by cloning the *aad*A cassette into a *Bst*XI site within the *psb*H coding sequence. The presence of an additional *Pst*I site within the gene creates two fragments with regions complementary to the probe which total 15.2 kb, 13.3kb wild type sequence and 1.9kb *aad*A cassette.

Figure 3.4 Southern analysis of the *C. reinhardtii* *psb*H mutants



Genomic DNA was prepared from :
1. Wild type
2-4. T3A
5-7. MluI
8-9. H-null same
10-11. H-null opp
The DNA was digested with *Pst*I and the filter was probed with a 700bp fragment containing the *psb*H coding sequence.

The pattern differs between H-same and H-opp because *aad*A is cloned in opposing orientations and the *Pst*I site cleaves the cassette approximately 450bp from its 3' end.

The site directed mutant was created from a plasmid carrying the *psb*H region by inserting the *aad*A cassette into a downstream *Mlu*I site in the opposite orientation to the gene then using a two step PCR procedure to replace the threonine codon. The sequence in the wild type gene is shown in Figure 3.5 below as is the altered sequence of the site directed mutant.

Figure 3.5

Wild type	Site directed change
ATG GCA ACA GGA	ATG GCT GCA GGA
M A T G	M A A G

By altering the codon for alanine at position one of the mature peptide an additional *Pst*I site is introduced. Both the *Mlu*I insertion and the site directed mutation were transformed into *C. reinhardtii*. When the DNA from these transformants is examined a single 3.25 kb band is seen in the MluI mutants. The site directed T3A cells have the additional *Pst*I site which further cleaves the 3.25kb band into fragments of 2.4 and 0.85kb. The appearance of these two bands confirms that the site directed changes have not reverted to the wild type sequence.

3.2.1 Confirmation of the site directed change

The southern analysis described above confirmed that the threonine to alanine change was still present in the mutant cells. This was periodically checked by PCR analysis to ensure that the loss of the phosphorylatable threonine was a persistent characteristic. A PCR reaction was performed using dilute QiaAmp purified genomic DNA from MluI and T3A cells and the PCR primers psbhnest and atpadown (Table 2.3). These primers hybridise between the *psb*H and *psb*N genes and at the 5` end of the *aad*A coding sequence respectively. The PCR

reaction was carried out for 30 cycles as described in the methods section with an annealing temperature of 70°C and yielded a 1.9 kb product. The amplified DNA was gel purified using the Qia Quick kit and 7µl (from a total of 50µl eluted from the column) was digested with *Pst*I. After incubation of the digests overnight at 37°C the products were run on a 1% agarose gel.

DNA from the MluI cells contains one *Pst*I site within the *aad*A cassette and yields fragments of 1090bp and 810bp. The site directed change introduces a further *Pst*I site cleaving the larger fragment giving DNA of 880, 810 and 210bp in size. The gel photo in Figure 3.6a shows that the restricted DNA is equivalent to the predicted sizes. Two of the fragments from the T3A digest are very similar in size and are not resolved on this gel.

3.3 Analysis of the PSII-H mutants

3.3.1 Growth requirements

The phenotypes of the mutants were examined by comparing their abilities to grow on different media. Tests were performed by dropping 5µl aliquots of stationary phase liquid culture onto TAP media with and without spectinomycin and HSM media which supports only photosynthetic growth. Three representatives of each class of mutant were tested in this way along with a wild type culture and a naturally occurring non photosynthetic mutant B4. Figure 3.7 shows that on TAP plates all cell types grow healthily and that all the H transformants can tolerate the 50µg/ml spectinomycin in the TAP Sp plates. Wild type and B4 cells have no resistance to this level of antibiotic as is to be expected. On the HSM media which contains no reduced carbon source, the site directed and control MluI mutants performed as well as the wild type cells showing that they are capable of photosynthetic function. In contrast B4, a known photosynthetic mutant and the *psb*H disruption mutants were incapable of photosynthetic growth. This finding contrasts with the situation in *Synechocystis* where the disruption of the *psb*H gene shows that it is not essential for photosynthetic growth (Mayes et al. 1993). A similar study in *C. reinhardtii* by Summer et al. (1997) supports our finding and concludes that PSII-H is necessary for the accumulation of the PSII core proteins.

Figure 3.6a
Gel photo showing the results of a *Pst*I digest of PCR products amplified from MluI and T3A genomic DNA.

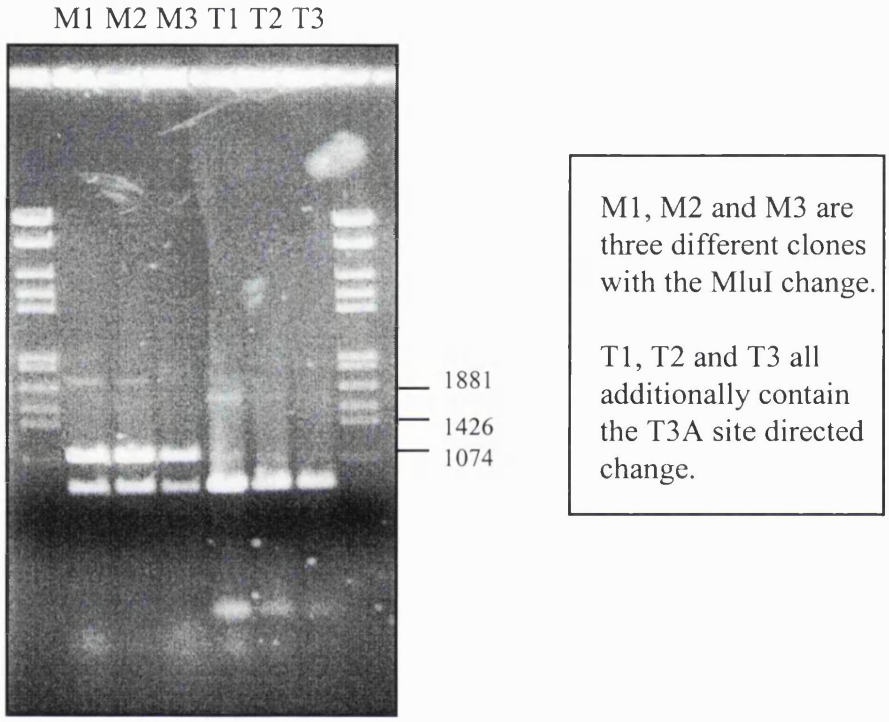


Figure 3.6b
A map showing the positions of the *Pst*I restriction sites on the two PCR produtcs.

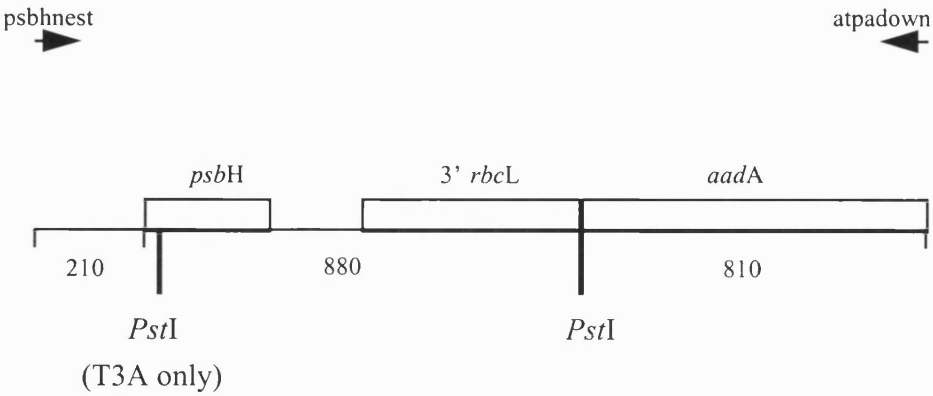
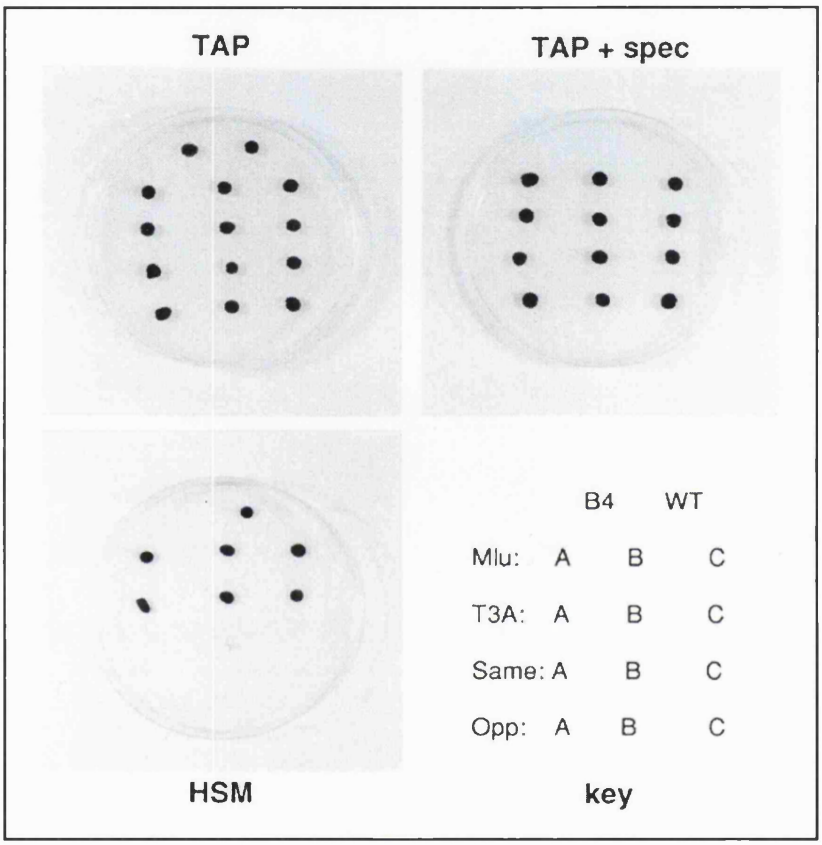


Figure 3.7 Spot tests of *C. reinhardtii* PSII-H mutants



The cultures used to set up these spot tests were grown in TAP media under 10μE/m^2/s. Three isolates of the mutant cell types were applied to each plate in the manner shown above, then they were placed for incubation under a light intensity of 40μE/m^2/s.

3.3.2 Oxygen evolution

Oxygen evolution measurements were made for wild type and all the PSII-H mutants in both cells and thylakoid membranes. The apparatus used was a Clarke type electrode which has a platinum wire cathode and a silver anode connected by a KCl salt bridge. The electrodes are covered by a teflon membrane, permeable only to oxygen, which protects the electrodes from the redox reagents. When a voltage is imposed in the presence of oxygen a current flows between the electrodes. Over the range of 1.4-1.8 volts the current output is linearly proportional to the oxygen concentration.

The cells or membranes were placed in the chamber, the amount used being equivalent of 50μg of chlorophyll. This amount was chosen because it gives the highest proportional rates of evolution per μg of chlorophyll present. Electron acceptors were added to promote the transfer of electrons away from PSII. In these experiments the quinone DMBQ, which accepts electrons primarily from the Qb site, is used in conjunction with potassium ferricyanide which keeps the quinone oxidised.

Table 3.1 shows the averaged results of oxygen evolution measurements from cells in the presence of 1mM of both acceptors. At no time was any oxygen evolution recorded for either of the *psb*H deletion mutants which indicates that either the cells are lacking in PSII or that their oxygen evolution is inactive. The control mutation, MluI, was tested to ensure that introducing the *aad*A sequence after the gene did not affect the cells in anyway. This is confirmed by oxygen evolution rates for these cells which were consistently equivalent to wild type rates. The site directed mutant which differs from MluI by only one codon of the *psb*H coding sequence however showed a slight decrease in the rate of oxygen evolution in comparison to wild type rates. These trends were mirrored when oxygen evolution rates were examined in thylakoid membranes (data not shown).

Table 3.1

Rates of oxygen evolution of wild type and PSII-H mutant cells in the presence of electron acceptors DMBQ and ferricyanide.

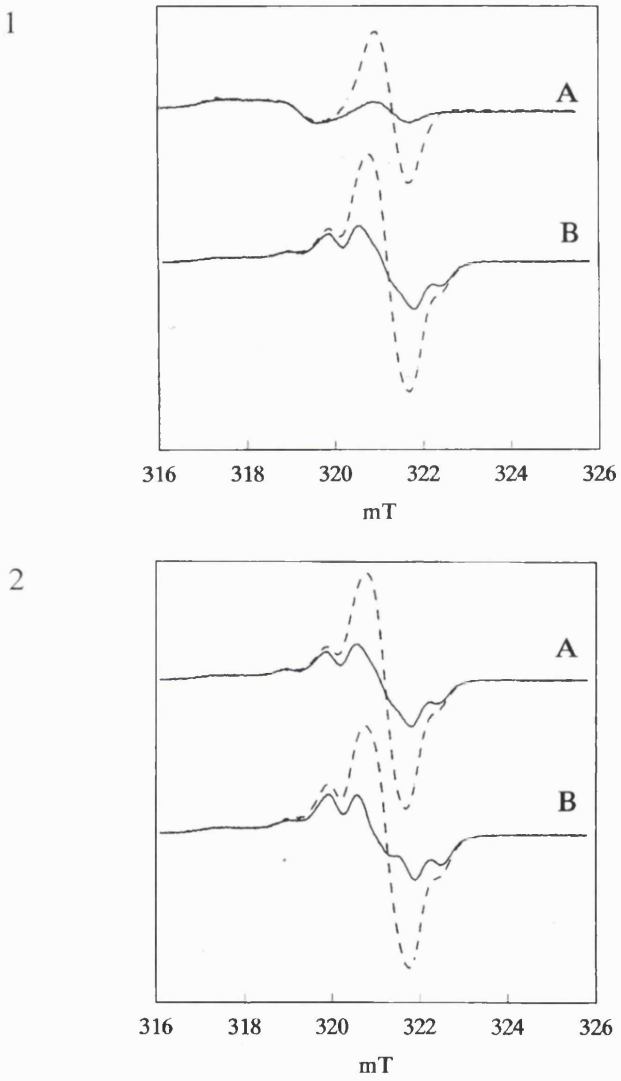
Results shown are an average taken from readings made on five separate occasions.

Cell Type	Oxygen Evolution ($\mu molO_2$/ mgchl/ hr)
Wild type CC1021	150
Mlu1	142
T3A	98
H null - same	0
H null - opp	0

3.3.3 Electron Paramagnetic Resonance analysis

Electron paramagnetic resonance (EPR) spectroscopy is used to study unpaired electrons (reviewed by Miller & Brudvig 1991). EPR signals have been identified for all the electron carriers within PSII from the manganese complex to Qb. In general only alternate oxidation states are paramagnetic and most photosynthetic electron carriers give a signal either after a flash of light or following a period of dark adaptation. In PSII the only dark stable signal visible by EPR is that of oxidised tyrosine D, Y_D^+. Y_D can donate an electron to $P680^+$ but unlike Y_Z is not thought to be involved in oxygen evolution. Y_D is oxidised during illumination but in a following dark period only 25% is re-reduced. It has been proposed (Styring & Rutherford 1987) that centres which remain in the S_0 state at the end of illumination, approximately 25%, use the electron from Y_D^+ to transfer to the S_1 state. EPR spectroscopy was used to analyse cells from all classes of mutant. Cells were grown to stationary phase in TAP media then EPR samples were prepared as described in Methods Section 2.18. Once the samples were placed into the EPR tubes the cells were dark adapted for at least 30 minutes before being frozen in liquid nitrogen.

Figure 3.8b
EPR data from the H null and T3A mutants



Panel 1 shows EPR spectra from A. PSII-H null and B. wild type cells
Panel 2 contains the spectra from A. T3A and B. wild type cells.
The solid line shows the dark adapted signal and the illuminated trace is displayed as a dashed line.

Figure 3.8 shows EPR traces from wild type, PSI minus and PSII minus *C. reinhardtii* cells. Both the dark adapted and illuminated traces are shown. The smaller signal in each case is due to the dark adapted state found in each cell type. Wild type and PSI minus cells show a typical Y_D^+ signal which is missing in the PSII mutant. When the samples are illuminated the $P700^+$ radical is formed partially obscuring the signal from PSII, except for the cells which lack PSI centres.

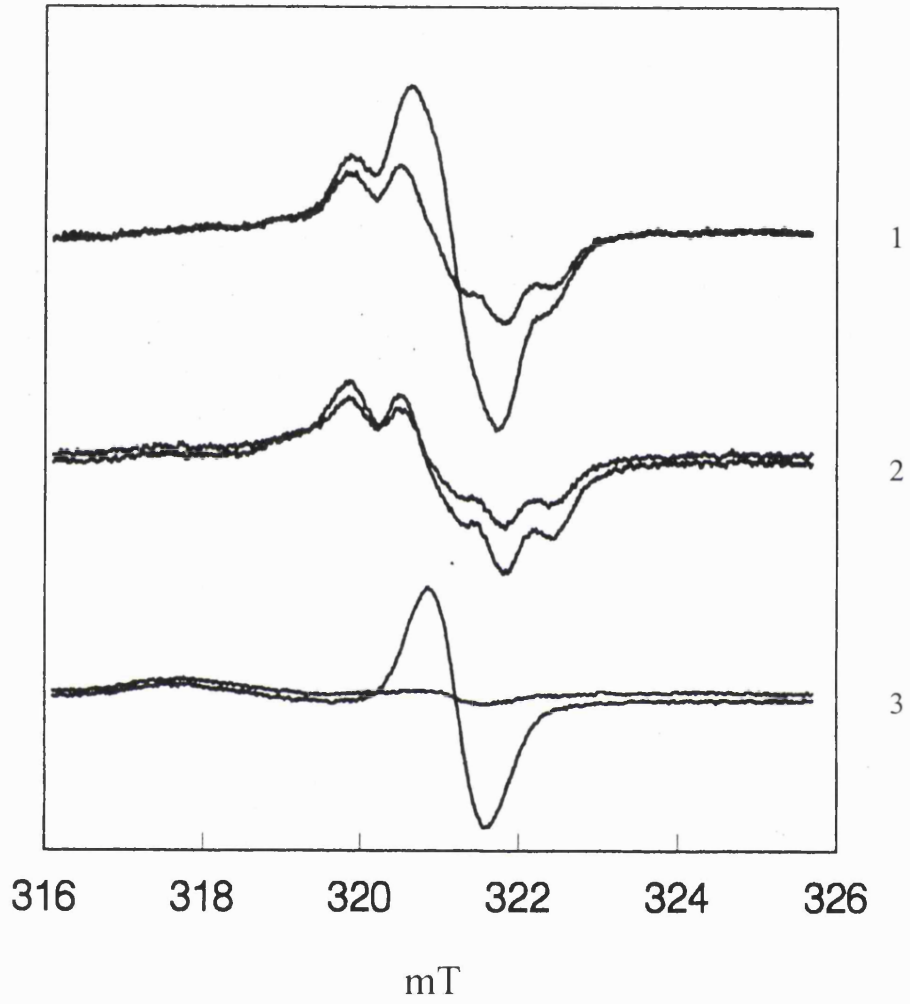
When PSII-H mutants were examined by this technique the signals recorded by the T3A and MluI cells were identical to traces from wild type cells while the PSII-H null mutants were able to produce the $P700^+$ signal but no amount of Y_D^+ was observed. This confirms that the absence of PSII-H leads to a PSII minus phenotype and is shown in Figure 3.8b

3.3.4 Phosphorylation analysis

The one characteristic of PSII-H which distinguishes it from most of the other subunits in the complex is its ability to become phosphorylated. The position of the phosphorylatable residue was found to be a threonine at position two of the mature protein (Dedner et al. 1988). This report determined that radiolabelled phosphate was incorporated into threonine two but not threonine four. In order to determine the function of this post translational modification we created a mutant with an alanine residue replacing the modified threonine. The PSII-H mutants were then examined in an *in vitro* phosphorylation analysis.

The first assays were carried out using thylakoid membrane preparations described in Section 2.15. These were prepared by opening the cells in a French press, separating the membrane components by centrifugation and then applying the membrane fraction to a sucrose density gradient. The thylakoid membranes which float between the two layers of sucrose were then collected and washed before being frozen in liquid nitrogen. The assays were carried out as detailed in Section 2.13. Samples were pre-incubated in either light or dark conditions which should mimic reduced or oxidised states seen in cells. After thirty minutes, the phosphatase inhibitor, NaF, and ^{32}P-γATP were added to the samples and incubation continued for a further 30 minutes. The reaction was stopped by the

Figure 3.8 EPR traces from wild type, PSI minus and PSII minus *C. reinhardtii*



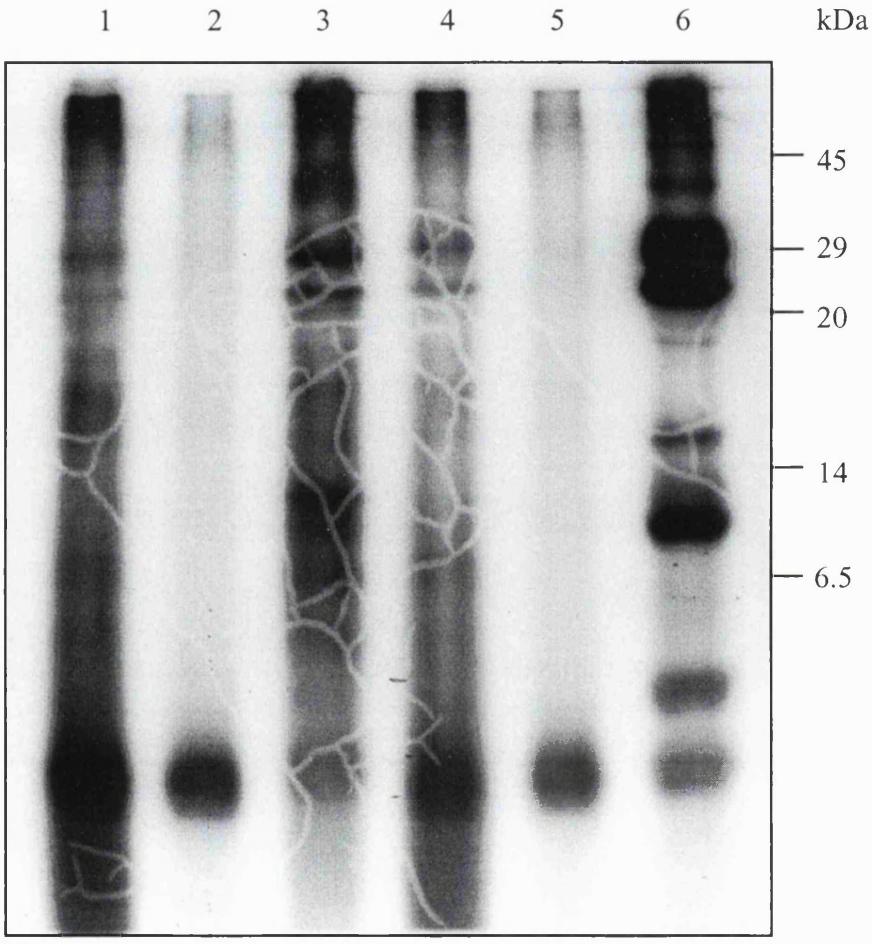
EPR traces obtained from :
1. Wild type
2. PSI minus
3. PSII minus
Each trace shows the dark adapted signal (the smaller of the two) and the effect of illumination on the sample

addition of EDTA and the samples were subjected to SDS-PAGE and subsequent autoradiography. The results of this experiment are shown in Figure 3.9.

The wild type samples, in tracks 3 and 6 show a large increase in the amount of incorporated ^{32}P in certain bands in the light incubated sample. The most notable of these are the light harvesting proteins which can be seen above the 20kDa marker. Another significant band can be seen between the 6.5 and 14 kDa marker proteins which I believe corresponds to the PSII-H protein. The PSII-H minus mutant in tracks 1 and 4 shows very low incorporation into the LHC bands and no visible PSII phosphoproteins. This is consistent with the PSII minus phenotype of these cells. The remaining two tracks, 2 and 5, contain the PSII-H site directed mutants which lack the modifiable threonine. These tracks show absolutely no incorporation of the labelled ATP. This result has been seen on several occasions and no bands have ever been visible. This would imply that in the absence of PSII-H phosphorylation no other protein can be phosphorylated.

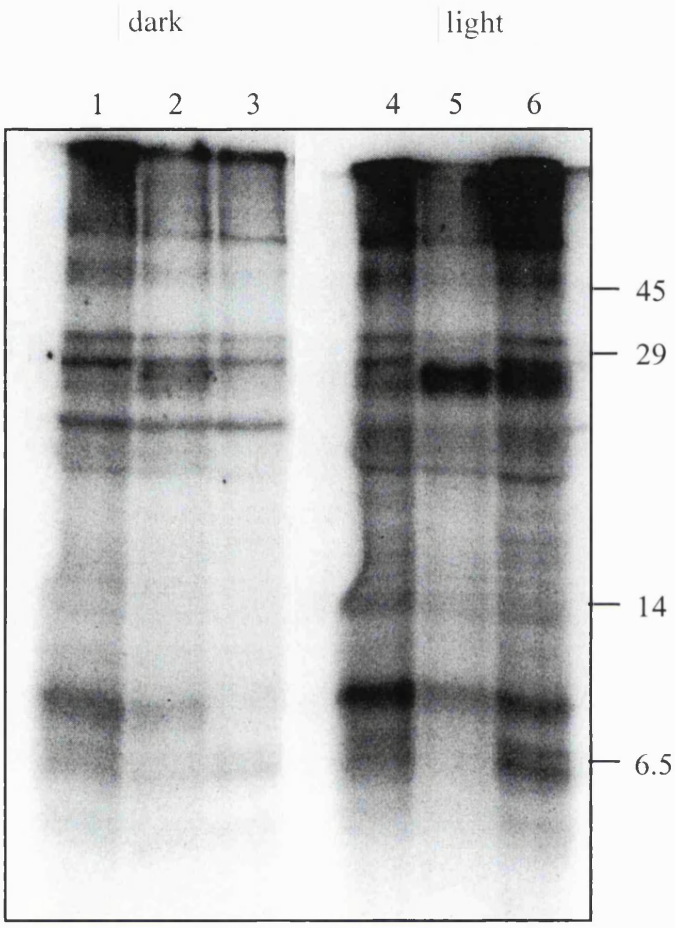
In addition to carrying out the assay using thylakoid membranes a broken cell experiment was also performed. This involved using a sonicator to lyse the cells then the membranes were separated by centrifugation from the supernatant. The membrane fraction was then used in an *in vitro* phosphorylation assay as described in Section 2.13. The results for this type of assay are shown in Figure 3.10. The most notable difference between this result and that shown in Figure 3.9 is the presence of phosphorylated bands in the T3A track. This implies that the effect shown in Figure 3.9 was not caused by the site directed change alone but that the preparation of the sample was also significant. The thylakoid membranes prepared from wild type cells showed clear phosphorylation patterns which behaved as expected so the technique by itself does not impair phosphorylation. However in the site directed mutant the extra sucrose density centrifugation step results in a sample which does not undergo phosphorylation. This effect must be caused by the single amino acid change or be related to the presence of the *aad*A cassette. MluI cells were not used in the thylakoid phosphorylation assay but the presence of these would confirm whether the amino acid substitution or the presence of exogenous DNA in the operon was the direct cause. It is possible that the changes found in the T3A cells alter the PSII complex in some unknown way

Figure 3.9 ***In vitro*** **membrane phosphorylation assay**



The autoradiogram shows the results of an *in vitro* phosphorylation assay carried out on *C.reinhardtii* PSII membranes. The membranes were prepared from wild type (3 & 6), T3A (2 & 5) and H null (1 & 4) cells. All samples contained 25μg of chlorophyll. Tracks 1-3 were incubated in the dark during the assay while the samples in tracks 4-6 were illuminated

Figure 3.10 *In vitro* broken cell phosphorylation assay



Cells, broken open by sonication, were used in an *in vitro* phosphorylation assay. Dark incubated samples were prepared from :
1. Wild type
2. MluI
3. T3A
Tracks 4-6 contain the same samples but were incubated in the light.

so that the final centrifugation step causes the loss of some factor from PSII. This may be the kinase enzyme which directly phosphorylates the proteins or some factor involved in the activation of the enzyme.

The banding patterns seen in the broken cell assays were not consistent and did not give results which help elucidate any further information about PSII-H. The only band in the correct position to correspond to the PSII-H protein is visible on Figure 3.10 above the position of the 6.5kDa marker. The banding pattern of this protein however does not reflect the expected result. The protein appears more heavily phosphorylated in the dark than in the light incubated sample which is at odds with the result shown in Figure 3.9. In addition the band is present in the T3A dark incubated sample so if this protein does correspond to PSII-H it would imply a second site for phosphorylation in this protein. The report by Dedner et al. (1988) found that in the N-terminal region of the first 15 residues only Thr-2 was phosphorylated. This does not rule out the possibility of another residue being capable of the same modification. An additional conserved threonine residue lies just outside the region which was examined and could potentially be modified. If the band discussed does not correspond to PSII-H then there are no other obvious candidates in the correct size region. No conclusions can be drawn from the *in vitro* phosphorylation experiments because of the lack of consistency and the problem of identifying the PSII-H protein. Western blots were also attempted on these preparations to confirm the position of the PSII-H band. Two antibodies were used in these experiments but neither bound to a PSII-H protein in a *C. reinhardtii* membrane preparation. One of the antibodies was raised against a peptide based on the spinach sequence. When this failed to recognise the *C. reinhardtii* protein we ordered one to be made specifically against the algal sequence but again antibodies did not hybridise.

In order to try and clarify this situation numerous attempts were made to carry out an *in vivo* phosphorylation assay. The results from these experiments are not shown here and did not give any further information. Problems were encountered with both the assay and the gel electrophoresis steps of the experiments and no usable information was gained. The cell cultures have been passed to other laboratories with more experience in these techniques. They have yet to report back with any improvement on the situation we found.

3.4 Analysis of PSII-N mutants

3.4.1 Growth requirements

Spot tests on different types of media were created, as for the *psb*H mutants, by dropping 5µl aliquots of stationary phase culture onto petri dishes. Once the culture had dried the plates were incubated at either 15°C or 25°C with illumination levels of 60 or 300µE/m^2/s. One isolate of each of the control mutations, PS6 and PS10 and four of the *psb*N disruption mutant were examined in this way together with wild type and PSII minus controls. The results of this experiment are shown in Figure 3.11.

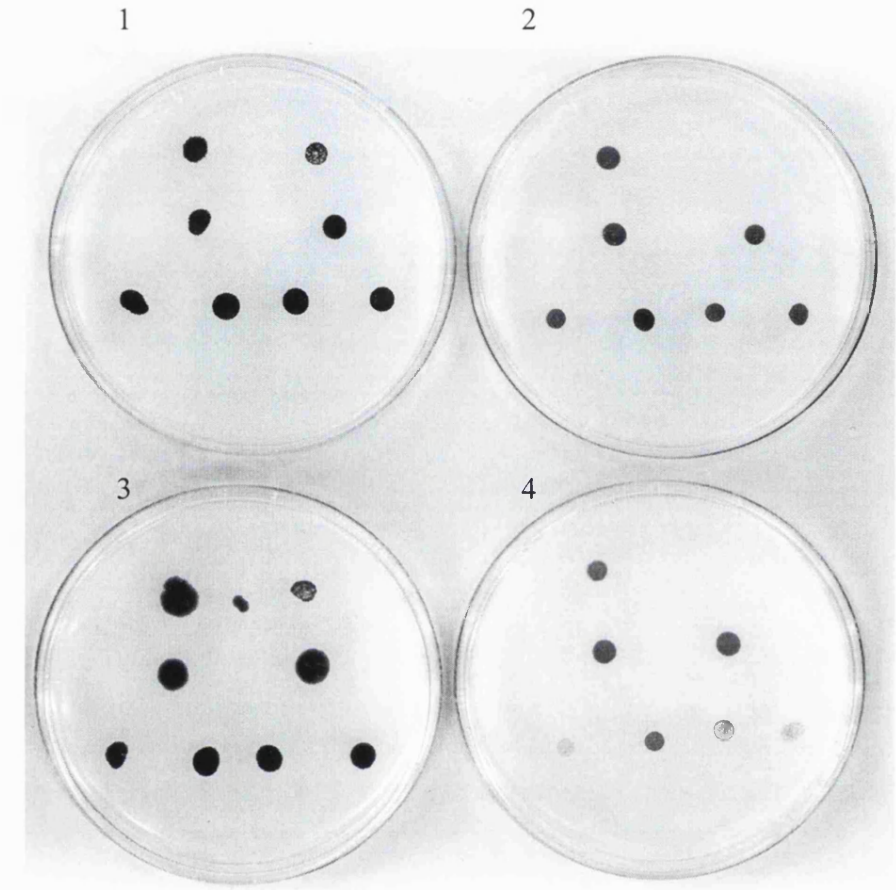
All three mutant cell types grew well on acetate containing media under all conditions tested. The insertion of the *aad*A cassette downstream of the gene does not appear to affect growth in any of these situations. The 'spots' of these mutants grew as strongly as wild type cells on all of the plates.

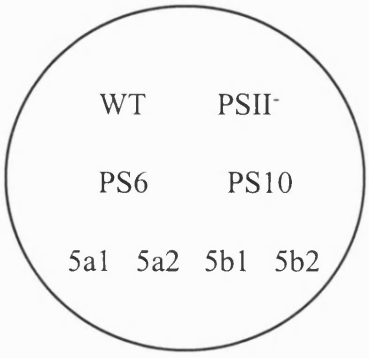
The PSII minus cells were incapable of supporting growth on HSM plates and at lower temperatures do not grow well even when supplied with acetate. The PSII-N disruption mutants were capable of photosynthetic growth under lower light intensities and a visual comparison suggests that they are as fit as wild type at this level of illumination. However at 250µE/m^2/s these cells do not appear to be as competent as wild type or the control insertional mutants. This suggests that PSII-N maybe stabilising PSII in some way and in its absence the cells are more susceptible to photoinhibition. An increase in the level of photoinhibition has been reported for many mutants which lack a PSII subunit. This characteristic may reflect the presence of an imperfect PSII complex rather than suggest that each of the missing subunits is directly involved in photoinhibition.

3.4.2 Fluorescence analysis

Plates were prepared, as described in Section 2.14, in duplicate for each sample to be tested. All were incubated overnight at 18°C and a light intensity of 4µE/m^2/s. The following day half the plates were transferred to a bright light incubator at 200µE/m^2/s and left for a further 48 hours. The plates were then analysed using the Plant Efficiency Analyser. Analysis of wild type cells using this instrument shows an increase in fluorescence from F_o to F_M followed by a decline

Figure 3.11 ***C. reinhardtii*** **PSII-N mutant spot tests**





The cultures were applied to the test plates in the pattern shown here. Plates 1 and 3 contain TAP media while plates 2 and 4 are HSM. The cultures on plates 1 and 2 were incubated under 50 uE/m^2/s while plates 3 and 4 were placed under high light intensity of 250 uE/m^2/s.

to a steady state value. Mutants without functional PSII show no variable fluorescence. The initial increase seen in a PSII mutant is caused by the chlorophyll *a* antenna but this level does not decline indicating a lack of variable fluorescence. Examples of wild type and PSII minus signals are shown in Figure 3.12 along side the results from the *psb*N mutants.

The signals from PS6 and PS10, which have the *aad*A cassette inserted after the gene, are identical. The plates incubated under low levels of illumination look exactly the same as wild type cells with an increase up to F_M followed by a region indicating variable fluorescence. Following the higher light incubation the signal varies slightly from that seen in the wild type cells.

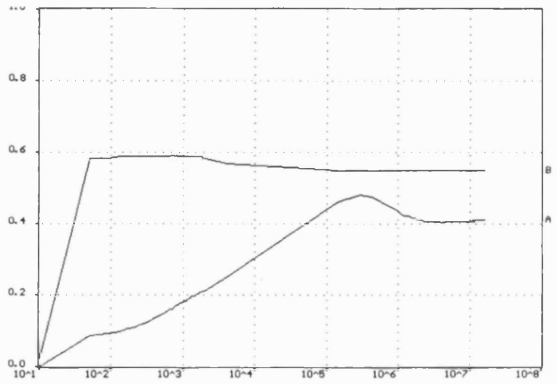
The results from the *psb*N disruption mutant, PB5, differ according to the light intensity they were incubated under. The signal resulting from the low levels of illumination resembles that of wild type, showing the same characteristic pattern. However after incubation at high light levels these cells show no variable fluorescence and the signal is characteristic of a cell lacking PSII. This evidence appears to strengthen the hypothesis that PSII-N has a role in stabilising the photosystem and under high light its absence leads to a loss of PSII centres.

3.4.3 Oxygen evolution rates

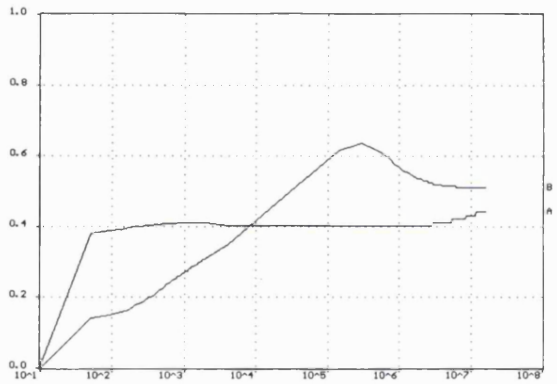
The rates of oxygen evolution created by the different PSII-N mutants were compared to the rates seen for unmutated wild type cells. The procedure employed was the same as described previously and cells were grown under low light intensity. The results, shown in Table 3.2, are the average values taken after five replicates had been performed. The results show that the mutants with the cassette inserted after the gene, PS6 and PS10, are affected in the rate of oxygen evolution. The cells are still able to function but are not capable of achieving wild type rates of oxygen evolution. In comparison the PSII-N disruption mutant PB5 show an even lower rate of oxygen evolution.

Insertion of the DNA for the *aad*A cassette after the *psb*N gene is causing a noticeable phenotype which was not seen for the equivalent PSII-H mutant. This could either be due to an effect on the *psb*N transcript or be destabilising the transcript on the opposite strand which encodes the genes of the *psb*B operon. The disruption of the *psb*N coding sequence has an even more severe phenotype

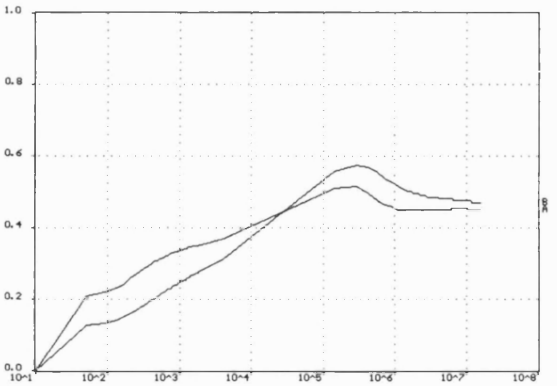
Figure 3.12 Fluorescence analysis of PSII-N mutants



A. wild type, B. PSII minus,
Line shape shown is typical
for these cell types and does not
vary with light intensity.



PSII-N minus cells
B. dim light, A. high light.
Under high levels of illumination
traces resemble PSII minus cells
while at low light intensity show
wild type characteristics.



Insertion downstream of *psb*N
A. dim light, B. high light.
Pattern produced does not vary
under incubation conditions.
PS6 and PS10 produce the same
pattern of results.

showing that this mutation has an additional effect. Some of the loss of oxygen evolution for this mutant may be a result of the destabilisation of the transcript on the opposite strand, as is potentially the case for the PS6 and PS10 mutants. The difference in the values between the PS cells and PB5 must be due to the loss of the PSII-N protein so we can conclude that the lack of this protein is affecting the level of PSII function seen.

Table 3.2

Rates of oxygen evolution of wild type and PSII-N mutants in the presence of electron acceptors DMBQ and ferricyanide.

Cell Type	Oxygen Evolution ($\mu molO_2$/ mgchl/ hr)
Wild type CC1021	150
PS6	67
PS10	62
N null - opp	36

3.5 Conclusions

The role of the PSII-H protein within the PSII complex has yet to be determined. A *Synechocystis* sp PCC 6803 deletion mutant for this subunit (Mayes et al. 1993) was shown to have a functional PSII but electron flow between the two quinones was impaired. The effect of deletion of some subunits has been shown to be more severe in eukaryotes than in prokaryotes such as *Synechocystis* sp PCC 6803. An example of this is the PSII-K subunit. The cyanobacterial deletion mutants are still capable of autotrophic growth whereas the *C. reinhardtii* deletion strain is unable to grow in the absence of acetate. This shows that while the complexes are basically similar in structure and function there are significant differences in the eukaryotic system.

A series of mutants were created in the *psb*H gene of *C. reinhardtii* with the aim of refining the knowledge of the function of this protein. Two deletion mutants were created by inserting the *aad*A cassette into an restriction site within the coding sequence. The extraneous DNA was added in both the same and opposite orientations as the coding sequence. The deletion mutants were found to lack any active PSII and hence were incapable of photoautotrophic growth. This differs from the situation found in *Synechocystis* but is not a unique example of this phenomenon. We attempted to confirm that the PSII-H protein was not expressed in these mutants, by using western blots, with PSII-H specific antibodies. We used an antibody raised against the spinach protein and one which we ordered against the *C. reinhardtii* sequence. Although numerous assays were performed neither antibody ever reacted to a protein present in any *C. reinhardtii* sample.

Two further mutants were created in *C. reinhardtii* to try and discover the significance of the phosphorylation of this subunit. The *aad*A sequence was inserted into a site downstream of the gene, in the opposite orientation (MluI). This mutant was to act as a control for all others in this region. The position of the *psb*H gene, in an operon with a number of others, led to the possibility that inserting DNA may disrupt the expression of the other genes. The results of all the experiments to characterise this strain showed that the phenotype of this mutant did not differ significantly from the wild type control so it is unlikely that any alteration in the other mutants is due to the presence of the *aad*A sequence.

The last PSII-H mutant constructed carried a site directed change in addition to the MluI addition of the *aad*A cassette. The altered residue was the threonine at position two in the mature protein. This residue is known to be phosphorylated in a light dependent manner but the role of this modification has yet to be conclusively determined. The site directed change altered the threonine to an alanine which cannot be modified by post translational phosphorylation. The initial characterisation of this mutant did not lead to any new conclusions. The cells were photosynthetically competent and capable of oxygen evolution but the rate was slightly lower than that seen in wild type or MluI cells.

The next experiments carried out on this mutant used an *in vitro* phosphorylation assay to determine whether any effect on the phosphorylation profiles could be seen. *In vitro* experiments were carried out on two different preparations. The first was a purified thylakoid membrane fraction and the second consisted of all the membrane fractions from *C. reinhardtii* cells. The difference between the two experiments in the T3A sample was dramatic. In the broken cell preparation phosphorylation of proteins was clearly obvious while in the thylakoid membrane sample no phosphorylation was observed. The wild type thylakoid sample behaved in the manner expected with significant phosphorylation of the LHCII and PSII-H proteins in the light. This implies that the preparation does not cause the effect observed in the T3A sample but some change inherent in the cells. There are two possible modifications which could have caused the effect; the presence of the *aad*A cassette and the site directed change. The MluI cells would act as a control in determining which of these factors is the most important in producing this result. The changes in the T3A cells must alter the PSII complex in some way that when the thylakoid membranes are purified some extra factors are lost. These could be involved at some stage in the activation of the kinase or could even be due to the direct loss of the kinase.

No further conclusions can be drawn from any of the phosphorylation assays carried out. It is possible that the PSII-H protein is still phosphorylated in the site directed mutant but this has not been confirmed. The results from the broken cell *in vitro* experiments did not show the characteristic strong phosphorylation patterns of LHCII and PSII-H in the light incubated samples so the possible phosphorylation of the mutant inferred from the same gel is suspect.

Further analysis has been commissioned in other laboratories which may help further our understanding of the phosphorylation of this protein.

The *psb*H gene lies in an operon with those of three other subunits of PSII, PSII-B, PSII-T and PSII-N. It was decided that while we were investigating one gene in this operon it would be easy to extend this work to another. The PSII-N subunit was chosen as mutants had previously been created in the other two genes. Four different mutants were created in the region around the *psb*N gene. Two were insertion mutants with the *aad*A cassette interrupting the coding sequence while in the other two the extra DNA was introduced outside of the coding region. The PSII-N null mutant, only one of the two created reached homoplasticity, is able to grow autotrophically but further analysis has shown that the level of PSII activity is not as great as in the wild type strain. The result from the deletion mutants is compared to that seen from the control mutants which contain the *aad*A cassette but contain an intact coding sequence. These cells were also photoautotrophic but were capable of higher levels of oxygen evolution than the deletion mutants. Despite this improvement the rate is still only 50% of that seen for wild type cells. This suggests that introducing DNA into the region around *psb*N is disrupting the expression of a protein which is required for optimal function. The *psb*N gene is on the opposite strand to all the others in the operon, and lies between *psb*T and *psb*H. The introduction of the *aad*A cassette may be influencing the expression of the genes on this other strand, most likely *psb*H as this lies downstream. The phenotype is not as severe as that seen for the PSII-H deletion mutants which tells us that PSII-H is transcribed but that the level of protein present may be less than seen in wild type cells.

When the *psb*N gene is disrupted the rate of oxygen evolution falls and the cells appear to be more susceptible to high light damage. This leads us to the conclusion that at low levels of light the PSII complex can function efficiently in the absence of this protein but at higher levels of illumination it is required to stabilise the complex. The exact role of PSII-N cannot be determined.

Chapter 4

Molecular examination of nuclear PSII mutants and the *ycf*9 gene

4.1 Introduction

The chloroplast is a DNA containing, semi autonomous organelle which is probably derived from an endosymbiotic cyanobacterium-type organism which subsequently lost most of its genetic information to the nuclear genome. In recent years a number of chloroplast genomes have been completely sequenced including those of tobacco (Shinozaki et al. 1986), liverwort (Ohyama et al. 1986) and rice (Hiratsuka et al. 1989). These studies show that over 100 genes are located in this plastid genome, of which about half are involved in the transcriptional and translational apparatus e.g. genes for rRNAs, tRNAs and approximately thirty encode subunits of the five photosynthetic complexes PSII, cytochrome b_6f, PSI, ATP synthase and ribulose bisphosphate carboxylase / oxygenase. The function of a number of genes remains to be determined.

The genes for the remainder of the proteins found within the chloroplast, including the other subunits of the photosynthetic complexes and many proteins involved in gene expression, are found within the nucleus. These nuclear encoded proteins are translated as precursors on cytosolic ribosomes and imported across the chloroplast envelope where they interact with chloroplast encoded proteins to form the functional complexes.

Other nuclear encoded gene products, which migrate to the chloroplast, are involved in the control of expression of some chloroplast genes enabling the nucleus to co-ordinate nuclear and plastid gene expression. These nuclear encoded factors have been shown to be able to act at the levels of transcription, RNA stability, RNA maturation and translation of the mRNA molecule.

C. reinhardtii has proved to be a popular organism for the study of these nuclear genes because of its ability to use acetate as an alternative energy source. Hence nuclear mutants, which cause a deficiency of an essential chloroplast protein, can be cultured on acetate containing media. Tagged nuclear mutants can easily be created by glass bead transformation of the nucleus then mutants selected for and screened for perturbed photosynthetic ability. The complex and chloroplast gene affected can be determined, then the interrupted nuclear gene identified and characterised. A number of nuclear encoded factors have been identified which affect the expression of PSII, $cytb_6f$ and PSI components, see Table 4.1.

Table 4.1

Examples of nuclear mutations affecting chloroplast gene expression

Gene	Mutant	Nature of mutation	Reference
***psb*A**	F35	No translation	Girard-Bascou et al. (1992)
***psb*B**	GE2.10	RNA stability	Sieburth et al. 1991)
***psb*B**	222E	RNA stability	Monod et al. (1992)
***psb*C**	6.2z5	RNA stability	Sieburth et al. (1991)
***psb*C**	F34	No translation	Girard-Bascou et al. (1992)
***psb*C**	F64	No translation	Rochaix et al. (1989)
***psb*D**	nac1-11/18	No translation	Kuchka et al. (1988)
***psb*D**	nac2	RNA stability	Kuchka et al. (1989)
***psa*A**	e.g. F1, M10	mRNA maturation	Choquet et al. (1988)
***atp*A**	F54	No translation	Drapier et al. (1992)
***atp*A**	ncc1	RNA stability	Drapier et al. (1992)
***atp*B**	thm24	RNA stability	Drapier et al. (1992)
***pet*A**	Mϕ11	RNA stability	Gumpel et al. (1995)
***pet*B**	Mϕ37	RNA stability	Gumpel et al. (1995)

Studies of nuclear encoded factors affecting PSII activity have found examples which affect both the stability of mRNA transcripts and their expression. One example by Sieburth et al. (1991) identifies two different mutations obtained by exposure to UV and enrichment with metronidazole for photosynthetic electron transport mutants. One mutant, 6.2z5 showed a lack of PSII subunits in an SDS PAGE analysis of a thylakoid preparation but a Northern analysis demonstrated the presence of all relevant transcripts except that of *psb*C. Further analysis showed that the *psb*C transcripts were synthesised at wild type rates indicating that they must suffer from transcript instability. The second mutant isolated showed no synthesis of the *psb*B encoded protein CP47 which was also due to the long term absence of its mRNA transcript.

One difference between the two mutants lies in the transcription of the other core component mRNAs. In mutant 6.2z5, polypeptides CP47, D1 and D2 are synthesised from their mRNAs at wild type rates then degraded. In mutant

GE2.10 a strong reduction in the translation of the *psb*A mRNA means that D1 is not synthesised, showing a coupling in the translation of CP47 and D1. Other evidence links the translation of D2 and CP47 in a similar fashion (Schmidt et al. 1987). In summary the expression of D1, D2 and CP47 is regulated in a concerted manner. The synthesis of CP43 is not required for the translation of other mRNAs but its presence is essential for ensuring the stabilisation of the other PSII polypeptides.

One commonly observed feature of post transcriptionally regulated genes is the binding of factors to the 3` end of an mRNA molecule. In different systems this can lead to either increased or decreased stability and can be a general or highly specific interaction. A specific example which could be relevant for this scenario is that of the human transferrin receptor mRNA (Liebold & Munro 1988) where a polypeptide binds to a specific 3` sequence conferring stability on the transcript. UV crosslinking has shown the presence of bound polypeptides on the *pet*D and *rbc*L transcripts (Stern et al. 1989). Other chloroplast mRNAs were unable to compete for the binding of these polypeptides suggesting a degree of transcript specificity. Sieburth et al. (1991) point out a 36bp perfect inverted repeat at the 3` end of the *psb*C transcript and put forward the hypothesis that a nuclear factor affected in their mutant, GE2.10, is no longer able to bind this structure causing instability of the mRNA.

Another class of nuclear encoded factors affects not the stability of the mRNA transcript but it translation. Mutants have been isolated which are again deficient in the CP43 protein but this time are not missing the mRNA transcript. There are three known nuclear loci which cause this effect, TBC1, TBC2 and TBC3 (Rochaix et al. 1989, Zerges et al. 1997). The first two nuclear mutants examined were from naturally occurring cell lines called F34 and F64. In pulse labelling experiments both were shown to lack the PSII-C protein. Northern analyses proved that these cells could accumulate wild type levels of the transcript so the nuclear mutation must affect a post transcriptional event. Complementation analysis confirmed that two different nuclear loci were causing the effect. A region of the 5` UTR, upstream of the *psb*C gene, which forms a stem loop structure, has been shown to be involved in the expression of this protein (Rochaix et al. 1989). A chloroplast mutant deficient in CP43 has been sequenced and the only changes

occur in the 5` UTR at this point which decreases the free energy of this structure making it more stable. Additionally, a partial chloroplast suppresser of the nuclear F34 mutation had changes in this region which caused a decrease in stability of the secondary structure. An early hypothesis put forward to explain these results proposed that the stem loop structure had to be destabilised before expression could occur.

The third nuclear locus, TBC3, involved in *psb*C expression (Zerges et al. 1997) also acts at the level of translation. The TBC3 product was shown to bind in the 5` UTR but at a site away from the stem loop structure. In contradiction to their earlier hypothesis the presence of this secondary structure was shown to be essential for gene expression. The group conclude that either TBC 1 and 3, binding at distinct sites, are both required to activate translation or that TBC1 may bind to the stem loop and prevent the TBC 3 repressor from acting at an alternative location. The TBC2 factor is also known to affect the translation of psbC but nothing is known of the nature of its action. The examples highlighted here show that the production of just one chloroplast protein CP43 requires the synthesis of at least four other genes to ensure both the stability of the RNA transcript and then the translation of the protein from it.

The first nuclear mutants identified as being involved in the expression of chloroplast genes came from naturally occurring or chemically mutated cells. An alternative approach was employed in our laboratory to generate further nuclear mutants of this nature (Gumpel et al. 1995). Transforming DNA recombines randomly within the nuclear genome so random mutants are easy to generate. A selectable phenotype is required to ensure that only transformant cells are capable of growth and in this study the arginine requiring mutant *arg7-8cw*$_d$ was rescued with a cloned *arg*7 gene. In order to allow the transforming copy of this gene to be distinguished from the endogenous one a 394bp fragment of ϕX174 DNA was cloned into an intragenic region. The arginine requiring mutants were transformed with this 'flagged' functional copy of *arg*7 gene by the glass bead method of Kindle (1990) and then transformants were selected for by growth in the absence of arginine. The 14000 colonies, recovered by this method, were screened for abnormal steady state fluorescence characteristics then the complex affected was identified by EPR spectroscopy. The result of this was that thirteen mutants were

found to lack the $P700^+$ signal indicative of PSI activity, seven clones showed altered PSII signals and five cytochrome b_6f mutants were identified.

Northern and western analyses of the cytochrome b_6f mutants revealed two distinct phenotypes for two of the mutants. One cell type, Mϕ11 failed to accumulate either the protein or the mRNA from the *pet*A gene encoding the cytochrome *f* subunit (Gumpel et al. 1995). Another, Mϕ37, was deficient in the cytochrome b_6 protein which was shown to be due to the instability of its transcript. These were the first nuclear mutants known to be affected in the synthesis of chloroplast encoded cytochrome b_6f subunits.

Of the thirteen PSI mutants identified six were shown to be affected in the ability to splice the three exons which make up the mature transcript of *psa*A. There are at least fourteen different nuclear loci which are required for this process to be completed successfully. Two further mutants displayed reduced levels of the mature *psa*A transcript and may therefore represent loci involved in the stability of this transcript.

Seven PSII mutants, which displayed less or none of the typical Y_D^+ EPR signal seen in wild type samples, were identified. Mϕ14 later proved to have no observable *psb*D transcript while Mϕ21 was affected in *psb*C expression also at the level of transcript stability. Mating experiments were performed to ensure that the phenotype was inherited together with the flagged copy of the gene. In Mϕ14 there was no evidence of linkage of the two characteristics so the mutation must be caused by the insertion of a partial copy of the transforming DNA with the functional gene inserted elsewhere on the genome. This would make identification of the nuclear gene affected difficult.

The five other PSII mutants were shown to accumulate wild type levels of the *psb*A, *psb*B, *psb*C, *psb*D and *psb*H transcripts. Western analyses using antibodies against the nuclear encoded subunits of the water oxidation complex showed that these proteins were present in these cells and that the phenotype was not due to their disruption. This chapter describes the further analysis of these mutants to try to determine the cause of the PSII minus phenotype. Two further mutants will be discussed, PM10 and PM26. These were isolated in a similar experiment where nuclear mutants were randomly generated using the *arg*7 marker and screened for both altered fluorescence characteristics and growth only in the

presence of acetate. These two mutants were shown to lack PSII activity but no further analysis was undertaken.

Some of the genes for the PSII subunits we were examining exist in operons with other genes e.g. *psb*B, *psb*T, *psb*N and *psb*H. In these instances a nuclear factor may affect the expression of one or all of the genes involved. One of the genes, *psb*M, which encodes a small PSII subunit has been recently sequenced (Higgs et al. 1996) and found in close proximity to the gene *ycf*9. The *ycf* nomination denotes a sequence which is conserved in one or more organisms but has no known function. In *C. reinhardtii* these two genes lie within 500 base pairs of each other and a single mRNA transcript is believed to carry both. The *ycf*9 gene has been found in all photosynthetic chloroplast containing organisms and the sequence is approximately 50% conserved between species. The predicted protein sequence has been analysed and two membrane spanning regions are thought to be present. The domains linking the two helices are believed to be small as are the N- and C-terminal extensions flanking the membrane spanning regions. There was a report that this protein may be a subunit of PSI based on a low degree of sequence homology to a protein found in the PSI complex of *Anabaena* (Bryant 1992). This hypothesis has not been substantiated and no further reports have been issued which address any potential function for this protein. In addition to the characterisation of the PSII nuclear mutants this chapter will describe the creation and characteristics of *ycf*9 deletion mutants in *C. reinhardtii* and *Synechocystis* sp PCC 6803.

Results

4.2 Analysis of PSII Function

Before any further characterisation was performed on these mutants we decided to confirm that they were still deficient in PSII and had not reverted to a wild type phenotype after many months of subculture. Growth tests were performed by placing 5µl aliquots of stationary phase culture onto the surface of acetate containing and minimal media (HSM) plates. The plates were then incubated as described in Section 3.3.13.

Wild type culture and a known PSII minus mutant were used as controls. Figure 4.1 shows the growth achieved by the different cell types under

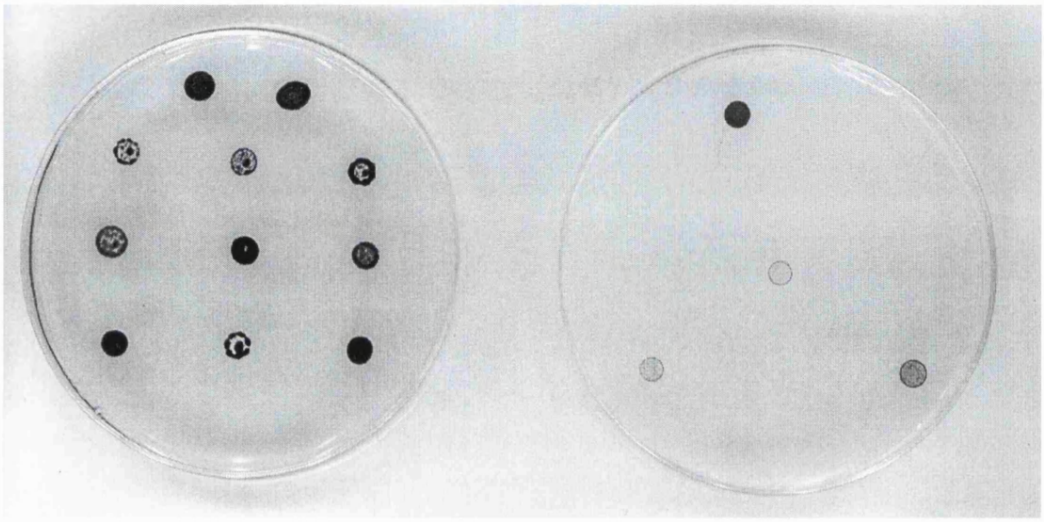
photoautotrophic and mixotrophic conditions. Wild type cells grew to a high density on both types of plate while the PSII deficient cells were incapable of supporting growth under the autotrophic conditions. The Mϕ and PM mutants fell into two different subgroups which describe their levels of growth in this test. The mutants PM10, PM26, Mϕ14, Mϕ16, Mϕ21 and Mϕ27 grew on acetate containing media but did not appear to be as healthy as wild type cells and were incapable of growing on the minimal media plates. The other three mutants Mϕ20, Mϕ25 and Mϕ38 grew as well as wild type when given a reduced carbon source and light and were capable of limited growth when presented with photoautotrophic conditions.

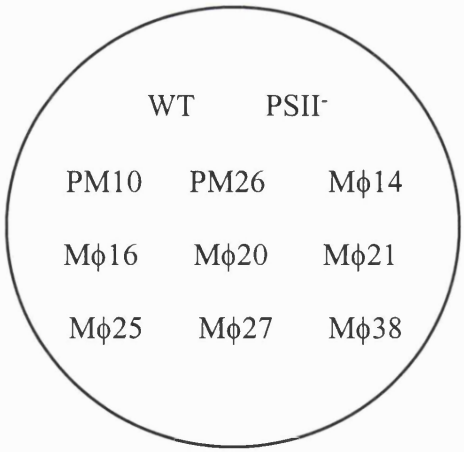
The level of PSII function was monitored by measuring oxygen evolution rates and by examining the EPR signal Y_D^+ for the mutants in comparison to a wild type sample. Oxygen evolution was measured using cell suspensions which contained 50μg chlorophyll in 3ml & 1mM DMBQ and potassium ferricyanide. The results are shown in Table 4.2.

The oxygen evolution data mirrors the results seen in the spot tests. The mutants which were unable to grow on the minimal media plates are the same ones which do not evolve oxygen. This shows that the photosynthetic lesion occurs in the PSII complex rather than elsewhere in the photosynthetic chain. The mutants which were capable of growth using light as an energy source are also able to evolve oxygen albeit at a reduced rate when compared to wild type cells.

Figure 4.1 Spot tests of *C. reinhardtii* Mϕ and PM nuclear mutants

A B





The cell spots are arranged in this order on both plates A and B. Plate A contains TAP medium while on plate B the cells are growing on HSM.

Table 4.2

Oxygen evolution rates for wild type, PM and Mϕ cell cultures

The values shown in Table 4.2 are averages taken over four separate occasions.

Cell Type	Oxygen Evolution (μmol O_2/ mg chl/ hr)
Wild type	168
PSII mutant	0
PM10	0
PM26	0
Mϕ14	0
Mϕ16	0
Mϕ20	35
Mϕ21	0
Mϕ25	43
Mϕ27	0
Mϕ38	85

Another technique used to determine the presence of functional PSII was low temperature EPR spectroscopy to look for the presence of radicals formed by the complex. The Y_D^+ radical is a dark stable and formed when active PSII is present. The samples used for this technique were concentrated cell suspensions prepared as described in Section 2.18. The wild type signal seen was a typical example of a functional PSII signal while the PSII deficient cells had no dark stable radical. The signals seen for the mutants varied as the results of the other tests have done (results not shown). There was no Y_D^+ signal from PM10, PM26, Mϕ14 or Mϕ21. The mutant Mϕ16 was not examined by EPR because it grew poorly in liquid culture and did not reach a high enough cell density to generate a sample. The mutants which were capable of evolving oxygen, Mϕ20, Mϕ25 and Mϕ38, would be expected to contain some active PSII centres and therefore to generate some of the Y_D^+ signal and this was seen to be the case. Another mutant, Mϕ27 was observed to contain a small amount of radical which may be derived from PSII activity. All of the samples were subsequently illuminated which causes

the formation of the signal P_{700}^{+} from active PSI centres. All the mutants and the wild type cells contained typical PSI signals.

The results of the characterisation are summarised in Table 4.3. All the mutants are affected in PSII function some being impaired while others are completely deficient in activity. Therefore we can conclude that the mutants have not reverted to a wild type phenotype during subculture.

Table 4.3

Summary of characterisation of PSII nuclear mutants

Cell type	Autotrophic growth	Oxygen Evolution	Y_D^+ EPR signal
PM10	x	x	None
PM26	x	x	None
Mϕ14	x	x	None
Mϕ16	x	x	n/d
Mϕ20	✓	✓	Small
Mϕ21	x	x	None
Mϕ25	✓	✓	Small
Mϕ27	x	x	None
Mϕ38	✓	✓	Small

4.3 Northern Analysis

4.3.1 Analysis of PM mutants

The mutants created by Dr N. Gumpel had already been screened for the presence of mRNA transcripts for the genes *psb*A, *psb*B, *psb*C, *psb*D and *psb*H. It was found that Mϕ14 failed to accumulate the transcript for *psb*D while Mϕ21 was lacking in the *psb*C mRNA. These mutants will not be discussed further as Mϕ21 is a mutant in the same complementation group as another reported mutant of this type. In Mϕ14, the nuclear insertion causing the PSII deficient phenotype is not linked to the functional copy of the gene which makes cloning the gene affected difficult. The PM cells have been determined to be PSII mutants but no further

work has been done to characterise the mutations. RNA was prepared from these cells and from a wild type CW10$^-$ strain using the Qiagen RNeasy kit. An aliquot of each was separated on a denaturing agarose gel as described in Section 2.8.2. The RNA was then transferred onto a nitro-cellulose filter and fixed by baking at 80°C for two hours. The filter was hybridised with random labelled DNA prepared as described in Materials and Methods and taken from the plasmids listed in Table 2.6 which contain the cloned genes.

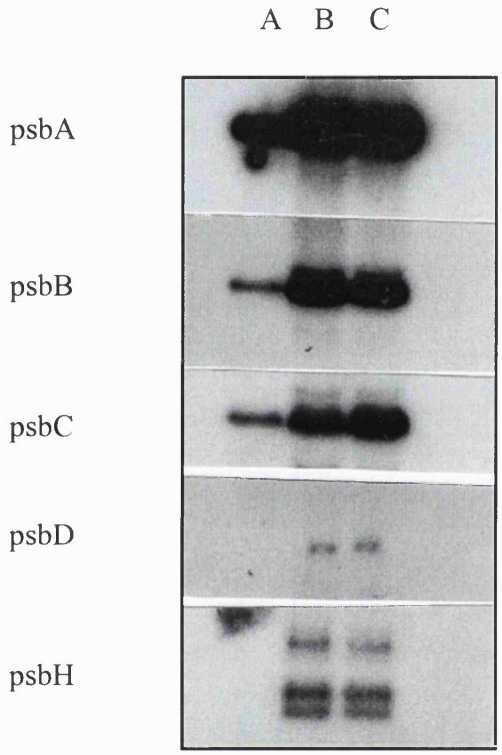
The results in Figure 4.2 show that the loading levels of the three samples were not equal with approximately ten times more of the mutant RNA than the wild type present on the filter. Clear signals are seen for all three samples with the *psb*A, *psb*B and *psb*C probes showing that the transcripts are stably accumulated. The wild type signal with the final two probes cannot be seen on the figure but when the filter was exposed for a long period a faint band was visible (data not presented). From this we can conclude that the mutants PM10 and PM26, like the rest of the Mϕ cells, are able to accumulate mRNA transcripts for these five genes.

4.3.2 Further analysis of Mϕ and PM mutants

There are known examples of nuclear mutants which affect the expression of four chloroplast PSII genes, *psb*A, *psb*B, *psb*C and *psb*D. We wished to try and discover if the expression of any other gene was controlled in this fashion. Good candidates would be the other components of the reaction centre core e.g. *psb*E, *psb*F and *psb*I but the expression of any or even all of the subunits could be under nuclear control.

The presence of each of the chloroplast encoded mRNA transcripts was examined as above by preparing RNA from each of the mutants and from wild type cells, separating it out on agarose denaturing gels then transferring it to nitrocellulose filters. These filters once they had been fixed by baking were used in Northern analyses. End labelled oligonucleotides were used as probes for each of the genes. The oligonucleotides were designed using published sequences for each gene and ordered from Perkin Elmer. The sequence for each of these probes is shown in Table 2.7. The hybridisation temperature used for each blot depends upon the melting temperature of the oligonucleotide probe and the oven was

Figure 4.2 Northern analysis of PM mutants.



Total RNA was prepared from A. wild type, B. PM10 and C. PM26 cells. The blots were successively probed with random labelled DNA prepared from plasmids carrying the five chloroplast genes *psb*A, *psb*B, *psb*C, *psb*D and *psb*H.

set at 5°C below the calculated T_M, determined as described in Section 2.7.10. The results of the series of Northern blots are summarised in Table 4.4.

Table 4.4

Summary of the results of all the Northern analyses performed on the PM and Mϕ mutants.

	PM10	PM26	Mϕ16	Mϕ20	Mϕ25	Mϕ27	Mϕ38
***psb*A**	✓	✓	✓	✓	✓	✓	✓
***psb*B**	✓	✓	✓	✓	✓	✓	✓
***psb*C**	✓	✓	✓	✓	✓	✓	✓
***psb*D**	✓	✓	✓	✓	✓	✓	✓
***psb*E**	✓	✓	✓	✓	n/d	✓	✓
***psb*F**	✓	✓	✓	✓	✓	✓	✓
***psb*H**	✓	✓	✓	✓	✓	✓	✓
***psb*I**	✓	✓	✓	✓	✓	✓	✓
***psb*J**	✓	✓	✓	✓	✓	✓	✓
***psb*K**	✓	✓	✓	✓	✓	✓	✓
***psb*L**	✓	✓	✓	✓	n/d	✓	✓
***psb*M**	✓	✓	✓	✓	1 band	✓	✓
***psb*N**	✓	✓	n/d	✓	✓	✓	✓
***psb*T**	✓	✓	✓	✓	✓	✓	✓

n/d = not done

These results show that no other PSII core subunit has a transcript which fails to accumulate in these mutants. When a transcript appeared to be missing for a certain cell type with a specific probe the experiment was always repeated using a new RNA preparation. In all cases except those highlighted the repeat experiment proved that the transcripts in question were indeed present and the initial result was due to the variability in the quality of RNA produced. The only exceptions to this were the *psb*N probe with the mutant Mϕ16 and *psb*L and Mϕ25. These samples would have to be repeated again to confirm whether or not the transcript is present or the apparent absence is due to a poor RNA preparation. The only other result of particular interest was seen with the *psb*M probe. The

autoradiogram showed that this probe hybridised strongly to two transcripts in the wild type sample and for all but one of the mutants. Mϕ25 however gave a signal for only the smaller of these two transcripts. The autoradiogram displaying this is shown in Figure 4.3.

This blot was repeated to ensure that the effect was not caused by a poor RNA preparation and the same pattern was seen. Another possible feature of *psb*M expression is highlighted by the Mϕ16 mutant. This appears to have a much lower concentration of the upper transcript in comparison to the amount of the smaller one which is present. The Northern blot was not repeated with fresh RNA from these cells so we cannot rule out this effect being caused by the RNA preparation rather than being a feature specific to this mutant.

4.4 *psb*M and *ycf*9

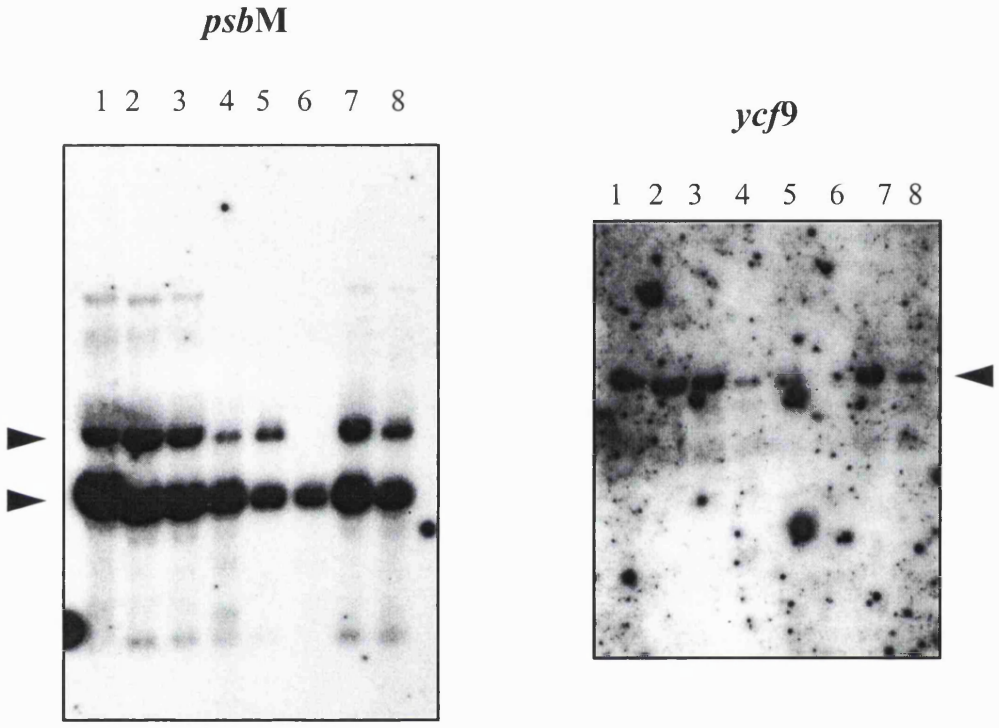
The information for the design of the *psb*M probe came from sequence data entered into Genbank (Acc. No. CRU81552 Higgs et al. (1996). The gene maps between exon 3 of *psa*A and the gene *rps*7. The authors report the presence of a polycistronic transcript which encodes the hypothetical Ycf9 protein and the PSII subunit PSII-M. They predict that the mRNA molecule covers 2705 base pairs of the DNA sequence and encodes both *ycf*9 and *psb*M.

We then designed a further oligonucleotide to use as a probe for the *ycf*9 sequence and stripped a filter, which had been previously hybridised with the *psb*M probe, by covering it with boiling SDS and allowing this to cool to room temperature. The filter was then used for a Northern analysis with the end labelled *ycf*9 probe, washed as described in methods Section 2.9.1 and placed for autoradiography next to an X-ray film. The results of this experiment can be seen in Figure 4.3.

The quality of the autoradiogram for this filter is poor but a single band can be seen in all tracks except for Mϕ25. When this film is overlaid with that taken using the *psb*M probe it is clear that the *ycf*9 band is in exactly the same position as the upper *psb*M band. This tells us that the larger of the two transcripts

Figure 4.3 Northern analysis of PM and Mϕ mutants probed with *psb*M and *ycf*9

Blots were probed with oligonucleotides designed against :



Total RNA was prepared from 1. wild type, 2. PM10, 3. PM26, 4. Mϕ16, 5. Mϕ20, 6. Mϕ25, 7. Mϕ27 and 8. Mϕ38 cells and run on a denaturing gel before being transferred to a nylon filter.

encodes both *ycf*9 and *psb*M while the lower band is specific to *psb*M. The mutant Mϕ25 has the smaller transcript which hybridises only to the *psb*M probe but is missing the larger transcript which carries both genes.

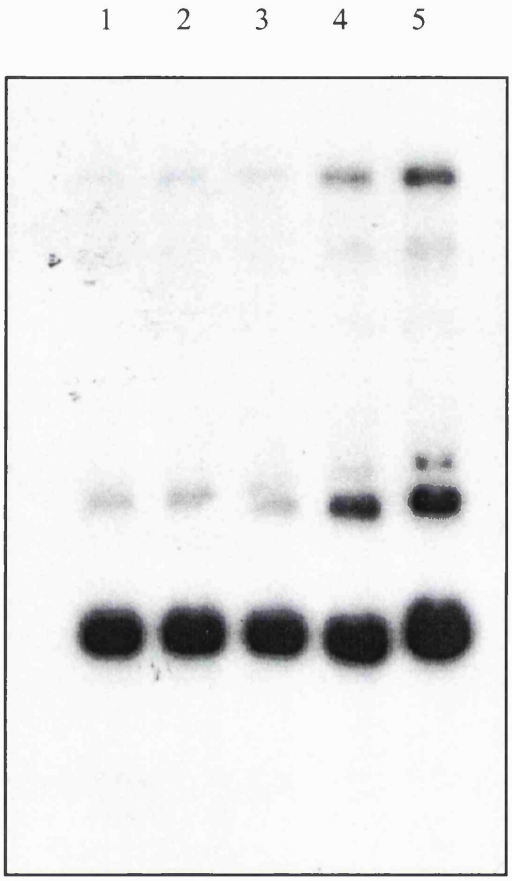
4.4.1 Size of the *psb*M transcripts

A further Northern analysis was carried out but this time RNA markers (Promega) were run alongside two tracks of wild type RNA. The filter was probed with the *psb*M oligo probe to determine the sizes of the two transcripts. The results from that experiment gave two transcripts of approximately 2900 and 1950 nucleotides (results not shown). The predicted polycistronic mRNA molecule was just over 2700 nucleotides which could be equivalent to the larger fragment which both *psb*M and *ycf*9 sequences hybridise to. The smaller 1950 nucleotide transcript is complementary to only the *psb*M sequence. This cannot represent a processed version of the larger transcript because both genes are located on the last 1000 nucleotides of the transcript and any shortened form would contain both. This suggests that a separate transcript is initiated after the region of the *ycf*9 gene used as the probe. If this hypothesis is correct then the Mϕ25 cells would have the smaller transcript with the *psb*M coding sequence but are missing the *ycf*9 open reading frame found only on the polycistronic molecule.

4.4.2 Pattern of *psb*M expression

The pattern of expression of the two *psb*M transcripts was investigated by using RNA prepared from wild type synchronised cultures which were grown under a twelve hour light twelve hour dark cycle. The cells were grown and the RNA prepared by Dr A. Watson in our laboratory. Samples were taken one hour before the end of the dark cycle, as the light was switched on and at hourly intervals after that. Five aliquots of this RNA were run on an agarose denaturing gel and then transferred to a filter for Northern analysis. The results can be seen in Figure 4.4. The smaller 1950 nucleotide transcript is present at approximately equal quantities throughout the light dark cycle but the amount of the larger transcript which encodes both the *psb*M and *ycf*9 genes varies. In the dark the transcript is present but at some point more than an hour into the light period the

Figure 4.4 Expression analysis of *psb*M



RNA was prepared from synchronized wild type cells, on a 12 hour light, 12 hour dark cycle, harvested
1. one hour before the end of the dark period,
2. at the end of the dark period,
3. one hour after the light was switched on,
4. & 5. three and four hours into the light period respectively.
The filter was probed with the end labeled oligonucleotide designed to be complementary to *psb*M.

level of the larger transcript increases. Other larger bands can be seen on this filter and others probed with the *psb*M gene. The intensity and appearance of these varies between experiments and they are likely to be due to binding of the probe to larger transcripts or due to hybridisation to contaminating DNA.

Finally, another Northern blot, using RNA from wild type cells and from a number of the mutants, was hybridised with the *psb*M oligo probe. The surprising result, seen in Figure 4.5, shows the two bands associated with the transcripts containing the *psb*M sequence in the first five tracks on the filter including that of Mϕ25. On all previous filters the Mϕ25 sample had shown only one band which hybridised to this probe. The last two lanes on the filter contain samples of wild type and Mϕ14 RNA, made on the same day, both of which show an altered pattern of expression. These tracks show that the upper *psb*M band is particularly unstable and that the absence of this band is not due to the mutation in the Mϕ25 cells. The bands which are smaller than the two typical *psb*M signals are likely to be due to hybridisation of the probe to degradation products caused by the instability of the larger transcript.

4.4.3 Conclusions from the examination of the nuclear PSII mutants

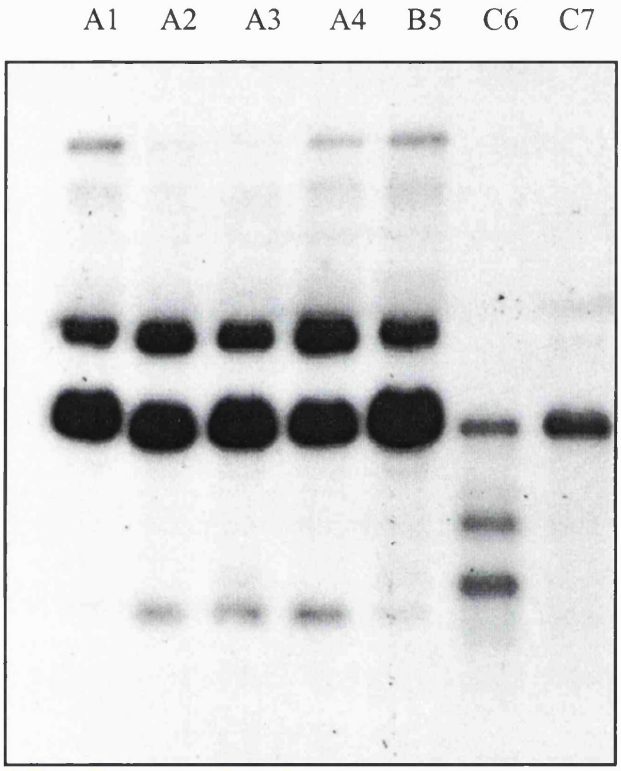
The results relating to the PM and Mϕ mutants which have been discussed show that the phenotypes of these cells are not caused by RNA instability of any of the chloroplast encoded subunits of PSII. The nuclear lesions which cause the PSII changes could be affecting the ability of the transcripts to be translated or possibly interfering with a post translational process.

4.5 *C. reinhardtii ycf*9 mutants

The gene for *ycf*9 lies between the gene, *psb*M and exon three of *psa*A. A plasmid, p207, which contains a 5kb insert of chloroplast DNA and encodes the region including the 5` end of *rps*7 and the entire genes for *psb*M and *ycf*9 was requested from the Chlamydomonas Genetics Centre. This can be seen in Figure 4.6.

The scheme designed for creating mutants involved cloning the *aad*A resistance marker (Goldschmidt-Clermont 1991) into a *Bgl*II site between the

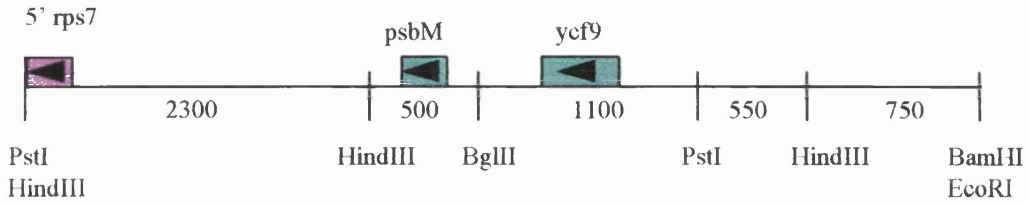
Figure 4.5 Northern analysis of wild type and Mϕ mutant RNA preparations



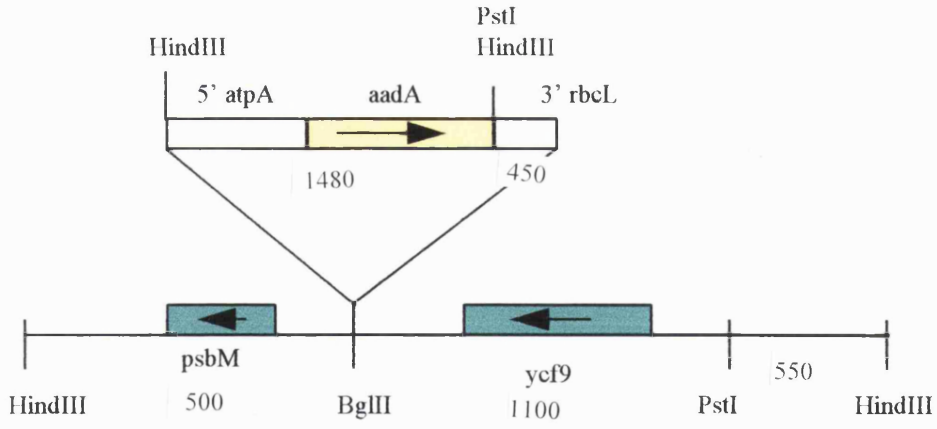
The RNA samples on this filter were made on three separate occasions A, B and C.
The samples are made from A1. wild type, A2. Mϕ14, A3. Mϕ25, A4. Mϕ38, B5. wild type, C6. wild type and C7. Mϕ14 cells.
The filter was probed with the labeled *psb*M oligonucleotide.

Figure 4.6 Restriction maps of plasmids used in the creation of *C.reinhardtii* Ycf9 mutants

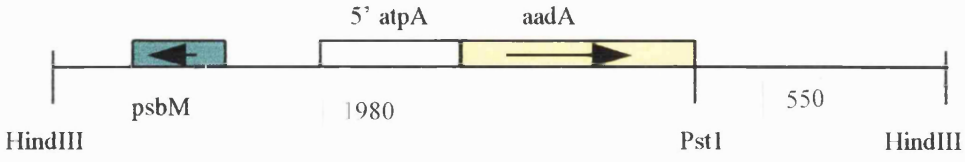
Wild type region on p207 plasmid



Insertion of aadA cassette between psbM and ycf9 genes in plasmid pCycfSp



Deletion of ycf9 coding sequence in plasmid pΔycf

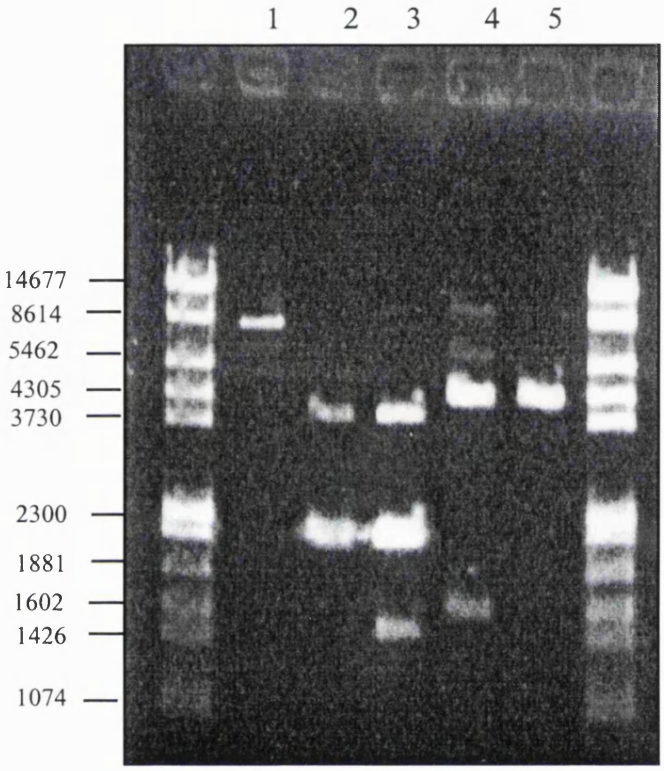


*psb*M and *ycf*9 genes but in the opposite orientation (Figure 4.6, plasmid pCysfSp). We wanted to determine the effect of inserting this extra DNA between the genes and to see if it altered the expression of either the polycistronic mRNA which covers both genes or the smaller transcript which carries only *psb*M. The deletion of *ycf*9 was then achieved by using the restriction enzyme *Pst*I to carry out a partial digest of the plasmid, pCycfSp, with *aad*A inserted between the genes to remove the 3` end of the resistance cassette and 1100bp of plasmid DNA including the gene coding sequence (Figure 4.6, plasmid pΔycf).

4.5.1 Creation of the insertion mutant

The insertional mutant was constructed by setting up a restriction digest of p207 with the enzyme *Bgl*II. The 5` phosphate groups were then removed by alkaline phosphatase treatment. The insert was prepared by removing the *aad*A coding sequence from the plasmid pSKaadA (Section 2.7.1) by *Bam*HI restriction enzyme digestion then purifying the desired segment using the Qia Quick gel purification kit. A ligation reaction was prepared using a 3:1 insert to vector ratio as described in methods Section 2.7.6 then this was used to transform competent JM109 cells. The transformants, named CycfSp, were selected for on media containing both spectinomycin and ampicillin and six were colonies were selected for analysis. Mini preps were carried out on these cells then enzyme digests were used to determine the correct insertion and orientation of the *aad*A cassette. Figure 4.7 shows the results of some of the digests carried out on the plasmid pCycfSp. The first track after the molecular weight markers shows the 7.7kb linear fragment obtained by *Bgl*II digestion of the plasmid p207 which was the vector used to construct the recombinant plasmid pCycfSp. The second digest shows the same plasmid, after digestion with *Hind*III, and gives fragments of 3.25, 2.3 and 2.15kb. The track next to this shows the results of a *Hind*III digest of pCycfSp. This has the *aad*A cassette present which also contains two additional *Hind*III sites, see Figure 4.6. The predicted sizes for this digest are 3.25, 2.3, 2.1, 1.48 and 0.5kb. The only visible difference on the gel photo between these *Hind*III digests is the appearance of the 1.48kb band in the track of the *aad*A containing plasmid. The two bands at just over 2kb are not resolved on this gel system and the small 500bp fragment is not visible.

Figure 4.7 **Gel photo of digested plasmids used in the construction of *C.reinhartdii ycf*9 mutants.**



Track 1. *Bgl*II digest of p207
Track 2. *Hind*III digest of p207
Track 3. *Hind*III digest of pCycfSp
Track 4. *Pst*I digest of pCycfSp
Track 5. *Pst*I digest of pΔycf

See text for further details

4.5.2 Creation of the deletion mutant

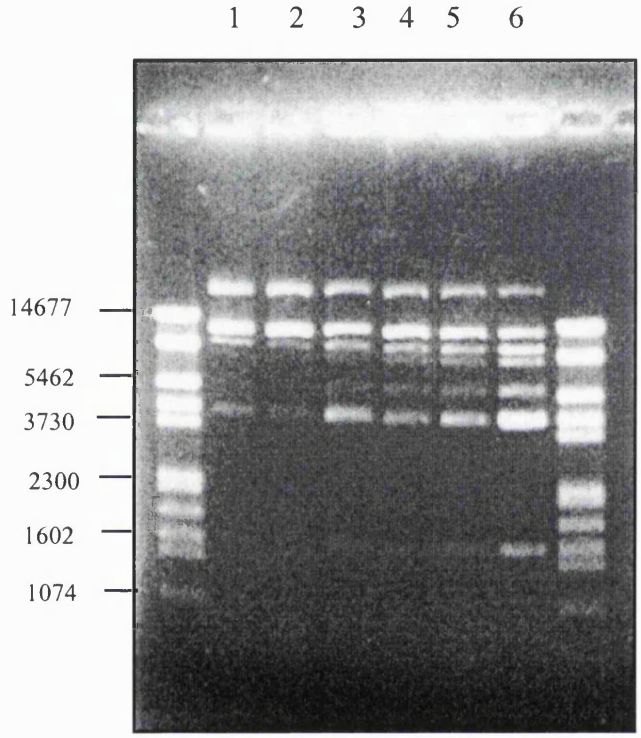
The deletion mutant was created from the plasmid pCycfSp by removing the *Pst*I fragment containing the 3` end of the *aad*A cassette and the *ycf*9 gene. A partial digest had to be used as there were more than two *Pst*I sites on the plasmid. This was achieved by diluting the enzyme stock by 1:10 then using 1µl of the dilute enzyme to set up the digest. Aliquots of 5µl were removed after 30s, 1, 2, 5, 10 and 20 minutes and added to 5µl of cold TE. Half of the sample was frozen and the other half examined on a 1% agarose gel shown in Figure 4.8. The aim was to selectively remove the 1.6kb fragment without also losing the larger *Pst*I fragment containing the *psb*M and *aad*A coding sequences. The aliquots removed after 10 and 20 minutes were used directly in ligation reactions which were then transformed into competent JM109 cells. The transformants were selected for on both ampicillin and spectinomycin to ensure that the *aad*A containing *Pst*I fragment had not been lost. Of the ten transformants selected seven were parental type plasmids while three were missing the 1.6kb *Pst*I fragment. This is demonstrated in Figure 4.7. The fourth digest track shows a complete *Pst*I digest of pCycfSp with expected fragments of 4.2, 3.9 and 1.6kb while the fifth digest shows the result of the pΔycf *Pst*I digest which is lacking the smallest fragment.

4.5.3 Transformation of *C. reinhardtii*

These mutations were introduced into the *C. reinhardtii* strain, CC1021, by gold particle bombardment by a helium powered gun. The cell culture to be transformed was restreaked every 3 days for a fortnight, then used to inoculate a liquid culture which was then grown to mid log phase. These cells were concentrated and the plated out on TAP medium containing spectinomycin at a concentration of 100µg per ml. The transformation procedure is described in Section 2.6.2.

Once the plated cells had been bombarded they were incubated under low light intensity of 5µE/m^2/s at 20°C until colonies were visible. 8 Cycfsp and 5 Δycf colonies were picked using a cocktail stick onto fresh TAP spectinomycin medium. Once the colonies had reached a suitable cell density on the plate, the cells were inoculated into a liquid culture also containing spectinomycin. This culture, once grown, was then used to select for individual colonies by spreading it

Figure 4.8 Gel photo of a partial *Pst*I digest of pCysfSp



A partial digest was required to remove the 1.6kb *Pst*I fragment from pCycfSp.
Aliquots of the digest were removed after
1. 30 s, 2. one min, 3. two mins, 4. five mins, 5. ten mins and
6. twenty mins and subject to gel electrophoresis

onto a TAP Sp plate. This procedure was repeated three times. The aim was to select for cells which contain only the mutated copy of this region of the genome i.e. cells which are homoplastic for the change. Of the selected colonies 7 Cycfsp9 mutants and 3 Δycf strains were available for characterisation.

4.6 *Synechocystis ycf9* mutants

In addition to the *C. reinhardtii* mutants in *ycf9* it was decided to create a deletion mutant in *Synechocystis* sp PCC6803 in a parallel experiment.

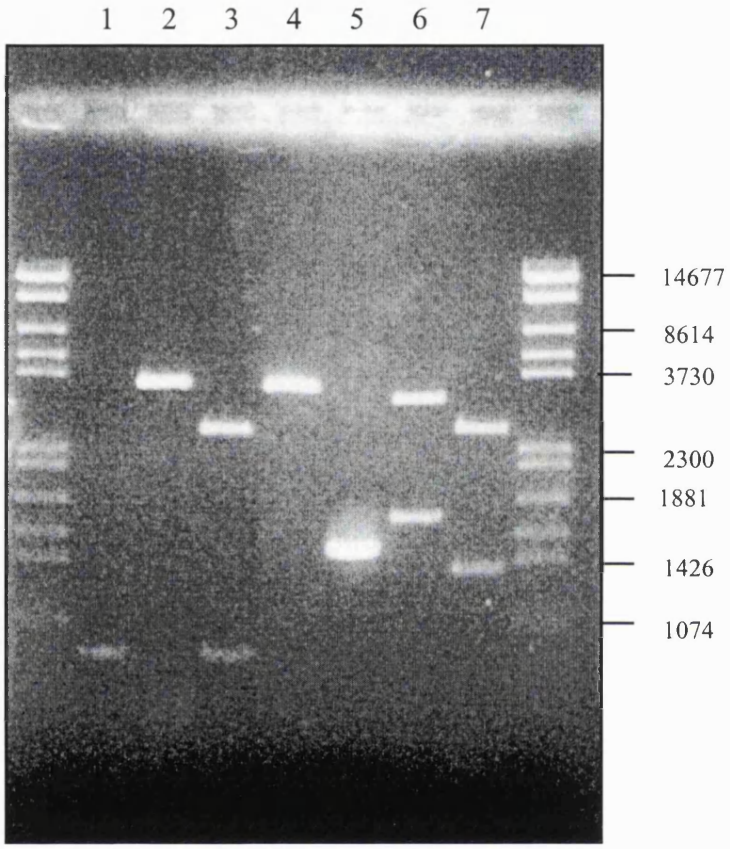
4.6.1 Cloning the *ycf9* gene from *Synechocystis* sp PCC 6803

The entire genome of this cyanobacteria has been sequenced (Kaneko et al. 1996) so the Cyanobase sequence database was used to design two PCR primers which would amplify the *ycf9* gene from purified genomic DNA. The PCR reaction was carried out using 2ng of genomic DNA with 100pmol of each primer and using both Vent and Taq polymerases and their relevant buffers. An annealing temperature of 54°C was used with the rest of the conditions and reagents listed in the methods section. Of the two PCR samples only the one using Vent polymerase gave a product under these conditions. A fragment of 886bp was predicted and the gel photo in Figure 4.9 shows the purified PCR product to be approximately 900bp.

The PCR reaction was repeated and the product gel purified. This was then blunt cloned into the *Hinc*II site of pUC19 to give the plasmid pSycf9. A digest of this plasmid is shown in tracks 2 and 3 of the gel photo in Figure 4.9. Track two shows a linear fragment of 3.9 kb from an *Eco*RI digest while track 3 shows the results of an *Eco*RI *Hind*III double digest. These two enzyme cut either side of the *Hinc*II site in the MCS so release the 900bp insert from the 2.7 kb vector.

Further restriction digests were carried out to determine the orientation of the PCR product in respect to the vector sequence. The results and both possible outcomes are displayed in Figure 4.10. *Xmn*I and *Ssp*I restriction digests were used to determine the direction of the gene relative to the vector sequence. These enzymes have sites within both the insert and vector DNA. Possibility A in Figure 4.10 gives predicted sizes which correspond best to the result seen from the actual digest so the plasmid must be in this proposed conformation.

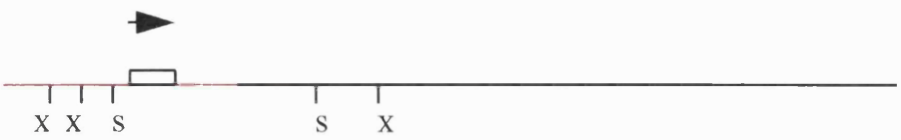
Figure 4.9 **Gel photo showing DNA used to create *Synechocystis* sp PCC 6803 *ycf*9 mutants**



Track 1. *ycf*9 PCR product
Track 2. *Eco*RI digest of pSycf9
Track 3. *Eco*RI/*Hind*III double digest of pSycf9
Track 4. *Bsm*FI digest of pSycf9
Track 5. Kanamycin cassette PCR product
Track 6. pSycfKn5 *Eco*RI digest
Track 7. pSycfKn5 *Eco*RI/*Hind*III digest

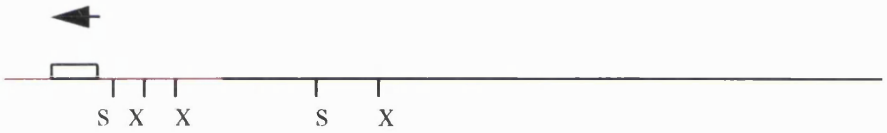
Figure 4.10 Gel analysis of the vector pSycf9
The PCR product containing the *ycf*9 gene was cloned into pUC19. *Xmn*I and *Ssp*I digests were used to determine the orientation of the gene. The two possible plasmids and the outcome of the actual digests are shown below.

A.



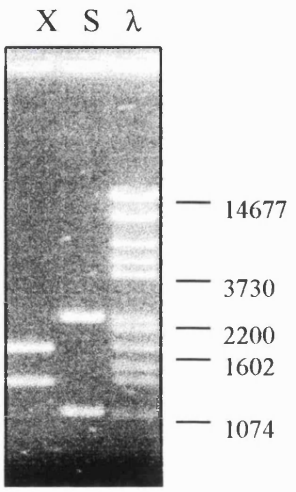
XmnI digest results in 2050, 1450 + 80 bp fragments
SspI digest results in 2440 + 1125 bp fragments

B.



XmnI digest results in fragments of 2450, 1030 + 80 bp
SspI digest results in fragments of 2560 + 1000 bp

Gel photo of XmnI and SspI digest



The results of the digests match the sizes predicted in plasmid A.

4.6.2 Creation of a *ycf*9 deletion mutant

Examination of the sequence of the *ycf*9 gene revealed the presence of two *Bsm*FI restriction sites, one 20 base pairs before the start of the gene and one within the coding sequence, see Figure 4.11. The strategy for creating the deletion mutants was to cut the cloned genomic DNA with *Bsm*FI to remove a 130 bp fragment then to remove the overhanging residues left by this enzyme with mung bean nuclease. Into this blunt ended molecule we attempted to clone the kanamycin resistance cassette which was purified from a PCR product.

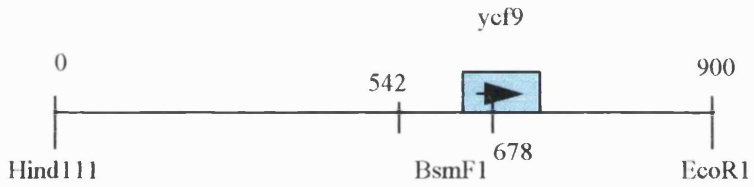
The *Bsm*FI digest had to be carried out at the enzyme's optimum temperature of 65°C and the digest was incubated for two hours. After this time the DNA was separated on a 1% agarose gel to ensure that the digest had been successful. An aliquot of this DNA is shown in track 4 of the gel in Figure 4.9. The small fragment removed by this enzyme is not visible on this gel but it is apparent that the plasmid has been linearised. The 3.7kb fragment created by this digest was gel purified and the treated with mung bean nuclease which removes any 5` overhanging bases.

The kanamycin resistance gene was purified from the plasmid pBSSK which is derived from a bluescript vector. The coding sequence was amplified in a PCR reaction using the T3 and T7 primers which hybridise either side of the pBluescript (Section 2.7.1) multiple cloning site. The reaction, carried out with an annealing temperature of 50.5°C, led to the production of a 1.4kb fragment shown in track 5 of Figure 4.9. The fragment shown matches the expected size.

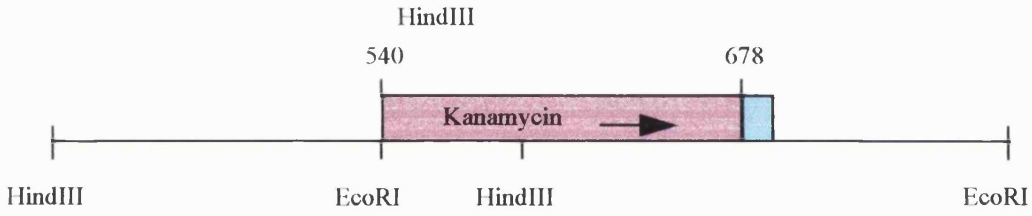
A ligation was then performed using the *Bsm*FI, mung bean nuclease treated vector and the kanamycin resistance encoding gene purified from the PCR reaction. A high ratio of vector to insert was required because both molecules were blunt ended. The products of the ligation were transformed into super competent JM109 cells and transformants were selected for on LB media containing ampicillin and kanamycin. Six transformants were selected, DNA prepared from them and initial digest showed that three appeared to contain a fragment of DNA which could correspond to the kanamycin cassette. Plasmids pKn2 and pKn5 were chosen for further evaluation and it was believed that they had the kanamycin cartridge inserted in opposing orientations. The gel photo on Figure 4.9 shows the

Figure 4.11
A schematic diagram showing the inserts in three plasmids carrying the *ycf9* gene from *Synechocystis* sp PCC 6803.
The numbers on the figures relate to the sequence of the intact PCR product which is cloned into plasmid pSycf9.

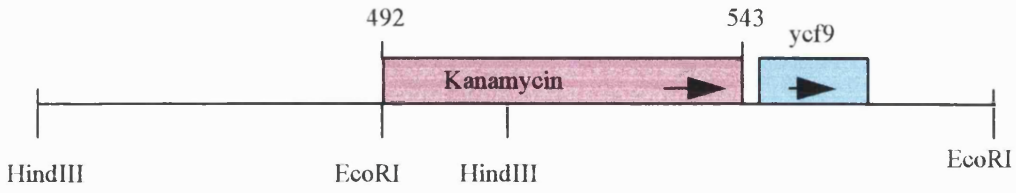
Wild type sequence in plasmid pSycf9



Kn5, kanamycin cassette replaces BsmF1 fragment



Kn2, kanamycin cassette inserts before the ycf coding sequence



plasmid pKn5 after incubation with both *Eco*RI alone and *Eco*RI and *Hind*III. These can be compared to the same digests carried out with the parental plasmid pSycf9. The kanamycin cassette introduces a site for both of these enzymes and the fragments seen on the gel photo match up to those expected if the cassette is introduced in the same orientation as the gene. The single *Eco*RI digest gives two predicted fragments of 3.2 and 1.7kb. The double digest should cut the DNA to give four fragments. The two largest of 2.7 and 1.3kb can be seen on the gel photo but two other small pieces of DNA of 400 and 350bp should have also been present. A different gel system would have provided the resolution required to visualised these but the fact that the two larger fragments are of the predicted sizes informs us that the digest has given the expected result.

Both plasmids were examined by automated sequencing to confirm that the cassette had inserted at the correct position. The sequencing primers pk7 and pk3 (Table 2.4) were used. These are designed against the sequence at either end of the kanamycin cassette. The sequence from pKn5 showed that the kanamycin sequence meets that of the genomic DNA at the expected site and that the cassette is inserted in the orientation predicted by the digests. Figure 4.11 shows the position of the insert relative to the gene.

The sequence data from pKn2 confirmed that the kanamycin sequence is also in the same orientation as *ycf*9 but that in this case the parental plasmid, pSycf9, cannot have been cut at both *Bsm*FI sites. The kanamycin cassette has inserted at the *Bsm*FI site which lies before the gene and fifty base pairs of sequence preceding this have been lost. The final 15 base pairs preceding the gene sequence are still in position and none of the coding sequence has been removed. It is possible that transcription could occur through the resistance gene and because the *ycf*9 gene is still in frame, a small level of expression may be possible.

4.6.3 Transformation of *Synechocystis* sp PCC 6803

Both the pKn2 and pKn5 plasmids were transformed into a wild type strain of *Synechocystis* sp PCC 6803 using the methods outlined in Section 2.6.4. The transformed cells were plated out on media both with and without glucose supplements and were incubated for 4 days. After this time a layer of kanamycin containing media was applied to the plates as method of selection for the

transformants. The cells were then incubated under 7μE /m^2/s illumination at 30°C until individual colonies were visible. Once the colonies had reached a transferable size they were removed using a cocktail stick onto fresh media and again incubated. The cells were cultured on kanamycin containing media until the homoplastic presence of the mutation could be confirmed.

4.7 Characterisation of the *ycf*9 mutants

4.7.1 Southern analysis of *C. reinhardtii* mutants

Southern analysis was performed after the cells had been taken through three rounds of selection to determine whether the mutant gene had completely replaced the wild type one or if there were still wild type copies left. The cells from which the DNA was prepared were grown under dim light at 8 μE /m^2/s then the Qia Amp kit was used to purify the genomic DNA. This was then used in overnight restriction digests to create a large selection of DNA of varying sizes which were separated by agarose gel electrophoresis. The DNA from the gel was then transferred to a nitrocellulose filter using the method described in Section 2.9.1.

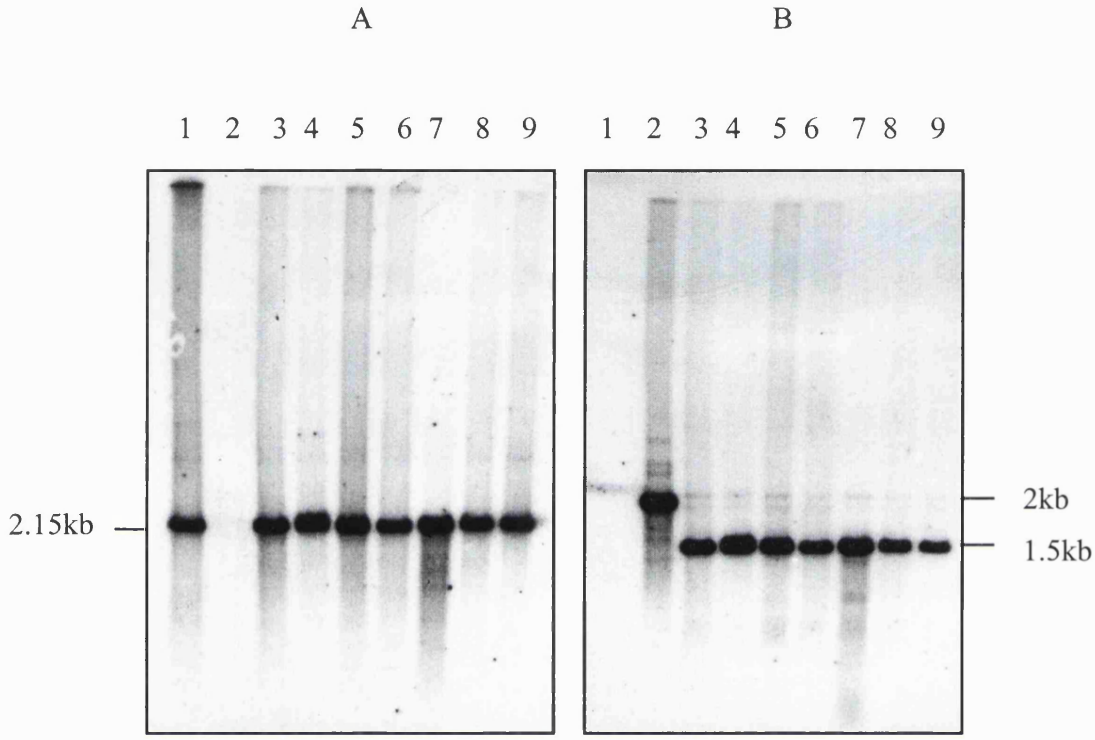
The cells which were examined in this way were a wild type control, one *ycf*9 deletion mutant and seven of the insertion mutants. Two restriction digests were used to try and determine whether any wild type copies of the gene were present and the blot for each was hybridised with both a *ycf*9 and an *aad*A specific probe. These probes were prepared by using restriction digests to cleave plasmids carrying the genes then the relevant fragment was purified. The *ycf*9 probe was the result of a *Pst*I / *Spe*I double digest of p207 and subsequent purification of the 1kb band which contains the gene. The *aad*A probe consisted of the coding sequence from the gene which is purified by *Pst*I / *Nco*I double digest of an *aad*A containing plasmid like pSKaadA and separation of the 800bp fragment. The first digest performed and subsequently blotted and probed used the enzyme *Hind*III. Figure 4.6 shows the maps of the three different cell types. The wild type fragment which the probe hybridised to is 2.15kb in length. The pCycfSp mutants have the *aad*A cassette cloned into a site on this 2.15kb fragment but this introduces an additional

*Hind*III site. The resulting fragment which the probe hybridised to consisted of 1.65kb between a *Hind*III site and the *Bgl*II insertion point and 0.4kb of the *aad*A cassette sequence. This makes a total of 2.05kb. The deletion mutant has lost the DNA containing the *ycf*9 gene so did not hybridise to the gene specific probe. The results of the hybridisation can be seen in Figure 4.12.

The first autoradiograph shows the results with the *ycf*9 probe. The predicted size of the wild type fragment is 2.15kb while the mutants with the insertion prior to *ycf*9 should give a band of approximately 2.1kb. The difference between bands of these sizes cannot be resolved on the gel system used here. However it is very clear that there is no band corresponding to the *ycf*9 probe in the track of the deletion mutant. This would be the expected result if all the wild type copies of this region had been removed. Figure 4.12B shows the same filter after it had been stripped and rehybridised with the *aad*A probe. On this filter no band is seen in the wild type track because the *aad*A cassette is not present. The deletion mutant does hybridise with this probe and the fragment seen corresponds to the predicted size of 2kb. All of the insertional mutants also hybridise to the *aad*A probe showing that the selection marker is present and expressed. The predicted size of 1480bp is equivalent to the actual size calculated from the blot. These *Hind*III digests tell us that the deletion mutant is homoplastic for the mutant phenotype but because the wild type and Cycfsp mutants give very similar size fragments we cannot be certain that all wild type copies of the gene have been removed by selection.

To confirm that these mutants were homoplastic a second digest, this time with *Pst*I was used. The fragments from this digest which hybridise to the *ycf*9 probe are predicted to be 3.9kb in wild type cells and 1.55kb in the mutants. The deletion strain would not hybridise to this probe. Figure 4.13 displays the results of this Southern blot. Track one contains the wild type DNA. Unfortunately the amount of DNA loaded into this track was much less than that seen in all the others. When the filter was exposed to film for a longer period a band of the predicted size was present but the background level becomes very high so this filter is not shown. The CycfSp insertion mutants all show a very definite band at a position which relates to the predicted size of 1.55kb. This result confirms that the mutated copy of the gene is present but we cannot state that there are no wild

Figure 4.12 Southern blots of *Hind*III digested DNA from *C.reinhardtii ycf*9 mutants



Filter A was probed with a random labelled 1kb *Pst*I/*Spe*I fragment of p207 containing the *ycf*9 gene.

Filter B is probed with the random labeled *Nco*I/*Pst*I fragment of the *aad*A cassette which contains the coding sequence.

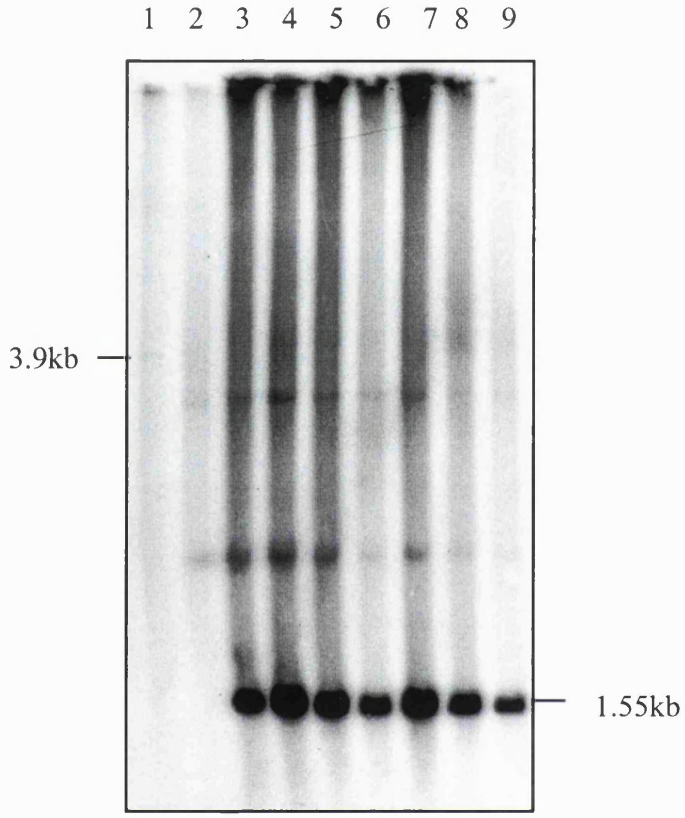
Track 1 contains wild type DNA and track 2 the deletion mutant Δ2. Tracks 3-9 contain examples of the insertion mutants Cycf 2,4,5,6,7 and 8.

copies of the gene left because the background signal obscures the region where a faint 3.9kb band would be. The cells are still cultured in the presence of antibiotic until the complete absence of the wild type gene can be confirmed. This filter was also stripped and reprobed with the *aad*A cassette coding sequence which confirmed that the mutants carry the resistance marker and that the wild type cells do not (not shown).

4.7.2 Southern analysis of *Synechocystis* mutants

A similar approach to that used of the *C. reinhardtii* mutants is required to determine whether all the wild type copies of the *ycf9* gene have been lost from the genome. DNA was prepared and then cut with the enzyme *Nco*I. The resulting DNA fragments were separated by electrophoresis then transferred onto a nitrocellulose filter. The probe for the *Synechocystis ycf9* gene used was the PCR product which was then cloned into a pUC vector, discussed in Section 4.6.1. The result of this Southern analysis, which is not shown here, confirmed that the wild type copy of the gene was still present in the mutants. The wild type band and a larger signal which relates to the copy of the gene disrupted by the kanamycin cassette were both seen on the filter. The cells required further rounds of selection to remove the wild type copy of the gene. The repeat of this experiment has yet to be completed so the results of the analysis of these mutants may still be influenced by the wild type gene.

Figure 4.13 Southern analysis of *Pst*I digest of DNA from *C.reinhardtii ycf*9 mutants



DNA was prepared from cells of 1. wild type, 2. ycfΔ2 and 3-9 insertion mutants 2, 4, 5, 6, 7 and 8.

The filter is probed with the *Pst*I/*Spe*I fragment containing the *ycf*9 coding sequence.

4.8 Analysis of the *ycf9* mutants

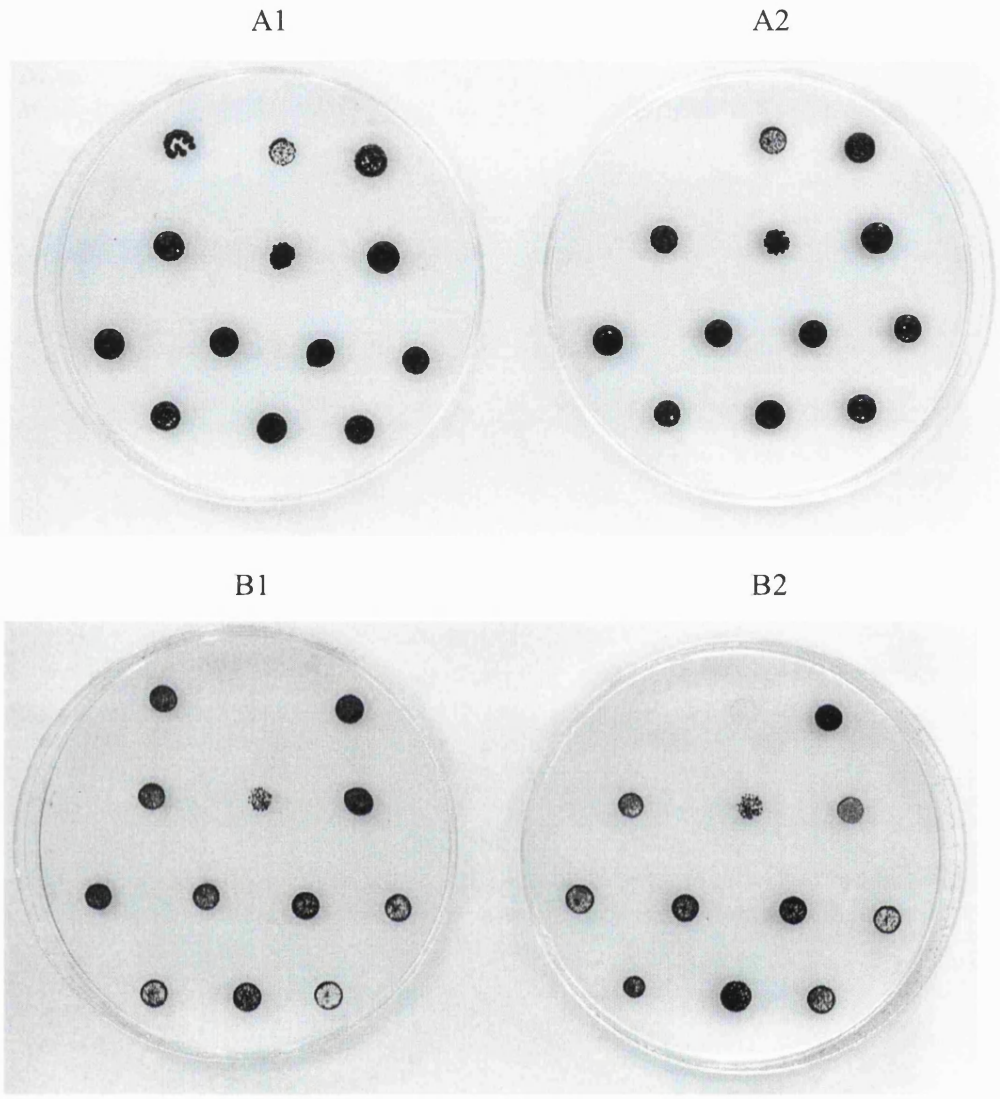
4.8.1 Growth tests of *C. reinhardtii* mutants

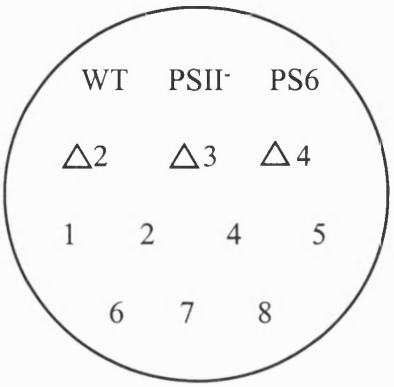
The *ycf9* mutants were grown in liquid culture under a light intensity of 45 $\mu E/m^2/s$ along with a number of controls. These cultures were used to set up spot tests where small aliquots of each cell type are placed onto different types of media. The cells used in this test were a wild type culture of CC1021, a PSII minus mutant, PSII-H null, Mlu1, a strain which contains the *aad*A cassette but does not affect its growth, three of the deletion mutants and all seven of the insertion mutants. The cultures were aliquoted onto TAP and HSM media, both with and without spectinomycin, then the plates were left to incubate at 20°C and light intensity of 45 $\mu E/m^2/s$. The results are shown in Figure 4.14. This figure shows that untransformed wild type cells are unable to grow on the antiobiotic containing plates and that the PSII minus cells do not grow on the HSM plates. All of the mutants appear to be able to grow under all the conditions tested. An additional set of TAP plates was incubated under very low light intensity of $5\mu E/m^2/s$ and in darkness because the plates upon which the cells were selected seemed to suggest that the cells grew better under slightly higher light intensity. The results from those plates, which are not displayed here, showed us that the cells were equally capable of growth under darkness and very low light as they were under higher levels of illumination.

4.8.2 Growth Tests of *Synechocystis ycf9* mutants

Similar experiments, to those previously described in Section 4.8.1, were carried out for the *Synechocystis ycf9* mutants. The mutant cells were cultured in liquid BG11 media containing 50μg/ml kanamycin under a light intensity of $25\mu E/m^2/s$ then 5μl aliquots were placed on BG11 and BG11 + glucose plates both with and without antibiotic supplements. Two control cultures were also placed on the plates. These were a wild type cell culture and a PSII minus mutant. The plates were then incubated at 30°C under a light intensity of $7\mu E/m^2/s$. The results of these tests are shown in Figure 4.15. The BG11 media supports only

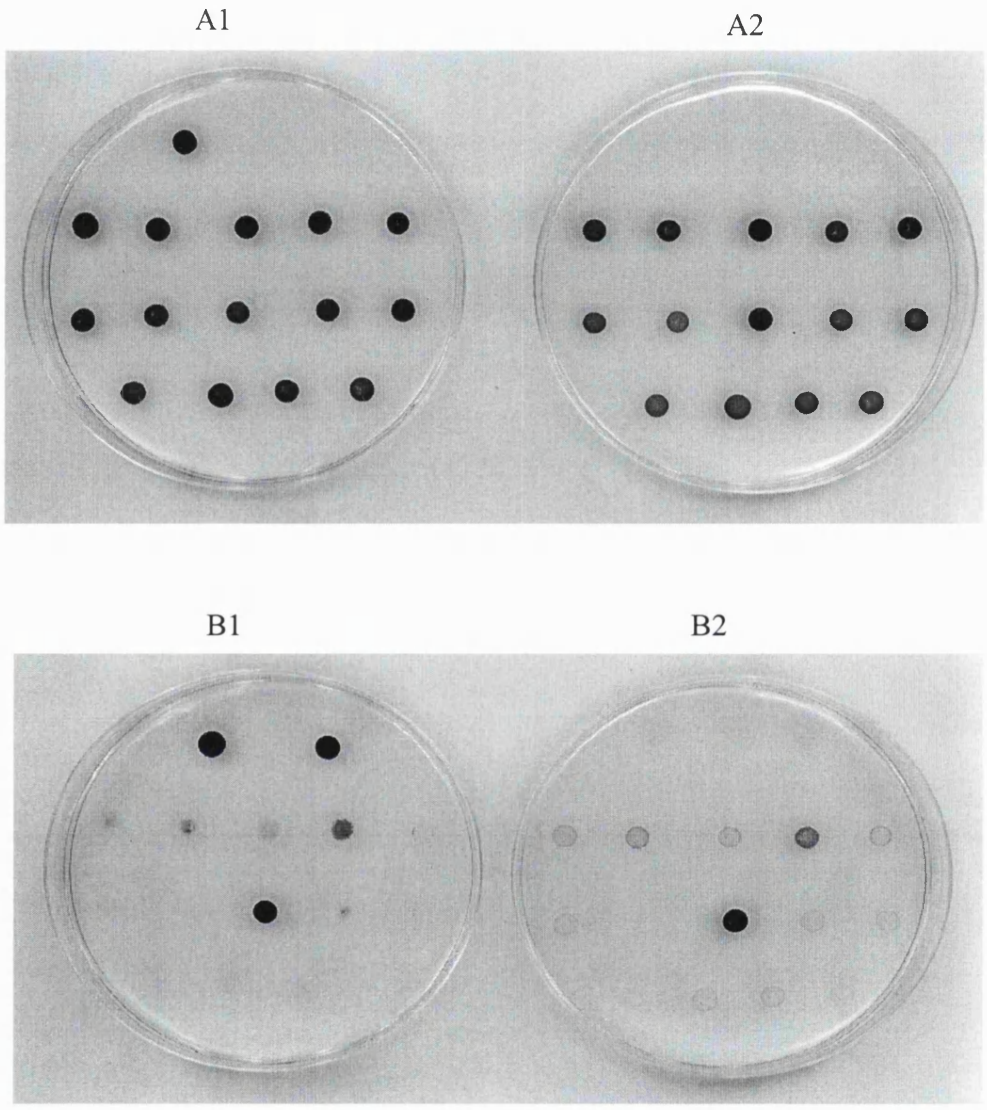
Figure 4.14 ***C. reinhardtii ycf9*** **mutant spot tests**

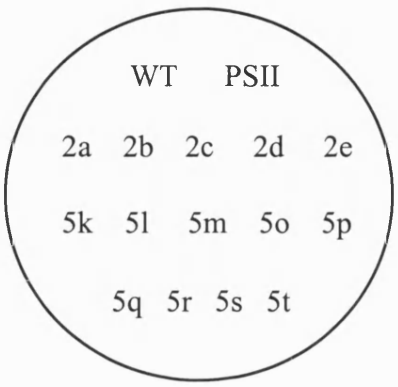




The cultures were placed on the plates in the manner shown.
Plates A contain TAP media with plate 2 supplemented with spectinomycin.
Plates B contain HSM and plate 2 again contains antibiotic.

Figure 4.15 *Synechocystis* sp PCC 6803 *ycf*9 mutant spot tests





The cultures were applied to the plates in the manner shown.
Plates A contain BG11 medium with plate A2 being supplemented with kanamycin.
Plates B contain BG11 + glucose again with plate 2 having added kanamycin.

photoautotrophic growth hence the PSII mutants are unable to grow under these conditions. The kanamycin plate also inhibits the growth of the untransformed wild type strain. All the mutants are capable of autotrophic growth to an equivalent level of that seen in the wild type cells. The BG11 + glucose plates allow the culture of non photosynthetic cells. The PSII minus mutant grows as well as wild type under these conditions. The mutants however seem to be unable to tolerate the presence of glucose and with the exception of Kn5m all grow very poorly or not at all on these plates. This was a surprising result because the BG11 plates show that these cells are fully competent under photoautotrophic conditions. The mutant Kn5m, which grows on the glucose plates when all others do not may be caused by the insertion of the kanamycin gene elsewhere on the genome. When grown on plates containing glucose the *Synechocystis* cells still require PSI activity for their metabolism. PSI must be present in the mutant cells because they are capable of unsupported photosynthetic growth but the presence of glucose must be inhibiting autotrophic growth while the mutation is in some way inhibiting glucose metabolism.

4.8.3 Oxygen evolution rates from the *ycf*9 mutants

Oxygen evolution measurements were taken using a cell suspension which contained 50μg/ml of chlorophyll in the presence of 1 mM of each of the electron acceptors DMBQ and potassium ferricyanide. The *Synechocystis* cells tested, wild type, Kn2a, Kn2b, Kn5k and Kn5m all gave rates of the same order of magnitude, see Table 4.5 for average rates, and all the mutants were capable of evolving oxygen at a rate equivalent to or greater than that seen for wild type cells. These cells have been shown to be capable of photoautotrophic growth so we would expect them to have a functioning oxygen evolving complex.

The rates observed for the *C. reinhardtii* cells showed more variety as can be seen in Table 4.6. The averages presented are the result of five separate measurements. Wild type cells give an average rate of nearly 200μmol O_2/ mg chlorophyll /hour which is equivalent to rates seen previously. The mutants all give rates which are significantly lower than this. The mutants with the *aad*A cassette inserted between *psb*M and *ycf*9 show rates of approximately half that seen from wild type cells. The presence of the *aad*A sequence in these cells may

disrupt the expression of both the *psb*M and *ycf*9 genes because they are expressed as a polycistronic unit, so we cannot precisely determine the cause of this phenotype.

The Δycf cells, in addition to having the *aad*A cassette inserted in the same place, are missing the *ycf*9 coding sequence. These cells show an even lower level of oxygen evolution, approximately a quarter of the wild type rate. This added effect must be due to the deletion of the *ycf*9 gene but the cells are still capable of limited autotrophic growth.

Table 4.5

The rate of oxygen evolution of *Synechocystis ycf*9 mutants in the presence of ferricyanide and DMBQ

Cell Type	Oxygen Evolution (μmol O_2/mg chl/hr)				Average
WT	35.4	31.7	31.7	36.3	33.8
Kn2a	40.5	43.3	43.3	46.4	43.4
Kn2b	32.5	38.6	32.1	36.4	34.9
Kn5k	29.6	38.3	32.8	35.6	34.1
Kn5m	28.7	41.1	40.0	37.4	36.8

Table 4.6

Oxygen evolution rates from *C. reinhardtii* wild type and *ycf*9 mutants in the presence of electron acceptors DMBQ and ferricyanide. The results shown are an average taken from five repeat experiments.

Cell Type	Oxygen Evolution (μmol O_2/mg chl/hr)
Wild type	193.0
Δycf 2	58.7
Δycf 4	41.8
Cycf Sp 5	106.0
Cycf Sp 7	110.4

4.8.4 EPR Analysis of the *ycf9* mutants

suspensions prepared as outlined in the methods section and dark adapted for two hours before being frozen in liquid nitrogen. The EPR spectra were measured at a temperature of 12K, 0.2mT modulation amplitude and $4x10^{-3}$ mW microwave power.

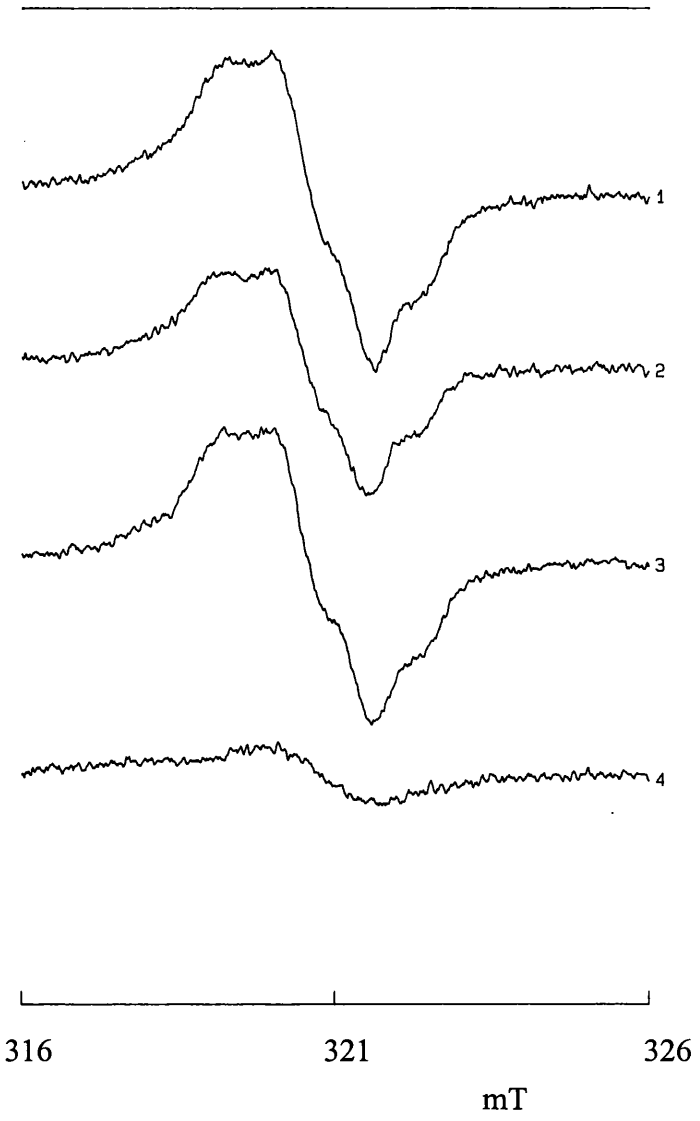
Figure 4.16 shows spectra recorded from dark adapted *Synechocystis* sp PCC 6803 cells. Trace one, from a wild type strain, shows a typical Y_D^+ spectra for this organism which indicates that PSII is present and active. The following two traces show the result from both type of deletion mutant*, Kn2 and Kn5. The basic shape of the spectra resembles that seen for wild type. The relative amounts of these signals are not relevant as the samples do not contain exactly the same concentration of chlorophyll. The final trace is from a PSII minus strain. It can be seen that the *ycf9* mutants in comparison have normal PSII signals. These cells were also tested to ensure that PSI is present and functioning. To achieve this the samples are illuminated in the EPR cavity to produce the $P700^+$ radical. All of the cells produced wild type PSI spectra (results not shown).

The *C. reinhardtii* cells were examined by EPR in the same way. The Y_D^+ signal was firstly examined in wild type cells. The chlorophyll concentrations of these samples were determined prior to examination by this technique so we are able to quantify the relative amounts of each functional complex. Figure 4.17 shows the Y_D^+ traces of wild type, Cycf 7 and ycfΔ2 cells. The deletion mutant (trace 3) shows only a small PSII signal which is equivalent of approximately 30% of the wild type signal based on the concentration of chlorophyll present. The trace from the insertion mutant shows a signal and the calculation of amplitude per mg chlorophyll gives a value of 80% of the wild type level. These values show a similar trend to the rates of oxygen evolution produced by these two different cell types.

The final figure (Figure 4.18) shows EPR traces of these mutants following illumination at 12K. Trace one, wild type cells shows a large peak superimposed on top of the Y_D^+ signal which is caused by the radical P_{700}^+. The deletion mutant

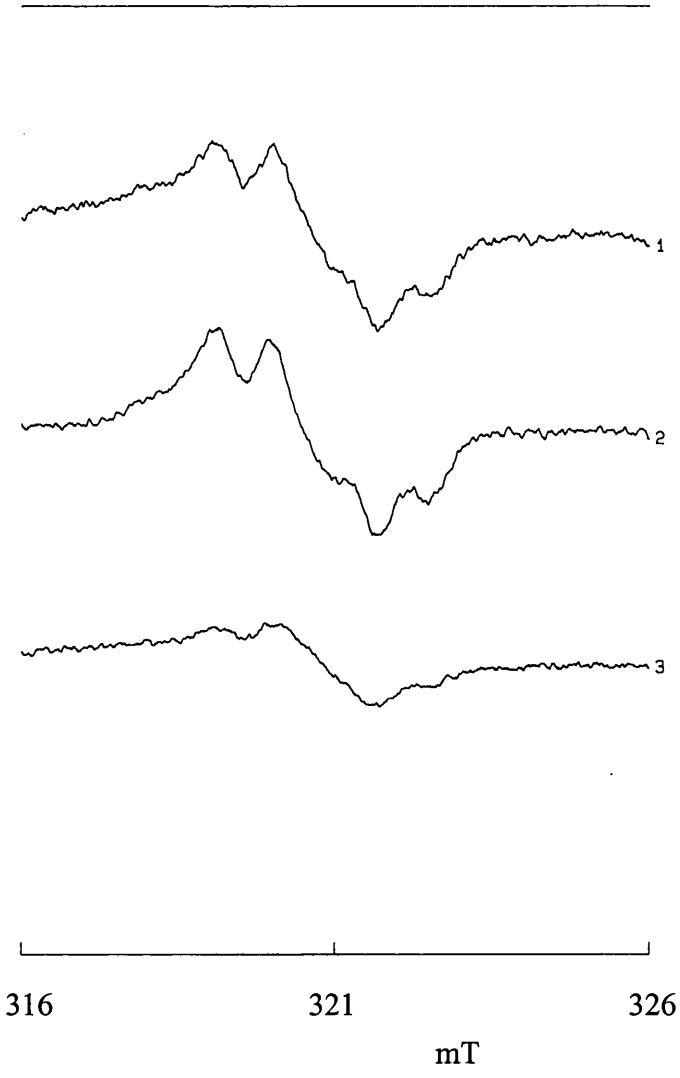
* These strains have been shown to be hetereplasmic so wild type copies of the gene are still present. They are referred to as deletion mutants as this was the characteristic we were trying to introduce.

Figure 4.16 EPR analysis of *Synechocystis* sp PCC 6803 wild type and *ycf*9 mutant cells



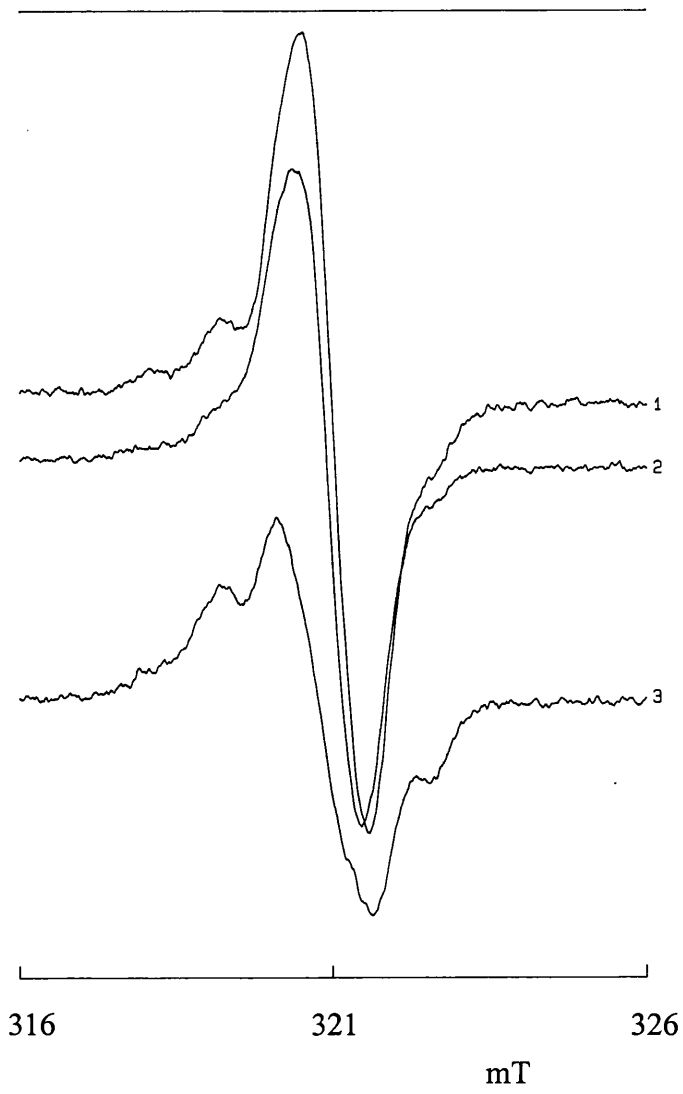
Trace 1. Wild type cells
Trace 2. Kn5k cells
Trace 3. Kn2a cells
Trace 4. PSII minus mutant

Figure 4.17 EPR analysis of Y_D^+ signal in *C. reinhardtii* wild type and *ycf*9 mutants



	[chlorophyll] mg/ml
Trace 1. Wild type cells	1.20
Trace 2. Cycf 7 cells	2.04
Trace 3. YcfΔ2 cells	2.06

Figure 4.18 EPR analysis of P_{700}^{+} signal in *C. reinhardtii* wild type and *ycf*9 mutant cells



Trace 1. Wild type cells
Trace 2. YcfΔ2 cells
Trace 3. Cycf7 cells

in trace two shows a similar amount of the PSI related peak but the shoulder which is caused by active PSII is much smaller. The final trace belongs to the insertion mutant cells. The result of illumination is much less dramatic with only a relatively small PSI signal present.

4.9 Conclusions

The range of work introduced within this chapter has been very broad. The first subject broached was the nature of a set of *C. reinhardtii* nuclear mutants which were already known to have altered PSII characteristics. It was hoped that the deficiency was caused by the absence of a nuclear factor required for the expression of a PSII gene. Examples of nuclear encoded proteins acting in this manner are known for the four chloroplast genes *psb*A, *psb*B, *psb*C and *psb*D. The transcription of these four genes had been monitored in most of the mutants and two were found to lack one of these transcripts. The aim of this investigation was to determine if the transcription of any other gene was affected in these mutants. Northern blots were performed using end labelled oligonucleotides to ascertain whether any deficiencies of this nature were present. The results of these extensive experiments were able to confirm, for almost all the mutants, that the transcripts for all known PSII chloroplast encoded genes were present. The only exceptions were due to poor RNA preparations which would require further repetition of the blots to confirm the absence of the transcript. The expression of PSII subunits may still be influenced by the absence of nuclear controlling factors in these mutants but they must act at another point in expression. Examples are known of nuclear proteins which are required for the translation of mRNA transcripts and it is possible that nuclear genes are required for the assembly of a functional PSII complex. Further experiments to ensure the translation of the transcripts would be required to determine if this was the cause of the altered PSII phenotypes in these cells. This would involve acquiring antibodies to the different subunits of PSII and carrying out Western blots to determine if the proteins were synthesised.

The series of Northern analyses highlighted the variability in the quality of RNA preparation. Any result which at first appeared to suggest a lack of any transcript had to be repeated and usually confirmed that the transcript was present in a different sample. One exception to this appeared to be one of the *psb*M related

transcripts which was consistently absent in several preparations. A polycistronic molecule is present in wild type cells which contains both the genes, *psb*M and *ycf*9. In addition a smaller transcript is also present which hybridises to the *psb*M probe but not the one for *ycf*9. The size of the transcripts and the positions of the genes rules out the possibility of the larger transcript being processed to form the shorter one. This suggests that a the second transcript must be initiated between the two genes. The Mϕ25 mutant initially appeared to contain only the smaller transcript which is specific to *psb*M and was lacking the polycistronic molecule covering both genes. A fourth repetition of the Northern blot with an Mϕ25 RNA sample and the *psb*M probe proved that both transcripts were present in this mutant and the previous absence may be due to the poor quality of the RNA preparation. It is possible that this transcript was particularly unstable because preparations from wild type and other mutant cells have also shown altered amounts of the larger *psb*M transcript and degradation products have been observed.

In the absence of any positive identification of the cause of the altered PSII phenotypes it was decided to investigate the role of *ycf*9. Deletion mutants were created in both *C. reinhardtii* . The eukaryotic mutants were created using a plasmid containing both the *psb*M and *ycf*9 genes. The *aad*A cassette was introduced at a *Bgl*II site which is positioned between the two genes. It was not known whether insertion between the genes would disrupt the expression of either or both the genes in the operon. A partial digest was then performed to remove the *ycf*9 coding sequence from the plasmid. The effect of this deletion was to be compared to the effect of inserting a large segment of DNA between the two genes. Both of the constructs were introduced into a wild type CC1021 strain of *C. reinhardtii.*

The prokaryotic mutants were prepared by first amplifying the region surrounding the gene using PCR and *Synechocystis* genomic DNA. The PCR product was cloned into a vector then a *Bsm*FI digest was used to remove a portion of the coding sequence. The kanamycin cassette, also amplified by PCR, was used to replace the coding sequence. Two different constructs resulted from this strategy. The first Kn5 had the kanamycin cassette in the predicted position replacing two thirds of the codons sequence and the 23 bases immediately

preceding the gene. The second construct, Kn2, had not been cleaved at the second *Bsm*FI site but had lost more of the upstream region. The kanamycin cassette was positioned 15 base pairs before the first codon of the gene and some expression through the kanamycin gene may be possible. Both constructs were transformed into a wild type strain of *Synechocystis* sp PCC 6803.

All the mutants were cultured to encourage the formation of homoplastic colonies then Southern blots were used to determine whether any of the cells still carried wild type copies of the gene. The *C. reinhardtii* mutants were shown to have the predicted sizes of fragment consistent with the mutant genotype. We cannot conclusively state that there are no copies of the wild type genome present in the mutants with the insertion between the genes, CycfSp, but there can be only very small quantities of wild type DNA present if any at all. The deletion mutants were confirmed to be totally homoplastic. The Southern analysis carried out on the *Synechocystis* mutants was not so conclusive. Wild type and mutant copies were both present so the cells were subjected to continued rounds of selection. The Southern analysis has yet to be repeated so the preliminary characterisation may be influenced by wild type copies of the gene.

The *C. reinhardtii* mutants proved to all be photosynthetically competent in spot tests on different media so all photosynthetic complexes must be present and active. However both oxygen evolution rates and EPR analysis suggested that PSII was adversely affected in all the mutant cell types. The mutants with the insertion between the genes showed a drop in the rate of oxygen evolution to approximately 50% of that seen in wild type cells. This suggests that the position of the *aad*A cassette is affecting expression of the *psb*M operon. The deletion mutants which in addition to carrying the *aad*A sequence have lost the *ycf*9 coding regions show an even lower rate of only 25% that seen in wild type samples. This effect implies that the absence of the *ycf*9 coding sequence is having an additional effect on PSII activity. The EPR spectra of the PSII signal Y_D^+ show a similar trend with the amount of PSII being lowest in the deletion mutants but the insertion mutants still show less than wild type cells. The EPR traces showing the effect of illumination on the same samples also highlight another effect. The deletion mutant has wild type levels of PSI activity as judged by the relative amount of the $P700^+$ signal. The insertion mutant however shows only a small $P700^+$ signal which suggests

that PSI activity is downregulated. The amount of each photosystem in each of the mutants must be sufficient to support photoautotrophic growth but each mutant appears to be affected in different complexes. The only difference between the two mutants is the lack of the *ycf9* coding sequence in the deletion mutant so the reason for the change in PSI activity in the insertion mutants is not at all clear. In the absence of any information relating to the expression of the two genes concerned, *psb*M and *ycf9*, we cannot draw any conclusions about which of the observable phenotypes is due to the disruption of the operon or to the deletion of *ycf9*. In light of this a Northern analysis would have to be performed before analysis of these mutants proceeds further.

The initial characterisation of the *Synechocystis ycf9* mutants gave differing results. The oxygen evolution and EPR experiments showed characteristics which did not differ significantly from wild type cells. Spot tests carried out on photoautotrophic and glucose supplemented media gave a puzzling result. All the mutants were capable of supporting growth on minimal media which implies that all the photosynthetic complexes are present and fully functional. BG11 media supplemented with glucose is used to culture PSII minus mutants which still possess active PSI. The mutants, which grew with no apparent difficulty under photosynthetic conditions, were unable to grow on the same medium when it was supplemented with glucose. This implies that in the presence of glucose linear photosynthetic electron transfer is suppressed and that these mutants are unable to metabolise glucose. The alteration may lie in the cyclic electron pathway around PSI, there may be a change in the pathway which detects glucose and alters the metabolism of the cell or glucose uptake and or metabolism may be affected.

In the prokaryotic mutants there appears no effect on PSII activity as was seen in the *Chlamydomonas ycf9* minus strain. There are many examples of eukaryotic deletion mutants which result in a complete lack of PSII activity while the equivalent mutant in a prokaryotic system leads to only an increase in photoinhibitory damage or no effect at all. The fact that these mutants have yet to be confirmed as homoplastic for the *ycf9* deletion means that I cannot draw any definite conclusions from these results. Until the wild type DNA for this gene has been shown to be absent the results of any experiments may be affected by the presence of wild type copies of the gene. Any further work carried out on these

mutants would have to include a Southern analysis to determine the state of the *ycf*9 gene.

The creation of deletion mutants has not led to the definition of the role of the Ycf9 protein. In eukaryotic cells its absence effects PSII activity and it may also play a role in the sensing of external conditions and the alteration of the cells metabolism as an adaptation to them. The deletion in *Synechocystis* PCC 6803 also perturbs the normal metabolism of the cells without any apparent effect on their photosynthetic ability. The presence of a confirmed homoplastic strain is required before any further comparison can be made.

Chapter 5

A Molecular Investigation of *psb*W in *C. reinhardtii*

5.1 Introduction

PSII is known to be composed of over twenty polypeptide subunits which are coded for by genes located in both the nucleus and chloroplast. Purification of the reaction centre complex (Nanba & Satoh 1987) led to the identification of five chloroplast encoded proteins D1, D2, the two cytochrome b_{559} subunits PSII-E and PSII-F and the small protein PSII-I present in this, the smallest known complex capable of charge separation. Larger purified PSII complexes have additional subunits including CP43 and CP47, the nuclear encoded extrinsic subunits of the oxygen evolving complex and many other small subunits of unknown function.

Initial identification of proteins as subunits of PSII was achieved by N-terminal sequencing of polypeptides from various purification protocols. One such subunit, known initially as the 6.1 kDa nuclear encoded protein, was sequenced by Ikeuchi et al. in 1989. Sequence data was obtained from both spinach and wheat and showed homology between the species. The absence of corresponding data from the sequenced chloroplast genomes of tobacco and liverwort suggested that this protein was encoded by a nuclear gene. This initial characterisation identified the 6.1kDa protein as being present in PSII membranes but not retained in the oxygen evolving core complex. The research discussed in this chapter was aimed at cloning and sequencing the gene for this protein in *C. reinhardtii.*

The sequence of the gene in *Arabidopsis thaliana* was determined from a randomly obtained cDNA (Hofte et al. 1993). Then, in 1995, two reports were published by Schröder's group in Sweden. They raised an antibody against a peptide corresponding to the 15 N-terminal residues of spinach PSII-W and used this to investigate the protein and isolate the gene. The antibody was used to create an immunoaffinity column which was then utilised in the purification of the protein (Irrgang et al. 1995). PSII core complexes were applied to the column and found to bind tightly. This implies that the N terminal epitope of PSII-W is exposed at the surface of the complex. The other protein subunits were eluted from the column by increasing NaCl concentration and adding Triton X-100. The PSII-W protein was then detached from the column in a low pH glycine buffer.

N-terminal protein sequencing of the purified fraction confirmed that the antibody had identified the PSII-W protein and extended the available sequence data. The absorption spectra of the purified protein indicated that no chromophores

were present (Irrgang et al. 1995). The antibody was also used to localise this subunit. A range of thylakoid membrane preparations and PSII complexes were screened for the presence of the PSII-W protein. Western blot analysis confirmed that the protein was in fact present in all PSII preparations including the reaction centre complexes. The 6.1kDa protein could not be removed by Tris washing at high pH or high salt concentrations so was proposed to be an integral membrane component (Irrgang et al. 1995). Analysis of the protein by electrophoresis revealed that the protein did not stain with Coomassie Blue and only reacted weakly with silver stain which could explain why its presence at the core of PSII had never before been reported.

The site directed antibody was also used to isolate the *psb*W gene (Lorkovic et al. 1995). A spinach cDNA expression library was immunoscreened and four phages were identified as containing the *psb*W coding sequence. Further cDNAs were isolated to provide as much 5` information as possible. The cDNAs were sequenced and the protein sequence deduced. This revealed that the mature protein contains 54 amino acid residues with a single transmembrane span. The coding sequence is preceded by a transit peptide of 83 residues which contains the characteristics of a bipartite transit sequence usually associated with lumenal proteins (Kieselbach et al. 1998). This type of transit peptide contains two domains. The first is responsible for import across the double envelope membrane and into the stroma where the polypeptide is processed into an intermediate form. After this, the second region is able to aid the protein as it crosses the thylakoid membrane. Once there, a peptidase cleaves the remainder of the signal peptide and the protein enters its mature form. It was confirmed that the transit peptide is capable of directing import of PSII-W which led to the accumulation of the mature protein (Lorkovic et al. 1995).

Nuclear encoded thylakoid membrane proteins are generally found with transit sequences which direct transport across the envelope and internal hydrophobic sequences which are required for insertion into the thylakoid membrane. PSII-W however has an additional hydrophobic targeting sequence, more characteristic of lumenal proteins which are fully translocated across the membrane. Lumenal proteins have been shown to be imported using a variety of mechanisms (reviewed Robinson & Mant 1997). The 33kDa WOC protein and

plastocyanin use a process which is related to the bacterial Sec pathway. A stromal equivalent of SecA has been identified and shown to be essential for import, as is the availability of ATP. The two smaller WOC proteins do not use this pathway and require only a pH gradient for import. The only proteins which use the second pH dependent pathway have been shown to be absent in prokaryotes, such as *Synechocystis* sp PCC 6803, so this mechanism may have evolved since the endosymbiotic event which gave rise to chloroplasts (Robinson & Mant 1997)

The two import pathways for lumenal proteins can be individually inhibited. Nigericin and other ionophores disrupt the pH gradient and azide inhibits the Sec-type pathway. Both inhibitors were used in the integration experiments with PSII-W and neither inhibited its import and maturation. This implies that although the protein has a similar transit sequence it uses neither of these pathways.

There are two other known examples of membrane proteins which have a bipartite transit sequence but are not affected by nigericin or azide. These are a subunit of the thylakoid ATP synthase CF_I and PSII-X, a small subunit of PSII. All three have a single transmembrane span whereas other known nuclear encoded membrane proteins have multiple spans e.g. PSII-S, light harvesting chlorophyll *a/b* binding protein. It has been proposed that the transit sequence provides a second temporary hydrophobic domain for PSII-W and PSII-X. The two hydrophobic regions would partition into the membrane and flip the intervening sequence across. A thylakoid processing peptidase would then complete the maturation process and cleave the transit sequence (Robinson & Mant 1997).

There have been two further reports from the Schröder group which extend our knowledge of PSII-W. The first looked into the composition and topology of this protein within the thylakoid membrane (Shi & Schröder 1997). In this they expanded on the localisation studies of their earlier publication (Irrgang et al. 1995). The localisation of PSII-W within the different fractions of the thylakoid membrane mirrored that of D1 i.e. 85% of the protein in the grana lamellae and 15% in the stromal lamellae. They attempted to analyse the amount of PSII-W associated with each reaction centre and found that the amount varied significantly between different types of preparation. The method of isolation appeared to influence the level of PSII-W retained and any use of Triton X-100 seemed to have

a particularly negative effect on the amount of this protein present. Further experiments confirmed those reported earlier which determined the orientation of PSII-W within the membrane. They were able to conclude that PSII-W lies in the opposite orientation to many other membrane proteins with the N-terminus in the lumen and the C-terminal residues exposed to the stroma.

The second report (Hagman et al. 1997) examined PSII-W to determine if it was subject to light induced proteolysis. They discovered that under photoinhibitory conditions the W subunit was degraded at a rate faster than that of D2 but slower than D1. Further examination led them to conclude that this was due to destabilisation caused by the loss of D1 rather than as a result of direct photodamage.

The precise role of PSII-W in the PSII complex is not known but its position as the only nuclear encoded subunit present at the heart of the reaction centre could be significant. It may be involved in the regulation of the amounts of PSII exerted by the nucleus. It could act as a nucleation point for the other core subunits to assemble around or could be needed for the attachment of the outer subunits e.g. WOC.

There is sequence data available for a number of species of higher plant and partial N-terminal sequence from the alga *Chlamydomonas reinhardtii*. An alignment of the known sequences is shown in Figure 5.1 and demonstrates the high level of homology in the N-terminal region. There has been no report of PSII-W in any prokaryote and the gene is not present on the sequenced genome of *Synechocystis* sp. PCC 6803 (Kaneko et al. 1996). The research discussed in this chapter was aimed at cloning and sequencing the PSII-W gene in *C. reinhardtii*.

Figure 5.1 An alignment of the known PSII-W sequences

Sequence data obtained by N-terminal protein sequencing (1) and deduced from DNA sequence data (2)
The solid line represents the position of the predicted transmembrane helix.

T.aestivum (1)	LVDERMSTEG	TGLSLGLSNN				
C.reinhardtii (1)	LVDERMNGDG	TGLPFG				
S.oleracea (2)	LVDERMSTEG	TGLPFGLSNN	LLGWILFGVF	GLIWALYFVY	ASGLEEDEES	GLSL
A.thaliana (2)	LVDERMSTEG	TGLPFGLSNN	LLGWILFGVF	GLIWTFFFVY	TSTLEEDEES	GLSL
	1 10	20	30	40	50	

References

T. aestivum	Ikeuchi et al 1989
C. reinhardtii	de Vitry et al 1984
S. oleacea	Lorkovic et al 1995
A. thaliana	Hofte et al 1993

Results

5.2 Amplification of *psb*W from a cDNA library

The first attempt to isolate the PSII-W gene sequence involved using a cDNA library as the DNA template in a PCR reaction. The library was a gift from Prof. Moroney (Louisiana State University) and was constructed from cells which had been growing photoautotrophically. The cDNA molecules were directionally cloned into a lambda-Zap vector between the *Eco*RI and *Xho*I sites as shown in Figure 5.3a. The PCR reaction was carried out using the T7 primer and a gene specific primer. The only information available for the *psb*W gene in *C. reinhardtii* is the partial N-terminal sequence reported by de Vitry et al. (1991). This was used to infer the most likely DNA codons which would represent this protein sequence. The codon usage data, used in this case, came from a review by Rochaix (1987) where the nucleotide sequence of eight nuclear genes was examined and the frequency of the different codons was determined. The consensus sequence used is shown below in Figure 5.2.

Figure 5.2 The N-terminal protein sequence of PSII-W and the inferred DNA sequence used to design a gene specific primer.

Protein	L	V	D	E	R	M	N	G
DNA	cug	guc/g	gac	gag	cgc	aug	aac	gg
% Frequency	94.5	(44 /52)	83.9	100	89	100	98.1	

A PCR reaction was carried out as described in Section 2.7.10, using the gene specific primer and T7, with an annealing temperature of 50°C for thirty cycles. The product amplified by this reaction is shown in Figure 5.3b. The fragment shown is approximately 900bp in length and contains DNA complementary to the gene primer designed against the PSII-W sequence. This was subsequently purified and blunt cloned into the vector pUC19 which had been

Figure 5.3a A diagram of a λ ZAP cDNA clone

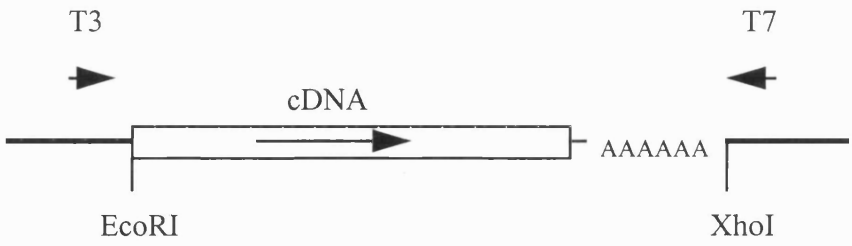
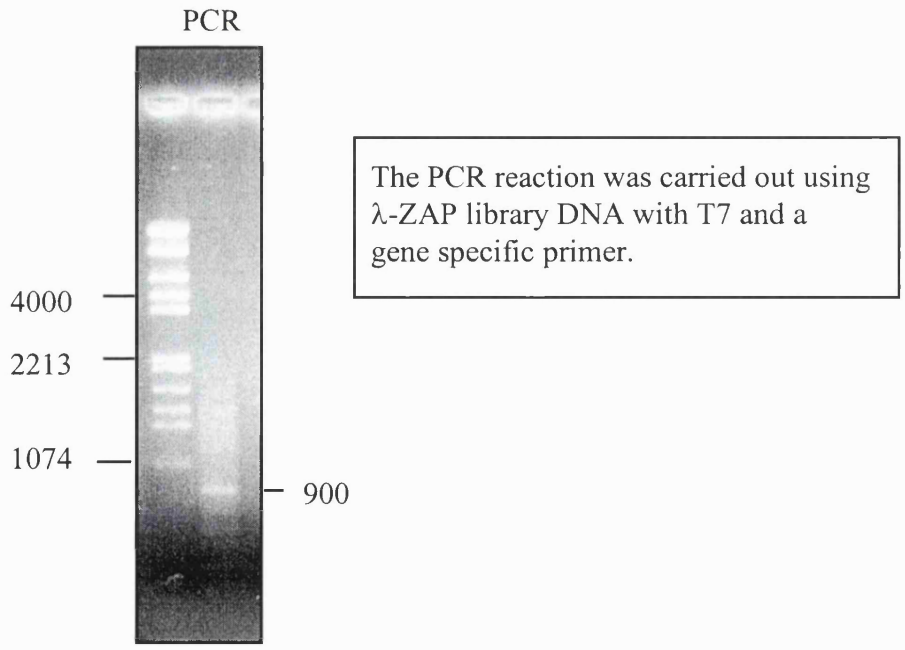


Figure 5.3b A gel photo of the PCR product amplified from the λ-ZAP library



cut with *Hinc*II. After transformation into DH5α and preparation of the plasmid (pPsbw) the insert was sequenced using an automated sequencer.

The 800bp of sequence data which was produced was GC rich indicating that it is likely to be due to a nuclear sequence but there was no homology to the *psb*W sequences published on the database nor any similarity to a particular gene type.

5.3 Rapid Amplification of cDNA Ends (RACE)

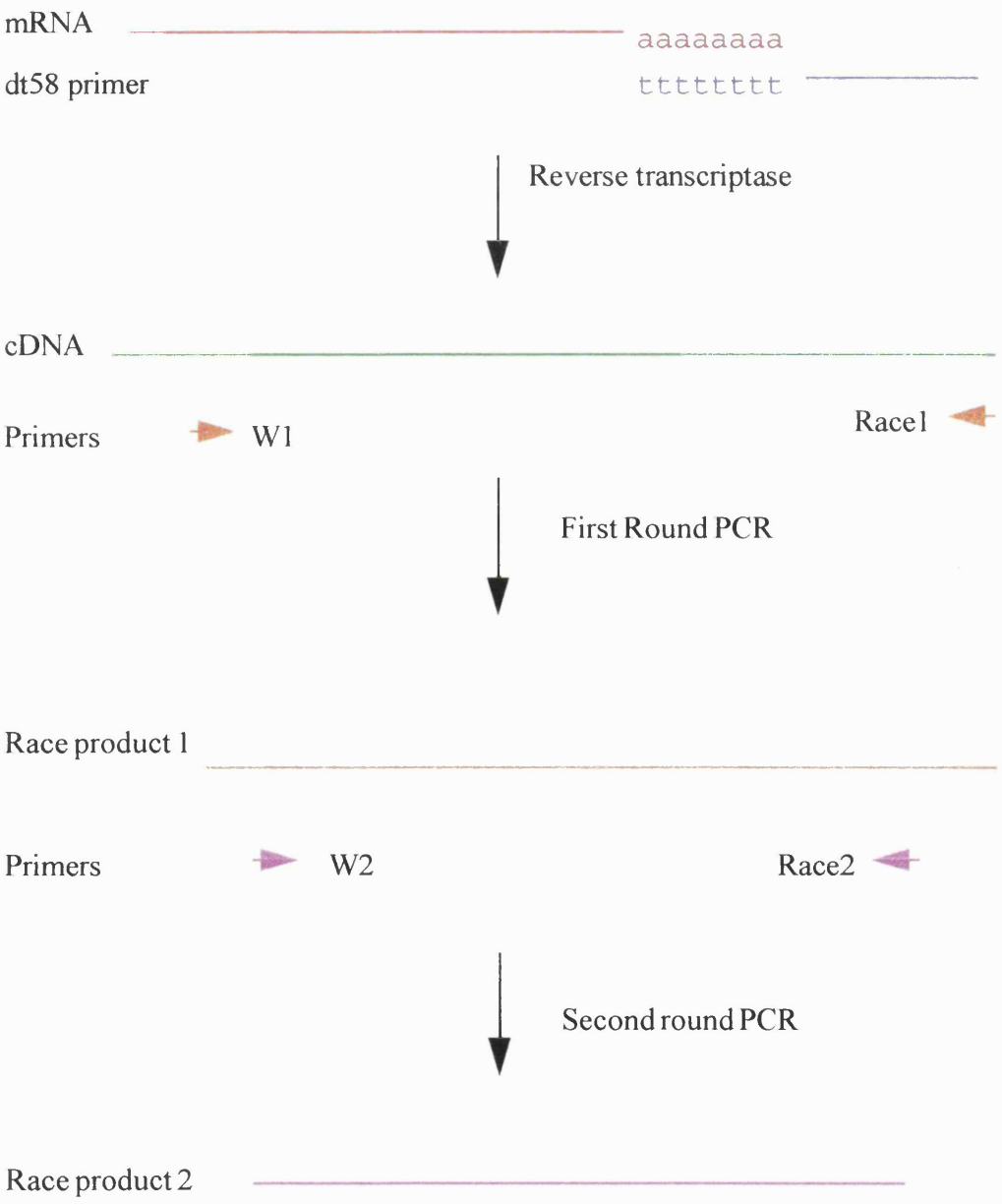
The technique known as RACE allows the researcher to amplify a desired cDNA from only a small length of known sequence (reviewed Frohman 1990, Schaefer 1995). The process requires the preparation of total cellular mRNA from the cell sample which is then used with reverse transcriptase to generate a corresponding population of cDNA molecules. The primer used in this synthesis is complementary to the poly A tail found on most mRNA molecules but it has additional nucleotides added after the thymines which are used in the next stage of the protocol. A summary of the amplification of sequence 3` to the known area can be seen in Figure 5.4. After synthesis of the cDNA pool the method involves amplification of the desired sequence by PCR. The primers used in this are complementary to the region of known sequence and to the introduced tag positioned after the poly-A tail. Any product amplified in this way can be screened by a second round of PCR using two internal primers. The purified PCR product is used as the template and the two primers lead to amplification of a slightly smaller product than the original.

The work to be described in this chapter used the 3` RACE reaction to amplify the genomic sequence of the *C. reinhardtii psb*W gene.

5.3.1 RACE primers

The process of RACE requires a region of known sequence in order to design primers. The only data available was the N-terminal protein sequence so this had to be the starting point in the planning of the experiment. When designing a new gene specific primer for this technique I decided to use a more degenerate primer to ensure that the correct sequence was not missed. In addition, I had

Figure 5.4 Schematic diagram to show the process of 3' RACE used to amplify the *psb*Wgene sequence



access to a different table of codon usage for nuclear genes which was compiled by using the data from 176 genes (www.dna.affrc.go.jp/nakamura-bin) whereas the older one, used in the previous section, was compiled from only eight sequences. Figure 5.5 shows the two overlapping gene specific primers designed for use in this protocol.

The first step of the RACE protocol requires the preparation of a population of cDNA molecules which is achieved using a poly T primer with an added tail section (DT-58). The 3` primers for the PCR sections of this method (race1 and race2) are complementary to the tag added at the cDNA synthesis stage. The sequence of each of these primers is given in the methods section, Table 2.5.

5.3.2 3' RACE to amplify the *psb*W gene

The RACE protocol requires the preparation of mRNA from *C. reinhardtii* cells and then synthesis of a corresponding cDNA pool. These two manipulations were carried out by Dr. N. Gumpel in our laboratory. I then used the cDNA as the template for the PCR steps of the *psb*W isolation initially with primers race 1 and W1. The details of the amplification are given in Section 2.8.3. The product of this reaction was run on an 2% agarose gel with a 100bp ladder and a fragment of approximately 670 bp was seen. This was subsequently gel purified and used as the template in the second PCR reaction which was designed to ensure that the product amplified corresponded to the *psb*W sequence. Internal primers race2 and W2 were used under the same conditions as the original amplification. Figure 5.6 shows a photo of a 2% agarose gel with both the first and second stage RACE products run against 100bp ladders. The product of the first reaction is approximately 50bp larger than the second product which is to be expected with the primers used.

Having potentially isolated a copy of the coding region for *psb*W we then needed to sequence the RACE product to ensure that it did correspond to the gene. This was achieved by direct sequencing of the purified PCR product on an automated sequencer. Details of the sequence will be given in a later section but it was obvious that the data did relate to the *psb*W gene through the levels of homology to published sequences from other species. The purified RACE product

Figure 5.5 The *psb*W specific primers used for RACE

Given below is the known protein sequence of PSII-W together with the codons which relate to the different amino acids. Where multiple codons are possible all alternatives were included in the primer design

Primer W1

Protein	L	V	D	E	R	M	N
Codons	cug c	gug c	gac u	gag	cgc g u	aug	aac
Primer	ts	gts	gay	gag	cgb	atg	aa

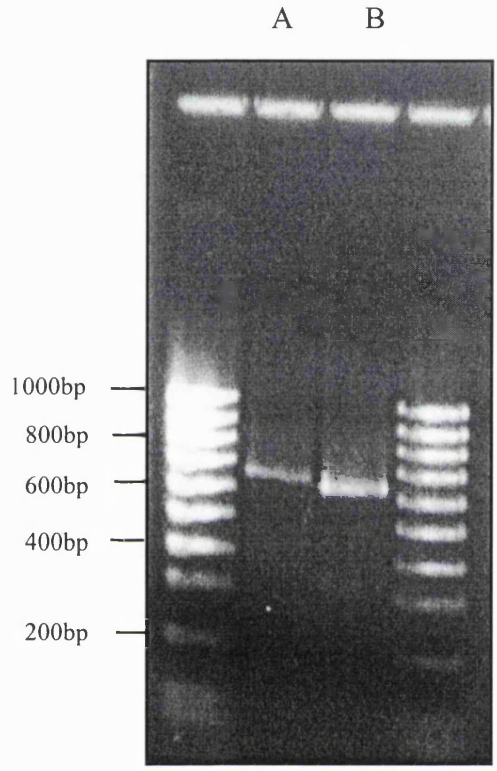
Primer W2

Protein	M	N	G	D	G	T	G
Codons	aug	aac u	ggc u g a	gac u	ggc u g a	acc g u a	ggc u g a
Primer	atg	aay	ggn	gay	ggn	acn	gg

Redundancy codes

s = gc
y = ct
b = cgt
n = acgt

Figure 5.6 A gel photo showing the results of the RACE reaction using *psb*W specific primers.



Track A shows the first RACE product which is approximately 660bp. This was then used as the template DNA with internal primers to amplify the second product shown in track B.

was blunt cloned into a pSK bluescript vector for ease of handling throughout the rest of the investigation. This plasmid will be referred to as pHW.

5.4 Southern Analysis

5.4.1 Determination of copy number of the *psb*W gene

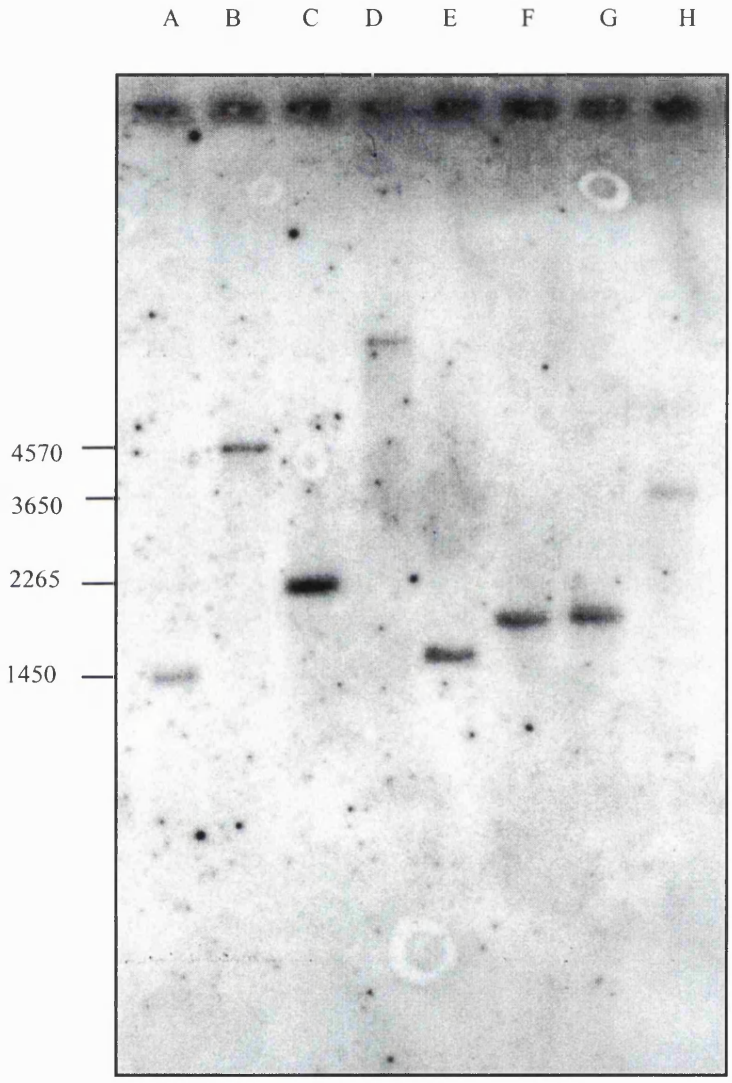
The cloned *psb*W DNA we had isolated contains no transit sequence because we designed the primer against the N-terminal sequence of the mature protein. In addition the copy which I cloned into the vector had been amplified by PCR which can introduce errors into the sequence. In order to address both of these issues we decided to isolate a genomic copy of the gene from a cosmid library. Before this was attempted we needed to ensure that there was only a single copy of the gene in the nucleus. The paper by Lorkovic et al. (1995) reported a single copy of *psb*W in the spinach nuclear genome and we hoped to confirm the same result for *C. reinhardtii*. All known *C. reinhardtii* nuclear photosynthetic genes exist in a single copy with the exception of *rbc*S which is present twice.

Genomic DNA was prepared from wild type cells grown under a light intensity of 40μE/m^2/s using the method of Rochaix et al. (1988) described in Section 2.7.8. Restriction enzyme digests were then performed using a variety of enzymes with varied recognition sites. The resulting fragments were separated on a 1% agarose gel and transferred to a filter. This was used in a Southern analysis for which the probe was a random labelled copy of the RACE product. Figure 5.7 shows the result of this analysis. Each of the different enzymes gave a single band on the autorad with the approximate sizes varying from over 8kb with *Not*I to 1.45kb with *Bam*HI. This result confirms that there is only a single copy of the *psb*W gene on the *C. reinhardtii* genome. If multiple copies were present then each digest would produce two or more fragments carrying the sequence which the probe recognises.

5.4.2 Southern analysis of PSII nuclear mutants

The nuclear mutants that were discussed in chapter 4 have an unidentified mutation in the nuclear genome which causes a PSII minus phenotype. It is possible that the transforming DNA has integrated into the *psb*W gene. There are

Figure 5.7 A Southern blot of wild type DNA used to determine the copy number of the *psb*W gene.



DNA was prepared from wild type *C.reinhardtii* then digested with a number of different restriction enzymes.
The enzymes used were A. *Bam*HI, B. *Hinc*II, C. *Nci*I, D. *Not*I, E. *Pst*I, F. *Pvu*II, G. *Rsa*I and H. *Sac*I.
The blot was probed with the labelled RACE product

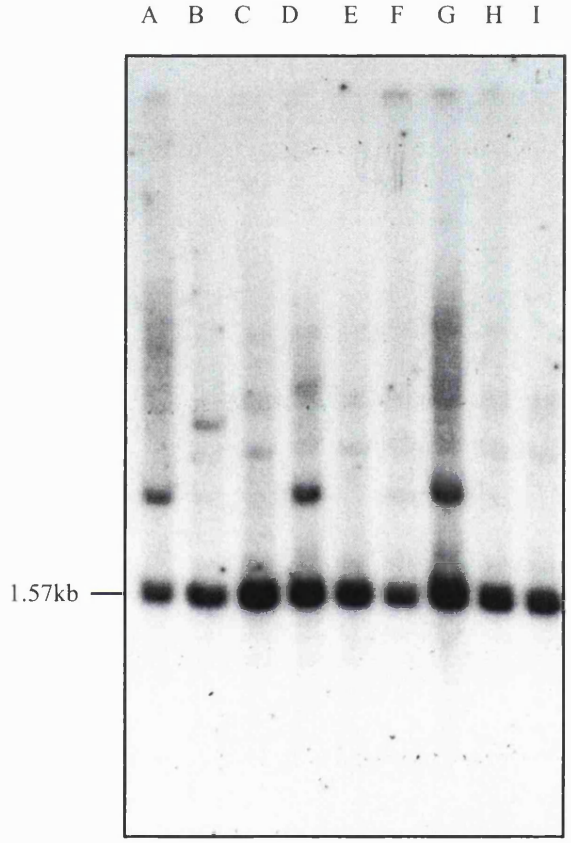
no reported PSII-W minus mutants but it is possible that the lack of this protein may lead to a phenotype similar to those of these mutants. The position of the PSII protein at the core of the reaction centre suggests it may be required for the assembly of a fully functional complex.

Total cellular DNA was prepared from each of these mutants and restriction digests were carried out using the enzyme *Pst*I. The DNA was then separated on an agarose gel and the fragments transferred to a filter for Southern analysis. The same probe, derived from the RACE product, was used and the result can be seen in Figure 5.8. The wild type digest shown in Figure 5.7 gave an estimated size of 1.57kb for the *Pst*I fragment carrying the *psb*W gene. If the nuclear mutants have a disrupted *psb*W gene we would expect to see a different size fragment which would include at least some of the transforming DNA. All the mutants have a band at the same position to the lower of the two bands in the wild type track. This relates to the size predicted from the last Southern blot so we can assume that all the mutants contain an uninterrupted copy of the *psb*W gene. The multiple bands seen in some tracks, including that of wild type DNA, are most likely due to incomplete digestion of the DNA.

5.5 Screening a cosmid library

The cosmid library used was prepared by Purton & Rochaix (1995) in the vector pARG7-8cos. The library contains $8.0x10^5$ independent clones with inserts ranging from 27 to 38kB. The library was plated out to a density of 20-50,000 colonies per plate by Dr. V. Lumbreras in our laboratory and had been stored at 4°C for four weeks. Duplicate lifts were taken from each of the ten plates. I then hybridised the filters with the random labelled RACE probe to identify clones which were carrying the *psb*W gene. The resulting film showed many positive clones were present but the density of the colonies on the plates made it impossible to identify the individual colonies responsible. Samples were taken using a toothpick to remove several colonies, in an area identified as containing a positive clone. These were placed into LB and grown overnight at 37°C. A second set of plates was then produced from these overnight cultures which contained between 100 and 200 colonies per 8cm plate. At this density it should be possible

Figure 5.8 Southern analysis of wild type and nuclear mutant genomic DNA



Genomic DNA was digested with the restriction enzyme *Pst*I then probed with the labelled *psbW* RACE product.
The tracks A-I contain DNA from wild type, PM10, Mϕ14, Mϕ16, Mϕ20, Mϕ21, Mϕ25, Mϕ27 and Mϕ38 respectively.

to isolate individual positive clones. The plates were incubated at 37°C to allow the bacteria to form colonies.

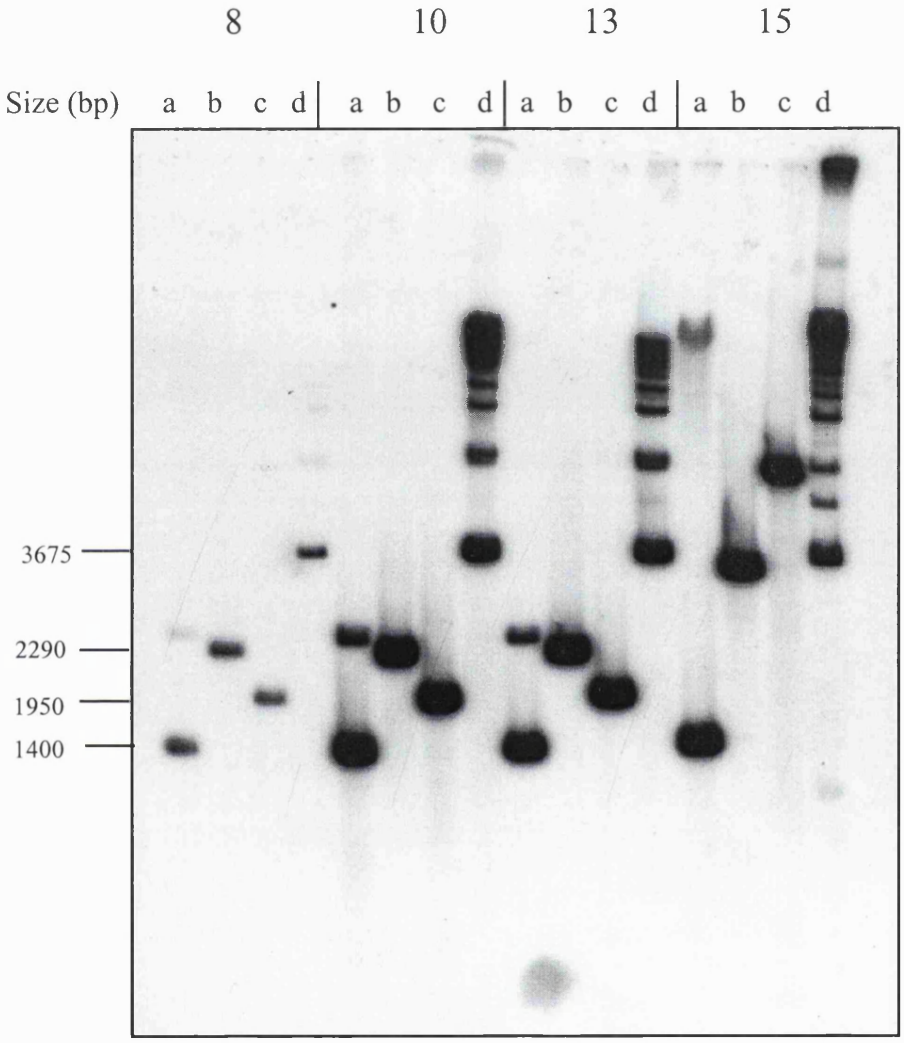
The following day filter lifts were taken from each plate as described in Section 2.9.3. These were placed in denaturation and neutralisation solutions then allowed to dry. The filters were then probed with the same RACE probe as used previously, washed and exposed to X-ray film. Each plate contained approximately 5-10 positive clones.

Sixteen colonies which appeared to match up to spots on the film were selected, picked into LB and grown overnight. DNA was then prepared from these cultures to identify which contain the desired *psb*W sequence. The cosmids were prepared then digested overnight with the enzyme *Pst*I. The fragments generated in this way were separated on a 1% agarose gel and from the gel photograph (not shown) it could be seen that all 16 contained a different pattern of bands. The gel was blotted onto a filter then used in a Southern analysis with the labelled RACE product as the probe. The result of this hybridisation (data not shown) was that four of the sixteen selected clones contained DNA complementary to the *psb*W probe. The cosmids were named pGW (after genomic *psb*W) and the positive clones were numbers 8, 10, 13 and 15.

An additional Southern analysis was carried out on these four positive clones. A larger scale DNA preparation was made for each, then the DNA was digested with four of the enzymes used in the analysis of wild type DNA seen in Figure 5.7. The fragments generated by these digests were separated by electrophoresis then blotted to a filter for Southern analysis. The sizes of the fragments generated in the earlier wild type Southern analysis had been calculated and similar values would be expected from these genomic cosmid clones. The result of the Southern hybridisation can be seen in Figure 5.9.

The expected band sizes, calculated from the wild type Southern, are shown in Table 5.1 along with the calculated sizes from the cosmid filter.

Figure 5.9 Southern analysis of cosmid clones hybridised with the RACE probe



The DNA from each of the cosmids pGW8, pGW10, pGW13 and pGW15 was digested with four different enzymes
a. *Bam*HI
b. *Nci*I
c. *Pvu*II
d. *Sac*I.
The filter was probed with the random labelled RACE product.

Table 5.1

Sizes of the bands (in kb) seen in genomic Southern analyses with the RACE probe.

Enzyme	Wild type	pGW8	pGW10	pGW13	pGW15
***Bam*HI**	1.45	1.40	1.40	1.40	1.40
***Nci*I**	2.24	2.29	2.29	2.29	3.43
***Pvu*II**	1.82	1.95	1.95	1.95	5.43
***Sac*I**	3.55	3.68	3.68	3.68	3.68

The results seen for the genomic cosmid clones confirm that the *psb*W sequence is present in all four. The banding pattern produced by the first three clones on the blot matches that seen from the wild type genomic DNA. All the values are calculated from the distance each band has migrated in comparison to the migration of known size markers. Some small variation, as seen in these results would be expected. The cosmid clones also show some additional bands which are not seen on the wild type filter. This could be due to incomplete digestion by the restriction enzyme for example in the tracks caused by the *Sac*I enzyme. Another alternative explanation is the presence of the *psb*W gene at the end of the insert on the cosmid. This may explain why pGW15 has two larger bands than the wild type cells. If the cosmid insert ends before the position of the *Nci*I and *Pvu*II sites on the wild type DNA then vector DNA would be included in the band produced on this filter.

This result confirmed that we have obtained four cosmid clones which contain a genomic copy of the *psb*W gene from *C. reinhardtii* which were then used in sequencing reactions.

5.6 Subcloning a *psb*W cosmid

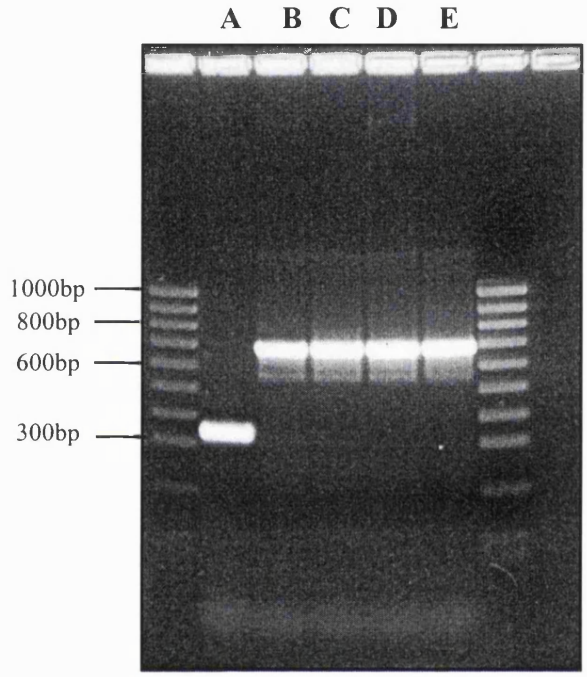
Attempts to sequence the cosmid clones containing the *psb*W gene sequence were initially unsuccessful. In order to facilitate the sequencing of the gene a subclone was made from the large cosmid clone. The *Pvu*II digest produces a 1.9kb band which carries the coding sequence for the *psb*W gene and this fragment was subcloned. The size of this insert means that there is a reasonable

chance of isolating the whole gene sequence and it will be easier to handle when cloned in a vector. A large scale *Pvu*II digest was carried out on pGW13 DNA and the resulting fragments were run on an agarose gel. The 1.9kb band was identified and excised so that the DNA could be purified. This was then cloned into a bluescript pSK vector cut with another blunt cutting enzyme *Hinc*II. A vector : insert ratio of 1:3 was used for the ligation reaction. After overnight incubation this was transformed into competent DH5α cells and antibiotic selection was used to identify transformants. Six colonies were chosen to be analysed and after plasmids from them had been prepared it became clear that five out of the six definitely contained the 1.9kb insert (gel photo not shown). The subclones were named pGWP 1-6 and were subsequently used in sequencing reactions to determine the genomic *psb*W sequence.

5.7 Presence of an intron in *psb*W

The presence of an intron within *psb*W was discovered by PCR analysis prior to sequencing. A PCR reaction was set up, using both the cloned RACE product, pHW, and the genomic cosmid clones as the template DNA, with primers which were complementary to the 5` end of the gene, W1 and an internal primer Wseq2. The positions of these primers relative to the gene sequence can be seen in Figure 5.12a. The product size was predicted, from the known sequence of the cDNA copy of the gene, to be 350bp. The PCR reaction was carried out at an annealing temperature of 51°C for 30 cycles. The products which were amplified were viewed on an 2% agarose gel using a 100bp ladder as the known size standards. Figure 5.10 shows the products amplified from the four different cosmid clones in comparison to that obtained when the RACE product is used as the template. The cDNA copy gives a band which matches the predicted 350bp size. The genomic copies of the gene give a band almost twice as large at approximately 680bp. This shows that the genomic DNA contains sequence which is removed when mature mRNA is formed. *C. reinhardtii* nuclear genes typically contain one or more introns so their presence in the *psb*W gene is not unexpected.

Figure 5.10 A gel photo showing the results of the PCR reaction using two *psb*W specific primers



The PCR products shown between the 100bp markers were amplified using primers which were complementary to the 5` sequence and an internal region of the gene. The template DNA used was :

A. pHW (cloned RACE product)
B. pGW8 (genomic cosmid clone)
C. pGW10 (genomic cosmid clone)
D. pGW13 (genomic cosmid clone)
E. pGW15 (genomic cosmid clone)

5.8 Northern analysis of *psb*W

RNA was prepared from wild type *C. reinhardtii* cells and run together with existing RNA samples from the PSII nuclear mutants on a denaturing gel. RNA markers were included on the gel so that the size of any *psb*W transcripts could be determined. The gel was visualised using ethidium bromide then the RNA transferred to a filter for Northern analysis. The filter was hybridised with the random labelled RACE product at 42°C overnight. The filter was then washed at first 42°C then at 65°C to remove any non specifically bound probe. The film which was developed after autoradiography is shown in Figure 5.11.

The first track contained the RNA markers and non-specific hybridisation has occurred with the smallest marker showing up as a faint band. The following three tracks contain RNA prepared from wild type, Mϕ16 and Mϕ38 respectively. The samples show a single band hybridising to the *psb*W probe. Other faint bands which are visible above this are due to non specific reaction with ribosomal RNA and contaminating DNA.

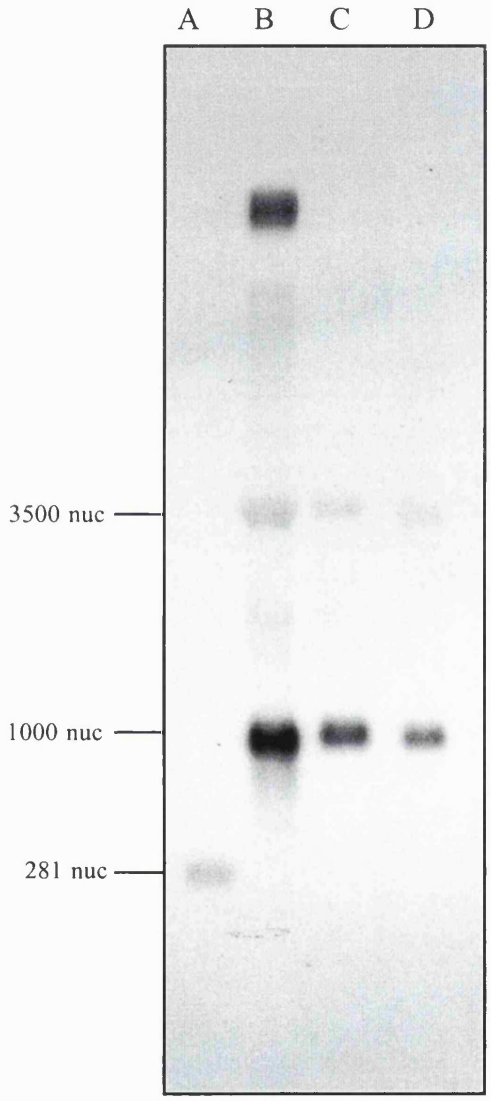
The size of the *psb*W specific band has been calculated using the migration of the molecular weight markers to draw a calibration curve which was used to infer the size of the gene transcript. The resulting transcript was calculated to be approximately 1000 nucleotides long.

5.9 Sequencing the *psb*W gene in *C. reinhardtii*

5.9.1 Sequencing the *psb*W RACE product

The *psb*W specific RACE product was sequenced to ensure that the amplified DNA did indeed correspond to the gene of interest. The initial sequencing reactions were performed using the primers, W1 and W2, which were used in the RACE amplification of the DNA. This data allowed us to confirm the identity of the purified DNA fragment and to design further primers with which to determine the whole sequence. Once the fragment had been identified as specific to the *psb*W gene it was cloned into a pSK bluescript vector. Cloning the DNA made it easier to manipulate and also allowed us to use the universal plasmid specific primers T3 and T7 to sequence from both ends of the fragment. Figure 5.12a shows the relative positions of all the primers employed in the sequencing of this gene.

Figure 5.11 Northern analysis of *psb*W probed with the labelled RACE product



RNA was prepared from B. wild type, C. Mϕ16 and D. Mϕ38 *C. reinhardtii* strains. RNA markers were run on the same gel in track A. The filter was probed with the labelled RACE product

Figure 5.12a
Diagram to show the primers used for sequencing the cloned RACE product

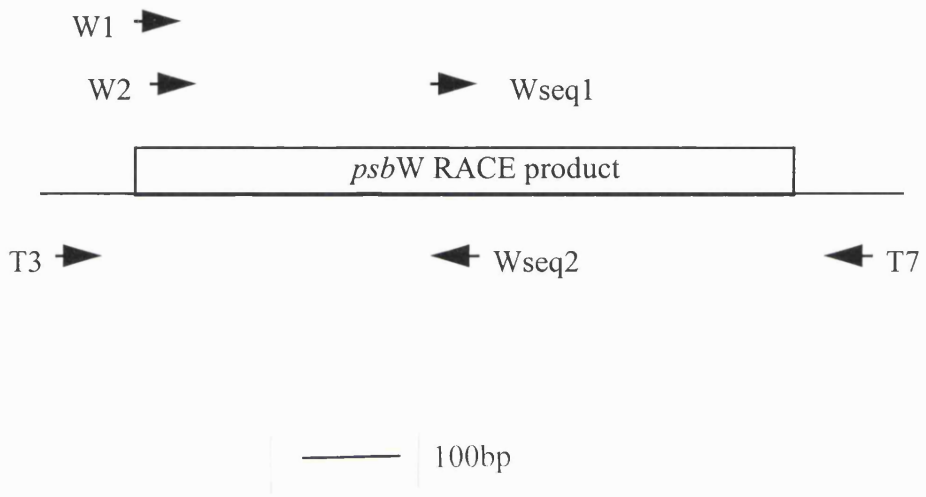
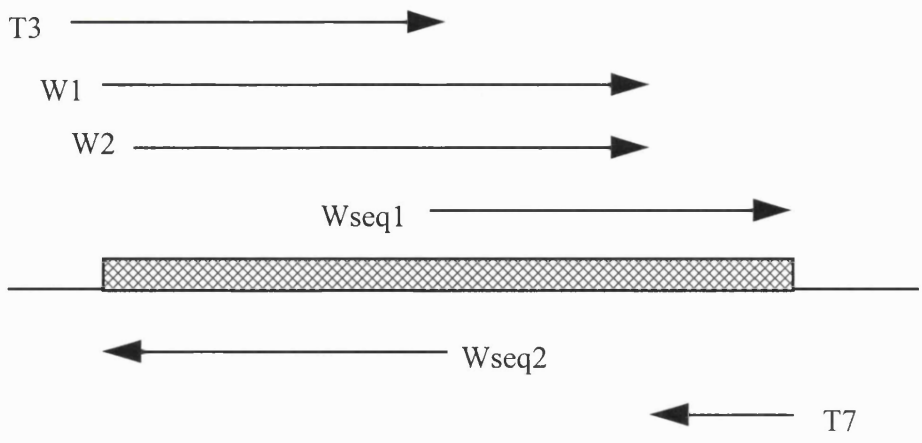


Figure 5.12b
Diagram to show the extent of the sequencing of the RACE product



The aim of the sequencing strategy was to obtain sequence data from both strands of DNA for the whole of the amplified region. This, as is shown in Figure 5.12b, has almost been achieved with only a 150 base pair gap in one direction remaining. The sequence determined for the RACE product is shown in Figure 5.13 along with predicted protein sequence. The DNA sequence from the T3 primer covers the region which is translated into the N-terminal region of the protein. This region, used to design the primers, may not contain a true reflection of the actual gene sequence because degenerate codons were used. The RACE product will contain the sequence of the primer which annealed with the greatest accuracy to the cDNA but the actual sequence will have to be determined from a genomic copy of the gene. However the protein sequence data used to design the primers came from a *C. reinhardtii* protein so we would not expect to find any alteration when this region is sequenced in the genomic copy. The protein sequence data available for *psb*W in *C. reinhardtii* included four further amino acids which were not used in the primer design and the translated DNA matches these extra sequence residues.

5.9.2 Sequencing the *psb*W cosmid clones

The amplified RACE sequence gave us some of the information we sought about *psb*W in *C. reinhardtii.* It was not able to tell us anything about the transit sequence because the primers used to obtain the fragment were designed against a region of DNA which follows the signal peptide. Another problem, relating to the method of isolation of the gene sequence, is the issue of inconsistencies introduced by PCR. The enzyme used was a mixture of thermostable Taq DNA and Pwo DNA polymerases which are marketed as Expand^{TM} High Fidelity PCR system (Boehringer Mannheim). The Expand ^{TM} High Fidelity PCR system has an error rate of 8.5 x 10^{-6} which compares to a rate for Taq of 2.6 x 10^{-5}. This threefold increase in fidelity is due to the inherent 3`-5` exonuclease proof-reading activity of Pwo DNA polymerase. Despite using an enzyme system with a low error rate the possibility that the DNA sequence has been altered by the amplification process cannot be excluded. In addition to these factors other experiments have made us aware of the presence of intron sequences in the *psb*W gene. These are

Figure 5.13 Sequence of the *psb*W RACE product

```
CTGGTCGATGAGCGGATGAACGGCGACGGCACTGGCCGCCCCTTCGGCGTGAACGACCCC   60
 L  V  D  E  R  M  N  G  D  G  T  G  L  P  F  G  V  N  D  P
 *
GTCCTGGGCTGGGTCCTGCTGGGCGTCTTCGGCACCATGTGGGCTATCTGGTTCATCGGC  120
 V  L  G  W  V  L  L  G  V  F  G  T  M  W  A  I  W  F  I  G
       *
CAGAAGGACCTGGGCGACTTCGAGGACGCCGACGATGGCCTGAAGCTGTAAATGGCTTCG  180
 Q  K  D  L  G  D  F  E  D  A  D  D  G  L  K  L

TAGTGCTTCCTTTTTTAGCAGCTTCGTAGTCGTGACTCGAATGGCCACGGTTCTTTGCGG  240

TCCGTAGCCTGCGGTGGAGCGGAGGGTGTGGATGCGGCAGCGGCAGTGCCCTCTTGAACC  300

GTCCTGGAGCGGTGGAGGTCATTCCAGGGTTTTGTCAGCAGCTCTGCGGTGGAGCTTGTG  360

GTGTGGTTGTGGATACTGGTGCGGCTGTAGCAGGCGCATGACGCGTGCACAGCAAGAAGC  420

ACGCACCACGATGTGGTGTGCGGGCAGGAGCAGGTGGCAGAGAAGAACAAGAAAGATAGA  480

TATAATTTTCAACGGCTATCAAGGCCGCTTGGAAAGCGCTCTAGGTTTTGCCTCGGTGCA  540

ACCATTGCACAGACCTGTTTAGGGAGAGCATGCGCCCAACTGTAATTCCCAAGTTTA     597
```

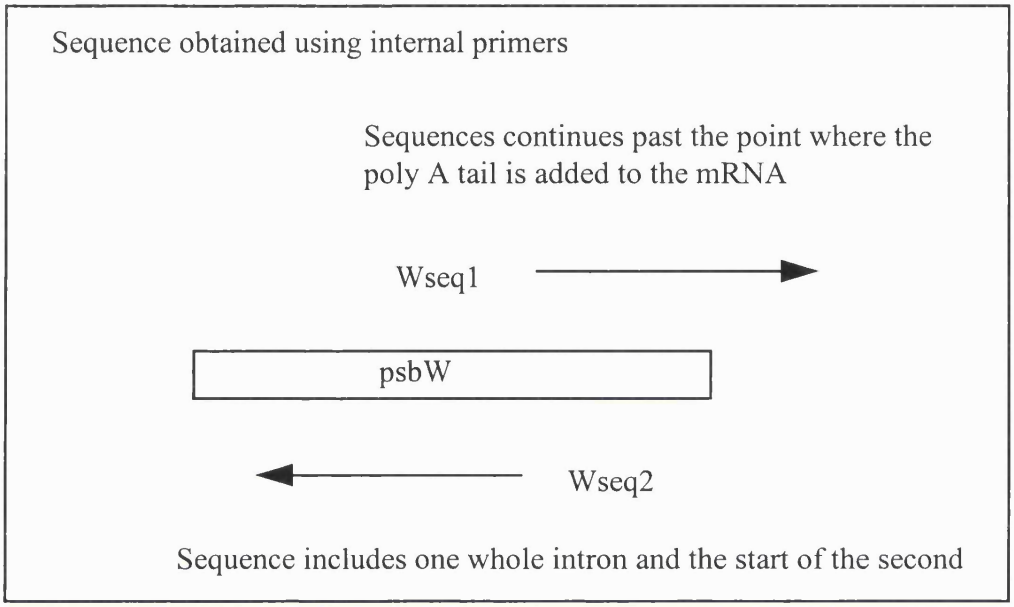
Where genomic sequence is available it has been compared to the sequence from the amplified RACE product. The data analysed to date has shown no discrepencies between the amplified and genomic sequence. The positions of the two introns present in the *psb*W gene have been identified as bases 61 and 128 shown by a green asterisk. The second intron is known to be 200 base pairs in length but the end of the first intron has not been sequenced. The arrows show the positions of the primers used in the RACE reaction. The red arrow highlights the sequence complementary to W1 while the blue denotes the position of W2.

spliced out during the process of RNA maturation so the cDNA molecules used in the RACE procedure were missing these sequences. All of these points make the sequencing of a genomic copy of *psb*W essential to fully characterise this gene in *C. reinhardtii.*

Initial sequencing reactions were carried out using the cosmid clones as the template DNA with the internal *psb*W sequencing primers. Figure 5.14 shows the extent of the sequence data achieved to date. The primer Wseq1 allowed us to examine the sequence at the 3` end of the coding sequence, as far as and through the position of the poly-A tail. This confirmed the sequence data which we had collected from the RACE product and showed that no errors had occurred during amplification. The other internal sequencing primer, Wseq2, gave information about the 5` end of the gene sequence. The presence of intron sequence was determined from PCR experiments described in Section 5.7 and the sequence data has confirmed their presence. The first to be sequenced from Wseq1 is exactly 200 base pairs in length and the sequence data relating to it is shown in Figure 5.15. The PCR experiments predicted an additional 350 base pairs of intron sequence so it was no surprise to discover the start position of another intron. The extent of the sequence determined from Wseq2 was not sufficient to reach the end of this second intron so we do not yet known precisely how large it may be but we can predict it should be in the order of 150bp long. Attempts were made to obtain sequence using the N-terminal primers W1 and W2. This would have located the end of the unmapped intron and confirmed the N-terminal protein sequence. However difficulties have been encountered in obtaining any further sequence from the cosmid clones. The size of the template DNA and the high GC content are suggested to be important factors in the failure to produce sequence data as they increase the likelihood of secondary structure formation.

A subclone of the cosmid, pGWP, was created to try and increase the success of the sequencing reactions. The subclone contained a 1.9kb insert derived from a *Pvu*II digest of one of the cosmid clones. The presence of the *psb*W gene on this plasmid was confirmed by carrying out a PCR reaction using the primers W1 (at the 5` end of the gene) and Wseq2 (an internal primer). The product of this reaction was a 650bp fragment which was identical in size to the product obtained

Figure 5.14
Figure showing the relative positions of sequence data collected from the genomic copy of the psbW gene.



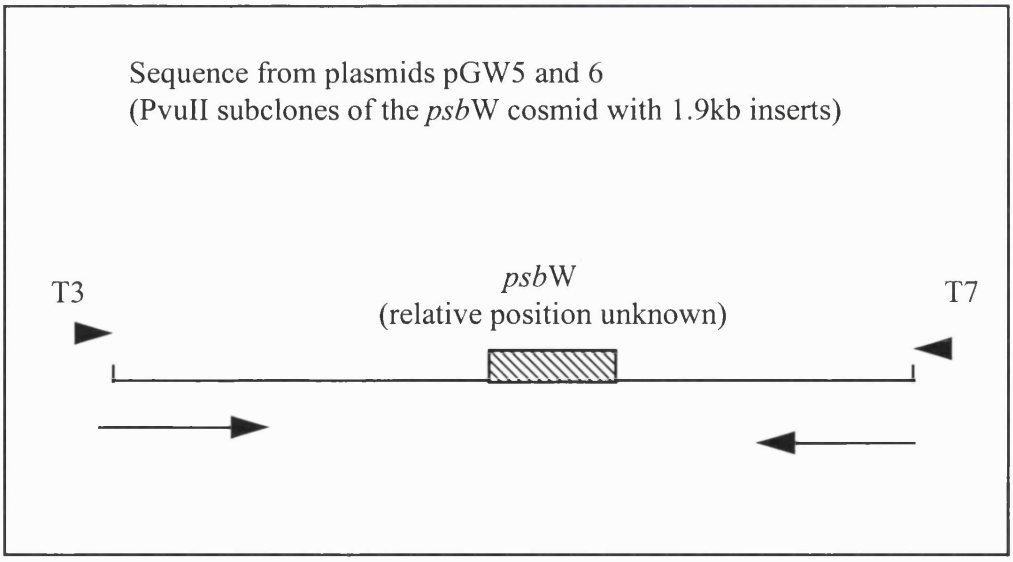


Figure 5.15 Known sequence of the introns in *psb*W

Intron 1 (5' sequence not yet available)

....gctgaatgcgcatgggctgcttcgctggctcgccacgtctcc
ttttcaagctcgtgcttgatcaaagtctgtgttcttctgcag

84 bases of known intron sequence available.
PCR experiment predict the total intron sequence to be in the order of 350bp. Intron 1 is expected to be approximately 150bp in length.

Intron 2

gtgagctccgcgccatgccacgtcgcggtgcttcccgtgtgtaaata
gtggtccccaagctatcctgggagggatccgatagccttgccacaat
cctgcacacgctctatgcgcggtcttgatattctcagtgtgacgcac
cgctgcttttgacacggctgctgaggcgactgtccccttcctacccc
ttctcaatgcag

Intron 2 is exactly 200bp in length and maps to position 128 of the sequenced RACE product (Figure 5.13).

using the same primers with the intact cosmid clone as the template (result not shown). This DNA was then used as the template for a range of sequencing reactions. Results were obtained using the T3 and T7 primers which amplify from the multiple cloning site of the vector. This data covered approximately half of the insert of the subclone, pGWP, but no gene sequence was found. This tells us that the gene lies on the unsequenced middle region of the cloned fragment. Further sequence information has yet to be obtained for this gene. Reactions have been carried out using all the primers available and a new primer which lies at the 5` end of the gene in the opposite orientation to W1. This primer should have enabled us to sequence the transit peptide region of *psb*W. The assumed reason for the failure of these reactions is the very high GC content of the DNA. The quality and quantity of the template DNA has been thoroughly checked and been discounted as the cause of the problem. An alternative protocol for the sequence reactions has been employed which includes the use of DMSO to destabilise any secondary structure which may form and increased the quantity of the terminator mix. This protocol has been successfully used in other situations to sequence GC rich regions of DNA which failed to produce data using the standard protocol. In the case of this gene however it was not sufficient to overcome the problems which have been encountered.

5.9.3 Summary of *psb*W sequence information

The sequencing strategy for the *psb*W gene in *C. reinhardtii* has yet to be completed due to the nature of the DNA. An amplified copy of the *psb*W gene has been sequenced and the protein sequence deduced from it. The C-terminal protein sequence has been confirmed from genomic data to be free of introduced errors but this has yet to be accomplished for the entire coding sequence. No information has been obtained relating to the transit peptide which is assumed to be present with this gene and the presence of two introns have been detected.

Figure 5.16 shows an alignment of all known PSII-W sequences including the deduced *C. reinhardtii* protein. The spinach and Arabidopsis sequences extend over both the transit and coding regions while other data is more limited. The ice plant transit sequence and the sorghum partial protein sequence are both derived

Figure 5.16
Alignment of known PSII-W protein sequences

Transit sequences

```
Spinach      MATITASSSA SLVARASLVH NSRVGVSSSP ILGLPSMTKR
Arabidopsis  MASFTASAST VSAARPALLL KPTVAI SAP VLGLPPMGKK
Ice plant    MATITASSSA SLVARASLVQ NRRVGVSSSP VLGLPAMTKK

Spinach      SKVT   CSIENK PSTTETTTTT NKSMGASLLAAAAAATI SNPAMA
Arabidopsis   K GGVRCSMETK QGNVSVMGAG      VSAAATAALTAVMSNPAMA
Ice plant    GKVT   CSMEEK AINQRKTTPA  MGMGSLMLRPAACQPPCSKP ..
```

Mature Protein Sequences

```
Spinach      LVDERMSTEG TGLPFGLSNN LLGWILFGVF
Arabidopsis  LVDERMSTEG TGLPFGLSNN LLGWILFGVF
C.reinhardtii LVDERMNGDG TGLPFGVNDP VLGWVLLGVF
Wheat        LVDERMDTEG TGLSLGLSNN
Sorghum                  ....GLSNN LLGWILLGVF

Spinach      GLIWALYFVY ASGL E EDEESGLSL
Arabidopsis  GLIWTFFFAY TSSL E EDEESGLSL
C.reinhardtii GTMWAIWFIG QKDLGDFEDADDGLKL
Sorghum      GRIWSLYTVY TSDL D EDEESGLSL
```

from EST sequences while the wheat data arose from N-terminal protein sequencing.

The coding regions show a high degree of homology particularly over the N-terminal residues. The predicted transmembrane helix which involves residues 21 to 40 also contains amino acids which are common to all four known species. The degree of similarity in the C-terminal region of the protein is not as strong. The higher plants show a greater relatedness to each other than they do to the *C. reinhardtii* sequence. In addition to the two extra residues seen in this region of the algal protein very few of the amino acids are directly conserved. This may imply that the N-terminal region of the protein is the functionally important domain so the sequence has been conserved.

5.10 Conclusions

The aim of the work discussed in this chapter was to isolate and characterise the *psb*W gene in *C. reinhardtii.* Two different methods were employed both of which used the known N-terminal protein sequence to design a primer which was specific to the *psb*W gene. The first attempt at isolating the gene sequence used a *psb*W specific primer to isolate the sequence from a cDNA library by PCR. Although this technique produced a fragment during amplification, sequence data showed that despite its high GC rich content, typical of nuclear DNA, it did not relate to known *psb*W sequences.

Following this, two further degenerate primers were designed against the known protein sequence and these were utilised in a RACE reaction. Total mRNA was prepared from wild type *C. reinhardtii* and used to create a cDNA pool using a tagged dT primer. The gene specific primers were then used in turn with primers complementary to the tagged region to amplify the *psb*W coding sequence. The fragments produced by this protocol were cloned into a plasmid vector and subjected to DNA sequencing. This confirmed that the DNA was related to other known *psb*W sequences.

The next step in the characterisation of this gene was to obtain a copy of the genomic sequence. This was required to ensure that the amplification stages had not introduced any errors and to obtain data for the transit sequence. Before any attempt was made to isolate a genomic copy of the gene a Southern analysis

was performed to determine the copy number. A number of different enzymes were used to cleave wild type genomic DNA and the resulting filter containing the digested DNA was probed with the labelled RACE product. The result of this experiment confirmed that there is only a single copy of this gene in the nuclear genome of *C. reinhardtii*. A Northern analysis of wild type RNA, hybridised with the same labelled RACE probe, showed the presence of a single transcript which complemented the *psb*W coding sequence. This band ran at a position equivalent to that of a 1000 nucleotide long transcript and showed that we could detect expression of the gene in wild type cells.

A further Southern analysis was also performed but this time the DNA was purified from the PM and Mϕ nuclear PSII mutants. The aim was to determine whether any of these mutants had a disrupted copy of the *psb*W gene which was causing their altered PSII phenotypes. All the mutants contained the *psb*W gene on a fragment of DNA which matched the size of the wild type band so we were able to conclude that none of them were missing the PSII-W gene.

The final aim of this work was to obtain sequence data for both the amplified and genomic copies of this gene. The sequence data for the RACE product is complete with the exception of a short stretch in one orientation. From this we can observe the considerable sequence homology between the PSII-W sequences of different species. This is particularly apparent in the N-terminal region where the greatest levels of conservation are seen. In the C-terminal domain the *C. reinhardtii* sequence differs markedly from the higher plant sequences. In addition to the changed amino acids this alga has two extra residues which are not seen in plant sequences. The PSII-H protein, which was discussed in chapter 3, and also the PSII-L protein all contain additional residues when compared to higher plant sequences.

The genomic sequence data has been difficult to obtain due to the nature of the DNA. The sequence we have collected confirms the C-terminal protein sequence, determined from the amplified copy of the gene, to be correct. The primer which leads to the N-terminal region of the protein also confirmed the RACE sequence but the presence of two introns in this region has meant that the bases at the start of the coding sequence have yet to be examined in the genomic

DNA. Introns are common in *C. reinhardtii* nuclear DNA and the size of the two present in the *psb*W sequence, 150bp and 200bp, are typical for this organism.

A feature of interest in the PSII-W protein is the presence of a bipartite transit sequence which is more typical of a lumenal protein than one found in the thylakoid membranes. The signal peptide has been sequenced in other species but we have yet to obtain the equivalent data in *C. reinhardtii*. The RACE product does not contain this information because the primers were designed to the region following it and the genomic DNA, which should possess the information we desire, has proved difficult to sequence. Further subclones could be prepared to try and remedy the sequencing difficulties or the second stage of the RACE protocol could be employed in an attempt to amplify the 5' sequence.

As well as the completion of the genomic sequence it is envisaged that there could be much future work on this gene. The expression pattern of the *psb*W gene could be examined. Northern analysis has proved that the transcript for this gene is detectable so preparations of RNA could be made from cells grown under different conditions to determine any regulatory conditions. The spinach study of *psb*W showed that the level of mRNA increased 10 fold in light incubation in comparison to dark cultured cells showing that the gene is light regulated in higher plants. A similar experiment could be conducted on *C. reinhardtii* cultures to see if this characteristic is conserved.

The function of the PSII-W protein within the photosystem is not known. Its central location suggests that it may play an important role which may be linked to control of assembly by the nucleus or be required at some point in the assembly of a fully functional complex. The function of some PSII proteins has been determined by the creation of deletion mutants and characterisation of the resulting PSII complexes. This procedure has become relatively routine for chloroplast encoded genes because insertion of transforming DNA can be directed. In the nuclear genome transformants arise randomly due to the large size of the genome in comparison to the chloroplast DNA. Therefore creating a deletion mutant for PSII-W would not be straightforward. Random mutants can be generated and the transformants can easily be screened for altered photosynthetic function but we have no way of easily detecting any PSII-W disruption mutants. To determine whether the incoming DNA was affecting the *psb*W gene, mutants with altered

fluorescence characteristics would first have to be identified. These would then be screened by EPR to confirm which complex was affected. Any PSII mutants would then have to be subjected to Southern analysis as described in Section 5.4.2 with a *psb*W specific probe to determine if the DNA had been altered. If the lack of PSII-W alters PSII function then it could be possible to isolate a mutant in this manner but the number of mutants screened could be very large. Since the function of this protein is unknown we do not know the effect its absence might have on PSII. If PSII-W mutants still had a functional PSII complex then this method may fail to identify them.

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