AN INVESTIGATION OF THE SMALL POLYPEPTIDES OF PHOTOSYSTEM II

A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
BY

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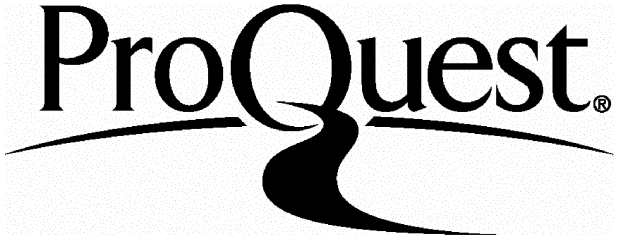

ABSTRACT

Photosystem II catalyses the light-driven electron transfer from water to plastoquinone. At present the complex is thought to contain at least 25 different polypeptides. In eukaryotes the components of PSII are encoded for both chloroplast and nuclear genes. Whilst information on the structure of the PSII complex is beginning to emerge, the role that the majority of the subunits play remains unknown. This thesis investigates three of these subunits, the nuclear-encoded subunit PSII-W, the chloroplast-encoded subunit PSII-M and PSII-Z (previously the hypothetical chloroplast protein Ycf9).

The gene that encodes the PSII-W protein has been cloned and sequenced from the photosynthetic alga *Chlamydomonas reinhardtii*. Analysis of the derived amino acid sequence has enabled the characterisation of the PSII-W protein. Antibodies to the mature form of the protein have been raised and used to perform initial expression analysis for the protein and to examine claims that the protein may also be present in a second thylakoid membrane complex, photosystem I. We establish that the PSII-W protein is located exclusively in the PSII complex of *C. reinhardtii*. Further work has been undertaken to clone and sequence the *psbW* gene from the moss *Physcomitrella patens*.

Disruption and deletion mutants in the *psbZ* (previously *ycf9*) and *psbM* genes of the cyanobacterium *Synechocystis* sp. PCC 6803 respectively, have been created by using the kanamycin resistance cassette as a selectable marker. Analysis of these mutants has been undertaken in an attempt to determine the role that each of the proteins plays in the PSII complex. Whilst no definitive role for either of the subunits is established a number of possible functions are ruled out.

FOR THOSE WHO SHOWED ME I COULD

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ABBREVIATIONS

~	approximately
1^o	primary
2^o	secondary
3'	3 prime
3D	three dimensional
5'	5 prime
α	alpha
β	beta
μE	microEinsteins
μg	microgram(s)
μL	microlitre(s)
μm	micrometer(s)
μM	microMolar
μmol	micromole(s)
μs	mircosecond(s)
al	allophycocyanin
A	adenine
Å	angstrom
ADP	adenosine diphosphate
A_{MAX}	maximum absorption
Amp	ampicillin
Amp^r	ampicillin resistant
AMPS	ammonium persulphate
AMV RTase	avian myeloblastosis virus reverse transcriptase
ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	base pair(s)
c	chloroplast
C	cytosine

°C	degrees Celsius
CAB	chlorophyll *a/b* binding protein
β-Car	β-Carotene
cDNA	copy deoxyribonucleic acid
Chl	chlorophyll
cm	centimetre(s)
CO_2	carbon dioxide
CTAB	hexadecyltrimethyl ammonium bromide
C-terminal	carboxy-terminal
cyt b_{559}	cytochrome b_{559}
cyt c_{550}	cytochrome c_{550}
d	day(s)
Da	Dalton
dCTP	2' deoxycytidine 5'-triphosphate
ddH_2O	double distilled water
DEPC	diethylpyrocarbonate
DMBQ	2, 5-dimethyl-*p*-benzoquinone
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DTT	dithiothreitol
e^-	electron
EDTA	ethylenediaminetetra-acetic acid
EDTA.Na_2	ethylenediaminetetra-acetic acid disodium salt
EPR	Electron Paramagnetic Resonance
EST	expressed sequence tag
EtOH	ethanol
EXAFS	Extended X-ray Absorption Fine Structure
fmol	femtomole(s)
Fd	ferredoxin
g	gram(s)
g	relative gravity
G	guanine
h	hour(s)

H^+	proton
H_2O	water
His	histidine
HSM	high salt minimal medium
IgG	Immunoglobulin G
IPTG	isopropylthio-β-D-galactosidase
K	degrees Kelvin
Kan	kanamycin
Kan^r	kanamycin resistant
kb	kilobase(s)
kDa	kilo Dalton
L	litre(s)
lb	pound
LB	Luria-Bertani medium
Leu	leucine
LHC	light-harvesting complex
LiDS-Urea PAGE	LiDS-Urea Polyacrylamide Gel Electrophoresis
LiDS	lithium dodecyl sulphate
Lut	Lutein
m	meter(s)
M	Molar
MBP	maltose-binding protein
MCS	multiple cloning site
mg	milligram(s)
min(s)	minute(s)
mL	millilitre(s)
mM	milliMolar
Mn	manganese
mRNA	messenger RNA
MSP	manganese stabilising protein
mt	mating type
mT	millitesla
mW	milliwatt

nm	nanometres
n	nuclear
$NADP^+$	nicotine adenine dinucleotide phosphate
Neo	Neoxanthin
ng	nanogram(s)
NPQ	non-photochemical quenching
N-terminal	amino terminal
O_2	molecular oxygen
OD	optical density
OEC	oxygen evolving complex
OEE	oxygen evolution enhancer
orf	open reading frame
p	phycocyanin
P680	special chlorophyll P680
P700	special chlorophyll P700
PAR	photosynthetically available radiation
PBS	phycobilisome
PC	plastocyanin
PCR	Polymerase Chain Reaction
PEG	polyethylene glycol
pheo	pheophytin
P_i	inorganic phosphate
pmol	picomole(s)
PQ	plastoquinone
ps	picosecond(s)
PSI	photosystem I
PSII	photosystem II
pSK	pBluescript SK(+)
Q_A	plastoquinone A
Q_B	plastoquinone B
*q*P	photochemical quenching
RACE	rapid amplification of cDNA ends
RC	reaction centre

RNA	ribonucleic acid
RNaseA	Ribonuclease A
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
s	second(s)
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sp	spectinomycin
SPP	Stromal Processing Peptidase
SSC	sodium saline citrate
T	thymine
T_m	melting temperature
TM	transmembrane
TAE	tris acetic acid EDTA
TAP	tris acetate phosphate medium
TEMED	*N,N,N',N*-tetramethylethylenediamine
TEN	tris EDTA NaCl
TPP	Thylakoid Processing Peptidase
tris	2-amino-2-hydroxy-methylpropane-1,3-diol
UCL	University College London
UV	ultraviolet
V	volts
Viol	Violoxanthin
v/v	volume for volume
WOC	water oxidising cluster
WT	wild type
w/v	weight for volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galatosidase
ycf	hypothetical chloroplast open reading frame
Y_D	tyrosine Y_D
Y_Z	tyrosine Y_Z

SPECIES LIST

Anabaena sp.	*Anabaena* sp. strain PCC 7120
A. thaliana	*Arabidopsis thaliana*
C. caldarium	*Cyanidium caldarium*
C. paradoxa	*Cyanophora paradoxa*
C. reinhardtii	*Chlamydomonas reinhardtii*
C. vulgaris	*Chlorella vulgaris*
E. coli	*Escherichia coli*
E. gracilis	*Euglena gracilis*
G. theta	*Guillardia theta*
H. vulgare	*Hordeum vulgare*
L. japonicus	*Lotus japonicus*
M. polymorpha	*Marchantia polymorpha*
M. viride	*Mesostigma viride*
N. olivacea	*Nephroselmis olivacea*
N. tabacum	*Nicotiana tabacum*
O. hookeri	*Oenothera elata subsp. hookeri*
O. sativa	*Oryza sativa*
O. sinensis	*Odontella sinensis*
P. patens	*Physcomitrella patens*
P. purpurea	*Porphyra purpurea*
P. sativum	*Pisum sativum*
P. thunbergii	*Pinus thunbergii*
S. costatum	*Skeletonema costatum*
S. oleracea	*Spinacia oleracea*
Synechocystis 6803	*Synechocystis* sp. PCC 6803
Synechococcus sp.	*Synechococcus* sp. strain Copeland
T. aestivum	*Triticum aestivum*
T. elongatus	*Thermosynechococcus* (formerly *Synechococcus*) *elongatus*
T. vulcanus	*Thermosynechococcus* (formerly *Synechococcus*) *vulcanus*
Z. mays	*Zea mays*

CHAPTER 1. INTRODUCTION

INTRODUCTION

1.1 THE IMPORTANCE OF PHOTOSYNTHESIS

Photosynthesis is the primary source of atmospheric oxygen. Oxygenic photosynthesis is the process by which solar energy is used to convert carbon dioxide and water into carbohydrates and oxygen. The reaction and its unique mechanism are of interest for a variety of reasons. This process forms the basis of the global food chain and nearly all food and fossil fuels are derived from it either directly or indirectly. Current predictions indicate that the Earth's environment is set to alter dramatically within the next century. It is unclear what impact such changes will have on photosynthetic organisms. Biomass, photosynthetic productivity, crop yields, and the nutritional value of plants are all affected by changes in the levels of carbon dioxide in the atmosphere. In addition to an increase in the levels of atmospheric carbon dioxide, comparatively drastic elevations of air temperature are predicted. With such a change, the migration of plant habitats towards the poles is a likely scenario. It is feared that plant communities will not be capable of migration or adaptation at a suitable rate. If this were to occur the impact on commercial agriculture could be catastrophic.

Compounding and contributing to the fears of global warming is the realisation that fossil fuel reserves are rapidly being depleted. This has led to an urgency for cleaner or alternative fuels, both to reduce the environmental side effects of burning fossil fuels and to act as a long-term replacement. Enough solar energy reaches the surface of the Earth every sixty seconds to provide the total annual energy requirement of world's population. When this fact is considered, the benefits of exploring solar power as a suitable alternative are evident (data obtained from the US Department of Energy National Centre for Photovoltaics, http//www.eren.doe.gov/pv/turning.html). Commercially available solar cells rely on photovoltaic cells, which convert solar energy directly into electricity, but in recent years intensive investigations have been undertaken to determine the possibility of applying the primary reactions of photosynthesis as a model for improved photovoltaic cells.

With an ever-increasing world population the interest in the products of photosynthesis is growing. Although new varieties of plants have been developed for centuries through selective breeding, the techniques of modern molecular biology have

speeded up the process substantially. This area of research, whilst still in its infancy, is critical, as recent studies show that agricultural production is levelling off, at a time when demand for food and other agricultural products is growing rapidly.

1.2 EVOLUTION OF PHOTOSYNTHESIS AND THE CONSEQUENCES

With the evolution of oxygenic photosynthesis about 2.3 billion years ago, oxygen, the element essential to catabolic processes, began to build-up in the atmosphere. This gradual accumulation led to the emergence and spread of organisms capable of efficient aerobic respiration, in the region of 1.5 billion years ago. Since this time the two metabolic processes of photosynthesis and respiration have been inextricably linked. These pathways, which characterise life processes today, are unified by shared products and by-products: organic food, water, carbon dioxide and oxygen (Figure 1.1).

1.3 THE SITE OF PHOTOSYNTHESIS

Located in the chloroplasts of photosynthetic eukaryotes is the thylakoid membrane. Embedded in this membrane is the photosynthetic machinery that is responsible for light harvesting and energy conversion (Figure 1.2). In plants the thylakoid membranes are arranged in stacks, termed grana, which are embedded in the stromal matrix of the chloroplast. A network of stroma lamellae interconnects the grana. The region within the thylakoid double membrane is the thylakoid lumen. During photosynthetic electron transfer protons (H^+) are transferred from the stroma to the thylakoid lumen through the thylakoid membrane. Whilst cyanobacteria lack chloroplasts they do possess thylakoid membranes, which are located in the cytoplasm of the cell.

Figure 1.2 also schematically details four of the complexes located in the thylakoid membrane. The process of oxygenic photosynthesis involves two photosystem reaction centres (RC), photosystem I (PSI) and photosystem II (PSII), that act in series via cytochrome b_6f. A RC is made up of proteins, pigments and co-factors and functions in the trapping and conversion of light energy into chemical energy. PSII, cytochrome b_6f and PSI are linked by the mobile components plastoquinone (PQ), located in the lipid phase of the thylakoid membrane and plastocyanin (PC) which is found in the lumen.

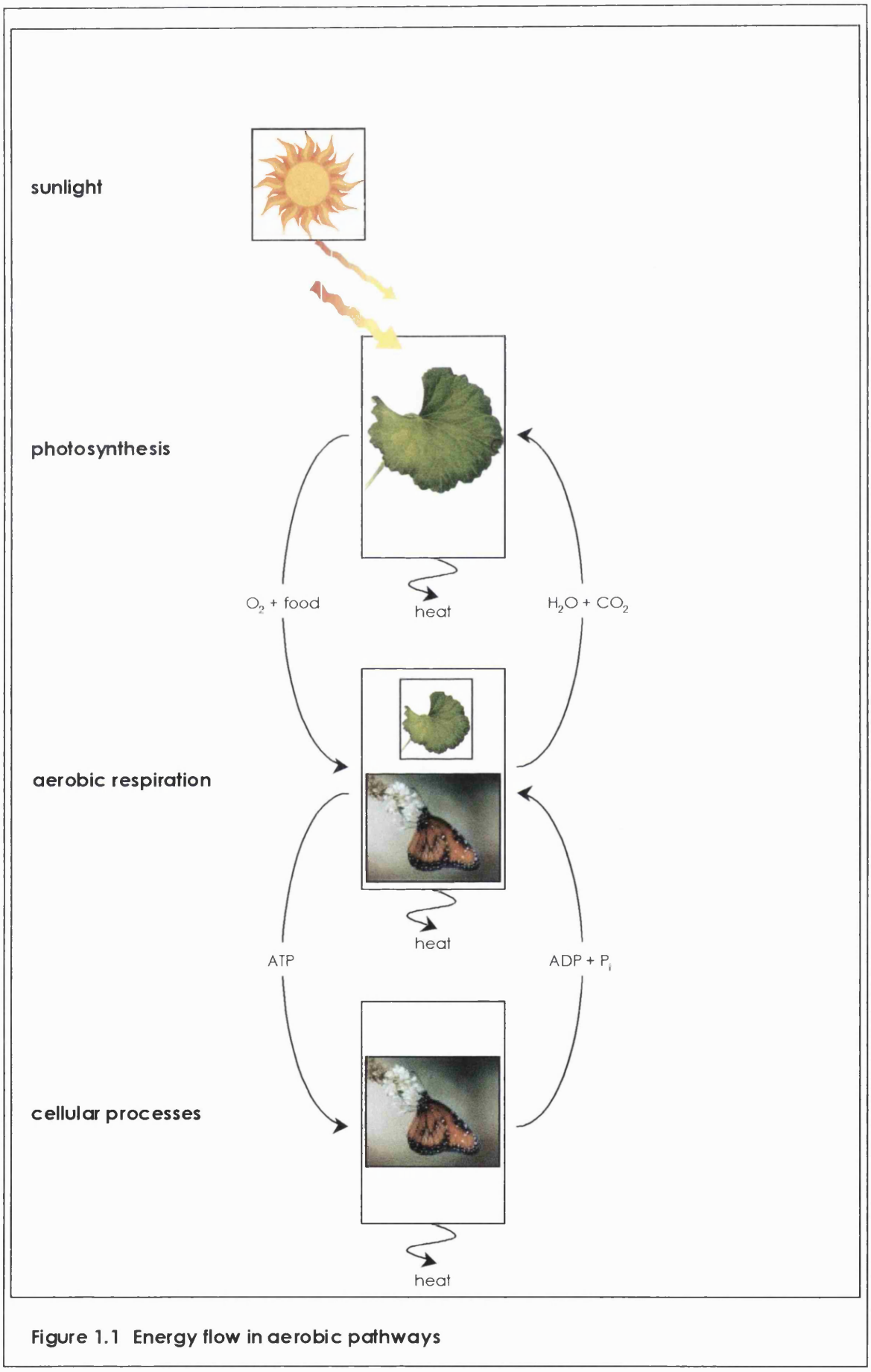


Figure 1.1 Energy flow in aerobic pathways

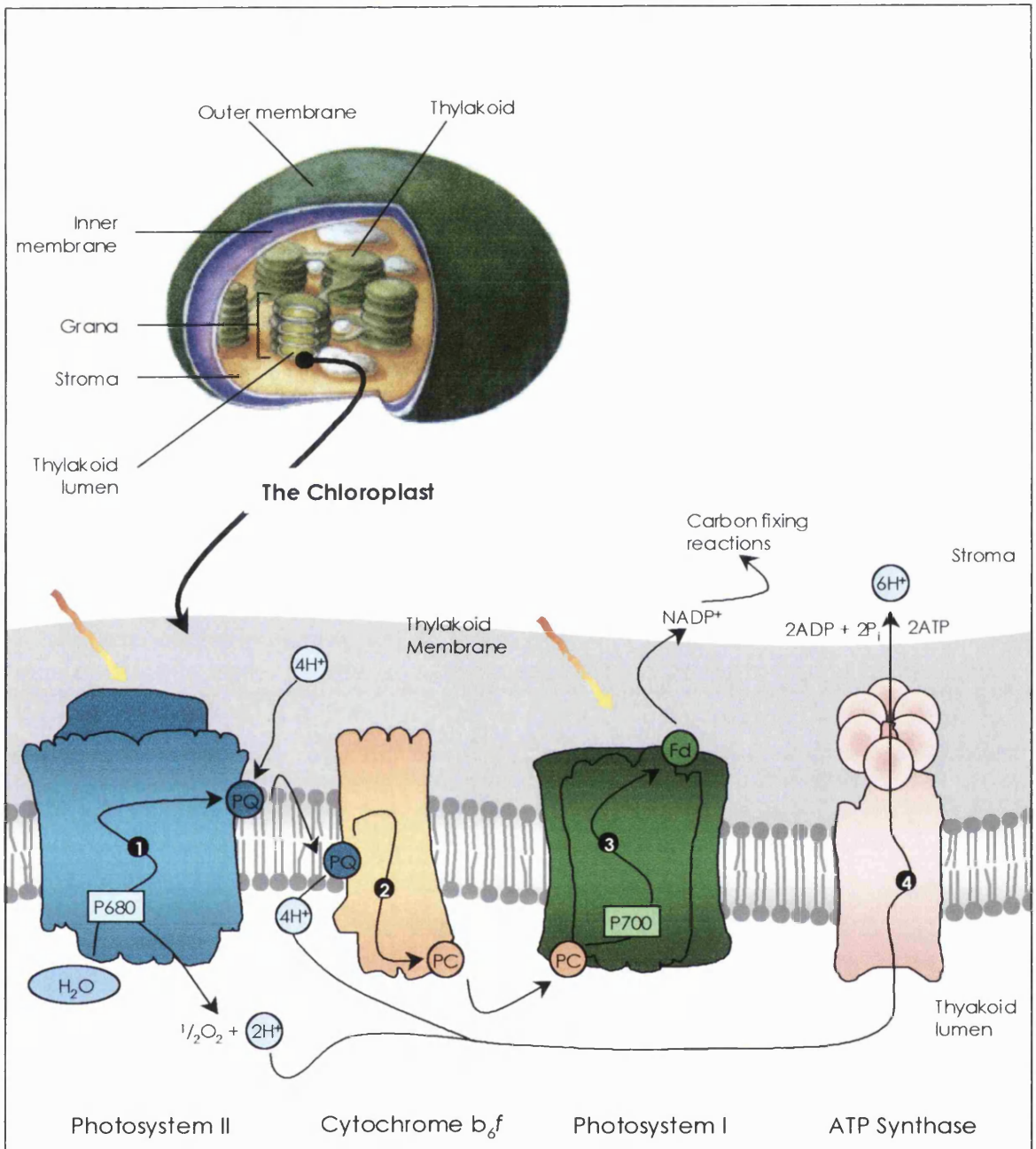


Figure 1.2 Schematic of the complexes located in the thylakoid membrane of oxygenic photosynthetic organisms
At the top of the figure is a cross section of the chloroplast, the organelle which houses the photosynthetic machinery in eukaryotes. Below this is a model cross section of the thylakoid membrane showing four of the complexes embedded in the membrane, together with the electron transfer chains which connect them.

NOTE
Arrows ❶ - ❹ indicate the direction that the electrons and protons flow through each of the electron transfer reactions.

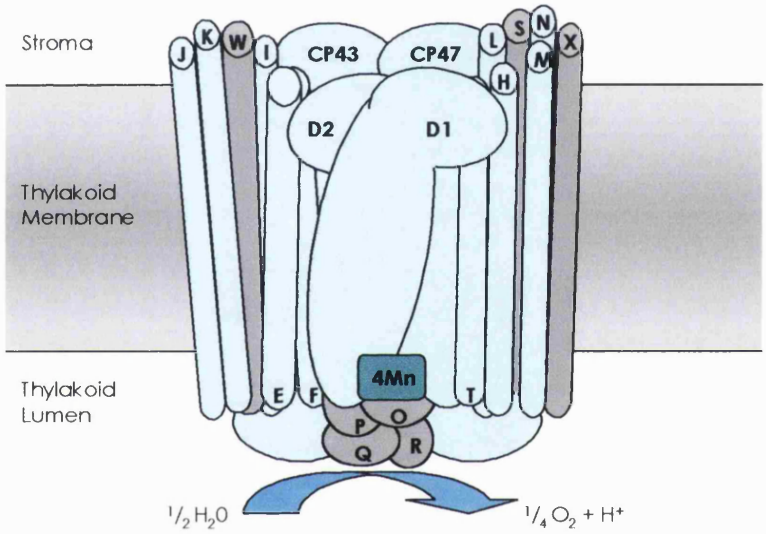
The first step in oxygenic photosynthesis is the absorption of a photon of light by the antenna complex of PSII, which excites an electron of the PSII RC special chlorophyll *a*, P680, producing $P680^*$. The electron is then rapidly transferred from $P680^*$ to the plastoquinone molecule Q_A via pheophytin, and then to Q_B, a second plastoquinone molecule. This leaves the P680 molecule in an oxidised state, termed $P680^+$. $P680^+$ recovers electrons from the oxidation of water. Four electrons, four protons and molecular oxygen are evolved for every two water molecules that are 'split'.

Once the plastoquinone Q_B has accepted two electrons and taken up two protons, the reduced Q_B (Q_BH_2 or plastoquinol) migrates to the lumenal side of the thylakoid membrane. Here it interacts with the cytochrome b_6f complex. This complex contains cytochromes and iron-sulphur proteins. Cytochrome b_6f accepts electrons from plastoquinol and passes them to PC. The oxidation of the plastoquinol results in release of two protons into the thylakoid lumen.

When a photon of light is absorbed by the antenna system of PSI the excitation energy is passed to P700, and results in a second electron transfer chain. Ferredoxin (Fd) is the direct recipient of electrons from the PSI complex. At this point the most likely fate of the electron is to reduce $NADP^+$, which in turn is used in the fixation of carbon and the synthesis of carbohydrates. The oxidised P700, termed $P700^+$, is in turn reduced by PC. During the reactions described, protons have been pumped across the thylakoid membrane into the lumen and derived from the splitting of water. This generates a powerful electrochemical gradient, which is used by the enzyme ATP synthase to generate ATP, the energy currency of the cell.

1.4 PHOTOSYSTEM II (PSII)

PSII is a multisubunit protein-chromophore complex responsible for carrying out the light-driven electron transfer from water to plastoquinone. In this process electron transfer to plastoquinone is coupled to proton transfer. This results in the reduction of plastoquinone, the oxidation of water and the formation of a transmembrane pH gradient. At present about 25 proteins have been assigned to the PSII complex. In eukaryotic photosynthetic organisms these subunits are encoded by both nuclear and chloroplast genes, as depicted in Figure 1.3 and in Table 1.1. The genes are generically referred to as the *psb* genes. Despite ongoing work the function of the



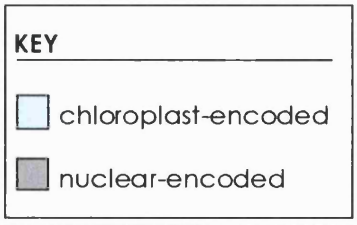


Figure 1.3 Schematic of the core of the photosystem II of higher plants and green algae, depicting the nuclear and chloroplast-encoded subunits of the complex
The chloroplast-encoded subunits are shown in colour, while the nuclear-encoded subunits are shown in grey. This model does not include the light-harvesting subunits Lhcb1-6.

Table 1.1 Table to summarise the PSII genes and proteins from oxygenic photosynthetic organisms

Complex components				Gene	Protein	Subunit	n/c	Mass (kDa)	TM span	Proposed function	Chla	Chlb	β-Car	Pheo	Lut	Neo	Viol
PSII RC	Full PSII core	PSII core & minor LHCII proteins	Full PSII & antenna complex	*psbA*	PSII-A	D1	c	38 (S)	5	Y_Z & binds P680, Q_B, pheo	6	0	2	2	0	0	0
				psbD	PSII-D	D2	c	39.5 (S)	5	Y_D & binds Q_A, P680							
				psbE	PSII-E	α- cyt b_{559}	c	9.3 (S)	1	Binds haem, photoprotection							
				psbF	PSII-F	β - cyt b_{559}	c	4.4 (S)	1	Binds haem, photoprotection							
				psbI	PSII-I	I protein	c	4.2 (S)	1	? Function							
				psbW	PSII-W	W protein	n	6.1 (S)	1	? Function Higher plants and algae only							
				psbB	PSII-B	CP47	c	56 (S)	6	Excitation energy transfer Binds 33kDa	10-25	0	3	0	?	0	0
				psbC	PSII-C	CP43	c	50 (S)	6	Excitation energy transfer Binds 33kDa	9-25	0	5	0	?	0	0
				psbH	PSII-H	H protein	c	7.7 (S)	1	? Photoprotection	-	-	-	-	-	-	-
				psbK	PSII-K	K protein	c	4.3 (S)	1	PSII assembly and stability	-	-	-	-	-	-	-
				psbL	PSII-L	L protein	c	4.4 (S)	1	Involved in Q_A function	-	-	-	-	-	-	-
				psbM	PSII-M	M protein	c	3.7 (P)	1	? Function	-	-	-	-	-	-	-
				psbN	PSII-N	N protein	c	4.7 (T)	1	? Function	-	-	-	-	-	-	-
				psbO	PSII-O	33kDa protein	n	26.5 (S)	0	Stabilises Mn cluster ? Ca^{2+} & Cl^- binding	-	-	-	-	-	-	-
				psbP	PSII-P	23kDa protein	n	20 (S)	0	? Ca^{2+} & Cl^- binding Higher plants and algae only	-	-	-	-	-	-	-
				psbQ	PSII-Q	16kDa protein	n	16.5 (S)	0	Ca^{2+} & Cl^- binding Higher plants and algae only	-	-	-	-	-	-	-
				psbR	PSII-R	10kDa protein	n	10.2 (S)	0	? Function Higher plants and algae only	-	-	-	-	-	-	-
				psbS	PSII-S	LHCII protein	n	22 (S)	4	? Antenna component ? Chl chaperone	5?	0	0	0	0	0	0
				psbT	PSII-T	Ycf8 protein	c	3.8 (S)	1	? Function	-	-	-	-	-	-	-
				psbT	PSII-T	5kDa protein	n	5	0	? Function Higher plants and algae only	-	-	-	-	-	-	-

Complex components	Gene	Protein	Subunit	n/c	Mass (kDa)	TM span	Function	Chla	Chlb	β-Car	Pheo	Lut	Neo	Viol
	psbV	PSII-V	cyt c_{550}	-	15.1 (Y)	0	Donor side stability Cyanobacteria only	-	-	-	-	-	-	-
	psbX	PSII-X	X protein	n	4.2 (S)	1	? Aids in Q_A functioning	-	-	-	-	-	-	-
	psbY	PSII-Y	Y protein	n	7 (S)	1	? Redox-active component	-	-	-	-	-	-	-
	lhcb4	Lhcb4	CP29	n	29	3	Excitation energy transfer and dissipation	9-10	3-4	0	0	1-2	1	1-2
	lhcb5	Lhcb5	CP26	n	26	3	Excitation energy transfer and dissipation	7-9	4-5	0	0	2	0.5-1	0.5-1
	lhcb6	Lhcb6	CP24	n	24	3	Excitation energy transfer and dissipation	6	7	0	0	2	1	1
	psbJ	PSII-J	J Protein	c	4.2 (P)	1	PSII assembly	-	-	-	-	-	-	-
	psbU	PSII-U	U protein	-	~10	0	? Function Cyanobacteria only	-	-	-	-	-	-	-
	lhcb1	Lhcb1	LHCII	n	25	3	Light harvesting	8	6	0	0	2	0.5-1	0.5
	lhcb2	Lhcb2	LHCII	n	25	3	Light harvesting	8	6	0	0	2	0.5-1	0.5
	lhcb3	Lhcb3	LHCII	n	25	3	Light harvesting	8	6	0	0	0	0	0
	psbZ	PSII-Z	Ycf9 protein	c	6.5	2	? Function	-	-	-	-	-	-	-

Table 1.1 Table to summarise the PSII genes and proteins from oxygenic photosynthetic organisms (continued)

This table presents the putative PSII proteins encoded by the genes *psbA-psbZ*, and the outer antenna proteins encoded by the *lhcb1-6* genes. The fraction of the complex with which each of the subunits co-purifies is detailed. The protein and subunit names for each gene product are given, together with which genome the gene is located in: nuclear (n) or chloroplast (c). Also detailed is the molecular mass for each of the subunits (kDa), from one of four organisms: spinach (S), *Synechococcus* (Y), pea (P) and tobacco (T). The apparent molecular masses are given for the Lhcb proteins and the PSII-U and PSII-Z subunits. The number of transmembrane (TM) spans for each subunit is then listed. Next, a putative function(s) for each of the PSII polypeptides and the cofactors associated with each of the subunits: Chlorophyll *a* (Chl*a*), Chlorophyll *b* (Chl*b*), β-Carotene (β-Car), Pheophytin (Pheo), Lutein (Lut), Neoxanthin (Neo) and Violoxanthin (Viol).

NOTE
PSII-G was originally assigned as a component of PSII, but it is actually part of the NADPH/quinone reductase.
Table adapted from Hankamer and Barber (1997).

majority of the subunits is still unknown. Table 1.1 summarises what is known about the *psb* gene products.

The centre of the PSII complex is composed of the D1, D2, PSII-I, PSII-W and cytochrome b_{559} (cyt b_{559}) proteins (Lindahl *et al.*, 2000). The highly conserved D1 and D2 subunits make up the PSII RC. These subunits have localised areas of identity with the non-oxygenic photosynthetic purple bacteria proteins L and M, as well as a high degree of identity with one and other (Ruffle *et al.*, 1992). The D1-D2 heterodimer binds the majority of the co-factors required for the electron transfer chain. Collectively they bind six chlorophyll molecules including the P680 and chlorophyll Z, two pheophytin molecules, the plastoquinone molecules Q_A and Q_B, β-carotene and the non-haem iron. Furthermore, it is now known that they might also act as ligands for the manganese atoms of the water oxidising complex (WOC). The gene products of *psbE* and *psbF*, the α-cyt b_{559} and β-cyt b_{559} proteins respectively, associate to form cyt b_{559}. The structure of the PSII RC has been solved to a resolution of 8Å by Rhee *et al.* (1997). In 1999 Hankamer *et al.* determined the structure of the PSII core dimers at a resolution of about 9Å. More recently Zouni *et al.* (2001) presented the X-ray crystal structure of PSII from *Thermosynechococcus* (formerly *Synechococcus*) *elongatus* at 3.8Å resolution.

Associated with the D1-D2 dimer are the antenna chlorophyll binding proteins, CP43 and CP47. When bound, the pigment molecules form a light-harvesting complex (LHC) that captures photons. CP43 and CP47 are involved in the transfer of energy from the LHC to the special chlorophyll P680. Deletion mutagenesis studies indicate that both these proteins are also essential for correct PSII assembly and function and in maintaining the water splitting reaction (reviewed in Barber *et al.*, 2000).

For higher plants, photosynthetic algae and cyanobacteria, the green pigment chlorophyll functions in the capture of the majority of the solar energy utilised by PSII. Chlorophyll *a* is the pigment molecule, or photoreceptor molecule, common to all photosynthetic cells. In addition to chlorophyll *a*, accessory pigments are present which absorb energy at other wavelengths. These include chlorophyll *b* (also *c*, *d*, and *e* in algae and protistans), xanthophylls, and carotenoids (such as β-carotene). Figure 1.4 shows the absorption spectra for these photoreceptive molecules. In addition to their role as light-harvesting accessory molecules, the pigments associated with the PSII RC also function in protecting the complex against the detrimental reactions associated with the splitting of water (Nugent, 1996).

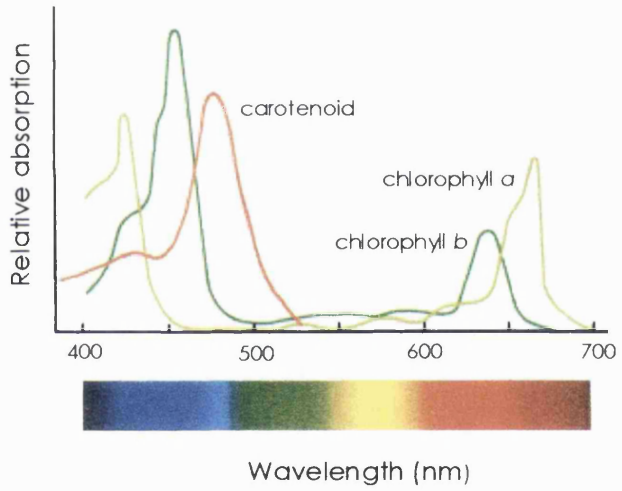


Figure 1.4 Absorption spectra of chlorophyll *a*, chlorophyll *b* and a carotenoid
Taken together the absorption spectra of the two chlorophylls and the carotenoid cover more of the range of wavelengths than the chlorophyll *a* does on its own.

1.4.1 The antenna systems

In general the PSII complex is very similar in all types of oxygenic photosynthetic organisms. One of the major differences is the light-harvesting system that is present. In cyanobacteria the phycobilisomes (PBSs) are responsible for capturing light, whilst in higher plants and algae the light-harvesting complex II (LHCII) performs this role (reviewed by Green and Durnford, 1996 and references therein).

1.4.1i Cyanobacterial antenna systems

The cyanobacteria and the algae of the Rhodophyta and Glaucocystophyta phyla have a PBS antenna system, composed of phycobiliproteins. This pigment-protein antenna system contains a fanned array of PBSs that are attached to the stromal side of the PSI and PSII cores by linker proteins. The arrangement of the PBSs of *Synechocystis* sp. PCC 6803 (referred to as *Synechocystis* 6803 in this text) relative to the PSII complex, is depicted in Figure 1.5. The three classes of PBS proteins can be distinguished by their absorption spectra and the number of tetrapyrrole pigment molecules present. These are allophycocyanin (A_{MAX} 650-665nm, binds two tetrapyrrole molecules), phycocyanin (A_{MAX} 490-625nm, binds two tetrapyrrole molecules) and phycoerythrin (A_{MAX} 490-570nm, binds more than three tetrapyrroles molecules) (Ducret *et al.*, 1998).

The PBS antenna system contains two, three or five allophycocyanin molecules that are positioned along the surface of the photosystem complex. The number of allophycocyanin molecules is dependent on the species examined (Ducret *et al.*, 1998). In the case of *Synechocystis* 6803 the PBSs contain three allophycocyanin molecules. Radiating out, and giving the characteristic fanned shape of the PBSs, are the rods of phycocyanin and/or phycoerythrin. In *Synechocystis* 6803 these rods only contain phycocyanin. The properties of the PBSs permit very efficient energy transfer from the rod pigments to the allophycocyanin core. Once received by the allophycocyanin molecules the energy passes to the chlorophyll antennae of the photosystems. It is not yet fully understood how the PBS units interact to form the antenna system or how they attach to the cores of the photosystems.

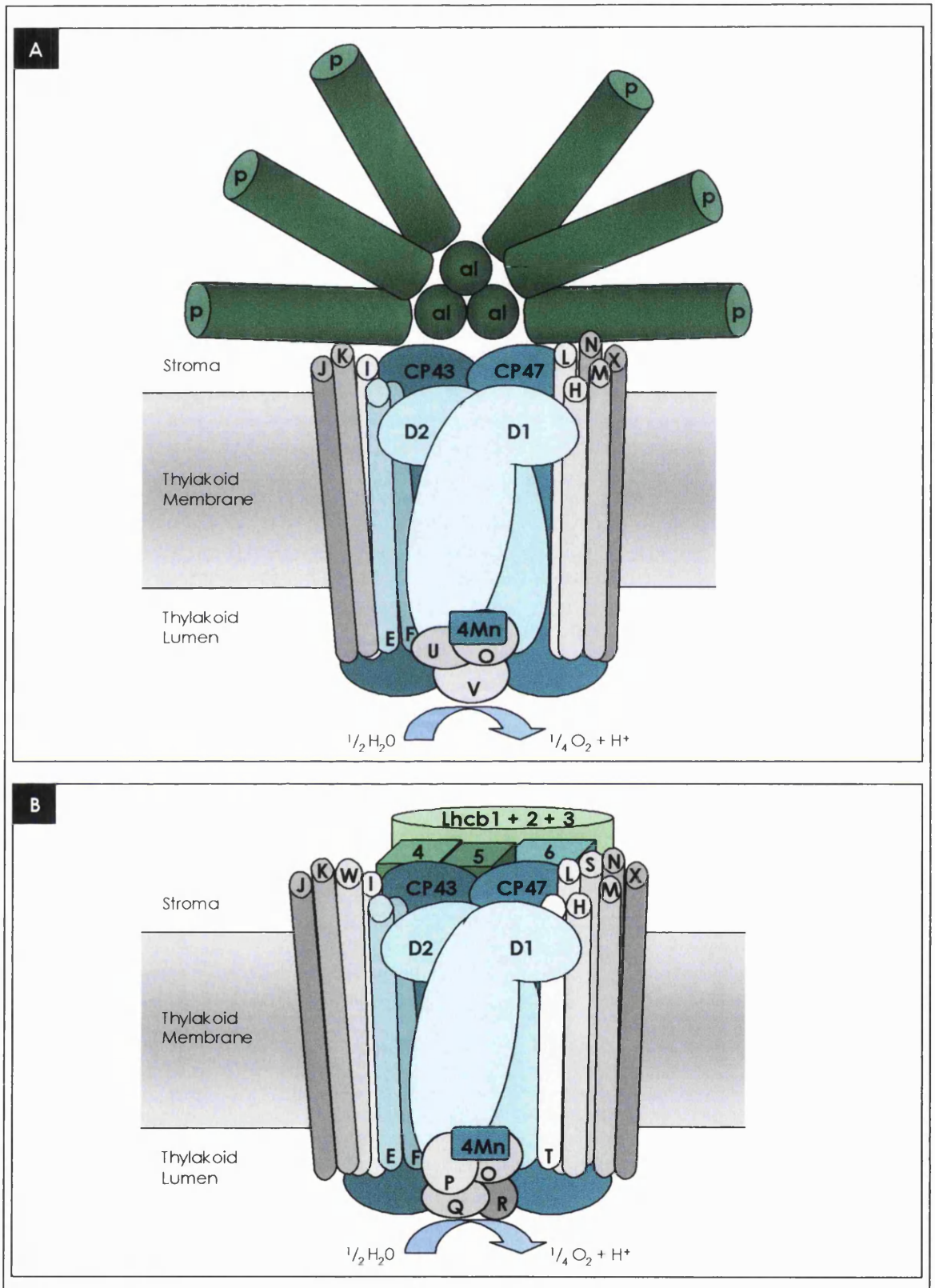


Figure 1.5 Model of the subunit composition of PSII
The subunits are annotated according to nomenclature detailed in Table 1.1, with the outer light-harvesting proteins in green.
A. The PSII of cyanobacteria: the PBS structure is shown for *Synechocystis* 6803, and consists of allophycocyanin (a) and phycocyanin (p).
B. Higher plants and green algae: 4, 5, and 6 refer to Lhcb4 - 6 respectively.

1.4.1ii Algal and higher plant antenna systems

In higher plants the membrane proteins Lhcb1-6 make up the outer antenna system of the PSII complex. These proteins are encoded by the nuclear genes *lhcb1-6* respectively. Similar proteins are present in the outer antennae of green algae. The proposed arrangement of these subunits is shown in Figure 1.5. The LHC proteins of the PSII complex are attached to the hydrophobic region of the PSII core, with the interaction occurring within the thylakoid membrane. The proteins Lhcb1-3 form a heterotrimer, whilst the remaining three subunits Lhcb4 (CP29), Lhcb5 (CP26) and Lhcb6 (CP24) are monomeric (Jansson, 1994; Kühlbrandt *et al.*, 1994). The LHCII proteins are capable of binding the pigment molecules chlorophylls *a* and *b* and carotenoids. Indeed, the binding of these pigments is essential for the correct folding of these polypeptides (Paulsen, 1995). Like the PBSs described above, the LHCII complex functions in the capture of light energy and its transmission to the PSII chlorophyll molecules.

1.4.2 The electron transport chain

1.4.2i P680

When light of the correct wavelength strikes the antenna pigments of the LHC of PSII, photons are absorbed by the pigment molecule of the PSII RC. This absorption causes the transition of the pigment molecule from a ground state to a 'higher energy' excited state, converting the pigment molecules into a powerful reactant. The captured energy is transferred to the special chlorophyll *a* molecule P680, termed as such because it absorbs light at 680nm. The start of the PSII electron transfer chain begins with this conversion of P680 to $P680^*$. How the transfer of light energy from the antenna pigments to P680 occurs is still not fully understood.

Currently the structure of P680 is hotly debated. Support for a chlorophyll *a* dimer stems from the sequence similarities between the D1-D2 and L-M polypeptide, where the structure has been determined for 'purple bacteria'. Contrasting data indicates that the special molecule consists of two or more monomeric chlorophyll molecules associated by weak interactions. The properties of P680 that support each of these theories are reviewed in Rochaix *et al.* (1998). It may indeed be the case that the composition of P680 is dependent of the state of P680, be it ground state (P680), excited state ($P680^*$), triplet

state ($P680^T$) or radical cation ($P680^+$). The observed state of P680 could therefore vary depending on the type of analysis that is performed.

1.4.2ii The electron acceptors pheophytin, Q_A and Q_B

On receiving the excitation energy from the pigments of the LHC, P680 is converted to $P680^*$. A single unpaired electron is immediately donated from $P680^*$ to the primary acceptor pheophytin resulting in the formation of the radical $P680^+Pheo^-$. This donation is a rapid one, estimated to occur within 21ps of the conversion of P680 to $P680^*$ (Hastings *et al.*, 1992). There are two pheophytin molecules per special chlorophyll in the PSII RC, only one of which actually participates in the electron transfer chain. Spectroscopic analysis shows that the two molecules have different absorbance properties, with absorption observed at 676nm for one and at around 680nm for the other. In 1990 van Kan *et al.* proposed that the 676nm form is consistently active in the transfer of electrons.

Within 200ps the electron is transferred from the $Pheo^-$ radical to the primary plastoquinone Q_A producing $Q_A^-P680^+$. Under non-stressed conditions Q_A accepts a single electron. However, during periods of high light intensity Q_A may accept two electrons, a process that promotes photoinhibition. Having donated the energised electron to pheophytin, the oxidised $P680^+$ is reduced by electrons derived from the splitting of water, via the redox active tyrosine residue at position 161 of the D1 subunit. This residue is referred to as tyrosine Z, or Y_Z.

Once reduced, Q_A^- transfers the electron to Q_B, the secondary plastoquinone, producing a semiquinone. Bicarbonate has been shown to influence the rate of electron transfer from Q_A^- to Q_B; in the absence of bicarbonate the transfer of electrons is slower (Nugent, 1996). A second electron is required for Q_B to undergo the transition from semiquinone to quinol, i.e. its fully reduced state. The doubly reduced Q_B plastoquinol is protonated (termed Q_BH_2) and released into the lipid matrix. Its place is rapidly filled by a member of the plastoquinone pool, present in the thylakoid membrane. Bicarbonate has also been implicated in the protonation of Q_B^- (Nugent, 1996).

The rate of first electron transfer from Q_A is estimated at 100-200μs, while the second transfer is completed within 300-500μs (Nugent, 1996). From a kinetics standpoint the second transfer is rather slow. It is made possible, however, by the fact that

the reduction of $P680^+$ by Y_Z is considerably faster than the backwards reaction between $P680^+$ and Q_A. It should be noted that Q_A and Q_B are identical plastoquinones, with the variance in their characteristics being a direct result of the protein environment in which each quinone is situated.

1.4.2iii The non-haem iron

The non-haem iron is situated between the Q_A and the Q_B sites of the complex, and is co-ordinated to the D1 and D2 subunits and a bicarbonate anion. The presence of the non-haem iron is not essential for electron transfer from Q_A to Q_B. However, its reduction is associated with proton binding, and it is thought to function as an electron carrier under oxidising conditions (Nugent, 1996). It has also been proposed that it may function in the stabilisation of Q_A^- and the structure of PSII (Rochaix *et al.*, 1998).

1.4.2iv The Water Oxidising Complex (WOC)

The manganese cluster together with PSII-O, PSII-P, PSII-Q (for further detail see Section 1.7), the intermediate electron carrier Y_Z and possibly the co-factors calcium and chloride constitute the functional unit that is referred to as the water oxidising complex (WOC) or oxygen evolving complex (OEC). It is assumed that the D1 and D2 subunits provide the majority of the ligands for the manganese cluster, but few have been categorically assigned. The tyrosine residue Y_Z acts as an intermediate electron carrier between the WOC and $P680^+$. When electron donation from the WOC to $P680^+$ via Y_Z is blocked, $P680^+$ is still capable of receiving electrons from the alternative donors, such as tyrosine Y_D (tyrosine 160 of the D2 subunit of *C. reinhardtii*), chlorophyll Z or cyt b_{559}.

Water acts as the substrate and electron donor for the WOC. The splitting of two molecules of water results in the production of molecular oxygen, four electrons and four protons. Consequently this reaction is a four-electron transfer process. To accomplish this the WOC passes cyclically through five redox states, termed S states, and denoted S_0 through to S_4. The S_4 state is unstable, and once achieved is spontaneously reduced to S_0 resulting in the release of molecular oxygen. Figure 1.6 depicts a model for the S-states of the WOC, detailing the products as each subsequent S state is achieved.

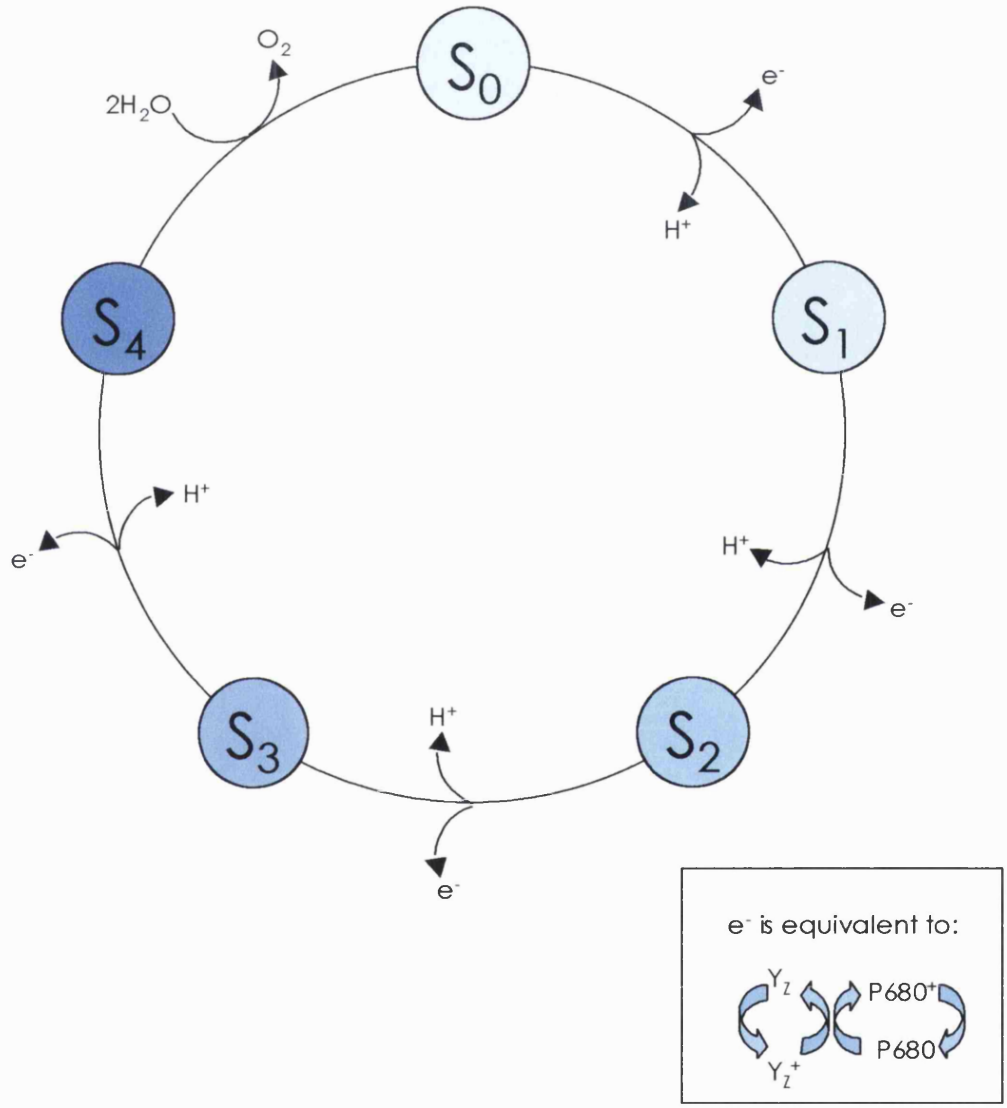


Figure 1.6 A model for the S-states of the WOC
The WOC splits water into protons and electrons, and releases molecular oxygen as a by-product. Electrons are removed by $P680^+$ via the tyrosine molecule Y_Z. The S-state number refers to the number of electrons stored. Once the transient S_4 state is achieved oxygen is released and the redox state returns to S_0.

Actively involved in the process of water oxidation is the manganese cluster. At present, there is immense controversy as to the structure and composition of this cluster. Various models have been proposed, but for them to be considered more than speculative a high-resolution three-dimensional structure is required. The models proposed have been reviewed (Yachandra *et al.*, 1996; Rüttinger and Dismukes, 1997; Nugent, 2001). At present the most popular structure contains a manganese tetramer. This structure is based predominately on data from EXAFS and is reviewed by Yachandra *et al.* (1996).

1.5 SUBUNITS OF THE PHOTOSYSTEM II REACTION CENTRE (PSII RC)

The subunits of the photosystem II complex have been reviewed in detail in a number of publications, for example Nugent (1996), Seidler (1996), Barber *et al.* (1997), Hankamer and Barber (1997) and Hankamer *et al.* (2001).

1.5.1 PSII-A (D1) and PSII-D (D2)

The D1 (PSII-A) and D2 (PSII-D) subunits form a heterodimer, which is at the core of the PSII complex. Work carried out by Chua and Bennoun was the first to identify the D1 subunit (Chua and Bennoun, 1975). Zurawski *et al.* (1982) isolated the *psbA* gene that encodes the D1 subunit from tobacco and spinach. Two years later the *C. reinhardtii* gene sequences for the *psbA* and *psbD* genes were determined by Erickson *et al.* (1984) and Rochaix *et al.* (1984) respectively. The two protein products of these genes share about 25% identity with each other in *C. reinhardtii*. When each of the protein sequences is compared for a number of oxygenic organisms the D1 subunit has 65% identity, whilst the D2 polypeptide conserves about 75% of the amino acid residues (Svensson *et al.*, 1991).

As well as having sequence identity with the reaction core subunits L and M of the PSII complex of purple bacteria (Williams *et al.*, 1983), the D1 and D2 subunits are functionally analogous. Like the L and M subunits, the D1 and D2 proteins are hydrophobic polypeptides, each with a predicted five α-helix transmembrane spanning domain. Conserved within these four proteins are residues that are believed to bind some of the co-factors of the electron transfer chain. Whilst this evidence is not conclusive, it does indicate a structural similarity between the L and M and the D1 and D2 subunits.

This led investigators to use the crystal structure of the purple bacterial RC (Deisenhofer *et al.*, 1985; Deisenhofer and Michel, 1989) as a blueprint for modelling the RC in oxygenic organisms, and resulted in the original models for the structure of the PSII complex (Svensson *et al.*, 1990; Ruffle *et al.*, 1992; Svensson *et al.*, 1996).

Both subunits are known to undergo post-translational modification to produce the mature forms of the proteins. Cleavage of a short C-terminal extension on the pre-D1 subunit, of between 12 and 16 amino acid residues (Takahashi *et al.*, 1988; Takahashi *et al.*, 1990; Nixon *et al.*, 1992) is essential for the assembly of the WOC into the PSII complex (Diner *et al.*, 1988; Nixon *et al.*, 1992). The region of the protein at this cleavage site is thought to adopt a conformation that is essential for oxygen evolution to occur. The protease *ctpA* is responsible for this processing, and has been cloned and sequenced (Shestakov *et al.*, 1994; Inagaki *et al.*, 1996; Trost *et al.*, 1997) and the 3D structure determined to 1.8Å resolution (Liao *et al.*, 2000). Following cleavage of the N-terminal methionine for both subunits, the N-terminal threonine at position two is exposed. In higher plants, but not algae or cyanobacteria, this residue can be acetylated and subjected to reversible phosphorylation (Michel *et al.*, 1988).

The D2 subunit is co-translationally inserted into the thylakoid membrane (Herrin and Michaels, 1985). In *C. reinhardtii* the successful synthesis of the D2 protein has been shown to require the protein products of at least three nuclear genes *nac1*, *nac2* and *ac115*. The *psbD* mRNA is stabilised by the protein product of the *nac2* gene, which binds within the 5'-untranslated region of the transcript. Synthesis of the D2 protein, following translation initiation, requires the presence of the *ac115* and *nac1* gene products. The *ac115* gene has been isolated from *C. reinhardtii* and it is postulated that it may be involved in either stabilising the D2 protein or in localising the mRNA~pre-D2 protein to the thylakoid membrane (Rattanachaikunsopon *et al.*, 1999).

During illumination, at any light intensity, the D1 subunit is continually damaged due to interaction with reactive oxygen species. To maintain PSII activity this subunit has to be replaced (Ohad *et al.*, 1984). Consequently the rate of activity of the complex is a result of the equilibrium between the rate of photoinactivation and protein degradation and rate of D1 protein synthesis and re-assembly of the PSII complex.

1.5.2 PSII-E and PSII-F (cytochrome b_{559})

A co-ordinated haem and the protein products of the *psbE* and *psbF* genes constitute cyt b_{559}. The α-subunit is encoded by the *psbE* gene, whilst the β-subunit is the product of the *psbF* gene. The protein products PSII-E and PSII-F are both thought to be maintained as single transmembrane proteins (Alizadeh *et al.*, 1995). Together with the D1-D2 heterodimer and the subunits PSII-I and PSII-W they form the RC of the PSII complex. The stoichiometry of the cyt b_{559} per RC is unclear. Currently it is thought that there are either one or two cyt b_{559} molecules per RC. Structural analysis of the PSII complex shows that cyt b_{559} is a single heterodimeric molecule, and not a combination of homodimers as previously suggested. How cyt b_{559} functions has not yet been ascertained, but current models suggest that its role is a protective one. It is proposed that the haem acts as an electron donor or acceptor during periods when electron flow is sub-optimal. As such it falls into the category of an alternative electron donor, together with Y_D and chlorophyll Z. Support for this comes from observations that cyt b_{559} is oxidised by $P680^+$ and reduced by plastoquinol (Barber and Andersson, 1992; Barber, 1994). Koulougliotis *et al.* (1994) propose that cyt b_{559} reduces a chlorophyll molecule, termed chlorophyll Z^+, whilst accepting electrons from reduced plastoquinone in a cyclic fashion.

1.5.3 PSII-I

PSII-I is a highly conserved subunit also located in the RC complex of PSII (Ikeuchi and Inoue, 1988; Webber *et al.*, 1989). Cross-linking studies established that this subunit is associated with the N-terminal fragments of the D2 protein and the α-subunit of cyt b_{559} (Tomo *et al.*, 1993) This component is estimated to be about 4.8kDa in size with a single transmembrane region. At present the function of this small component is unknown, but it is not believed to be associated with any of the electron transfer cofactors.

Unlike the *psbA*, *psbD*, *psbE* and *psbF* gene products, disruption or deletion of the *psbI* gene does not result in complete inactivation of the PSII complex. Disruption of the *psbI* gene in *C. reinhardtii* results in a reduced amount of PSII assembly and oxygen evolving capacity of about 10 to 20% that of wild type (WT) cells (Künstner *et al.*, 1995). This led the authors to propose that the PSII-I subunit may play a role in PSII stability or in modulation of electron transfer in the complex. Deletion of the *psbI* gene in the

cyanobacterium *Synechocystis* 6803 did not result in a destabilisation of the PSII complex, or in a reduction of the levels of other PSII subunits. However, a decrease of about 25% to 30% of PSII activity was observed (Ikeuchi *et al.*, 1995).

1.5.4 PSII-W

PSII-W is encoded by the nuclear gene *psbW*. This gene appears to be present throughout higher plants and green algae, but is absent from the fully sequenced genome of *Synechocystis* 6803 (Kaneko *et al.*, 1996). The gene is also absent from non-green algae and all other prokaryotes examined. An unrelated gene, also termed 'psbW', is present in the plastid genomes of the non-green algae *Cyanophora paradoxa*, *Odontella sinensis* and *Porphyra purpurea*. There is no evidence to indicate that this gene is even a *psb* gene. In 1989 Ikeuchi *et al.* published the N-terminal sequence for the mature PSII-W protein in spinach (Ikeuchi *et al.*, 1989a), and this was followed by the N-terminal sequence for *C. reinhardtii* in 1991 (de Vitry *et al.*, 1991). In 1995 PSII-W was isolated from spinach PSII and similarity to the translated sequence of an *Arabidopsis thaliana* gene was shown (Irrgang *et al.*, 1995). Sequencing of the spinach gene showed that the mature polypeptide has 54 amino acids containing one predicted transmembrane span (Lorković *et al.*, 1995). In the pre-protein this is preceded by an 83 amino acid residue transit peptide that has many of the characteristics of a bipartite transit sequence.

Lorković *et al.* (1995) showed that the pre-PSII-W protein is capable of integration into the thylakoid membrane in a ΔpH independent manner. They also found that the insertion was azide-insensitive, leading them to conclude that the protein inserts itself into the thylakoid membrane in a spontaneous manner or via an as yet unknown mechanism. This finding has led to the proposal that the protein is transported by a potentially new mechanism (Thompson *et al.*, 1998). Compositional and topological studies, also of spinach PSII, indicate that the polypeptide is located close to the core of the PSII complex, in close proximity to the D1-D2 heterodimer (Shi and Schröder, 1997).

Investigations into the rates of light-induced proteolysis concluded that the PSII-W protein is degraded at a comparable rate to that of the D1 polypeptide. Furthermore, its degradation was greater when compared to rates for D2 and CP43. The authors concluded that the PSII-W protein is not degraded directly as a result of photodamage, as is the case for the D1 subunit. Instead they suggest that it is more likely the result of a general

destabilisation of the PSII complex following degradation of the D1 polypeptide (Hagman *et al.*, 1997).

Shi *et al.* (2000) recently proposed a role for PSII-W in the stabilisation of the PSII dimer. In this work *A. thaliana* was transformed with an antisense gene to *psbW*. Seedlings were produced with >96% reduction in PSII-W content. Whilst no discernible phenotype could be detected for the antisense plants, the PSII dimeric supercomplex band was absent following sucrose gradient separation of thylakoid membranes. Furthermore, the rate of oxygen evolution for isolated thylakoids was about 50% that of WT *A. thaliana*. Therefore, in the absence of the PSII-W protein, the stability and levels of the PSII complex appear to be affected. The authors propose that PSII-W may play a role in the biogenesis and regulation of the PSII complex.

A recent paper by Hiyama *et al.* (2000) called into question the long held view that PSII-W is a protein located exclusively within the PSII complex. In this work PSI was isolated from a number of plants, and subjected to analysis. In addition to the core PSI polypeptides, a small protein of 5-6kDa was also isolated with each of the PSI preparations. The N-terminal sequence of these small proteins from the different PSI preparations maintained a high level of sequence identity with the PSII-W protein of spinach, wheat and *C. reinhardtii*. The authors concluded that PSII-W is also a subunit of the PSI complex.

1.6 OTHER SUBUNITS OF THE PHOTOSYSTEM II COMPLEX

1.6.1 PSII-B (CP47) and PSII-C (CP43)

The subunits PSII-B and PSII-C form the internal antenna system of the PSII complex. The chloroplast genes *psbB* (Morris and Herrmann, 1984) and *psbC* encode these subunits respectively, which are commonly referred to as the CP47 and CP43 proteins. Together with the D1, D2, cyt b_{559} and a number of small hydrophobic subunits they form the core of the complex. It should be noted that the calculated molecular masses of the spinach proteins are 56kDa and 50kDa, rather that the 47kDa and 43kDa predicted by SDS-PAGE. Both mature proteins bind about 15 chlorophyll *a* molecules and two to three β-carotenes. Each has a number of highly conserved histidine residues, many of which lie within predicted transmembrane regions. These residues are proposed

to act as chlorophyll ligands. The associated pigments form the core light harvesting system for the PSII complex.

Both proteins are predicted to contain six transmembrane helices with five hydrophobic loops, termed loops A to E; loops A, C and E are exposed on the lumenal side of the thylakoid membrane (Bricker, 1990; Shen *et al.*, 1993). The C and N-termini of these two proteins are located in the stroma. Electron crystallography has produced a model for the transmembrane helical domains of these proteins, to a resolution of about 8Å (Rhee *et al.*, 1997; Rhee *et al.*, 1998; Hankamer *et al.*, 1999) and more recently by Shen and Kamiya (2000) to a resolution of 3.5Å.

Most of the investigations to date have focused on the CP47 subunit. Work performed by Wu *et al.* (1996) indicated that CP47 might play a role in PSII assembly and/or stability. Loop E of both subunits has been shown to interact with the WOC and the 33kDa protein (Wu *et al.*, 1999 and references therein; Barber *et al.*, 2000 and references therein). These large lumenal loops may function directly, or indirectly, in water oxidation. Mutants in which specific amino acids of loop E have been deleted in *Synechocystis* 6803 fail to assemble PSII centres (Eaton-Rye and Vermaas, 1991). Both deletion and site directed mutagenesis studies of the CP47 protein suggest that it is essential for photoautotrophic growth (Vermaas *et al.*, 1986) and disruption of the *psbB* gene results in a loss of oxygen evolving activity (Wu *et al.*, 1999).

Less work has been reported for CP43, but deletion and inactivation mutants of the *psbC* gene of spinach result in an elimination of oxygen evolution and photoautotrophic growth. However, so called 'subcomplexes' of PSII, which contain all the core subunits except CP43, are still capable of evolving oxygen. Therefore it is thought that CP43 does not maintain ligands for the manganese cluster and is not essential for the binding of the WOC (Büchel *et al.*, 1999).

CP43 contrasts from CP47 in three major ways. It is post-translationally modified, the mature protein maintains an N-terminal threonine that is reversibly phosphorylated in higher plants and algae (Michel *et al.*, 1988) and it is biochemically 'easier' to remove.

1.6.2 PSII-G

The chloroplast gene originally designated *psbG* (Steinmetz *et al.*, 1986) was later shown to be located in the NADPH/quinone oxidoreductase. It has subsequently been renamed *ndhK* (Nixon *et al.*, 1989).

1.6.3 PSII-H

The PSII-H protein is present in all oxygenic organisms. In higher plants the chloroplast-encoded protein contains 73 amino acids. The *C. reinhardtii* protein is slightly larger with 88 residues. From work performed by Koike *et al.* (1989) it is believed that the PSII-H subunit is associated with the oxygen-evolving core of the PSII complex. It is predicted to be present as a single transmembrane protein, and undergoes reversible phosphorylation. Precisely which residues were phosphorylated was uncertain for a number of years. However, Vener *et al.* (2001) used mass spectrometry to analyse thylakoid membranes from *A. thaliana* and established that the PSII-H subunit can be phosphorylated at least on threonine-2 and threonine-4. The function that this polypeptide plays, either in its phosphorylated or un-phosphorylated state, is still to be determined.

In an attempt to determine the role of the phosphoprotein, deletion mutants have been created. Deletion mutants of *C. reinhardtii psbH* fail to accumulate the core proteins of the PSII complex, do not evolve oxygen and are unable to grow photoautotrophically (Summer *et al.*, 1997; O'Connor *et al.*, 1998; Cain, 1998). These results suggest that PSII-H may play a structural role in the complex. In contrast, a deletion mutant created by Mayes *et al.* (1993) in *Synechocystis* 6803 was capable of photoautotrophic growth, but electron transfer from Q_A to Q_B was affected. Packham (1988) has proposed that this protein may play a role in the regulation of Q_A to Q_B electron transfer.

1.6.4 PSII-J

The *psbJ* gene encodes a protein of 39 amino acids. The gene has been sequenced for a number of photosynthetic organisms. Until 1993 it was unclear if the *psbJ* gene actually codes for a PSII subunit, as no direct evidence had been reported. Lind *et al.* (1993) created a *Synechocystis* 6803 deletion mutant that grew photoautotrophically. However, the rate of PSII activity was about 45% that of WT 6803. Oxygen evolution was also impaired, when compared to WT 6803. The authors confirmed that PSII mediated electron transfer was effected, whilst rates through PSI were not. This was the first direct evidence that PSII-J was indeed a PSII subunit. Despite this work, no direct conclusions could be drawn about the role that the PSII-J polypeptide plays, and to date its function is still unknown. However, the authors propose that PSII-J may control the levels of PSII that are fully assembled and functional.

1.6.5 PSII-K

The nucleotide sequence for the *psbK* gene has been obtained for a number of species. Examination of the protein sequence of the ~4kDa PSII-K protein of *C. reinhardtii* shows high sequence similarity of about 80% to the post-translationally modified PSII-K protein of higher plants. N-terminal sequencing of the proteins contained in the oxygen-evolving core of PSII from *Thermosynechococcus* (formerly *Synechococcus*) *vulcanus* indicates that the PSII-K protein is associated with this fraction of the complex (Koike *et al.*, 1989).

The *psbK* gene from *Synechocystis* 6803 has been cloned and sequenced and the N-terminal sequence for the mature PSII-K protein determined by Ikeuchi *et al.* (1991). Eight amino acid residues are cleaved from the pre-protein to produce the processed mature protein. The authors also constructed a mutant which lacked *psbK*, that was capable of photoautotrophic growth, but at a reduced rate. Furthermore, the mutant had decreased levels of PSII RC, based on chlorophyll assays. In *Synechocystis* 6803 PSII-K does not appear to be involved in PSII assembly, or activity, but does seem to be required for optimal functioning of the complex.

The *psbK* gene has also been disrupted in *C. reinhardtii* by Takahashi *et al.* (1994). In contrast to the *Synechocystis* 6803 mutant, this disruption mutant could not grow photoautotrophically. The authors propose that the PSII-K subunit may be necessary for assembly and stability of PSII. The results of work by Ikeuchi *et al.* (1991) and Takahashi *et al.* (1994) indicate that the PSII stability requirements of the two species are different.

1.6.6 PSII-L

The PSII-L polypeptide is a 4.5kDa protein with a predicted single transmembrane α-helical domain. Deletion of the *psbL* gene in *Synechocystis* 6803 resulted in a mutant that was not capable of photoautotrophic growth (Anbudurai and Pakrasi, 1993). The mutant has WT 6803 levels of PSI activity, but fails to exhibit PSII-mediated oxygen evolution. The phenotype of this mutant has led to the belief that the PSII-L subunit is required for PSII to function normally. Analysis of re-constituted PSII reaction core complexes suggests the lumenal C-terminus of the protein may function in the transfer of electrons to the primary electron acceptor Q_A (Kitamura *et al.*, 1994; Ozawa *et al.*, 1997).

Further work by Hoshida *et al.* (1997) proposes that the subunit may enable the formation of Y_Z^+P680Pheo$^-$ following the oxidation of Y_Z by P680$^+$.

1.6.7 PSII-M

The *psbM* gene has been shown to encode a 31 amino acid peptide in *C. reinhardtii*, which maintains about 74% sequence identity with the PSII-M protein of tobacco. The *psbM* sequence is also available for a number of other vascular plants, together with the cyanobacterium *Synechocystis* 6803. Characterisation of the PSII-M subunit from *T. vulcanus* indicates that it has an apparent molecular mass of ~4.7kDa (Ikeuchi *et al.*, 1989b). There are no reports in the literature of a mutant in the *psbM* gene, and as such there is no indication of the role that this subunit plays within the PSII complex.

Higgs *et al.* (1996) published the sequence for the *psbM* gene in *C. reinhardtii* and noted that the *psbM* gene is expressed on a dicistronic transcript that encodes the PSII-Z (formerly Ycf9) and the PSII-M proteins. (Higgs *et al.*, 1996). Further work detected the presence of a 2.7kb *psbZ-psbM* dicistronic transcript and a 0.7kb monocistronic transcript in WT CC-1021 (Higgs *et al.*, 1998). Work performed by Cain (1998) detected a *psbZ-psbM* dicistronic transcript of ~2.9kb and a monocistronic *psbM* transcript of 1.95kb. The smaller transcript of 1.95kb does not represent a processed form of the ~2.9kb dicistronic transcript, as both the *psbZ* and the *psbM* genes lie within the last 1kb of the ~2.9kb RNA. It is unclear why a discrepancy in the transcript sizes between these workers is observed.

1.6.8 PSII-N

The subunit PSII-N is thought to contain 43 amino acids, with a putative single transmembrane domain. Analysis of the oxygen-evolving complex of *T. vulcanus* determined that the PSII-N subunit is a 4.7kDa polypeptide (Ikeuchi *et al.*, 1989b). When the sequence of the PSII-N protein of *C. reinhardtii* is compared with those from vascular plants a similarity of 50 to 62% is observed (Johnson and Schmidt, 1993). The *psbN* gene of *C. reinhardtii* has been disrupted to produce a mutant which is not capable of photoautotrophic growth, but which does exhibit low levels of PSII activity when grown under heterotrophic conditions (Ruffle and Sayre in Rochaix *et al.*, 1998).

1.6.9 PSII-S

The PSII-S protein appears to be present in all green plants examined to date. The spinach *psbS* gene encodes a pre-protein of 274 amino acid residues (Kim *et al.*, 1992; Wedel *et al.*, 1992). The 200 amino acid mature protein is preceded by a 74 amino acid bipartite transit peptide. This is responsible for the import of the pre-protein across the chloroplast envelope and its insertion into the thylakoid membrane. Hydropathy plot analysis of the protein sequence indicates that the mature protein has four hydrophobic membrane spanning helices. Analysis of the protein sequence highlights a number of similarities with the LHC proteins. It also reveals high levels of sequence similarity between the two halves of the 200-residue mature protein, suggesting that the *psbS* gene is the result of a gene duplication event.

Funk and co-workers established that the 22kDa PSII-S protein is capable of binding chlorophyll (Funk *et al.*, 1994; Funk *et al.*, 1995a). In contrast to other chlorophyll-binding proteins the PSII-S protein is stable in the absence of the pigment molecules (Funk *et al.*, 1995b). Their results suggest that the PSII-S protein may function as a pigment chaperone, aiding chlorophyll biogenesis and mediating the binding of chlorophyll to CP43 and CP47 (Li *et al.*, 2000). More recently it has been shown that the PSII-S protein is not essential for efficient light harvesting or photosynthesis. However, it has been shown that this subunit appears to have a regulatory role in non-photochemical quenching (NPQ) (Section 1.9). This process helps minimise the damage caused by excess light.

With the modelling of the spinach LHCII PSII supercomplex it has emerged that the PSII-S subunit is not situated within the supercomplex but is located in the LHCII regions that interconnect the supercomplex within the membrane (Nield *et al.*, 2000a). This would add further weight to the proposed function of the *psbS* gene product in NPQ.

1.6.10 PSII-T

Also referred to in the literature as *ycf8* and orf31, the *psbT* gene encodes a 31 amino acid protein with a predicted mass of 3.3kDa in *C. reinhardtii*. In eukaryotes this gene is located in the chloroplast genome. Observations made from a deletion mutant in

C. reinhardtii indicate that the subunit, while not essential for photoautotrophic growth, does play a role in the maintenance of photosynthetic activity under adverse growth conditions (Monod *et al.*, 1994). Comparison of the PSII-T sequences in *C. reinhardtii* and *Synechocystis* 6803 shows about 40% identity.

In 1995 a second gene, also termed *psbT* was detected in the nuclear genome of cotton (Kapazoglou *et al.*, 1995). The 28 amino acid mature protein maintains a high degree of sequence identity with the PSII-T subunit of spinach, but no detectable sequence identity with the previously termed PSII-T polypeptides of *C. reinhardtii* and *Synechocystis* 6803. Furthermore, no homology to this nuclear form of the *psbT* gene can be detected in the nuclear genome of *C. reinhardtii*. No function has been proposed for this second PSII-T subunit.

1.6.11 PSII-X

The *psbX* gene encodes a mature protein of about 4.1kDa in size, which is predicted to have a single transmembrane helix. N-terminal sequences were initially generated for the nuclear-encoded PSII-X protein products from spinach and *C. reinhardtii* (Ikeuchi *et al.*, 1989b; de Vitry *et al.*, 1991 respectively). Like the PSII-W subunit, this subunit appears to undergo spontaneous insertion into the thylakoid membrane (Kim *et al.*, 1996; Thompson *et al.*, 1998). Analysis of PSII preparations has established that the PSII-X subunit is not present in the PSII RC or in the LHCs. However, the discovery that it can cross link with cyt b_{559}, suggests that it is located near to the core of the complex (Shi *et al.*, 1999).

A deletion mutant has been created in the cyanobacterium *Synechocystis* 6803 (Funk, 2000). The mutant appeared as WT 6803 when growth rates, electron transfer and water oxidation rates were examined. In contrast, when PSII complex levels were compared, the mutant had about 30% that of WT 6803. This led to the proposal that the PSII-X protein plays a role in the regulation of the levels of functionally active PSII, be it directly or indirectly.

A *psbX* disruption mutant has also been created in *T. elongatus* (Katoh and Ikeuchi, 2001). This mutant was capable of photoautotrophic growth, supporting the view that the PSII-X subunit does not have an essential role. Analysis of the composition of the mutant PSII complex confirmed that only the PSII-X subunit was absent. The authors also show that the levels of oxygen evolution are reduced in the mutant. They propose that the

PSII-X subunit may function in the binding or turnover of quinones at the Q_B site of the PSII complex.

It should be noted that there is a second gene, also referred to as 'psbX', from tomato. This too is a nuclear-encoded gene that encodes a pre-protein of 257 residues, 185 of which make up the mature protein. The protein has a predicted mass of 23kDa. Analysis of the protein sequence indicates that this gene has been wrongly assigned, and that it is in fact the tomato homologue for the *psbP* gene.

1.6.12 PSII-Y

The PSII-Y subunit is encoded in eukaryotes by the nuclear gene *psbY*, previously termed *ycf32*, and is also present within the genome of cyanobacteria. The mature protein is about 7kDa in size and is thought to be inserted into the thylakoid via a spontaneous insertion mechanism, similar to that described for PSII-W and PSII-X (Mant and Robinson, 1998; Thompson *et al.*, 1999). The protein was originally isolated by Gau *et al.* (1995) from PSII preparations of spinach and tobacco. Analysis of these preparations showed that the PSII-Y subunit could be localised to the core of the complex.

In cyanobacteria the *psbY* gene is present in a single copy. Interestingly in higher plants two highly homologous, yet distinct polypeptides are derived from a polyprotein (Gau *et al.*, 1998; Mant and Robinson, 1998). The two species of the PSII-Y protein are referred to in the literature as PsbY-A1 and PsbY-A2. The polyprotein appears to be imported in the chloroplast where it undergoes a number of proteolytic processing steps, resulting in the N-termini of PsbY-A1 and PsbY-A2 proteins positioned in the lumen of the thylakoid (Thompson *et al.*, 1999). This was the first chloroplast-targeted polyprotein to be identified and characterised in higher plants. Analysis of the derived PSII-Y sequence for *C. reinhardtii* indicates that the *psbY* also encodes a polyprotein (Prof. Jonathan Nugent, UCL, personal communication).

The role that the PSII-Y subunit plays within the complex is not yet clear. It has been proposed that it may act as a ligand for the manganese complex. PSII-Y appears to bind manganese effectively and may also contain a redox-active component (Gau *et al.*, 1998). However, work performed by Meetam and co-workers (Meetam *et al.*, 1999) in *Synechocystis* 6803 has refuted this theory. An inactivation mutant of the *psbY* gene was generated and the mutant capable of photosynthetic growth and oxygen evolution. The authors conclude that, in *Synechocystis* 6803 at least, PSII-Y is not essential for oxygenic

photosynthesis. The PSII-Y subunit in cyanobacteria may not be a true homologue of that found in higher plants. Consequently PsbY-A1 and PsbY-A2 may play a different role to the PSII-Y of *Synechocystis* 6803.

1.6.13 PSII-Z (Ycf9, orf62)

Until recently this protein has been referred to in the literature as Ycf9 and orf62. However, following very recent work performed by Swiatek *et al.* (2001) the authors propose that this protein be renamed PsbZ, or PSII-Z. This new nomenclature has been adopted for this text. The *psbZ* gene is present both in chloroplast and cyanobacterial genomes. It encodes a protein of ~6.5kDa in size, with two predicted transmembrane regions. Protein alignment of the sequences from a number of species indicates a high degree of sequence identity for the PSII-Z protein within photosynthetic organisms (Mäenpää *et al.*, 2000). This protein has been of increasing interest, as mutants of the *psbZ* gene in different species give a spectrum of phenotypes.

In *C. reinhardtii* the *psbZ* gene is expressed as part of a 2.7kb-2.9kb dicistronic transcript, which also contains the *psbM* gene (Higgs *et al.*, 1998; Cain, 1998). Cain (1998) created insertion and deletion mutants for the *psbZ* gene of *C. reinhardtii*. While the deletion mutants were successfully segregated, the insertion mutants remained heteroplasmic. These mutants were capable of photosynthetic growth, but had decreased rates of oxygen evolution. The deletion mutants showed an oxygen evolution rate of only 25% that observed for WT CC-1021 cells. EPR analysis indicated that the absence of the dicistronic transcript had an adverse effect on PSII. The isolation of revertants and a *psbZ* deletion mutant, both of which lack the dicistronic *psbZ-psbM* transcript, has also reported by Higgs *et al.* (1998). It is, however, hard to draw conclusions about the function of the PSII-Z subunit without first knowing the effect that the absence of the dicistronic transcript has on the accumulation of PSII-M in the thylakoid.

In tobacco the *psbZ* gene is transcribed on the same transcription unit as the *psbC* and *psbD* genes. The targeted inactivation of the *psbZ* gene in tobacco has been reported (Mäenpää *et al.*, 1998; Mäenpää *et al.*, 2000; Baena-González *et al.*, 2001). Homoplasmic transformants could not be isolated, indicating an essential role for the protein in tobacco. The mutant showed an increased rate of flux of electrons to PSI, with no comparable effect on the electron transfer capacity of PSII. It was proposed from the observations made in this work that the PSII-Z subunit may be responsible for 'fine-tuning' electron

transfer through the photosystems and may be associated solely with PSI. Through this work the PSII-Z protein has also been localised to the stromal thylakoid membrane of chloroplasts (Mäenpää *et al.*, 2000).

Subsequently, a homoplasmic disruption mutant has been isolated for the *psbZ* gene in tobacco (Ruf *et al.*, 2000). Under normal growth conditions a WT phenotype was observed. When plants are grown under low light conditions, the growth rate of the mutant was significantly impaired, when compared to that of WT plants. The authors propose that in the absence of PSII-Z there is a reduction in a pigment-protein complex of the light-harvesting apparatus of PSII. Under normal growth conditions this reduction does not have a detrimental effect. However, when light is a limiting factor the result is a decreased efficiency of photon capture. They go on to suggest that PSII-Z may play a role in the assembly and/or maintenance of stable light-harvesting antennae.

Using antibodies raised to the PSII-Z protein of *C. reinhardtii* and tobacco, Swiatek *et al.* (2001) have established, for the first time, that the protein is present within PSII cores and have proposed that the proteins Ycf9 and orf62 be renamed PSII-Z. Homoplasmic inactivation mutants of the *psbZ* gene were isolated for *C. reinhardtii* and tobacco. Characterisation of these mutants supports the theory that the subunit controls the interaction of the light-harvesting antenna with the core of PSII. When both sets of mutants were grown under photoautotrophic conditions, the levels of the CP26 were substantially decreased, accompanied by a reduction of the CP29 subunits to a lesser extent. It has been suggested that the CP26 and CP29 proteins play a role in NPQ (Section 1.9). In both mutants there was a reduced capacity for NPQ under stress conditions (increased light intensity and/or decreased temperature). The results obtained indicate that, in addition to functioning in the interaction between PSII and LHCII, the PSII-Z subunit may also play a role in NPQ under conditions that give rise to photoinhibition.

1.7 THE EXTRINSIC POLYPEPTIDES

In addition to the *psbO* gene product, two other extrinsic proteins are associated with PSII. In higher plants and green algae these are the PSII-P and PSII-Q subunits. Cyanobacteria, red algae and almost certainly diatoms lack these two polypeptides. Instead the protein products of the *psbU* and *psbV* genes associate with the 33kDa protein.

1.7.1 PSII-O (33kDa protein, MSP, OEE1)

The *psbO* gene encodes the PSII subunit referred to in the literature as the 33kDa protein, manganese-stabilising protein (MSP), oxygen evolving enhancer I (OEE1) or PSII-O. The *psbO* gene is present in all oxygenic photosynthetic organisms, and is a nuclear-encoded gene in eukaryotes (De Las Rivas and Heredia, 1999). The mature protein is located on the donor side of the PSII complex, and is thought to stabilise the function of the manganese cluster (reviewed by Seidler, 1996). Work performed by Hutchison *et al.* (1999) concluded that PSII-O acts as a negative charge accumulator and as a donor of protons during early state transitions. They propose that it is this role which suggests that while the PSII-O subunit it not essential for the process of water oxidation, it may act as a catalyst for the reaction.

The *psbO* gene has been deleted in the cyanobacterium *Synechocystis* 6803 (Burnap and Sherman, 1991). The mutant is capable of photoautotrophic growth and evolves oxygen at a rate of about 70% that of WT 6803 cells. However, these mutants are more sensitive to photoinhibition (Philbrick *et al.*, 1991) and have an increased rate of D1 turnover accompanied by a efficient rate of recovery from photodamage when the cells are returned to normal light conditions (Komenda and Barber, 1995). Furthermore, photoautotrophic growth is lost in the absence of calcium, indicating that the WOC is less stable in the *psbO* mutant (Philbrick *et al.*, 1991). The authors propose that in cyanobacteria the PSII-O subunit either protects the RC at low calcium concentrations or is involved in aiding or enhancing the binding of calcium. Bockholt *et al.* (1991) reported the inactivation of the *psbO* gene in *Synechococcus* sp. PCC 7942. This mutant was also capable of photoautotrophic growth.

In *C. reinhardtii* the nuclear mutant FUD44 lacks the PSII-O mRNA and protein and, in contrast to the mutants described above, is not able to perform photosynthetic oxygen evolution. The accumulation and assembly of other nuclear-encoded PSII subunits do not appear to be affected in this mutant. However, the relative turnover of the chloroplast-encoded subunits appears to be accelerated in FUD44 cells, and the authors report a deficiency of the core subunits of the PSII complex in this mutant (Mayfield *et al.*, 1987a).

1.7.2 PSII-P (23kDa protein, OEE2)

The nuclear-encoded *psbP* gene is present in all photosynthetic eukaryotes. The PSII-P subunit, also referred to as the 23kDa protein or OEE2 subunit, is absent from cyanobacterial PSII. The PSII-P protein sequence of *C. reinhardtii* maintains about 60% sequence identity with that of vascular plants. Mayfield *et al.* (1987b) characterised the *C. reinhardtii* mutant BF25, which failed to accumulate the *psbP* transcript. This mutant was unaffected in the levels of other PSII subunits. The BF25 mutant evolved oxygen at rates of about 5% that observed for WT CC-1021 cells. The authors show that while PSII-P is necessary for normal levels of oxygen evolution the BF25 mutant could still grow photoautotrophically. Miyao and Murata (1989) showed the presence of PSII-P is required for the assembly of the PSII-Q subunits into the complex.

From other data it is thought that the PSII-P subunit is also involved in maintaining calcium in a bound form during S-state turnover and in providing a high affinity binding site for chloride ions. Other roles proposed include a function in the stabilisation and in the prevention of chemical attack of the manganese cluster (reviewed by Seidler, 1996).

1.7.3 PSII-Q (16kDa protein, OEE3)

Like *psbP,* the *psbQ* gene encodes a PSII subunit that is unique to photosynthetic eukaryotes. The mature protein is referred to as the PSII-Q subunit, the 16kDa protein or OEE3. The level of protein identity, estimated at about 30% between the *C. reinhardtii* subunit and that of higher plants, reflects the fact that this subunit is probably the most loosely associated of the extrinsic proteins. The role of this polypeptide within the WOC is unclear. The PSII-Q subunit is only incorporated into the PSII complex in the presence of the PSII-P polypeptide (Miyao and Murata, 1989). Thus, PSII-P is either involved in aiding the binding of PSII-Q or PSII-Q binds directly to PSII-P.

1.7.4 PSII-R

Apparently unique to oxygenic photosynthetic eukaryotes, the PSII-R subunit is about 10kDa in size. Very little is known about this polypeptide. It is thought that it is an extrinsic protein, which integrates onto the lumenal side of the complex with a high affinity (Barber *et al.*, 1997).

1.7.5 PSII-U

This subunit is unique to cyanobacteria, red algae and probably diatoms (Shen *et al.*, 1998), and is an extrinsic protein of the PSII complex. Analysis of the PSII structure indicates that the PSII-U subunit is located on the lumenal surface of the thylakoid membrane over the N-terminal region of the D1 polypeptide (Nield *et al.*, 2000b). The subunit cytochrome c_{550} (cyt c_{550}) (PSII-V) (Section 1.7.6) needs to be present for the PSII-U subunit to be incorporated into the complex.

In *Synechocystis* 6803, the *psbU* gene encodes a pre-protein of 131 amino acid residues, with the mature protein having a predicted molecular mass of 12kDa. The first 36 residues are thought to function in the insertion of the pre-protein into the thylakoid membrane. A deletion mutant in the *psbU* gene is capable of photoautotrophic growth at rates comparable to WT 6803 (Shen *et al.*, 1997). However, the rates of oxygen evolution are impaired in the mutant, with about a 20% decrease observed relative to WT 6803 rates. Furthermore, when the mutant is grown in medium depleted of calcium or chloride ions the growth rate of the mutant is also reduced, when compared to WT 6803 rates. Thus, the PSII-U subunit is not essential for PSII function. It may, however, function in the optimisation of the ion environment and in the maintenance of the structure of the WOC. Further work on this mutant has led to the proposal that the PSII-U subunit has a role in maintaining normal S-state transitions in cyanobacterial photosystems (Shen *et al.*, 1998).

Further evidence in support of a structural stability role for the PSII-U protein was provided by Nishiyama and co-workers (Nishiyama *et al.*, 1997; Nishiyama *et al.*, 1999). The authors propose that the PSII-U subunit and the PSII-V polypeptide are involved in the protection the oxygen-evolving complex from heat-induced inactivation in the cyanobacterium *Synechococcus* sp. PCC 7002.

In the red alga *Cyanidium caldarium* the *psbU* gene is located in the nuclear genome. The gene encodes a pre-protein of 154 amino acids, containing the characteristics of a bipartite transit peptide (Ohta *et al.*, 1999). The mature protein is 93 amino acid residues in length and has an expected molecular mass of 10.5kDa.

1.7.6 PSII-V (cytochrome c_{550})

The extrinsic PSII-V protein, encoded by the *psbV* gene, is a haem protein that is also referred to as cyt c_{550}. Like PSII-U, the protein product of the *psbV* gene is unique to cyanobacteria, red algae and probably diatoms (Shen *et al.*, 1998).

The *psbV* gene has been deleted in the cyanobacterium *Synechocystis* 6803 (Shen *et al.*, 1995; Shen *et al.*, 1998). When grown photoautotrophically the deletion strain had a doubling time of more than twice that observed for WT 6803 cells, and an oxygen-evolving activity of 42% that of WT 6803. As with the *psbO* deletion mutant (Philbrick *et al.*, 1991) the *psbV* mutants could not grow in the absence of calcium or chloride ions. The number of PSII RCs was about half that of WT 6803. From the results observed the authors propose that the PSII-V subunit plays a role in regulating the conformational integrity of the PSII complex and the stability of the manganese cluster.

Katoh *et al.* (2001) have disrupted the *psbV* gene in *T. elongatus* and expressed it heterologously in a *psbV* mutant of *Synechocystis* 6803. The *psbV* mutant was capable of photoautotrophic growth, but grew consistently slower than the WT *T. elongatus* at various temperatures and CO_2 concentrations. The mutant was not capable of growth under low CO_2 conditions, a phenotype that was also observed for a *psbU* mutant of *T. elongatus* (Katoh and Ikeuchi, unpublished results, in Katoh *et al.*, 2001). Neither the *psbV* mutant nor the WT *T. elongatus* cells were capable of growth in the absence of calcium ions, indicating that *T. elongatus* may lack the mechanism present in *Synechocystis* 6803 for the regulation of calcium concentration. The rates of oxygen evolution for the mutant were shown to be about 30% that of the WT *T. elongatus*. Expression of the *psbV* gene from *T. elongatus* in the *psbV* mutant of *Synechocystis* 6803 returned growth rates to those observed for WT 6803 cells. The ability of the cells to grow in the absence of calcium or chloride ions was also reported. The results presented by this paper suggest that the PSII-V subunit has a regulatory role in the WOC, and support those proposed by Shen and co-workers (Shen *et al.*, 1995; Shen *et al.*, 1998) above.

1.8 STATE TRANSITIONS

Oxygenic photosynthetic organisms adjust to changes in the abundance and spectrum of available light. They achieve this by moving their antenna systems (Section 1.4.1) between PSII and PSI. The movement of these mobile LHCs are termed state transitions. State transitions are performed to maintain a balance in the electron transport between the two complexes (recently reviewed by Haldrup *et al.*, 2001; Wollman 2001), and also as a photoprotection mechanism minimising the potential for damage of the PSII complex in high light conditions (Schroda *et al.*, 1999). The redox state of the plastoquinone pool is generally considered to be a regulatory point. When the two photosystems are harvesting equal amounts of light energy the electrons transported from PSII to PSI are balanced. If the available light favours one photosystem over the other this balance is disrupted. State transitions function to redistribute the available excitation energy to the complex that it favours less. State 1 occurs when the available light preferentially excites PSI. In this situation the antenna system is bound to PSII. State 2 is induced by illumination that predominantly excites PSII. In this situation PSI cannot keep up with the electrons from PSII which accumulate between the two photosystems. Under these conditions the light antenna detach from PSII and associate with PSI. Therefore the light-limited photosystem receives more energy.

1.9 NON-PHOTOCHEMICAL QUENCHING (NPQ)

When chlorophyll absorbs light it is excited from its ground state to a singlet excited state Chl^*. From there it has several ways to relax back to the ground state. It can relax by emitting light as fluorescence. Chl^* excitation can be used to start electron transfer, this process being termed photochemical quenching (qP). Alternatively Chl^* can be de-excited by the dissipation of heat, termed non-photochemical quenching of chlorophyll fluorescence (NPQ) (reviewed by Ruban and Horton, 1995; Niyogi, 1999). Finally the excited chlorophyll can form $^3Chl^*$ which in turn, through interaction with O_2 can produce singlet oxygen and toxic oxygen radicals.

Photosynthetic organisms have protective mechanisms that can detoxify such active oxygen species. However, under excess light conditions these radicals reach elevated levels that in turn can saturate the processes responsible for this detoxification process. In reality the level of qP is maintained at a constant 'maximum' level, and as

such it does not constitute a channel for the dissipation of excess excitation energy. In fact because *q*P is at a constant high level, funnelling of any excess energy would be detrimental to the photosystems and would result in photodamage. Instead, photosynthetic organisms prevent the production of the active oxygen species, under conditions of excess light, by limiting the amount of energy that reaches the photosystem RCs. This in turn protects the photosystems from damaging radicals. To achieve this, light energy absorbed by the chlorophyll molecules of the LHC under these excess conditions is channelled through the NPQ process. Thus the level of activity of the NPQ pathway, or the level of heat dissipation, fluctuates in response to varying levels of light intensity. It should be noted that the level of energy dissipation through NPQ is limited, and reaches a maximum. If this circumstance arises then the level of *q*P decreases and the organism is prone to photoinhibition (Section 1.10).

Under light stress an increase in the ΔpH across the thylakoid membrane is observed. This increase is thought to lead to the protonation of the CP29 and CP26 subunits of the LHC complex (Section 1.4.1). The change in ΔpH strongly correlates with the conversion of the pigment molecule violaxanthin to zeaxanthin via the xanthophyll cycle. The process of NPQ is thought to be mediated by this reaction (Demmig-Adams and Adams, 1992). The conversion from violaxanthin to zeaxanthin lowers the energy level of the carotenoid. In this lower energy state it is thought to be able to act as a trap for the energy of the excited chlorophyll. It has also been proposed that the formation of zeaxanthin induces a structural change in the antenna system, which in turn promotes the conversion of the excitation energy to heat.

1.10 PHOTOINHIBITION

When photosynthetic organisms are exposed to high light for extended periods of time (hours or days) the photosynthetic flow of electrons is impaired. This leads to a situation termed photoinhibition or photoinactivation, and results in a decrease in PSII activity and oxygen evolution rates (reviewed by Niyogi, 1999). At high light intensities charge separation rates and the resulting flow of electrons exceeds the capacity of the plastoquinone pool to accept electrons from PSII. Under these conditions there is also a rapid flux of electrons from $Pheo^+$ to Q_A. When this occurs it is possible for Q_A to receive an extra electron, forming Q_AH_2. The presence of Q_AH_2 results in a temporary block on

electron transfer from the PSII RC to Q_A and the generation of $^3Chl^*$. Under aerobic conditions $^3Chl^*$ reacts with O_2 to form singlet oxygen. Under photoinhibitory conditions the production of this highly active oxygen species can bleach P680 and triggers the degradation of the D1 subunit.

This degradation of the D1 subunit has been shown both *in vivo* and *in vitro* (Schroda *et al.*, 1999 and references therein). Associated with the degradation of D1 under these conditions is the dissociation of the WOC and manganese cluster from the complex (Eisenberg-Domovich *et al.*, 1995). The repair of the PSII complex can take minutes or even hours. Repair of these damaged complexes takes place in the stromal-exposed thylakoid membranes. It is widely accepted that the mechanism for the repair of PSII is functional even in dim and normal light conditions. The turnover of the D1 protein is rapid under non-stress conditions. Thus the repair mechanism is promoted under light stress conditions. Photoinhibition could be viewed as a situation when the rate of damage exceeds the rate of repair.

1.11 MODEL ORGANISMS

1.11.1 *Synechocystis* sp. PCC 6803

The cyanobacteria are a group of ancient oxygenic photosynthetic prokaryotes, present in both marine and fresh water environments. There is a good base of evidence to suggest that chloroplasts evolved from a cyanobacterium-like ancestor. Isolated from freshwater, one of the model cyanobacteria commonly used is *Synechocystis* 6803. Figure 1.7 shows an electron micrograph of a cyanobacterial cell, together with a picture of *Synechocystis* 6803 cells in culture.

In 1996 the complete genome sequence for *Synechocystis* 6803 was published (Kaneko *et al.*, 1996). This is a major advantage as it permits the targeted deletion, disruption or mutagenesis of previously uncharacterised genes. This sequence information is freely available via the Cyanobase Database (http://www.kazusa.or.jp/cyano/cyano.html).

Synechocystis 6803 is particularly suitable for the study of photosynthesis, as it is capable of photoautotrophic and heterotrophic growth. The ability of this organism to grow in the presence of a suitable carbon source, typically glucose, enables the isolation

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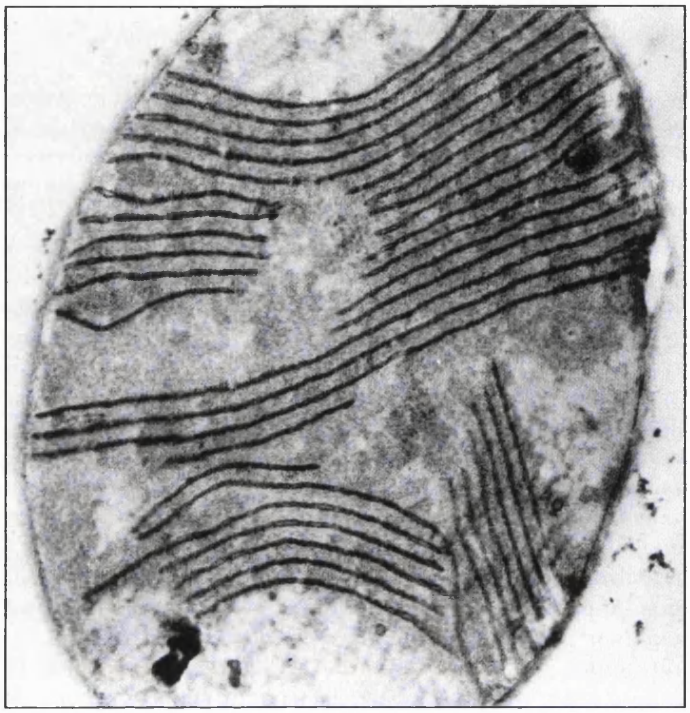

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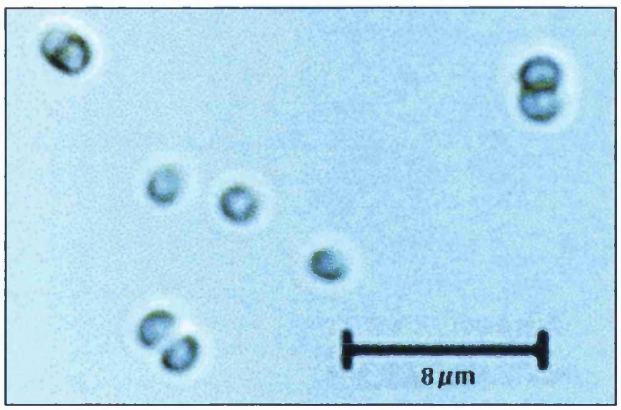


Figure 1.7 ***Synechocystis*** **sp. PCC 6803**
A. Electron micrograph of a cyanobacterium.
Adapted from Keeton and Gould (1993).
B. *Synechocystis* 6803 cells. A single cell is ~1µm in diameter.
Adapted from Cyanobase (http://www.kazusa.or.jp/cyano/about.html).

and characterisation of potentially lethal photosynthetic mutations. It should be noted that *Synechocystis* 6803 is not capable of heterotrophic growth in the dark. *Synechocystis* 6803 cells are also naturally transformable (reviewed by Haselkorn, 1991). How the DNA is taken up by the cells is not fully understood, but once inside the cell it undergoes homologous recombination. In this way the mutated DNA replaces a region of the WT 6803 DNA.

1.11.2 *Chlamydomonas reinhardtii*

C. reinhardtii has been used for about 40 years as a model organism for the study of photosynthesis (reviewed recently by Grossman, 2000; Dent *et al.*, 2001). It is a unicellular green alga that varies from 8μm to about 22μm in length. A diagrammatic cross section of the cell is shown in Figure 1.8. As well as photosynthesis, a number of other areas of research have benefited from the study of *C. reinhardtii*. These include cell wall synthesis, flagellar function, metabolism and phototaxis. The number and spectrum of subject areas presented in the literature represent the suitability of this organism for genetic research.

C. reinhardtii contain two flagella at the anterior pole of the cell. The cells contain an eyespot that senses incoming light, and permits phototaxis to regions of optimal light conditions. The eyespot also registers sudden increases in light intensity, enabling the cells to move away to areas of more suitable light intensity. Contained within the cell is a single large cup shaped chloroplast that contains about 80 copies of the chloroplast genome.

There are a number of characteristics of *C. reinhardtii* which make it an ideal organism for the study of photosynthesis. Firstly, the photosynthetic apparatus of *C. reinhardtii* is highly analogous to that found in vascular plants. Consequently discoveries made through research on this organism can be applied to higher plants. Secondly, it is a eukaryote, and as such the genes which encode the subunits of the photosynthetic apparatus are coded for by both the chloroplast and nuclear genomes, as they are in higher plants. As a unicellular organism it comes without the complications associated with maintaining multicellular stocks and WT CC-1021 cultures have a doubling time of less than ten hours.

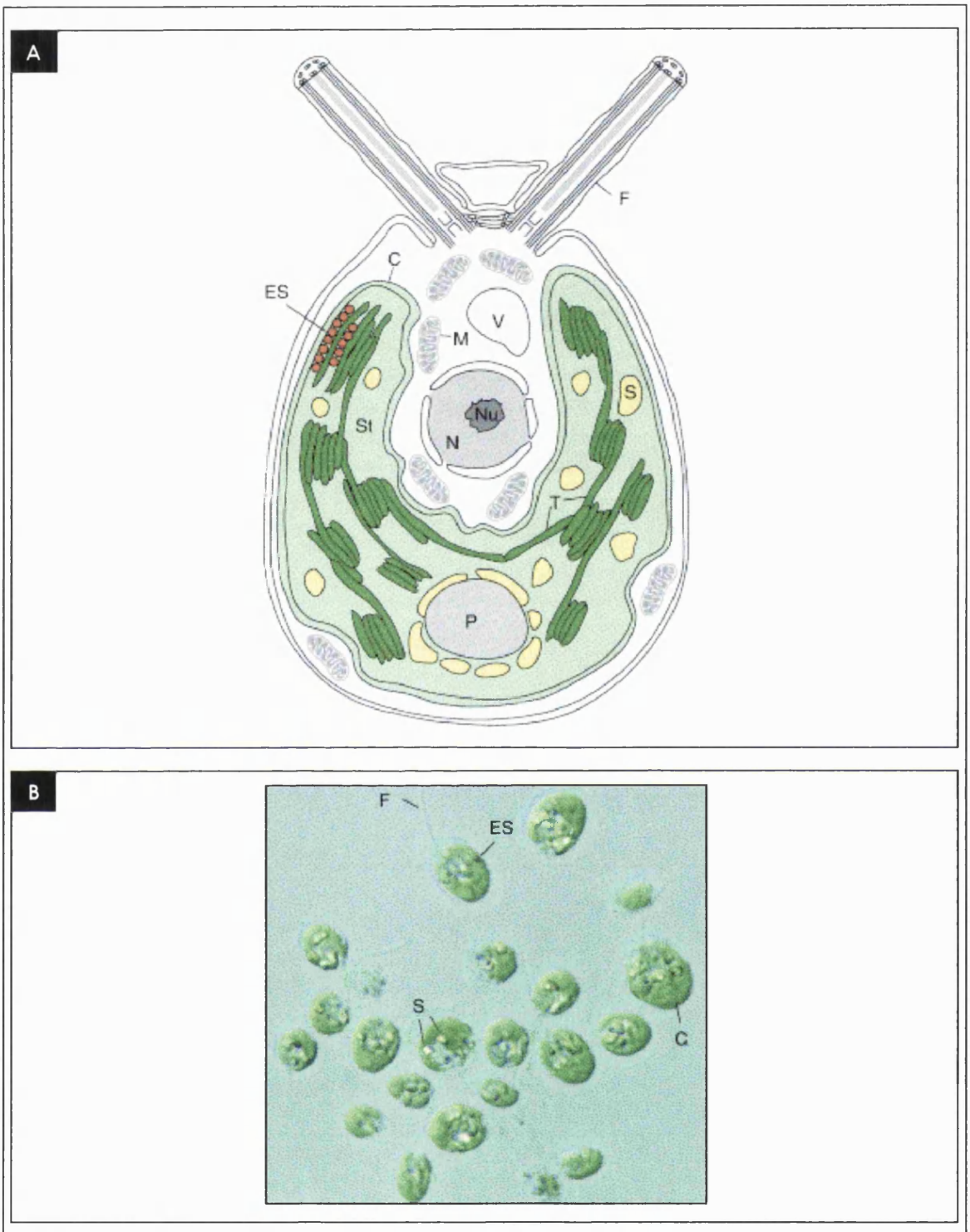


Figure 1.8 ***Chlamydomonas reinhardtii***
A. A cross section of a *Chlamydomonas* cell. Detailed in the figure are the following: the nucleus (N) with nucleolus (Nu), the single cup-shaped chloroplast (C) containing thylakoid membranes (T), starch grains (S) and pyrenoid (P), positioned within the stroma (St); located against the inner envelope membrane of this chloroplast is the eyespot (ES); two bipolar flagella (F) project from the apical region of the cell, and vacuoles (V) are visible in the cytoplasm.
B. *Chlamydomonas* cells (x 500). A single *Chlamydomonas* cell can vary from 8 to ~22μm in length. The same abbreviations apply as in Figure 1.8A.
Adapted from Dent *et al.* (2001).

Under conditions of nitrogen starvation, *C. reinhardtii* enters a cycle of sexual reproduction. *Chlamydomonas* cells can exist as haploid cells, and are differentiated by their mating type (mt), denoted mt (+) or mt (-). Mixing two cultures of opposite mating types results in the formation of zygotes. Following meiosis these zygotes produce four haploid progeny, two of mt (+) and two that are mt (-). These progeny are referred to as a tetrad. This feature of the *C. reinhardtii* life cycle has been used to establish in which genome a given mutation is located and in the study of gene linkage.

When provided with an alternative carbon source, typically acetate, *C. reinhardtii* cells can dispense with photosynthetic growth. This ability to grow heterotrophically makes the isolation and maintenance of potentially lethal photosynthetic mutants a reality. Furthermore, *Chlamydomonas* can synthesise chlorophyll and assemble the apparatus of photosynthesis in the absence of light. This characteristic makes it possible to analyse dark-grown photosynthetic mutants biochemically and biophysically.

Techniques have been developed to facilitate the transformation of the three genomes of *C. reinhardtii.* This is a unique feature, and has been exploited extensively in the study of photosynthesis. Because *C. reinhardtii* contains a single chloroplast, the isolation of homoplasmic transformants is considerably easier than it is in plant models. The most widely used selectable markers are the *aadA* cassette (Goldschmidt-Clermont, 1991) and the *aphA-6* marker (Bateman and Purton, 2000). As a result, site directed mutagenesis of chloroplast-encoded photosynthetic genes has been widely undertaken, providing information on the relationship between structure and function of the proteins of photosynthesis.

1.11.3 *Physcomitrella patens*

As early as the 1920s the moss *Physcomitrella patens* was being routinely used as a model organism. Since this early research the study of *P. patens* has led to information on the formation of tetrads, aspects of mutagenesis and most prominently plant development.

P. patens is an anatomically simple species with few cell types and is composed of a single sheet of cells. Combined with an ability to regenerate from any cell type and to grow either on solid media or in liquid culture, these characteristics make it an easy organism with which to work. It has been proposed that moss may represent a genetic link between the algae and higher plants such as *Arabidopsis.* Indeed a very young moss plant

strongly resembles a green algal filament. Examination of the moss EST database shows a high degree of sequence similarity between *P. patens* and vascular plants (Reski, 1998).

The moss life cycle has two predominant stages, and is detailed in Figure 1.9. The first is a dominant 'leafy' haploid gametophyte stage followed by a diploid sporophyte stage. Gametes are produced by mitosis within the gametophyte. The zygote, produced by the fertilisation of an egg, divides mitotically to produce a diploid sporophyte. The sporophyte is a simple structure consisting of a foot that is embedded in the gametophyte, a stalk and a capsule - the sporangium. Whilst it contains chloroplasts and performs photosynthesis, the sporophyte also obtains nutrients from the gametophyte. Meiosis occurs within the sporangium, producing haploid spores. When conditions are favourable the spores germinate to produce a gametophyte plant, thus completing the life cycle.

The field in which *P. patens* is clearly an invaluable model is the area of nuclear transformation. Whilst techniques are available for the transformation of the chloroplast genome of most photosynthetic model organisms, the transformation of the nuclear genome is more complicated. The degree of homologous recombination within the nuclear genomes of *C. reinhardtii* and *A. thaliana* is so low that introduced DNA appears to integrate randomly. The reverse genetics of these genomes required the generation and screening a large numbers of mutants – a laborious pursuit. Homologous recombination rates amongst the transformants of up to 90% have been reported for moss (Schaefer and Zrÿd, 1997; reviewed by Schaefer, 2001) making it an excellent genetic tool for the study of nuclear-encoded genes and their products.

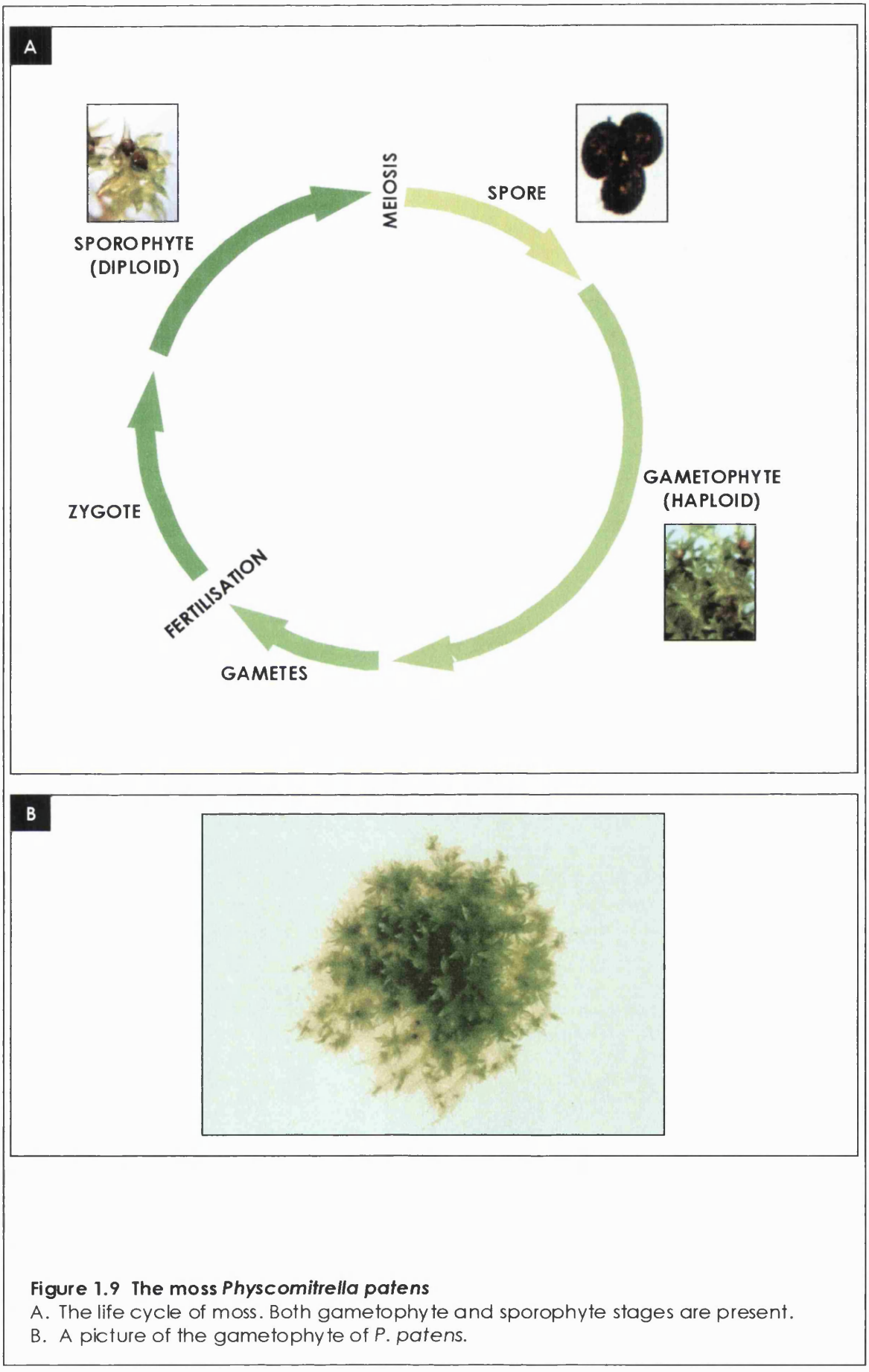


Figure 1.9 The moss *Physcomitrella patens*
A. The life cycle of moss. Both gametophyte and sporophyte stages are present.
B. A picture of the gametophyte of *P. patens*.

1.12 AIMS OF THIS RESEARCH

This thesis describes the investigation of three of the small polypeptides of the PSII complex, the nuclear-encoded subunit PSII-W and the proteins PSII-M and PSII-Z. The foundations for this work were established by previous studies performed in our laboratory, as part of ongoing research into the polypeptides of PSII (Cain, 1998).

The PSII-W protein (Section 1.5.4) is the only nuclear encoded subunit located in the core of the complex. Furthermore the protein is only found in the PSII complex of higher plants and green algae. At the start of this project there was no firm evidence to enable a function to be suggested for this protein. This made PSII-W protein an interesting target for investigation. Previous work had isolated a number of *C. reinhardtii* genomic clones for the *psbW* gene (Cain, 1998). From this starting point the following aims were set out:

- to clone and sequence the nuclear-encoded *psbW* gene of *C. reinhardtii* and examine the derived amino acid sequence. In addition, to undertake the cloning and sequencing of the same gene in *P. patens*; and

- to raise antibodies to the mature PSII-W protein of *C. reinhardtii* and use these to study the expression patterns of the PSII-W protein *in vivo*. To establish whether the PSII-W protein is also located in the PSI complex.

The results of this work are presented in Chapters 3 and 4.

Sequence data is available for the *psbM* gene of a number of organisms. However, almost nothing is known about the role of the PSII-M subunit. To date there are no reports of a deletion mutant in either cyanobacteria or photosynthetic eukaryotes. This makes the PSII-M subunit a good candidate for investigation. Previous work in our laboratory had set out to investigate the PSII-M subunit of *C. reinhardtii* together with what was then the hypothetical chloroplast protein Ycf9, now termed PSII-Z. In *C. reinhardtii* the *psbM* gene is expressed both monocistronically and as a dicistronic transcript with the upstream *psbZ* gene (Cain, 1998; Higgs *et al.*, 1998). The observed sizes of these transcripts differ between laboratories (Cain, 1998; Higgs *et al.*, 1998). Previous work in our laboratory successfully isolated a *psbZ* deletion mutant of *C. reinhardtii* (Cain, 1998). Because of

the dicistronic expression of *psbZ* with *psbM* it is possible that the disruption of one of these genes could have an effect on the expression of the other. Consequently the interpretation of the phenotype of the *psbZ* mutant is complicated by the arrangement of these two genes, and relating the phenotype directly to *psbZ* is difficult.

It was decided to continue the investigation of the PSII-M and PSII-Z subunits in tandem in a different model organism. In the cyanobacterium *Synechocystis* 6803 the *psbZ* and *psbM* genes are not located in the same region of the genome. Thus the deletion of one gene is very unlikely to have a direct effect on the other. For this reason *Synechocystis* 6803 was selected for the investigation of the *psbZ* and *psbM* genes. The following aims were set out:

- to study the role of the PSII-M subunit by constructing and characterising a *psbM* deletion mutant in *Synechocystis* 6803; and

- to investigate the function of the PSII-Z subunit by creating and analysing a *psbZ* disruption mutant also in *Synechocystis* 6803.

The results of the investigations into the PSII-M subunit are presented in Chapter 5. Chapter 6 presents the results of the studies of the PSII-Z protein. At the end of each of these chapters (i.e. Chapters 3-6), immediate further work arising from the results obtained is presented. Chapter 7 describes the long term avenues of investigation arising from the work presented in this thesis.

CHAPTER 2. MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 CHEMICALS

All chemicals used were of the highest analytical grade available. Unless otherwise stated they were purchased from Sigma Chemical Co. (Dorset), and all solutions were made using double distilled water (ddH_2O) from a Maxima water filtration unit (Elga, High Wycombe). Where appropriate, solutions were autoclaved (121°C, 15 lb/inch^2, 15 min) and aseptic technique employed. Restriction enzymes were purchased from New England Biolabs (Hitchin).

2.2 STRAINS EMPLOYED AND THEIR MAINTENANCE

2.2.1 *Escherichia coli* bacterial strains

The *E. coli* strains used, together with their genotypes, are listed in Table 2.1.

2.2.2 Growth and storage of bacterial strains

E. coli were cultured using Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). Short-term storage was achieved by overnight growth in liquid medium, or on solid medium (LB medium supplemented with 2% (w/v) agar). When required antibiotics were added to a final concentration of 100μg/mL, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) to a final concentration of 50μg/mL, and IPTG (isopropylthio-β-D-galactoside) to a final concentration of 10μg/mL. All medium supplements were added after autoclaving and cooling to <50°C.

Cultures were grown at 37°C, with liquid cultures grown with aeration at 150 rpm, then stored at 4°C. For long-term storage, *E. coli* strains were maintained as glycerol stocks. A volume of 1mL of an overnight culture was supplemented with 0.5mL LB medium (60% (v/v) glycerol), snap frozen in liquid nitrogen and stored at –70°C or below.

Table 2.1 ***E. coli*** **strains**

Genotype	Growth medium	Source and Reference
DH5α		
F'/*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*laclZYA-argF*)*U169 deoR* (ϕ80*dlac*Δ(*lacZ*)*M15*)	LB	New England Biolabs (Hitchin); Sambrook *et al.*, (1989).
DH10B		
F- *mcrA del* (*mrr-hsdRMS-mcrBV*) ϕ80 *lacZdelM15 del lacX74 deoR recA1endA1 araD139 del*(*ara,leu*)7697 *galU galK* λ- *rpsL nupG*	LB	Invitrogen Life Technologies, Holland.
JM109		
F' *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^{+}$*B*$^{+}$/e14^{-} (McrA^{-}) Δ(*lac-proAB*) *thi gyrA96*(Nal^{r}) *endA1 hsdR17 rtelA1 supE44 recA1*	LB	New England Biolabs (Hitchin); Sambrook *et al.*, (1989).
XL1-Blue MRA (P2)		
Δ(*mA*)*183* Δ(*mCB-HSRM-m*)*173d A1p E44h-1g A96e A1 lac*	LB + 10mM$MgSO_4$ + 0.2% (w/v) maltose	Stratagene (California, USA).

2.2.3 *Synechocystis* 6803 strains and mutants created

Table 2.2 details the *Synechocystis* strains together with their genotypes used in this work.

Table 2.2 *Synechocystis* strains

Genotype	Growth medium	Source
***Synechocystis* sp. PCC 6803 WT**		
Wild type.	BG-11	Dr. Conrad Mullineaux, UCL.
***Synechocystis* Δycf9 (Kn2)**		
Disruption of the *Synechocystis* 6803 *ycf9* orf with the kanamycin resistance cassette[(a)] in the opposite orientation to the *ycf9* orf via a *Bsm*F1 site.	BG-11 Kan^{100}	This thesis (Chapter 6).
***Synechocystis* Δycf9 (Kn5)**		
Null mutant of *ycf9* orf by replacement of a *Bsm*F1 fragment with the kanamycin resistance cassette[(a)] in the same orientation.	BG-11 Kan^{100}	This thesis (Chapter 6).
***Synechocystis* ΔpsbM**		
deletion of *psbM* orf and replacement with the kanamycin resistance cassette[(a)].	BG-11 Kan^{100}	This thesis (Chapter 5).

NOTE

a. The kanamycin resistance cassette was isolated from the pUC4K plasmid vector (Amersham Pharmacia Biotech, Amersham).

b. Kan^{100}: kanamycin was supplemented to a final concentration of 100μg/mL.

2.2.4 Growth and storage of *Synechocystis* strains

Synechocystis strains were grown photoautotrophically in BG-11 medium (Castenholz, 1988). Heterotrophic growth was achieved by the supplementation of the BG-11 medium with glucose (5mM). Cells were grown in liquid culture at 30°C with aeration at 150 rpm and with 10μE/m^2/s of PAR (Orbital Incubator, Gallenkamp, Loughborough).

Solid medium was prepared by mixing equal volumes of 2 x BG-11 medium with 3% (w/v) agar after autoclaving. Cultures on agar plates were re-streaked every three to four weeks, and were grown at 30°C with a light intensity of 7μE/m^2/s. When appropriate the medium was supplemented with kanamycin to 100μg/mL. For long term storage, cells were maintained in glycerol stocks. Initially 40mL of stationary cell culture was pelleted and re-suspended in 2mL BG-11 medium. Sterile glycerol was added to 20% (v/v), 200μL aliquots were snap frozen in liquid nitrogen, and stored at –70°C or in liquid nitrogen.

2.2.5 Quantification of *Synechocystis* cultures

The OD of a liquid culture at 750nm was measured using a UV-Visible Spectrometer, (Unicam, Cambridge). The concentration (cells/mL) was calculated by multiplying this figure by 1.15 x 10^8.

2.2.6 Establishing the doubling time of *Synechocystis* strains

Synechocystis strains were grown in BG-11 medium under very low light conditions (3μE/m^2/s), low light conditions (10μE/m^2/s) and moderate light condition (50μE/m^2/s) to a cell density of approximately 1.5 x 10^7 cells. The optical density (OD_{750}) of the cell cultures was then measured at intervals. Measurements were taken every 6 h for cells grown at 3μE/m^2/s, every 4 h for cells grown at 10μE/m^2/s and every hour for cells grown at 50μE/m^2/s.

The doubling time for each culture was then calculated using non-linear regression analysis of the exponential growth phase of the culture, as determined from the OD_{750} measurements. The standard error was also calculated, and represents the accuracy of the

determined doubling time for a given culture. The package Sigma Plot for Windows, Version 5.0 was used to perform these calculations.

2.2.7 C. *reinhardtii* strains

The strains employed throughout this research are listed in Table 2.3.

2.2.8 Growth and storage of C. *reinhardtii* strains

C. reinhardtii strains were grown heterotrophically on or in Tris Acetate Phosphate (TAP) medium, whilst photoautotrophic growth was achieved using High Salt Minimal (HSM) medium with 50μE/m^2/s illumination. Liquid cultures were grown at 25°C. Solid medium was prepared by the addition of 2% (w/v) bactoagar to medium prior to autoclaving. Strains were maintained on plates at 18°C under 8μE/m^2/s, and re-streaked every three to four weeks. Table 2.4 details the quantities of each of the stock solutions required for each of the two media.

2.2.9 Quantification of C. *reinhardtii* cultures

Culture cell density was determined by the use of a haemocytometer. The cell culture was diluted as appropriate, then a 1mL aliquot was killed by the addition of 10μL iodine solution (25mg/mL in EtOH). 10μL of this was then placed on each side of the haemocytometer (Weber Scientific International Ltd., Teddington). An average cell count was obtained from the number of cells on each of the two grids. This number was multiplied by 1 x 10^4 and any dilution factor to give the number of cells/mL.

Table 2.3 ***C. reinhardtii*** **strains**

Genotype	Growth medium	Source
WT CC-1021		
Wild type.	TAP, HSM	Dr. Elizabeth Harris, Duke University.
CC-5		
ac35 mt (-).	TAP	Dr. Elizabeth Harris, Duke University.
CC-520		
ac7a mt (-).	TAP	Dr. Elizabeth Harris, Duke University.
CC-673		
ac7 mt (-).	TAP	Dr. Elizabeth Harris, Duke University.
CC-1727		
ac7 eryl b mt (+).	TAP	Dr. Elizabeth Harris, Duke University.
C575D+ (PSI^-)		
psaA-3: C575D mt (+). Derived from CC-1021.	TAP, TAP Sp^{100}	Hallahan *et al.* (1995).
***psbH* minus ($PSII^-$)**		
psbH : : *aadA* mt (+). Derived from CC-1021.	TAP, TAP Sp^{100}	O'Connor *et al.* (1998).

NOTE

Sp^{100}: spectinomycin was supplemented to a final concentration of 100μg/mL.

Table 2.4 Recipes of media for *C. reinhardtii*

To make 1 litre	Tris Acetate Phosphate (TAP) Medium	High Salt Minimal (HSM) Medium
Tris	2.42g	-
4 x Beijerinck Salts [(a)]	25mL	25mL
1M (K)PO_4, pH 7.0 [(b)]	1mL	-
2 x PO_4 for HSM [(c)]	-	50mL
Trace elements [(d)]	1mL	1mL
Glacial Acetic Acid	1mL	-
ddH_2O	To 1 litre	To 1 litre

NOTE

a. 4 x Beijerinck Salts

16g NH_4Cl

2g $CaCl_2$

4g $MgSO_4$

dissolve in 1L ddH_2O

b. 1M (K)PO_4

250mL 1M K_2HPO_4

170mL KH_2PO_4

titrate to pH 7.0

c. 2 x PO_4 for HSM

14.34g K_2HPO_4

7.26g KH_2PO_4

Table 2.4 Recipes of media for *C. reinhardtii* (continued)

d. Trace elements

1. Dissolve in 550mL ddH_2O in the order listed overleaf, then heat to 100°C.

    11.4g H_3BO_4

    22.0g $ZnSO_4.7H_2O$

    5.06g $MnCl_2.4H_2O$

    4.99g $FeSO_4.7H_2O$

    1.61g $CoCl_2.6H_2O$

    1.57g $CuSO_4.4H_2O$

    1.1g $(NH_4)_6Mo_7O_{24}.4H_2O$

2. Dissolve 50g EDTA.Na_2 in 250mL ddH_2O by heating and add to the solution above. Reheat to 100°C and then cool to 80-90°C. Adjust the pH to 6.5-6.8 with 20% KOH.

3. Adjust the volume to 1L. Incubate at room temperature for 2 weeks. A rust coloured precipitate will form, and the solution will change from green to purple.

4. Filter through three layers of Whatman No. 1 (Whatman, Maidstone) under suction until the solution is clear.

2.2.10 Growth and storage of *P. patens* strains

P. patens was grown in liquid culture at 25°C with illumination at 50μE/m^2/s for preparation of genomic DNA and for long term storage. Stocks were maintained at room temperature (RT) on liquid medium supplemented with 1% (w/v) agar. Table 2.5 details the quantities of each of the stock solutions required for the liquid medium. The *P. patens* used in this work was obtained from Moss Technology, Leeds Innovations (Leeds).

Table 2.5 Recipe of medium for *P. patens* – Minimal medium

Solution	Volume
Solution A [a]	10mL
Solution B [b]	10mL
Solution C [c]	10mL
Solution D [d]	10mL
Trace elements [f]	1mL
ddH_2O	to 998mL

Autoclave, then supplement with 2mL/L of Solution E [e].

NOTE

a. Solution A

25g $MgSO_4.7H_2O$

ddH_2O to 1L

b. Solution B

25g KH_2PO_4 pH 6.5 with 4M KOH

ddH_2O to 1L

Table 2.5 Recipe of medium for *P. patens* – Minimal medium (continued)

c. Solution C

101g KNO_3

1.25g $FeSO_4.7H_2O$

ddH_2O to 1L

d. Solution D

96g NH_4 tartate

ddH_2O to 1L

e. Solution E

73.5g $CaCl_2$

ddH_2O to 1L. Autoclave.

f. Trace elements

614mg H_3BO_3

55mg $CuSO_4.5H_2O$

28mg KBr

389mg $MnCl_2.4H_2O$

55mg $CoCl_2.6H_2O$

28mg LiCl

55mg $Al_2(SO_4).H_2SO_4.24H_2O$

55mg $ZnSO_4.7H_2O$

28mg $SnCl_2.2H_2O$

28mg KI

ddH_2O to 1L

2.3 DNA TECHNIQUES

2.3.1 Plasmid DNA vectors utilised

Appendix 1 details the plasmids utilised and created in this work.

2.3.2 Isolation of plasmid DNA

Small scale plasmid DNA preparations were performed using 1.5mL of an overnight cell culture and employed a modified version of the plasmid mini preparation method from Sambrook *et al.* (1989). If sequencing of the plasmid DNA was to be undertaken then the DNA was prepared using the Qiagen (Crawley) Plasmid Mini Kit. Large quantities of plasmid DNA were obtained using the Qiagen Maxi Prep Kit, and produced yields of about 0.2μg of DNA from 100mL of cell culture. When a Qiagen kit was employed the manufactures' instructions were followed, and columns were incubated at RT for 5 mins with ddH_2O/Ribonuclease A (RNaseA) (10μg/mL) before the final elution spin. In all methods the DNA was re-dissolved in ddH_2O/RNaseA (10μg/mL).

2.3.3 Isolation of lambda DNA

Day 1. *E. coli* XL1-Blue MRA (P2) cells infected with a lambda clone were plated out as described in the Stratagene instruction manual, and incubated overnight at 37°C to permit plaque formation to occur.

Day 2. The lambda plaques were solubilised in 6mL of SM medium (100mM NaCl, 10mM $MgSO_4.7H_2O$, 5mL 1M Tris•Cl pH 7.5, 0.5mL 2% (w/v) gelatin, ddH_2O to 100mL) which was gently pipetted onto the plate. The plate was then incubated overnight at 4°C with gentle agitation to allow the lambda particles to diffuse in the SM medium.

Day 3. The lambda containing SM medium was transferred to a 15mL Falcon tube (Cell Star, Germany). Chloroform (250□L) was added, and the tube incubated at RT for 15 mins to kill the *E. coli* cells present in the medium. RNaseA was then added to a final concentration of 1μg/mL to facilitate the digestion of *E. coli* RNA present. Following the addition of NaCl to 1M, the tubes were incubated on ice for 1 h to permit dissociation on the lambda particles from the *E. coli* cell debris. Centrifugation at 2,110*g*

(Mistral 1,000, MSE Scientific Instruments, Loughborough) for 15 mins pelleted the *E. coli* cell debris and any agar present. The supernatant was then transferred to sterile SS34 tubes. PEG-8,000 (BDH, Germany) was added to a final concentration of 10% (w/v) and incubated on ice for 1 h to allow precipitation of the lambda particles. The lambda particles were pelleted by centrifugation at 14,650*g* (Sorvall, Du Pont Instruments, USA) for 10 mins and the supernatant discarded. The pellet was then re-suspended in 2mL of SM medium and transferred to a new Falcon tube (Cell Star, Germany). Chloroform (2mL) was then added and the tube vortexed for 30 s. Centrifugation at 1,352*g* (Mistral 3,000i, MSE Scientific Instruments, Loughborough) for 15 mins removed the PEG-8,000 from the solution and allowed separation of the lambda particles to the upper aqueous phase. This top phase was transferred to a glass Corex tubes suitable for use in the Beckman ultra-centrifuge. The lambda particles were pelleted by centrifugation in a bench top ultra-centrifuge at 206,020*g* (TL-100 Ultra Centrifuge, Beckman, USA) for 2 h. The supernatant was removed to reveal a glassy pellet that was dissolved in 50µL of SM medium by incubation overnight at 4°C on a rocking platform.

2.3.4 Preparation of genomic DNA from *Synechocystis* strains

Genomic DNA was isolated from 12mL of a stationary *Synechocystis* cell culture with an OD at 750nm of ≥1.5. The cells were harvested by centrifugation (Mistral 1,000, MSE Scientific Instruments, Loughborough) in a bench top centrifuge at 2,110*g* for 15 mins. The supernatant was discarded and the pellet re-suspended in 400µL TES Buffer (5mM Tris•Cl pH 8.5, 50mM NaCl and 5mM EDTA) then transferred to a 1.5mL Eppendorf tube. The cells were lysed by the addition of lysozyme to 10mg/mL and incubation at 37°C for 45 mins. Following the addition of 50µL sarkosyl and 600µL saturated phenol pH 8.0 (BDH, Germany) to the tube, the tube was vortexed once a min for 10 mins. Then 100µL of 5M NaCl, 100µL CTAB-NaCl (700mM NaCl, 10% (w/v) CTAB) and 600µL chloroform:isoamylalcohol (24:1) were added. The tube was then vortexed once a minute for 10 mins. Centrifugation in a bench top micro-centrifuge (Heraeus, Brentwood) at 16,060*g* for 2 mins produced a nucleic acid containing top phase. This top phase was transferred to a fresh 1.5mL Eppendorf tube. A second phenol-chloroform extraction was performed using equal volumes of saturated phenol pH 8.0 and chloroform:isoamylalcohol (24:1). Precipitation of the genomic DNA was achieved by

the addition of 1mL cold EtOH, incubation at –20°C for at least 20 mins and micro-centrifugation in a bench-top centrifuge (Heraeus, Brentwood) at 16,060*g* for 10 mins. The pellet was washed with 70% (v/v) EtOH for 5 mins at RT then dried at 37°C for 5 mins. The DNA was dissolved in 50µL of ddH_2O/RNaseA (10µg/mL).

2.3.5 Preparation of genomic DNA from *C. reinhardtii* strains

Genomic DNA was isolated from 10mL of cell culture at 3 x 10^6 cells/mL. The cells were pelleted by centrifugation at 2,110*g* for 5 mins in a bench top centrifuge (Mistral 1,000, MSE Scientific Instruments, Loughborough). The pellet was re-suspended in 1mL TEN Buffer (50mM EDTA, 20mM Tris•Cl pH 8.0, 0.1M NaCl), and transferred to a 1.5mL Eppendorf tube. The cells were then re-pelleted by micro-centrifugation (Heraeus, Brentwood) at 16,060*g* for 2 mins, the supernatant discarded and the cells re-suspended in 0.35mL TEN Buffer. Then 5µL of Proteinase K (20mg/mL) and 50µL of 10% (w/v) SDS were added, and cell lysis achieved by incubation at 55°C for 2 h. Following the addition of 2µL diethylpyrocarbonate (DEPC) the tube was incubated at 70°C for 15 mins. The tubes were cooled briefly on ice and then 50µL 5M potassium acetate was added and the tube incubated on ice for 30 mins. Once the cell debris had been pelleted by micro-centrifuge in a bench-top centrifuge (Heraeus, Brentwood) at 16,060*g* for 15 mins, the nucleic acid containing supernatant was transferred to a fresh tube. The samples were then subjected to phenol:chloroform extraction by the addition of 0.4mL phenol pH 8.0 (BDH, Germany), followed by a 10 s vortexing, the addition of 0.4mL chloroform:isoamylalcohol (24:1 v:v) and a final vortex for 10 s. This extraction was performed three times. Following the final extraction the top phase was transferred to a fresh 1.5mL Eppendorf tube and the DNA precipitated by the addition of an equal volume of isopropanol and incubation on ice for 5 mins. Micro-centrifugation (Heraeus, Brentwood) at 16,060*g* yielded a DNA pellet, which was washed with 70% (v/v) EtOH for 5 mins at RT and then dried at 37°C for 5 mins. The DNA was dissolved in 50µL ddH_2O/RNase (10µg/mL).

2.3.6 Preparation of genomic DNA from *P. patens*

Genomic DNA was isolated from about 0.5g 'dry weight' of *P. patens* per column using the Qiagen (Crawley) DNeasy Plant Maxi Kit. The moss was thoroughly ground in a mortar and pestle containing liquid nitrogen. Once ground, the manufacturers' instructions were followed and the DNA eluted in 2 x 750µL of ddH_2O/RNase (10µg/mL). The DNA was then concentrated by drying down in a 'Speedivac' vacuum centrifuge (Uni Equip, Germany) to a volume of 50µL.

2.3.7 Restriction analysis of DNA

To digest bacterial plasmid DNA, 1µg of DNA was cut with at least 1 unit of restriction enzyme in the recommended buffer (New England Biolabs, Hitchin) diluted to 1 x in ddH_2O, and with 1 x BSA (0.1mg/mL) if required. The total reaction volume of 10µL was incubated for 2 h at the appropriate temperature. Polymerase Chain Reaction (PCR) products were digested with the same components but were incubated overnight to ensure complete digestion. Digestion of genomic DNA was achieved with an excess of restriction enzyme in 1 x recommended buffer and, if required, 1 x BSA in a total volume of 50µL, overnight at the appropriate temperature.

2.3.8 Agarose gel electrophoresis

Samples for gel electrophoresis were supplemented with the appropriate volume of 6 x Agarose Gel Loading Buffer (0.1M EDTA.Na_2 pH 8.0, 40% (v/v) glycerol, 0.01% (w/v) SDS, 0.01% (w/v) bromophenol blue). The appropriate percentage (w/v) of agarose for the DNA to be separated was added to 1 x TAE Buffer (40mM Tris acetate, 10mM EDTA.Na_2 pH 8.0) (Sambrook *et al.*, 1989) and dissolved by heating in a microwave (Table 2.6). PCR products and restriction enzyme digest products were separated according to size in agarose gels in 1 x TAE Buffer with ethidium bromide at 40ng/mL. DNA ladders employed were the 1kb and 100bp plus ladders (MBI Fermentas, Sunderland). Agarose minigels were run in 1 x TAE Buffer in a minigel apparatus (Hoefer, Newcastle-under-Lyme) pre-chilled to 4°C, at either 100V constant voltage for 1 to 2 h, or overnight at a constant voltage of 12V. Genomic digest products were separated overnight, also in 1 x TAE Buffer, in the maxigel apparatus (Hybaid, Teddington) at 50V

constant voltage. DNA bands were visualised on a UV transilluminator (UVP, California). The gel documentation system (UVP, California) was employed to produce a record of each gel.

Table 2.6 The range of separation achievable in gels containing different percentages of agarose

Percentage of agarose (w/v)	Efficient range of separation of linear DNA molecules (kb)
0.3	5 – 60
0.6	1 – 20
0.7	0.8 – 10
0.9	0.5 – 0.7
1.2	0.4 – 0.6
1.5	0.2 – 0.3
2.0	0.1 – 0.2

2.3.9 Recovery of DNA from agarose gels

The region of the agarose gel that contained the DNA of interest was visualised using a UV Plate (UVP, California) and removed using a razor blade. The DNA was recovered from the agarose gel using the QIAquick Gel Purification Kit (Qiagen, Crawley) according to the manufacturers' instructions. The column was incubated with 50μL of ddH_2O for 5 mins prior to the final elution spin.

2.3.10 Construction of recombinant DNA plasmids

A linearised plasmid, previously digested with the appropriate restriction enzyme or enzymes, acted as the vector for the ligation reaction. The insert was either the product of a second restriction enzyme digestion, a PCR product or a PCR product which had also be subjected to restriction digestion. The vector and insert maintained compatible 'sticky' or 'blunt' ends. In both cases the vector and insert were separated from other fragments by agarose gel electrophoresis (Section 2.3.8) and then gel purified (Section 2.3.9). The resulting products were mixed at a molar ratio of vector:insert of either 1:3 in the case of

'sticky-ended' ligations or 1:1 or 1:2 for 'blunt-ended' ligations. The molar ratio was established by employing the equation below.

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

The ligation reaction contained the vector and insert linear DNA, 5 units of T4 DNA Ligase and 1 x Ligase Buffer in a total volume of 10μL. 'Sticky-ended' ligations were incubated at 37°C for 1 h, whilst 'blunt-ended' ligations were incubated overnight at 16°C.

2.3.11 Polymerase Chain Reaction (PCR)

PCR was employed to amplify DNA from bacterial plasmid and genomic DNA. Reactions were performed in a total volume of 50μL and contained 1 x Reaction Buffer (10mM KCl, 20mM Tris•Cl pH 8.8, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% (w/v) Triton X-100), 0.4mM of each dNTP (Amersham Pharmacia Biotech, Amersham), 1μM of each primer (MWG Biotech, Germany) and approximately 10ng of template DNA. Following a 'hot start', 1 unit of Vent DNA Polymerase was added. To determine the optimum concentration of $MgSO_4$ the reaction was also supplemented with between 1mM and 5mM of $MgSO_4$. The total reaction volume was made up with ddH_2O.

PCR was performed using a programmable thermocycler (Techne Progene, Cambridge). 'Hot start' PCR was achieved by the following steps: the DNA template was denatured by incubation at 95°C for 4 mins, at this point the Vent DNA Polymerase was added to the reaction. Following a further 1 min incubation at 95°C, the next three steps were performed 20 to 30 times. The DNA was denatured at 95°C for 1 min, then the primers were annealed at a suitable temperature, typically 5°C below the T_m of the primers for 1 min. Extension was performed at 72°C for 1 min per kb of DNA to be extended. Once the appropriate number of cycles had been performed, this was followed

by a final extension at 72°C for 5 mins. The PCR product(s) were analysed by submitting 5μL of the reaction to agarose gel electrophoresis (Section 2.3.8).

The annealing temperature (T_m) for a given primer was estimated by the following equation:

$T_m - 5°C$

where: $T_m = 69.3 + (0.41 \times GC\%) - (650/\text{primer length})$

2.3.12 DNA sequencing reactions

DNA sequencing was performed using an ABI Prism 377 Genetic Analyser Automated Sequencer (Perkin Elmer, California). For each sequencing reaction 100fmol of template DNA was used. The sample for sequencing was prepared by cycle sequencing using the BigDye labelling system (Perkin Elmer, California). The returned data was checked manually for base-calling errors and was analysed using the Lasergene computer package (DNA Star, USA).

2.3.13 Oligonucleotide primers

Appendix 2 details the primers used in this work, the experiments for which they were used, and their T_m. All primers were provided by MWG Biotech (Germany). Stock solutions were maintained at 100pmol/μL and working solutions used at 20pmol/μL.

2.4 RNA TECHNIQUES

2.4.1 Extraction of total cellular RNA from C. *reinhardtii* strains

Total RNA was extracted from 10mL of *C. reinhardtii* cells at 2 - 4 x 10^6 cells/mL. The cells were harvested by centrifugation at 2,110*g* for 5 mins in a bench top

centrifuge (Mistral 1,000, MSE Scientific Instruments, Loughborough). The supernatant was discarded and the cells re-suspended in 0.6mL TEN.SDS Buffer (0.2M Tris•Cl pH 8.0, 0.5M NaCl, 0.01M EDTA, 0.2% (w/v) SDS) and then transferred to 1.5mL Eppendorf tubes. The samples were freeze-thawed three times using liquid nitrogen and a water bath set to 37°C. The samples were then subjected to phenol:chloroform extraction by the addition of 0.4mL phenol pH 8.0 (BDH, Germany), followed by a 10 s vortexing, the addition of 0.4mL chloroform:isoamylalcohol (24:1 v:v) and a final vortex for 10 s. Centrifugation in a bench-top micro-centrifuge (Heraeus, Brentwood) at 16,060*g* for 2 mins yielded a nucleic acid containing top phase which was transferred to a fresh 1.5mL Eppendorf tube. Addition of 1.4mL cold absolute EtOH facilitated the precipitation of the nucleic acid, which was pelleted by micro-centrifugation (Heraeus, Brentwood) at 16,060*g* for 10 mins. The pellet was washed with 70% (v/v) EtOH, then dried at 37°C and re-suspended in 50µL DEPC treated ddH_2O. The yield and purity of the RNA obtained was estimated by running a tenth of total volume on an agarose gel (Section 2.3.8).

2.4.2 Extraction of mRNA from *C. reinhardtii* strains

The MicroPoly(A) Pure Kit (Ambion, Texas) was used for the extraction of high quality mRNA from *C. reinhardtii* cells. The manufacturers' instructions were followed for mRNA isolation from 5 x 10^6 cells. The mRNA was eluted from the oligodT columns in 2 x 100µL DEPC treated ddH_2O, and then precipitated by the addition of 555µL cold absolute EtOH, 20µL sodium acetate pH 5.2 and 2µL glycogen. Following an overnight incubation at –70°C to permit efficient precipitation the mRNA was recovered by centrifugation in a bench top micro-centrifuge (Heraeus, Brentwood) at 16,060*g* for 20 mins. The supernatant was discarded and the pellet re-suspended in 10µL DEPC treated ddH_2O. The mRNA samples were stored at –70°C.

2.4.3 3' Rapid Amplification of cDNA Ends (RACE)

The major steps in the production of a 3' RACE product are diagrammatically represented in Figure 2.1. The methodology employed was adapted from White (1997). Between 2 - 5µg of mRNA was added to 20pmol of the oligodT primer (Appendix 2). DEPC treated ddH_2O was used to make the volume to 11.5µL. The sample was incubated

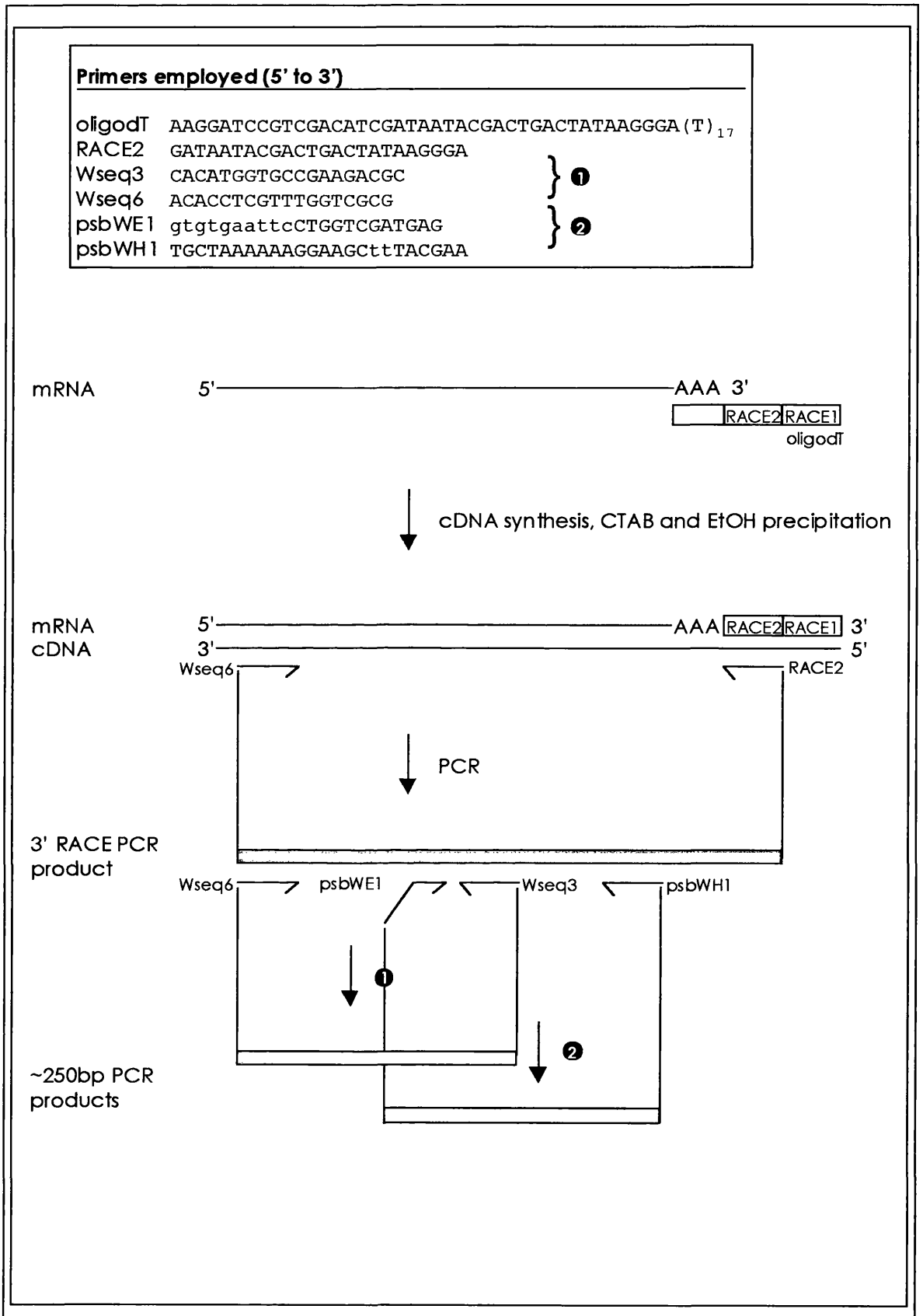


Figure 2.1 A diagrammatic summary of the 3' RACE method

at 70°C for 5 mins and then centrifuged on a bench top micro-centrifuge (Heraeus, Brentwood) at 16,060*g* for 30 s to collect any condensation. The following was then added to produce a total volume of 20µL:1 x Reverse Transcriptase Buffer, 10mM DTT, 1mM each dNTP (Amersham Pharmacia Biotech, Amersham) and 50 units of AMV-RTase. First strand cDNA synthesis was achieved by incubation at 42°C for 1 h. Then 2.5µL 3.5M NaCl and 2.5µL 10% (w/v) CTAB was added and the sample centrifuged in a bench top micro-centrifuge (Heraeus, Brentwood) for 30 mins at 16,060*g* to recover CTAB:nucleic acid complex. The supernatant was discarded and the pellet re-suspended in 100µL 1.2M NaCl by incubation at RT for 5 mins. Cold absolute EtOH (2.7 volumes) was then added and the solution incubated at RT for 10 mins. The nucleic acids were pelleted by centrifugation in a bench top micro-centrifuge (Heraeus, Brentwood) at 16,060*g* for 10 mins. The pellet was then washed with 1mL of 70% (v/v) EtOH by incubation at RT for 5 mins. Following a final centrifugation step in a bench top micro-centrifuge (Heraeus, Brentwood) at 16,060*g* for 5 mins, the supernatant was discarded, the cDNA containing pellet was dried at 37°C for precisely 5 mins and re-suspended in 15µL ddH_2O. Then 1µL of this product was subjected to PCR as described in Section 2.3.11 using the RACE1 primer (Appendix 2) and an appropriate 5' primer. If required nested PCR was performed with the RACE2 primer (Appendix 2) and a second internal 5' primer.

2.4.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using the Access RT-PCR System (Promega, Southampton) and employed 1µg of mRNA (isolated as described in Section 2.4.2). The mRNA together with the appropriate primers was subjected to RT-PCR. Synthesis of the second DNA strand and amplification of the DNA was performed over 40 cycles, and the magnesium concentration optimised by the addition of 1 - 3mM $MgSO_4$ in 0.5mM increments.

2.5 TRANSFORMATION TECHNIQUES

2.5.1 Bacterial transformation

Day 1. Competent cells were prepared employing a method adapted from Sambrook *et al.* (1989). An aliquot of 100μL of an overnight culture of *E. coli* (Table 2.1) was inoculated into 10mL LB medium and grown at 37°C for 3 h with aeration at 200 rpm. The cells were pelleted by centrifugation in a bench top centrifuge (Mistral 1,000, MSE Scientific Instruments, Loughborough) at 2,110*g* for 5 mins, the supernatant discarded and the cells re-suspended in 10mL 100mM $MgCl_2$ (autoclaved). The cells were incubated on ice for 5 mins, and then pelleted by centrifugation in a bench top centrifuge (Mistral 1,000, MSE Scientific Instruments, Loughborough) at 2,110*g* for 5 min. The supernatant was discarded and the cells re-suspended in 1mL 100mM $CaCl_2$ (autoclaved). The competent *E. coli* cells were stored at 4°C for at least 30 mins, typically overnight, and were used for bacterial transformation within 24 h.

Day 2. Transformation of competent *E. coli* cells was performed based on the observations of Mandel and Higa (1970). Between 5 to 20ng of plasmid DNA or a total ligation reaction (Section 2.3.10) was added to 100μL of competent *E. coli* cells, and the cells incubated on ice for 30 mins. The cells were heat shocked at 42°C for 90 s and then allowed to recover on ice for 2 mins. LB medium (0.8mL) was then added, and the cells incubated at 37°C for 1 h with aeration at 200 rpm. Following this incubation period 100μL of the cells were plated out onto the appropriate plates, which included LB and LB supplemented with the appropriate antibiotic or alternative supplements. The remaining cells were centrifuged in a bench top micro-centrifuge (Heraeus, Brentwood) for 16,060*g* for 2 mins. The pellet was re-suspended in 100μL of the supernatant and this was then plated onto the most selective of the plates employed. The plates were supplemented with 100μg/mL of the appropriate antibiotic, and if blue white selection was employed, X-gal was added to 50μg/mL and IPTG to 10μg/mL. The plates were inverted and incubated overnight at 37°C. The following day the plates were examined for putative transformant colonies.

2.5.2 Transformation of *Synechocystis* 6803

The methodology employed was adapted from Williams (1988) and Ermakova *et al.* (1993). Wild type (WT) *Synechocystis* 6803 was grown in liquid culture to an OD at 750nm of ~0.5. This culture was then diluted two fold and grown overnight. The following day 40mL of this overnight culture was harvested by centrifugation in a bench top centrifuge (Mistral 1,000, MSE Scientific Instruments, Loughborough) at 2,110*g* for 5 mins. The cells were then washed in fresh BG-11 medium (Section 2.2.4) and spun down a second time. The washed cell pellets were re-suspended in BG-11 medium to a final concentration of 4 x 10^8 cells/mL. In duplicate, 5μg of the DNA to be transformed was added to 100μL of the re-suspended cells. Following incubation at 30°C in the light for 4 h, the cells were spread onto a BG-11 plate and a BG-11 glucose plate. The plates were allowed to dry overnight. The following day the plates were overlain with 2mL of 0.6% (w/v) agar supplemented with kanamycin to 600μg/mL, giving a final kanamycin concentration for the plates of 40μg/mL. The plates were incubated for about 2 weeks in dim light (7μE/m^2/s) at 30°C and then examined for the presence of putative transformants.

2.5.3 Obtaining homoplasmic *Synechocystis* strains

Transformants typically appeared after 2 weeks. Colonies were picked with sterile cocktail sticks and transferred to fresh BG-11 Kan^{100} plates with and without glucose, as appropriate. Single colonies were taken through at least three rounds of selection. At this time sufficient selection pressure will have been applied by the antibiotic for all of the WT copies of the gene/orf to have been replaced. However, if the product of the gene of interest was essential the colonies will not have been driven to homoplasmicity. The transformants will be heteroplasmic maintaining both copies of the disrupted or modified gene in conjunction with WT copies of the gene of interest.

The homoplasmic status of the transformants was determined by Southern blot analysis (Section 2.7.1).

2.6 LAMBDA EXPERIMENTAL TECHNIQUES

2.6.1 Screening of a Lambda Fix II (Ppg) DNA replacement library

A *P. patens* genomic library in the Lambda Fix II (Ppg DNA) replacement vector and the host strain XL1-Blue MRA (P2) (Table 2.1) were obtained from Stratagene (California, USA). Once the library had been titered and amplified according to the manufactures' instructions, the resulting plaques were lifted. Following overnight incubation to permit plaque formation the plates were chilled at 4°C for 2 h. The plates were marked to permit orientation. Two DNA filters (Hybond, Amersham Pharmacia Biotech, Amersham) were sequentially placed on the plate and left for 2 min to permit the transfer of the plaques. The orientation of the filters relative to the plates was marked. The filters were incubated, plaque-side face up, in Denaturing Solution (1.5M NaCl and 0.5M NaOH) and Neutralising Solution (1.5M NaCl and 0.5M Tris•Cl pH 7.5) for 5 mins each. The filters were then washed for 2 mins in Washing Solution (2 x SSC [300mM NaCl, 30mM sodium citrate pH7.0] with 0.2M Tris•Cl pH 7.5) and air dried for up to 1 h at RT. Finally the filters were layered between 3MM Whatman Paper (Whatman, Maidstone) and dried for a further 1 h at 80°C. Southern analysis of each of the filters was then undertaken, as described in Section 2.7.1. The probe employed was the gel purified (Section 2.3.9) *Eco*RI-*Xho*I fragment produced by the digestion of the Moss EST AW155929 cloned into pSK. This EST was cloned into the plasmid vector as an *Eco*RI-*Xho*I fragment (maintained in *E. coli* DH10B (Table 2.1), supplied from Invitrogen Life Technologies, Holland). The screening process was repeated, with appropriate dilution of the Lambda Fix II Library until single positive plaques could be visualised.

2.6.2 Preparation of a Lambda Fix II positive clone stock solution

Day 1. A single putative positive plaque was picked using the top of a sterile 10µL tip (Gilson, Luton) and was transferred to 400µL SM medium (100mMg NaCl, 10mM $MgSO_4.7H_2O$, 5mL 1M Tris•Cl pH 7.5, 0.5mL 2% (w/v) gelatin, ddH_2O to 100mL). The lambda plaque plug was incubated overnight at 4°C to allow diffusion of the phage into the medium. An overnight culture of XL1-MRA (P2) in 10mL of LB medium was also set up and was incubated overnight at 37°C with aeration at 150 rpm.

Day 2. The medium into which the plaques had dissolved was centrifuged in a bench top micro-centrifuge (Heraeus, Brentwood) at 16,060*g* for 20 mins to pellet the agar and bacteria. The phage containing supernatant was transferred to a fresh 1.5mL Eppendorf tube (Mistral 1,000, MSE Scientific Instruments, Loughborough), and two drops of chloroform were added to kill any remaining *E. coli*. A second micro-centrifuge step at 16,060*g* for 5 mins pelleted any retained *E. coli*. The resulting supernatant was transferred to a second fresh 1.5mL Eppendorf tube. The titering of the phage stock solution was performed as described by the Stratagene (California, USA) instruction manual.

2.7 FILTER HYBRIDISATION OF NUCLEIC ACIDS

2.7.1 Southern blotting

The methodology employed was adapted from that established by Southern (1975) and described in Sambrook *et al.* (1989). Following separation of the DNA of interest by maxigel gel electrophoresis (Section 2.3.8) the agarose gel was soaked, with agitation, once in Denaturing Buffer (1.5M NaCl, 0.5M NaOH) for 45 mins and twice in Neutralising Buffer (1M Tris•Cl pH 8.0, 1.5M NaCl) for 30 mins and 15 mins. The agarose gel was washed briefly in ddH_2O. The DNA was irreversibly transferred to Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Amersham) by capillary action following incubation overnight at RT. The nylon membrane was rinsed in ddH_2O and then baked, sandwiched between 3MM Whatman Paper (Whatman, Maidstone) for 1 h at 80°C.

2.7.2 Random primer radiolabelling of DNA probes

Either the gel purified DNA from a restriction enzyme digest or a PCR product was used as the DNA template for the production of a DNA probe. About 25ng of the template DNA was used for the probe synthesis, using the Prime It II Random Primer Labelling Kit (Stratagene, California, USA). The DNA probes were radiolabelled with α-^{32}P dCTP (Amersham Pharmacia Biotech, Amersham). Prior to the hybridisation of the

probe to nylon membrane (Section 2.7.3) the radiolabelled DNA probe was denatured by boiling at 95°C for 5 mins.

2.7.3 Hybridisation of DNA probes

The nylon membrane with the nucleic acid irreversibly bound to it, produced in Sections 2.7.1 was used for this procedure. The nylon membrane was incubated in a hybridisation oven (Hybaid, Teddington) at 65°C for at least 4 h with 20mL of Pre-hybridisation Solution (5 x SSC [750mM NaCl, 75mM sodium citrate pH7.0], 5 x Denhardts [5 x Denhardts: 0.5g Ficoll 400, 0.5g polyvinylpyrrolidone, 0.5g BSA, ddH_2O to 500mL, filter sterilised], 0.5% (w/v) SDS, 2mg sheared salmon sperm DNA). Following this period the Pre-hybridisation Solution was replaced with 20mL fresh Pre-hybridisation Solution supplemented with the radiolabelled DNA probe (Section 2.7.2). The probe was bound overnight at 65°C. The following day the filter was washed with a suitably stringent Wash Solution. A low stringency wash was achieved using 'Wash I' (2 x SSC [300mM NaCl, 30mM sodium citrate pH7.0], 0.1% (w/v) SDS) at RT for two washes each of 20 mins. A high stringency wash was performed using 'Wash II' (0.2 x SSC [30mM NaCl, 3mM sodium citrate pH7.0], 0.1% (w/v) SDS) at 65°C for 30 mins and again for 15 mins washes. The filter was transferred to a sealed plastic bag and exposed to X-ray film (Kodak, New York, USA) at –70°C in cassettes containing an intensifying screen (Kodak, New York, USA or Genetic Research Instruments, Dunmow).

2.8 MEMBRANE AND PROTEIN TECHNIQUES

2.8.1 Over expression and isolation of an MBP fusion protein

2.8.1i Amylose affinity chromatography

Small scale amylose affinity chromatography experiments were performed according to the manufacturers' instructions (New England Biolabs, Hitchin) to establish the behaviour of the MBP~PSII-W fusion protein. The resulting samples (pre-induction crude cell lysine, 1, 2, and 3 h post-induction crude cell lysate, purified protein and

periplasmic fraction) were subjected to Laemmli SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) to determined the optimal conditions for the expression and isolation of the fusion protein.

A large scale amylose affinity chromatography experiment was undertaken, again according to the manufacturers' instructions, to isolate a quantity of the MBP~PSII-W fusion protein suitable for the production of rabbit antibodies. Long term storage of the fusion protein was achieved by the addition of sterile glycerol to 50% (v/v).

2.8.1ii Factor Xa cleavage

The Factor Xa cleavage was performed at a w/w ratio of 0.5% fusion protein to Factor Xa at 25°C in the Factor Xa Buffer (20mM Tris•Cl pH 8.0, 100mM NaCl, 2mM $CaCl_2$). This ratio took into account the fact that Factor Xa functions at 50% efficiency in 50% (v/v) glycerol. A time course experiment with samples taken at 0, 2, 4, 8 and 24 h established the optimal cutting time for the enzyme. Laemmli SDS-PAGE (Section 2.8.3) was used analyse the cleavage reactions.

2.8.1iii Production of rabbit antibodies to the MBP~PSII-W fusion protein

Nine samples, each of 100μg of lyophilised MBP~PSII-W fusion protein, were provided to Eurogentec (Belgium) who raised rabbit antibodies to this fusion protein. The optimum antibody concentration required was determined by varying the dilution of the primary and secondary antibodies employed in western analysis (Section 2.8.5).

2.8.2 Solubilisation of samples for SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.8.2i Solubilisation of bacterial whole cell extracts, *C. reinhardtii* membranes and photosystem preparations

Bacterial crude cell lysate of ~5 x 10^6 cells, *C. reinhardtii* thylakoids (prepared by Mrs. Susan Jones, UCL, as described in Diner and Wollman, 1980), His-tagged PSII preparations (prepared by Dr. Sarah. Husain, UCL, as described in Ray, 2001) and PSI preparations (prepared by Mrs. Susan Jones, adapted from Boardman, 1970) were

solubilised by the addition of an equal volume of 2 x Laemmli Gel Loading Buffer (0.3M Tris•Cl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.01% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol) and boiling at 100°C for 5 mins or incubation at 37°C for 4 h. Samples were subjected to a short micro-centrifuge step (Heraeus, Brentwood) at 16,060*g* for 1 min, and the supernatant loaded onto the protein gel.

During the optimisation of the protein gel system for western analysis of *C. reinhardtii* thylakoid and PSII samples acetone precipitation of PSII samples was explored. This method of sample preparation aimed to remove the chlorophyll molecules that hindered detection of the PSII-W protein by western analysis following sample separation by Laemmli SDS-PAGE. The PSII samples were washed with an excess of 100% acetone (i.e. a PSII sample with a chlorophyll content of 5μg was washed with 100μL of 100% acetone). The precipitated proteins were pelleted by micro-centrifugation at 16,060*g* for 1 min. The supernatant was removed and the pellet washed three times with 80% acetone. Following the third 80% acetone wash the pellet was dried at 37°C for 1 min and then solubilised in 2 x Laemmli Gel Loading Buffer by boiling at 100°C for 5 mins.

2.8.2ii Solubilisation of C. *reinhardtii* whole cell extracts

The following steps were performed to prepare whole cell samples of *C. reinhardtii* for protein analysis. The OD of 1mL of a healthy *C. reinhardtii* culture at 750nm was measured. An aliquot of 20mL of the culture was then pelleted by centrifugation in a bench top centrifuge (Mistral 1,000, MSE Scientific Instruments, Loughborough) at 2,110*g* for 5 mins. Each pellet was re-suspended in x mL of cell Re-suspension Solution (0.8M Tris•Cl pH 8.3, 0.2M sorbitol), where x = OD at 750nm. The re-suspended cells were divided into 200μL aliquots and stored at –70°C. When the samples were required, they were thawed, supplemented with SDS and β-mercaptoethanol both to 1% (v/v), and solubilised by boiling at 100°C for 1 min. Following micro-centrifugation at 16,060*g* for 1 min, the supernatant was loaded onto the protein gel.

2.8.3 Laemmli SDS-PAGE

Solubilised protein samples were subjected to SDS-PAGE according to the method published by Laemmli (1970). Acrylamide maxigels were prepared with a fixed acrylamide concentration, determined by the size of the protein to be resolved (Table 2.7). Samples were electrophoresed with 20μL Full-Range Rainbow Molecular Weight Markers (Amersham Pharmacia Biotech, Amersham). Protein separation was performed using the maxigel apparatus (Cambridge Electrophoresis Ltd., Cambridge) overnight at 50V constant voltage.

Table 2.7 The relationship between acrylamide percentage and protein resolution

% Total acrylamide	% Bisacrylamide	Range of sample polypeptides resolved (kDa)
5	2.6	25-300
10	2.6	15-100
10	3.0	10-100
15	2.6	10-50

2.8.4 LiDS-Urea PAGE

For the resolution of small polypeptides (<12kDa) the LiDS-Urea PAGE technique was employed. A 16% (w/v) resolving gel (acrylamide:bisacrylamide stock at 10% (w/v) AMPS and 0.3μL/mL TEMED) was overlain with the 5% (w/v) stacking gel (acrylamide:bisacrylamide stock at 37:1, 0.15M Tris•Cl pH 6.8, 0.1% (w/v) LiDS supplemented with 1.25μL/mL 10% (w/v) AMPS and 0.5μL/mL TEMED). The samples were run along side 20μL Full-Range Rainbow Molecular Weight Markers (Amersham Pharmacia Biotech, Amersham). The protein samples were separated using the maxigel apparatus (Cambridge Electrophoresis Ltd., Cambridge) overnight at 50V constant voltage, in presence of the Electrode Buffer (200mM glycine, 25mM Tris•Cl pH 8.3, 0.001% (w/v) LiDS).

2.8.5 Western blot analysis

2.8.5i Transfer of proteins to nitrocellulose membrane

Proteins were irreversibly transferred to Immobilon PVDF membrane (Millipore (U.K.) International, Watford) using a semi-dry blotter and the adapted methodology of Towbin *et al.* (1979). Prior to transfer, six pieces of 17 CHR Whatman paper (Whatman, Maidstone) were soaked in Transfer Buffer (0.25M Tris, 1.93M Glycine) and the Immobilon membrane in methanol. Transfer was achieved by applying a constant current of 25V. A transfer time of 40 s per cm^2 was employed.

2.8.5ii Immunological probing of nitrocellulose membranes

Immunological probing of the protein blots was performed according to the ECL protocol (Amersham Pharmacia Biotech, Amersham). Antibody dilutions were optimised. The secondary antibody employed was anti-rabbit IgG, peroxidase-linked species-specific whole antibody (raised in donkey) (Amersham Pharmacia Biotech, Amersham). Light emission was achieved with the ECL chemilluminescence detection kit and was detected by Hyperfilm ECL (Amersham Pharmacia Biotech, Amersham).

2.9 CHARACTERISATION TECHNIQUES

2.9.1 Spot Tests

An aliquot of 10μL of a culture in exponential growth was spotted onto suitable solid medium, supplemented where necessary with the appropriate antibiotic to 100μg/mL. The plates were incubated in the light conditions described in Sections 2.2.4 and Section 2.2.8 for *Synechocystis* and *C. reinhardtii* respectively.

2.9.2 Growth curves of *Synechocystis* strains

A volume of 100mL of BG-11 medium, supplemented with kanamycin to 100μg/mL where necessary, was inoculated with cells to 1 x 10^7 cells/mL and grown as described in Section 2.2.4. Cell density was determined by OD at 750nm using a UV-

Visible Spectrometer (Unicam, Cambridge). Measurements of cells grown under moderate light conditions (50μE/m^2/s) were taken approximately every 12 h, whilst those grown under low light (10μE/m^2/s) and very low light (3μE/m^2/s) were taken every 24 h.

2.9.3 Chlorophyll assay of *Synechocystis*

An aliquot of 20ml of *Synechocystis* cells in exponential phase was centrifuged at 2,110*g* for 5 mins (Mistral 1,000, MSE Scientific Instruments, Loughborough), and the pellet re-suspended in 2ml of BG-11 medium. Then 50μL of the re-suspended cells was added to 950μL methanol, mixed, and incubated at RT for 5 mins. The supernatant obtained by micro-centrifugation at 16,060*g* (Heraeus, Brentwood) was subjected to spectroscopic analysis at 665nm. Multiplication of the OD_{665} by 14 (coefficient, derived from the methodology and equations of Porra *et al.*, (1989) assuming that *Synechocystis* only has chlorophyll *a*) and then by 20 (dilution factor) produced the concentration (μM) of chlorophyll in the original cell sample.

2.9.4 Chlorophyll Assay of *C. reinhardtii*

Chlorophyll assays were performed on cell suspensions and preparations of thylakoids and photosystems. The method of Porra *et al.* was employed to calculate the chlorophyll concentration of a given sample (Porra *et al.*, 1989).

2.9.5 Oxygen evolution analysis

Oxygen evolution was performed for whole cells using a Clark type electrode. A volume of cells equivalent to 50μg of chlorophyll (Section 2.9.3 and Section 2.9.4) was made to a total volume of 3mL with Resuspending Medium (20mM MES, 15mM NaCl, 5mM $MgCl_2$, pH 6.3). The electron acceptors 2, 5-dimethyl-*p*-benzoquinone (DMBQ) and potassium ferricyanide, each at a final concentration of 1mM were employed. Levels of oxygen evolution were determined from measurements of 1 min without illumination, and then for 1 to 2 min under saturating light conditions, i.e. when light is not a limiting factor for the rate of oxygen evolution. To determine saturating light conditions the following was done: The rate of oxygen evolution for whole cells at a range of

chlorophyll concentrations (3-30μg/mL) was measured. These rates were then plotted against chlorophyll concentration. The region of saturating light is the initial portion of the graph in which there is a linear increase in oxygen evolution relative to chlorophyll concentration.

2.9.6 77K fluorescence emission spectroscopy and room temperature fluorescence analysis

The measurements obtained from 77K fluorescence emission spectroscopy enable the energy transfer to and between PSII and PSI to be monitored, as well as the determination of the PSII/PSI ratio for a given sample. For these measurements whole *Synechocystis* cell samples containing 10μM chlorophyll (Section 2.9.3) were transferred to 4mm diameter silica tubes. The samples were then either dark adapted (state 2 samples) for at least 5 mins and frozen by lowering into liquid nitrogen or frozen immediately (state 1 samples). Room temperature fluorescence measurements were performed on whole cell liquid cultures in a 3mL curvette. 77K fluorescence emission spectra and room temperature fluorescence experiments were performed using a Perkin Elmer LS50 luminescence spectrometer (Perkin Elmer, California) with excitation and emission slit widths of 5nm and 10nm respectively. For 77K fluorescence emission spectroscopy spectra were taken following excitation of the samples at 435nm (excitation of chlorophyll *a*) and 600nm (excitation of allophycocyanin). The results for both types of analysis were analysed using Sigma Plot for Windows Version 5.0. All spectra were normalised to the PSI fluorescence maximum at around 725nm. The main peaks observed following excitation at 435nm were the result of PSI fluorescence together with two peaks originating from PSII with maxima at 685nm (fluorescence of chlorophyll molecules associated with PSII) and 695nm (fluorescence of the chlorophyll molecule associated specifically with CP47). An additional two peaks were visualised following excitation at 600nm. These were at 645nm (originating from the phycocyanin) and 660nm (resulting from allophycocyanin fluorescence).

2.9.7 Electron Paramagnetic Resonance (EPR) analysis

EPR analysis was performed on whole cell *Synechocystis* samples concentrated from 200mL of exponentially growing cell culture. The pellet was re-suspended in as small a volume of TES Buffer (5mM Tris•Cl pH 8.5, 50mM NaCl and 5mM EDTA) as possible. Then 0.3 - 0.4mL of the concentrated samples were transferred to EPR tubes, and dark adapted by incubation on ice in the absence of light for at least 1 h. The dark adapted samples were frozen by lowering into liquid nitrogen, and either used immediately or stored in liquid nitrogen until required. A REIX spectrometer (Jeol, Germany) with an Oxford Instrument liquid helium cryostat (Oxford Materials, Oxford) was used for the EPR analysis. The EPR conditions were as follows: microwave power 10^{-3}mW, modulation amplitude 0.2mT and temperature at 10K.

2.10 Mating of *Chlamydomonas* strains

Days 1-3. The cells to be mated were re-streaked daily onto TAP plates. This ensures that the cells were growing exponentially.

Day 4. Cells were then re-streaked onto TAP $^1/_{10}$ Nitrogen plates. The plates were incubated for 4 d at 25°C with a light intensity of 50μE/m^2/s. The low levels of nitrogen stimulate the formation of gametes.

Day 8. A loopful of the cells was re-suspended in Na phosphate Buffer (10mM Na_2HPO_4, 10mM NaH_2PO_4 pH 7.0) to approximately 2 x 10^7 cells/mL. The cell suspension was then incubated in the light for ~30 min to permit regeneration of the flagella. Following this period 2mL of cells of opposite mating type were mixed in a sterile 50mL flask. At 1, 2 and 3 h intervals 100□L of cells was spotted onto TAP plates supplemented with 3% (w/v) agar. The plates were incubated overnight at 25°C and with a light intensity of 50μE/m^2/s.

Day 9. The plates were covered with aluminium foil and stored at 25°C for at least 1 week.

Day 16. Towards the end of the day the unmated cells were removed from the surface of the agar using a sterile razor blade. The zygotes were then transferred to fresh TAP plates (1.5% (w/v) agar) and spread out to defined positions. To kill any vegetative cells the plates were held over chloroform for 30 s precisely, and then incubated overnight at 25°C and with a light intensity of 8μE/m^2/s.

Day 17. The following morning the plates were examined for the presence of daughter cells. The four daughter cells were moved to new defined positions on the same plate. The plates were incubated at 25°C and under a light intensity of 8μE/m^2/s for 1 week, after which time the daughter cells were characterised (Section 2.2.8).

CHAPTER 3. CHARACTERISATION OF *psbW* IN *C. reinhardtii* AND *P. patens*

CHARACTERISATION OF *psbW* IN *C. reinhardtii* AND *P. patens*

3.1 INTRODUCTION

The subunit PSII-W is encoded by the nuclear gene *psbW* (Section 1.5.4). Ikeuchi *et al.* published the N-terminal sequence for the mature PSII-W protein in spinach (Ikeuchi *et al.*, 1989a), and this was followed by the N-terminal sequence for *C. reinhardtii* (de Vitry *et al.*, 1991). In 1995 PSII-W was isolated from spinach PSII and this showed a similarity to the translated sequence of an *Arabidopsis* gene (Irrgang *et al.*, 1995). Previous work in our laboratory had established that the *psbW* gene is present as a single copy in the nuclear genome of *C. reinhardtii* (Cain, 1998).

The function of this subunit is not known, although a number of possible roles have been proposed (Section 1.5.4). The subunit is not present in any prokaryote examined or in non-green algae. It is the only nuclear-encoded subunit present within the core of the PSII RC. For these reasons it is of particular interest.

This is the first of two chapters investigating the *psbW* gene and its protein product. Presented here is work carried out initially on the *psbW* gene of *C. reinhardtii* and subsequently on the same gene of *P. patens*.

3.2 AIMS

The aims of this chapter are twofold. Firstly, to characterise the *psbW* gene and the PSII-W protein in *C. reinhardtii*. To achieve this the following goals were set:

- to clone and sequence the *psbW* gene;
- to establish the coding sequence and the structure of the *psbW* gene;
- to investigate the topology of the PSII-W protein; and
- to map the location of the *psbW* gene in the nuclear genome of *C. reinhardtii*.

Secondly, similar work was undertaken in the moss *P. patens* that aimed:

- to identify *psbW* cDNA from the moss EST database;
- to determine the copy number of the *psbW* gene in the nuclear genome of *P. patens*; and

- to screen a lambda library to isolate a genomic clone that could be used to determine the sequence of the *psbW* gene.

3.3 RESULTS

3.3.1 Cloning and sequencing of the *psbW* gene from *C. reinhardtii*

3.3.1i Screening of a genomic cosmid library

Previous work performed by Dr. Amanda Cain, UCL isolated four positive genomic clones containing the *psbW* gene from a cosmid library prepared by Purton and Rochaix (1995) in the vector pARG7-8cos (Cain, 1998). The library contains 8.0 x 10^5 independent clones with inserts in the range of 27kb to 38kb. It was screened using a 3' RACE product. The synthesis of template cDNA was achieved using mRNA isolated from *C. reinhardtii* and the primer oligodT, which hybridises to the polyA tail of mRNA (Appendix 2). The 3' RACE reaction used a primer designed to the oligodT primer and a degenerate primer derived from the N-terminal sequence published for *psbW* (de Vitry *et al.*, 1991). Four positive cosmid clones were isolated and named pGW (for genomic *psbW*) and numbered 8, 10, 13 and 15. Restriction analysis of the clone pGW10 with *Bam*HI produced two bands of 1.4kb and 2.2kb. These bands were also produced by digestion of WT CC-1021 DNA with *Bam*HI, confirming that the clone had not undergone any rearrangements in the *E. coli* host. This clone, pGW10, was selected to further work.

3.3.1ii Subcloning the *psbW* genomic sequence

In order to determine the *psbW* genomic sequence the 1.4kb and 2.2kb fragments, produced by the digestion of pGW10 with *Bam*HI, were subcloned into the plasmid vector pBluescript SK (Appendix 1). A large-scale digestion of pGW10 was performed with *Bam*HI. The digest was separated in an agarose gel, the resulting 1.4kb and 2.2kb fragments were excised and the DNA subjected to the Qiagen Gel Extraction protocol (Section 2.3.9). The plasmid vector pBluescript SK was also digested with *Bam*HI and subjected to the same 'clean up' protocol. The purified vector and inserts were subjected

to a ligation reaction and transformed into the bacterial host *E. coli* JM109. This reaction resulted in the generation of the plasmids pWBam1.4 and pWBam2.2, containing the 1.4kb and 2.2kb *Bam*HI pGW10 fragments respectively. Figure 3.1 shows the arrangement of the inserts in the plasmids. These plasmids were used to determine the genomic sequence of the *psbW* gene.

3.3.1iii Sequencing of the genomic clones

Initially the plasmid specific primers T3 and T7 (Appendix 2) were employed to generate genomic sequence data from the clones pWBam1.4 and pWBam2.2. The sequence data obtained was used to generate new primers, which were used to generate a total of 2,295bp of sequence data which included 845bp upstream of the start codon and 50bp downstream of the stop codon. Both strands were sequenced in duplicate. Figure 3.2 shows the primers employed for each of these sequencing reactions together with the region of the gene for which each primer generated genomic sequence data.

3.3.1iv Amplification of *psbW* mRNA by 3' RACE

3' RACE was used to amplify the coding sequence for the *psbW* gene of *C. reinhardtii* (Section 2.4.3). Messenger RNA was isolated from WT CC-1021 cells grown under photoautotrophic conditions using the Poly(A) Pure Kit (Ambion). Amplification of a cDNA pool employed the oligonucleotide primer oligodT (Appendix 2) and AMV reverse transcriptase (AMV RTase). The second phase employed an oligonucleotide primer designed against the 5' end of the oligodT primer, RACE2 (Appendix 2), and a second degenerate oligonucleotide primer, derived from the N-terminal protein sequence published by de Vitry *et al.* (1991), Wseq6. Two separate nested PCR reactions were then performed, using the 3' RACE product as a template. The first used the primers Wseq3 and Wseq6, and the second the primers psbWE1 and psbWH1 (Appendix 2). Both PCR reactions produced products of ~250bp. Figure 3.3A details these steps together with the primer sequences. The PCR products produced by the two nested PCR reactions are shown in Figure 3.3B. It should be noted that the original 3' RACE product produced was identical to that which was employed for the screening of the cosmid library by Cain (1998; Section 3.3.1i).

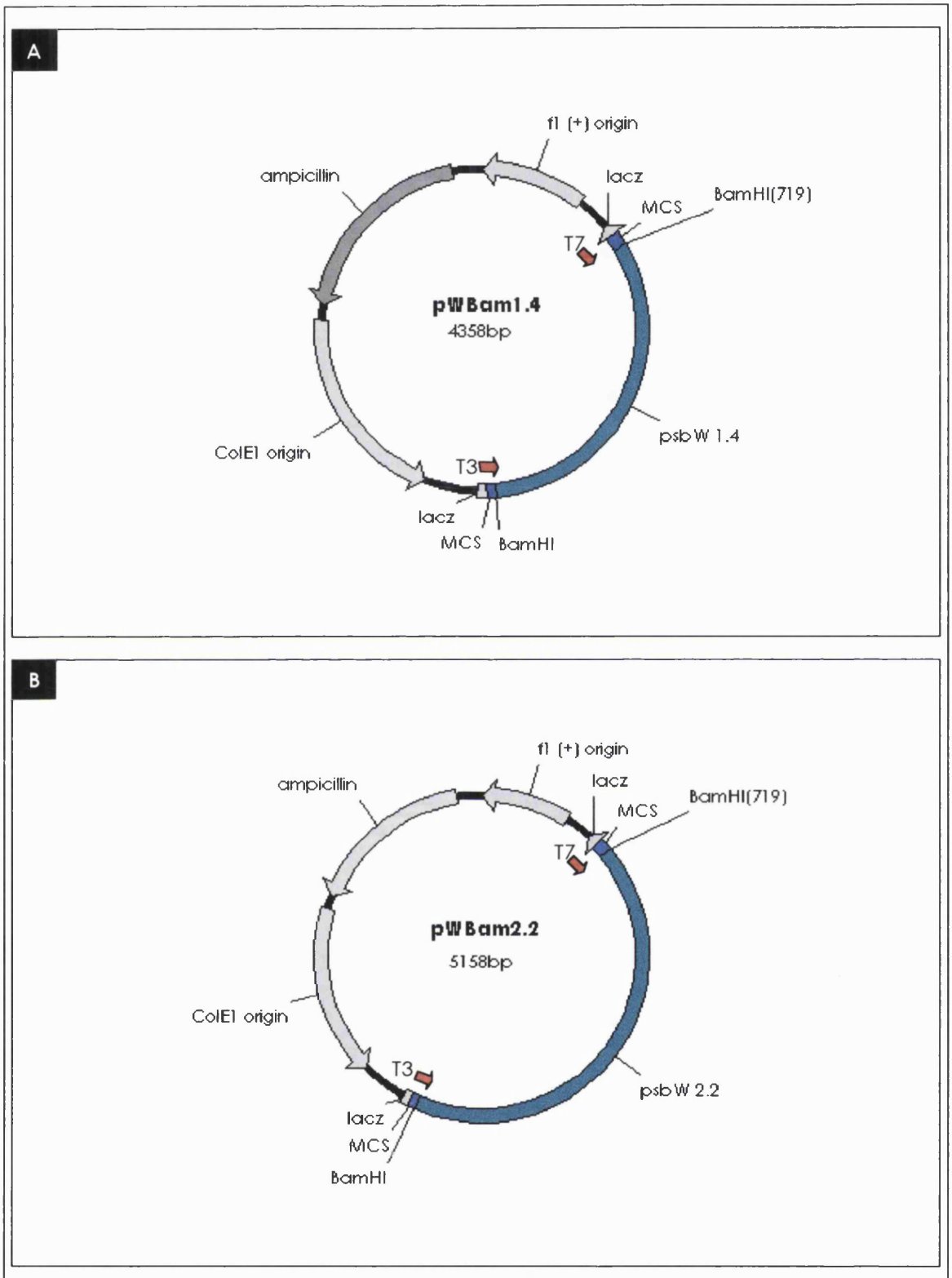
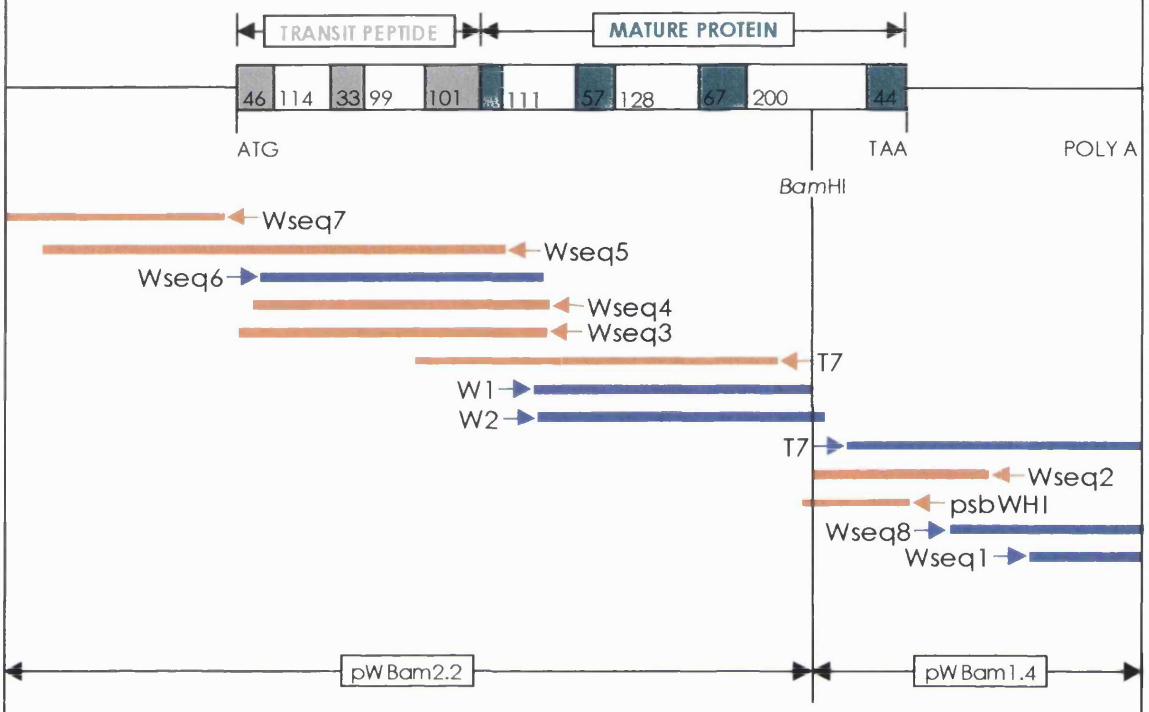


Figure 3.1 Subcloning two *psbW BamHI* fragments
A. Diagram of the plasmid pWBam1.4. The 1.4kb *Bam*HI fragment containing a portion of the *psbW* gene from C. *reinhardtii* was cloned into the *Bam*HI site of pBluescript SK.
B. Diagram of the plasmid pWBam2.2. The 2.2kb *Bam*HI fragment containing a portion of the *psbW* gene from C. *reinhardtii* was cloned into the *Bam*HI site of pBluescript SK.



Primers employed (5' to 3')

psbWH1	TGCTAAAAAAGGAAGCttTACGAA
T7	GTAATACGACTCACTATAGGGC
W1	TSGTSGAYGAGCGBATGAA
W2	ATGAAYGGNGAYGGNACNGG
Wseq1	TTGTCAGCAGCTCTGCGGTG
Wseq2	CACCGCAGAGCTGCTGACAA
Wseq3	CACATGGTGCCGAAGACGC
Wseq4	GAACCAGATAGCCCACAT
Wseq5	CCAGTGCCGTCGCCGTTCAT
Wseq6	ACACCTCGTTTGGTCGCG
Wseq7	GCGCGACCAAACGAGGTGT
Wseq8	TTCGTAGTGCTTCCTTTTTTAG

KEY

- intron
- transit peptide exon
- mature peptide exon
- 5' primer
- 3' primer
- 100bp scale

Figure 3.2 The primers used in the generation of the genomic sequence of *psbW* and the region of sequence that each generated

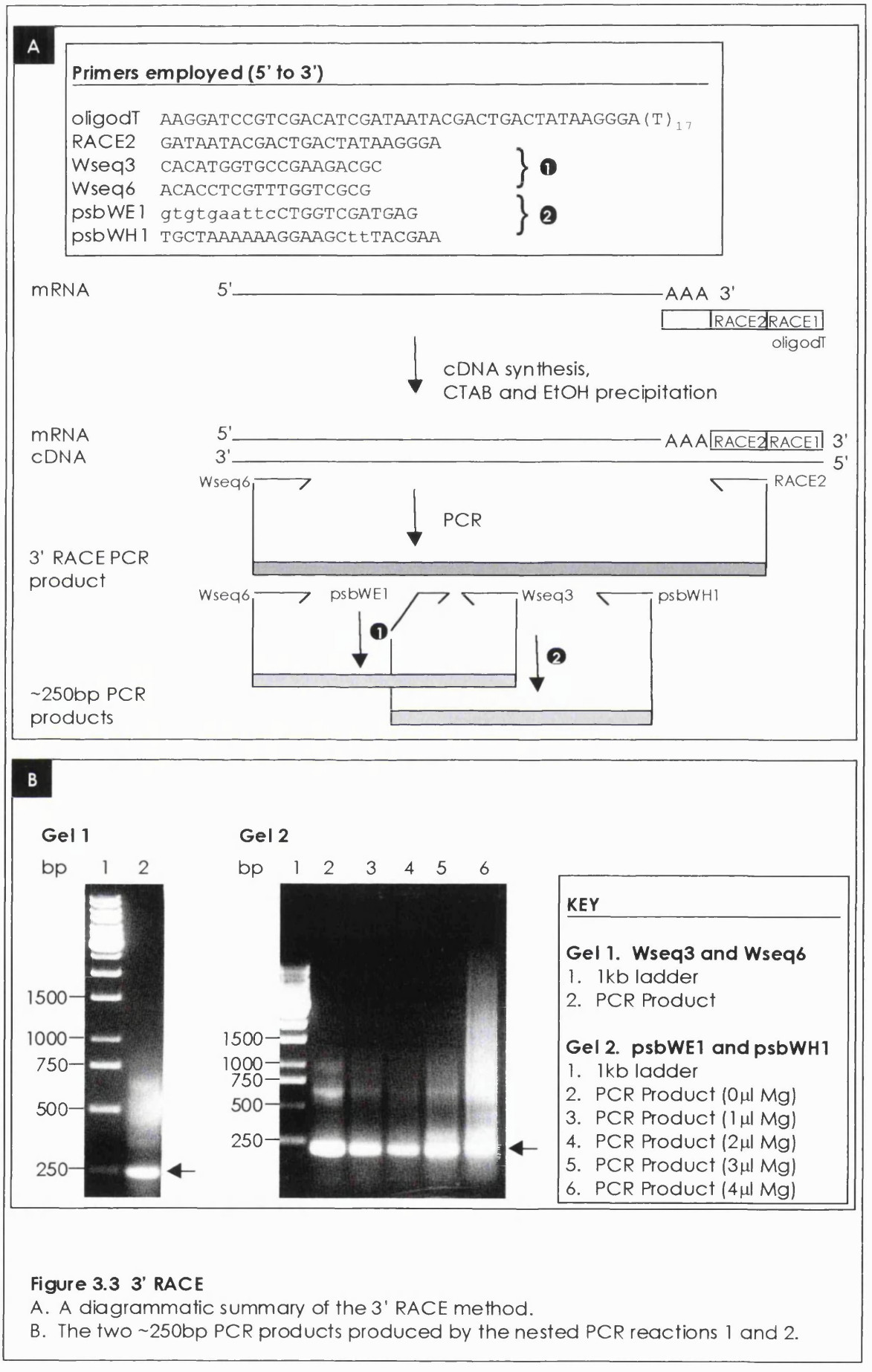


Figure 3.3 3' RACE
A. A diagrammatic summary of the 3' RACE method.
B. The two ~250bp PCR products produced by the nested PCR reactions 1 and 2.

3.3.1v Sequencing of the 3' RACE product

The products produced by the nested PCR reactions were sequenced. The PCR product produced with the primers psbWE1 and psbWH1 (Appendix 2), supplemented with 2μL of Mg^{2+} (Gel 2, Lane 4, Figure 3.3B), was selected for further work. The products were sequenced using both of the primers used to generate them. This resulted in the generation of both 5' and 3' sequence data for the coding sequence of the *psbW* gene. The coding sequence of the gene, together with the intron/exon boundaries was established by the alignment of the genomic and 3' RACE sequences. Analyses of the nucleotide and protein sequences are presented below in Section 3.3.1vi and Section 3.3.2 respectively.

3.3.1vi Analysis of the *psbW* nucleotide sequence

Figure 3.4 shows the genomic sequence data generated, the primers used, together with the exon and intron regions of the genes. A total of 2,000bp of sequence data was obtained. The coding region of the *psb*W gene spans 1,000 nucleotides and contains six exons and five introns (Accession No. AF170026). Introns range from 99bp to 200bp in size, whilst the smallest exon was 33bp in length. The regions upstream of the translation start site, and downstream of the stop site, were also sequenced. Comparison of the available *psbW* cDNA sequences with the *psbW* genomic sequence indicates that there are no additional introns in these regions. In excess of 500bp of 5' sequence data was obtained (not shown). No evidence of a transcription start could be found, nor were the presence of other regulatory elements such as the GC or CAAT promoter element or the TATA box detected. Further work, which could be undertaken to determine the transcription start site of the *psbW* gene experimentally, is described in Section 7.2.1.

Comparison of the 5' and 3' splice sites for the *psbW* exons with other nuclear-encoded genes of *C. reinhardtii* confirms that the *psbW* gene contains the same consensus splice site sequences (Rochaix *et al.*, 1998). At least three of the *psbW* introns contain putative branch points for splicing (Lorković *et al.*, 2000). Table 3.1 shows the 5' exon/intron and 3' intron/exon splice sites for each of the introns.

Previously the consensus start and stop codons of *Chlamydomonas* nuclear genes have been described: start - (A/C) A (A/C) (A/C) ATG (G/C); stop - (G/C) TAA (G/A) (Ikeda and Miyasaka, 1998; Dalphin *et al.* in Rochaix *et al.*, 1998). Both the deduced start

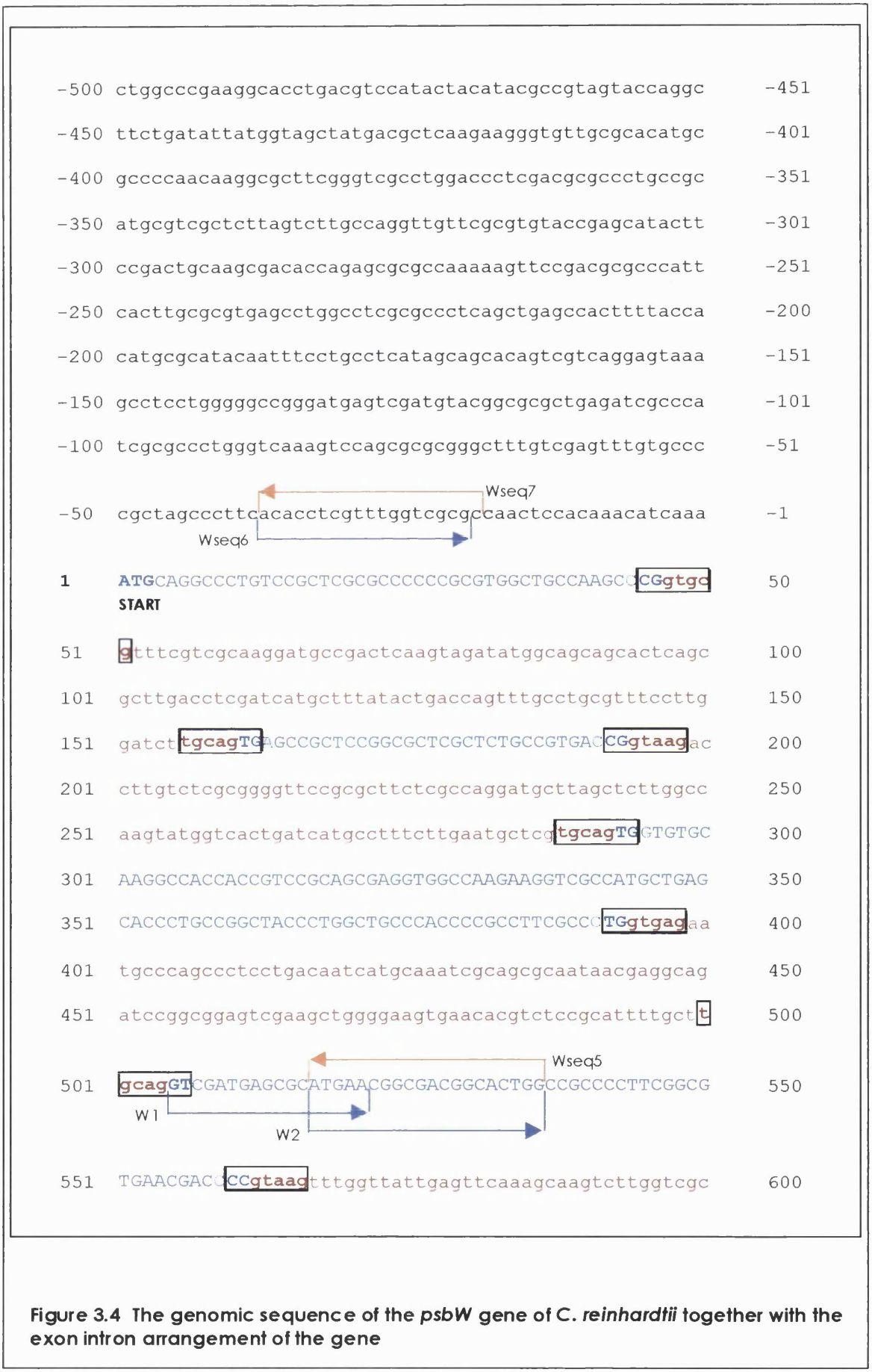


Figure 3.4 The genomic sequence of the *psbW* gene of *C. reinhardtii* together with the exon intron arrangement of the gene

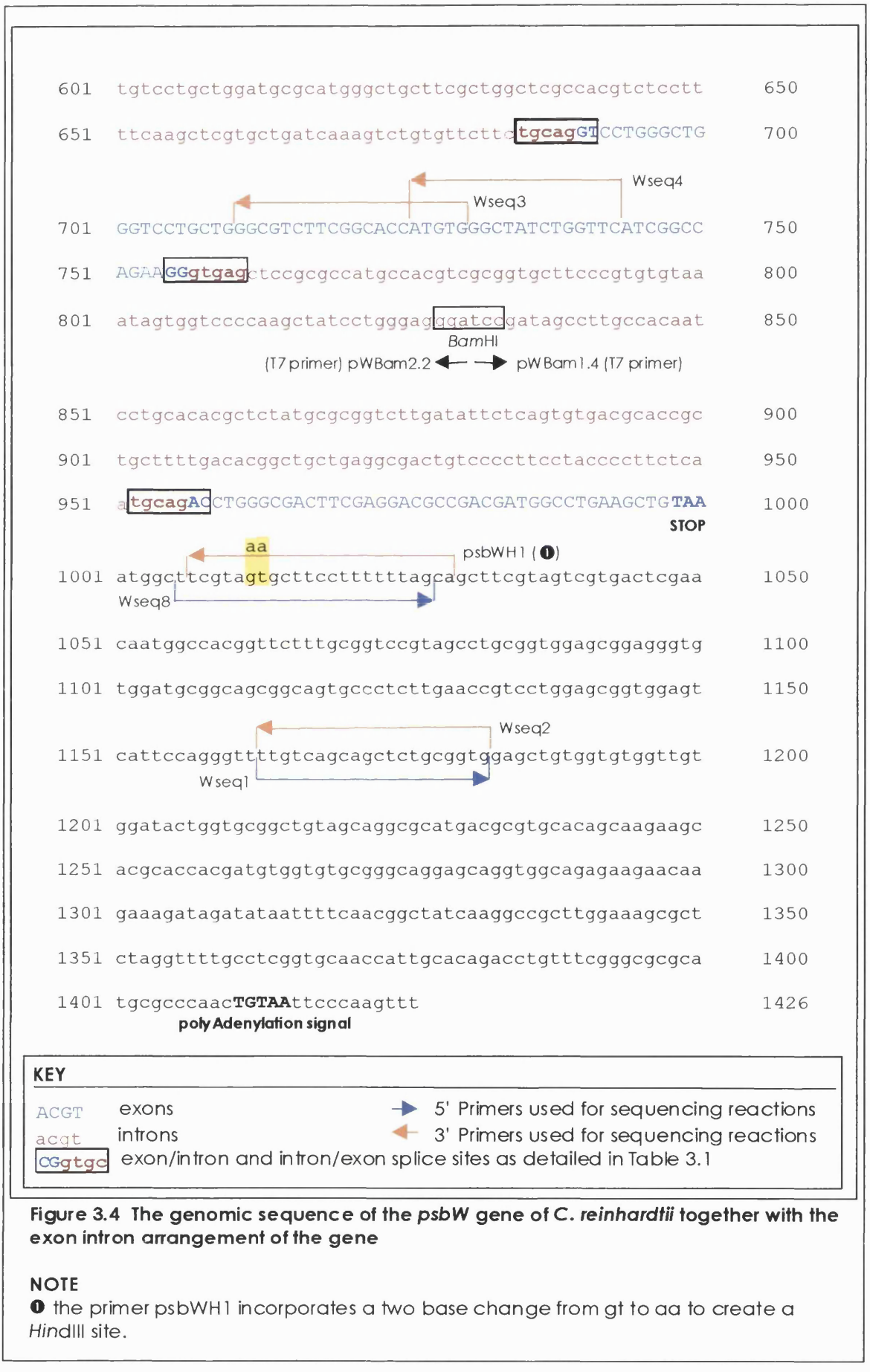


Figure 3.4 The genomic sequence of the *psbW* gene of *C. reinhardtii* together with the exon intron arrangement of the gene

NOTE
❶ the primer psbWH1 incorporates a two base change from gt to aa to create a *Hin*dIII site.

and stop codons of the *psbW* gene maintain the same consensus. These are presented, together with those of other nuclear-encoded proteins of *C. reinhardtii* in Table 3.2. A conventional polyadenylation signal (TGTAA) was located at position 1,411, 16bp upstream of the polyA tail. This same polyadenylation sequence is found in most other *Chlamydomonas* genes (Rattanachaikunsopon *et al.*, 1999 and references therein).

Table 3.1 The 5' and 3' splice sites at the exon/intron boundaries of the *psbW* gene of *C. reinhardtii*

Intron number	5' splice site (exon/intron)	3' splice site (intron/exon)
1	(CG) ↓ GTGCG	TGCAG ↓ TG
2	(CC) ↓ GTAAG	TGCAG ↓ TG
3	(TG) ↓ GTGAG	TGCAG ↓ GT
4	(CC) ↓ GTAAG	TGCAG ↓ GT
5	(GG) ↓ GTGAG	TGCAG ↓ AC
CONSENSUS	(C/A)(A/C) G ↓ GTG(A/C)G	(G/A)CAG ↓ (G/A)

Table 3.2 The start and stop codons of nuclear-encoded PSII proteins for *C. reinhardtii*

PSII Protein	Start codons	Stop codons
PSII-O	caaagATG	TAAgcg
PSII-P	caaaaATG	TAAgcg
PSII-Q	caaagATG	TAAgcg
PSII-W	caaaATG	TAAatg
PSII-X	cgaaATG	TAAatg
PSII-Y	caaaATG	TAAttg

3.3.2 Topological analysis of PSII-W

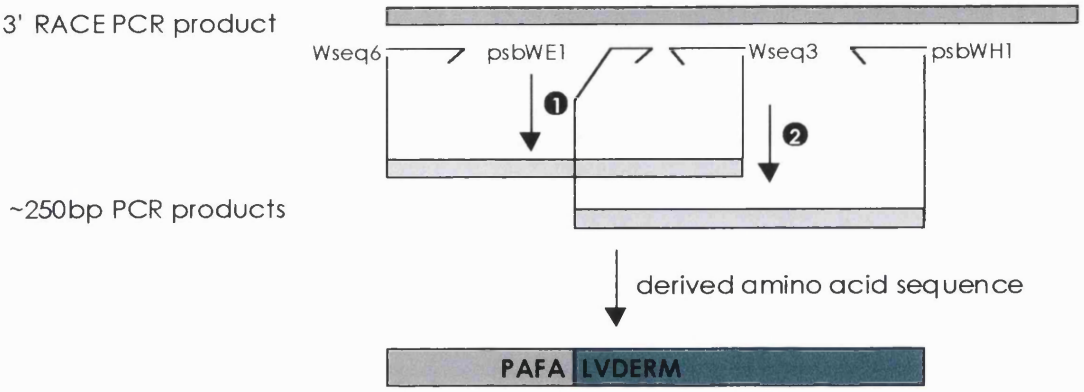
3.3.2i The PSII-W protein amino acid sequence

The *psbW* gene encodes a protein of 115 residues. Figure 3.5 shows the coding sequence, together with the derived amino acid sequence. It is known from N-terminal sequencing performed by de Vitry *et al.*, (1991) that the first six amino acids of the

A

Primers employed (5' to 3')

Wseq3	CACATGGTGCCGAAGACGC	❶
Wseq6	ACACCTCGTTTGGTCGCG	
psbWE1	gtgtgaattcCTGGTCGATGAG	❷
psbWH1	TGCTAAAAAAGGAAGCttTACGAA	



B

```
    ATG CAG GCC CTG TCC GCT CGC GCC CCC CGC GTG GCT GCC AAG CCC
1     M   Q   A   L   S   A   R   S   P   R   V   A   A   K   P   15

    GTG AGC CGC TCC GGC GCT CGC TCT GCC GTG ACC GTG GTG TGC AAG
16    V   S   R   S   G   A   R   S   A   V   T   V   V   C   K   30

    GCC ACC ACC GTC CGC AGC GAG GTG GCC AAG AAG GTC GCC ATG CTG
31    A   T   T   V   R   S   E   V   A   K   K   V   A   M   L   45

    AGC ACC CTG CCG GCT ACC CTG GCT GCC CAC CCC GCC TTC GCC CTG
46    S   T   L   P   A   T   L   A   A   H   P   A   F   A   L   60

    GTC GAT GAG CGC ATG AAC GGC GAC GGC ACT GGC CGC CCC TTC GGC
61    V   D   E   R   M   N   G   D   G   T   G   R   P   F   G   75

    GTG AAC GAC CCC GTC CTG GGC TGG GTC CTG CTG GGC GTC TTC GGC
76    V   N   D   P   V   L   G   W   V   L   L   G   V   F   G   90

    ACC ATG TGG GCT ATC TGG TTC ATC GGC CAG AAG GAC CTG GGC GAC
91    T   M   W   A   I   W   F   I   G   Q   K   D   L   G   D   105

    TTC GAG GAC GCC GAC GAT GGC CTG AAG CTG TAA
106   F   E   D   A   D   D   G   L   K   L   *                   116
```

Figure 3.5 The amino acid sequence of the PSII-W protein of *C. reinhardtii*
A. Representation of how the coding sequence was determined.
B. The coding sequence for the PSII-W protein determined by sequencing of the two 3' RACE PCR products, together with the derived amino acid sequence.

mature PSII-W protein are LVDERM. This knowledge enabled the assignment of the boundary between the transit peptide and the mature protein of the PSII-W pre-protein. Therefore the complete sequence comprises a 56 amino acid mature protein with a predicted molecular mass of 6.155kDa, preceded by a 59 amino acid bipartite transit peptide. This transit peptide enables import of the protein across the chloroplast membrane, and insertion into the thylakoid membrane. The targeting pre-sequences for *C. reinhardtii* are unlike those from higher plants (Franzén, 1995; Krimm *et al.*, 1999).

3.3.2ii Alignment of PSII-W sequences

Figure 3.6A shows the alignment of the targeting sequence for the PSII proteins PSII-W, PSII-O (33kDa protein), PSII-P (23kDa protein) and PSII-Q (16kDa protein) of *C. reinhardtii.* Examination of the protein sequences of transit peptides from different photosynthetic organisms shows that they are poorly conserved at the primary sequence level. Furthermore, there are clear structural differences between the transit peptides of *C. reinhardtii* and higher plants (Franzén, 1995). Consequently, Figure 3.6A does not include the transit peptides of other photosynthetic organisms.

Figure 3.6B shows the alignment of the mature *C. reinhardtii* PSII-W amino acid sequence, together with the sequences for *A. thaliana* (Accession No. X90769), *Spinacia oleracea* (Accession No.Q41387) and *Triticum aestivum* (P55967). The *P. patens* EST (Accession number AW155929; Section 3.3.4i) was used to derive a potential amino acid sequence for the mature PSII-W protein in this species. The sequence identity of the mature protein with that of the spinach sequence is 52%, but overall there is a high degree of similarity between the sequences.

3.3.2iii TMPred topology

Using the TMPred database, which predicts transmembrane regions and indicates orientations, the mature PSII-W protein was predicted to have a single transmembrane span (http://www.ch.embnet.org/cgi-bin/TMPRED_form_parser). The presence of tryptophan residues towards each side of the proposed transmembrane span suggests that it is firmly anchored in the membrane. The transit peptide lacks such anchoring residues. However, the data from the TMPred database does not provide information about the

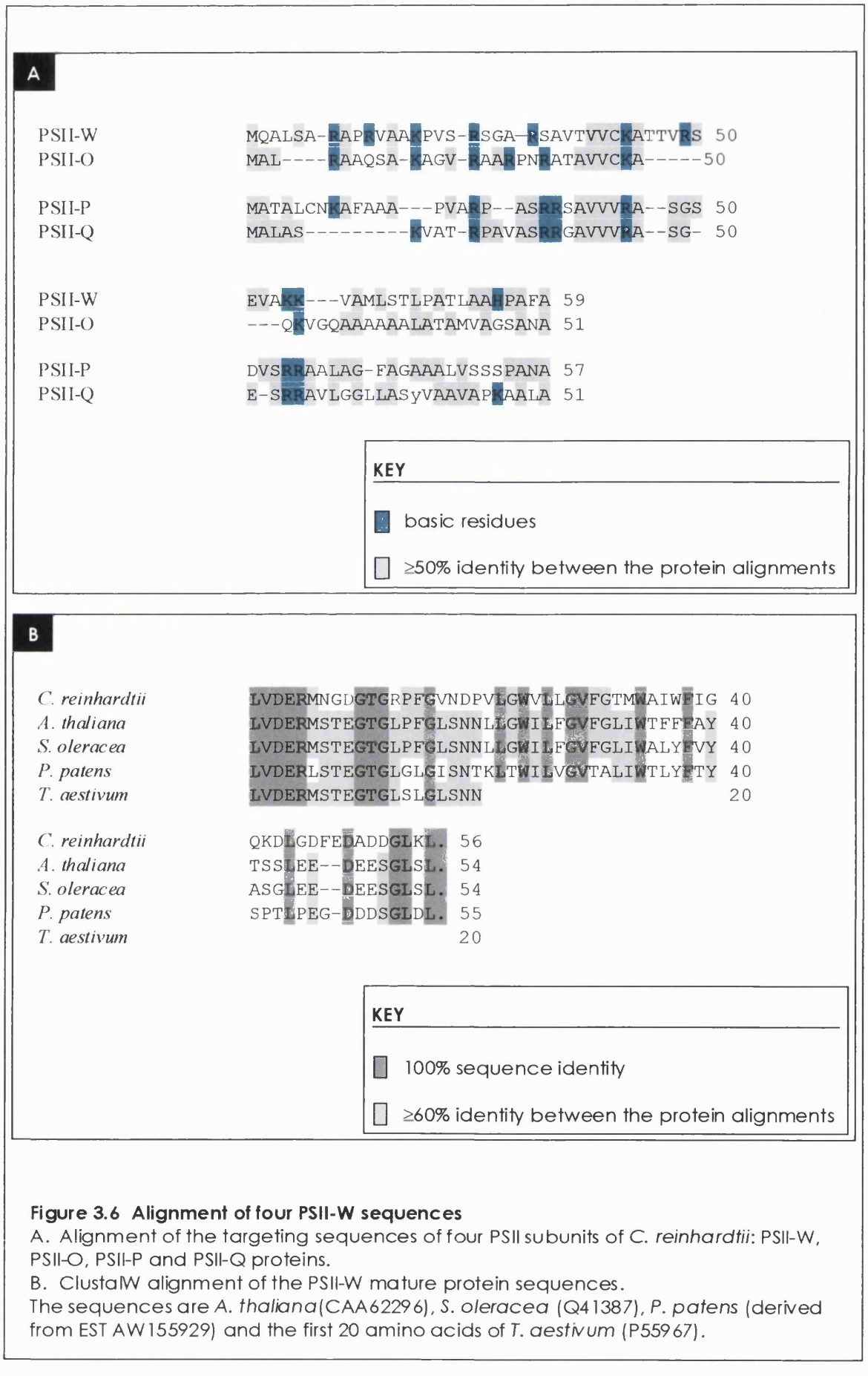


Figure 3.6 Alignment of four PSII-W sequences
A. Alignment of the targeting sequences of four PSII subunits of *C. reinhardtii*: PSII-W, PSII-O, PSII-P and PSII-Q proteins.
B. ClustalW alignment of the PSII-W mature protein sequences.
The sequences are *A. thaliana*(CAA62296), *S. oleracea* (Q41387), *P. patens* (derived from EST AW155929) and the first 20 amino acids of *T. aestivum* (P55967).

orientation of the mature protein within the thylakoid membrane. Figure 3.7 shows the proposed model for the transmembrane topology of the PSII-W protein suggested by the amino acid sequence.

3.3.2iv ChoroP predictions and model for the import of the pre-PSII-W protein

Using the results from analysis of the protein sequence with the thylakoid prediction programme ChloroP the following organisation of the transit peptides is proposed (http://www.cbs.dtu.dk/services/ChloroP/). Residues one to 30 form the first portion of the transit peptide, and facilitate import of the pre-protein across the chloroplast membrane via the chloroplast import machinery. Cleavage by the Stromal Processing Peptidase (SPP) is predicted to occur between residues 30 and 31 at a site very similar to that of other chloroplast targeted proteins including other PSII proteins. Amino acids 31 to 59 form the remainder of the transit peptide and contain a putative transmembrane span for insertion of the protein into the thylakoid membrane. Insertion of the pre~PSII-W protein into the thylakoid membrane is proposed to occur via a spontaneous insertion method (Thompson *et al.*, 1998; reviewed by Robinson *et al.*, 2000). The twin-arginine targeting motif of the twin-arginine translocase pathway that are clearly present in pre~PSII-P and pre~PSII-U is absent in pre~PSII-W. The terminal three amino acids of the pre~PSII-W transit peptide (AFA) constitute the recognition motif for the Thylakoid Processing Peptidase (TPP). It is after the last alanine that the TPP cleaves the protein, releasing the mature PSII-W protein into the thylakoid membrane. A model for the proposed import of the pre~PSII-W protein and its insertion into the thylakoid membrane is shown in Figure 3.8.

3.3.3 Mapping the location of the *psbW* gene

3.3.3i Southern analysis of WT and S1D2 C. *reinhardtii* genomic DNA

This next section describes how the location of the *psbW* gene within the nuclear genome of *C. reinhardtii* was mapped. This work was carried out in collaboration with Dr. Carolyn Silflow, University of Minnesota. To map the location of the *psbW* gene the sub strain S1D2 was utilised. S1D2 maintains a number of polymorphisms with respect to

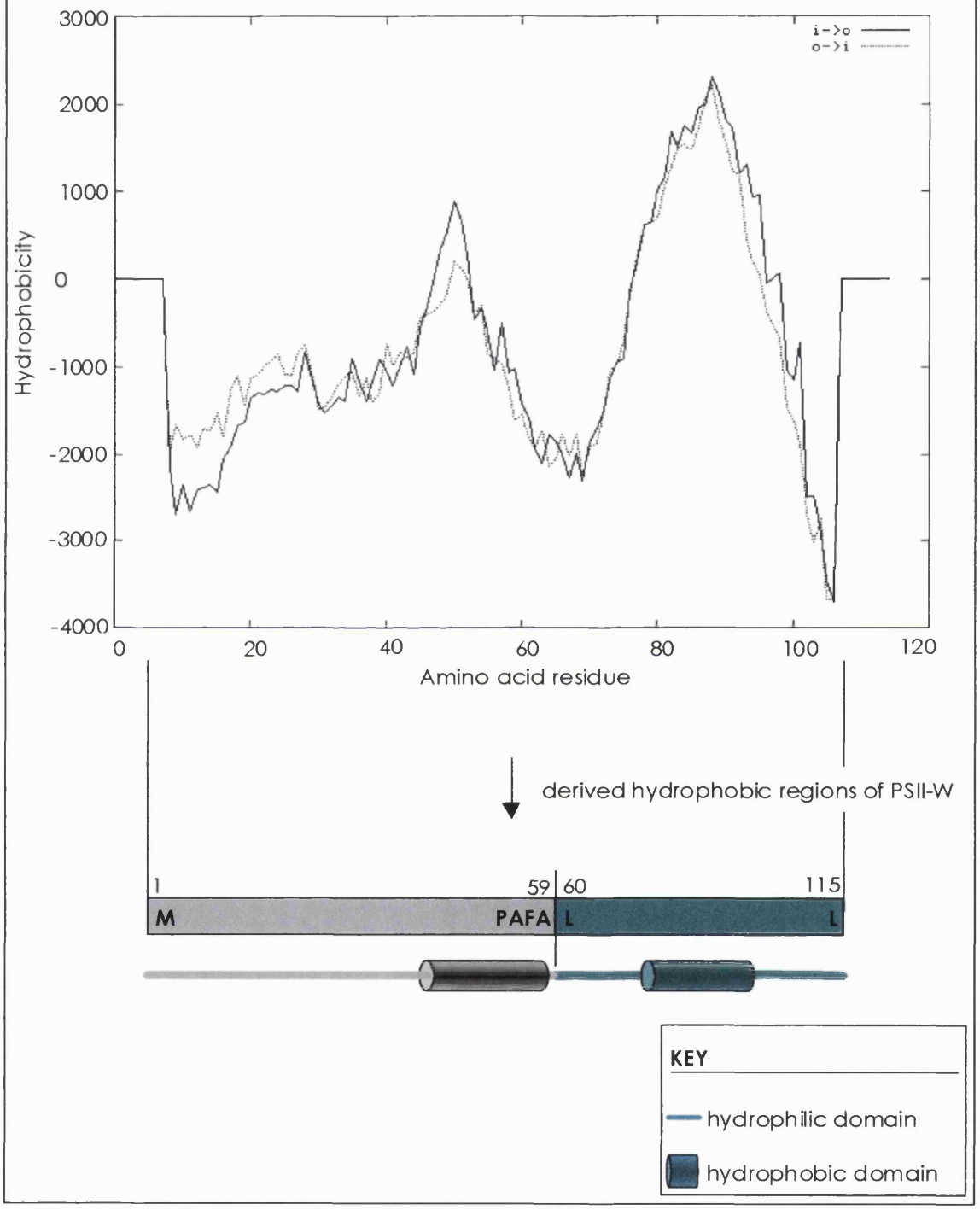
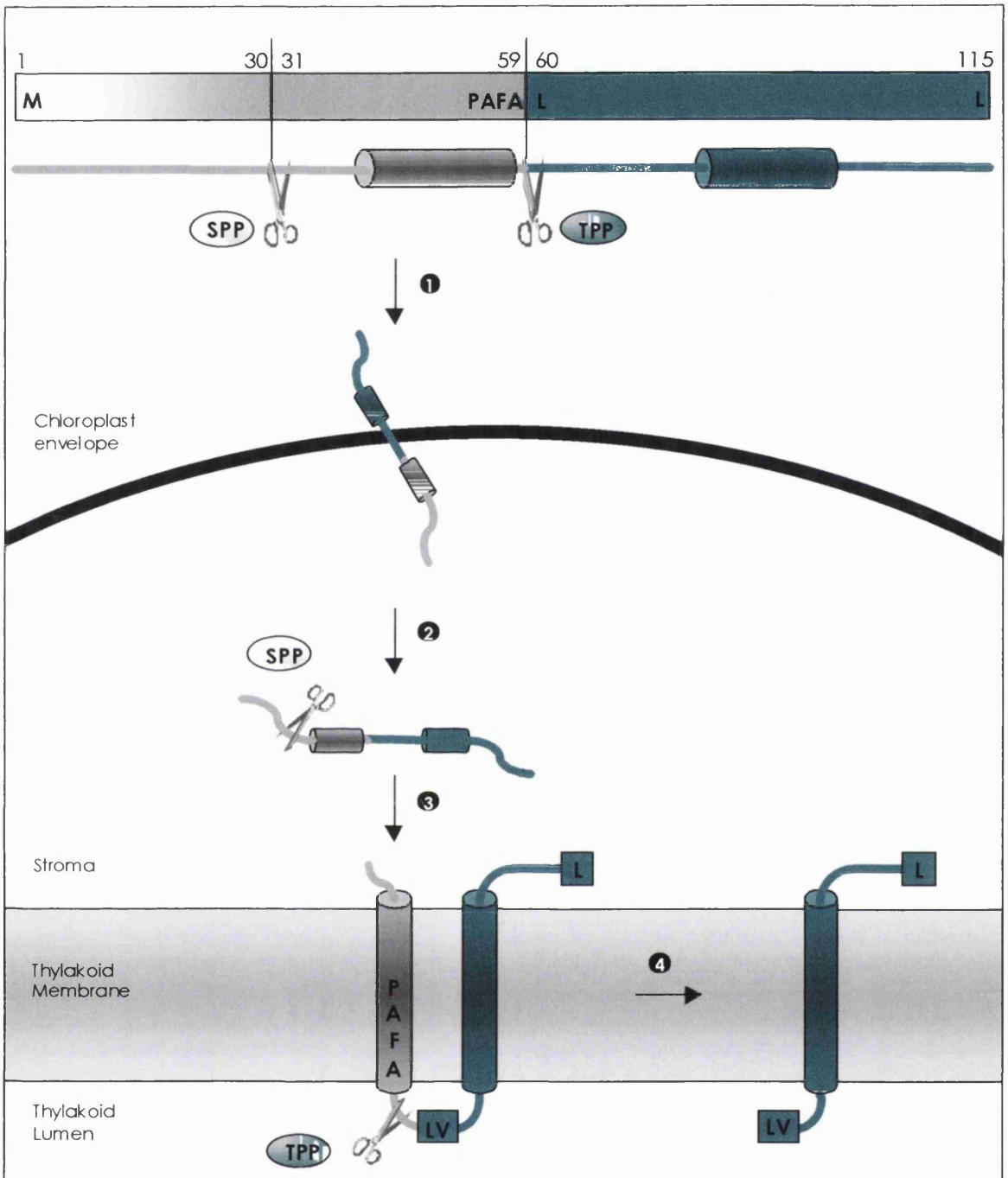


Figure 3.7 TMPred suggested model for transmembrane topology of PSII-W
This figure shows the hydrophobicity of the PSII-W protein from the N-terminus to the C-terminus, together with the derived hyrophobic regions for the protein.



KEY

1. Pre~PSII-W is imported across the chloroplast envelope via the chloroplast import machinery.
2. Primary processing is achieved by the Stromal Processing Peptidase (SPP).
3. Insertion into the thlyakoid membrane is thought to occur via a spontaneous insertion mechanism.
4. Secondary cleavage by the Thylakoid Processing Peptidase (TPP) at the AXA motif, releases the mature PSII-W protein into the membrane.

Figure 3.8 Model for the import of pre~PSII-W into the chloroplast and its insertion into the thylakoid membrane

the WT CC-1021 genome. Together with the knowledge of genetic markers within the nuclear genome, these polymorphisms enable the mapping of a gene of interest. Initially Southern analysis was performed on genomic DNA from WT CC-1021 and S1D2. This would determine if there was a polymorphism within the *psbW* gene for the two strains.

Total DNA was isolated from WT CC-1021 and S1D2 strains and subjected to restriction enzyme analysis with a number of enzymes. The digested DNA was separated in an agarose maxigel and transferred to Hybond N+ nylon using the Southern blotting technique. The ~300bp PCR product produced by the amplification of the cDNA insert of pHW5 (Appendix 1) with Wexp3' and Wexp5' was radiolabelled and used to probe the blot. Figure 3.9A shows the resulting autoradiogram for the digestion of the DNA with *Sma*I. A band of ~9kb was seen in the lane containing WT DNA, whereas the S1D2 DNA showed a slightly larger band of ~9.5kb. The autoradiogram confirms the presence of a genetic polymorphism for the *psbW* gene between WT CC-1021 and the sub strain S1D2.

Following this result the location of the *psbW* gene in the nuclear genome of *C. reinhardtii* was mapped using the molecular markers known for WT CC-1021 and S1D2. This work was performed by Dr. Carolyn Silflow's laboratory, University of Minnesota. By mating WT CC-1021 cells with S1D2 cells this group has isolated 136 random progeny. PCR-based DNA markers have been scored for each of the progeny. The results enable construction of a molecular map. If a polymorphism was present between the WT CC-1021 and S1D2 DNA for a gene of interest, this together with the score for each of the molecular markers, could be used to map the location of a gene of interest.

3.3.3ii Determination of the location of the *psbW* gene in the nuclear genome of *C. reinhardtii*

The genetic mapping of *psbW* was performed by Dr. Carolyn Silflow, University of Minnesota. The *psbW* gene was mapped to Linkage Group XI, between the genetic markers GP49 and Cry1. Figure 3.9B details the arrangement of the established genetic markers for this Linkage Group. Also mapped to the same region of Linkage Group XI were three acetate requiring mutants: ac7, ac21 and ac35. Acetate requiring mutants are only capable of heterotrophic growth, and are dependent on the presence of acetate in the medium. The mutants were generated in 1978 by Ebersold by treatment of WT *C. reinhardtii* with UV radiation and are maintained by the *Chlamydomonas* Genetics Center, Duke University (http://www.biology.duke.edu/chlamy). The details of these

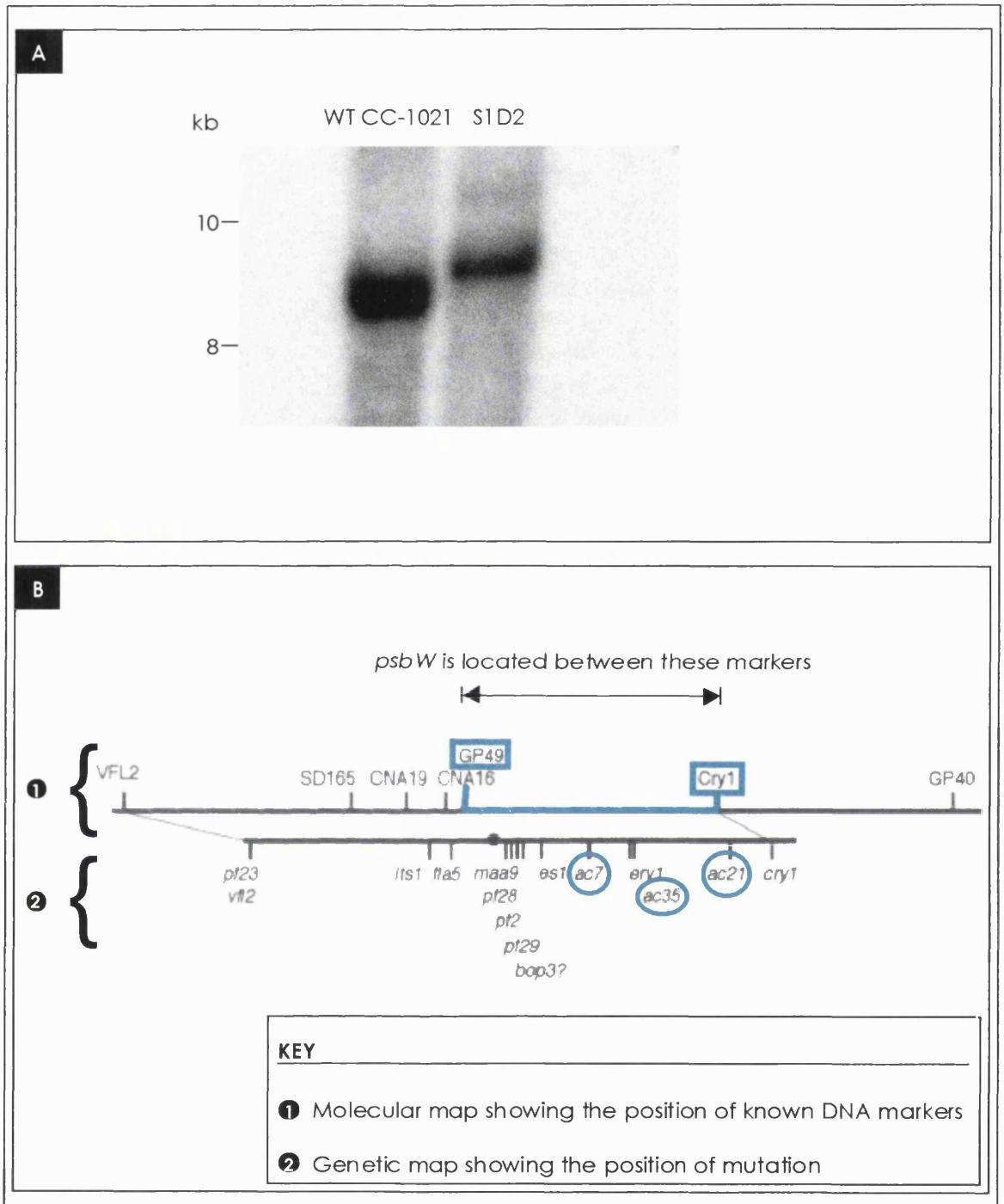


Figure 3.9 Establishing the presence of a polymorphism for the *psbW* gene of WT CC-1021 and S1D2 and Linkage Group XI of the nuclear genome of C. *reinhardtii*

A. Southern analysis to establish if there is a polymorphism present in WT CC-1021 and S1D2 C. *reinhardtii* for the *psbW* gene

Genomic DNA was prepared from WT CC-1021 and S1D2 and digested with *Sma*I. The filter was probed with the ~300bp PCR product produced by the amplification of the cDNA insert of pHW5 with Wexp3' and Wexp5'.

B. Linkage Group XI

The *psbW* gene of C. *reinhardtii* maps to the region between the molecular markers GP49 and Cry1. The three acetate requiring mutants ac7, ac35 and ac21 also map to the same region.

mutants are given in Table 3.3. It has been established that mutants at the ac21 locus are deficient in the Rieske iron-sulphur protein (de Vitry *et al.*, 1999). The strains CC-5, CC-520, CC-673 and CC-1727 were ordered from the *Chlamydomonas* Genetics Center.

Table 3.3 The acetate requiring mutants ac7, ac21 and ac35

Locus	Strain	Mating type	Phenotype
ac7	CC-673	(mt-)	acetate requiring, somewhat light sensitive
ac7a	CC-520	(mt-)	acetate requiring, somewhat light sensitive
ac7 eryl b	CC-1727	(mt+)	acetate requiring
ac21	CC-2115	(mt+)	acetate requiring, cytochrome b_6f mutant
ac35	CC-5	(mt-)	acetate requiring

3.3.3iii Characterisation of the strains CC-5, CC-520, CC-673 and CC-1727: Growth on minimal medium

To establish the phenotype of each of the four acetate requiring mutants, spot tests were performed for each of mutants and WT CC-1021. An aliquot of 10μL of culture, in log phase, was spotted onto TAP and HSM plates and grown in moderate light. Figure 3.10 shows the results of these spot tests. Each of the 'acetate requiring' mutants was capable of growth on HSM medium, i.e. in the absence of acetate. Consequently it was concluded that the mutant phenotype had been lost or suppressed and each of the four mutant strains had reverted to a WT phenotype.

3.3.3iv Attempt to recover the acetate requiring phenotype of CC-5, CC-520, CC-673 and CC-1727

In an attempt to recover the original acetate requiring phenotype, a mating experiment was performed (Section 2.10). Mating experiments with each of the 'acetate requiring mutants' were performed and selected for on TAP plates. The resulting progeny were then transferred to HSM plates to determine if there were still capable of photoautotrophic growth or if the acetate requiring phenotype had been recovered. All of the resulting progeny were capable of photoautotrophic growth (not shown). The mating

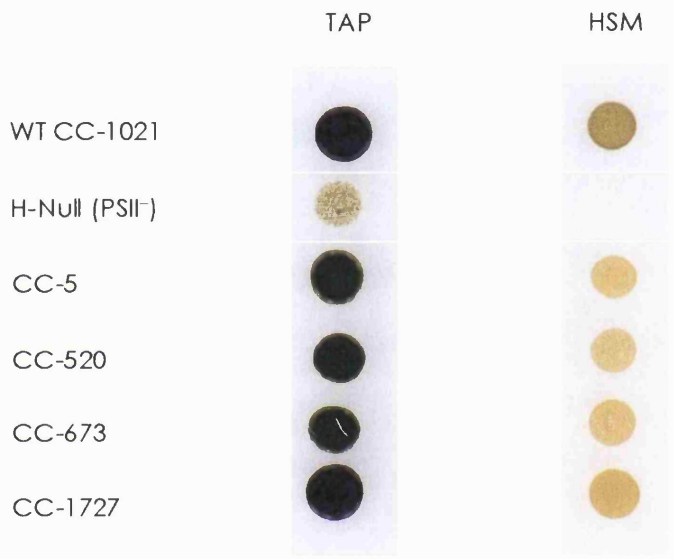


Figure 3.10 Spot tests of WT CC-1021, H-Null (PSII⁻) and the acetate requiring mutants CC-5 (ac35), CC-520 (ac7a), CC-673 (ac7) and CC-1727 (ac7ery1b) on TAP and HSM
An aliquot of 10µl of each culture was spotted onto each of the plates, and grown at 25°C at a light intensity of 8µE/m^2/s.

experiments were not successful, and the acetate requiring phenotype could not be recovered. Therefore, no further work was performed with these strains.

3.3.4 Attempts to clone and sequence the *psbW* gene from *P. patens*

This work was the result of a joint project with Dr. Sarah Husain, UCL. *P. patens* is an ideal candidate for the generation of nuclear deletion and/or disruption mutants (Section 1.11.3). To this end work was undertaken to clone and sequence the *psbW* gene of *P. patens*.

3.3.4i Identification of *psbW* in the *P. patens* EST database

The nuclear genome of *P. patens* is currently being characterised by an EST approach. ESTs are partial sequences generated by single-pass sequencing of random cDNA clones. The cDNA library is maintained as *Eco*RI-*Xho*I fragments cloned into the plasmid vector pBluescript SK (Appendix 1). The EST database was searched in order to identify a cDNA for *psbW* that could be used as a probe to screen a genomic library for the *psbW* gene of *P. patens*. A BLAST search was performed using the cDNA sequence derived for the *psbW* gene of *C. reinhardtii* (http://www.ncbi.nlm.nih.gov/BLAST/). Four *P. patens* ESTs were identified. These are shown together with the clone recognition in Table 3.4. The four cDNAs were supplied by Dr. Stavros Bashiardes as part of the *Physcomitrella* EST Programme at the University of Leeds and Washington University, St. Louis.

Table 3.4 *P. patens psbW* EST Accession numbers and corresponding clone recognitions

Accession Number	Clone recognition
AW155929	PPU030513
AW477229	PPU051217
AW497003	PPU071414
AW561533	PPU150424

3.3.4ii Sequencing the cDNA clones

Sequencing of the four clones in Table 3.4 confirmed that they each contained the cDNA for the *psbW* gene. Figure 3.11 shows a ClustalW alignment of the four ESTs (http://www2.ebi.ac.uk/clustalw/). While there was a high degree of identity between the EST sequences, further examination of the alignment indicated that there may be two sets of EST sequences. The ESTs AW155929 and AW497003 form the first set, with the second potentially distinct group containing the ESTs AW477229 and AW561533.

The amino acid sequence for each of the four ESTs was derived, and are shown in Figure 3.12. The differences observed at the DNA level effect the protein sequence. The derived amino acid sequences can also be divided into two groups. This would suggest that either there were consistent transcription errors in the generation of the cDNAs or that there may be more than one *psbW* gene in the nuclear genome of *P. patens*.

An alignment of the derived amino acid sequence of the EST AW155929 with other PSII-W protein sequences is presented in Figure 3.6. There appears to be no precedence for the conservation of the amino acids that were different in the two putative sets of *P. patens* cDNA sequences (Figure 3.12). The clone AW155929 was selected for future work.

3.3.4iii Screening a genomic Lambda library

The sequencing and analysis of the *psbW* ESTs provided the coding sequence for the PSII-W protein and the derived amino acid sequence. In order to determine the genomic sequence for the gene a *P. patens* genomic library in Lambda Fix II (Ppg DNA) was screened with a radiolabelled probe made from the ~400bp *Eco*RI-*Xho*I fragment of AW155929. The library was a gift from Dr. Stavros Bashiardes, University of Leeds and Washington University, St Louis. Eight positive clones were obtained from the initial screening. A second screening confirmed that all eight were positive clones (lambda 1-8), and that this group contained no false positives.

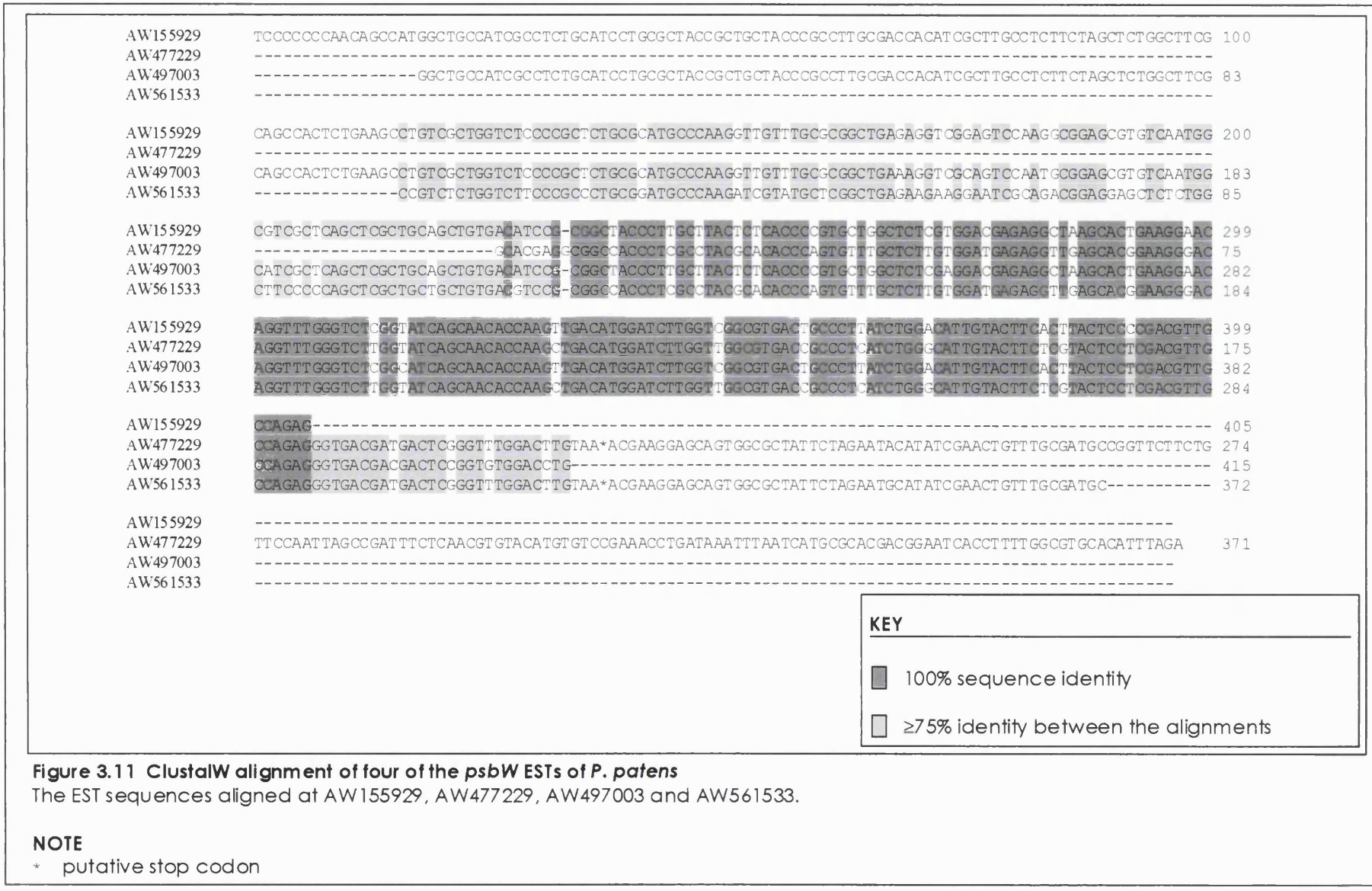


Figure 3.11 ClustalW alignment of four of the *psbW* ESTs of *P. patens*
The EST sequences aligned at AW155929, AW477229, AW497003 and AW561533.

NOTE
* putative stop codon

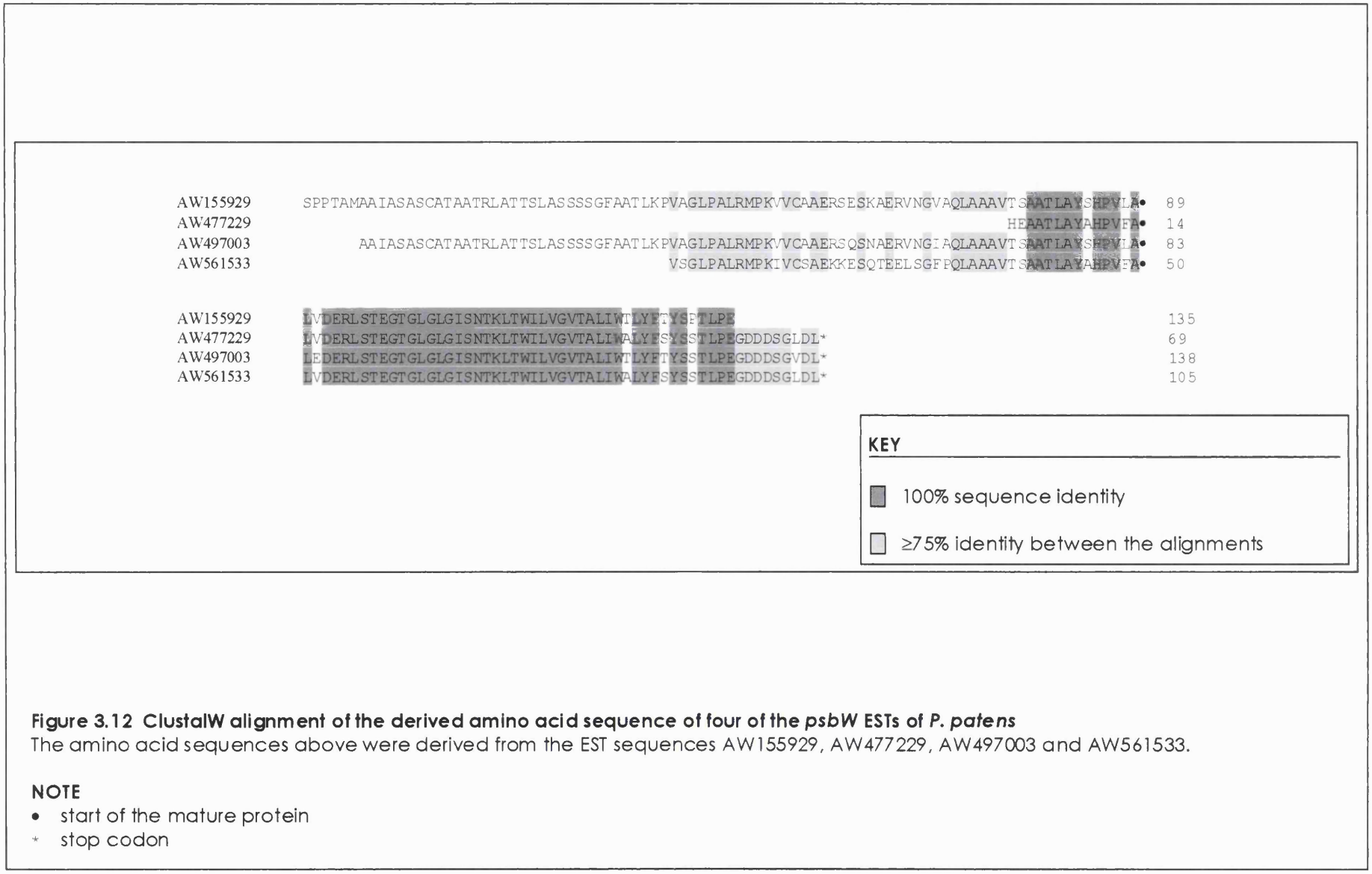


Figure 3.12 ClustalW alignment of the derived amino acid sequence of four of the *psbW* ESTs of *P. patens*
The amino acid sequences above were derived from the EST sequences AW155929, AW477229, AW497003 and AW561533.

NOTE
- • start of the mature protein
- * stop codon

3.3.4iv Attempts to subclone the *psbW* genomic sequence

To determine if the eight clones were each distinct, and to isolate a genomic fragment suitable to subclone, analysis of lambda clones 1-8 was performed. Lambda DNA was prepared for each of the eight positive clones and digested with a number of restriction enzymes. The digests were separated in a maxigel and transferred to nylon membrane, as detailed for the Southern blotting technique. The radiolabelled ~400bp *Eco*RI-*Xho*I fragment of AW155929 was used to probe the resulting blot. Several attempts were made to identify a genomic fragment containing the *psbW* gene. Despite investigating ways to optimise digestion of the DNA we were unsuccessful in obtaining complete digestion of the DNA. This was reflected in the resulting autoradiograms (data not shown).

3.3.4v Attempts to amplify the *psbW* genomic sequence by PCR

PCR was used to try to amplify the *psbW* genomic sequence from each of the eight positive lambda clones. A combination of the 5' primers mossW1 and mossW2 and the 3' primers mossW2 and mossW4 (Appendix 2) were used. A PCR product of ~1kb was produced following amplification of lambda clone 8 with the primers mossW1 and mossW4 (data not shown). However, although several attempts were made, this PCR product failed to sequence.

3.3.4vi Attempts to determine the copy number of the *psbW* gene of *P. patens*

To date all reports show that the *psbW* gene is maintained as a single copy gene in the nuclear genome of oxygenic photosynthetic eukaryotes. However, examination of the EST sequence data (Figure 3.11) and the derived amino acid sequences (Figure 3.12) from four *psbW* ESTs indicates that there may be more than one copy of this gene present in *P. patens*. Analysis of genomic DNA was undertaken to determine the *psbW* copy number in *P. patens*. Genomic DNA was isolated from *P. patens*. Restriction enzyme digests with a spectrum of enzymes were performed and the resulting fragments separated in a maxigel and transferred to nylon membrane using the Southern blotting technique. The blot was probed with the radiolabelled ~400bp *Eco*RI-*Xho*I fragment of AW155929.

Despite several attempts reproducible results were not obtained. The banding patterns obtained for the restriction enzymes selected were not consistent (data not shown). The copy number of the *psbW* gene of *P. patens* could not categorically be determined.

It has been reported in the literature that a number of factors affect the digestion of moss DNA, and that achieving complete digestion of a DNA sample is often not possible (Krogan and Ashton, 1999). The main factor appears to be the number of cytosine residues susceptible to methylation in the recognition sequences of restriction enzymes.

3.4 CONCLUSION

The initial aim of the work presented in this chapter was to determine the genomic sequence for the *psbW* gene of *C. reinhardtii*. Previous work performed by Dr. Amanda Cain, UCL had isolated four positive clones from a cosmid library prepared by Purton and Rochaix (1995). Two *Bam*HI fragments containing the genomic sequence of the *psbW* gene of *C. reinhardtii* were subcloned. The generated genomic sequence was used to design primers to determine a total of 2,000bp of genomic sequence. To establish the structure of the *psbW* gene the genomic and coding sequences were compared.

The *psbW* gene of *C. reinhardtii* contains six exons and five introns, and the region from the start to the proposed stop codon is contained within 1,000bp of sequence. The gene contains the consensus splice sites of nuclear-encoded genes of *C. reinhardtii*, with at least three introns containing putative branch points for splicing characterised by Lorković *et al.* (2000). Furthermore, the deduced start and stop codons maintain the pattern described for nuclear-encoded PSII proteins of *C. reinhardtii*. The resulting sequence data was submitted to GenBank (Accession Number AF170026).

The PSII-W protein of *C. reinhardtii* contains 115 amino acid residues. Examination of the derived protein sequence for pre~PSII-W confirmed the N-terminal sequence presented by de Vitry *et al.* (1991). The putative cleavage sites for the stromal and thylakoid processing peptidases have been assigned to the pre-protein, together with two hydrophobic transmembrane domains, the second of these being located within the mature protein. Modelling of the import of the pre-protein from the cytoplasm across the chloroplast envelope and its insertion into the thylakoid membrane supports the view that the N-terminus of the protein is located in the thylakoid lumen. This orientation of the PSII-W protein is indicated for all PSII-W sequences found to date and is atypical of the subunits of PSII, most of which have their N-termini located on the stromal side of the

membrane. The amino acid sequence determined for the PSII-W protein of *C. reinhardtii* maintains ~50% identity with other published PSII-W sequences.

The location of the *psbW* gene within the nuclear genome of *C. reinhardtii* has been mapped to the region of Linkage Group XI between the molecular markers GP49 and Cry1. This work was performed in collaboration with Dr. Carolyn Silflow, University of Minnesota. Also mapping to this same region are three acetate-requiring mutations. The function of one of these, ac21, has been assigned to the Rieske iron-sulphur protein (de Vitry *et al.*, 1999). The strains corresponding to the loci ac7 and ac35 were received from the *Chlamydomonas* Genetics Centre. Initial characterisation of these mutants established that none are acetate requiring, as each is capable of photoautotrophic growth. Attempts to recover the original acetate requiring phenotype, by mating, were not successful.

The acetate requiring mutants were generated over 30 years ago by treatment of WT *C. reinhardtii* with UV radiation. Exposure of DNA to UV radiation causes the formation of intrachain pyrimidine dimers. Excision and repair of the dimers can result in point mutations at these sites. At present there is no reliable low temperature method for the long term storage of *Chlamydomonas* and strains are therefore maintained by frequent re-streaking on nutrient agar plates that are grown under low light conditions. This method of maintenance increases the likelihood of a reversion or suppression of this type of mutation, since there is a selective pressure for the restoration of photosynthetic function. During the time that these stocks have been maintained there is also the potential for a 'mix up' of different strains. It is therefore not entirely surprising that the acetate requiring phenotype of the strains CC-5, CC-520, CC-673 and CC-1727 has been lost with time.

The WT phenotype could have been restored by a number of mechanisms. For example a reversion could have occurred, which would have restored the original sequence. Alternatively a secondary mutation may have occurred that relieved the effect of the first mutation. If the recovery of a WT phenotype was the results of a secondary mutation it might be possible to determine if the original and/or secondary mutation had occurred in the *psbW* gene. Comparison of the sequence the *psbW* gene of each of the mutants with the data generated with the WT CC-1021 sequence would achieve this and identify any point mutations.

In a joint project with Dr. Sarah Husain, UCL, attempts were made to clone the *psbW* gene of the moss *P. patens*. Eight positive genomic lambda clones were isolated from the screening of a lambda library. Attempts to subclone the *psbW* gene or to amplify it by PCR were unsuccessful. Investigation of the *psbW* copy number did not yield reproducible results. However, examination of the four *psbW* EST sequences, and their derived amino acid sequences, shows the presence of two distinct groups. Although further work is required (outlined in Section 7.2.1), initial analysis suggests that there may be two copies of the gene in *P. patens*. If this were the case, it would be the first instance in which this gene had more than a single copy number.

CHAPTER 4. EXPRESSION ANALYSIS OF PSII-W FROM *C. reinhardtii*

CHAPTER 4. EXPRESSION ANALYSIS OF PSII-W FROM C. *reinhardtii*

4.1 INTRODUCTION

This is the second of two chapters that investigate the nuclear-encoded subunit PSII-W (Section 1.5.4). While Chapter 3 focused on the characterisation of the *psbW* gene, this chapter sets out to discover more about the PSII-W protein. To date there are no reports in the literature which analyse the expression of the PSII-W protein. A paper was recently published by Hiyama *et al.* (2000) that called into question the long held view that PSII-W is a protein located exclusively within the PSII complex. The authors proposed that PSII-W is also a subunit of the PSI complex. The objectives of this chapter are to determine if the PSII-W protein is also a component of the PSI complex and to examine the expression patterns of this protein in *C. reinhardtii*.

4.2 AIMS

The aims of this chapter are:

- to raise antibodies to the mature PSII-W protein of *C. reinhardtii*;
- to examine if PSII-W is a component of the PSII complex of *C. reinhardtii*;
- to determine if the PSII-W protein is also present within the PSI complex; and
- to investigate the expression patterns of the PSII-W protein of *C. reinhardtii*.

4.3 RESULTS

4.3.1 Production of antibodies to the mature PSII-W protein

4.3.1i Construction of pMAL-c2.psbW

The first stage in the generation of antibodies to a protein is to clone all or part of the coding sequence for the protein of interest into an *E. coli* expression vector. The expression vector chosen was pMAL-c2 (NEB, Hitchin; Appendix 1). The coding

sequence for the protein of interest is cloned downstream and in-frame with the *malE* gene, which encodes the maltose-binding protein (MBP). The vector exploits the P_{tac} promoter and the translation initiation signals of *malE*. In the presence of IPTG the expression of large amounts of the fusion protein is induced. This fusion protein can be purified by affinity purification on an amylose column.

Figure 4.1 shows the steps taken to amplify the coding sequence for the mature PSII-W protein. The cloning of this coding sequence into pMAL-c2 is detailed in Figure 4.2. WT CC-1021 cells were grown photoautotrophically and the mRNA isolated. Then 3' RACE was performed using the primer oligodT (Appendix 2). Amplification with this primer generated a cDNA pool. The primers Wseq6 and RACE2 (Appendix 2) were then employed to generate a PCR product that contained the coding sequence of the *psbW* gene. Two primers were designed to amplify the coding sequence for the mature portion of the PSII-W protein (Appendix 2). The 5' primer, psbWE1, has a 5' overhang into which was incorporated a unique *Eco*RI site. The 3' primer, psbWH1, has a two base pair change that resulted in the creation of a unique *Hin*dIII site. Nested PCR was performed using these primers. The nested PCR product and the plasmid vector pMAL-c2 (Appendix 1) were then digested with *Eco*RI and *Hin*dIII. Both digestions were separated in an agarose gel and the cleaved ends were then removed using the Qiagen gel extraction protocol. A ligation reaction with the gel purified products was set up and then transformed into competent *E. coli* DH5α cells. Sequencing with the primer psbWH1 confirmed that the coding sequence for the mature PSII-W protein had been cloned downstream and in-frame with the *malE* gene of the pMAL-c2 vector. The resulting plasmid was called pMAL-c2.psbW.

4.3.1ii Purification of the MBP~PSII-W fusion protein

To confirm that expression of the MBP~PSII-W fusion protein could be induced and that the fusion protein could be purified, a pilot purification was undertaken according to the manufacturers' instructions. For *E. coli* transformants containing the pMAL-c2 and the pMAL-c2.psbW constructs, samples were taken prior to induction with IPTG and then at 1, 2 and 3 h intervals post induction. Aliquots of these samples were separated in a 10% (w/v) SDS-PAGE gel. Figure 4.3 shows the resulting gel following staining with Coomassie Blue. For both the vector control and the pMAL-c2.psbW

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TGGCCCGAAGGCACCTGACGTCCATACTACATACGCCGTAGTACCAGGCTTCTGATATT
ATGGTAGCTATGACGCTCAAGAAGGGTGTTGCGCACATGCGCCCCAACAAGGCGCTTCG
GGTCGCCTGGACCCTCGACGCGCCCTGCCGCATGCGTCGCTCTTAGTCTTGCCAGGTTG
TTCGCGTGTACCGAGCATACTTCCGACTGCAAGCGACACCAGAGCGCGCCAAAAAGTTC
CGACGCGCCCATTCACTTGCGCGTGAGCCTGGCCTCGCGCCCTCAGCTGAGCCACTTTT
ACCACATGCGCATACAATTTCCTGCCTCATAGCAGCACAGTCGTCAGGAGTAAAGCCTC
CTGGGGGCCGGGATGAGTCGATGTACGGCGCGCTGAGATCGCCCATCGCGCCCTGGGTC
AAAGTCCAGCGCGCGGGCTTTGTCGAGTTTGTGCCCCGCTAGCCCTTCACACCTCGTTT
GGTCGCGCCAACTCCACAAACATCAAAATGCAGGCCCTGTCCGCTCGCGCCCCCCGCGT
GGCTGCCAAGCCCGTGAGCCGCTCCGGCGCTCGCTCTGCCGTGACCGTGGTGTGCAAGG
CCACCACCGTCCGCAGCGAGGTGGCCAAGAAGGTCGCCATGCTGAGCACCCTGCCGGCT
ACCCTGGCTGCCCACCCCGCCTTCGCC CTG GTC GAT GAG CGC ATG AAC GGC
                  GTGTGAATT/  L   V   D   E   R   M   N   G

GAC GGC ACT GGC CGC CCC TTC GGC GTG AAC GAC CCC GTC CTG GGC
 D   G   T   G   R   P   F   G   V   N   D   P   V   L   G

TGG GTC CTG CTG GGC GTC TTC GGC ACC ATG TGG GCT ATC TGG TTC
 W   V   L   L   G   V   F   G   T   M   W   A   I   W   F

ATC GGC CAG AAG GAC CTG GGC GAC TTC GAG GAC GCC GAC GAT GGC
 I   G   Q   K   D   L   G   D   F   E   D   A   D   D   G

CTG AAG CTG TAAATGGCTTCGTAaaGCTTCCTTTTTTAGCAGCTTCGTAGTCGTGA
 L   K   L   *

CTCGAACAATGGCCACGGTTCTTTGCGGTCCGTAGCCTGCGGTGGAGCGGAGGGTGTGG
ATGCGGCAGCGGCAGTGCCCTCTTGAACCGTCCTGGAGCGGTGGAGTCATTCCAGGGTT
TTGTCAGCAGCTCTGCGGTGGAGCTGTGGTGTGGTTGTGGATACTGGTGCGGCTGTAGC
AGGCGCATGACGCGTGCACAGCAAGAAGCACGCACCACGATGTGGTGTGCGGGCAGGAG
CAGGTGGCAGAGAAGAACAATGCGCCCAACTGTAATTCCCAAGTTT(A)17TCCCTTATA
GTCAGTCGATATTATCGATGTCGACGGATCCTT                polyA tail
```

Primers employed (5' to 3')

oligodT	AAGGATCCGTCGACATCGATAATACGACTGACTATAAGGGA(T)$_{17}$
RACE2	GATAATACGACTGACTATAAGGGA
Wseq6	ACACCTCGTTTGGTCGCG
psbWE1	gtgtgaattcCTGGTCGATGAG
psbWH1	TGCTAAAAAAGGAAGCttTACGAA

Figure 4.1 Amplification of the coding sequence for the mature PSII-W protein
The coding sequence of the *psbW* gene of *C. reinhardtii*, including 499bp upstream of the start codon is presented here. The 5' primers Wseq6 and psbWE1 (shown in blue) and the 3' primers oligodT, RACE2 and psbWH1 (shown in orange) were employed in the amplification of the coding sequence of the mature PSII-W protein.

NOTE
The following restriction enzyme sites are present, and underlined, within the sequence presented above:
*Eco*RI G↓AATTC (primer psbWE1);
*Hin*dIII A↓AGCTT (primer psbWH1).

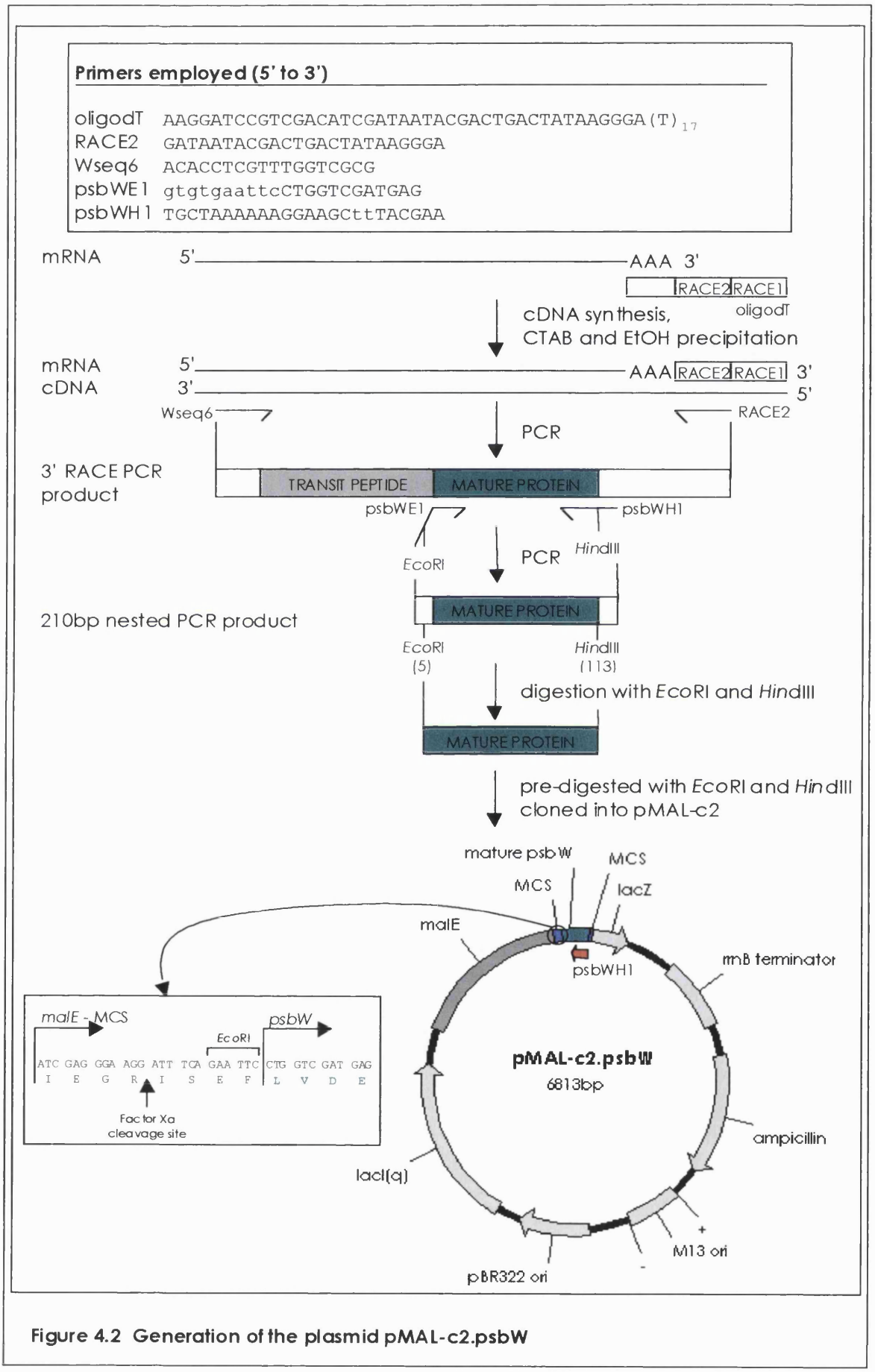
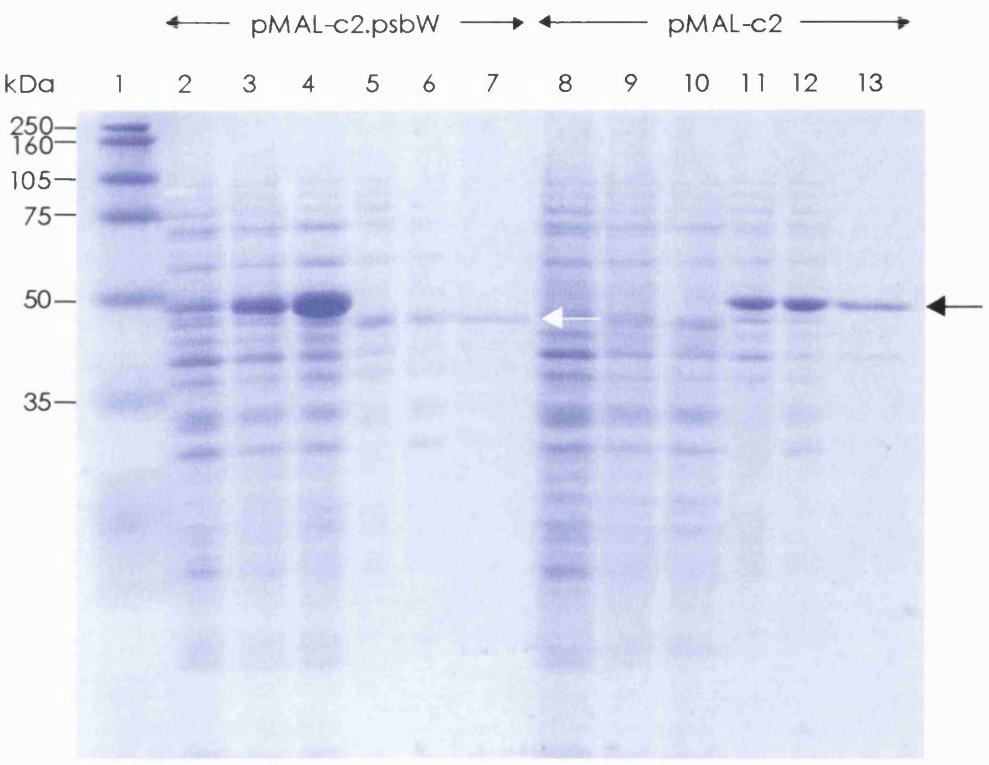


Figure 4.2 Generation of the plasmid pMAL-c2.psbW



LANE ORDER

1. protein markers
2. pMAL-c2.psbW pre-induction
3. pMAL-c2.psbW 1 h post IPTG induction
4. pMAL-c2.psbW 2 h post IPTG induction
5. pMAL-c2.psbW soluble fraction
6. pMAL-c2.psbW insoluble fraction
7. pMAL-c2.psbW amylose purification: MBP~PSII-W
8. pMAL-c2 pre-induction
9. pMAL-c2 1 h post IPTG induction
10. pMAL-c2 2 h post IPTG induction
11. pMAL-c2 soluble fraction
12. pMAL-c2 insoluble fraction
13. pMAL-c2 amylose purification: MBP

Figure 4.3 Coomassie stained SDS-PAGE of bacterial cells, and the soluble and insoluble fractions following the induction of the expression of the MBP~PSII-W and MBP proteins with IPTG
Transformants containing either the pMAL-c2.psbW or pMAL-c2 constructs were subject to induction of the MBP~PSII-W (~48kDa) or MBP (50kDa) proteins respectively. Samples were taken prior to induction (Lanes 2 and 8) and then 1 and 2 h post induction (Lanes 3,4 and 9,10). A second sample following 2 h IPTG induction was subject to sonication. The soluble and insoluble fractions were collected (Lanes 5,6 and 11,12 respectively). The MBP~PSII-W proteins (white arrow) and MBP (black arrow) were isolated by affinity to an amylose column from the soluble fractions (Lanes 7 and 13).

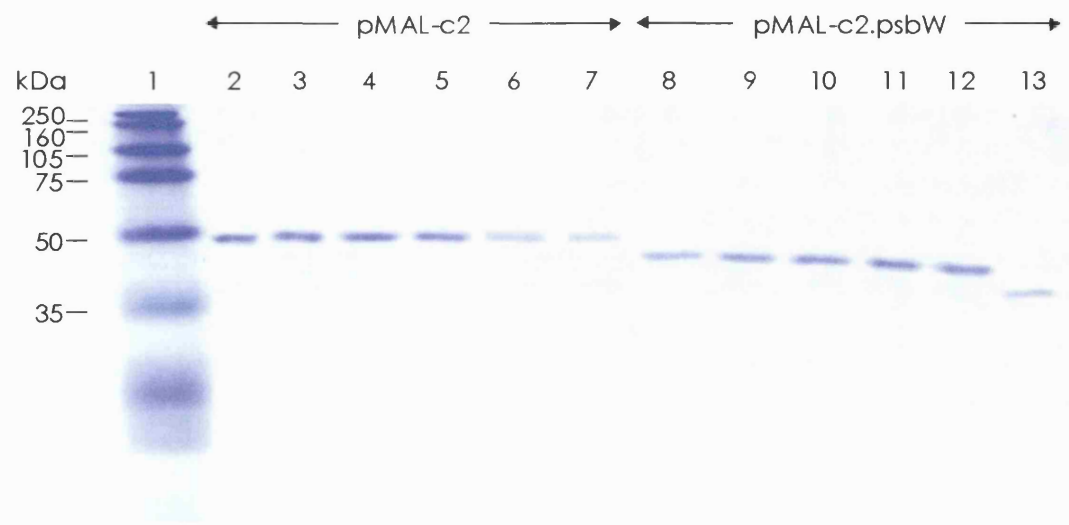
transformants, the MBP and MBP fusion protein were expressed following induction with IPTG. It should be noted that the MBP was expressed as a fusion with the LacZα protein, and resulted in a protein of 50kDa in size. When the MBP (42kDa) is fused to the PSII-W protein (~6kDa) the resulting MBP~PSII-W fusion protein is smaller with a predicted molecular mass of ~48kDa. From this pilot experiment, it was decided to harvest the cells 2 h post IPTG induction. A large-scale purification of the MBP~PSII-W protein was then undertaken as detailed in the manufacturers' instructions. The lyophilised protein was sent to Eurogentec (Belgium) who raised rabbit antibodies to the fusion protein.

4.3.1iii Cleavage of the MBP~PSII-W fusion protein with Factor Xa

Present at the C-terminus of the MBP was a cleavage site for the protease Factor Xa. Following digestion of the fusion protein with Factor Xa, the protein of interest can be isolated. To establish if Factor Xa cleaves the MBP~PSII-W protein a time course digestion over a 24 h period was set up according to the manufacturers' instructions. The resulting samples were separated in a 10% (w/v) SDS-PAGE gel, and then stained with Coomassie Blue. Figure 4.4 shows the stained digest products. From this experiment it can be concluded that the optimal time for digestion of the MBP~PSII-W fusion protein is 24 h. Because of the tertiary structure of MBP, it was not amenable to cleavage with Factor Xa.

4.3.1iv Optimisation of αMBP~PSII-W antibodies

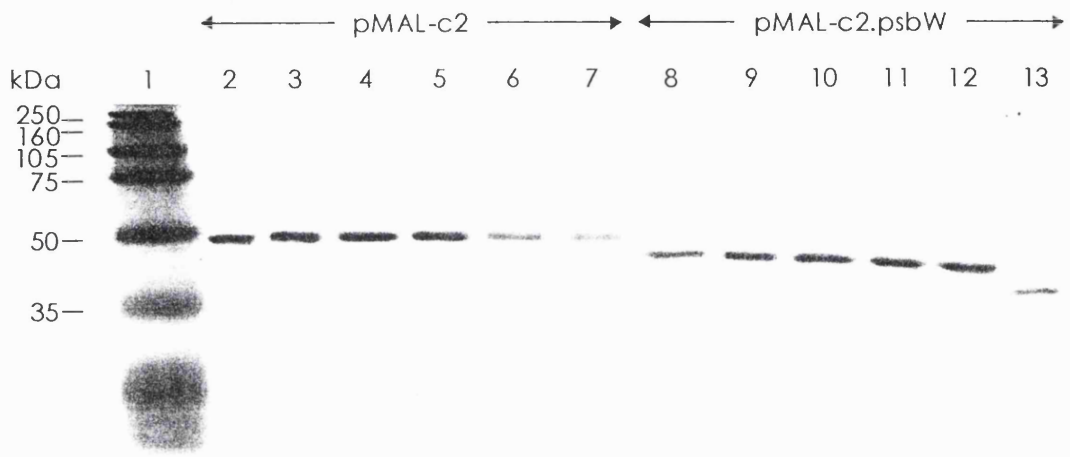
Two antibody samples were received from Eurogentec - SK26 and SK27. To determine which of these has the higher affinity for the PSII-W protein, a series of optimisation experiments were performed. Figure 4.5 shows the western blots that resulted from the establishment of a reliable gel system and from the antibody optimisation. Initial western analysis was performed with the isolated MBP~PSII-W fusion protein. This established that the antibody SK26 (Blot 1) produced cleaner blots with lower background than those obtained with the antibody SK27 (data not shown). The SK26 antibody was selected for further experiments. Following optimisation of the antibody concentrations for the immunological probing of the protein blots, the primary



LANE ORDER

1. protein markers
2. MBP undigested control
3. MBP 1 h digestion with Factor Xa
4. MBP 2 h digestion with Factor Xa
5. MBP 4 h digestion with Factor Xa
6. MBP 8 h digestion with Factor Xa
7. MBP 24 h digestion with Factor Xa
8. MBP~PSII-W undigested control
9. MBP~PSII-W 1 h digestion with Factor Xa
10. MBP~PSII-W 2 h digestion with Factor Xa
11. MBP~PSII-W 4 h digestion with Factor Xa
12. MBP~PSII-W 8 h digestion with Factor Xa
13. MBP~PSII-W 24 h digestion with Factor Xa

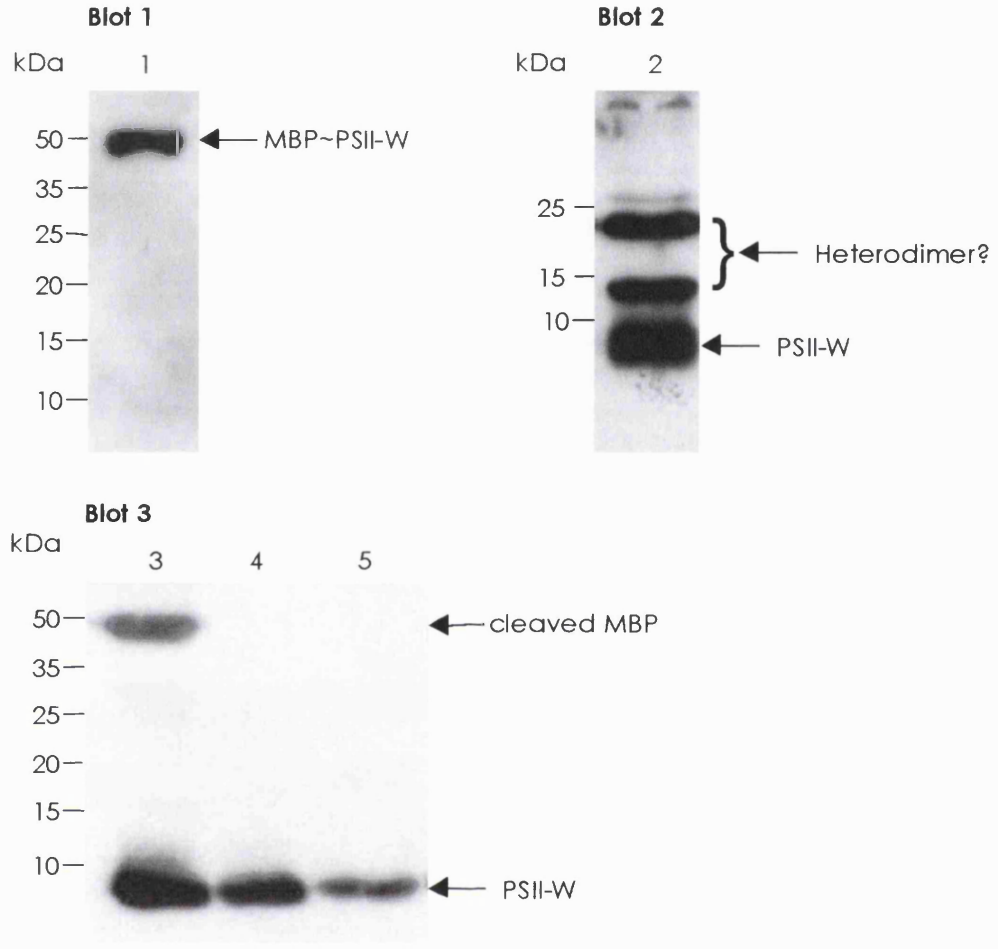
Figure 4.4 Coomassie stained SDS-PAGE of the products of a time course cleavage of MBP and the MBP~PSII-W fusion protein with Factor Xa
MBP and MBP~PSII-W were isolated using the amylose column from cells following 2 h IPTG induction. A time course digestion with Factor Xa (Section 2.8.1ii) was set up with samples taken at 1 h, 2 h, 4 h, 8 h and 24 h intervals for both proteins. The samples were then separated by SDS-PAGE. Lanes 2-7 show the MBP control. Lanes 8-12 show the fusion protein MBP~PSII-W. Only after 24 h is the fusion protein cleaved (Lane 13). The hydrophobic PSII-W protein does not stain with Coomassie Blue and the MBP is not amenable to cleavage with Factor Xa.



LANE ORDER

1. protein markers
2. MBP undigested control
3. MBP 1 h digestion with Factor Xa
4. MBP 2 h digestion with Factor Xa
5. MBP 4 h digestion with Factor Xa
6. MBP 8 h digestion with Factor Xa
7. MBP 24 h digestion with Factor Xa
8. MBP~PSII-W undigested control
9. MBP~PSII-W 1 h digestion with Factor Xa
10. MBP~PSII-W 2 h digestion with Factor Xa
11. MBP~PSII-W 4 h digestion with Factor Xa
12. MBP~PSII-W 8 h digestion with Factor Xa
13. MBP~PSII-W 24 h digestion with Factor Xa

Figure 4.4 Coomassie stained SDS-PAGE of the products of a time course cleavage of MBP and the MBP~PSII-W fusion protein with Factor Xa
MBP and MBP~PSII-W were isolated using the amylose column from cells following 2 h IPTG induction. A time course digestion with Factor Xa (Section 2.8.1ii) was set up with samples taken at 1 h, 2 h, 4 h, 8 h and 24 h intervals for both proteins. The samples were then separated by SDS-PAGE. Lanes 2-7 show the MBP control. Lanes 8-12 show the fusion protein MBP~PSII-W. Only after 24 h is the fusion protein cleaved (Lane 13). The hydrophobic PSII-W protein does not stain with Coomassie Blue and the MBP is not amenable to cleavage with Factor Xa.



LANE ORDER

1. 5µg MBP~PSII-W
2. Acetone treated PSII preparation
3. 5µg MBP~PSII-W treated with Factor Xa for 24 h
4. 4µg chlorophyll equivalent of a PSII preparation
5. 2µg chlorophyll equivalent of a PSII preparation

Figure 4.5 Western blots showing the optimised antibody concentration of the PSII-W antibody SK26, and evidence that the antibodies recognise the PSII-W protein
Blot 1. Western analysis of the isolated MBP~PSII-W protein with the optimised antibody concentrations.
Blot 2. Western analysis of acetone precipitated PSII preparation.
Blot 3. Western analysis of the cleaved MBP~PSII-W protein, and two aliquots of a PSII preparation.

NOTE
Antibody dilutions are as follows:
1° antibody (SK26) 1:2,000 (v/v); and
2° antibody 1:10,000 (v/v).

SK26 antibody was diluted 1:2,000 (v/v) and the secondary antibody was diluted 1:10,000 (v/v) prior to use.

In our hands the Laemmli SDS-PAGE gel system (following sample preparation with 2 x Laemmli Gel Loading Buffer) was not capable of resolving the PSII-W protein from chlorophyll present in *C. reinhardtii* thylakoid or PSII preparations. The proximity of the chlorophyll band to the PSII-W band hindered immunological detection of the PSII-W protein. A number of alternative sample preparation methods were explored. One of these, acetone precipitation of the thylakoid and PSII preparations (Section 2.8.2i), facilitated the removal of the chlorophyll molecules prior to separation by Laemmli SDS-PAGE. This method of sample preparation enabled the detection of the PSII-W protein from PSII preparations (Blot 2). Interestingly, when acetone precipitation is used the PSII-W protein also binds strongly to either itself and/or to other subunits of the PSII complex producing several bands (Blot 2). This method of sample preparation was not used in further western analysis.

Following the optimisation of the protein gel system protein samples were solubilised in 2 x Laemmli Gel Loading Buffer and resolved using the LiDS-Urea PAGE gel system. This gel system resulted in suitable separation of the PSII-W and chlorophyll to enable the detection of a single PSII-W band. Aliquots of MBP~PSII-W (5μg), Factor Xa digested fusion protein (5μg) and purified PSII (2μg and 4μg chlorophyll) preparation were subjected to the western blotting protocol. Blot 3 shows the optimised blots for the Factor Xa cleaved fusion protein following immunological probing with the SK26 antibody. It confirms that antibodies have been raised to both the MBP and PSII-W portions of the fusion protein. The detection of a PSII-W band in the purified PSII shows that PSII-W is a component of the PSII complex, and that the protein is processed to the mature size predicted from the cDNA sequence. The remainder of this chapter presents the western analysis of the PSII-W protein of *C. reinhardtii* using the SK26 antibody.

4.3.2 Western analysis of PSII-W in *C. reinhardtii*

4.3.2i Western analysis of whole cell, thylakoid and photosystem preparations

A paper was recently published by Hiyama *et al.*, (2000) which suggested the PSII-W protein might also be a component of the PSI complex. To confirm the location

of the PSII-W protein within the thylakoid membrane of *C. reinhardtii*, western analysis was performed on whole cell extracts, thylakoid membranes and photosystem preparations. Western analysis was initially performed on whole cell extracts from WT CC-1021 cells, PSII$^-$ cells (O'Connor *et al.*, 1998) and PSI$^-$ cells (Hallahan *et al.*, 1995). Whole cell samples were loaded on an equal cell basis, with thylakoid and photosystem preparations loaded on an equal chlorophyll basis. The blots were probed with the αPSII-W antibodies SK26.

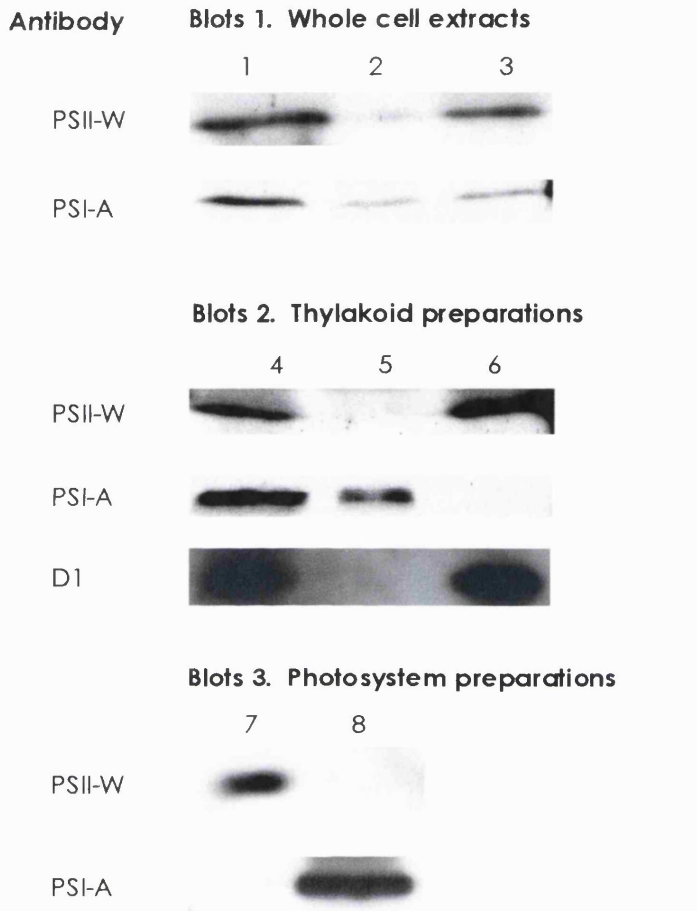
Figure 4.6 shows the resulting western blots. The presence of the PSII-W was detected in all three whole cell samples. Although PSII-W could be detected in cells lacking PSII, the levels were considerably lower than those of the WT CC-1021 and PSI$^-$ samples. When thylakoid preparations from the same strains were subjected to western analysis, the PSII-W could again be identified in the WT CC-1021 and PSI$^-$ thylakoids. However, PSII-W could not be detected in thylakoid samples from cells deficient in PSII. Western analysis of photosystem preparations resulted in detection of the PSII-W protein in PSII preparations. An equivalent band could not be detected in pure PSI preparations.

Blots 1 – 3 (Figure 4.6) were stripped and re-probed with antibodies to the PSI subunit PSI-A (a gift from Dr. Kevin Redding, University of Alabama) (1° antibody 1:1,000 (v/v), 2° antibody 1:10,000 (v/v)). The presence of this protein, and by inference the PSI complex, could be detected in each of the three whole cell samples. It could also be detected in WT CC-1021 thylakoids, PSII$^-$ thylakoids and PSI preparations. As expected this protein could not be detected in PSI$^-$ thylakoids or preparations of PSII.

Finally, and as a control, Blot 2 was stripped and re-probed with an anti-D1 antibody (a gift from Dr. Peter Nixon, Imperial College) (1° antibody 1:1,000 (v/v), 2° antibody 1:10,000 (v/v)). This western analysis further confirms the presence of the PSII complex in both the WT CC-1021 and PSI$^-$ thylakoids samples, and its absence in the PSII$^-$ thylakoids. This result also indicates that the levels of PSII in PSI$^-$ and WT CC-1021 thylakoids are similar, but not equivalent.

4.3.2ii Expression patterns of PSII-W in *C. reinhardtii* grown at 25°C and 37°C under varying light conditions

To investigate under what conditions the PSII-W protein is expressed in *C. reinhardtii*, the effects of light intensity and temperature were studied. WT CC-1021 cells



LANE ORDER

1. WT CC-1021 whole cell extracts
2. PSII⁻ (psbH⁻) whole cell extracts
3. PSI⁻ (C575D+) whole cell extracts
4. WT CC-1021 thylakoid preparation
5. PSII⁻ (psbH⁻) thylakoid preparation
6. PSI⁻ (C575D+) thylakoid preparation
7. PSII preparation
8. PSI preparation

Figure 4.6 Western analysis shows that the PSII-W protein is exclusive to PSII in *C. reinhardtii*

Western analysis of CC-1021, PSII⁻ and PSI⁻ whole cell extracts, WT CC-1021, PSII⁻ and PSI⁻ thylakoid preparations and photosystem preparations. Blots were immunologically probed with antibodies to both the PSII-W and PSI-A subunits (Section 2.8.5). Blot 5 was probed with an antibody to D1.

NOTE

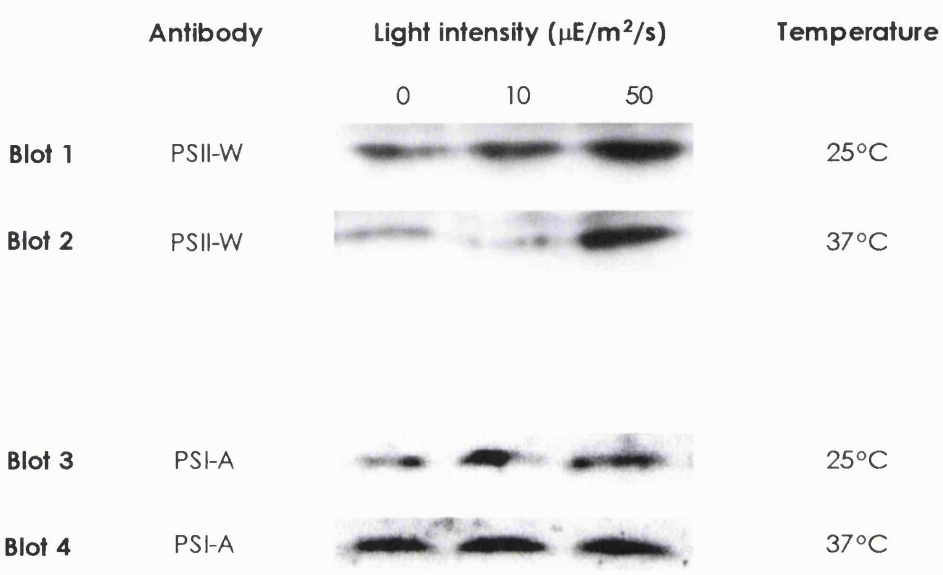
Antibody dilutions are as follows:

1° antibody (SK26)	1:2,000 (v/v);
1° antibody (αPSI-A)	1:1,000 (v/v);
1° antibody (αD1)	1:1,000 (v/v); and
2° antibody	1:10,000 (v/v).

were grown at both 25°C (standard conditions) and 37°C (stress conditions) in the dark, under low light (10μE/m^2/s) and moderate light (50μE/m^2/s) conditions. Solubilised whole cell extracts were prepared. The samples were loaded on an equal cell basis, subjected to LiDS-Urea PAGE and were then transferred to Immobilon PVDF membrane using the western blotting protocol. Figure 4.7 shows the western blots produced following immunological probing of the blots with the SK26 antibody (1:2,000 (v/v)), and then re-probing with the αPSI-A antibody (1:1,000 (v/v)). In both cases the 2° antibody was diluted 1:10,000 (v/v). The *psaA* transcript is constitutively expressed, and as such re-probing with antibodies to PSI-A provides a means of confirming that the samples were equally loaded.

Examination of the levels of PSII-W at 25°C indicates that as the light levels increase so does the level of the PSII-W protein. A similar, but more exaggerated pattern was observed for PSII-W expression when cells were grown under temperature stress conditions at 37°C. The levels of PSI-A expression remain constant, regardless of the light intensity or temperature, confirming that the samples were loaded equally. Thus any changes in the levels of the PSII-W protein were a result of changes in the steady state level of this subunit, determined by the rates of PSII-W synthesis and turnover.

The levels of PSII-W expression and accumulation were also compared for the two temperature conditions. When cells were grown under moderate light conditions (50μE/m^2/s) the levels of PSII-W protein were comparable at the two temperatures examined. The levels of PSII-W accumulation appeared lower for cells grown at 37°C in the dark and under low light conditions (10μE/m^2/s) than for cells grown at 25°C and under the same light regimes.



BLOT ORDER

1. WT CC-1021 cells grown at 25°C
2. WT CC-1021 cells grown at 37°C
3. WT CC-1021 cells grown at 25°C
4. WT CC-1021 cells grown at 37°C

Figure 4.7 Western analysis of the expression of PSII-W shows variation following growth of *C. reinhardtii* under different light and temperature conditions
Blots 1 and 2 were immunologically probed with the 1° antibody SK26.
Blots 3 and 4 were immunologically probed with the 1° antibody αPSI-A (Section 2.8.5).

NOTE
Light conditions are as follows:

0μE/m²/s	dark;
10μE/m²/s	low light;
50μE/m²/s	moderate light.

Antibody dilutions are as follows:

1° antibody (SK26)	1:2,000 (v/v);
1° antibody (αPSI-A)	1:1,000 (v/v);
2° antibody	1:10,000 (v/v).

4.4 CONCLUSION

The coding sequence for the mature PSII-W protein was cloned into the expression vector pMAL-c2; antibodies were raised to a purified fusion protein MBP~PSII-W. Once optimised, no non-specific bands were observed following immunological probing of whole cell WT CC-1021 samples with the SK26 antibodies. This observations shows that there are no MBP-like proteins present in *C. reinhardtii*, and further that the pMAL-c2 expression system is highly suitable for raising antibodies to PSII and other *C. reinhardtii* proteins.

Western analysis with the SK26 antibody was successful in the detection of the PSII-W protein in WT CC-1021, PSII$^-$ and PSI$^-$ cell extracts, in WT CC-1021 and PSI$^-$ thylakoid preparations, and in PSII preparations. The presence of this protein could not be detected in PSII$^-$ thylakoid preparations or PSI preparations. A weak band corresponding to the mature PSII-W protein was detected in the PSII$^-$ cell extracts. This suggests that the PSII-W pre-protein is still expressed and processed and that low levels of unassembled mature protein are present even in the absence of an assembled PSII complex. One explanation for the decreased levels relative to the WT and PSI$^-$ samples is that, in the absence of PSII, the turnover of the mature PSII-W subunit is accelerated. The PSII-W band was not detected in thylakoid preparations from the PSII$^-$ cells. It is unclear why the presence of the PSII-W protein could be detected in PSII$^-$ whole cells samples but not in the PSII$^-$ thylakoid samples, since import into the membrane precedes thylakoid processing (Figure 3.8). This may indicate that the protein detected in the whole cell samples is not located in the thylakoid membrane and is lost during thylakoid purification.

Future investigations could be performed to establish if the unassembled protein is detectable in the PSII$^-$ cells and whether processing intermediates can be detected. Initially work using northern analysis would establish if the *psbW* transcript accumulated in the PSII$^-$ mutant or whether a feedback mechanism operates to down-regulate *psbW* expression in the absence of PSII assembly. If this was the case the next step would be to detect the presence of the protein in isolated chloroplasts from WT CC-1021 and PSII$^-$ cells (Sültemeyer *et al.*, 1988; Sültemeyer *et al.*, 1995).

To analyse the PSII-W processing intermediates, western analysis of isolated chloroplasts could be performed to determine if the partially processed PSII-W protein is

present in the stroma. Alternatively immunoprecipitation of the lysate of either whole cells or chloroplasts which had been briefly pulse-radiolabelled could be employed. This strategy, described by Howe and Merchant (1993), would be used to detect the presence and/or absence of the pre-protein in WT CC-1021 and PSII$^-$ cells and chloroplasts.

The PSI-A subunit was detected, as expected, in WT CC-1021 and PSII$^-$ cell extracts. It was also present in PSI$^-$ cell extracts. Analysis of the thylakoid and photosystem II preparations with αPSI-A antibodies confirmed the presence of the PSI-A protein, and by inference the PSI complex, in WT CC-1021 and PSII$^-$ cells and thylakoids, and in PSI preparations. As expected the PSI-A protein could not be detected in PSI$^-$ thylakoids and in the PSII preparation, the latter confirming the purity of the PSII preparation.

Western analysis of the thylakoid samples was also performed with the anti-D1 antibody. The presence of this protein, and by inference the presence of the PSII complex, was detected in both WT CC-1021 and PSI$^-$ thylakoid samples. No D1 band was produced for the PSII$^-$ thylakoid samples. This result confirmed the integrity of both of the mutants. It also established that while the levels of D1 (PSII) loaded in WT CC-1021 and PSI$^-$ thylakoids were not equivalent, they were similar.

Work by Hiyama *et al.* (2000) proposed that the PSII-W protein is also present within PSI. It should be noted that the presence of the PSII-W protein was not monitored with antibodies by Hiyama *et al.* (2000). Furthermore, the PSI preparations used were not well characterised. From the sequence analysis of PSII-W, it can be concluded that it is a strongly bound membrane protein that may bind to crude preparations of thylakoid complexes through hydrophobic interactions.

Using the SK26 antibodies raised to the mature PSII-W protein of *C. reinhardtii* western analysis was undertaken to investigate if PSII-W could be detected in PSI. Preliminary western analysis with the SK26 antibodies of purified PSII pre-treated with acetone demonstrated that the PSII-W protein binds strongly to either itself and/or to other subunits of the PSII complex (Section 4.3.1iv). Our explanation of the detection of PSII-W in PSI preparations by Hiyama *et al.* (2000) is therefore that this represents protein released from PSII that has adventitiously bound to their PSI. From our results, it can be concluded that the PSII-W protein is detected when PSII is present and PSI absent in a sample. The protein could not be detected in samples with PSI solely or when PSII

fwas lacking. These results show that PSII-W is present exclusively in the PSII complex of *C. reinhardtii*, and is absent from the PSI complex.

Examination of the expression of the PSII-W yielded some interesting results. Cells were initially grown under three light regimes at normal growth temperature. When the light intensity is changed from darkness to low light (10μE/m^2/s) and then moderate light (50μE/m^2/s) there is a gradual increase in the levels of PSII-W. A similar pattern is observed when the same light conditions are studies at the stress temperature of 37°C. These results support the findings at 25°C. Thus the expression and/or accumulation of this protein appear to occur in a light dependent manner.

The levels of the PSII-W protein at the same light conditions for the two different temperatures, 25°C and 37°C, can also be compared. There are two possible explanations for the results observed. Firstly, the expression and/or accumulation of PSII-W under stress temperature (37°C) may be down regulated when cells are grown in the dark or under low light (10μE/m^2/s) relative to the same light conditions at 25°C. Alternatively, under these light conditions at 37°C the rate of PSII turnover may be increased, relative to the PSII turnover rate at 25°C. Under moderate light conditions (50μE/m^2/s) the levels are similar for the two temperatures examined. While it is tempting to speculate why this might occur, more experiments are required to examine this question.

CHAPTER 5. CREATION AND ANALYSIS OF A *psbM* DELETION MUTANT IN *SYNECHOCYSTIS*

CREATION AND ANALYSIS OF A *psbM* DELETION MUTANT IN *SYNECHOCYSTIS*

5.1 INTRODUCTION

The function of the majority of the small subunits of the PSII complex is poorly understood, despite the creation and isolation of mutants for many of these. One of the small subunits of interest is the chloroplast-encoded subunit PSII-M (Section 1.6.7). To date there is no report of a *psbM* mutant in the literature, and very little is known about the PSII-M protein or the role that it plays within PSII. As part of this investigation of the small polypeptides of the PSII complex, a *psbM* deletion mutant was constructed. As discussed in Section 1.6.7 the *psbM* gene of *C. reinhardtii* is co-transcribed with the *psbZ* gene (formerly *ycf9*). Consequently it is plausible that a deletion in the *psbM* gene of *C. reinhardtii* may have an effect on the transcription of *psbZ* and the accumulation of the PSII-Z subunit. For this reason the model organism *Synechocystis* 6803 was selected for this work. This chapter details the creation, isolation and characterisation of a *psbM* deletion mutant in this cyanobacterium.

5.2 AIMS

The aim of the work undertaken in this chapter is to study the role of the PSII-M subunit by:

- creation of a deletion mutant in the *psbM* orf of *Synechocystis* 6803; and
- characterisation and analysis of the *psbM* mutant.

5.3 RESULTS

5.3.1 Alignment of the PSII-M protein sequences

The PSII-M protein sequence is available for a number of organisms. Figure 5.1 shows the alignment of these sequences with ClustalW (http://www2.ebi.ac.uk/clustalw/). As shown in Figure 5.1 there is high degree of identity for the PSII-M subunit at the

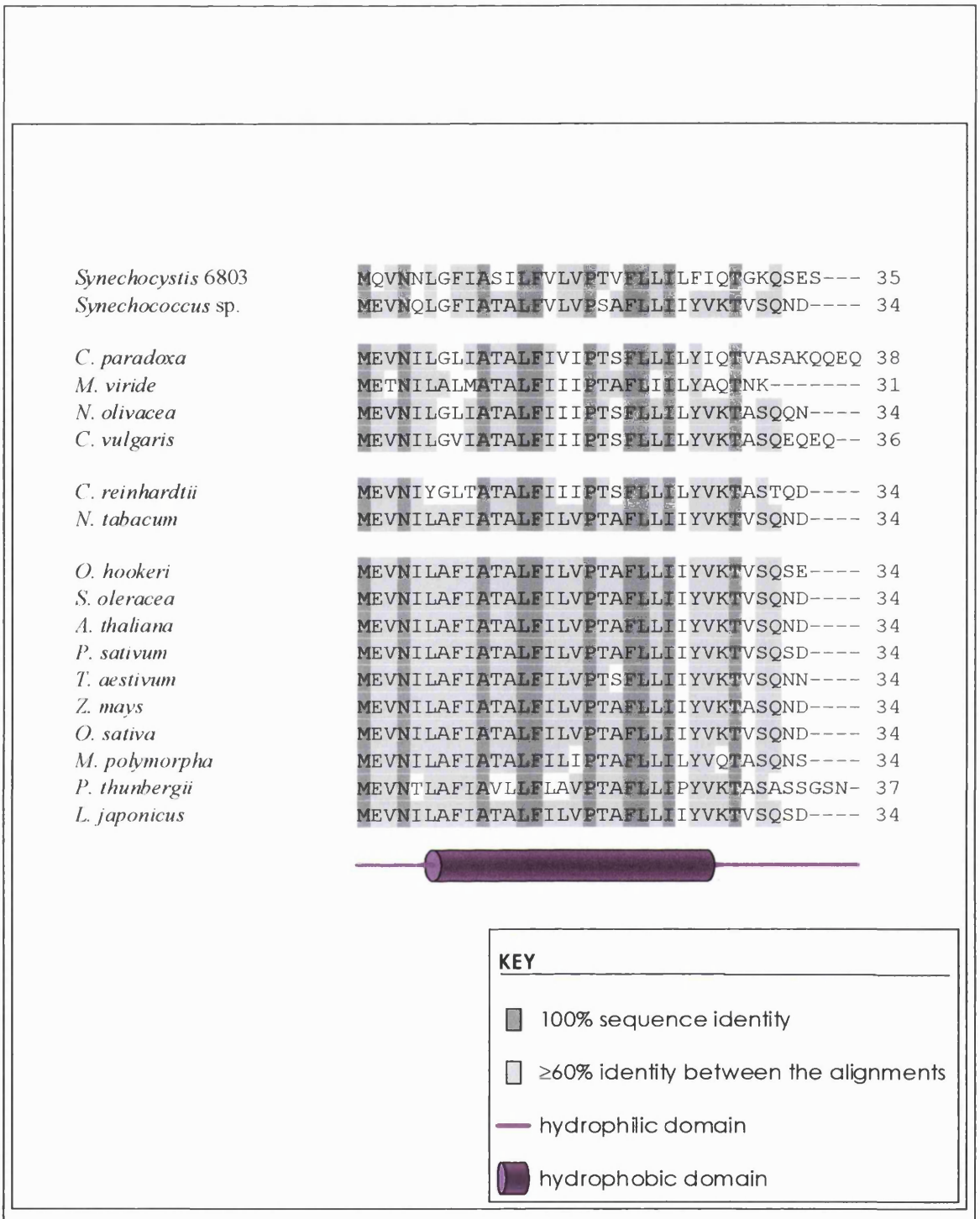


Figure 5.1 ClustalW alignment of the known PSII-M protein sequences
The sequences presented are *Synechocystis* 6803 (NP_440028), *Synechococcus* sp. (S05215), *C. paradoxa* (P48107), *M. viride* (Q9MUQ7), *N. olivacea* (AAD54779), *C. vulgaris* (BAA57919), *C. reinhardtii* (AAB39941), *N. tabacum* (P12169), *S. oleracea* (CAB88719), *A. thaliana* (NP_051053), *P. sativum* (BAA02098), *T. aestivum* (BAA78038), *Z. mays* (NP_043013), *O. sativa* (NP_039371), *M. polymorpha* (P12168), *P. thunbergii* (NP_042376), *L. japonicus* (P34833) and *O. hookeri* (CAB67147).

protein level, particularly for those sequences known for the higher plant protein. The PSII-M protein of *Synechocystis* 6803 has between 42-54% identity with those of *C. reinhardtii, A. thaliana, S. oleracea* and *N. tabacum*. The degree of similarity of the PSII-M protein of *Synechocystis* 6803 with the other PSII-M sequences presented in Figure 5.1 makes it a good candidate for the disruption/deletion of the *psbM* orf. The function of a number of the PSII subunits is conserved between photosynthetic prokaryotes and eukaryotes. However, there is evidence in the literature to indicate that some of subunits perform different or additional roles in eukaryotic PSII versus prokaryotic PSII. These subunits include PSII-K (Ikeuchi *et al.*, 1991; Takahashi *et al.*, 1994; Section 1.6.5) and PSII-Y (Gau *et al.*, 1998; Meetam *et al.*, 1999; Section 1.6.12). Consequently the phenotype of a given prokaryote photosynthetic mutant and that of a eukaryotic mutant may not be the same.

5.3.2 Generation of a *Synechocystis psbM* deletion mutant

5.3.2i Cloning *psbM* from *Synechocystis* 6803

The first stage in the generation of a deletion mutant in the *psbM* orf of *Synechocystis* 6803 was to clone the orf together with a suitable length of 5' and 3' flanking sequence. Using the fully sequenced genome for *Synechocystis* 6803 (Kaneko *et al.*, 1996), the region upstream and downstream of the *psbM* orf was examined. The *psbM* orf is assigned to the orf sml0003 of the genome. Two primers were designed to amplify the *psbM* orf together with 5' and 3' flanking regions (Appendix 2). The 5' primer, psbMH1 has a single base change to incorporate a unique *Hin*dIII site, and annealed 669bp upstream of the *psbM* orf. The 3' primer, psbMX1 annealed 975bp downstream of the *psbM* stop codon Genomic DNA was isolated from *Synechocystis* 6803 cells, and subjected to PCR with the primers psbMH1 and psbMX1. A PCR product of 1,751bp in length was produced; at position 1,402 of the PCR product was a unique *Xba*I site. The PCR product was subjected to the Qiagen gel extraction protocol followed by digestion with *Hin*dIII and *Xba*I to yield a product of 1,396bp. The plasmid vector pBluescript SK (Appendix 1) was also digested with the same two enzymes. Both digestions were separated in an agarose gel and subjected to the Qiagen gel extraction protocol. A ligation reaction with the gel purified products was set up and the product of this reaction was

transformed into competent *E. coli* DH5α cells. Sequencing with the primers T3 and T7 (Appendix 2) confirmed that the correct region of the *Synechocystis* genome had been amplified and cloned. Figure 5.2 shows the region of the *Synechocystis* 6803 genome used to generation of the 1,751bp PCR product. Figure 5.3 presents the annotated PCR product together with the map of the plasmid pSK.MHX.

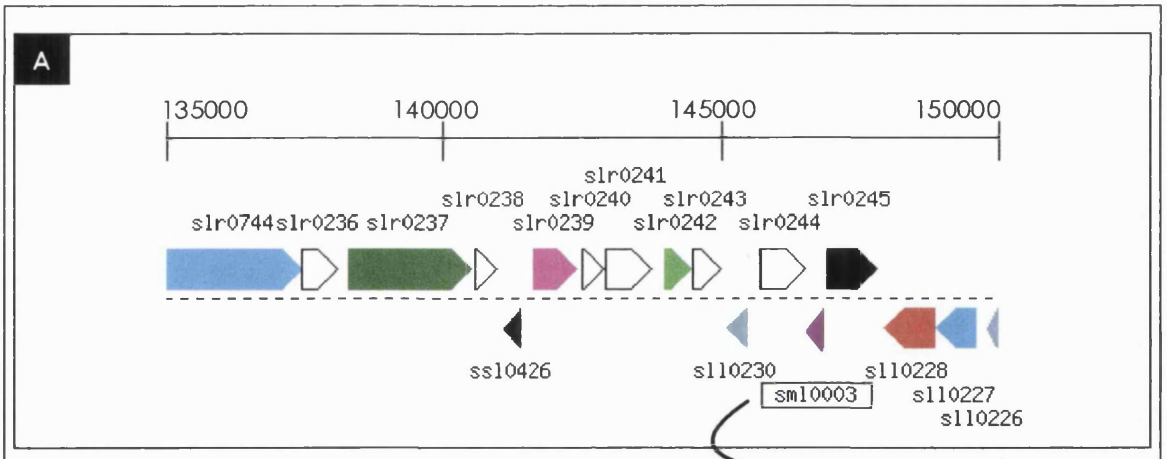
5.3.2ii Creation of pSK.MHX.Kn

Having cloned the *psbM* orf of *Synechocystis* 6803, together with 5' and 3' flanking regions, the next stage was to delete the orf to create a *psbM* mutant. A kanamycin resistance cassette (Appendix 2) was selected for this procedure. Present within the plasmid pSK.MHX are two *Hpa*I sites. The first was located at position 9 of the *psbM* orf (position 678 of the PCR insert). The second *Hpa*I site was located 9bp after the stop codon of the *psbM* orf (position 785 of the *psbM* PCR product). The plasmid pSK.MHX was digested with *Hpa*I to remove this 107bp region. At the same time the plasmid pUC4K (Appendix 1), which houses the kanamycin cassette, was also digested with *Hin*cII. Both the digested pSK.MHX plasmid and the 1,291bp fragment from the digestion of pUCK4K were subjected to the Qiagen gel extraction protocol. A blunt ligation reaction was set up with the two gel purified DNA fragments and resulted in the production of the plasmid pSK.MHX.Kn. The product of this reaction was transformed into competent *E. coli* DH5α cells. A schematic diagram showing the production of the plasmid pSK.MHX.Kn is presented in Figure 5.4.

The primers pK3 and pK7 (Appendix 2), which anneal to the 5' and 3' regions of the kanamycin resistance cassette, were used to confirm that the cassette had replaced the 107bp *Hpa*I fragment. The sequence data generated also established that the kanamycin resistance cassette had been inserted in the same orientation as the *psbM* containing insert.

5.3.2iii Transformation of *Synechocystis* 6803

The plasmid pSK.MHX.Kn was transformed into WT 6803, as detailed in Section 2.5.2. Transformants were selected on BG-11 supplemented with glucose and kanamycin (100μg/mL) under 7μE/m^2/s illumination at 30°C. When individual colonies were visible



B

147600 5'

AAGCACATTGTCGTGTTGACCCCGATCGCCAGCGGCCCCTGTGCCGGGATAGCAGGGAAA
TTGGTGCAGGGAACAATAGAAAATCCGGGGGTTATGGTCCACCAAaGCTTCAGTGCCATT
GCCATGGTGTACATCCCAATCTAAGATGGCTACCCGCTCCACCCCTGGTCTAGTTAAGGC
GTAATGGGCG → **500bp** ← ATTGCCCCAGGGCCATGACCCACCAATGCTATTCTAGG
TAGGGTATTGAGCCAAATTTAAGATCGAGGCAGTTTAGGCTATGCAAGTTAACAATCTCG
GCTTTATAGCAAGTATTTTATTTGTGTTGGTGCCAACGGTTTTCCTGTTGATCCTGTTTA
TTCAAACCGGGAAACAAAGCGAAAGCTAAGCCTTG**GTTAAC**CATTGTCAAACAGAACAGA
GTCAGGGGCGGAGTTGATCCAGCCCCTTTTTTTGCCGATAGTTAAACCCTGGGTA →
500bp ← TGATTGGTCAGTTGAAATACATACTGACTAACAGAATTT**TCTAGA**ATGGC
TTCCAACCGTTTCAAGCCCCGGGAACCCATAATAATCAGGTCCGCATCAATTTCGTTGGC
CACATCACACACCACTCCCTTGGGGTCTCCTTGGCGCAACACGGTGGAAACCTTACTGGG
TTCGATGGCCACATCCTCCAACAAGTCCGCCAGGATTTTACCGCCCTCGGCCCATTTTTC
CGTGAATGCCTCCGTGGTAATTTGGGGCGGAACCACGTGCAAAATCGTGATCGATGCCTT
TTGCACCGCCGGAAAGTCCATCATGGCCTTGAGCATTTCTTGGGTTTGGCTGGTGCCAGA
ATCTGCGTATAAAATTTTGCTAAGCATGATAATTGAGTTCCTTGAATCGGTTCAGGGAGA
GTCTCACCGTGCCATGGAGATTGATAACGGCAAGCCCTCCTCGGTTAAGCCTTATCCCTT
CAGAATAAGGCCAATGCAAGCGCAGAAATTTAACGACTTGTTGCGGACTTTCTCAGCCTT
GGTGGGCC 3' 145600

Primers employed (5' to 3')
psbMH1 CACCAAaGCTTCAGTGCCA
psbMX1 ACTCAATTATCATGCTTAGC

Figure 5.2 Generation of a PCR product containing the *psbM* orf, assigned to orf sml0003, together with 5' and 3' flanking sequence

A. The genome of *Synechocystis* 6803 from 135,000 to 150,000, annotated with the genes present in this region. (Cyanobase, http://www.kazusa.or.jp/cyano/cyano.html).

B. The nucleotide sequence of the *Synechocystis* 6803 genome on the reverse strand from 147,600 to 145,600. Annotated are the primers psbMH1 (in blue) and psbMX1 (in orange). The orf sml0003 is shown in purple.

NOTE

The following restriction enzyme sites are present, and underlined, within the 2,000bp presented above:

*Hin*dIII	A ↓AGCTT	(primer psbMH1);
*Hpa*I	GTT ↓AAC	(a. position 7 of the *psbM* gene),
		(b. 7bp after the stop codon of the *psbM* gene);
*Xba*I	T ↓CTAGA	(126bp after the stop codon of the *psbM* gene).

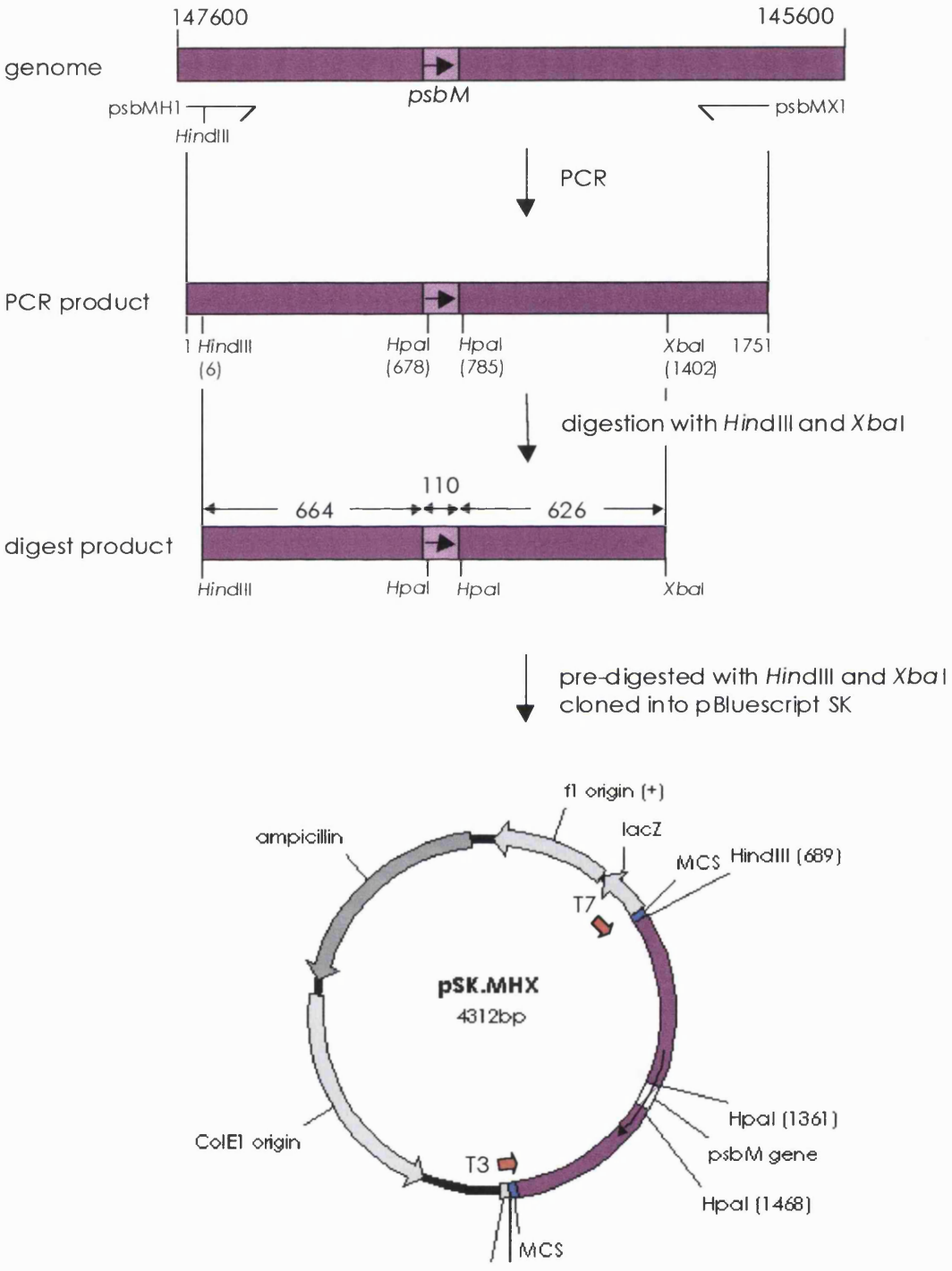


Figure 5.3 Generation of the plasmid pSK.MHX
The 1,396bp digested PCR product produced by the primers psbMH1 and psbMX1, containing the *psbM* orf of *Synechocystis* 6803, was cloned into the *Hin*dIII and *Xba*I sites of pBluescript SK.

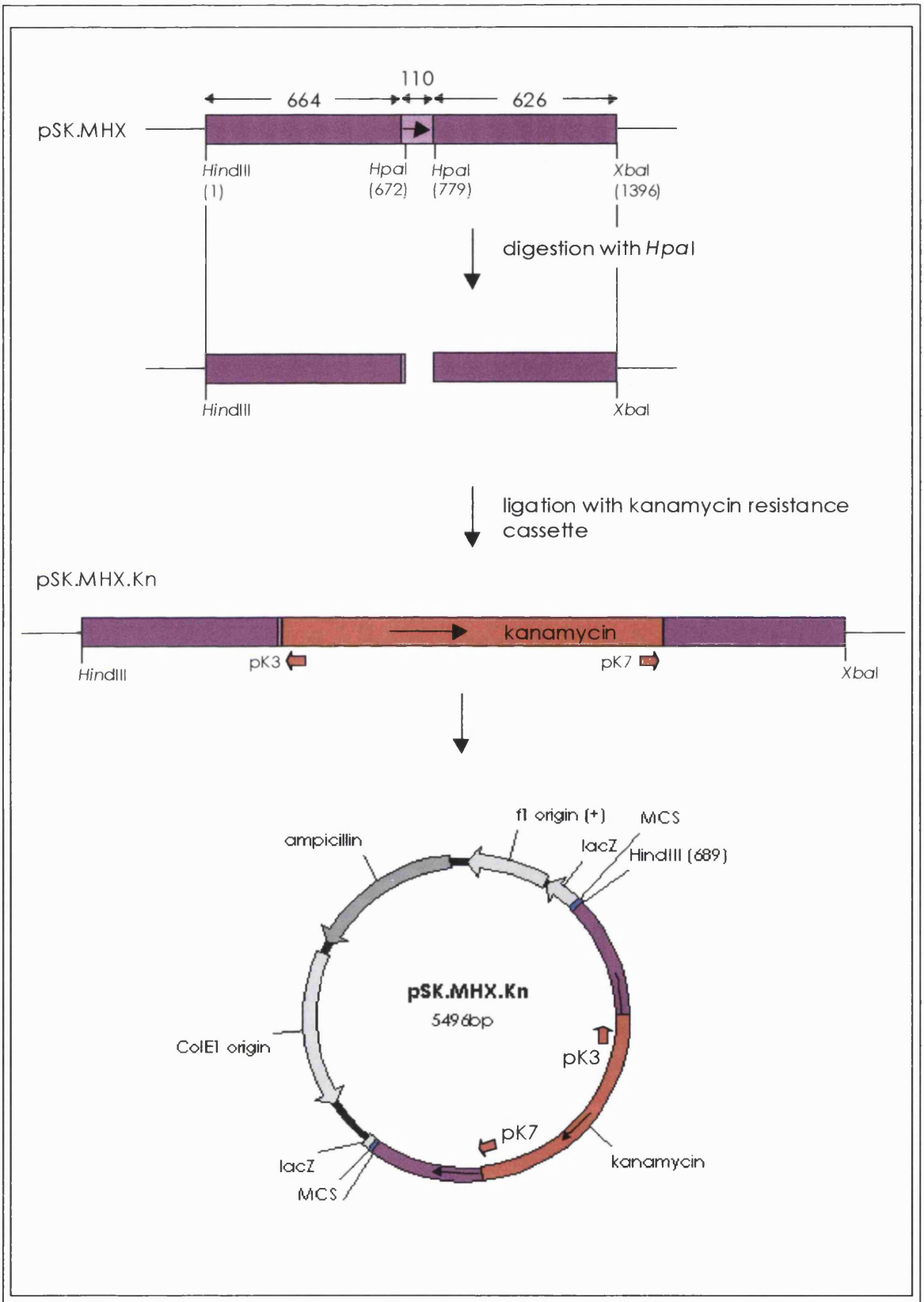


Figure 5.4 Generation of the plasmid pSK.MHX.Kn
The 1,291bp kanamycin reisitance cassette replaces the *Hpa*I fragement.

they were transferred to fresh plates and taken through three rounds of selection. The resulting transformants were called ΔpsbM8.1-14. Analysis was then undertaken to establish if the *psbM* orf had been replaced with the kanamycin resistance cassette and if the transformants were homoplasmic.

5.3.3 Characterisation and analysis of the *psbM* mutants

5.3.3i Southern analysis of the *psbM* mutants

Southern analysis was performed to establish if the ΔpsbM8.1-14 transformants isolated contained the *psbM* orf replaced by the kanamycin resistance cassette, and to establish if the transformants had reached a state of homoplasmicity. Total DNA was extracted from WT 6803 and the transformants ΔpsbM8.1-14. The DNA was digested with the restriction enzyme *Mfe*I. The digested DNA was then separated by gel electrophoresis in a maxigel and transferred to nylon membrane using the Southern blotting methodology. A radiolabelled probe was made from the 1,751bp *psbM* orf containing PCR product and used to probe the blot. Figure 5.5 shows the resulting autoradiogram. A single band of 2.8kb was present in the lane containing the WT 6803 DNA. The transformants ΔpsbM8.1-8.14 showed a larger band of 4.0kb. The increase in the band for the transformants was due to the presence of the kanamycin resistance cassette. The absence of a band at 2.8kb for each of the transformants confirmed that no WT copies of the *psbM* orf had been retained and that all of the transformants were homoplasmic. Transformants ΔpsbM8.3, 8.4 and 8.13 were chosen for further analysis.

5.3.3ii Growth tests of the *psbM* mutants

In order to compare the growth of the ΔpsbM transformants with WT 6803, spot tests were performed. WT 6803, a $PSII^-$ mutant and the transformants ΔpsbM8.3, ΔpsbM8.4 and ΔpsbM8.13 were grown to log phase. An aliquot of 10μL of each culture was spotted onto one of four plates: BG-11, BG-11 supplemented with glucose, BG-11 supplemented with kanamycin (100μg/mL), and BG-11 supplemented with glucose and kanamycin (100μg/mL). The cells were grown at 30°C under a light intensity of 7μE/m^2/s and the results are presented in Figure 5.6. The WT 6803 cells were capable of growth on

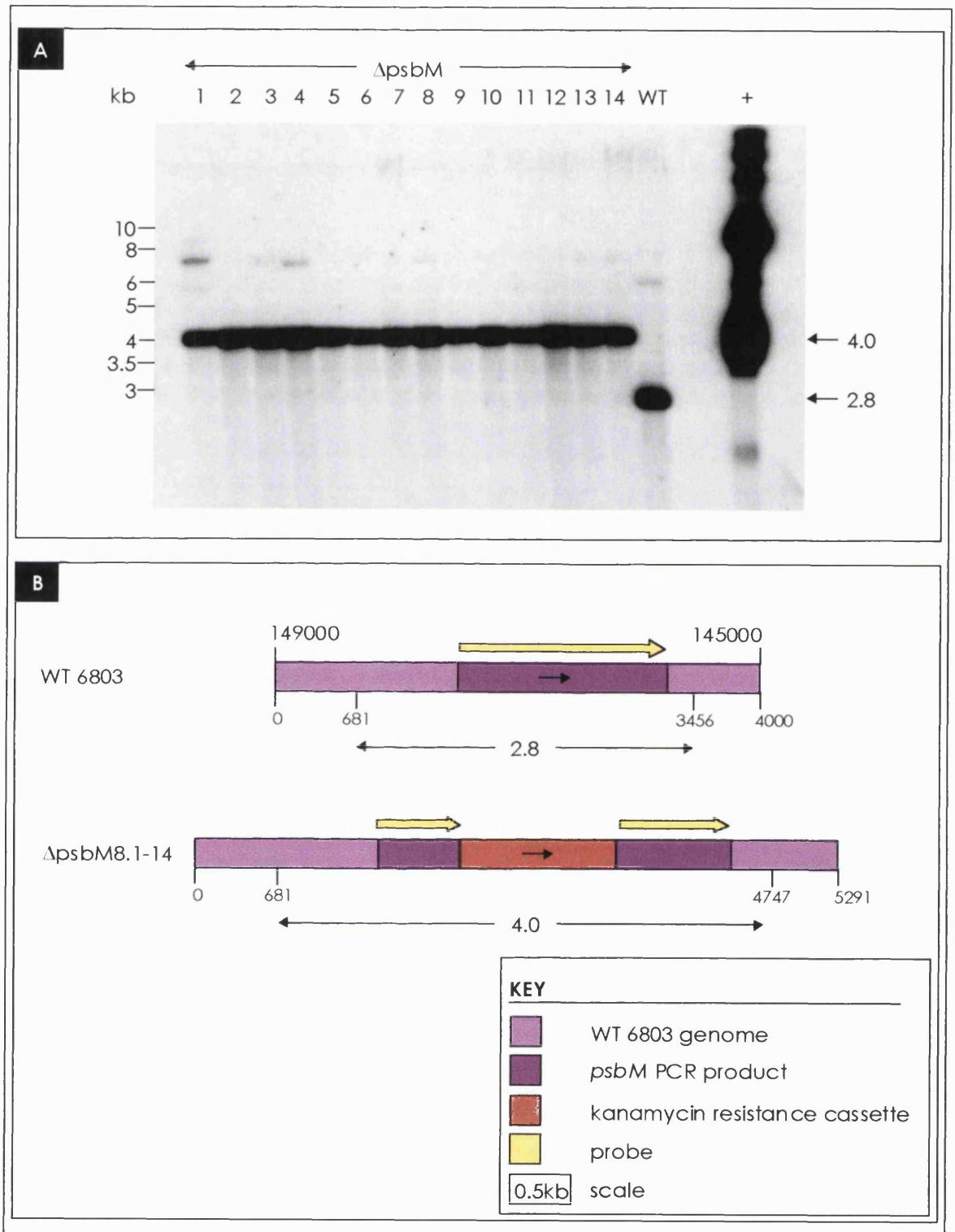


Figure 5.5 Southern analysis of WT 6803 and the ΔpsbM8.1-14 transformants with *Mfe*I
A. The Southern blot
Genomic DNA was prepared from WT 6803 cells and the transformants ΔpsbM8.1-8.14, and digested with *Mfe*I. The filter was probed with the 1,751bp PCR product containing the *psbM* orf. + is the lane containing <1ng of the plasmid psK.MHX.Kn.
B. The banding pattern
The predicted banding pattern following analysis of the genome of WT 6803 and the transformants ΔpsbM8.1-14 with the restriction enzyme *Mfe*I.

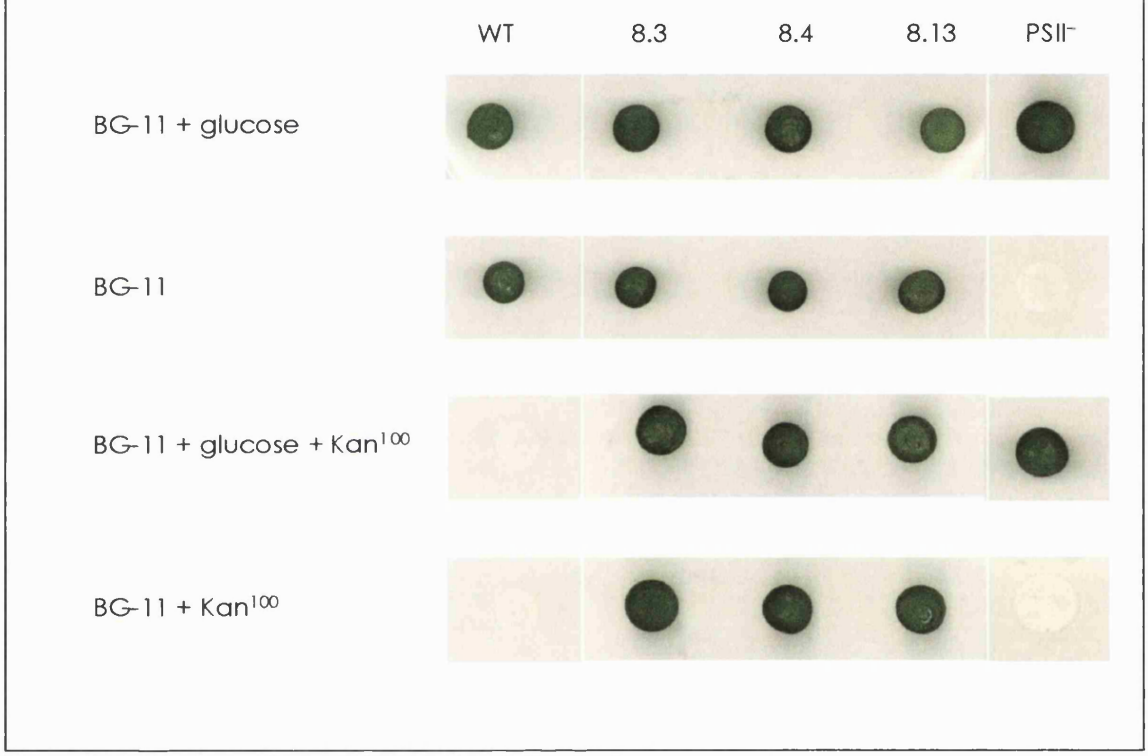


Figure 5.6 Spot tests of WT 6803 and the mutants ΔpsbM8.3, 8.4, 8.13 and PSII⁻
Spot tests for WT 6803 and the mutants ΔpsbM8.3, 8.4, 8.13 and PSII⁻ on BG-11, BG-11 supplemented with glucose, BG-11 supplemented with kanamycin (100μg/ml) and BG-11 supplemented with glucose and kanamycin (100μg/ml). An aliquot of 10μl of each culture was spotted onto each of the plates, and grown at 30°C at a light intensity of 7μE/m^2/s.

NOTE
Kan^{100}: refers to the kanamycin concentration (100μg/ml) in the corresponding plate.

BG-11 plates and BG-11 plates supplemented with glucose. They could not grow in the presence of kanamycin. The PSII$^-$ mutants were not capable of growth in the absence of glucose. The *psbM* mutants were capable of growth on all four plates. It can be concluded that the *psbM* mutants were capable of photoautotrophic growth and were resistant to kanamycin.

5.3.3iii Doubling times for the *psbM* mutants

WT 6803 and the *psbM* mutants ΔpsbM8.3, 8.4 and 8.13 were grown in BG-11 medium under very low light conditions (3μE/m^2/s), low light conditions (10μE/m^2/s) and moderate light conditions (50μE/m^2/s) to a cell density of approximately 1.5 x 10^7 cells. The optical density (OD_{750}) of the cell cultures were then measured at intervals as described in Section 2.2.6.

The doubling times for the WT 6803 and *psbM* mutant cell cultures were determined in triplicate and the averages of these times are presented in Table 5.1. Under low and moderate light regimes the doubling times of the *psbM* mutants were comparable to those observed for WT 6803 cells. This indicates that the mutants were as 'fit' as the WT 6803 cells under these conditions. When cells were grown at very low light intensities (3μE/m^2/s) the mutant cells have a doubling time which was faster than the WT 6803 rate.

5.3.3iv Oxygen evolution rates of the *psbM* mutants

The rates of oxygen evolution for the *psbM* mutants ΔpsbM8.3, 8.4 and 8.13 were compared to those of WT 6803 cells. A cell suspension for each of the cell types standardised to 50μg/mL of chlorophyll was supplemented with 100mM of electron acceptors DMBQ and ferricyanide, and used for the oxygen evolution measurements. Table 5.2 shows the average rates obtained from three independent measurements. All three mutants were capable of rates of oxygen evolution comparable to that of WT 6803 cells.

Table 5.1 Doubling times for WT 6803 and the *psbM* mutants 8.3, 8.4 and 8.13 under constant light regimes

Light Intensity ($\mu E/m^2/s$)	Strain	Doubling time (h)	Standard error
3	WT 6803	94.6	±0.6
	Δ*psbM* 8.3	81.8	±0.5
	Δ*psbM* 8.4	83.2	±0.7
	Δ*psbM* 8.13	82.7	±0.6
10	WT 6803	25.6	±0.6
	Δ*psbM* 8.3	24.8	±0.9
	Δ*psbM* 8.4	25.0	±0.9
	Δ*psbM* 8.13	24.6	±0.9
50	WT 6803	15.3	±0.7
	Δ*psbM* 8.3	15.7	±0.5
	Δ*psbM* 8.4	16.2	±0.9
	Δ*psbM* 8.13	15.9	±0.6

Table 5.2 The average rate of oxygen evolution for WT 6803 and the *psbM* mutants in the presence of DMBQ and ferricyanide

Strain	Average oxygen evolution rate (μmol O_2/mg Chl/h)
WT 6803	38.6 ± 0.8
ΔpsbM8.3	42.7 ± 1.2
ΔpsbM8.4	33.9 ± 1.3
ΔpsbM8.13	32.3 ± 1.6

5.3.3v EPR analysis of the *psbM* mutant ΔpsbM8.13

EPR samples were prepared as detailed in Section 2.9.7. Figure 5.7 shows the resulting spectra. Figure 5.7A shows traces for dark adapted WT 6803 cells together with the *psbM* mutant ΔpsbM8.13 and a $PSII^-$ mutant. A typical Y_D^+ trace was produced by the WT 6803 cells and showed that the PSII complex was assembled and active. A similar

trace was observed for the mutant ΔpsbM8.13, indicating that the PSII complex was present and assembled in these cells. The PSII$^-$ cells lack the Y_D^+ signal. From this data it can be concluded that the absence of the PSII-M protein in the mutant ΔpsbM8.13 does not affect the assembly or stability of the PSII complex.

Figure 5.7B shows that $P700^+$ traces for WT 6803 cells and *psbM* mutant ΔpsbM8.13. The spectra confirm that PSI was also assembled in both cell types.

5.3.3vi 77K fluorescence emission spectroscopy of the *psbM* mutant ΔpsbM8.13

77K fluorescence emission spectroscopy was performed to follow the energy transfer to and between the photosystems. The samples used were WT 6803 and ΔpsbM8.13 cells grown at 3μE/m^2/s and adjusted to 10μM chlorophyll. Section 2.9.6 details the assignment of the fluorescence peaks. Figure 5.8 shows the 77K fluorescence emission spectra for WT 6803 and the *psbM* mutant ΔpsbM8.13 following excitation at 435nm (excitation of chlorophyll *a*) and 600nm (excitation of phycocyanin). Following excitation at 435nm there was a decrease in the peaks at 685nm and 695nm relative to the WT 6803 peaks. These peaks correspond to the fluorescence of the chlorophyll molecules associated with PSII, and as such indicate that the PSI:PSII ratio was altered in the mutant. The results support the view that the mutant has less PSII relative to PSI than the WT 6803 cells. The same conclusion can be drawn following excitation of the cells at 600nm.

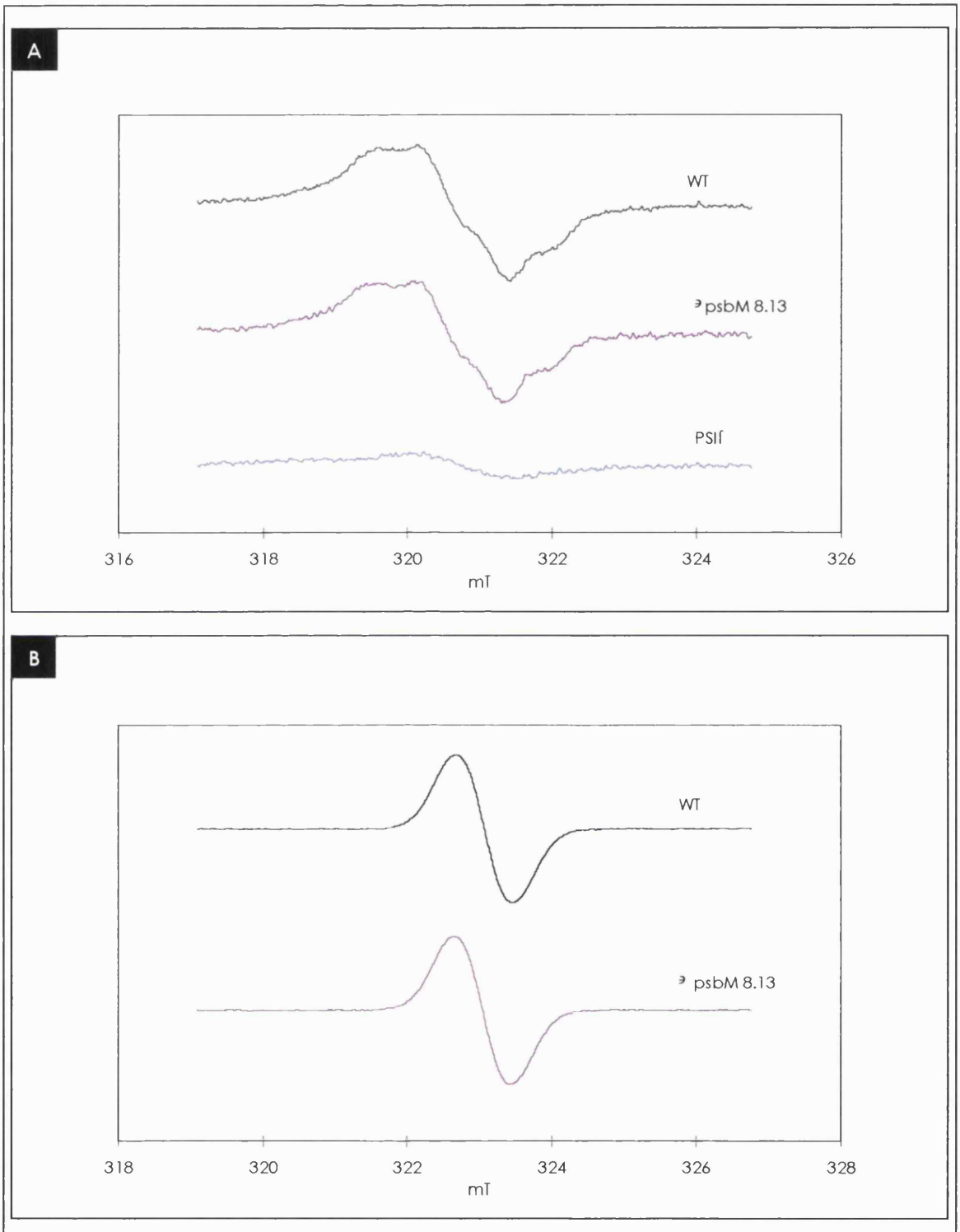


Figure 5.7 EPR analysis of the WT 6803 cells, and the mutants ΔpsbM8.13 and PSII⁻
A. EPR analysis of the Y_D^+ signal in WT 6803, ΔpsbM8.13 and PSII⁻ dark adapted *Synechcocystis* cells.
B. EPR analysis of the $P700^+$ signal in WT 6803 and ΔpsbM8.13 *Synechcocystis* cells.
The spectrum is the illuminated spectrum minus the dark spectrum.

NOTE
EPR conditions: microwave power 10^{-3}mW, modulation amplitude 0.2mT, temperature 10K.

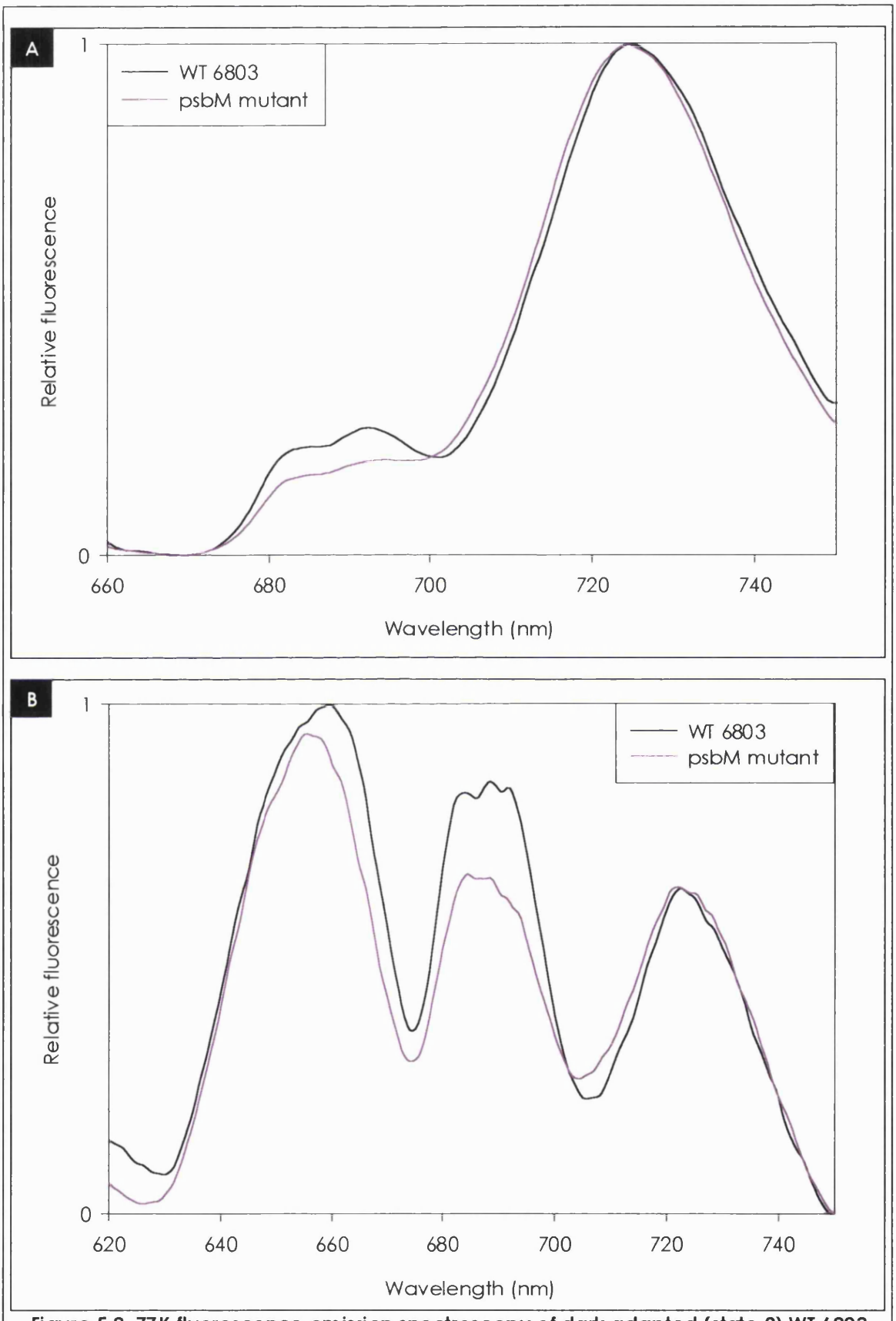


Figure 5.8 77K fluorescence emission spectroscopy of dark adapted (state 2) WT 6803 and ΔpsbM8.13 cells
A. Excitation at 435nm. B. Excitation at 600nm.
Both spectra are normalised to the PSI maximum at ~725nm.

5.4 CONCLUSION

The aim of this chapter was to investigate the role of the PSII-M subunit in *Synechocystis*. To this end, a deletion mutant was created in which an *Hpa*I fragment, which spanned most of the *psbM* orf, was replaced by the kanamycin resistance cassette. Three homoplasmic transformants were selected for characterisation. The mutants were capable of photoautotrophic growth and had oxygen evolution rates comparable to those of WT 6803 cells. Likewise the doubling times for the *psbM* mutants under low and moderate light conditions (10μE/m^2/s and 50μE/m^2/s respectively) were similar to those observed for WT 6803 cells. However, when cells were grown at very low light intensities (3μE/m^2/s) the mutant cells have a doubling time that was faster than the WT 6803 rate. Further work is required to be able to explain this result.

EPR analyses confirmed that the mutants were capable of assembly of functional PSII. Analysis of the 77K fluorescence emission spectra shows that the *psbM* mutants have an altered PSI:PSII ratio, relative to the WT levels, suggesting that in the *psbM* mutants there is a reduction in the levels of PSII relative to PSI. Further studies are required to confirm that the change in the PSI:PSII ratio is the result of a reduction in PSII rather than an increase in PSI. Comparison of the relative levels of each of the photosystems of WT and mutant thylakoid membranes could be achieved using non-denaturing 'green gel' electrophoresis. This sensitive method of electrophoresis enables the separation of the two photosystems. The levels of each of the photosystems can be compared by the relative intensities of the green bands. This strategy has been used successfully by Zak *et al.* (2001) for the analysis of *Synechocystis* thylakoids.

Characterisation of the individual photosystem bands could then be undertaken, to further confirm this quantification. Following excision of each of the green bands from the gel, the photosystems would be subjected to denaturing SDS-PAGE and the relative levels of photosystem subunits examined by immunoblotting. Finally, the excised photosystem fragments could be subjected to 77K fluorescence emission spectroscopy. This approach would be used as an alternative method to confirm the purity of each of the photosystem bands.

The ability of the *psbM* mutants to grow photoautotrophically indicates that PSII-M is not essential for PSII activity in *Synechocystis*. Moreover, the PSII-M subunit does not appear to be necessary for stable assembly of the PSII complex. The comparable

oxygen evolution rates and doubling times for the mutants indicate that this protein is not necessary for PSII function.

This work shows that the PSII-M subunit is a non-essential component of the PSII complex in *Synechocystis*. The role that some of the PSII subunits play within the complex appears to differ between oxygenic photosynthetic prokaryotes and eukaryotes. These include the subunits PSII-K (Ikeuchi *et al.*, 1991; Takahashi *et al.*, 1994; Section 1.6.5) and PSII-Y (Gau *et al.*, 1998; Meetam *et al.*, 1999; Section 1.6.12). This may also be the case for PSII-M. While this work does not provide specific information about the role of this protein, it does represent the first report of the generation and characterisation of a *psbM* mutant.

CHAPTER 6. CREATION AND ANALYSIS OF *psbZ* MUTANTS IN *SYNECHOCYSTIS*

CREATION AND ANALYSIS OF *psbZ* MUTANTS IN *SYNECHOCYSTIS*

6.1 INTRODUCTION

While it has been thought for some time that the protein encoded by the *ycf9* gene is a component of the PSII complex, definitive evidence has only been provided very recently (Swiatek *et al.*, 2001). Following this work the gene has been renamed *psbZ* and the gene product renamed PSII-Z (Section 1.6.13) (for consistency referred to in this text as *psbZ*). Previous work at UCL resulted in the creation of insertion and deletion mutants for the *psbZ* orf in *C. reinhardtii* (Cain, 1998). These mutants remained heteroplasmic, and were capable of photoautotrophic growth, but had reduced rates of oxygen evolution when compared to WT CC-1021 cells. In *C. reinhardtii* the *psbZ* gene is expressed on a dicistronic transcript with the *psbM* gene, which also codes for a subunit of the PSII complex. It could not be established if the effect of the mutations in the *psbZ* gene disrupted the expression and accumulation of the PSII-M protein. At the time there were no other reports of *psbZ* mutants.

This chapter details the generation and characterisation of two *psbZ* mutants derived from *Synechocystis* 6803. The genome of this cyanobacterium has been fully sequenced (Kaneko *et al.*, 1996). From examination of the genome it is clear that the *psbZ* gene, assigned as orf sll1281, is expressed as a monocistronic transcript, and that there are no other photosynthetic genes in the proximity of the gene. Thus the result of any mutations in the *psbZ* orf of *Synechocystis* can be examined without the potential for an indirect effect on other gene products.

In the work performed by Swiatek *et al.* (2001) the authors suggest a number of roles for the PSII-Z subunit. Their results indicate that the protein may act in the interaction of the light-harvesting antenna and the PSII complex. They go on to propose that the PSII-Z subunit may play a role in NPQ under photoinhibitory conditions. These theories have been addressed in this work.

6.2 AIMS

The aim of the work undertaken in this chapter is to investigate the function of the PSII-Z subunit by:

- the creation of *psbZ* (initially *ycf9*) mutants in *Synechocystis* 6803; and
- the characterisation of the *psbZ* mutants using both biochemical and biophysical analysis.

6.3 RESULTS

6.3.1 ALIGNMENT OF PSII-Z PROTEIN SEQUENCES

Figure 6.1 shows the alignment for the known protein sequences of the PSII-Z protein. The complete protein sequence of orf sll1281 is given in this alignment. It has been thought for some time that the start of translation was incorrectly assigned for this region of the genome. Evidence has recently emerged to confirm this (Kashino *et al.*, unpublished). N-terminal sequencing of PSII components isolated from His-tagged CP47-containing PSII has shown that the first six amino acids of the PSII-Z protein are MSIVFQ. Further support comes from the examination of the PSII-Z protein of *Anabaena* sp.. This protein is of a similar size to the other PSII-Z proteins presented in Figure 6.1, and lacks the extended N-terminus present in the *Synechocystis* 6803 amino acid sequence. Thus the first 50 amino acids encoded by orf sll1281 are not part of the PSII-Z protein.

As is evident in Figure 6.1 there is a high degree of identity between most of the PSII-Z proteins, particularly amongst those from higher plants. The common feature of these sequences is the presence of two distinct hydrophobic stretches, indicating that the PSII-Z protein is a protein with two transmembrane domains. These are annotated in Figure 6.1. The PSII-Z protein of *Synechocystis* 6803 has between 35-40% identity with those of *C. reinhardtii*, *A. thaliana*, *S. oleracea* and *N. tabacum*. This fact supports the use of *Synechocystis* 6803 for the study of the disruption of the *psbZ* orf.

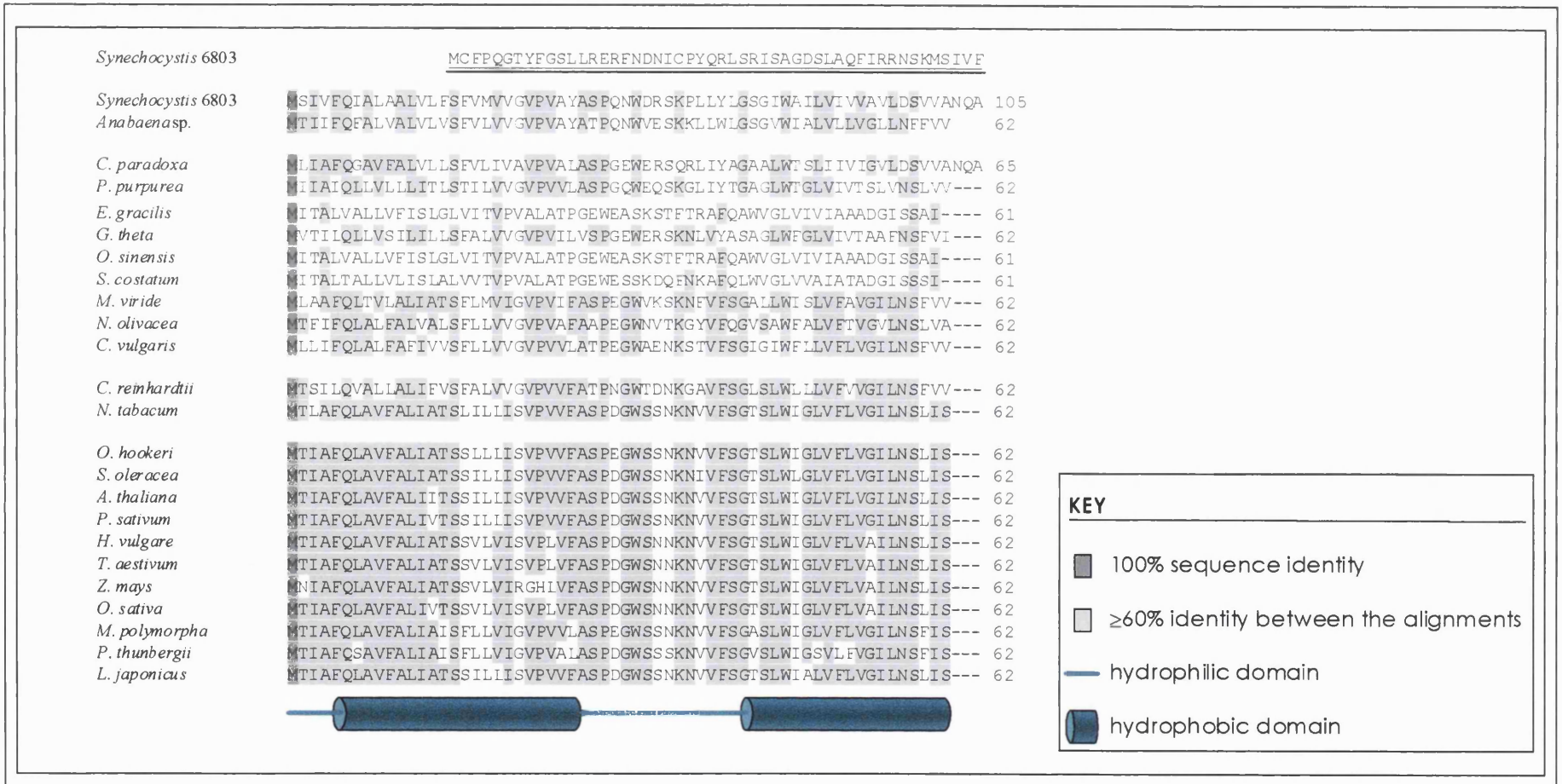


Figure 6.1 ClustalW alignment of the known PSII-Z protein sequences
The sequences presented are *Synechocystis* 6803 (BAA17568) (the derived protein sequence for orf sll1281 is presented. The first 50 amino acids (underlined) are not thought to be part of the PSII-Z protein sequence), *Anabaena* (AP003595), *C. paradoxa* (AAA81235), *P. purpurea* (P51316), *E. gracilis* (CAA50118), *G. theta* (O78503), *O. sinensis* (P49528), *S. costatum* (CAA10619), *M viride* (NP_038411), *N. olivacea* (NP_050873), *C. vulgaris* (NP_045822), *C. reinhardtii* (P92276), *N. tabacum* (P09974), *O. hookeri* (NP_084675), *S. oleracea* (NP_054930), *A. thaliana* (NP_051056), *P. sativum* (Q32902), *H. vulgare* (AAA79707), *T. aestivum* (NP_114246), *Z. mays* (Q33300), *O. sativa* (P12194), *M. polymorpha* (P09973), *P. thunbergii* (P41642) and *L. japonicus* (BAB33189).

6.3.2 Creation of *Synechocystis psbZ* mutants

This section presents the generation of two sets of mutants in the *psbZ* orf of *Synechocystis* 6803.

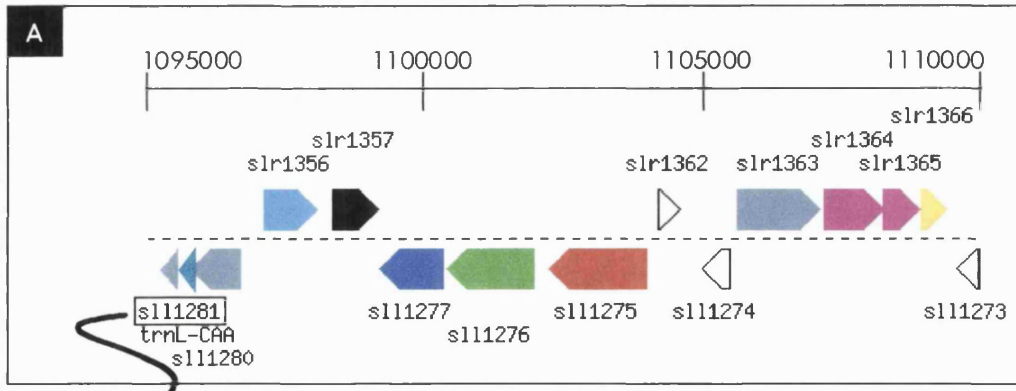
6.3.2i Cloning *psbZ* from *Synechocystis* 6803

The first stage in the generation of a mutant in the *psbZ* orf of *Synechocystis* 6803 was to clone the orf together with a suitable length of 5' and 3' flanking sequence. Genomic DNA was isolated from 12mL of *Synechocystis* 6803 cells. Using the fully sequenced genome for *Synechocystis* 6803 (Kaneko *et al.*, 1996) the region upstream and downstream of the *psbZ* orf was examined. The *psbZ* orf is assigned to the orf sll1281 of the genome (Figure 6.2). The primers Sycf93' and Sycf95' (Appendix 2) were designed to amplify the *psbZ* orf together with 3' and 5' flanking regions. Sycf93' annealed to a region 245bp downstream of the last codon of the *psbZ* orf, whilst Sycf95' annealed 452bp upstream of the start codon. These primers amplify a region of 886bp of the *Synechocystis* 6803 genome. The PCR product contains 5' and 3' sequence which flanks the 189bp *psbZ* orf. Figure 6.2 shows the region of the *Synechocystis* 6803 genome used to generate the 886bp PCR product.

Once the plasmid vector pUC19 (Appendix 1) had been digested with *Hin*cII, the digested plasmid and PCR product were run in an agarose gel and purified using the Qiagen Gel Extraction Kit. Blunt ligation of the gel purified products resulted in the production of the plasmid pSycf9, which was transformed into competent *E. coli* JM109 bacterial cells. Sequencing of the plasmid pSycf9 confirmed that the correct region of the *Synechocystis* 6803 genome had been amplified and cloned, and established the orientation of the insert. Figure 6.3 presents the annotated PCR product together with the map of the plasmid pSycf9.

6.3.2ii Creation of pKn2 and pKn5

Having cloned the *psbZ* orf of *Synechocystis* 6803, together with 5' and 3' flanking regions, the orf was disrupted to create a *psbZ* mutant. The kanamycin resistance cassette was selected for this procedure. The cassette would permit disruption of the *psbZ*



B

1096000 5' (anti-sense strand)

TGCTTTCATTGACCAAGCCCGGGAATACGATCGCCTGGGGGAAGATGAAATGGGGGCAAT
GCTGAGGAATATGCAGACCCAAAACCTGACCCATCCGGTGCCGGTACTCCGGGCCAGGGA
AATTGATCGTTGGAGTGATTCCCAAACCTATCAAAACTTGCTCAAGGGCCGCAAAAATGC
GTATAATCTGAACACCGAAGAAAACAAGGGCGGCTGGCGGAATTGGTAGACGCACCACAC
TCAAAATGTGGCGACTTCGGTCGTGAGAGTTCGAGTCTCTCGCTGCCCATCCTAAAACCT
AGATATGAACCGGTTCTGCTTAGGTCAACTCGTCTCCTTCGGGGTGAGTCCTTAATTACC
CTGGTTTCATTGACCGGGGAAAAATTTCCCACCCACAGAGCTAGGCGATCGCCTCTGGGT
AATCCGGCTATGTGCTTCCCTCAGGGCACATATTTTGGTAGCTTACTTAGGGAAAGATTC
AACGATAATATTTGCCCATATCAGCGACTGTCGAGGATTTCAGCCGGGGACAGTCTTGCT
CAATTTATAAGGAGAAACTCTAAAATGTCCATTGTTTTTCAGATTGCGTTGGCGGCCCTG
GTGCTTTTTTCCTTCGTGATGGTGGTGGGGGTGCCCGTAGCCTATGCTTCTCCCCAGAAC
TGGGACCGTTCTAAGCCTTTGCTGTATTTAGGTTCCGGCATTTGGGCCATTTTGGTAATT
GTGGTGGCCCTGTTGAACTTCCTGGTCGTCTAGGGGAGGCGATCGCCCTATCCCGGTAAT
TTCCCCATGGCCTGGGCTGTGGGGGATAGCCCCGAAAAACTCTGATCTACCGCTAACCCG
ATCTAGGGAACTACACCATGACAGTTTATGAAGGGTCGTTTACTCCTCCGGCCCGGCCAT
TTCGTTTTGCCCTTGTAATTGCCCGTTTCAATGATTTAGTAACGGAAAAGCTACTTTCTG
GTTGCCAAGATTGCCTCAAACGTCATGGCATTGATGTGGACCCCGCCGGTACCCAGGTGG
ACTATATTTGGGTTCCCGGTAGCTTTGAGGTGCCATTGGTAACCCGTAAGTTGGCAGTGT
CGGGGCAGTATGATGCCATTATCTGCCTAGGGGCGGTGATTCGGGGACAAACCCCCCATT
TTGATTTTGTGGCCGGAGAAGCGGCCAAGGGCATTGCGGCGATCGCCAGCCAGACTGGGG

3' 1094801

Primers employed (5' to 3')

Sycf95' GCCAGGGAAATTGATCGTTGGAGTG
Sycf93' CACATCAATGCCATGACGTTTGAGGC

Figure 6.2 Generation of a PCR product containing the *psbZ* gene, assigned to orf sll1281, together with 5' and 3' flanking sequence

A. The genome of *Synechocystis* 6803 from 1,095,000 to 1,110,000, annotated with the genes present in this region.
(Cyanobase, http://www.kazusa.or.jp/cyano/cyano.html).

B. The nucleotide sequence of the *Synechocystis* 6803 genome on the reverse strand from 1,096,000 to 1,094,801. Annotated are the primers Sycf9 5' (in blue) and Sycf93' (in orange). orf sll1281 is shown by the green text followed by the shaded *psbZ* sequence.

NOTE

The recognition site for *Bsm*FI is 5' GGGAC$(N)_{10}\downarrow$ 3'. The two *Bsm*FI sites present within this region of the genome are underlined in the sequence above; cleavage is at 1,095,459 and 1,095,324.

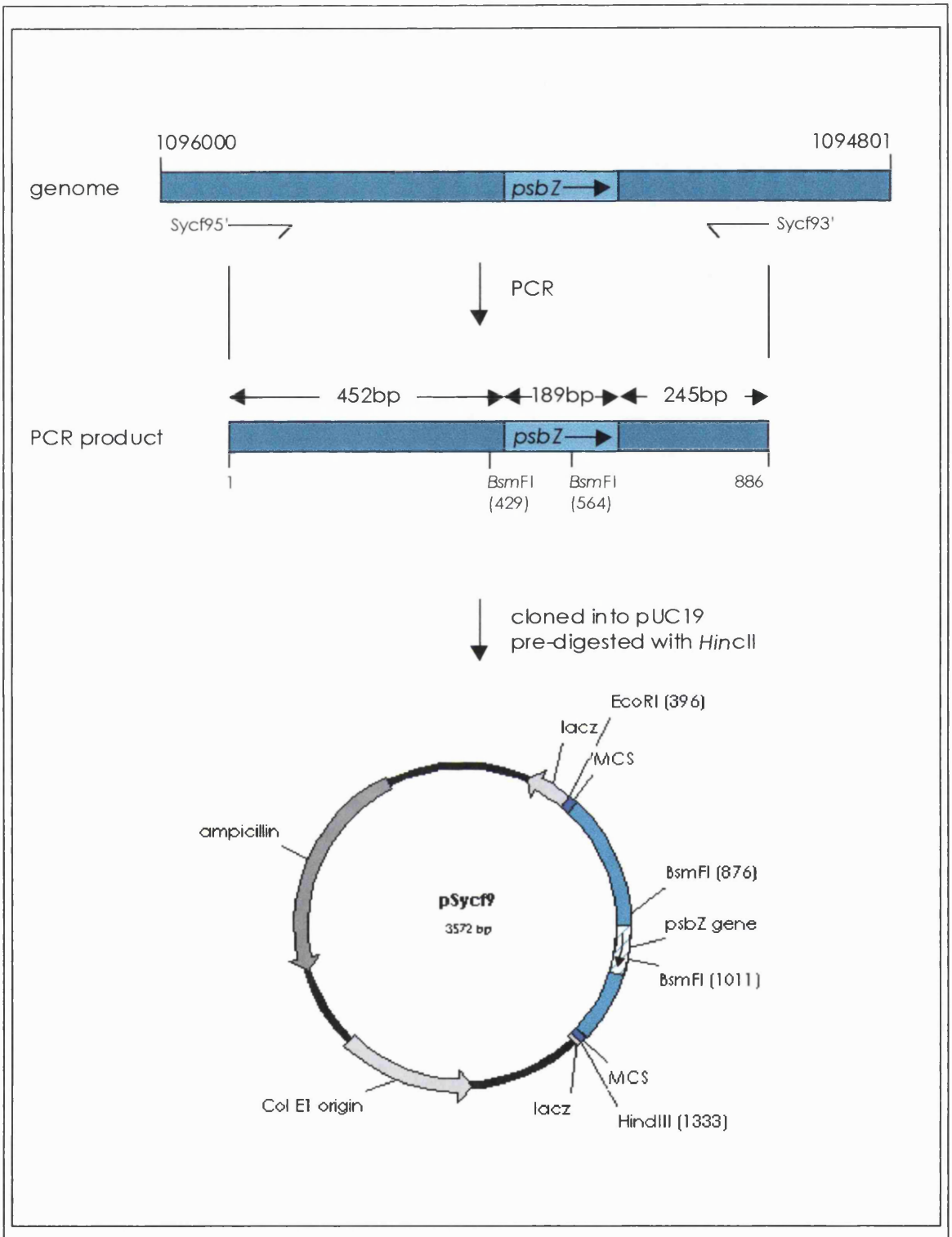


Figure 6.3 Generation of the plasmid pSycf9
The 886bp PCR product produced by the primers Sycf95' and Sycf93', containing the *psbZ* gene of *Synechocystis* 6803, was cloned into the *Hinc*II site of pUC19.

orf, and provides a suitable method for the isolation of transformants. Present within the region amplified by the primers Sycf93' and Sycf95' were two *Bsm*FI sites at positions 429 and 564 of the PCR product. These sites are annotated in Figure 6.3. The two sites were selected as targets for the disruption of the *psbZ* orf. Following digestion of the plasmid pSycf9 with *Bsm*FI, the plasmid was separated in an agarose gel and the resulting linear band was subjected to the gel extraction protocol. The gel purified product was then treated with mung bean nuclease to remove any 5' overhanging bases. At the same time the kanamycin resistance cassette was excised from the plasmid pUC4K (Appendix 1) by digestion with the blunt cutting enzyme *Hinc*II, and subjected to the gel extraction protocol. A ligation reaction was performed with the gel purified, mung bean nuclease treated linear pSycf9 and the kanamycin resistance cassette. The products of the ligation reaction were transformed into competent *E. coli* JM109 bacterial cells.

Sequence analysis of the *E. coli* transformants established that two populations of plasmids were produced. In the first instance the *Bsm*FI site at position 564 had failed to cut, and the kanamycin resistance cassette had inserted into the cleavage site at position 429. This resulted in production of the plasmid pKn2. Examination of the second population of *E. coli* transformants revealed that the kanamycin resistance cassette replaced the region between the two *Bsm*FI sites, at positions 429 and 564 of the *psbZ* containing insert. Consequently the deletion of 135bp of the *psbZ* orf was achieved. This plasmid was called pKn5. A schematic diagram showing the production of the plasmids pKn2 and pKn5 is presented in Figures 6.4 and Figure 6.5 respectively.

Sequencing of the plasmids pKn2 and pKn5 confirmed that in both plasmids the kanamycin resistance cassette was present in the opposite orientation to the *psbZ* orf. The *psbZ* PCR product specific primers Sycf93' and Sycf95' were used for these reactions.

6.3.2iii Transformation of *Synechocystis* 6803

The plasmids pKn2 and pKn5 were transformed into WT 6803, as detailed in Section 2.5.2. Transformants were selected on BG-11 supplemented with kanamycin (100μg/mL) under 7μE/m^2/s illumination at 30°C. When individual colonies were visible they were transferred to fresh plates and taken though three rounds of selection, again in the presence of kanamycin. The resulting mutants were called Kn2a-j and Kn5k-q. Analysis was then undertaken to establish if the mutants were homoplasmic.

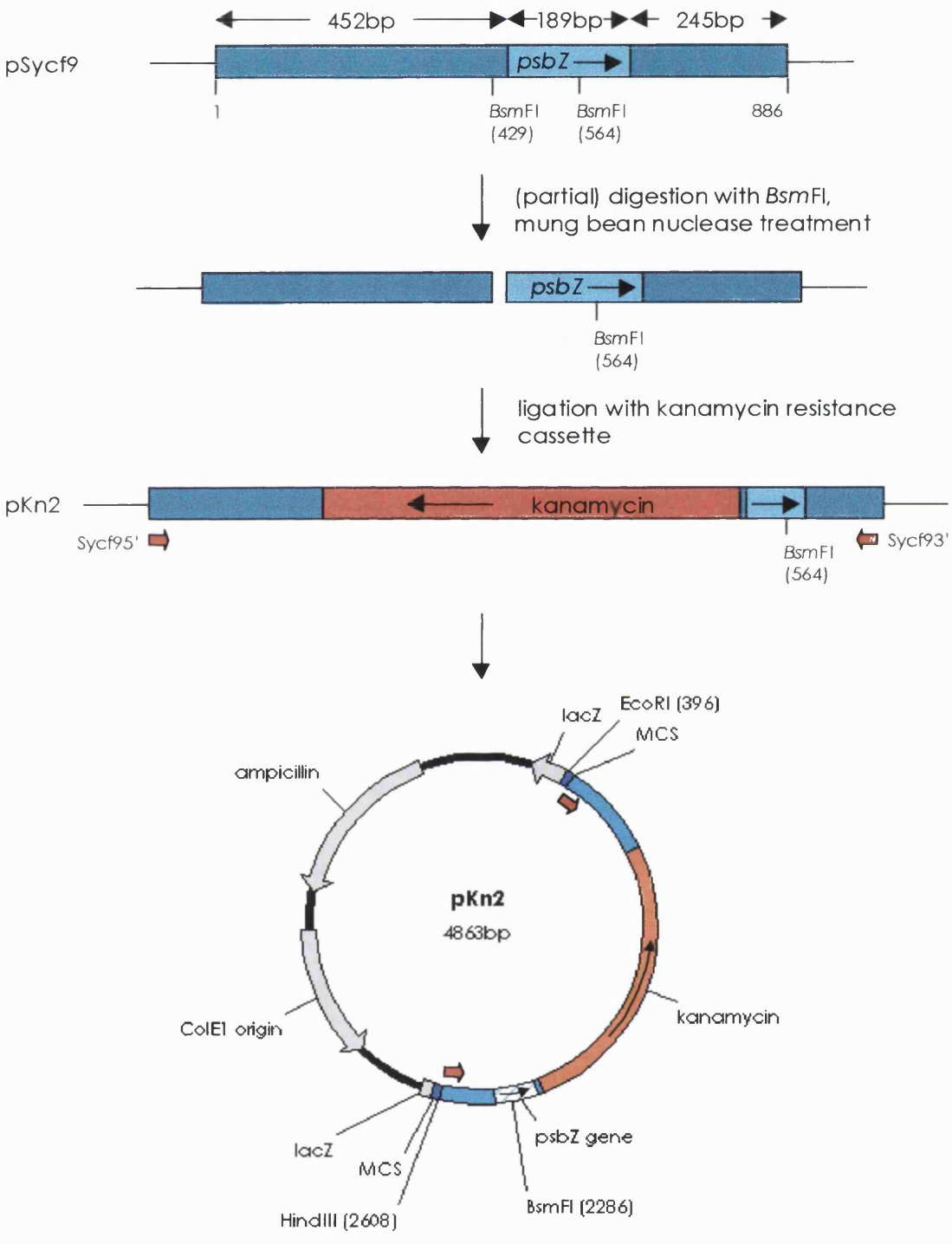


Figure 6.4 Generation of the plasmid pKn2
The 1,291bp kanamycin resistance cassette inserts into the *Bsm*FI site at position 429 of the *psbZ* containing insert of pSycf9.

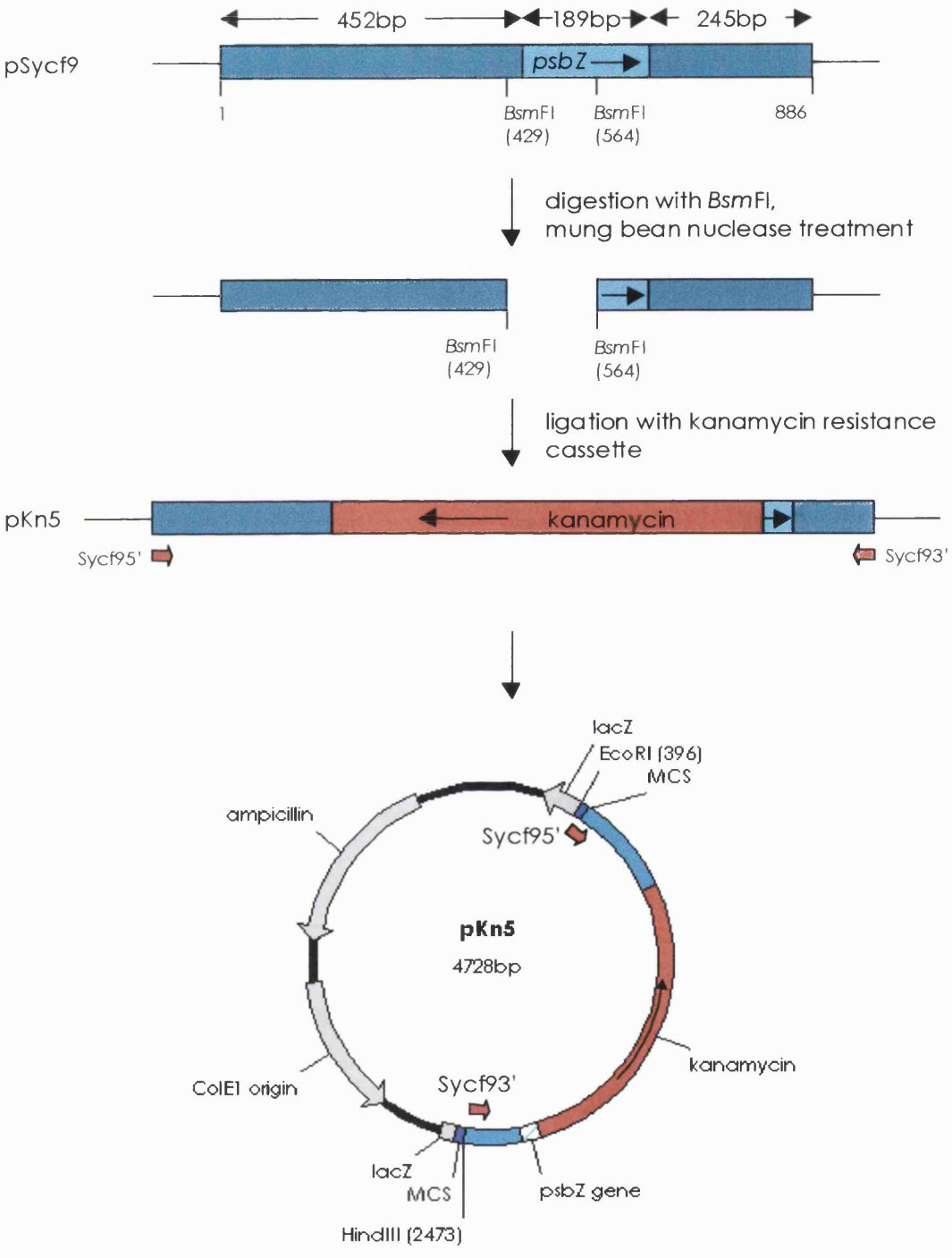


Figure 6.5 Generation of the plasmid pKn5
The 1,291bp kanamycin resistance cassette replaces the *Bsm*FI fragment.

6.3.3 Characterisation and analysis of the *psbZ* mutants

6.3.3i Southern analysis of the *psbZ* mutants

Southern analysis was performed to establish if the Kn2a-j and Kn5k-q transformants isolated were homoplasmic. Total DNA was extracted from WT 6803 and the Kn2 and Kn5 transformants. The DNA was digested with the restriction enzyme *Cla*I. The digested DNA was then separated by gel electrophoresis in a maxigel and transferred to nylon membrane using the Southern blotting methodology. A radiolabelled probe was made from the 886bp *psbZ* orf containing PCR product and used to probe the blot. Figure 6.6 shows the resulting autoradiogram.

The digestion of the WT 6803 DNA produces a single band of 7.1kb. Analysis of the band produced and the genomic sequence, leads to the conclusion that the *Cla*I site located at 8,296 of the WT 6803 genomic fragment presented in Figure 6.6 has not been cleaved. This *Cla*I site may be subject to methylation, or may be the result of a sequencing error. Consequently the 7.1kb fragment produced is the result of successful digestion at sites 2,283 and 9,423 of the WT 6803 genome. The transformants Kn2a-j, Kn5m and Kn5p show two bands. The first at 2.6kb was the same for both sets of mutants. The second band of ~4.8kb was slightly different depending of which mutant, Kn2 or Kn5 is examined. The Kn2 mutants produced a band of 4.9kb, whilst the Kn5 mutants, which lack 135bp of the *psbZ* PCR product have a second band of ~4.8kb. The presence of two *Cla*I sites within the kanamycin resistance cassette results in the production of these two bands, instead of the single band produced by the WT 6803 DNA. There were no other bands present within these lanes, and it can be concluded that these transformants were homoplasmic. The remaining Kn5 transformants (Kn5k-l, Kn5n-o, Kn5q) were heteroplasmic. This blot also confirms the presence of the kanamycin cassette in all of the transformants. The transformants Kn2a and Kn5m were selected for further analysis. In the text that follows Kn2a is referred to as Kn2 and Kn5m as Kn5.

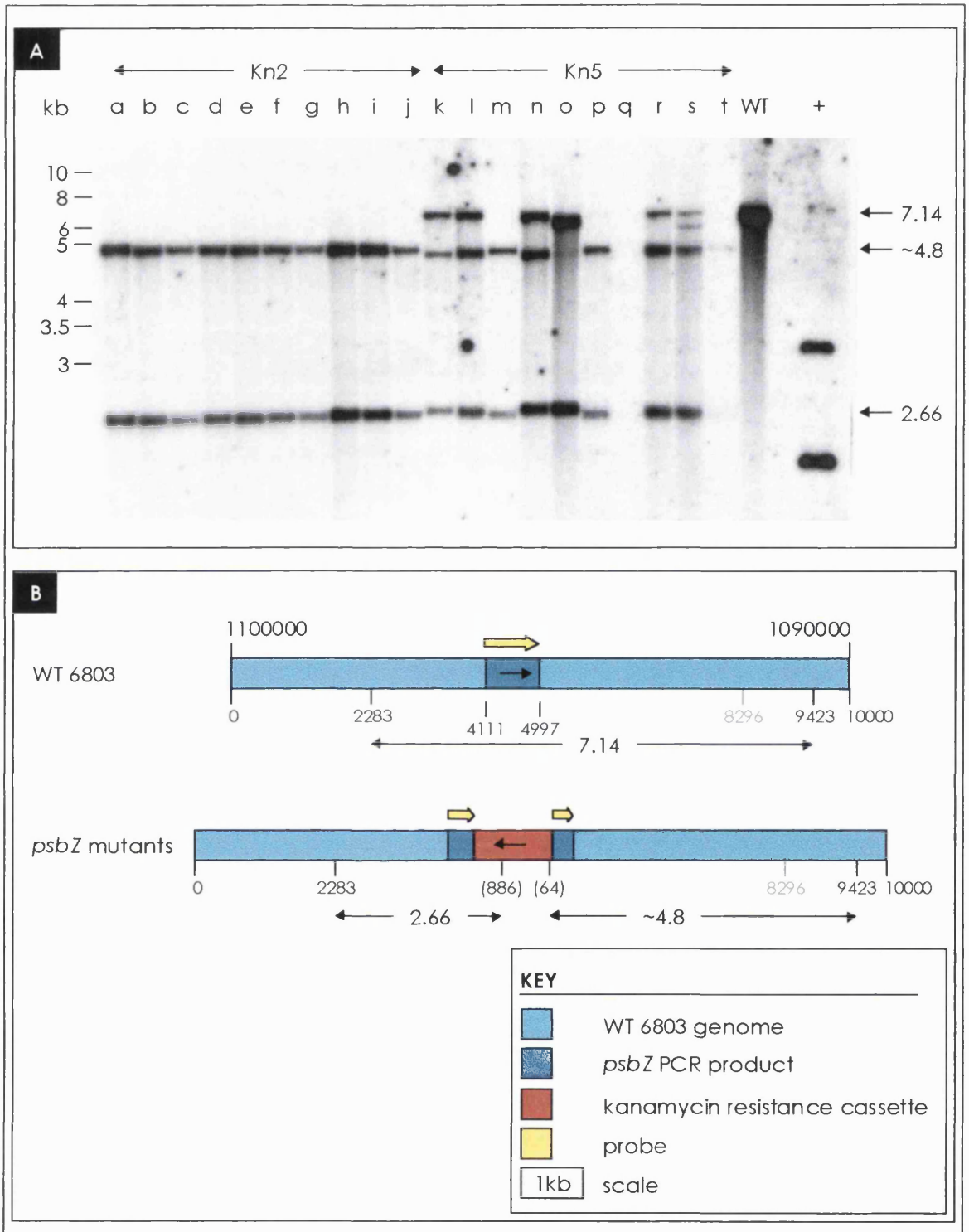


Figure 6.6 Southern analysis of WT 6803 and the transformants Kn2a-j and Kn5k-t with *Cla*I

A. The Southern blot

Genomic DNA was prepared from WT 6803 cells and the transformants Kn2a-j and Kn5k-t, and digested with *Cla*I. The filter was probed with the 886bp PCR product containing the *psbZ* gene. + is the lane containing <1ng of the plasmid Kn2.

B. The banding pattern

The predicted banding pattern following analysis of the genome of WT 6803 and the transformants Kn2 and Kn5 with the restriction enzyme *Cla*I. The band at ~4.8 is different for the mutants: Kn2 (4.947kb); Kn5 is 135bp smaller (4.812kb).

6.3.3ii Growth tests of the *psbZ* mutants

In order to compare the growth of the transformants Kn2 and Kn5 to WT 6803, spot tests were performed. WT 6803, Kn2, Kn5 and a PSII$^-$ mutant were grown to log phase. An aliquot of 10μL of each culture was spotted onto one of four plates: BG-11, BG-11 supplemented with glucose, BG-11 supplemented with kanamycin (100μg/mL), and BG-11 supplemented with glucose and kanamycin (100μg/mL). The plates were grown at 30°C under a light intensity of 7μE/m^2/s. The results are presented in Figure 6.7. The WT 6803 cells were capable of growth on BG-11 plates and BG-11 plates supplemented with glucose. They could not grow in the presence of kanamycin. The mutants Kn2 and Kn5 were able to grow on BG-11 plates and BG-11 plates supplemented with kanamycin. It can therefore be concluded that Kn2 and Kn5 were capable of photoautotrophic growth. However, the mutants grew poorly or not at all in the presence of glucose. This result was an unexpected one as the spot tests on BG-11 plates confirmed that Kn2 and Kn5 were capable of photoautotrophic growth. Southern analysis showed that the kanamycin resistance cassette has only inserted once into the genome of these mutants (data not shown). Thus, this phenotype was unlikely to be the result of a secondary integration of the cassette. It is possible that the mutations created in the Kn2 and Kn5 mutants had a knock on effect on the metabolism of glucose.

Other researchers have reported a glucose sensitive mutant phenotype. A *Synechocystis* mutant has been isolated by Hihara and Ikeuchi (1997), which arose from a base substitution in the gene designated *pmgA*. When cultured under mixotrophic conditions, in the presence of glucose and light, the growth of this mutant was severely inhibited. This phenotype is very similar to that observed for the *psbZ* mutants. The *pmgA* mutant outgrew WT 6803 cells under short-term exposure to high light. This contrasts with the phenotype of the *psbZ* mutants. The *psbZ* mutants were fitter than WT 6803 cells under very low light conditions but the *pmgA* mutant was not capable of sustaining prolonged growth under high light conditions.

Using the derived protein sequence for the PmgA protein bioinformatic analysis has been undertaken (this work). A Blast 2.2.1 search was performed against a database of 818,655 sequences (http:www4.ncbi.nlm.nhi.gov). The results suggest that the C-terminus of the PmgA protein has weak identity with a histidine kinase from *Methanothermobacter thermautotrophicus* (gi|15678202, e value = 0.25) and very weak identity with a second histidine kinase from *Bacillus halodurans* (gi|15613317 e value =

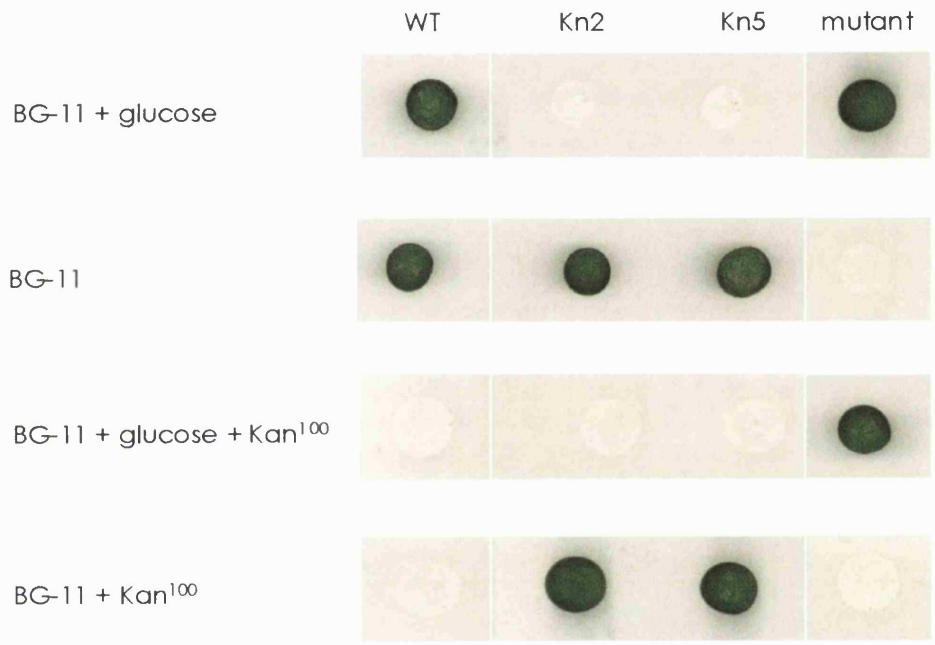


Figure 6.7 Spot tests of WT 6803, the *psbZ* mutants Kn2 and Kn5 and a photosynthetic mutant
Spot tests for WT 6803, Kn2, Kn5 and PSII$^-$ on BG-11 supplemented with glucose, BG-11, BG-11 supplemented with glucose and kanamycin (100μg/ml) and BG-11 supplemented with kanamycin (100μg/ml). An aliquot of 10μl of each culture was spotted onto each of the plates, and grown at 30°C at a light intensity of 7μE/m^2/s.

NOTE
Kan^{100}: refers to the kanamycin concentration (100μg/ml) in the corresponding plate.

1.2). While further studies would be required, this result may indicate a role for the PmgA protein in a signal transduction pathway. The absence of a histidine residue in the PSII-Z protein sequence rules out the possibility of a direct interaction between this potential histidine kinase and the PSII-Z subunit.

6.3.3iii Doubling times for the *psbZ* mutants

WT 6803 and the *psbZ* mutants Kn2 and Kn5 were grown in BG-11 medium under very low light conditions (3μE/m^2/s), low light conditions (10μE/m^2/s) and moderate light condition (50μE/m^2/s) to a cell density of approximately 1.5 x 10^7 cells. The optical density at 750nm of the cell cultures was then measured at intervals during the exponential growth phase as described in Section 2.2.6. It should be noted that after the initial exponential phase (about 24 to 48 hours) the doubling time of the cells cannot be accurately determined.

The average doubling times were calculated from three separate experiments using non-linear regression analysis of the exponential growth phase of a culture, and utilised Sigma Plot for Windows Version 5.0. The doubling times for the WT 6803, Kn2 and Kn5 cell cultures are presented in Table 6.1. Under low and moderate light regimes (10μE/m^2/s and 50μE/m^2/s respectively) the doubling times of the *psbZ* mutants were comparable to that of the WT 6803 cells. This indicates that the mutants were as fit as the WT 6803 cells under such conditions. When the doubling time for cells grown under very low light conditions (3μE/m^2/s) was investigated the mutant cells had a doubling time which was faster than that of the WT 6803 cells.

Table 6.1 Doubling times for WT 6803 and the *psbZ* mutants Kn2 and Kn5 under constant light regimes

Light Intensity (μE/m²/s)	Strain	Doubling time (h)	Standard error
3	WT 6803	94.6	±0.6
	Kn2	84.3	±0.6
	Kn5	83.1	±1.4
10	WT 6803	25.7	±0.5
	Kn2	26.2	±0.7
	Kn5	26.6	±0.6
50	WT 6803	15.9	±0.8
	Kn2	16.9	±0.5
	Kn5	16.2	±0.7

6.3.3iv Growth curves for the *psbZ* mutants

To compare the overall pattern of growth of the *psbZ* mutants at different light conditions, growth curves were set up for WT 6803 cells and the *psbZ* mutants Kn2 and Kn5 (Section 2.9.2). Measurements of cells grown under moderate light conditions (50μE/m^2/s) were taken approximately every 12 h, whilst those grown under low light (10μE/m^2/s) and very low light (3μE/m^2/s) were taken every 24 h. Figure 6.8 shows the growth curves produced. At light intensities of 50μE/m^2/s and 10μE/m^2/s the WT 6803 cells grow at an overall faster rate than the *psbZ* mutants. Under both these conditions the Kn5 mutant was the least 'fit'. Interestingly at very low light intensities (3μE/m^2/s) overall growth rates of the *psbZ* mutants exceeded those of the WT 6803 cells. Under these conditions the Kn5 cells were the fittest of the three cell types examined. Thus, the mutants were able to grow better in conditions where light was limiting. However, growth was impaired at light intensities of 10μE/m^2/s and above when compared to WT 6803. This data supports the results of the doubling time experiments (Section 6.3.3iii).

It should be noted that the measurements for the growth curves (Section 2.9.2) and the doubling times (Section 2.2.6) were taken at different time points, and were performed as separate experiments. Consequently the early exponential growth phase, used to determine the doubling time of the cells, in not evident on the graphs presented in Figure 6.8.

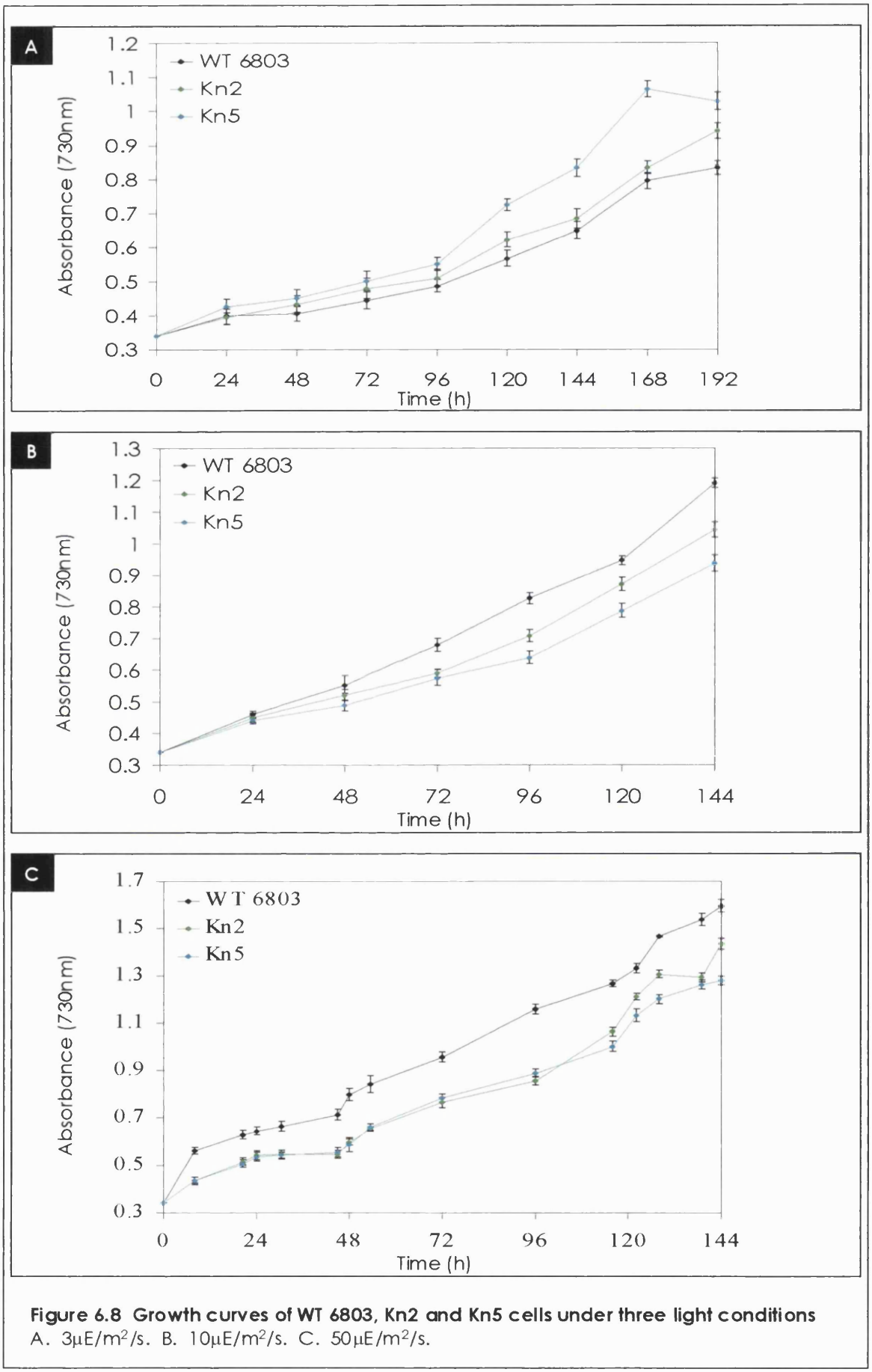


Figure 6.8 Growth curves of WT 6803, Kn2 and Kn5 cells under three light conditions
A. 3μE/m²/s. B. 10μE/m²/s. C. 50μE/m²/s.

6.3.3v Oxygen evolution rates of the *psbZ* mutants

The rates of oxygen evolution for the *psbZ* mutants Kn2 and Kn5 were compared to those of WT 6803 cells. A cell suspension for each of the cell types containing 50μg/mL of chlorophyll was supplemented with 100mM of both DMBQ and ferricyanide, and used for the oxygen evolution measurements. Both mutants were capable of oxygen evolution rates comparable to those obtained for WT 6803 cells (Table 6.2).

Table 6.2 The average rate of oxygen evolution for WT 6803 and the *psbZ* mutants in the presence of DMBQ and ferricyanide

Strain	Oxygen evolution rate (μmol O_2/mg Chl/h)
WT 6803	38.6 ± 0.8
Kn2	36.5 ± 1.9
Kn5	39.7 ± 0.7

6.3.3vi EPR analysis of the *psbZ* mutants

EPR samples were prepared as detailed in Section 2.9.7. Figure 6.9 shows the resulting spectra. Figure 6.9A shows traces for dark adapted WT 6803 cells together with the *psbZ* mutants Kn2 and Kn5 and a PSII$^-$ mutant. A typical Y_D^+ trace was produced by the WT 6803 cells, showing that the PSII core complex was assembled and active. For both of the *psbZ* mutants similar traces were observed, indicating that the PSII core complex is not compromised in these cells. The PSII$^-$ cells lack the Y_D^+ signal. From the EPR and oxygen evolution data (Section 6.3.3v) it can be concluded that the absence of the PSII-Z protein in the mutants Kn2 and Kn5 does not significantly affect the assembly of the RC core or water oxidation. The detection of the Y_D^+ signal confirms that PSII is able to oxidise Y_D via P680.

Figure 6.9B shows the $P700^+$ photoinduced traces for WT 6803 cells and both of the *psbZ* mutants. The spectra confirm that PSI was also functional in all three cell types. Comparative quantitative analysis was achieved by normalising the EPR signals to the same chlorophyll concentration. This showed that the levels of PSII (measured by the Y_D^+ signal) relative to PSI (measured by the $P700^+$ signal) were lower in the mutants Kn2 and Kn5, than in the WT.

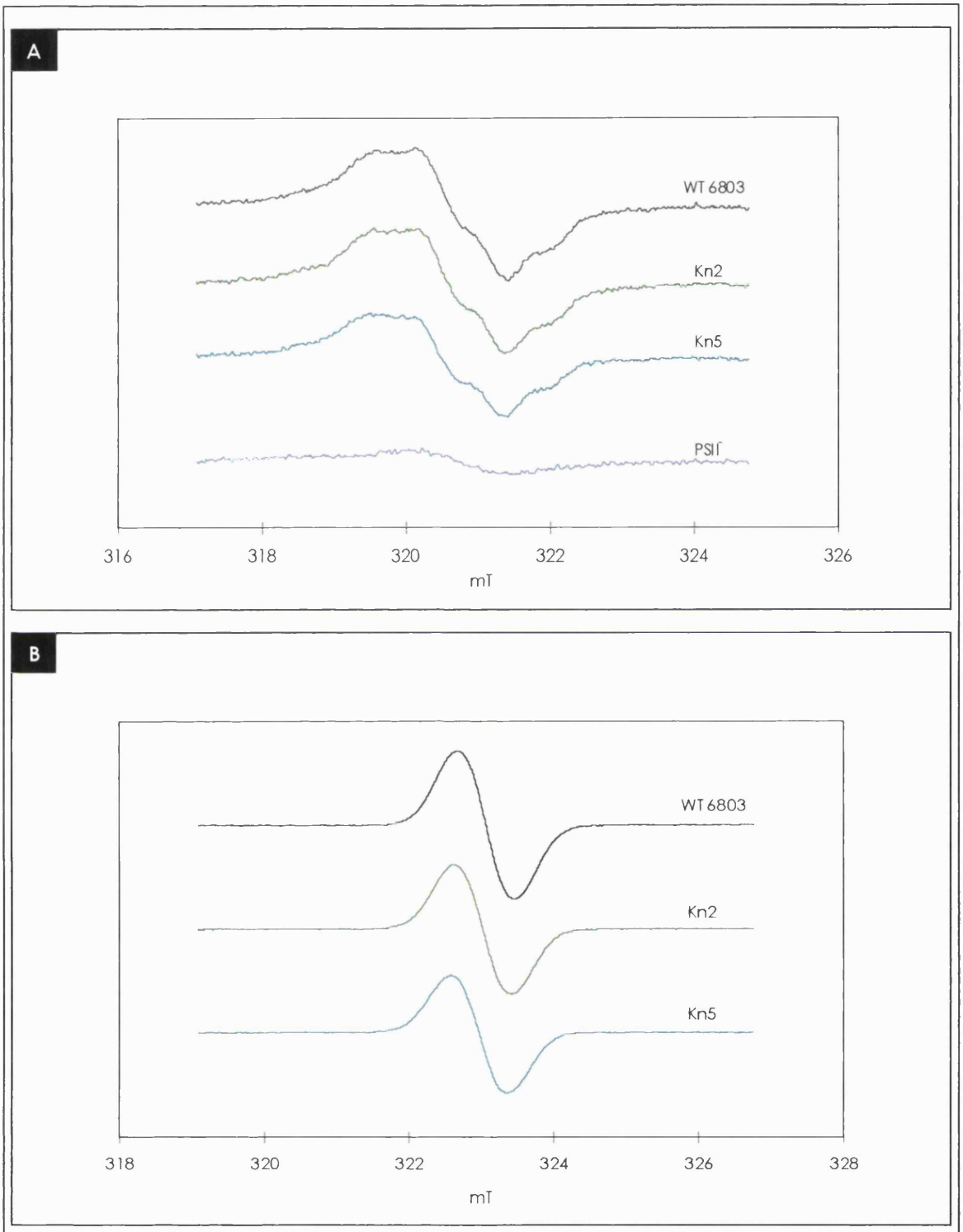


Figure 6.9 EPR analysis of WT 6803 cells and the mutants Kn2, Kn5 and PSII⁻
A. EPR analysis of the Y_D^+ signal in WT 6803, Kn2, Kn5 and PSII⁻ dark adapted *Synechocystis* cells.
B. EPR analysis of the $P700^+$ signal in WT 6803, Kn2 and Kn5 *Synechocystis* cells. The spectrum is the illuminated spectrum minus the dark spectrum.

NOTE
EPR conditions: microwave power 10^{-3}mW, modulation amplitude 0.2mT, temperature 10K.

6.3.3vii NPQ assay of the *psbZ* mutants

In the recent work published by Swiatek *et al.* (2001) the authors propose that the PSII-Z subunit may function in non-photochemical quenching (NPQ). To determine if this was the case in *Synechocystis* cells the *psbZ* mutants were subjected to an NPQ assay. Two sets of samples were prepared for 77K fluorescence emission spectroscopy (Section 2.9.6). Prior to freezing in liquid nitrogen the first set was dark adapted for a minimum of 5 mins. The second set of samples was illuminated by light treatment at 3,000μE/m^2/s for 1 min, conditions that induce NPQ. 77K fluorescence emission spectroscopy was then performed for each sample with excitation at 435nm. Figure 6.10 shows the resulting spectra. It should be noted that the difference in relative fluorescence for the dark adapted WT 6803 and dark adapted mutant samples was a result of an alteration of the PSII:PSI ratio in the mutants. The same applies to the illuminated samples. What is compared in these results is the relative change in the spectra following high light illumination (3,000μE/m^2/s for 1 min) of the cells.

EPR analysis of the yield of $P700^+$ indicated that the PSI level is almost constant. Thus any changes observed are more likely to be a result of a change in PSII. More measurements of the PSII and PSI content are needed to confirm this. Possible future experiments are described in Section 5.4.

Currently there is no definitive evidence in the literature to confirm that NPQ occurs in cyanobacteria. Following illumination of the WT 6803 cells there was a decrease in the level of PSII fluorescence. This result strongly supports the view that NPQ does occur in cyanobacteria. The same pattern was observed for both of the *psbZ* mutants. Following illumination of the mutants Kn2 and Kn5 there was also a decrease in the relative fluorescence of PSII. The degree of the reduction in PSII fluorescence was comparable to that of the WT 6803 cells. Thus these results lend support for the theory that NPQ occurs in cyanobacteria. However, they also showed that there was no difference in the degree of NPQ when WT 6803 cells were compared to the *psbZ* mutants Kn2 and Kn5. This suggests that in cyanobacteria the PSII-Z subunit is not involved in the regulation of NPQ.

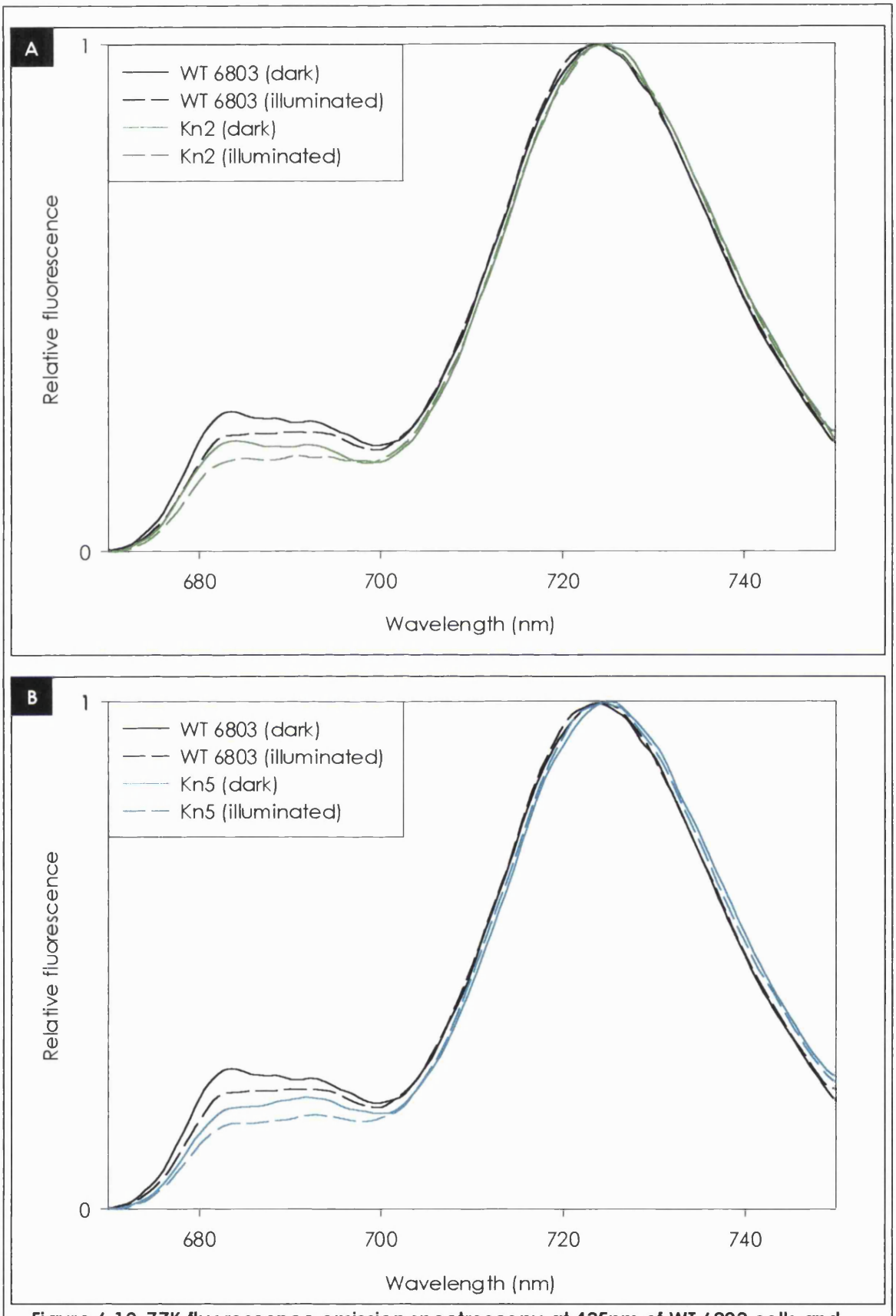


Figure 6.10 77K fluorescence emission spectroscopy at 435nm of WT 6803 cells and the *psbZ* mutants Kn2 and Kn5 following induction of NPQ
A. WT 6803 and Kn2 cells. B. WT 6803 and Kn5 cells.
Both spectra are normalised to the PSI maximum at ~725nm.

6.3.3viii State transition assay of the *psbZ* mutants

Swiatek *et al.* (2001) proposed that the PSII-Z subunit might play a role in the interaction between the LHC and PSII. If this is the case it is plausible that state transitions in the *psbZ* mutants may be compromised. It should not be discounted that the antenna system of prokaryotes and eukaryotes are not the same and interact with the PSII complex differently. The following experiment was undertaken to establish if the *psbZ* mutants were capable of state transitions. WT 6803 cells and the *psbZ* mutants Kn2 and Kn5 were grown photoautotrophically under very low light conditions (3μE/m^2/s). State transitions are more readily observed when cells are grown under light limiting conditions. Whole cell samples at RT were prepared at 5μM chlorophyll, and subjected to room temperature fluorescence analysis (Section 2.9.6). Cells were allowed to dark adapt (state 2) for at least 5 mins before a spectrum was recorded. Transition from state 2 to state 1 was initiated by illumination with red light after 60 s. The cells were illuminated for 140 s, and then returned to state 1. Figure 6.11 shows the resulting spectra for WT 6803, Kn2 and Kn5 cells.

Initially the dark adapted cells were in state 2, and a stable level of relative fluorescence was observed for the WT 6803 spectrum. In this state the PBSs are associated with PSI and the plastoquinone pool is reduced. Following illumination of the samples at 60 s there was a temporary decrease in the relative fluorescence of the WT 6803 spectrum followed by a rise in relative fluorescence. This increase levelled off before 200 s. The initial dip in fluorescence following illumination was the result of a rapid oxidation of the plastoquinone pool. There was then a slower increase in fluorescence as the PBSs moves from PSI to PSII. When the cells were returned to the dark after 140 s of illumination there was an immediate sharp increase in the level of relative fluorescence. This fluorescence gradually decreased as the PBSs return to PSII. The same overall pattern was observed for both of the mutants. Figure 6.11 clearly shows that both mutants were capable of performing state transitions. The short decrease in fluorescence observed for the WT 6803 cells at 60 s was absent in both of the mutant spectra. This is the result of a shift in the ratio of PSII to PSI in the cells. The decreased levels of PSII relative to PSI in the mutants means that the plastoquinone pool is not as reduced in these cells, relative to WT 6803 cells, when they were in state 2. Thus on illumination the same rapid oxidation of the plastoquinone pool is not observed. These results support those of Sections 6.3.3vi and Section 6.3.3vii.

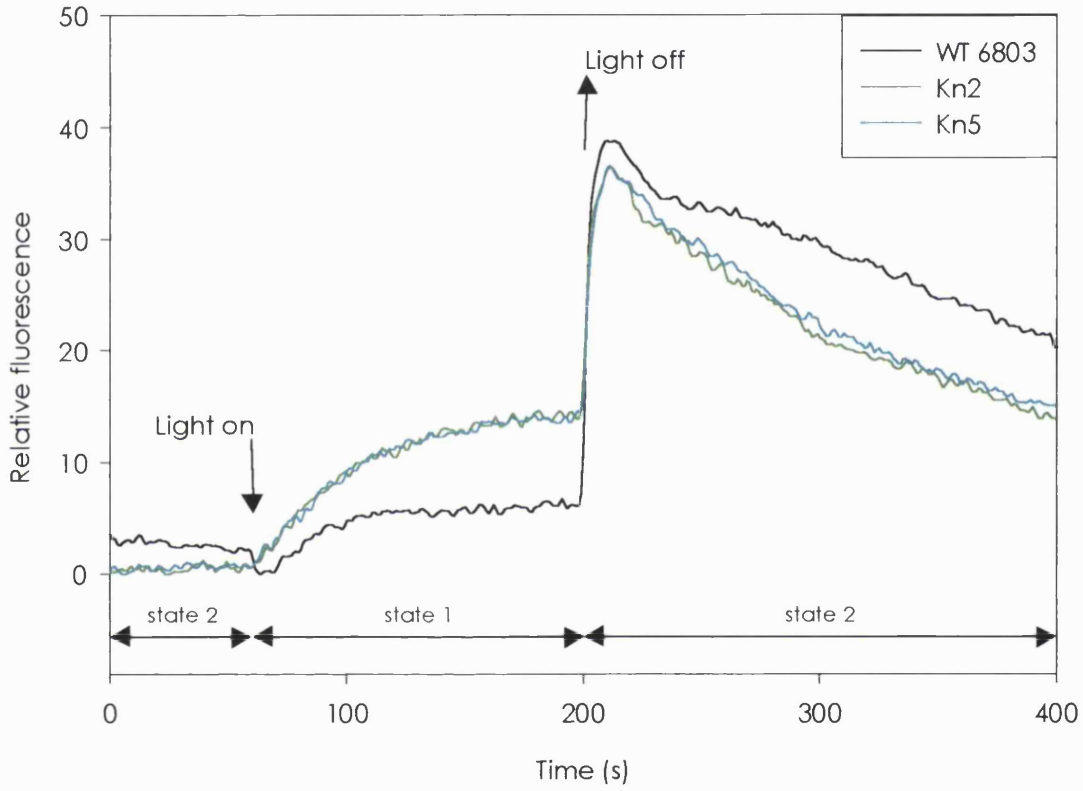


Figure 6.11 Room temperature fluorescence time course for WT 6803 cells and the *psbZ* mutants Kn2 and Kn5
Excitation was at 600nm (phycocyanin absorption) and fluorescence measured at 680nm (PSII chlorophyll *a*). Cells were dark adapted (state 2). The light was switch on at 60 s to induce a transition to state 1. The light was switched off at 200 s.

6.3.3ix 77K fluorescence emission spectroscopy analysis of the *psbZ* mutants

To follow the energy transfer to and between the photosystems 77K fluorescence emission spectroscopy was performed. The samples used were WT 6803, Kn2 and Kn5 cells grown at 3μE/m^2/s and adjusted to 10μM chlorophyll. The assignment of the fluorescence peaks is detailed in Section 2.9.6. Figure 6.12 shows the resulting spectra for the cells in state 2 (dark adapted) following excitation at 435nm and 600nm. Examination of the 435nm fluorescence emission spectra reveals a decrease in peaks at 685nm and 695nm for both Kn2 and Kn5 relative to those observed for WT 6803. The reduction in both peaks was greater for the Kn5 cells than it was for the Kn2 cells. This result indicates that the ratio of PSI:PSII has altered, with the Kn2 and Kn5 having less PSII compared to PSI relative to the WT 6803 ratio. Following excitation at 600nm the shoulder at 695nm was less obvious in the mutants, indicating a minor disturbance in PSII. This was consistent with a lower level of PSII. It should be noted that it is harder to estimate PSII to PSI ratio with excitation at 600nm.

The same fluorescence measurements were performed for the cells in state 1. Again the cell samples were excited at 435nm and 600nm, and the resulting fluorescence emission spectra are presented in Figure 6.13. The results confirm those observed for the state 2 measurements. The mutants appear as WT 6803 cells, except for the alteration in the PSII:PSI ratio. These results support the findings of the EPR studies (Section 6.3.3vi).

6.3.3x Photoinhibition assay of the *psbZ* mutants

Finally, to address the theory that the PSII-Z subunit may be involved in minimising the effects of damage under photoinhibitory conditions, a preliminary assay was performed to examine light-induced PSII photoinhibition. WT 6803, Kn2 and Kn5 cells were grown under moderate light conditions (50μE/m^2/s) to early log phase. A total volume of 200mL of cells was employed. The cells were then transferred to high light conditions (900μE/m^2/s). The level of high light used was limited by the apparatus available in the laboratory. Samples were taken every hour for 10 h and the cultures were then returned to moderate light conditions. A further two samples were then taken, again at hourly intervals. For each of the samples the rate of oxygen evolution was determined (Section 2.9.5) and 77K fluorescence emission measurements (Section 2.9.6) were made for each of the three cultures. The high light conditions employed in this experiment will

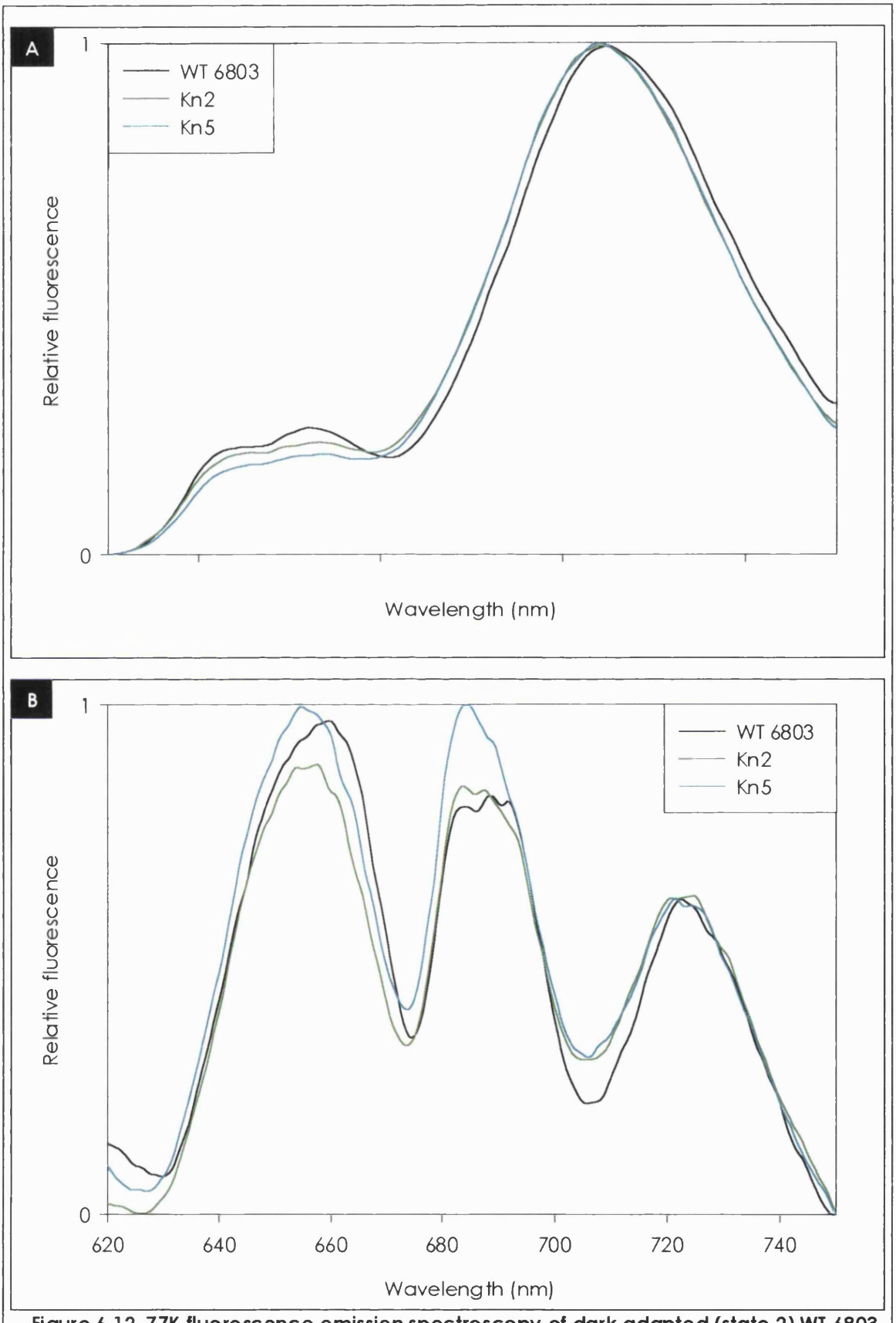


Figure 6.12 77K fluorescence emission spectroscopy of dark adapted (state 2) WT 6803 cells and the *psbZ* mutants Kn2 and Kn5
A. Excitation at 435nm. B. Excitation at 600nm.
Both spectra are normalised to the PSI maximum at ~725nm.

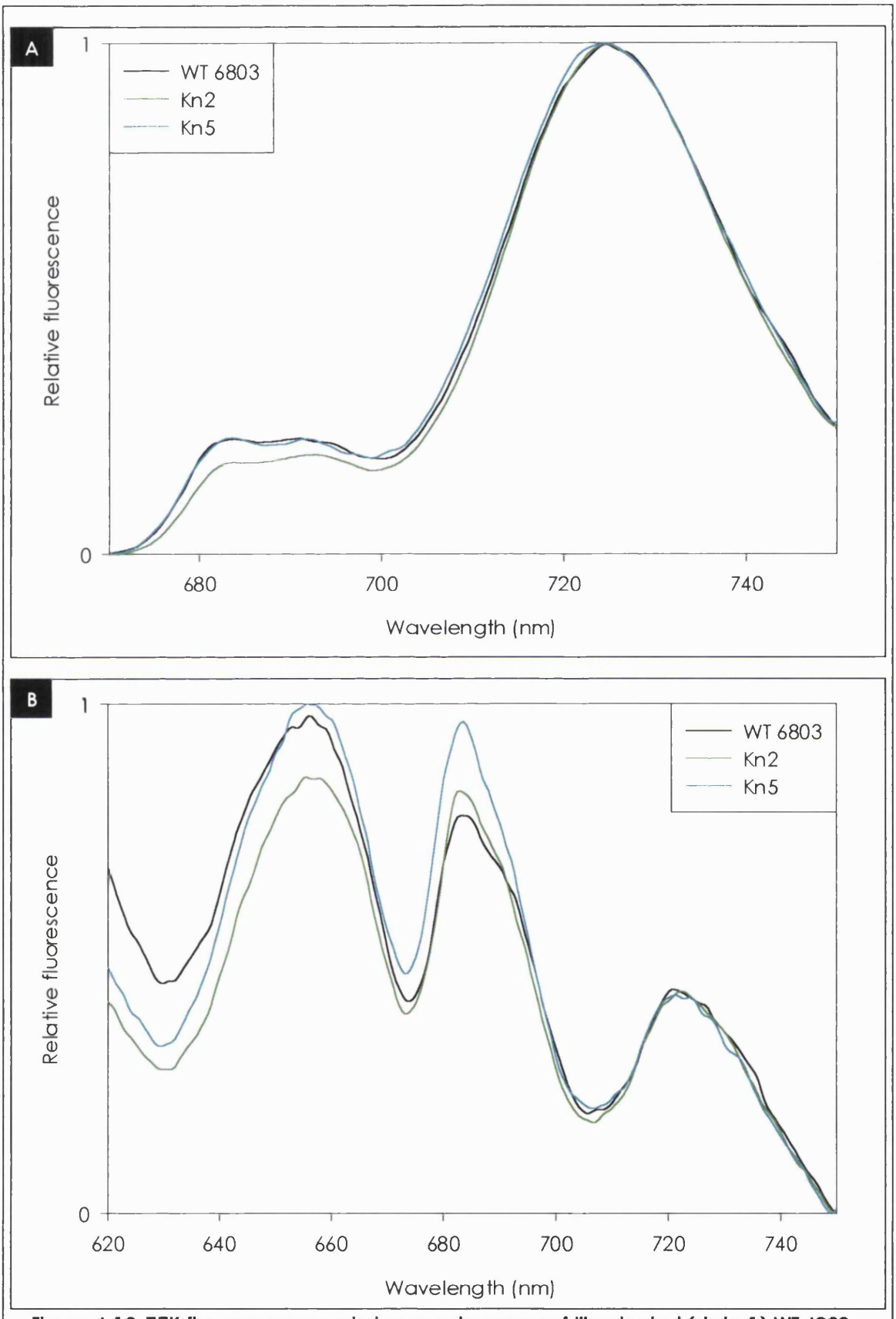


Figure 6.13 77K fluorescence emission spectroscopy of illuminated (state 1) WT 6803 cells and the *psbZ* mutants Kn2 and Kn5
A. Excitation at 435nm. B. Excitation at 600nm.
Both spectra are normalised to the PSI maximum at ~725nm.

not induce photoinhibition in the WT 6803 cells (E. Thompson, personal communication). It is known that many PSII mutants show greatly increased levels of photoinhibition (reviewed by Rochaix *et al.*, 1998; Niyogi, 1999). If the Kn2 and Kn5 cells were more susceptible to photoinhibition than the WT 6803 cells, it is possible that in this preliminary experiment a phenotype might be observed. If this is the case, a decrease in the rate of oxygen evolution and a comparable decrease in the PSII signal with time in the 77K fluorescence emission spectrum would be observed. The rates of oxygen evolution for all three cell types remained constant for each of the 12 samples taken during this assay (data not shown). The PSII signal did not decrease in any of the samples, and remained similar to those observed in Section 6.3.3ix (data not shown). With this preliminary photoinhibition assay we were unsuccessful at inducing photoinhibition in the mutants. It can be concluded from these results that the mutants Kn2 and Kn5 behave as WT and do not appear susceptible to photoinhibition at 900μE/m^2/s.

6.4 CONCLUSION

This work describes the creation of two *psbZ* mutants in *Synechocystis*. In the first, Kn2, the cassette conferring resistance to kanamycin has been inserted into a *Bsm*FI site 23bp upstream of the start of the *psbZ* orf. While the cassette does not directly disrupt the *psbZ* orf it is likely that the presence of the cassette so close to the start of the orf will affect its transcription and thus the levels of translated protein. The second mutant characterised in this work was the transformant Kn5. A *Bsm*FI fragment has been replaced with the kanamycin resistance cassette. The *Bsm*FI fragment contains the 23bp upstream of the start of the *psbZ* orf together with the first 112bp of the *psbZ* orf. In both mutants the cassette is in the opposite orientation to the *psbZ* orf. Both Kn2 and Kn5 are homoplasmic transformants, are capable of photoautotrophic growth and have oxygen evolution rates comparable to those observed for WT 6803 cells. Interestingly, the growth of these two mutants in the presence of glucose is highly impaired. The gene directly upstream of the *psbZ* gene is orf sll1282, or *ribH* and encodes the β subunit of riboflavin synthase. An impact on the expression of *ribH* expression is unlikely to give rise to the glucose intolerance phenotype observed. While this phenotype cannot be explained, the

absence of the PSII-Z protein may have a downstream effect on the metabolism of glucose. This is discussed further in Section 7.2.3.

This work conclusively shows that the PSII-Z protein does not play an essential role in photosynthetic growth or in the activity of PSII in *Synechocystis* 6803. Both EPR and 77K fluorescence emission spectroscopy confirm that the mutants have fully functional PSII complexes. However, the ratio of PSII to PSI is altered, with the mutants having less PSII relative to PSI when compared to WT 6803 cells. Estimates show that the mutant Kn5 has about 80% PSII, relative to WT 6803. As discussed in Section 5.4 comparisons of the relative levels of PSI and PSII for WT and mutant thylakoids could be achieved using non-denaturing 'green gel' electrophoresis.

When light is limiting ($3\mu E/m^2/s$) the mutants Kn2 and Kn5 have growth rates that exceed those of the WT 6803. Under these very low light conditions the mutant Kn5 has the fastest growth rate and doubling time. Once the light intensity is increased to $10\mu E/m^2/s$ or above, the mutants grow more slowly than the WT 6803 cells. These results suggest that in the mutants the PSII functions optimally at very low light conditions.

Further characterisation of the mutants shows that the PSII-Z subunit is not directly involved in NPQ. This contrasts with the findings of Swiatek *et al.* (2001), who propose that in *C. reinhardtii* PSII-Z may play a role in NPQ. However, the results of the NPQ experiments provide evidence, for the first time, that NPQ occurs in cyanobacteria. In the absence of PSII-Z the mutants are capable of performing state transitions.

To examine the effect of high light treatment of the *psbZ* mutants a preliminary photoinhibition assay was performed at a light intensity of $900\mu E/m^2/s$. This light intensity is not adequate to induce photoinhibition in WT cells. However, it was hoped that if the *psbZ* mutants were more susceptible to photoinhibition than WT cells, it might be adequate to induce photoinhibition in these mutants. This assay was unsuccessful in inducing photoinhibition at this light intensity. This result does not rule out the possibility that at higher light intensities, or with prolonged exposure to this level of irradiance, a phenotype might be observed for the mutants.

Two possible conclusions can be drawn from the results obtained. First, the PSII-Z subunit may have a non-essential function, or an important role under conditions not investigated in this study. Second, when the results of mutagenesis studies are compared for cyanobacterial and eukaryotic models, the phenotypes observed are not always the same. It may be that the PSII-Z subunit plays a more important role in green algae and

higher plants. Although speculative, these results do indicate that the absence of the PSII-Z protein has an effect on the functional efficiency and/or the proficiency of PSII assembly. Further studies would be required to address this. While this work has not established a function of the PSII-Z subunit in *Synechocystis* 6803 it has allowed a number of proposed functions to be ruled out.

CHAPTER 7. GENERAL DISCUSSION

GENERAL DISCUSSION

7.1 SUMMARY OF FINDINGS

This thesis presents an investigation of three of the small polypeptides of PSII – PSII-W, PSII-M and PSII-Z. The work described was undertaken to gain further information about these proteins and in the case of PSII-W, the gene that encodes it.

Chapter 3 details the cloning and sequencing of the *psbW* gene of *C. reinhardtii*, together with the characterisation of the gene and its derived amino acid sequence. In collaboration with Dr. Carolyn Silflow's laboratory, University of Minnesota, the location of the *psbW* gene within the nuclear genome of *C. reinhardtii* was mapped to Linkage Group XI, between the molecular markers GP49 and Cryl. Following on from this work, attempts were made to clone the *psbW* gene of the moss *P. patens*. *P. patens* is an excellent model for the study of nuclear-encoded genes and their protein products since the techniques for knockout of nuclear genes is now well established. This work was undertaken with the long-term aim of generating a *psbW* mutant in this species. Although eight positive clones were isolated from the screening of a genomic Lambda library, the gene was not isolated.

Chapter 4 sets out to characterise the PSII-W protein and is a continuation of the work undertaken in the Chapter 3. Using antibodies raised to the mature protein, we are able to refute the proposal by Hiyama *et al.* (2001) that the PSII-W protein may also be present within the PSI complex. Our results show that in *C. reinhardtii* the PSII-W protein is exclusive to PSII. Preliminary expression analysis showed that the PSII-W protein is expressed in a partly light dependent manner. In the dark and under low light conditions (10μE/m^2/s), at the stress temperature of 37°C, the accumulation of this protein is lower than the levels observed under the same light conditions at 25°C.

Chapter 5 presents the production and characterisation of a *psbM* deletion mutant in *Synechocystis* 6803. When grown at very low light (3μE/m^2/s), the *psbM* mutants have a doubling time that is faster than the rate observed for WT 6803 cells. However, the results show that the PSII-M protein is not required for the assembly or function of the PSII complex. To our knowledge, this is the first report of the generation of a *psbM* deletion mutant in any organism.

Chapter 6 details the creation and analysis of a *psbZ* disruption mutant. Interestingly, the mutants are sensitive to the presence of glucose; no explanation for this can be provided. A similar result to that observed for *psbM* was obtained when the doubling time and growth of the *psbZ* mutants were examined at very low light conditions (3μE/m^2/s). Under these growth conditions the *psbZ* mutants appear fitter than the WT 6803 cells. All other characterisations of the *psbZ* mutants indicate that the PSII-Z protein is a non-essential subunit of the PSII complex of *Synechocystis* 6803. This work also provides evidence that NPQ occurs in cyanobacteria.

7.2 FUTURE WORK

7.2.1 PSII-W

The work undertaken to characterise the *psbW* gene of *C. reinhardtii* is almost complete. The sequence data obtained in this research indicates the start site for translation. With this information a number of strategies could be explored to determine the transcription start site of the *psbW* gene.

1. An S1-exonuclease assay could be undertaken. From the available sequence data the restriction enzyme sites located in the region of the ATG of the *psbW* gene would be determined. This information would be used to generate an end-labelled DNA product which would anneal to the 5' region of the *psbW* mRNA. Treatment of the hybridised DNA-mRNA complex with S1-exonuclease would remove any unpaired DNA. The size of the remaining DNA-mRNA could be determined by gel electrophoresis, from which the location of the transcription start could be deduced.

2. Using the available sequence data, a primer extension assay could be performed. This technique uses a radiolabelled DNA oligonucleotide which binds specifically to the *psbW* mRNA. Extension of this primer using reverse transcriptase to the beginning of the mRNA molecule followed by gel electrophoresis of the product would establish the length of the extension and thus the start of transcription.

3. A transcription assay could also be performed. Initially nascent RNA molecules are radiolabelled. Restriction enzyme digestion of the *psbW* DNA can be used to produce known fragments of the gene. These fragments are then employed in

DNA-RNA hybridisation experiments to determine the initiation of transcription start site.

4. Finally, *psbW* cDNA generated by RT-PCR could be sequenced to establish the transcription start site of the gene. However, this would rely on the production of a full-length cDNA.

As new molecular markers for the nuclear genome of *C. reinhardtii* become available further mapping experiments could be performed, providing more information about the location of the *psbW* gene and its neighbours. In addition, work is well underway to sequence the *C. reinhardtii* genome, with a projected finishing date of June 2002 (http://www.biology.duke.edu/chlamy_genome/cgp.html). The completion of this project will provide a full map of the *psbW* gene and its exact location in the genome.

Given more time, the back crosses of the acetate requiring mutants, which mapped to the same region of the genome as the *psbW* gene, could be repeated. If successful, characterisation of these progeny would establish if the expression of PSII-W protein is compromised. As discussed in Section 3.4, if the acetate requiring phenotype had been the result of a secondary mutation it might be possible to determine if the original mutation had occurred in the *psbW* gene. Similarly, if one or more of the mutants had maintained their acetate requiring phenotype the first step in their characterisation would also have been to determine if the mutation had occurred in the *psbW* gene. Comparing the *psbW* gene sequences of each of the mutants with the data generated for the WT CC-1021 *psbW* gene could identify any point mutations. If the phenotype was the result of a mutation in the *psbW* gene it would suggest that the PSII-W subunit is essential for photosynthetic growth. The levels of oxygen evolution and growth rates of the mutant(s) would be examined relative to WT CC-1021. The effect of the mutation(s) at the protein level would be also examined.

Northern analysis could also be used to compare the *psbW* transcript sizes and levels between the mutants and WT CC-1021 cells. This strategy has been used successfully to characterise the acetate requiring mutant ac208 and a number of other plastocyanin mutants generated by UV mutagenesis (Li *et al.*, 1996). These mutants arose from frameshift mutations in the gene *pcy-1*, which encodes plastocyanin. Northern analysis detected the accelerated degradation of the *pcy-1* transcript due to inefficient translation of the mutant mRNA.

To confirm that the acetate-requiring phenotype of the mutant is due to a defective *psbW* gene a complementation experiment would then be performed. To achieve this the nuclear genome of the mutant would be transformed with a plasmid containing the WT copy of *psbW*. If the phenotype is due purely to the absence of the PSII-W protein this transformation should restore the WT photoautotrophic phenotype.

The attainment of a PSII-W mutant is pivotal in determining the function of the protein. To this end, the generation of an anti-sense and/or an over-expression mutant for the *psbW* gene of *C. reinhardtii* could be undertaken. This strategy would involve the cloning of the cDNA for the *psbW* gene in both anti-sense and sense orientations downstream of a strong *Chlamydomonas* promoter, e.g. the *RBCS2* promoter (Khrebtukova and Spreitzer, 1996). These constructs together with the *ARG-7* gene would then be co-transformed into an arginine auxotroph. At the time of writing limited success has been achieved with *C. reinhardtii* anti-sense strains. Day and Rochaix (1991) have reported between a two-fold and fourfold reduction in WT levels of the mRNA of the transposon *tocl*. Schroda *et al.* (1999) have achieved a 20-40% reduction in WT levels of the *hsp70B* mRNA levels. It is also unclear what the phenotype of a *psbW* anti-sense or over-expression mutant would be. Shi *et al.* (2000) isolated a *psbW* anti-sense mutant in *A. thaliana* and found that the mutant was capable of photoautotrophic growth. However, the isolation and characterisation of a *psbW* anti-sense mutant of *C. reinhardtii* might provide clues about the role of the PSII-W protein. Comparisons could be made with the findings of Shi *et al.* (2000), who propose that in *Arabidopsis* PSII-W is involved in the formation and stabilisation of the PSII dimer *in vivo*. The monomeric and dimeric forms of PSII can be isolated from detergent digests of thylakoid membranes using density gradient centrifugation (Zheleva *et al.*, 1998).

In tandem, a library of random insertional mutants could be generated and screened with the aim of isolating a *psbW* deletion mutant. The initial generation of the library would be reasonably simple. The nuclear genome of WT CC-1021 would be transformed with DNA containing a selectable marker, and transformants selected under suitable growth conditions (Mayfield and Kindle, 1990). There are a number of disadvantages for using *C. reinhardtii* to isolate a null mutant. For example, integration of transforming DNA into the nuclear genome of this green alga occurs via non-homologous recombination. Consequently a large number of random mutants

would need to be screened, either by Southern analysis or PCR, in order to isolate a potential *psbW* mutant. It is estimated that 46,000 transformants would need to be screened to have a 99% chance of finding a mutation in a desired gene (Pazour and Witman, 2000). As stated above it is also unclear what phenotype a *psbW* mutant would have. This fact would make the isolation process even more difficult.

If isolated the availability of a PSII-W null mutant would permit structure-function studies to be undertaken. For example, the site-directed mutagenesis of specific residues of the PSII-W protein could be investigated. This would be achieved by transformation of the nuclear genome of *C. reinhardtii* with the altered copy of the *psbW* gene. There are no discernible motifs present within the protein sequence. However, the availability of a *psbW* mutant would allow the truncation of the protein at either the N or C-termini to be investigated, as described by de Vitry *et al.* (1999). Again, this would be achieved by the transformation of the null mutant with the altered copy of the *psbW* gene. The mutant could also enable the investigation of the mechanism of import of the PSII-W protein by using site directed mutagenisis or truncation of the transit peptide. A similar approach has been used by Lawrence and Kindle (1997) to investigate the import of plastocyanin in *Chlamydomonas.*

Further work is required to clone the *psbW* gene of *P. patens*. The main hurdle in this project was isolating *P. patens* genomic DNA and DNA from the eight genomic clones that could be completely digested with restriction enzymes. Future work would investigate alternative methods for the isolation of this DNA and the optimisation of restriction enzyme digests. Once suitable techniques are in place the *psbW* gene should be cloned and sequenced.

The sequencing of nuclear-encoded genes of *P. patens* is in its infancy. From the limited information available, it is clear that the genes possess introns that are typically in the region of 200bp to 400bp in length. It could be postulated that the small *psbW* gene is unlikely to contain a number of large introns. This would support the use of PCR as an alternative strategy for the amplification of the genomic sequence.

Examination of the cDNA sequence data and the derived amino acid sequence from four ESTs indicates that there may be more than one *psbW* gene present within the nuclear genome of *P. patens*. Characterisation of the 3' UTR for each of the cDNA sequences would provide more information. This region of a gene is not

subject to the same sequence conservation pressures, and is the most divergent amongst different isoforms of the same gene. Once an optimised strategy for the isolation and digestion of the genomic DNA of *P. patens* has been established, Southern analysis can be undertaken to determine the copy number of the *psbW* gene.

The long term aim would be the creation and characterisation of a deletion mutant for the *psbW* gene in this organism, although this strategy may be complicated if it is confirmed that there are two copies of the *psbW* gene in *P. patens*. The *Physcomitrella* EST Programme has generated a number of transformation vectors and selectable markers suitable for the targeted gene inactivation of *psbW* (http://www.moss.leeds.as.uk). An appropriate region of the *psbW* gene and flanking region would be cloned such that the gene is disrupted by a suitable selectable marker, for example the 35S promoter-driven *npt*II gene (Töpfer *et al.*, 1988). This plasmid would be linearised and used to transform *P. patens* protoplasts. The construct will undergo targeted integration by homologous recombination and transformants selected on appropriate growth medium. Isolation of resistant transformants takes about 50 days. The high levels of homologous recombination observed for the nuclear genome of *P. patens* make selection of transformants much less arduous than the selection of nuclear mutants of *C. reinhardtii*. This approach has been used successfully to disrupt several *P. patens* genes. For example Strepp *et al.* (1998) created a *ftsZ* null mutant that was defective in the division of the chloroplast. The *Physcomitrella* EST Programme provides a transformation service, which could be useful for workers who are new to *P. patens* research.

In addition to the generation of a *psbW* null mutant in *P. patens*, an anti-sense strategy could also be explored. The generation of an anti-sense mutant of *P. patens* has already been successfully reported (Leech *et al.*, 1993). The steps taken would be similar to those described above for *C. reinhardtii*.

The initial expression analysis of the *C. reinhardtii* PSII-W protein yielded some interesting results. From the conditions investigated the PSII-W protein is expressed in a light-dependent manner. The decrease in the level of accumulation of the PSII-W protein in darkness and at light intensities of 10μE/m^2/s at 37°C relative to those observed under the same light conditions at 25°C is a puzzling result. To obtain a clearer idea of the factors that influence the levels of the PSII-W protein further expression studies should be performed. For example, are the same patterns of

light-dependent expression observed at light intensities above 50μE/m^2/s. To complement such experiments, the expression pattern of the PSII-W protein could be investigated under a 12 h light/12 h dark regime. This would give an insight into the light and/or circadian expression mechanisms for this protein.

7.2.2 PSII-M

From the results obtained in Chapter 5, it can be concluded that the PSII-M protein is a non-essential subunit of the PSII complex in the cyanobacterium Synechocystis 6803. The psbM mutants are capable of oxygen evolution rates that are comparable to those of WT CC-1021 under saturating light conditions. Interestingly the growth experiments indicate that the mutants are fitter than WT 6803 cells under very low light conditions (3μE/m2/s). With this in mind the oxygen evolution measurements should be repeated in more detail. Future experiment would involve generating saturation curves for each of the mutants and WT 6803 cells using a range of light levels, rather than just the saturating light level used in Chapter 5. This data could then be analysed using the Michaelis-Menten equation in order to determine the enzyme kinetics of the PSII complex. The Michaelis-Menton equation can be mathematically transformed to generate linear plots that enable the determination of V_{MAX} and K_M. These transformations include the Eadie-Hofstee and Lineweaver-Burk plots, which could be applied to the saturation curves to look for differences between mutants and WT.

A more detailed study of this mutant is required to establish the conditions in which the PSII complex is altered. Analysis of the assembly of monomeric and dimeric PSII could be investigated (Section 7.2.1). The accumulation of the *psbM* transcript under different growth conditions could be examined. This would also produce information about the conditions under which this protein is expressed.

Eventually the next step would be to investigate the role of this subunit in a eukaryotic model. In *C. reinhardtii,* the *psbM* chloroplast gene is located on an operon with the gene that encodes PSII-Z. The *psbM* gene is expressed monocistronically and also dicistronically with *psbZ* (Cain, 1998; Higgs *et al.*, 1998). There are a number of strategies that could be used to circumvent the problems with the arrangement and transcription of the *psbZ* and *psbM* genes. A stop codon could

be introduced into the *psbM* gene, with a selectable marker located outside the operon. This way transcription of the operon should not be affected, but the effect of the absence of the *psbM* gene product can be investigated. A PCR based strategy would be employed to introduce a stop codon into the *psbM* gene. The resulting construct together with a second construct containing a suitable selectable marker would be used for the biolistic co-transformation into the chloroplast genome of *C. reinhardtii*. Other workers investigating *Chlamydomonas* chloroplast genes (Newman *et al.*, 1991) have used similar co-transformation strategies.

Alternatively a two-stage transformation experiment could be explored for the deletion of the *psbM* gene, whilst maintaining the *psbZ* gene. The first step would involve knocking out the *psbZ-psbM* operon. A plasmid containing the appropriate region of the genome would be used to generate a construct in which the *psbZ-psbM* genomic sequence was replaced with a suitable selectable marker. This construct would be used for the first transformation of the chloroplast genome. The homoplasmic transformants would then undergo a second transformation with a plasmid containing the *psbZ* gene and a second selectable marker. The resulting transformants would contain the *psbZ* gene but lack the *psbM* gene. The phenotypes of both the *psbM* and the double *psbZ-psbM* mutants could then be characterised.

Although RNA interference has yet to be applied to chloroplast genes, this strategy could possibly be used as a means of investigating the gene silencing of the *psbM* gene by dsRNA suppression. RNA interference is a technique that results in the post-transcriptional silencing of a chosen gene by targeting and destroying the gene's mRNA product, thus revealing the gene's null phenotype. A short RNA duplex is introduced into the cell or expressed from an introduced transgene. The cell's RNA interference machinery detects the presence of the dsRNA which is then bound in a RNA-protein complex that mediates the detection and destruction of mRNAs containing the same sequence as the mRNA. This technique has been successfully employed in the nuclear genome of *C. reinhardtii* recently (Fuhrmann *et al.*, 2001). The authors achieved a 95-98% reduction in the WT expression of the *cop* gene following transformation of WT *C. reinhardtii* with a construct that expressed a self-complementary hairpin RNA molecule.

When characterising any mutants isolated by the strategies outlined above care should be taken that the accumulation of the *psbZ* mRNA and the subsequent translation of the *psbZ* protein is not affected. It should be also noted that although

the *Synechocystis* 6803 Δ*psbM* mutants generated in this work were capable of photoautotrophic growth, the photosynthetic phenotype of a *C. reinhardtii* Δ*psbM* mutant should be characterised, since the loss of minor PSII subunits can have differing effects in these species. For example, loss of *psbK* does not prevent photoautotrophic growth in *Synechocystis* (Ikeuchi *et al.*, 1991) but does in *C. reinhardtii* (Takahashi *et al.*, 1994).

7.2.3 PSII-Z

As with the PSII-M subunit, the PSII-Z protein does not appear to be essential to the PSII complex of *Synechocystis* 6803. Recently Swiatek *et al.* (2001) isolated *psbZ* mutants of *C. reinhardtii* and tobacco. The characterisation of these mutants led the authors to propose that PSII-Z functions in the interaction between PSII and LHCII, and that the subunit may also play a role in NPQ under conditions that give rise to photoinhibition.

In light of these findings, initial further work would focus on establishing if the *Synechocystis psbZ* mutants generated in this work were more sensitive to photoinhibition that WT 6803 cells. Experiments similar to those performed in this work (Section 6.3.3x) would be repeated to establish the light intensity that is necessary to observe photoinhibition in WT 6803 cells. The preliminary photoinhibition experiment was performed at a light intensity of 900μE/m^2/s over a 10 h period. Other researchers have reported a 29% reduction in the level of the D1 protein following treatment of WT 6803 cells at a light intensity of 1,500μE/m^2/s for 160 mins (Kanervo *et al.*, 1995). Thus the timescale previously described should provide adequate time in which to observe photoinhibition. However, experiments at higher light levels may detect differences between mutants and WT 6803 cells.

Following this, a variety of lower light intensities and duration of exposure could be examined. This would establish the minimum light intensity and amount of time required to induce photoinhibition in the WT 6803 and *psbZ* mutants. In addition to measuring oxygen evolution and the 77K fluorescence emission spectra of time points through these experiments, alternative strategies could be employed for the detection of photoinhibition. For example western analysis of the rate of D1 turnover in the presence of lincomycin could be investigated. As well as examining

the susceptibility of the *psbZ* mutants to photoinhibition, the rate of recovery from a photoinhibited state could also be investigated. This could initially be established by determining the rate of recovery of oxygen evolution, and then in more detail, for example by examining the level of the D1 protein by western analysis.

Similar studies to those outlined for PSII-M (Section 7.2.2) could be undertaken. The *psbZ* mutants are capable of oxygen evolution rates that are comparable to those of WT 6803 under saturating light conditions. Interestingly the growth experiments indicate that the mutants are fitter than WT 6803 cells under very low light conditions ($3\mu E/m^2/s$). As described in Section 7.2.2 saturation curves at varying light intensities could be performed and results obtained for the *psbZ* mutants compared with those of WT 6803 cells.

The glucose sensitive phenotype of the *psbZ* mutants is a puzzling result. It is possible that the absence of the PSII-Z subunit may have a downstream effect on glucose metabolism. As outlined in Section 6.3.3ii, Hihara and Ikeuchi (1997) have also isolated a mutant with a glucose sensitive phenotype. The mutation arose from a base substitution in the *pmgA* gene. When cultured under mixotrophic conditions the growth of the *pmgA* mutant was severely inhibited, a phenotype very similar to that observed for the *psbZ* mutants. Following short-term exposure to high light the *pmgA* mutant outgrew the WT 6803 cells. In contrast the *psbZ* mutants were fitter than WT 6803 cells under very low light conditions. The *pmgA* mutant was not capable of sustaining prolonged growth under high light conditions. More recent work suggests that the *pmgA* mutants are incapable of regulating photosystem stoichiometry (Sonoike *et al.*, 2001). Further work on the *psbZ* mutants, using illumination by light preferentially exciting PSI or PSII, would be required to determine if they are also affected in the regulation of photosystem stoichiometry.

Using the derived protein sequence for the PmgA a Blast search was undertaken (Blast 2.2.1, http:www4.ncbi.nlm.nhi.gov; Section 6.3.3ii). This showed that the PmgA protein has a weak and very weak identity with the histidine kinases from *Methanothermobacter thermautotrophicus* and *Bacillus halodurans* respectively. This may indicate a role for the PmgA protein in a signal transduction pathway. The absence of a histidine residue in the PSII-Z protein sequence rules out the possibility of a direct interaction between this potential histidine kinase and the PSII-Z subunit.

Swiatek *et al.* (2001) propose that the PSII-Z subunit is involved in interaction of the light-harvesting complex with the PSII complex. From the findings presented here, this does not appear to be the case in *Synechocystis*. Further work would involve the isolation of thylakoid membranes and PSII preparations from the *psbZ* mutant. These would be characterised by immunoblotting and compared to those obtained from WT *Synechocystis*, to determine if the PSII composition was altered in these mutants. The use of His-tagged PSII preparations (for example Bricker *et al.*, 1998) to purify PSII under different conditions that would gradually strip subunits from the complex could enable the localisation of the PSII-Z subunit within the PSII complex. Antibodies could be raised to PSII-Z and, together with existing PSII antibodies, could be used to characterise the composition of such preparations. Information about the position of PSII-Z within the complex may provide an insight into the role that this protein plays.

As more detailed information becomes available about the structure of PSII the location of the subunits within the complex will be resolved. Such information may provide vital clues necessary for the assignment of protein function. In the future, the high resolution crystal structure of the PSII of eukaryotic organisms will be achieved. The recent advances in the isolation of His-tagged PSII may provide a good source for crystallisation work. Furthermore, the viability of *C. reinhardtii* in the absence of cyt b_6f, PSI and LHC would make the purification of PSII from this alga even more effective. With the wealth of DNA sequence data being generated, the field of proteomics will become increasingly important. Developments in this area will enable, amongst other things, investigations into the post translational modification of proteins and the levels of translational control of a protein of interest. We stand on the brink of the post-genomic era. The next few years in photosynthetic research will be challenging and exciting.

APPENDICES

APPENDICES

Appendix 1 Plasmid DNA vectors utilised

Cloning vectors

Details	Selection	Host	Source
pBluescript SK(+)			
F1 origin, *lacZ* gene, MCS, Amp^r. Referred to as pBluescript SK in this work.	LB Amp^{100}	DH5α and JM109	Stratagene (California, USA).
pUC19			
lacZ gene, MCS, Amp^r.	LB Amp^{100}	DH5α	New England Biolabs, (Hitchin).
pUC4K			
Kanamycin resistance. Digestion with *Hinc*II excises the 1291bp fragment containing the Kan^r cassette.	LB Kan^{100}	XL1-blue	Amersham Pharmacia Biotech, (Amersham).
pMAL-c2			
M13 origin, *lacZ* gene MCS Amp^r.	LB Amp^{100}	JM109	New England Biolabs, (Hitchin).

psbM plasmids

Details	Selection	Host	Source
pSK.MHX			
PCR product of *Synechocystis* 6803 *psbM* gene with 5'and 3' flanking sequence. Cloned into the *Hin*dIII and *Xba*I sites of pSK.	LB Amp^{100}	JM109	This thesis (Chapter 5).
pSK.MHX.Kn			
Derived from pSK.MHX. The Kan^r cassette replaces the *Hpa*I fragment of the pSK.MHX insert, and results in the deletion of the *psbM* gene.	LB Amp^{100} Kan^{100}	JM109	This thesis (Chapter 5).

Appendix 1 Plasmid DNA vectors utilised (continued)

***psbW* plasmids**

Details	Selection	Host	Source
pGW10			
Genomic cosmid clone containing *psbW* gene from library created by Purton and Rochaix.	LB Amp^{100}	DH5α	Cain (1998).
pHW5			
~600bp 3' RACE product of *psbW* coding sequence blunt cloned into pSK via *Hin*cII site.	LB Amp^{100}	DH5α	Cain (1998).
pMAL-c2.psbW			
Derived from pMAL-c2. The coding sequence for the mature PSII-W protein was cloned into the *Eco*RI and *Hin*dIII sites.	LB Amp^{100}	JM109	This thesis (Chapter 4).
pWBam1.4			
1.4 kb *Bam*HI fragment containing a portion of the *psbW* gene from the pGW10. cloned into the *Bam*HI site of pSK MCS.	LB Amp^{100}	JM109	This thesis (Chapter 3).
pWBam2.2			
2.2 kb *Bam*HI fragment containing a portion of the *psbW* gene from the pGW10. cloned into the *Bam*HI site of pSK MCS.	LB Amp^{100}	JM109	This thesis (Chapter 3).

Appendix 1 Plasmid DNA vectors utilised (continued)

ycf9 plasmids

Details	Selection	Host	Source
pKn2			
Derived from pSycf9. The Kan^r cassette is inserted into the *Bsm*FI site at position 429 of the pSycf9 insert.	LB Amp^{100} Kan ^{100}	DH5α	This thesis (Chapter 6).
pKn5			
Derived from pSycf9. The Kan^r cassette replaces the *Bsm*F1 fragment between positions 429 and 564 of the pSycf9 insert.	LB Amp^{100} Kan ^{100}	DH5α	This thesis (Chapter 6).
pSycf9			
PCR product of *Synechocystis* 6803 *ycf9* gene with 5'and 3' flanking sequence cloned into the *Hinc*II site of pUC19.	LB Amp^{100}	DH5α	This thesis (Chapter 6).

NOTE

a. Amp^{100} and Kan^{100} - each antibiotic was supplemented to a final concentration of 100μg/mL.

Amp^r - ampicilin resisitant.

Kan^r: kanamycin resistant.

MCS: Multiple Cloning Site.

Appendix 2 The oligonucleotide primers used in this work

psbM primers

Sequence (5' to 3')	Direction and T_M	Comments	Reference
psbMH1			
CACCAAaGCTTCAGTGCCA	sense 56.7°C	674bp upstream of the *Synechocystis* 6803 *psbM* start codon. 1bp change to incorporate *Hin*dIII site.	Creation of pSK.MKX.
psbMX1			
ACTCAATTATCATGCTTAGC	antisense 51.2°C	637bp downstream of the *Synechocystis* 6803 *psbM* stop codon.	Creation of pSK.MKX.

psbW primers

Sequence (5' to 3')	Direction and T_M	Comments	Reference
psbWE1			
GTGTGAATTCCTGGTCGATGAG	sense 60.3°C	Anneals at Leu codon of *C. reinhardtii psbW* mature sequences. 5' non specific sequence with *Eco*RI site introduced.	Creation of pMAL-c2.*psbW*. Sequencing of *psbW*.

Appendix 2 The oligonucleotide primers used in this work (continued)

***psbW* primers (continued)**

Sequence (5' to 3')	Direction and T_M	Comments	Reference
psbWH1			
TGCTAAAAAAGGAAGCttTACG AA	antisense 55.9°C	29bp downstream of *C. reinhardtii psbW* stop codon. 2bp changes to incorporate *Hin*dIII site.	Creation of pMAL-c2.*psbW*. Sequencing of *psbW*.
W1			
TSGTSGAYGAGCGBATGAA	sense 54.5°C		Creation of 3'RACE product subsequently amplified to create insert of pHW5. Sequencing of *psbW*.
W2			
ATGAAYGGNGAYGGNACNGG	antisense 55.3°C	Complementary to *W*seq6.	Creation of 3'RACE product subsequently amplified to create insert of pHW5. Sequencing of *psbW*.
Wexp3'			
CCATCTCGAGCTTCAGGCCATC GTCG	antisense 64.1°C		With Wexp5' amplification of pHW5 insert. This PCR product was used as a probe for Southern analysis.

Appendix 2 The oligonucleotide primers used in this work (continued)

psbW primers (continued)

Sequence (5' to 3')	Direction and T_M	Comments	Reference
Wexp5'			
GGACATATGCTGGTCGATGAG CGGATG	sense 62.6°C		With Wexp3' amplification of pHW5 insert. This PCR product was used as a probe for Southern analysis.
Wseq1			
TTGTCAGCAGCTCTGCGGTG	sense 55.7°C	Complementary to *W*seq2.	Sequencing of *psbW*.
Wseq2			
CACCGCAGAGCTGCTGACAA	antisense 61.4°C	Complementary to *W*seq1.	Sequencing of *psbW*.
Wseq3			
CACATGGTGCCGAAGACGC	antisense 55.2°C		Sequencing of *psbW*.
Wseq4			
GAACCAGATAGCCCACAT	antisense 53.7°C		Sequencing of *psbW*.

psbW primers (continued)

Sequence (5' to 3')	Direction and T_M	Comments	Reference
Wseq5			
CCAGTGCCGTCGCCGTTCAT	antisense 63.5°C	Complementary to W2.	Sequencing of *psbW*.
Wseq6			
ACACCTCGTTTGGTCGCG	sense 58.2°C	Virtually complementary to *W*seq7.	Sequencing of *psbW*.
Wseq7			
GCGCGACCAAACGAGGTGT	antisense 61.0°C	Virtually complementary to *W*seq6.	Sequencing of *psbW*.
Wseq8			
TTCGTAGTGCTTCCTTTTTTAG	sense 54.7°C		Sequencing of *psbW*.
mossW1			
CTGGTCTCCCCGCCCTG	sense 62.4°C		Attempts to amplify the *P. patens psbW* gene from a Lambda library.

Appendix 2 The oligonucleotide primers used in this work (continued)

***psbW* primers (continued)**

Sequence (5' to 3')	Direction and T_M	Comments	Reference
mossW2			
TACAAGTCCAAACCCGAGTC	antisense 57.3°C		Attempts to amplify the *P. patens psbW* gene from a Lambda library.
mossW3			
TGGCTGCCATCGCCTCTG	sense 60.5°C		Attempts to amplify the *P. patens psbW* gene from a Lambda library.
mossW4			
CATCGCAAACAGTTCGATATG	antisense 55.9°C		Attempts to amplify the *P. patens psbW* gene from a Lambda library.

***ycf9* primers**

Sequence (5' to 3')	Direction and T_M	Comments	Reference
Sycf9 3'			
CACATCAATGCCATGACGTTTG AGGC	antisense 64.8°C	200bp downstream of *Synechocystis* 6803 *ycf9* stop codon.	Creation of pS*ycf9*.

Appendix 2 The oligonucleotide primers used in this work (continued)

ycf9 primers (continued)

Sequence (5' to 3')	Direction and T_M	Comments	Reference
Sycf9 5'			
GCCAGGGAAATTGATCGTTGG AGTG	sense 64.6°C	300bp upstream of *Synechocystis* 6803 *ycf9* start codon.	Creation of pS*ycf9*.

Others

Sequence (5' to 3')	Direction and T_M	Comments	Reference
oligodT			
AAGGATCCGTCGACATCGATA ATACGACTGACTATAAGGGAT TTTTTTTTTTTTTTT	70.8°C		3' RACE.
pK3			
GATTTTGAGACACAACGTGGC	sense 57.9°C	Anneals to 5' region of the Kan^r cassette.	Sequencing of pSK.MHX.Kn.
pK7			
GACTTGACGGGACGGCGGC	antisense 65.3°C	Anneals to 3' region of the Kan^r cassette.	Sequencing of pSK.MHX.Kn.

Appendix 2 The oligonucleotide primers used in this work (continued)

Others (continued)

Sequence (5' to 3')	Direction and T_M	Comments	Reference
RACE1			
AAGGATCCGTCGACATCGATAAT	58.9°C	Internal to oligodT primer.	3' RACE.
RACE2			
GATAATACGACTGACTATAAGGGA	57.6°C	Internal to oligodT primer.	3' RACE.
T3			
AATTAACCCTCACTAAAGGG	sense 53.2°C	Flanks MCS of pSK.	Sequencing.
T7			
GTAATACGACTCACTATAGGGC	antisense 56.6°C	Flanks MCS of pSK.	Sequencing.

NOTE

A= adenine, C= cytosine, G = guanine, T = thymine, S = GC, Y = CT, B = CGT and N = ACGT.

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