Site-directed mutagenesis of the D2 protein in the green alga *Chlamydomonas reinhardtii*

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A dissertation submitted to the University of London in partial fulfilment of the requirements for the degree of Doctor of Philosophy

September 1996

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To my parents

To Angie

ABSTRACT

The D2 polypeptide together with the D1 polypeptide are key components of the photosystem II (PS II) complex which is involved in photosynthetic oxygen evolution. This thesis describes the use of site-directed mutagenesis to introduce specific amino acid changes in the D2 protein of *Chlamydomonas reinhardtii*. The mutations were constructed at positions D2-Leu205 and D2-Thr2. Plasmids carrying mutated copies of the *psbD* gene (which encodes D2) were introduced into the chloroplast genome of *C. reinhardtii* by means of a particle gun. In order to identify the transformants, a selectable marker encoding resistance to spectinomycin, was inserted either upstream or downstream of the *psbD* gene. Genetic characterisation of the mutants was carried out using Southern blotting, PCR and DNA sequencing.

D2-Leu205 is thought to be analogous to residue Tyr210 of the M subunit of the reaction centre (RC) of the purple photosynthetic bacterium *Rhodobacter sphaeroides*. Previous mutagenesis experiments have shown that the replacement of M-Tyr210 by leucine reduces the rate of the primary charge separation in purple bacterial RCs. Thus, the mutation Leu205Tyr was constructed in order to study whether the rate of primary charge separation is increased in this mutant. The Leu205Tyr mutant could grow photoautotrophically at wild type rates. However, its light-saturated PS II activity was reduced to ~62% compared to the wild type. PS II RCs isolated from a "wild type" control transformant and from the Leu205Tyr mutant showed in ultrafast transient absorption measurements that the rate of formation of the radical pair was slowed down in the mutant (lifetime of 40±7 ps compared to 21±2 ps for the wild type PS II RCs). Also the quantum yield of charge separation was reduced by about 50%. Fluorescence and thermoluminescence measurements in whole cells were also consistent with a reduced quantum yield of charge separation in PS II.

Mutations Thr2Ala and Thr2Ser were constructed in order to study the role of D2 phosphorylation in *C. reinhardtii*. Biochemical characterisation of the Thr2Ala and Thr2Ser mutants indicated that both strains could grow photoautotrophically at wild type rates. Moreover, oxygen evolution, fluorescence and thermoluminescence measurements suggested that these site-directed mutations have only minor effects on PS II function. In addition to these experiments, attempts were carried out to find out whether D2 can be phosphorylated in *C. reinhardtii*. These attempts included *in vivo* labelling of whole cells with [^{32}P]-orthophosphate, *in vitro* labelling of thylakoid membranes with [γ-^{32}P]-ATP, pulse-chase labelling of cells with [^{14}C]-acetate and the use of a monospecific antibody against phosphothreonine. The data obtained from these experiments did not provide any indication that D2 can be phosphorylated in *C. reinhardtii*.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Peter Nixon, for his help, guidance and constructive criticism during the course of this work. I would also like to thank Prof. Jim Barber for giving me the opportunity to work in this lab as a member of the Photosynthesis Research Group.

I should also extend my thanks to the following people who have contributed to the production of experimental data, necessary for the characterisation of the site-directed mutants presented here: Dr. James Durrant, Dr. David Klug, Dr. Linda Giorgi, Steve Merry and Melissa Joseph from the Molecular Dynamics Group at Imperial College (for their femtosecond measurements); Dr. Bruce Diner from the Central Research and Development Department at E. I. du Pont de Nemours, U.S.A (for carrying out the fluorescence decay measurements); Dr. Imre Vass and Dr. Zsuzsanna Deak from the Biological Research Centre, Szeged, Hungary (for their fluorescence induction and thermoluminescence measurements).

Many people have given me help, support and encouragement throughout the complexities associated with a Ph.D. Particularly, I would like to thank Dr. Alison Telfer for all her invaluable advices concerning various aspects of photosystem II function and her practical help in fluorescence and spectroscopy, Dr. Olaf Kruse (also for his help with the phosphorylation experiments), Francisco Morais, Dr. Pedro Canovas, Dr. Giulia Friso, Dr. Marta Dalla Chiesa, Dr. Sedigeh Alizadeh (also for her help with the isolation of PS II RCs), Dr. Ben Hankamer, Dr. Daniella Zheleva, Jon Nield, Dr. Markella Ponticos, Dr. Emanuel Franco, Maria Sarcina, Dr. Leo Sazanov, Paul Burroughs, Deniz Stewart and all members of the Photosynthesis Research Group both past and present. I am also grateful to Dr. Javier De Las Rivas (University of Bilbao, Spain) for his help with fluorescence, spectroscopy and for proof-reading a part of the thesis, Dr. James Durrant for patiently explaining me the femtosecond data and also for proof-reading part of the thesis and also Dr. Josef Komenda (Institute of Microbiology, Trebon, Czech Republic) for his help with the pulse-chase experiments. I should also thank Dr. Saul Purton (University College, University of London) for introducing me to the use of the particle gun.

I am also indebted to Karim Maghlaoui, Susie van Acker, David Frith and Caroline Woollin for their excellent technical support and Roisin Smith and Lyn Barber for all the help and patience.

Finally, I would like to acknowledge the State Scholarship Foundation of Greece (I.K.Y.) for providing me with financial support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Å	Ångstrom
ADP	adenosine-5'-diphosphate
AP	alkaline phosphatase buffer
ATP	adenosine-5'-triphosphate
BChl	bacteriochlorophyll
BCIP	5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt
BPheo	bacteriopheophytin
BSA	Bovine Serum Albumine
C-terminal	carboxy-terminal
C. moewusii	*Chlamydomonas moewusii*
C. reinhardtii	*Chlamydomonas reinhardtii*
CAB polypeptides	chlorophyll *a/b*-binding polypeptides
CAP	chloramphenicol
CF_0	membrane integral part of the chloroplast ATP synthase
CF_1	extrinsic protein of the chloroplast ATP synthase
CHI	cycloheximide
Chl	chlorophyll
ctDNA	chloroplast DNA
Cyt	cytochrome
Cyt b_{HP}	high-potential b-type cytochrome
Cyt b_{LP}	low-potential b-type cytochrome
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone
DCBQ	2,6-dichloro-*p*-benzoquinone
DCMU	3-[3,4-dichlorophenyl]-1,1-dimethylurea
dCTP	2'-deoxycytidine-5'-triphopshate
dH_2O	distilled H_2O
DM	n-dodecyl-β-D-maltoside
DMBQ	2,5-dimethyl-*p*-benzoquinone
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside triphopshate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ENDOR	electron nuclear double resonance
EPR	electron paramagnetic resonance
EtBr	ethidium bromide

F_A, F_B, F_X	low potential iron-sulphur centres in PS I
F_D	ferredoxin
Fe	non-haem iron
Fe-S	iron-sulphur centre
F_m	maximal level of fluorescence obtained when all photosystem II reaction centres are "closed" for photochemistry
F_o	minimal level of fluorescence obtained when all photosystem II reaction centres are "open" for photochemistry
F_v	F_m-F_o
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HSM	High salt minimal medium
IPTG	isopropylthio-β-D-galactoside
LHCI	light-harvesting complex I
LHCII	light-harvesting complex II
MES	2-[morpholino]ethane-sulphonic acid
MOPS	morpholinopropane-sulphonic acid
mRNA	messenger RNA
mt^+ or mt^-	mating type (+) or (-)
N-terminal	amino-terminal
$NAD(P)^+$	β-nicotinamide adenine dinucleotide phosphate
NAD(P)H	reduced β-nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium chloride (2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-[3,3'-dimethoxy-4,4'-diphenylene] ditetrazolium chloride)
OD	optical density
ORF	open reading frame
P	primary electron donor in purple bacterial reaction centre
P680	primary electron donor of PS II
P700	primary electron donor of PS I
PAGE	polyacrylamide gel electrophoresis
PBS	phycobilisome
PC	plastocyanin
PCR	Polymerase Chain Reaction
PEG	polyethylene glycol
Pheo	pheophytin
PMSF	phenylmethanesulphonyl fluoride
PQ	plastoquinone

PS I	photosystem I
PS II	photosystem II
Q_A	plastoquinone molecule bound to the D2 protein
Q_B	plastoquinone molecule bound to the D1 protein
Q_BH_2	plastoquinol
Rb. capsulatus	*Rhodobacter capsulatus*
Rb. sphaeroides	*Rhodobacter sphaeroides*
RC	reaction centre
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
Rps. viridis	*Rhodopseudomonas viridis*
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SiMo	silicomolybdate
S_n	S-state or oxidation state of the oxygen-evolving complex, where n indicates the number of positive charges accumulated ($0 \leq n \leq 4$)
$spec^R$	spectinomycin resistance
SSC	saline sodium citrate
ssDNA	single-stranded DNA
TAE	Tris acetate EDTA buffer
TAP	Tris acetate phosphate medium
TBE	Tris borate EDTA
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TE	Tris-EDTA buffer
TL	thermoluminescence
Tris	2-amino-2-hydroxy-methylpropane-1,3-diol
Tween 20	polyoxyethylene-sorbitan monolaureate
UTR	untranslated region
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
Y_D (or D)	redox-active tyrosyl residue of the D2 protein
Y_Z (or Z)	redox-active tyrosyl residue of the D1 protein

1. Introduction

Photosynthesis is the process by which living organisms convert the energy of light into the chemical energy of organic molecules. The photosynthetic process involves many intermediate steps. The photosynthetic reactions are usually described by the following generalised equation:

$$CO_2 + 2H_2A + \text{light} \rightarrow [CH_2O] + H_2O + 2A$$

where $2H_2A$ represents an oxidisable substrate, $[CH_2O]$ a general carbohydrate and A the oxidised by-product (van Niel, 1962).

Photosynthetic organisms can be divided into oxygenic and anoxygenic types. Oxygenic organisms, such as higher plants, algae and cyanobacteria, produce oxygen and therefore, use H_2O as the hydrogen donor. The anoxygenic types utilise a variety of inorganic and organic compounds as hydrogen donors ranging from hydrogen and hydrogen sulphide (H_2S) to organic acids. This group includes various genera of purple and green bacteria.

A convenient way to consider photosynthesis is to divide it into light-requiring and non light-requiring reactions ("dark reactions"). The former ("light reactions") are responsible for the photochemical oxidation of the hydrogen donor with the concomitant production of high energy compounds and reducing equivalents, in the form of ATP and NAD(P)H, respectively. Both ATP and NAD(P)H are then utilised in the reductive synthesis of carbohydrates in the so-called dark reactions.

The general aim of the work described here is the study of structure-function relationships within photosystem II, which is a major pigment-protein complex involved in the light reactions. This was accomplished by the protein engineering of D2, one of the proteins that provide the site for primary photochemistry in photosystem II. The organism used for this purpose was the unicellular green alga *Chlamydomonas reinhardtii*.

1.1 Overview of the photosynthetic apparatus in oxygenic organisms

In plants and algae, the photosynthetic processes are localised in organelles called chloroplasts. Chloroplasts are semiautonomous in the sense that they carry their own DNA and the protein synthesis machinery for translation of the appropriate messenger RNAs. A chloroplast has a freely-permeable outer membrane and a selectively-permeable inner membrane. The latter encloses a soluble phase, the stroma, which is analogous to the mitochondrial matrix. Immersed in the stroma are membrane structures called thylakoid membranes, which are often stacked to form units called grana. The latter are irregularly

interconnected by thylakoid extensions called stroma lamellae. Thylakoids enclose an interior space, called lumen. Light reactions occur on or within the thylakoid membranes. Dark reactions take place in the stroma.

The prokaryotic cyanobacteria do not contain chloroplasts, but instead have thylakoids, located adjacent to the cell membrane, that are sometimes arranged in parallel to form concentric layers (reviewed by Bryant, 1994).

Light reactions are mediated by four thylakoid membrane-bound protein complexes: photosystem I (PS I), photosystem II (PS II) the cytochrome (Cyt) *b/f* complex and the CF_0-CF_1 ATP synthase complex (Figure 1.1) (reviewed by Andersson and Barber, 1994). In plants and green algae, associated with PS I and PS II are light harvesting complexes (LHCI and LHCII, respectively) that gather the excitation energy needed for photochemistry within the two photosystems. In cyanobacteria and red algae the LHC is replaced by the phycobilisome (PBS), which is water-soluble and is located on the surface of the thylakoid membrane in such a way as to preferentially transfer energy to PS II (reviewed in Grossman *et al.*, 1995).

The structural differentiation of thylakoids into grana and stroma lamellae reflects a functional differentiation and a lateral heterogeneity (Anderson and Andersson, 1988). Thus, the ATP synthase and the PS I complexes are located only in the stroma-exposed thylakoid regions (Miller and Staehelin, 1976; Olive and Vallon, 1991). In contrast, most of the PS II complexes and LHCII are confined to the appressed thylakoid regions. The cytochrome *b/f* complex appears to be distributed in both thylakoid regions (Anderson, 1992). Several studies have suggested that there is heterogeneity among both PS II and PS I. The major population of PS II (PS IIα) is located in the grana and has antennae which are about twice as large as the one attached to the stroma PS II (PS IIβ) (Anderson and Melis, 1983). Other differences between the two types of PS II include the absence of the high potential form of cytochrome b_{559} and of the 22-kDa subunit (encoded by the *psbS* gene) from PS IIβ (reviewed in Andersson and Barber, 1994). PS I is also heterogeneous and can be divided into α- and β-forms (Svensson *et al.*, 1991). PS Iβ contains a smaller antenna and is located in the stroma lamellae regions, while PS Iα is located in the grana margins extending into the peripheral annulus of the actual appressed portions of the grana.

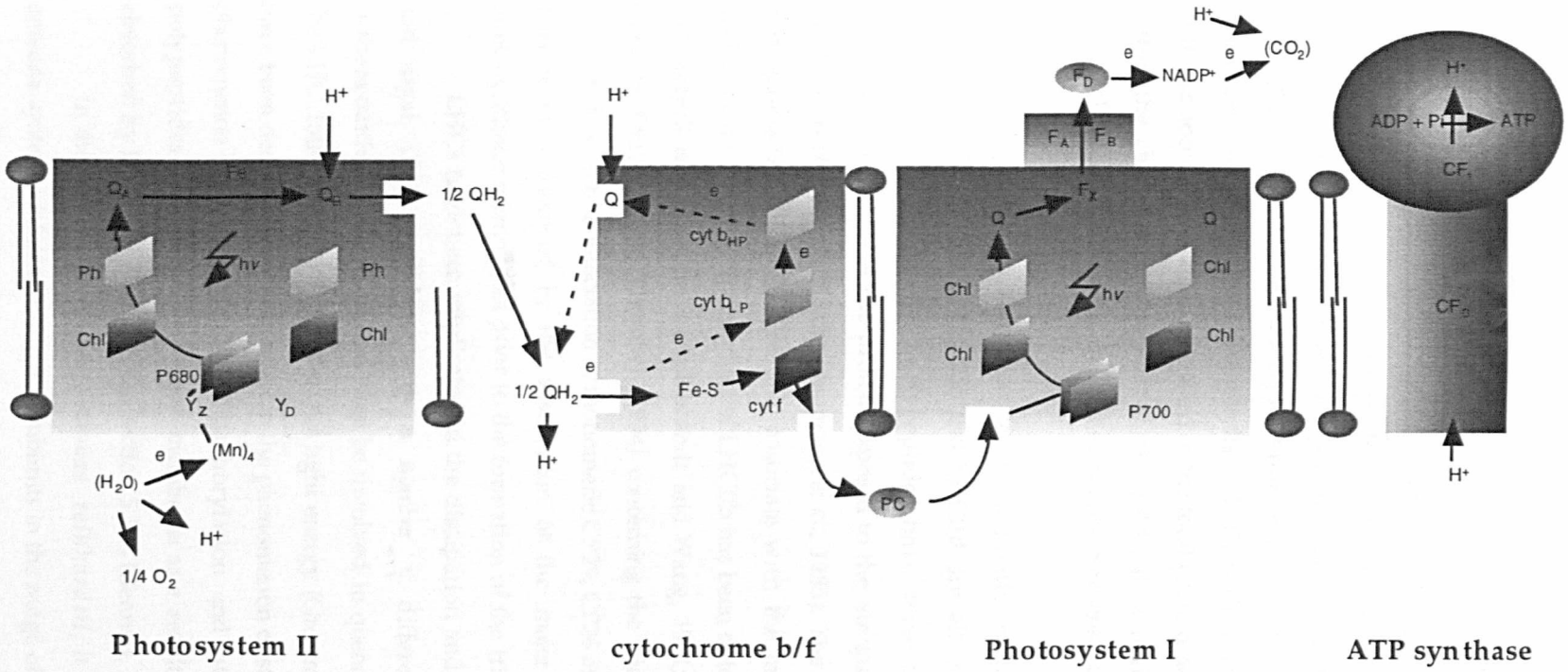


Figure 1.1: The photosynthetic apparatus within the thylakoid membrane. Arrows indicate the electron transport pathway. $(Mn)_4$, manganese cluster; Y_Z and Y_D, redox active tyrosines; Chl, chlorophyll *a*; Phe, pheophytin; P680, primary electron donor of PS II; Q, quinones, plastoquinones (Q_A and Q_B) in PS II and phylloquinone in PS I; Fe, non-haem iron; QH_2, plastoquinol; Fe-S, iron sulphur centre; Cyt b_{LP}, low potential b-type cytochrome; Cyt b_{HP}, high potential b-type cytochrome; Cyt f, cytochrome f; PC, plastocyanin; P700, primary electron donor of PS I; F_X, F_A, F_B, low potential iron sulphur centres; F_D, ferredoxin (adapted from a diagram in Barber and Andersson, 1994)

1.1.1 Antenna pigment-protein complexes of higher plants and green algae

In plants and green algae, both LHCI and LHCII are composed of a family of related proteins. These polypeptides, which bind chlorophylls *a*, *b* and xanthophylls, are called the LHC or CAB polypeptides (for chlorophyll *a/b*-binding) (reviewed in Thornber *et al.*, 1994; Grossman *et al.*, 1995).

In higher plants, at least four different LHC components are associated with LHCI (LHCIa-d) with polypeptides ranging from 11 to 24 kDa (Thornber *et al.*, 1994). In analogy with LHCI, the LHCII pigment-protein subcomplexes, are LHCIIa, LHCIIb, LHCIIc and LHCIId (Thornber *et al.*, 1994). The polypeptides of LHCIIa, c and d (also designated as CP29, CP26 and CP24, respectively) are not very abundant constituents of LHCII and exist as monomers associated with chlorophylls. LHCIIb is the most abundant chl-protein complex of PS II and accounts for about one third of the total protein and about 45% of total chlorophyll within the chloroplast (Thornber *et al.*, 1994). It is peripherally located relative to the other LHC polypeptides and in higher plants contains very similar polypeptides of 28, 27 and 25 kDa.

LHC apoproteins are encoded by a family of nuclear genes (*cab* genes). LHCIa, LHCIb, LHCIIa, LHCIIb, LHCIIc and LHCIId are all encoded by this multigene family (Green *et al.*, 1991). The LHC polypeptides have three α-helical transmembrane domains with the N-terminus of the protein exposed to the stroma and the C-terminus in the lumen (Andersson *et al.*, 1982; Karlin-Neumann *et al.*, 1985). The polypeptides of LHCIIb assemble into trimers (Kühlbrandt, 1988) that associate with the monomeric LHCII polypeptides and the PS II core. The atomic structure of LHCIIb has been determined at a resolution of 3.4 Å by Kühlbrandt and co-workers (Kühlbrandt and Wang, 1991; Kühlbrandt *et al.*, 1994). Recent work by Dreyfuss and Thornber (1994) concerning the biogenesis of LHCII in barley, has suggested an initial association of monomeric CP29, CP26 and CP24-chl complexes with PS II. This is then followed by the association of the major LHCIIb polypeptides with the photosynthetic membranes prior to the formation of the trimers.

LHCs have been implicated in the dissipation and distribution of light energy. Plant and algal LHCs associate with a number of different xanthophylls. Violoxanthin, antheraxanthin and zeaxanthin may be involved in quenching excited chl molecules within the LHC, following exposure to excess light energy (Grossman *et al.*, 1995). Moreover, LHCII have been described to be involved in the phenomenon of state transitions (Allen, 1992). This phenomenon involves reversible phosphorylation and lateral migration of specific LHCII polypeptides within the thylakoid membrane as a result of light energy that is unevenly absorbed by PS II and PS I (see also section 5.1.3) (Bennett, 1977).

In the green alga *Chlamydomonas reinhardtii*, it has been suggested that the PS II antenna system contains four LHCII subunits in the range of 31 to 25 kDa, designated p11, p13, p16 and p17 in addition to the monomeric CP29, CP26 and CP24 polypeptides (designated as

p9, p10 and p22, respectively) (Bassi and Wollman, 1991). However, Allen and Staehelin (1994) recently showed that these antenna systems contain at least 10 polypeptides associated with LHCII, 3 with CP29 and 2 with CP26.

1.1.2 Photosystem II

Photosystem II (PS II) is a multi-subunit pigment-protein complex that catalyses the light-dependent oxidation of H_2O and reduction of plastoquinone (PQ) with the concomitant production of oxygen. This complex together with the D2 polypeptide, which is the subject of this study, will be discussed in detail, later in this chapter (section 1.2).

1.1.3 The cytochrome *b/f* complex

The cytochrome (Cyt) *b/f* complex catalyses the oxidation of plastoquinol and the reduction of plastocyanin (or cytochrome *c6*) and participates in the establishment of the proton motive force used in the synthesis of ATP. This protein complex comprises four major subunits, a *c*-type cytochrome (Cyt *f*), two *b*-type cytochromes (low and high potential forms of Cyt b_6), a ~17 kDa polypeptide called subunit IV and a Rieske [2Fe-2S] centre (Cramer *et al.*, 1991, 1994). In addition to these polypeptides, three more subunits with apparent molecular weights of around 4 kDa have been associated with the Cyt *b/f* complex, the polypeptide products of genes *petG* (Haley and Bogorad, 1989), *petX* (de Vitry *et al.*, 1995) and *petL* (Breyton *et al.*, 1995).

Reduced plastoquinone, produced by PS II, reduces the Rieske iron-sulphur centre, while oxidised plastocyanin (or cytochrome *c6*) extracts electrons from Cyt f. Electrons then flow from the Rieske centre to the oxidised Cyt f. At the same time, the protons associated with plastoquinol are released into the lumen. As the oxidation of plastoquinol is a two electron/two proton event, the second electron passes to a low-potential form of Cyt b_6 (Cyt b_{LP}). This second electron is then passed to a high-potential form of Cyt b_6, according to the Q-cycle hypothesis of Mitchell (1975), giving rise to a quinone-dependent proton pump (Figure 1.1).

1.1.4 Photosystem I

Photosystem I (PS I) is a pigment-protein complex that catalyses the light-induced transmembrane electron transfer, from either the copper protein plastocyanin (in higher plants) or the haem protein cytochrome *c6* (in cyanobacteria and green algae) to the iron-sulphur protein ferredoxin or the flavoprotein flavodoxin (in cyanobacteria). Light absorbed by the antenna of PS I is rapidly transferred to the primary donor P700 (probably a Chl *a* dimer). An electron is then transferred to the primary acceptor A_0 (a monomeric Chl *a*

molecule) and then through a series of secondary acceptors, denoted A_1 (a phylloquinone), F_X, F_B and F_A ([4Fe-4S] iron sulphur centres) it reaches the soluble ferredoxin on the stroma side of PS I (reviewed in Golbeck and Bryant, 1991).

In the past few years, structural information on PS I has been made available, following the successful crystallisation of PS I from the thermophilic cyanobacterium *Synechococcus elongatus*, and resolution of the structure by X-ray crystallography to 6 Å (and recently to 4.5 Å) by Witt and co-workers (Krauss *et al.*, 1993; Schubert *et al.*, 1995). PS I consists of at least 13 individual polypeptides (Golbeck and Bryant, 1991; Golbeck, 1992, 1993). The reaction centre core of PS I is a heterodimeric complex of two related polypeptides of around 83 kDa, called PsaA and PsaB. PsaA and PsaB coordinate the primary electron donor P700 and early electron acceptors A_0, A_1 and F_X. The electron acceptors, F_A and F_B, are coordinated by a 9 kDa polypeptide, PsaC, located on the stromal side of the thylakoid membrane. In addition to the three cofactor binding polypeptides, the crystallised PS I complex contains seven other proteins (Schubert *et al.*, 1995). PsaD and PsaE are stroma-exposed polypeptides, located on the reducing side of PS I which are involved in the docking of ferredoxin to PS I and in the cyclic electron flow around PS I, respectively (Golbeck, 1992, 1993). PsaF is located at the lumenal side and has been suggested to be a plastocyanin-docking protein (Wynn and Malkin, 1988). PsaI and PsaJ are two small intrinsic membrane proteins that have been suggested to be involved in the proper assembly of PsaL and PsaF, respectively (Xu *et al.*, 1994, 1995). PsaL has been suggested to be the connecting protein for the formation of PS I trimers (Golbeck, 1993), as seen in the crystal structure of PS I (Krauss *et al.*, 1993). PsaK and PsaM are two small hydrophobic polypeptides that appear to be tightly bound to the PS I reaction centre, but whose function is still unknown (Golbeck and Bryant, 1991).

1.1.5 The CF_0-CF_1 ATP synthase complex

The electrochemical potential gradient of protons resulting from the photochemical electron transport provides the free energy to drive the conversion of ADP to ATP. This phosphorylation process is catalysed by a complex known as the coupling factor, or the CF_0-CF_1 complex (Glaser and Norling, 1991, Barber, 1994). The membrane integral part of the ATP synthase complex (CF_0) is responsible for proton translocation and consists of four different, largely hydrophobic subunits (I, II, III and IV). The extrinsic hydrophilic subcomplex CF_1 forms the catalytic activity and consists of nine subunits: three α-subunits, three β-subunits and one each of the subunits γ, δ and ε.

1.2 Structure and function of the photosystem II complex

1.2.1 Electron transfer in Photosystem II

Photosystem II is that part of the photosynthetic apparatus that splits water into its elemental constituents (Figure 1.3). The electrons released from water are passed through a series of acceptor/donor molecules, finally reducing the terminal PS II quinone electron acceptor, Q_B. The initial photochemical events occur when light energy, absorbed by the light-harvesting complex, LHCII, is funnelled to the primary electron donor (a chlorophyll *a* dimer) of the PS II reaction centre, generating the excited state P680*. Within a few picoseconds, an electron is transferred to a pheophytin *a* molecule (Klimov *et al.*, 1977) generating the radical pair $P680^+Pheo^-$. The primary quinone electron acceptor Q_A (Stiehl and Witt, 1969) receives an electron from $Pheo^-$, within 250-300 ps (Nuijs *et al.*, 1986) further stabilising the charge separation across the complex. In order to couple the one-electron process of the primary reactants (P680 and Pheo) with the two-electron process needed for plastoquinone (PQ) reduction and the four-electron process involved in H_2O oxidation (O_2 evolution), PS II has evolved unique binary and quartenary gating functions on the reducing and oxidising sides, respectively. Thus, on the reducing side, Q_B (a bound plastoquinone) accepts sequentially two electrons from Q_A (in the time scale of 100-500 μs) (Hansson and Wydrzynski, 1990) and two protons from the stroma to become the fully reduced plastoquinol (PQH_2) which then leaves the PS II complex. PQH_2 is then replaced by another plastoquinone available in the plastoquinone pool in the thylakoid membranes.

It is the strong oxidising potential of $P680^+$ [$E_m \approx +1.2$ mV (Klimov *et al.*, 1979)] that in conjunction with a protein-bound cluster of four manganese atoms, drives the photo-oxidation of water to molecular oxygen providing electrons for the reduction of $P680^+$. The latter is reduced by a secondary electron donor, Z (or Y_Z), a redox-active tyrosyl residue (Tyr161) on the D1 protein (Debus *et al.*, 1988b, Metz *et al.*, 1989). A second redox-active tyrosine (D or Y_D), located at position 160 of D2 (*Chlamydomonas* and cyanobacterial numbering, Tyr161 in higher plants) (Debus *et al.*, 1988a; Vermaas *et al.*, 1988a) is not involved in the main electron transfer chain nor is essential for H_2O oxidation (see also section 1.4).

After electron donation to $P680^+$, the oxidised Y_Z is re-reduced by the water-splitting complex composed of a cluster of manganese ions and possibly also involving organic radicals. After four electron donations to the oxidised Y_Z, O_2 is evolved (see reviews of Rutherford *et al.*, 1992; Nixon *et al.*, 1992a). It is now well established that the water-oxidising complex exists in five redox states, denoted S_0, S_1, S_2, S_3 and S_4 where the subscript is the number of the positive charges stored (Kok *et al.*, 1970). Among them, S_0 represents the lowest and S_4 the highest oxidation state. During subsequent photoacts, four electrons are sequentially extracted from the water-oxidising complex which advances to the next higher S-state. In dark-adapted samples the S-state cycle starts from the dark-stable S_0 and S_1 states. The S_2

and S_3 states are stable for tens of seconds in the dark. S_4 is a short-lived, intermediate state whose spontaneous conversion to S_0 is accompanied with the release of molecular oxygen (Rutherford *et al.*, 1992). It is well established that the site of water oxidation is a cluster of (probably) four manganese ions (Rutherford *et al.*, 1992). Mn undergoes redox changes upon each S-state transition, perhaps with the exception of the S_2 to S_3 transition, where an amino acid, such as histidine (Boussac *et al.*, 1990) or Y_Z itself (Hallahan *et al.*, 1990) may be oxidised. Calcium and chloride ions have been suggested to be involved in the formation, stability and function of the manganese cluster (reviewed in Rutherford *et al.*, 1992). Although, considerable amount of data have increased our understanding concerning the structure and function of the O_2-evolving complex, important aspects of the mechanism of water splitting remain unresolved.

1.2.2 Primary electron transfer in the purple bacterial and PS II reaction centres

1.2.2.1 Primary electron transfer in the purple bacterial reaction centre

The study of electron transfer in PS II has benefited from the determination of the X-ray structure of the related purple bacterial reaction centre (RC). The crystal structures of RCs from the purple non-sulphur bacteria *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* have been solved to atomic resolution (Deisenhofer *et al.*, 1984, 1985; Chang *et al.*, 1986; Allen *et al.*, 1987a, b). The RC from *Rb. sphaeroides* contains three protein subunits referred to as L, M and H, according to the their respective mobilities in SDS-polyacrylamide gels. Associated with the L and M subunits are the cofactors, consisting of four bacteriochlorophyll (BChl) *a*, two bacteriopheophytin (BPhe) *a*, one atom of non-haem ferrous iron, two quinones (Q_A and Q_B) and in some species one carotenoid (reviewed in Schiffer and Norris, 1993). The L and M subunits are transmembrane proteins with similar structures. Each protein contains five transmembrane helices and other short helical segments (Deisenhofer *et al.*, 1985). The cofactors are arranged in two branches, A and B with an approximate C2 axis of symmetry. This axis runs from a dimer of BChl *a* molecules (the primary electron donor, P) at the periplasmic side of the membrane, to the iron atom at the cytoplasmic side. Each branch contains, in addition to one BChl of P, a BChl molecule (the accessory BChl) located near P, a BPhe molecule and a quinone. Spectroscopic studies have revealed that electron transfer proceeds only through chain A (reviewed in Kirmaier and Holten, 1987). The cofactors of the active branch (A) and Q_B are associated with the L subunit whereas those of the inactive branch (B) and Q_A are associated with the M subunit.

Electron transfer within the purple bacterial RC proceeds as follows: absorption of light by the RC generates the excited electronic state P*. After ~3 ps, an electron arrives to the BPhe on the active branch, A, forming the state P^+BPhe^-. In a subsequent step the electron

is transferred in ~200 ps from $BPhe_A^-$ to Q_A. In a final slower reaction (~100 μs) the electron is transferred from Q_A^- to Q_B. In the intact organism, reducing equivalents leave the RC via Q_B and a *c*-type cytochrome reduces P^+ to P (Kirmaier and Holten, 1987).

The role of the accessory BChl located between P and $BPhe_A^-$ ($BChl_A$), in primary charge separation has not been definitely assigned. There are two competing ideas trying to address this question (reviewed in Kirmaier and Holten, 1993). One is that an initial transfer of an electron from P* to $BChl_A$ creates an intermediate radical pair $P^+BChl_A^-$, which then passes an electron to $BPhe_A$ (the "two step" mechanism) (Holzapfel *et al.*, 1989; Schmidt *et al.*, 1994; Zinth *et al.*, 1995). The second possibility most often discussed for the role of $BChl_A$ is that it provides orbitals through which electron transfer from P* to $BPhe_A$ is facilitated but $BChl_A$ is not actually reduced (the "superexchange" mechanism) (Woodbury *et al.*, 1995). Additional intriguing points concerning the process of primary electron transfer in the bacterial RC include the basis for the unidirectionality of electron transfer, as well as the influence of the protein side groups surrounding the chromophores. In order to study the role of certain amino acid residues in primary electron transfer, side-directed mutagenesis has been used extensively (e.g., Bylina and Youvan, 1988; Gray *et al.*, 1990; Nagarajan *et al.*, 1990; Williams *et al.*, 1992; Chirino *et al.*, 1994; Shochat *et al.*, 1994).

One of the most studied residues in connection with a role in primary charge separation is a tyrosine at position 210 (*Rb. sphaeroides* numbering) of the M subunit. M-Tyr210 is in the direct vicinity of P, $BChl_A$ and $BPhe_A$. This residue is conserved in *Rb. sphaeroides*, *Rps. viridis*, *Rb. capsulatus* and *Rhodospirillum rubrum* (Williams *et al.*, 1986). The symmetry-related amino acid in the L polypeptide is a phenylalanine at position 181. In the RC of the green photosynthetic bacterium *Chloroflexus aurianticus*, a leucine is found at M-210 (Shiozawa, 1989). Primary charge separation in this bacterium has been shown (Becker *et al.*, 1991) to be slower than in *Rb. sphaeroides* (7 ps compared with 3 ps). Parson *et al.* (1990) suggested that the interactions of M-Tyr210 with P and $BChl_A$ may lower the energy of the $P^+BChl_A^-$, thereby facilitating electron transfer along the A branch. To understand the role of M-Tyr210 in primary electron transfer, a series of site-directed mutants has been constructed. Thus, tyrosine has been replaced by phenylalanine (Gray *et al.*, 1990; Nagarajan *et al.*, 1990; Finkele *et al.*, 1990; Jones *et al.*, 1994), leucine (Gray *et al.*, 1990; Finkele *et al.*, 1990; Jones *et al.*, 1994), isoleucine (Nagarajan *et al.*, 1990), histidine (Jones *et al.*, 1994) and tryptophan (Shochat *et al.*, 1994) in *Rb. sphaeroides* and to phenylalanine, histidine and threonine in *Rb. capsulatus* (Chan *et al.*, 1991). Except for histidine, it was found that in all cases the rate of charge separation was lower in the mutants than in the wild type, indicating that M-Tyr210 plays an important role in the initial electron transfer. The time constants found were approximately 16 ps, 22 ps, and 16 ps for the Tyr210Phe, Tyr210Leu and Tyr210Ile mutants, respectively. The M-Tyr210Leu mutation also resulted in non-photoheterotrophic growth under low light intensities, which did not coincide with a decrease in the amount of RC present in the mutant (measured by steady-state

photobleaching spectra of whole cells and pigments) (Gray *et al.*, 1990). The fact that the Tyr210Leu colonies appeared only under high light indicated that there was a problem with the mutant in utilising light efficiently (Gray *et al.*, 1990).

Chan *et al.* (1991) suggested that both the hydroxyl group and the aromatic ring of the tyrosine are important for efficient electron transfer through the A branch. Initially, Allen *et al.* (1987a) had suggested that tyrosine could be hydrogen-bonded to P, however, Mattioli *et al.* (1991) using Raman spectroscopy excluded this possibility. Shochat *et al.* (1994) postulated that the phenolic residue acts on electron transfer by influencing the polarity of the electrostatic environment of P. Nagarajan *et al.* (1990) performed studies on the temperature dependence of the rate of charge separation in two mutants of *Rb. sphaeroides* (M-Tyr210Phe and M-Tyr210Ile). Their results suggested that electrostatic interactions of the phenolic OH group of tyrosine with pigments in its vicinity and electron conduction via the phenyl ring could both be important factors in determining the rate of primary charge separation.

1.2.2.2 *Primary electron transfer in the isolated PS II reaction centre*

Although the three dimensional structure of the PS II RC has not yet been determined, the similarities between the L/M subunits of the bacterial RC and D1/D2, in their primary structures (Michel and Deisenhofer, 1988), has allowed various groups to construct models of the D1/D2 heterodimer (Svensson *et al.*, 1990; Ruffle *et al.*, 1992; Svensson and Styring, 1995). These models are helping to provide the structural framework guiding the choice of site-directed mutations designed to probe structure-function relationships (e.g., Nixon *et al.*, 1992a; Pakrasi and Vermaas, 1992; Vermaas 1993).

Moreover, the isolation of a photochemically active PS II RC (Nanba and Satoh, 1987) has provided researchers with valuable material for the study of the primary electron transfer process in PS II. The isolated PS II RC can carry out a very limited number of the functional activities associated with PS II, because many PS II constituents are missing (section 1.2.3). Thus, in the absence of artificial electron donors and acceptors, light-induced reactions within the isolated PS II RC are limited to the production of the radical pair, $P680^{+}Phe^{-}$, and subsequent charge recombination pathways. The primary charge separation in the isolated PS II RC takes place in a few picoseconds. Initially, Wasielewski *et al.* (1989) using laser transient absorption spectroscopy proposed that primary charge separation in isolated PS II RCs occurs with a lifetime of 3 ps at 277K. However, the transient absorption kinetic studies of Hastings *et al.* (1992) and Durrant *et al.* (1993) at room temperature showed that the formation of the radical pair state occurred primarily with a 21 ps time constant. Recent studies of Klug *et al.* (1995) have also indicated the presence of a 3 ps component; however, its amplitude is approximately three times lower than that of the 21 ps component. In addition, Kumazaki *et al.* (1995) identified another minor component of charge separation

with a lifetime of approximately 100 ps. The initial discrepancies in the reports for primary charge separation between different groups (3 ps compared to 21 ps) appear now to be resolved with a consensus forming for the presence of a dominant kinetic component of 21 ps (Donovan *et al.*, 1995; Greenfield *et al.*, 1995; Klug *et al.*, 1995). The problems in measuring the exact lifetimes of primary charge separation in the PS II RC, originated mainly from the fact that the spectral resolution between the different reaction centre pigments in the Q_y (680 nm) region of the PS II RC is not as clear as in the purple bacterial RCs. Moreover, as the P680 excited singlet state is not likely to be a deep trap for excitation energy (in contrast to the special pair of the purple bacterial RC), energy-transfer equilibration is expected to take place between P680 and the accessory chlorins following excitation of either chlorin pool (Durrant *et al.*, 1992). This energy-transfer equilibration takes place at a rate of ~100 fs^{-1} (Durrant *et al.*, 1992) and must therefore be taken into account in studies of primary electron transfer reactions in PS II.

The kinetics of charge recombination of the primary radical pair have been shown to be multiexponential, exhibiting at least two lifetimes of 20 and 52 ns (Booth *et al.*, 1991; Roelofs *et al.*, 1993).

In purple bacterial RCs primary charge separation takes place with a lifetime of 3 ps. It has been shown that the replacement of a specific amino acid residue of the M subunit (M-Tyr210) with leucine can slow down this process to 22 ps (see section 1.2.2.1). The analogous amino acid to M-Tyr210 in the PS II RC is a leucine at position 205 of D2 (*Chlamydomonas* numbering, 206 in higher plants) (Michel and Deisenhofer, 1988; Ruffle *et al.*, 1992) (Figure 1.2).

```
                199
M               NPFHGLSIAFLYSAGLLFAMHGAT
                **** ||| *  |  |* |**  * |****
                194
D2C             NPFHMMGVAGVLGAALLCAIHGAT
                ************************
                195
D2S             NPFHMMGVAGVLGAALLCAIHGAT
```

Figure 1.2: Alignment of the sequences in the Leu205Tyr area of the D2 polypeptide from *C. reinhardtii* (D2C), with those from D2 of *Spinacea oleracea* (D2S) and the M subunit from *Rhodobacter sphaeroides* (M) (data from Michel and Deisenhofer, 1988; Shiozawa *et al.*, 1989)

Assuming that the correct lifetime for primary charge separation in the isolated PS II RC is 21 ps, it has been suggested (Hastings *et al.*, 1992; Barber, 1993) that the difference observed between purple bacteria and PS II concerning the primary electron transfer process, could be attributed to the fact that the analogous amino acid to M-Tyr210, is a leucine in PS II, rather than a tyrosine. This suggestion has been addressed in this thesis by constructing

the D2-Leu205Tyr mutant in C. *reinhardtii*, with a view to study the influence of such a replacement in primary charge separation (see chapter 4).

1.2.3 Photosystem II structure

Photosystem II (PS II) is a pigment-protein complex consisting of more than twenty different proteins whose primary structures are known by DNA sequencing of chloroplast and nuclear genes (reviewed in Andersson and Barber, 1994; Barber, 1989, 1993; Barber and Andersson, 1994; Cogdell and Malkin, 1992; Diner *et al.*, 1991; Erickson and Rochaix, 1992; Hansson and Wydrzynski, 1990; Nixon *et al.*, 1992a; Pakrasi, 1995; Pakrasi and Vermaas, 1992; Vermaas, 1993; Vermaas *et al.*, 1993). Figure 1.3 shows a schematic diagram of the PS II complex. Table 1.1 lists the genes and proteins of PS II.

As can be seen from Table 1.1, at least 14 of the subunits of PS II are encoded by chloroplast DNA. Apart from 4 subunits, PS II polypeptides are hydrophobic and are integral membrane proteins. Altogether, protein components of the PS II complex are predicted to have a total of at least 35 membrane-spanning regions (Andersson and Barber, 1994; Vermaas *et al.*, 1993). Despite its functional importance, structural information about PS II is scarce. Consequently, a significant part of research has recently been directed towards understanding how the protein components of PS II are arranged within the complex (reviewed in Rögner *et al.*, 1995). One of the main questions that has arisen from this area of research is whether PS II exists as a monomer or dimer *in vivo* (Holzenburg *et al.*, 1993; Boekema *et al.*, 1995). The possibility that PS II exists as a dimer *in vivo* has been supported by recent data from work with higher plants by Lyon *et al.* (1993), Santini *et al.* (1994), Boekema *et al.* (1995) and Marr *et al.* (1996). In contrast, the electron micrographs of PS II-ordered arrays obtained by Holzenburg *et al.* (1993), Ford *et al.* (1995) and Tsiotis *et al.* (1996) have led these authors to suggest that PS II exists as a monomer *in vivo*.

Although diffracting crystals of PS II have not yet been obtained, elucidation of the structure of the bacterial reaction centre complex which shares many functional similarities with PS II has provided useful information on their structure (Michel and Deisenhofer, 1988).

As discussed in the previous section, light energy absorbed by the antenna complex is transferred to a reaction centre within PS II, which contains a photochemical trap where primary energy conversion occurs. Before the determination of the structure of the reaction centre from purple non-sulphur photosynthetic bacteria it was generally believed that the reaction centre of PS II was located on the CP47 protein (Nakatani *et al.*, 1984). Hints that this dogma might not be correct emerged when the genes for the L and M subunits of the purple bacterial reaction centre were sequenced (Williams *et al.*, 1984). These sequences showed similarities with the corresponding sequences from the D1 and D2 proteins of PS II. When the structure of the *Rps. viridis* reaction centre was published, it became evident that the PS II reaction centre was probably composed of a heterodimer of the D1 and D2 proteins (Barber,

1987; Trebst, 1986; Michel and Deisenhofer, 1988). This idea was supported by the biochemical isolation of a PS II reaction centre (RC) complex capable of performing primary photochemistry and containing the D1/D2 heterodimer but not CP47 (Nanba and Satoh, 1987). Apart from the D1 and D2 proteins the, PS II RC contains the α and β subunits of cytochrome b_{559} and a small subunit encoded by the product of the *psbI* gene (reviewed in Satoh, 1993).

The purple non-sulphur photosynthetic bacteria do not evolve oxygen. Therefore, there is no homology between the secondary and tertiary electron donors of their reaction centres and those of PS II. However, the purple non-sulphur photosynthetic bacteria show striking homologies with respect to the remaining redox components of the reaction centre. The primary donor, a pair of bacteriochlorophyll molecules and the electron acceptors bacteriopheophytin and ubiquinone , play homologous roles to the chl *a*, pheo *a* and plastoquinones found in PS II (Barber, 1993). Like its bacterial counterpart, the PS II reaction centre contains more cofactors than actually participate in the primary photochemistry. Moreover, it is thought that these components are bound in a bilaterally symmetrical fashion with each complex (Trebst, 1986; Michel and Deisenhofer, 1988). The isolated PS II RC does not retain any bound plastoquinones corresponding to the secondary electron acceptors Q_A and Q_B (Satoh, 1993). However, it is highly probable that the binding sites for these two plastoquinones are located on the D1/D2 heterodimer (Trebst, 1986; Barber, 1987; Michel and Deisenhofer, 1988). The loss of Q_A and Q_B during the isolation procedure could be due to the removal of the non-haem iron atom, which is a component that is common to the PS II and purple bacterial reaction centres (Barber, 1993). The cofactor composition of the isolated PS II reaction centre is still a matter of debate (reviewed in Satoh, 1993). However, in its most active and stable form, the isolated PS II RC complex has been shown to bind six chlorophylls per two β-carotenes per two pheophytins (Gounaris *et al.*, 1990; Kobayashi *et al.*, 1990).

The main polypeptide components of the PS II complex will be presented in the following sections. The D1 and D2 polypeptides will be described in greater detail because of their central role within the PS II reaction centre complex.

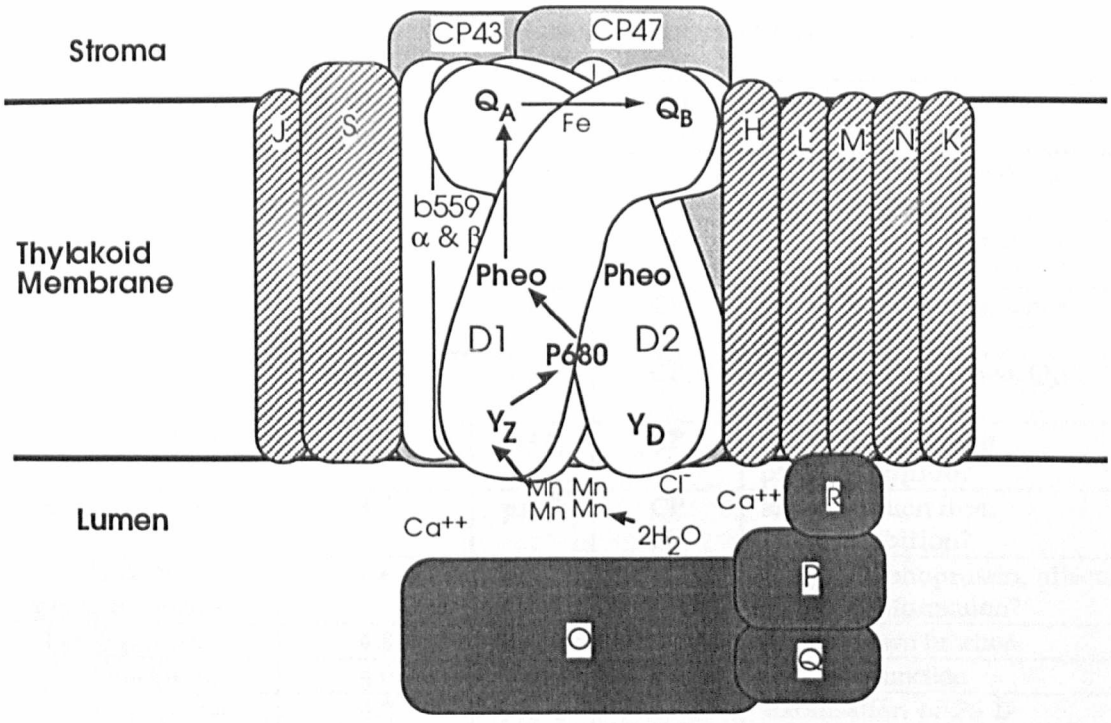


Figure 1.3: Schematic diagram of the PS II core complex of higher plants. Polypeptides are designated by their most common name or the letter of their designated gene. Cofactors mediating electron transport are depicted: P680 is probably a chlorophyll pair forming the primary electron donor; Pheo, are pheophytin *a* molecules; Q_A and Q_B are quinones; Fe is the non-haem iron; Y_Z and Y_D are redox-active tyrosines on D1 and D2, respectively.

Table 1.1: Summary of PS II genes and corresponding polypeptides

Protein	Molecular mass (kDa)[1]	Gene	Location of gene	Location and proposed function
D1	38.0	*psbA*	CP	RC, binds P680, Pheo, Q_B, Mn(?), contains Z
CP47	55.6	*psbB*	CP	Core, Chl*a* binding, inner antenna
CP43	50.1	*psbC*	CP	Core, Chl*a* binding, inner antenna
D2	39.4	*psbD*	CP	RC, binds P680, Pheo, Q_A, Mn(?), contains D
cyt b_{559} α-subunit	9.0	*psbE*	CP	RC, protection from photoinhibition?
cyt b_{559} β-subunit	4.5	*psbF*	CP	RC, protection from photoinhibition?
10-kDa phosphoprotein	7.6	*psbH*	CP	core, phosphoprotein, affects D1/D2 conformation?
I-polypeptide	4.8	*psbI*	CP	RC, unknown function
J-polypeptide	4.0	*psbJ*	CP	unknown function
K-polypeptide	4.3	*psbK*	CP	stabilisation of PS II complex?
L-polypeptide	4.3	*psbL*	CP	regulates Q_A binding?
M-polypeptide	3.8	*psbM*	CP	unknown function
N-polypeptide	4.7	*psbN*	CP	unknown function
33-kDa extrinsic polypeptide	26.5	*psbO*	N	O_2-evolving complex, Mn stabilising
23-kDa extrinsic polypeptide	20.2	*psbP*	N	O_2-evolving complex, regulating Ca^{2+}
16-kDa extrinsic polypeptide	16.5	*psbQ*	N	O_2-evolving complex, regulating Cl^-
10-kDa polypeptide	10.0	*psbR*	N	O_2-evolving complex, regulatory component
S-polypeptide	22.0	*psbS*	N	intrinsic, Chl-binding, CAB-like protein
ycf8 gene product	31 amino[2] acids	*psbT*	CP	protects PS II against light stress
cyt c550	160 amino[3] acids	*psbV*	-	lumenal side, present in cyanobacteria only
PsbW	6.1	*psbW*	N	unknown function

Data from Barber (1993); Nixon *et al.* (1992a); Pakrasi (1995); Lorkovic *et al.* (1995).

Abbreviations: CP, chloroplast encoded; N, nuclear encoded; RC, reaction centre; P680, primary electron donor; Pheo, pheophytin; Q_A and Q_B, plastoquinones bound to PS II RC; Z and D, Tyr 161 of D1 and D2, respectively.

[1]molecular mass predicted from higher plant gene sequence.

[2]: from C. *reinhardtii* gene sequence (Monod *et al.*, 1994)

[3]: from *Synechocystis* PCC 6803 gene sequence (Shen *et al.*, 1995)

1.2.4 Polypeptides of the Photosystem II reaction centre

1.2.4.1 The D1 and D2 proteins

As mentioned before, despite the structural complexity of PS II, the D1 and D2 proteins appear to carry all the necessary redox components for the primary photochemistry and possibly the water oxidation reactions. These polypeptides were originally visualised in autoradiograms, after pulse labelling *Chlamydomonas reinhardtii* cells with [^{14}C]-acetate under conditions that block all cytoplasmic translation. After fractionating the thylakoid membrane proteins by SDS-PAGE, two broad, diffuse bands with apparent molecular weights of 30-34 kDa were observed and designated D1 and D2 (for diffuse bands 1 and 2) (Chua and Gillham, 1977). D1 migrates more slowly than D2 in a standard SDS-PAGE system but the relative mobility of these two proteins is reversed in gels containing urea (Delepelaire, 1984). D1 and D2 also stain very poorly with Coomassie blue and are therefore difficult to detect in the absence of label.

D1 is encoded by the chloroplast gene *psbA*. Its nucleotide sequence was initially determined by Zurawski *et al.* (1982) for spinach and *Nicotiana debneyi*. The *psbA* gene was subsequently identified and sequenced from a number of higher plants, green algae and cyanobacteria (reviewed in Erickson and Rochaix, 1992). The *psbA* transcript encodes a translation product of 353 amino acids (352 in *Chlamydomonas* and 360 in cyanobacteria) although, the mature protein contains 344 amino acids as a result of post-translational processing (discussed later in this section). In cyanobacterial genomes, *psbA* exists in multiple copies. In addition, though not split in higher plants and cyanobacteria, the *psbA* genes of both *Euglena* and *Chlamydomonas* are interrupted by four introns (Karabin *et al.*, 1984; Erickson *et al.*, 1984).

The D2 protein is encoded by the chloroplast gene *psbD*. The nucleotide sequence of *psbD* has been determined for a variety of organisms (reviewed in Erickson and Rochaix, 1992) (see also section 1.3.2). The primary translation product of *psbD* encodes 353 amino acids (352 in *Chlamydomonas* and cyanobacteria), however, post-translational removal of the first *N*-formylmethionine results in a mature protein with 352 amino acid residues (Michel *et al.*, 1988). Two copies of the *psbD* gene have been identified in cyanobacteria (see Erickson and Rochaix, 1992).

The deduced amino acid sequences of D1 and D2 are highly conserved among a wide range of oxygenic organisms. In a detailed comparison of the primary structures of these two proteins, Svensson *et al.* (1991) have shown that around 65% of the residues in D1 and 75% of the residues in D2, are totally conserved among higher plants, algae and cyanobacteria. The strong local similarities in their primary structures between the L/M subunits of purple bacteria and D1/D2 (reviewed in Barber, 1987; Michel and Deisenhofer, 1988) coupled with hydropathy analyses (Trebst, 1986), antibody binding (Sayre *et al.*, 1986) and site-directed

mutagenesis of the relevant proteins (reviewed in Diner *et al.*, 1991) have allowed the construction of folding models and also the identification of potentially important residues in the D1 and D2 proteins (Michel and Deisenhofer, 1988). Thus, in analogy to the L and M subunits, both D1 and D2 were predicted to have five transmembrane helices (Trebst, 1986) that adopt an α-helical conformation. Moreover, it was found that localised homologies were particularly strong in the central helical core of the proteins corresponding to the fourth transmembrane segment of the purple bacterial reaction centre (Barber, 1987). This central helical core most probably binds several of the redox components that are involved in the primary photochemistry of PS II (Barber, 1987; Michel and Deisenhofer, 1988; Svensson *et al.*, 1991).

The primary donor of PS II, P680, is thought to be a chlorophyll dimer with its central Mg ions coordinated by histidine residues (His198) in both D1 and D2 (Barber, 1987; Michel and Deisenhofer, 1988). The corresponding amino acids in the purple bacterial reaction centres are also histidines at positions 173 and 200 of the L and M subunits, respectively. Similarly, potential ligands to the non-haem iron of the bacterial reaction centre L190 and M217 are conserved in His 215 and 214 of D1 and D2, respectively. Additional ligands to the non-haem iron on purple bacteria, are histidines at positions 230 and 264 of the L and M subunits. The equivalent PS II ligands are probably His272 of D1 and His269 of D2 and possibly a bicarbonate molecule (Michel and Deisenhofer, 1988; Vermaas *et al.*, 1993). The primary electron acceptor pheophytin is believed to be ligated to the D1 protein via hydrogen bonding to a glutamate residue (Glu130, but glutamine in *Synechocystis* PCC 6803) (Möenne-Loccoz *et al.*, 1989; Nadebryk *et al.*, 1990; Giorgi *et al.*, 1996). The primary quinone acceptor Q_A binds to the D2 protein, presumably to the peptide loop which is exposed to the stromal side of the thylakoid membrane and which connects helices IV and V (Trebst, 1986). On the basis of the analogy with the purple bacterial reaction centre, His214 of D2 has been implicated in the binding of Q_A as a His214Asn mutation in *Synechocystis* 6803 has resulted in loss of PS II from thylakoids (Vermaas *et al.*, 1988a). The secondary quinone acceptor Q_B is bound to the corresponding loop on the D1 protein. From analogies with the bacterial reaction centre, it has been proposed that Q_B forms hydrogen bonds with D1 residues His215, Ser264 and a peptide bond with Ala263 (Pakrasi, 1995).

As mentioned before (section 1.2.1) the redox-active tyrosine which is the primary electron donor to P680 has been identified as Tyr161 (Y_Z) on the D1 protein (Debus *et al.*, 1988b, Metz *et al.*, 1989). The manganese cluster that acts as an electron transfer intermediate between H_2O and Y_Z, is also probably in close association with the PS II RC heterodimer. Each of the (possibly) four Mn atoms of the cluster can have up to six ligands, which means that there are potentially 24 ligands, of which 12 may come from the cluster itself (Vermaas, 1993; Vermaas *et al.*, 1993; Pakrasi, 1995). Current expectations are that most of the remaining ligands are amino acid residues of various PS II proteins. X-ray absorption analysis indicated that at least some of the ligands for the Mn cluster must be nitrogen or

carboxylate groups (Debus, 1992). Thus, Glu69 of D2 (Vermaas *et al.*, 1990b) and later Asp170 of D1 (Boerner *et al.*, 1992; Nixon and Diner, 1992; Whitelegge *et al.*, 1995) were identified by site-directed mutagenesis to be possible Mn-ligands. Among other putative ligands are His190, His332, His337, Asp342 and the carboxyl terminus of the mature D1 protein (Debus, 1992; Nixon *et al.*, 1992a, b).

The C-terminus of the D1 protein has been shown to be post-translationally processed in higher plants (Marder *et al.*, 1984), in the green alga *C. reinhardtii* (Lers *et al.*, 1992; Schrader and Johanningmeier, 1992) and cyanobacteria (Nixon *et al.*, 1992b). The site of processing has been located on the carboxy side of the alanine at position 344 of D1 (Takahashi *et al.*, 1988; Nixon *et al.*, 1992b). Interestingly, in *Euglena*, the *psbA* coding region terminates at the predicted processing site (reviewed by Erickson *et al.*, 1985). Ala344 has been implicated in the stabilisation of the Mn complex (Nixon *et al.*, 1992b). C-terminal sequencing of the mature D2 polypeptide of spinach did not reveal any evidence for C-terminal processing of this protein (Takahashi *et al.*, 1990).

D1 and D2 have been shown to be post-translationally modified at their N-termini in higher plants. Tandem mass spectrometric analysis of the N-terminus of D1 and D2 from spinach indicated the presence of an *N*-acetylphosphothreonine, which is exposed on the stromal side of the membrane (Michel *et al.*, 1988). The functional role of this phosphorylation is nevertheless still uncertain (Allen, 1992). Phosphorylation of D1 and D2 has not been observed in cyanobacteria (reviewed in Allen, 1992). Phosphorylation of D2 but not of D1, has been suggested (but not conclusively shown) to occur in *C. reinhardtii* (Delepelaire, 1984; de Vitry *et al.*, 1991). The question of whether D2 is indeed phosphorylated in *C. reinhardtii* and the possible role of this phosphorylation have been addressed in the present work by creating the mutants D2-Thr2Ala and D2-Thr2Ser (chapter 5). Mattoo and Edelman (1987) have also proposed a palmitoylation step in the maturation of D1, which may regulate its distribution between the stroma lamellae and the grana regions.

1.2.4.2 Cytochrome b_{559}

Cytochrome (Cyt) b_{559} is an integral, haem-containing protein consisting of two low-molecular-weight subunits, α and β, with molecular weights of around 9 and 4 kDa, respectively. These subunits are encoded by the chloroplast genes, *psbE* and *psbF*, which are co-transcribed, in higher plants and cyanobacteria, from a common promoter upstream of *psbE*, to produce a primary transcript that includes *psbE*, *psbF*, *psbL* and *psbJ* (Erickson and Rochaix, 1992). The only exceptions to this gene organisation are *C. reinhardtii* and *C. moewusii*, where the two genes are located in two different regions of the plastid genome and are not transcribed together (Alizadeh *et al.*, 1994; Boudreau *et al.*, 1994; Mor *et al.*, 1995). Each of the two polypeptide subunits has one membrane span with the N-terminus exposed at

the stromal thylakoid surface (Tae and Cramer, 1989). Cyt b_{559} appears to be tightly bound to the D1/D2 heterodimer as judged by its presence in the PS II RC complex (Nanba and Satoh, 1987). Each subunit of Cyt b_{559} has only one histidine residue which is therefore thought to be the haem ligand. Replacement of the histidines with either Leu (Pakrasi and Vermaas, 1992) or Tyr (Pakrasi, 1995) in *Synechocystis* PCC 6803 has resulted in destabilisation of the PS II RC complex.

Despite its close association with D1/D2, the physiological function of Cyt b_{559} remains elusive. Inactivation of the genes encoding the two subunits of Cyt b_{559} in *Synechocystis* results in a loss of the structural integrity and function of PS II (Pakrasi and Vermaas, 1992). Among other suggestions, Cyt b_{559} has been involved in the protection of PS II against photoinhibition (Thompson and Brudvig, 1988; Nedbal *et al.*, 1992; Barber and De Las Rivas, 1993).

1.2.4.3 The PsbI polypeptide

This polypeptide is encoded by the chloroplast *psbI* gene. It has an apparent molecular weight of 4.8 kDa and has been identified as a component of the isolated PS II RC (Ikeuchi and Inoue, 1988; Webber *et al.*, 1989). Recent mutagenesis studies in *C. reinhardtii* (Künster *et al.*, 1995) and *Synechocystis* 6803 (Pakrasi, 1995), where the *psbI* gene has been inactivated, have shown that this protein is not essential for PS II function. The *Chlamydomonas* mutant was found to be photosensitive when grown photoautotrophically or in media containing acetate and was suggested to be involved in the stability of PS II and in the modulation of energy or electron transport in the PS II complex (Künster *et al.*, 1995).

1.2.5 CP47 and CP43

CP47 and CP43 are chlorophyll *a*-binding proteins encoded by the chloroplast genes *psbB* and *psbC*, respectively. Both proteins are associated with the PS II RC but are peripheral to the primary electron transfer reactions (reviewed in Nixon *et al.*, 1992a; Erickson and Rochaix, 1992; Pakrasi, 1995). They function as internal antenna to capture and transfer light energy to the P680 RC chlorophylls but also have other structural and functional roles. Both proteins are predicted to have six membrane-spanning regions (Bricker 1990; Sayre and Wrobel-Boerner, 1994). The number of chlorophylls bound to each protein has been estimated to be between 12 and 25 (de Vitry *et al.*, 1984; Gounaris *et al.*, 1990).

Both CP47 and CP43 contain a large hydrophilic region in the C-terminal half of the respective protein, located in the lumenal side of the thylakoid membrane (Bricker, 1990), which may contribute to the environment of the water-splitting complex. Indeed, cross-linking studies have confirmed a direct interaction between CP47 and the manganese-stabilising protein (encoded by the *psbO* gene) (Enami *et al.*, 1991; Odom and Bricker, 1992).

Mutants bearing short deletions in various parts of this lumenal region have shown variable degrees of destabilisation of the water-splitting complex (Pakrasi, 1995). In addition, a mutant strain of *Synechocystis* bearing the mutation Arg448Gly on CP47 showed oxygen evolution only in the presence of elevated levels of chloride, suggesting a role for CP47 in binding chloride ions to PS II (Putnam-Evans and Bricker, 1994).

The CP43 protein also contributes to the activity of the water-splitting complex (Vermaas *et al.*, 1993). Inactivation of the *psbC* gene (the gene encoding CP43) in *Synechocystis* 6803 leads to complete inhibition of water oxidation (reviewed in Vermaas *et al.*, 1993). Interestingly, the remaining PS II RC proteins and CP47 are present in such a mutant. Moreover, the PS II complex is assembled and mediates electron transfer from Y_Z to Q_A. As for the D1 protein of higher plants, CP43 is phosphorylated at a threonine residue at the N-terminus of the protein (Michel *et al.*, 1988) (see also section 5.1).

1.2.6 Extrinsic polypeptides associated with the oxygen-evolving complex

In photosynthetic eukaryotes, three extrinsic proteins, located in the lumenal side of the thylakoid membrane have been shown to influence the properties of the water-splitting complex (Erickson and Rochaix, 1992; Vermaas, 1993; Vermaas *et al.*, 1993; Pakrasi, 1995). These proteins have apparent molecular weights of 33, 23 and 16 kDa (by SDS-PAGE) and are encoded by the nuclear genes *psbO*, *psbP* and *psbQ*, respectively. These three proteins are not catalytically essential components of PS II, although in their absence a number of properties of the PS II complex are altered (Vermaas *et al.*, 1993). Consistent with this non-essential role for the extrinsic proteins in water oxidation is the fact that the 23 and 16 kDa proteins are not detected in cyanobacteria.

In eukaryotes, the 33 kDa extrinsic protein (also called the manganese-stabilising protein) is synthesised as a precursor protein which is processed in two steps to its mature form (Erickson and Rochaix, 1992). The main role of this protein is in the stabilisation of the manganese cluster, although direct metal ligation is unlikely (Vermaas *et al.*, 1993). From the experiments of Miyao and Murata (1984) and Ono and Inoue (1984) a role for this protein in the binding of Ca^{2+} and Cl^- was suggested. The non-catalytic role of the 33 kDa protein was confirmed by construction of a 33 kDa-deficient mutant of *Synechocystis*, which could grow photoautotrophically although PS II activity was light-sensitive (Burnap and Sherman, 1991; Mayes *et al.*, 1991; Philbrick *et al.*, 1991). This mutant showed perturbations in the redox cycling of the water-splitting system and retardations in the formation of its higher S-states, indicating a regulatory role of the 33 kDa protein in the process of oxygen evolution (Vass *et al.*, 1992; Boichenko *et al.*, 1993). In contrast to the results in cyanobacteria, a *C. reinhardtii* mutant, FuD44, lacking the 33 kDa protein was found to be an obligate heterotroph which could not evolve oxygen (Mayfield *et al.*, 1987a). Although the reason for

this discrepancy between the two systems is unknown, it serves to present a case for some differences between prokaryotic and eukaryotic PS II.

The 33 kDa extrinsic protein has been shown to interact with isolated PS II RCs (Gounaris *et al.*, 1990) and with the chlorophyll *a*-binding protein CP47 (Enami *et al.*, 1991; Odom and Bricker, 1992). This binding of the 33 kDa protein to PS II probably involves carboxylic groups located at the N-terminal region of the protein (Eaton-Rye and Murata, 1989).

The extrinsic 23 and 16 kDa proteins are less well characterised than the 33 kDa protein. As mentioned before, these two proteins are absent from cyanobacteria, although two other proteins have been detected in the thermophilic cyanobacterium *Synechococcus vulcanus*, a 10-12 kDa protein and a low-potential cytochrome c-550 (encoded by the *psbV* gene) which in some functional aspects seem similar to the 23 and 16 kDa proteins of eukaryotic organisms (Shen and Inoue, 1993). Both the 23 and 16 kDa proteins have been implicated in the binding of Ca^{2+} and Cl^- ions to the water-splitting complex (Vermaas *et al.*, 1993). A *C. reinhardtii* mutant lacking the 23 kDa extrinsic protein was shown to stably assemble PS II and to evolve oxygen, although at reduced levels, in contrast to the situation with the *psbO*-deficient mutant of the same organism (Mayfield *et al.*, 1987b).

1.2.7 Other PS II proteins

The chloroplast *psbH* gene codes for a 9 kDa phosphoprotein (Farchaus and Dilley, 1986) which was first detected by Bennett (1977) as being reversibly phosphorylated at a threonine residue near its N-terminus (Michel and Bennett, 1987) (see also section 5.1). N-terminal sequencing of the PsbH polypeptide from *C. reinhardtii* (Dedner *et al.*, 1988) and *Synechococcus vulcanus* (Koike *et al.*, 1989) and DNA sequencing of the *psbH* gene from *Synechocystis* PCC 6803 (Abdel-Mawgood and Dilley, 1990; Mayes and Barber, 1991) have shown that the phosphorylation site is conserved in the green alga but not in cyanobacteria. However, phosphorylation of PsbH in cyanobacteria has recently been reported (Race and Gounaris, 1993). Although the function of this protein is unknown, it is presumably related to the reversible phosphorylation that the protein undergoes. Thus, it has been proposed that it plays a role in regulating and stabilising electron transfer between Q_A and Q_B (Packham *et al.*, 1988). A mutant of *Synechocystis* 6803 lacking the PsbH polypeptide could grow photoautotrophically and was able to assemble a functional PS II complex but was sensitive to photoinhibition (Mayes *et al.*, 1993). The latter effect was later found to stem from the inability of this mutant to regulate the rate of turnover of the D1 protein (Komenda and Barber, 1995). In contrast to the situation in cyanobacteria, a PsbH-deficient mutant of *C. reinhardtii* has recently been shown to be unable to assemble an active PS II complex (Ruffle *et al.*, 1995).

A conserved open reading frame (ORF) having 39-42 codons is located downstream of the *psbE-F-L* operon in higher plants and cyanobacteria (Erickson and Rochaix, 1992; Nixon *et al.*, 1992a) but not in *C. reinhardtii* (Fong and Syrzycki, 1992). Based on the fact that it is conserved between species and that it is co-transcribed with the *psbE-F-L* operon, the gene containing this ORF was designated as *psbJ*. A *Synechocystis* 6803 mutant deficient in the *psbJ* gene product was shown to be able to grow photoautotrophically, although at reduced rates compared to the wild type (Nyhus and Pakrasi, 1989). In another mutagenesis study, Lind *et al.* (1993) reported that the absence of the PsbJ polypeptide from *Synechocystis* 6803 lowers the PS II/chlorophyll ratio, suggesting, according to the authors, that this protein may have a regulatory role in the biogenesis or stability of the PS II complex.

The *psbK* gene product is another low molecular weight component of PS II predicted to contain a single transmembrane helix (Erickson and Rochaix, 1992). Directed disruption of the *psbK* gene in *C. reinhardtii* destabilises the PS II RC complex and as a result mutants cannot grow photoautotrophically (Takahashi *et al.*, 1994). Therefore, these authors suggested that the *psbK* gene product is required for the stable assembly and/or stability of the photosystem II complex and is essential for photoautotrophic growth. However, no detectable change in the structure and function of PS II was observed when the same gene was inactivated in *Synechocystis* 6803 (Ikeuchi *et al.*, 1991; Zhang *et al.*, 1993).

PsbL is a small integral membrane protein. The *psbL* gene is located downstream of the *psbF* gene (Erickson and Rochaix, 1992). Inactivation of this gene in *Synechocystis* 6803 resulted in loss of PS II function (Anbudurai and Pakrasi, 1993). Biochemical reconstitution experiments have shown that the presence of this protein is necessary for the normal functioning of Q_A (Kitamura *et al.*, 1994).

The *psbM* and *psbN* genes have been identified in the chloroplast genome by comparison with the partial N-terminal sequence of two small polypeptides found in O_2-evolving complexes from *Synechococcus vulcanus* (Ikeuchi *et al.*, 1989). The *psbN* gene has been identified in *Synechocystis* 6803 and is located adjacent to the *psbH* gene (Mayes and Barber, 1991).

The nuclear *psbR* gene codes for a 10 kDa protein that is located in the lumen of higher plants (Erickson and Rochaix, 1992). Extraction studies have suggested that it is an extrinsic polypeptide but of a more hydrophobic nature than the other three extrinsic proteins involved in water-splitting (Ljunberg *et al.*, 1986). Potato plants expressing an anti-sense *psbR* RNA have been produced by Stockhaus *et al.* (1990). Although, these plants were almost deficient in the PsbR polypeptide, this did not affect the growth rates or the levels of the other extrinsic PS II proteins, suggesting that PsbR is not essential for PS II activity.

The nuclear *psbS* gene (Kim *et al.*, 1992; Wedel *et al.*, 1992) encodes a hydrophobic 22-kDa protein (Ljunberg *et al.*, 1986) which has been predicted to possess four membrane-spanning regions (Andersson and Barber, 1994). The sequence of *psbS* shows a clear homology with chlorophyll *a/b*-binding (CAB) proteins (Kim *et al.*, 1992; Wedel *et al.*, 1992) and the

protein itself has recently been shown to bind both chlorophyll *a* and *b* (Funk *et al.*, 1994). The protein has also been detected immunochemically in cyanobacterial thylakoids (Nilsson *et al.*, 1990), although the latter do not possess CAB proteins but instead have phycobilisomes containing only chlorophyll *a*.

The chloroplast ORF, *ycf8*, designated as *psbT* by Pakrasi (1995), has been shown to be expressed as part of the *psbB-psbH* operon in *C. reinhardtii* and its gene product to be specifically associated with PS II (Monod *et al.*, 1994). This polypeptide has been suggested to be a stress protein because its role manifests itself only under certain stress conditions, such as reduced chloroplast protein synthesis and high light (Monod *et al.*, 1994).

Recently, Lorkovic *et al.* (1995) and Irrgang *et al.* (1995) have described a 6.1 kDa integral membrane polypeptide associated with the isolated PS II RC from spinach. This protein, which does not stain well with Coomassie stain, is encoded by the nuclear *psbW* gene (Lorkovic *et al.*, 1995). The protein has not been detected yet in other photosynthetic organisms and its function in PS II is unknown.

1.3 *Chlamydomonas reinhardtii* as a model system to study photosynthesis

The genus *Chlamydomonas* (from the Greek *chlamys*, a cloak or mantle and *monas*, unit) comprises a group of unicellular green algae having an overall polar structure, with paired apical flagella and a single basal chloroplast surrounding one or more pyrenoids. Cells are usually free-swimming in liquid media but on solid substrates they may be non-flagellated. Most species are ellipsoid or ovate in shape with a distinct cell wall, usually closely appressed to the plasma membrane. The pyrenoids (containing the enzyme RuBP carboxylase/oxygenase) are located either at the basal region or along the side of the cell, often seen surrounded by starch bodies. The nucleus is usually centrally located. All but a few species have a coloured eyespot and most have one or more contractile vacuoles.

Chlamydomonas reinhardtii cells are oval-shaped, typically 10 μm in length and 3 μm in width and possess two flagella at their anterior end (Figure 1.4). Cells contain a single, cup-shaped chloroplast that occupies close to 40% of the cell volume and several smaller mitochondria. Thylakoid membranes appear as flat vesicles which can be arranged into stacks of 2-10 discs, although they do not differentiate into multidisc grana as in higher plants. The cell wall of *C. reinhardtii* consists of hydroxyproline-rich glycoproteins with arabinose, mannose, galactose and glucose being the predominant sugars, arranged in up to seven layers. The cell wall is not particularly difficult to disrupt and a simple freeze-thawing is usually sufficient to obtain soluble enzymes. To obtain particulate fractions, the more frequently used methods are sonication, the French press, the Yeda press and homogenisation with glass beads. For the preparation of nucleic acids, cells are usually lysed by the use of detergent (Harris, 1989 and references within).

C. reinhardtii has been extensively utilised for the study of the photosynthetic process. An important feature of this organism is that its photosynthetic function is dispensable when the cells are grown in the presence of a reduced carbon source, such as acetate. Thus, the alga can be grown photoautotrophically (light with CO_2 as carbon source), mixotrophically (light with acetate) and heterotrophically (dark with acetate). This latter property has allowed for the isolation of numerous chloroplast and nuclear mutants deficient in functions associated with all thylakoid protein complexes (Harris, 1989). Other reasons for using *C. reinhardtii* as a model system to study photosynthesis include the facts that its photosynthetic apparatus is quite similar to that of higher plants; it is amenable to analysis by standard genetic, molecular and biochemical methods; large quantities of cells can be produced on simple, inexpensive media; the cell cycle can be synchronised by a variety of light/dark regimes and a single life cycle can be completed within 4 to 10 days (Weeks, 1992). These features, together with the recent technical advances of chloroplast (Boynton *et al.*, 1988) and nuclear (Debuchy *et al.*, 1989; Mayfield and Kindle, 1990) transformation (discussed in section 3.1) have enabled researchers to dissect structure-function relationships within the photosynthetic apparatus and the regulation of gene expression of photosynthetic genes (reviewed in Rochaix, 1995; Webber *et al.*, 1995).

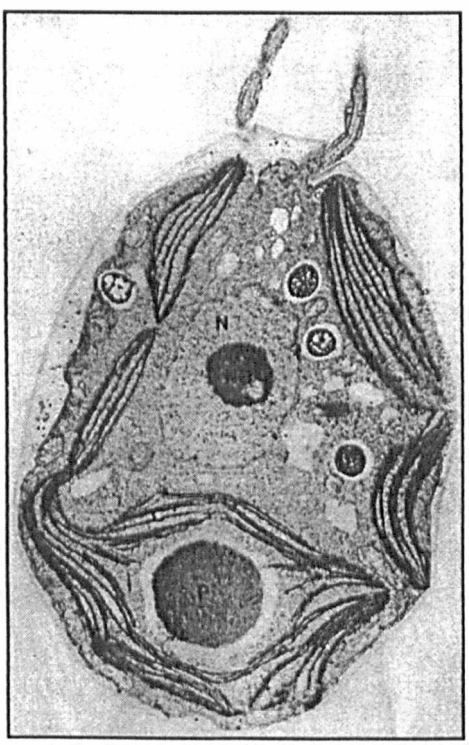


Figure 1.4: Median section through mixotrophically grown wild-type cell of *C. reinhardtii*. n, nucleus; NU, nucleolus; C, chloroplast; P, pyrenoid (taken from Harris, 1989)

Cyanobacteria are in many respects similar to the eukaryotic plants and algae in terms of the photosynthetic function. Therefore, they have been widely used for the study of structure-function relationships within the photosynthetic electron transport chain, especially in PS II (reviewed in Nixon *et al.*, 1992a; Pakrasi and Vermaas, 1992). However, there are several features that are unique to the structure, function, regulation and stability of the chloroplast. For example, in eukaryotes, genes encoding photosynthetic proteins are contained in two distinct genetic compartments (nucleus and chloroplast). Therefore, assembly of the chloroplast photosynthetic apparatus requires the coordinated expression of both chloroplast and nuclear genes. This is in contrast with cyanobacteria which lack intracellular organelles and which contain only one type of genome. Cyanobacteria also lack the organisation of thylakoids into highly ordered stacks that are differentiated into appressed and non-appressed regions. Another major difference between cyanobacteria and eukaryotic organisms lies in the light-harvesting antenna associated with the photosystems (section 1.1). Differences have also been found in PS II polypeptide composition, especially on the lumenal side of PS II and function (see section 1.2).

Given the similarities of *C. reinhardtii* to higher plants, in terms of the chloroplast photosynthetic apparatus, and its ability to be used for molecular, genetic and biochemical analyses, it is obvious that this green alga provides an excellent model plant cell system. Therefore, it has been used as the experimental system of choice for the construction and characterisation of the site-directed mutants of the D2 protein of PS II described in this thesis.

1.3.1 The chloroplast genome of *C. reinhardtii*

The genomic organisation of *C. reinhardtii* is very similar to that of the other photosynthetic eukaryotes. *C. reinhardtii* contains three genetic systems located in the nucleus, chloroplast and mitochondria (reviewed in Rochaix, 1995). The genetic loci of different mutations can be readily distinguished after genetic crosses between the two mating types (+ and -), as nuclear genes segregate according to a Mendelian fashion, whereas chloroplast and mitochondrial genomes are usually transmitted uniparentally to the progeny, from the mt(+) and mt(-) parent, respectively (Harris, 1989).

The chloroplast of *C. reinhardtii* contains approximately 80 copies of its 196-kb circular genome. This genome is considerably larger than the one found in most land plants (120-160 kb) (Sugiura, 1989). The extra size of the *Chlamydomonas* chloroplast genome relative to its land plant counterpart is mainly accounted for by the presence of enlarged spacers between coding regions and also by the presence of long genes featuring introns (Boudreau *et al.*, 1994). To date, a single gene (*psaA*) has been found to be interrupted by group II introns (Kück *et al.*, 1987) and one gene, the *psbA gene*, has been shown to be interrupted by group I introns (Erickson *et al.*, 1984).

It is generally believed that the chloroplast (ct) DNA molecules in *C. reinhardtii* are organised into 8-10 discrete bodies, consisting of DNA-protein complexes, called nucleoids (Harris, 1989). The *C. reinhardtii* ctDNA is generally AT-rich (around 65%) and has a density of 1.695 $g.cm^{-3}$. 75 genes have been mapped until recently on the chloroplast genome of *C. reinhardtii* (Boudreau *et al.*, 1994), including 26 genes encoding subunits of the photosynthetic apparatus. However, a collaborative effort is now under way to complete the sequencing of the chloroplast genome of *C. reinhardtii*.

The *C. reinhardtii* chloroplast genome comprises two single-copy regions which are separated by inverted repeat sequences containing the ribosomal RNA genes (as in most higher plants) and the *psbA* gene (Harris, 1989). Unlike higher plants where the single-copy regions are unequal in size, in *C. reinhardtii* they are roughly equivalent. A major difference between the higher plant and *C. reinhardtii* chloroplast genome is the way genes are organised. In plant chloroplasts many genes are co-transcribed, for example the *psbB-psbH-petD-petB* or *psbK-psbI-psbD-psbC* gene clusters (Erickson and Rochaix, 1992), a situation which is not common in *C. reinhardtii*. Moreover, the relative positions of the genes involved in photosynthetic functions are quite different (Harris, 1989, Sugiura, 1989, Boudreau *et al.*, 1994). Finally, there are a few genes that appear to be unique to the *Chlamydomonas* genus and which have not been reported in any higher plant ctDNA (such as the *tscA* gene, see section 1.3.2.1) (Boudreau *et al.*, 1994).

1.3.2 The *psbD* gene in *C. reinhardtii*

The *psbD* gene encodes the D2 protein of the photosystem II reaction centre. In all chloroplast genomes examined to date (with the exception of *C. reinhardtii* and *Euglena gracilis*), the *psbD* gene is part of the *psbD-psbC* operon, with the last 14 nucleotides of *psbD* coding region overlapping with the 5' end of the *psbC* gene (Erickson and Rochaix, 1992). A similar situation exists in cyanobacteria where one of the two copies of *psbD* (*psbD1*) is co-transcribed with *psbC* (Carpenter *et al.*, 1990). In higher plants, the *psbD-psbC* operon is transcribed from four promoters which are active at different stages of chloroplast development (Sexton *et al.*, 1990; Christopher and Mullet, 1994). Three of those promoters are active in dark-grown and illuminated seedlings producing mRNAs that are utilised for the build-up and assembly of functional PS II during thylakoid formation (Sexton *et al.*, 1990). Once PS II is assembled and mature chloroplasts are formed, the activity of these promoters and the levels of the corresponding mRNAs gradually decrease. A fourth promoter (Christopher *et al.*, 1992) is then differentially activated by blue light and UV-A and assumes the dominant role of *psbD-psbC* transcription in mature chloroplasts (Christopher and Mullet, 1994).

The nucleotide sequence of *psbD* for *C. reinhardtii* was determined initially by Rochaix *et al.* (1984) and later amended by Erickson *et al.* (1986). Transcription from a

promoter upstream of *psbD* results in a polycistronic message containing *psbD* and the second exon of *psaA* (the gene encoding one of the two apoproteins of the reaction centre of PS I) (Kück *et al.*, 1987; Choquet *et al.*, 1988).

The putative promoter is located 80-90 nucleotides upstream of the translation initiation codon and contains a bacterial-like -10 sequence (TATAAT) in a palindromic motif of two overlapping -10 elements (TATAAT and TAATAT) (Erickson *et al.*, 1986). Primer extension experiments revealed that the 5' end of the *psbD* transcript is located 74 nucleotides (nt) upstream of the translation initiation codon (Nickelsen *et al.*, 1994). The *C. reinhardtii psbD* gene codes for two methionine residues in the first 11 amino acids of the same ORF. The second of these is preceded by the ribosomal binding site. Translation from this second methionine gives a protein of 352 amino acids which is similar to the 353 amino acids found in the higher plant D2 protein. Overall there is a very high degree of similarity (>90%) of the D2 from *C. reinhardtii* with that of higher plants (Erickson *et al.*, 1985; Svensson *et al.*, 1991).

The regulation of expression of the *psbD* gene in *C. reinhardtii* has been reviewed by Nickelsen and Rochaix (1994). A number of nuclear mutants affected in distinct post-transcriptional steps have been isolated and characterised. Mutant *nac2-26* (Kuchka *et al.*, 1989) is deficient in PS II and fails to accumulate *psbD* mRNA, although *psbD* is transcribed, indicating that the corresponding nuclear locus, *NAC2*, affects *psbD* mRNA stability. Nickelsen *et al.* (1994) fused the 74 nt 5' untranslated region (UTR) of *psbD* to the bacterial *aadA* gene, which confers resistance to spectinomycin when expressed in the chloroplast (Goldschmidt-Clermont, 1991) and used this construct to transform wild type *C. reinhardtii* cells. After crossing the resulting transformant to *nac2-26*, they found that the *psbD* leader region was sufficient to destabilise the *aadA* chimeric message in the *nac2-26* background. Thus, they concluded that this region contained the target site for rapid turnover of its transcript. They also correlated the instability of the *psbD* mRNA with the loss of binding of a 47 kDa protein to the *psbD* leader in the mutant, suggesting that this factor acts as a message stabiliser in the wild type strain (Nickelsen *et al.*, 1994). However, contrasting results have been obtained by Mayfield *et al.* (1995), who reported that the 47 kDa protein is present and binds to the *psbD* 5'-UTR in a *nac2-26* background. In contrast to the 5'-UTR, the 3'-UTR was not found to influence the stability of the *psbD* transcript (Nickelsen *et al.*, 1994).

The nuclear mutations *nac1-11* and *nac1-18* of *C. reinhardtii*, which belong to the same complementation group, have been shown to affect a nuclear gene whose product controls the translation and/or degradation of the chloroplast-encoded D2 polypeptide (Kuchka *et al.*, 1988). In addition, these two mutants were shown to accumulate higher amounts of total and polysome-associated *psbD* mRNA than the wild type despite the absence of D2 synthesis. Mayfield *et al.* (1995) suggest that the stability of the *psbD* mRNA in the mutants may be influenced by association of the mRNA with ribosomes. Recently, a nuclear suppressor (*sup4b*), was shown to be able to suppress the two different mutations in the *NAC1* locus and

one mutation in the *AC-115* locus (another nuclear locus involved in *psbD* gene expression; Kuchka *et al.*, 1988) (Wu and Kuchka, 1995). This suppressor was suggested by the authors to identify an additional nuclear locus involved in the synthesis of the D2 polypeptide.

Chloroplast mutant FuD47 contains a 46 bp direct repeat within the coding region of *psbD* which results in a *psbD* transcript coding for 186 amino acid residues instead of 352. As a result, there is no detectable synthesis of full length D2. Apart from D2, the following proteins are absent or present in very small amounts in FuD47: D1, CP47 and CP43, the three extrinsic proteins of the oxygen-evolving complex (33 kDa, 23 kDa and 16 kDa) and a small 6-10 kDa protein, most likely one of the subunits of Cyt b_{559} (Erickson *et al.*, 1986).

1.3.2.1 The *psaA* gene

190 bp downstream of the termination codon of *psbD* lies the second exon of the *psaA* gene (*psaA*-2, 198 bp). This gene encodes one of the two related polypeptides that are the apoproteins of CP1, the protein-pigment complex containing the reaction centre chlorophylls, P700, and the first electron acceptors of photosystem I. The gene for the other related polypeptide, *psaB*, has a normal continuous structure (Kück *et al.*, 1987).

The *psaA* gene in *C. reinhardtii* has an unusual structure: it is formed of three exons that are widely separated on the circular genome and are transcribed from opposite strands (Kück *et al.*, 1987; Choquet *et al.*, 1988) (see Figure 3.1). The *psaA* gene has drawn much attention because of its unusual mechanism of splicing. Thus, mutants that are defective in the assembly of the mature mRNA have been isolated and characterised (Choquet *et al.*, 1988; Goldschmidt-Clermont *et al.*, 1990). The analysis of these mutants has led to the suggestion that the three exons of the *psaA* gene are transcribed separately to produce three individual precursors. The precursors are then assembled to produce the mature mRNA in two consecutive steps of *trans*-splicing (Goldschmidt-Clermont *et al.*, 1990; Goldschmidt-Clermont *et al.*, 1991). Goldschmidt-Clermont *et al.* (1991) showed that a small chloroplast RNA, encoded by the *tscA* gene, is required for efficient splicing. The authors also suggested that the *tscA* RNA functions as part of the splicing machinery, contributing to the formation of the characteristic structure of group II introns and participating in the assembly of the catalytic core.

The fact that *psaA*-2 lies so close to the *psbD* gene has been utilised in the present work for the construction of site-directed mutants of D2 that also lack photosystem I (by inserting a selectable marker in *psaA*-2, see chapter 3).

1.3.3 Protein engineering of Photosystem II in *C. reinhardtii*

Since the first report of chloroplast transformation by Boynton and co-workers (Boynton *et al.*, 1988), *C. reinhardtii* has been increasingly used for studying the regulation of

chloroplast gene expression and the relationship between structure and function of various thylakoid membrane proteins (reviewed in Rochaix, 1995; Webber *et al.*, 1995). This section focuses on the application of chloroplast transformation in *C. reinhardtii* for protein engineering of PS II. In order to dissect the function of chloroplast-encoded proteins, two strategies have been used. One of them uses site-directed mutagenesis to target specific amino acids or functional domains. The second strategy involves inactivation of the gene encoding the protein of interest either by interrupting the open reading frame or by deleting a part or the whole gene.

1.3.3.1 Site-directed mutagenesis

The D1 protein of PS II is one of the most studied thylakoid membrane proteins (see section 1.2.4.1). Thus, most of the site-directed mutations of *C. reinhardtii* chloroplast genes have been made to the *psbA* gene (encoding D1).

Many commercially applied herbicides have their primary target site on the D1 protein (Oettmeier, 1992). These herbicides compete with Q_B for binding to the binding pocket. Przibilla *et al.* (1991) introduced mutations into the *psbA* gene so as to produce resistance to the herbicide metribuzin, which was additionally used for the selection for transformants. A double mutant Val219Ile/Ser264Ala of D1 was constructed (Heiss and Johanningmeier, 1992) by transforming the chloroplast of the *psbA* deletion mutant FuD7 (Bennoun *et al.*, 1986) with a cloned *psbA* gene fragment containing the two mutations. The resulting mutant showed an increased tolerance to the herbicides DCMU and atrazine. Interestingly, copies from both the recipient (FuD7) and the donor genome were found to persist in the transformant, indicating that this heteroplasmic state was required for photoautotrophic growth (Heiss and Johanningmeier, 1992).

As mentioned in section 1.2.4.1, the D1 precursor is synthesised with a C-terminal extension which is post-translationally removed by a processing protease. Lack of this protease in the LF1 mutant of *Scenedesmus obliquus* results in the inability of the mutant to assemble a functional oxygen-evolving complex (Taylor *et al.*, 1988). Investigation of the possible function of the C-terminal extension of D1 in *C. reinhardtii* was carried out by replacing Ser345 (the first residue removed by the protease) with a UAA stop codon (Schrader and Johanningmeier, 1992; Lers *et al.*, 1992). These truncation mutants accumulated normal levels of PS II and exhibited no impairment in photoautotrophic growth. These results were in line with similar mutagenesis experiments in *Synechocystis* 6803 (Nixon *et al.*, 1992a).

The accessory chlorophylls of the PS II RC have been proposed to be ligated by histidine residues (His118) located on the second membrane-spanning helix of D1 and D2 (Michel and Deisenhofer, 1988). Thus, His118 of D1 has been replaced by leucine and arginine (Hutchison and Sayre, 1995). Initial characterisation of these mutants showed that

mutagenesis of the D1-His118 residue disrupts the functional and structural integrity of the PS II RC. According to the authors, the results obtained by the characterisation of the mutants are consistent with the possible participation of His118 in coordination of an accessory chlorophyll (Hutchison and Sayre, 1995).

Bicarbonate is known to have an important role in electron transfer within PS II (Govindjee and van Rensen, 1993). Depletion of bicarbonate or its replacement by formate slows the kinetics of electron transfer from Q_A^- to Q_B (Eaton-Rye and Govindjee, 1988). The identity of the bicarbonate binding site is unknown, but it is expected to be near the non-haem iron (reviewed by Govindjee and van Rensen, 1993). In an attempt to identify possible ligands to bicarbonate, Xiong *et al.* (1995) have replaced an arginine at position 269 of D1 with a glycine. The mutant was unable to evolve oxygen , had a slower Q_A^- to Q_B electron transfer and a lower amount of functional PS II complexes. However, this residue is probably not involved in bicarbonate binding since formate could induce a structurally unperturbed (although at reduced levels) Q_A^-/Fe EPR signal (Xiong *et al.*, 1995).

A conserved histidine at position 195 of D1 is located near the N-terminal end of the α-helical transmembrane span that contains one of the histidines (His198) predicted to be a ligand to P680. His195 was replaced by tyrosine (Roffey *et al.*, 1991), asparagine and aspartate (Roffey *et al.*, 1994a). The mutations at His195 did not disrupt oxygen evolving activity, although the electron transfer kinetics from Y_Z to $P680^+$ were slower in the His195Asp and His195Tyr mutants, as measured by fluorescence induction and decay kinetics. Site-directed mutagenesis was used to replace His190 with phenylalanine (Roffey *et al.*, 1994a) and tyrosine (Roffey *et al.*, 1994b). This histidine residue has been predicted to be near the lumenal thylakoid surface of D1, in close proximity to Y_Z (Svensson *et al.*, 1990). The His190Phe mutant was severely impaired in oxygen evolution, lacked the A_T thermoluminescence band (Kramer *et al.*, 1994) and exhibited 100-1000 fold slower electron transfer from Y_Z to $P680^+$. Based on these results His190 was suggested to be important for the assembly of the manganese cluster (Roffey *et al.*, 1994a). The slower electron transfer kinetics in the His190 mutants suggested a close interaction between Y_Z and $P680^+$. However, EPR spectral characteristics of the oxidised Y_Z were identical to the wild type, indicating that they are not in hydrogen-bonding distance (Roffey *et al.*, 1994b).

An aspartate residue at position 170 of D1 has been shown by Nixon and Diner (1992) to be involved in the early ligation and assembly of the manganese cluster in cyanobacteria. In order to examine the role of this residue in *C. reinhardtii*, D1-Asp170 was replaced by histidine, threonine (Whitelegge *et al.*, 1992, 1995), asparagine and proline (Whitelegge *et al.*, 1995). All mutants (apart from the Asp170Pro) retained normal levels of PS II RCs (50% in the Asp170Pro) and were able, to different extents, to evolve oxygen. Kinetic analysis of chlorophyll fluorescence indicated that the mutants were impaired in donor side function (Whitelegge *et al.*, 1995). However, measurements of oxygen flash yield in the Asp170His mutant showed that those reactions centres capable of evolving oxygen, function normally.

This led the authors to conclude that as in cyanobacteria, Asp170 of D1 in *Chlamydomonas* also plays a critical role in the initial binding of manganese during the assembly of the water-splitting complex (Whitelegge *et al.*, 1995), in line with the suggestions of Nixon and Diner (1992) for the analogous amino acid residue of cyanobacteria.

1.3.3.2 Gene inactivation

The chloroplast *aadA* expression cassette (Goldschmidt-Clermont, 1991), consisting of the bacterial *aadA* (aminoglycoside adenyl transferase) coding sequence conferring resistance to spectinomycin/streptomycin, flanked by chloroplast regulatory sequences, has been widely used for inactivating chloroplast genes (reviewed in Webber *et al.*, 1995). This system has provided valuable information, especially on the assembly and function of small PS II polypeptides.

In order to gain insight into the role of the α subunit of Cyt b_{559} (encoded by the *psbE* gene, see section 1.2.4.2) within the PS II RC, the *psbE* gene has been inactivated by insertion of the *aadA* cassette inside the coding region of the gene (Morais *et al.*, 1995). The mutant was unable to grow photoautotrophically and evolve oxygen and was deficient in the D1, D2, CP47 and 33 kDa proteins. These results indicated a structural role of this polypeptide in the assembly and/or stability of the PS II RC (Morais *et al.*, 1995).

The *psbH* gene encodes a 9-kDa phosphoprotein (Bennett, 1977) whose role in PS II is unknown (see section 1.2.7). This small phosphoprotein has recently been studied in *C. reinhardtii* by both inactivation of the relevant gene and site-directed mutagenesis of the conserved phosphorylation site at Thr3. Thus, disruption of the *psbH* gene resulted in a mutant that could not grow photoautotrophically or evolve oxygen (Ruffle *et al.*, 1995). This result contrasts with the situation in cyanobacteria where the analogous mutant could grow photoautotrophically, although at a slower rate than the wild type (Mayes *et al.*, 1993). A threonine at position 3 of the PsbH polypeptide, has been predicted to be the phosphorylation site in *C. reinhardtii* (Dedner *et al.*, 1988). Replacement of Thr3 with an alanine residue (Cheater *et al.*, 1995) apparently results in a complete loss of phosphorylation of thylakoid membrane proteins.

Genes *psbI*, *psbK* and *psbT* (*ycf8* ORF) have also been inactivated in order to study their function in PS II (discussed in sections 1.2.4.3 and 1.2.7). The *psbI* mutant (Künster *et al.*, 1995) was able to grow photoautotrophically but PS II activity and accumulation of PS II subunits were reduced to 10-20% of the wild type levels. These results, together with an observed light sensitivity of the mutant, prompted the authors to suggest that the PsbI polypeptide might be involved in the stability and electron or energy transfer in PS II (Künster *et al.*, 1995). Directed inactivation of the *psbK* gene resulted in loss of photoautotrophic growth and PS II activity and reduced accumulation of PS II polypeptides (Takahashi *et al.*, 1994). Pulse-labelling experiments indicated that polypeptides of the PS

II complex were synthesised normally in the mutant, suggesting the PsbK polypeptide may have a role in PS II assembly and/or stability in *C. reinhardtii*. Finally, the *psbT* gene-product represents a new PS II subunit that has been found (by directed disruption of the relevant gene) to be essential for maintaining high photosynthetic activity under adverse growth conditions, such as high light intensity (Monod *et al.*, 1994).

1.4 Site-directed mutants of D2

All the site-directed mutants of D2 that have been reported in the literature up to now, have been created in the cyanobacterium *Synechocystis* sp. PCC 6803 (reviewed in Nixon *et al.*, 1992a; Pakrasi and Vermaas, 1992; Vermaas, 1993; Pakrasi, 1995). The reason for this was the lack, until recently, of an efficient chloroplast transformation system in *C. reinhardtii*. However, the use of *Synechocystis* 6803 for the study of the function of D2, through protein engineering, is complicated for two reasons: the *psbD* gene in *Synechocystis* 6803 exists as two copies (*psbD1* and *psbD2*). This situation increases the difficulty of creating deletion strains. In addition, the 3' end of *psbD1* overlaps with the 5' end of *psbC*. Therefore, it is difficult to create mutations at the C-terminus of *psbD* without interfering with expression of the *psbC* gene (Eggers and Vermaas, 1993). Despite these difficulties, a wealth of information concerning the function of specific residues of D2, has been obtained by using this organism in recent years.

Ligands to the non-haem iron. In bacterial reaction centres the non-haem iron is coordinated by four His (two from the L subunit and two from the M subunit) and by Glu232 contributed by the M subunit (Michel and Deisenhofer, 1988). The His are conserved in PS II. Two of them are on D1 and the rest on D2: His214 and His268. Replacement of His214 with asparagine resulted in complete loss of the PS II RC (Vermaas *et al.*, 1987a). Whereas the replacement with lysine and methionine gave no PS II photoactivity (Nixon *et al.*, 1992a). Replacement of His268 with aspartate resulted in the loss of PS II activity (Nixon *et al.*, 1992a). Vermaas *et al.* (1994) have exchanged His268 with glutamine. This mutant could not grow photoautotrophically and also showed a loss of the characteristic Q_A^-/Fe_2^+ EPR signal and altered electron transport between Q_A and Q_B and herbicide binding. The amount of PS II centres present in the mutant was approximately 20-30% of the amount found in the wild type. These effects in both the Q_A and Q_B pockets suggested not only a functional loss of the non-haem iron, caused by the mutation, but also that D2 may have a significant influence on the properties of the Q_B/herbicide binding environment (Vermaas *et al.*, 1994). The M-Glu232 residue of the bacterial reaction centre is not conserved in PS II. Chisholm and Diner (unpublished results) targeted residues Glu224, Asp225 and Glu227 for mutagenesis. However,

replacement of these residues with phenylalanine and tyrosine did not result in a very significant alteration in the rates of Q_A/Q_B electron transfer (Nixon *et al.*, 1992a).

Electron transfer to Q_A. In purple bacteria, a Trp residue on the M subunit (M-Trp250) is in Van der Waals contact with both $BPheo_A$ and Q_A and has been suggested to be involved in the electron transfer between the primary electron acceptor and Q_A (Plato *et al.*, 1989). In PS II the homologous residue is D2-Trp253. Replacement of Trp with Phe (another aromatic residue) has led to a phenotype similar to wild type (Vermaas, 1993). However, replacement of Trp253 with Leu resulted in loss of the PS II RC (Vermaas *et al.*, 1990a). In contrast, in purple bacteria such a mutation leads to loss of Q_A but the mutant retains reaction centres (Coleman *et al.*, 1990). These results led to the suggestion that Q_A binding is required for the stability in PS II, but not for that of the reaction centre of purple bacteria (Pakrasi and Vermaas, 1992).

Bicarbonate binding. Bicarbonate has a major effect on the acceptor side of PS II, since in its absence electron transfer between Q_A and Q_B is slowed down (Blubaugh and Govindjee, 1988). Michel and Deisenhofer (1988) suggested that bicarbonate could be the PS II homologue of M-Glu232 and that consequently it could serve as the fifth ligand to the non-haem iron. In an attempt to find potential ligands to bicarbonate, residues Lys264 and Arg265 of D2 were targeted for mutagenesis. All the mutations performed at these sites (Nixon *et al.*, 1992a) were characterised by a considerable slowing of Q_A/Q_B electron transfer, reminiscent of that observed following bicarbonate depletion of PS II using formate (Eaton-Rye and Govindjee, 1988; Cao and Govindjee, 1990). Replacement of Lys264 with either Arg or Leu resulted in a decrease in the affinity of bicarbonate for the site by an order of magnitude. Mutations of residues Arg233 and Arg251 of D2, also led to alterations in bicarbonate and formate sensitivity (Cao *et al.*, 1991). These results suggested a bicarbonate binding site associated with D1 and D2 and a close interaction of bicarbonate with residues in the area involved in herbicide-binding (see Vermaas, 1993).

Bicarbonate has also been implicated in PS II photoinactivation (Sundby, 1990) as part of the process of photoinhibition. As mentioned before, bicarbonate is predicted to be located near the non-haem iron (Michel and Deisenhofer, 1988). A glycine residue (Gly215) located next to the histidine ligand to the non-haem iron (His214), was replaced by tryptophan (Vermaas *et al.*, 1990a). The mutant showed a very poor photoautotrophic growth and was more sensitive to light than the wild type (Vermaas *et al.*, 1990a; van der Bolt and Vermaas, 1992). The mutation was later found to affect the binding characteristics of bicarbonate to PS II (Schansker *et al.*, 1995). The authors then suggested that the higher sensitivity of the mutant to light might be explained by a less tight binding of bicarbonate to PS II and correlated the loss of bicarbonate binding with the initial events of PS II photoinactivation (Schansker *et al.*, 1995).

The D-de loop region of D2. The region that contains the stroma-exposed loop between transmembrane helix *D* and the parallel helix *de (D-de* loop region) is divergent between D1 and D2. An important question about the function of D1 and D2 in this area concerns the relevant structural difference between these two proteins that yields different functional properties in terms of quinone/herbicide binding. The role of this area was studied by constructing a chimeric D2 protein, where a portion of D1 was introduced into D2 (Kless *et al.*, 1992). The resulting mutant had a nearly normal photoautotrophic phenotype. However, after introduction of a frame-shift into the chimeric gene (leading to a photoheterotrophic phenotype), a number of different photoautotrophic pseudorevertants could be isolated with additional frame-shift mutations in different parts of the D2 gene. The sequences in the restored *D-de* loop varied widely amongst the mutants: changes ranged from a deletion of one amino acid residue to the insertion of 31. These results suggested that the *D-de* loop region of D2 can accommodate viable alterations of length, composition and charges without a dramatic functional impairment (Kless *et al.*, 1992).

Ligands to P680. From sequence comparisons with the L and M subunits of the bacterial reaction centre, it has been suggested that His198 of D1 and His197 of D2 could be ligands to the primary donor P680. D2-His197 has been replaced by Tyr (Vermaas *et al.*, 1987a) leading to a loss of PS II RCs. This suggested that without a proper ligand for Mg in the chlorophyll, the PS II complex is not stable. Mutation of His197 to Gln and Asn allows a relatively stable assembly of the PS II complex, but PS II electron transport is rapidly inhibited in the light (Pakrasi and Vermaas, 1992). Moreover, the His197Gln mutant shows a shift of about 20-30 mV negative in the midpoint potential of the $P680^+/P680$ couple. There is also an enhancement in the misses observed in the oscillatory pattern of oxygen flash yields upon illumination of the dark adapted mutant, indicating that one of the oxidation steps of the oxygen evolving complex has a midpoint potential close to that of $P680^+/P680$. These results support the assignment of His197 of D2 as a ligand to P680 (Nixon *et al.*, 1992a).

Ligands to the manganese cluster. In an attempt to locate carboxylic residues on D2 that could serve as ligands to the manganese cluster, Vermaas *et al.* (1990b) replaced Glu69 by Gln and Val. Both mutants were non-photosynthetic. Thylakoid membranes isolated from the Glu69Gln mutant were capable of oxygen evolution but were quickly photoinhibited. This impairment was slowed down specifically in the presence of exogenous Mn^{2+}. The Glu69Val could not accumulate PS II RCs. Taken together, these results indicated a function of Glu69 as a potential ligand to the manganese cluster.

Mutations at D2-Tyr160 and D2-His189. Barry and Babcock (1987) showed that the secondary electron donor D was a tyrosine residue. Tyr160 of D2 was then assigned to D (reviewed in Vermaas *et al.*, 1993). Tyr160 was replaced by phenylalanine (Debus *et al.*,

1988a; Vermaas *et al.*, 1988b). As a result, the characteristic EPR Signal II_s arising from the oxidised secondary donor D^+ was lost, implicating Tyr160 as donor D. However, the mutant remained photoautotrophic, indicating the non-essential nature of this species. The assignment of Tyr160 as D was enhanced when Tyr160 was replaced by Trp (Barry *et al.*, 1990). The typical EPR II_s signal was replaced by a much narrower signal which the authors attributed to the oxidised free radical of tryptophan.

According to the model of the donor side of PS II produced by Svensson *et al.* (1990), His189 of D2 points into the cavity occupied by D, with the nitrogen on the imidazole side-chain turned towards the phenolic oxygen of D. Upon oxidation, D could give its phenolic proton to His190, forming a neutral radical (Svensson *et al.*, 1990). To test this hypothesis, His189 (the *Synechocystis* homologue of His190 of spinach) was replaced by glutamine, asparagine or leucine (Tang *et al.*, 1993). Spectroscopic analyses of PS II core complexes isolated from these mutants showed narrower EPR D^+ signals compared to the wild type. These signals had normal characteristics. Specific deuteration in one of these mutants resulted in a loss of hyperfine structure of the EPR signal, proving that the signal arose from tyrosine. These results, coupled with proton-ENDOR studies supported the model for the functional role of His190 (spinach numbering) proposed by Svensson *et al.* (1990). D2-His189 was also mutated to Leu and Tyr by Tommos *et al.* (1993). Slight differences in the results obtained from the above studies were attributed to the different methods employed by the two groups (Tang *et al.*, 1993).

Termination and deletion mutants near the C-terminus of D2. A number of site-directed mutations that have been introduced near the C-terminus of D1 have been shown to affect significantly PS II activity (Nixon *et al.*, 1992a). On the basis of the apparent 2-fold symmetry in the PS II D1/D2 reaction centre structure, Eggers and Vermaas (1993) have introduced a number of truncations and deletions near the C-terminus of D2 in order to determine the role of the large hydrophilic C-terminal domain of D2 in the function and stability of PS II. The results obtained from these mutations indicated, according to the authors, that specific domains near the C-terminus of D2 were involved in oxygen evolution and in the stability and activity of the PS II complex.

Table 1.2: Site-directed mutants of D2, all constructed in *Synechocystis* PCC 6803. The number given under "photoautotrophic growth" for some of the mutants, shows the doubling time compared to the wild type. References are given in the text (section 1.4)

Mutant	Photoautotrophic growth	Oxygen evolution (% WT)	PS II content (% WT)	Comments
Glu69Gln	-	30	50	O_2 rate slows in bright light
Glu69Val	-	0	0	
Tyr160Phe	+		75	D^+ signal lost; Tyr160 is D
Tyr160Trp	poor	35-45	80	replacement of stable D^+ Tyr free radical with Trp free radical
Tyr160Met	very poor	25-35	60	
His189Gln	poor	30-40	73	altered D^+ EPR signal; His189 H-bond to Tyr160?
His189Leu	very poor	35-40	45	altered D^+ EPR signal; His189 H-bond to Tyr160?
His189Tyr	+			
His189Asp	very poor	15-30	45	altered D^+ EPR signal; His189 H-bond to Tyr160?
His197Asn	+			e^- transport rapidly inhibited in the light
His197Gln	+	~40	80	ligand to P680?
His197Leu	-		0	
His197Tyr	-		0	
His214Asn/Lys /Met	-		0	
Gly215Trp	very poor	~30	40	light sensitive; affects binding of bicarbonate to PS II
Glu224Phe	+		100	
Asp225Phe	+		100	
Asp225Tyr	+		100	Q_A/Q_B e^- transfer slightly slower
Glu227Phe/Tyr	+			Q_A/Q_B e^- transfer slightly slower
Arg233Gln	+			reduced affinity for bicarbonate
Arg251Ser	+			reduced affinity for bicarbonate
Trp253Phe	+			
Trp253Leu	-		0	
Lys264Arg/Leu /Glu/Gln	+		100	Q_A/Q_B e^- transfer has large slow phase
Arg265His/Asn /Met/Gln	+		100	Q_A/Q_B e^- transfer has large slow phase
His268Asp	-		0	
His268Gln	-	0	20-30	functional loss of the non-haem iron
ET-9	-	0	0	truncation of 9 aa from the C-terminus
ET-11	+ (x2.0)	35	~70	truncation of 11 aa from the C-terminus

Table 1.2 (continued)

Mutant	Photoautotrophic growth	Oxygen evolution (% WT)	PS II content (% WT)	Comments
ET-13	-	<5	25-30	truncation of 13 aa from the C-terminus
ET-14	-	0	25-30	truncation of 14 aa from the C-terminus
ET-15	-	<10	25-30	truncation of 15 aa from the C-terminus
ET-16	-	0	25-30	truncation of 16 aa from the C-terminus
ET-57	-	0	0	truncation of 57 aa from the C-terminus
Δ(11-19)	+ (x2.5)	60-80	40	deletion of 8 aa; from the 11th to 19th aa away from 3' end
Δ(11-15)	-	0	0	deletion of 4 aa; from the 11th to 15th aa away from 3' end
Δ(11-14)	-	~45	~40	deletion of 3 aa; from the 11th to 14th aa away from 3' end
Δ(12-18)	+(x2.2)	60-80	40	deletion of 6 aa; from the 12th to 18th aa away from 3' end
Δ(12-14)	-	0	<10	deletion of 2 aa; from the 12th to 14th aa away from 3' end

1.5 Aims of the project

At the outset of this work, all the *C. reinhardtii* mutants of chloroplast and nuclear origin that affect the synthesis of D2, have been generated by random mutagenesis. The first attempts to produce site-directed mutants of D2 in *C. reinhardtii* are presented in this report. The work undertaken has three aims: (a) to develop a genetic system for constructing site-directed mutants of D2 in *C. reinhardtii* in either a wild type or a PS I$^-$ background (chapter 3), (b) to elucidate the role of a specific residue of D2 (Leu205) in the primary electron transfer process (chapter 4) and (c) to study the role of phosphorylation of the N-terminal threonine (Thr2) of D2 (chapter 5).

The amino acid residues targeted in this work for site-directed mutagenesis, are shown in Figure 1.5 within the proposed folding model of D2 .

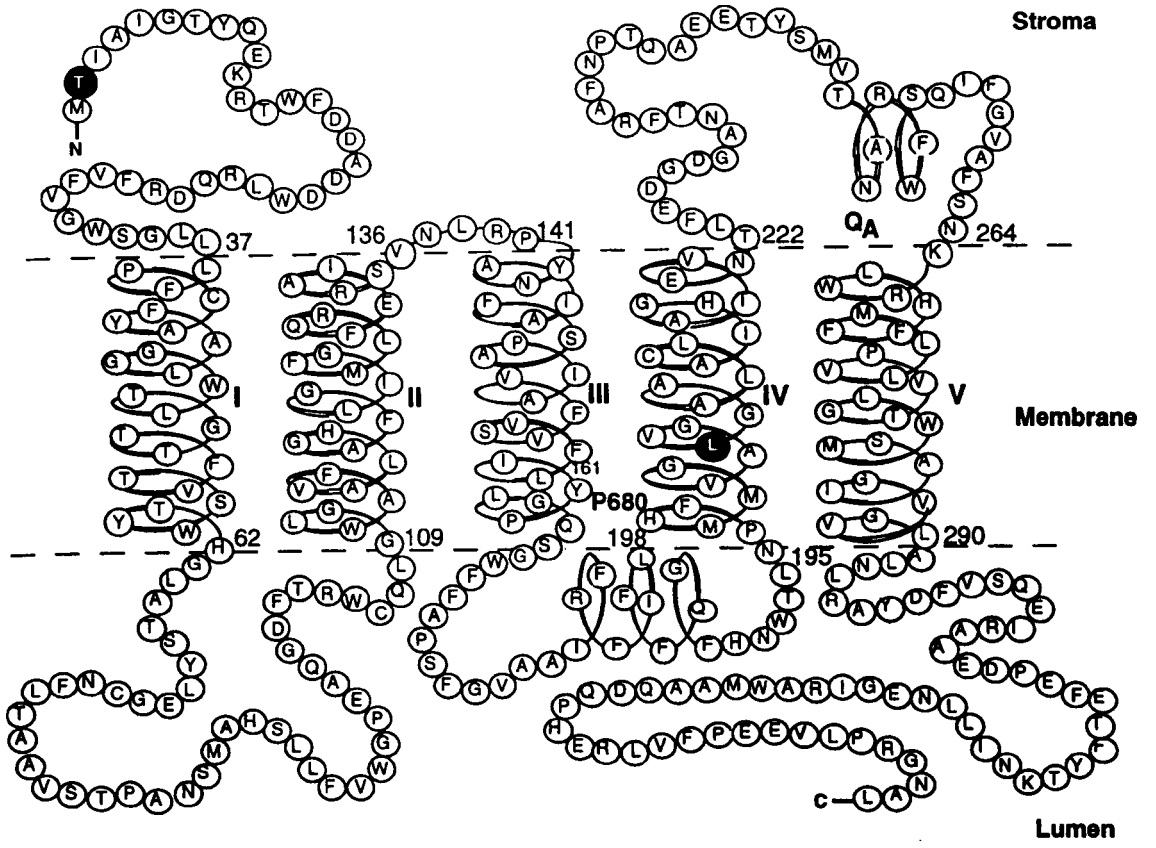


Figure 1.5: Folding model of the D2 protein of *C. reinhardtii*. Amino acids targeted for site-directed mutagenesis are shown in bold.

2. Materials and Methods

2.1 Buffers and reagents

All standard buffers and reagents (such as media) were prepared according to Sambrook *et al.* (1989), unless specifically stated in the text. Solutions and media were sterilised by autoclaving for at least 30 minutes at 15 lb/in^2 and at 121^{0}C. Thermolabile solutions and antibiotics were sterilised by filtration through a 0.2 μm filter (Sartorius Minisart N).

2.2 Strains and growth conditions

2.2.1 *E.coli* strains

E.coli strain DH5-α was used throughout this work for the majority of recombinant DNA techniques. Strain CJ236 was used for the *in vitro* mutagenesis experiments.

Table 2.1: *E.coli* strains

Strain designation	Genotype	Reference
DH5-α	F$^-$, ϕ80d*lac*ZΔM15, *rec*A1, *end*A1,*gyr*A96,*thi*-1,*hsd*R17 (r_k^-, m_k^+), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZYA-*arg*F)U169	Hanahan, 1983
CJ236	*dut*-1, *ung*-1, *thi*-1, *rel* A-1; pCJ105 (Cm^r, F′)	Kunkel *et al.*, 1987

E.coli strains were grown overnight at 37^{0}C in either 2xYT or TB (Terrific Broth) media (Sambrook *et al.*, 1989). When required, the following antibiotics were added at the specified concentrations: ampicillin, 100 μg/ml; spectinomycin, 25 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 50 μg/ml. Stock cultures for long term storage were kept at -80^{0}C in 15% (v/v) glycerol.

2.2.2 *Chlamydomonas reinhardtii* strains

All *Chlamydomonas* strains used in this work are listed in Table 2.2. Strains CC125, CC373 and CC2655 were obtained from the Chlamydomonas Genetic Centre (CGC), Department of Botany, Duke University, Durham, N. Carolina, USA.

Table 2.2: *Chlamydomonas reinhardtii* strains

Strain designation	Characteristics	Reference
CC125	wild type; mt^+	Harris, 1989
CC373 (*ac-u-c-2-21*)	mt^+; 2.5 kb deletion in *atpB* gene (lacks ATPase)	Woessner *et al.*, 1984
CC2655	double mutant of *F54* and *F14*, deficient in both photosystem I and ATPase	Diner and Wollman, 1980
Nsi16	wild type; $spec^R$ (spectinomycin cassette inserted into an *Nsi* I restriction site 261 bp upstream of the initiation codon of *psbD*)	This work
Xba9	PSI^-; $spec^R$ (spectinomycin cassette inserted into an *Xba* I restriction site 295 bp downstream of the termination codon of *psbD*)	This work
D2-Leu205Tyr	replacement of Leu at position 205 of D2 with Tyr; $spec^R$ (spectinomycin cassette inserted into an *Nsi* I restriction site 261 bp upstream of the initiation codon of *psbD*)	This work
D2-Leu205Tyr/PSI^-	replacement of Leu at position 205 of D2 with Tyr; $PS\ I^-$; $spec^R$ (spectinomycin cassette inserted into an *Xba* I restriction site 295 bp downstream of the termination codon of *psbD*)	This work
D2-Thr2Ala	replacement of Thr at position 2 of D2 with Ala; $spec^R$ (spectinomycin cassette inserted at an *Nsi* I restriction site 261 bp upstream of the initiation codon of *psbD*)	This work
D2-Thr2Ser	replacement of Thr at position 2 of D2 with Ser; $spec^R$ (spectinomycin cassette inserted at an *Nsi* I restriction site 261 bp upstream of the initiation codon of *psbD*)	This work

CC125 is of mating type (+) (mt^+) and was used as the reference wild type strain. Cells were grown in Tris Acetate Phosphate (TAP) medium (Gorman and Levine, 1965) or High Salt Minimal (HSM) medium (Sueoka, 1960) and handled as described by Harris (1989). Where necessary, the media were solidified with 1.5% agar (Merck) and supplemented with spectinomycin (Sigma) at 100 μg/ml, ampicillin (Sigma) at 50 μg/ml and 10 μM 3-[3,4-dichlorophenyl]-1,1-dimethylurea (DCMU) (British Greyhound).

Small scale (up to 1 litre) liquid cultures were grown at 25-30°C in an orbital shaker incubator either at an incident light intensity of 30-50 $\mu E.m^{-2}.s^{-1}$ or in the dark.

Large scale (20 litre) liquid cultures were grown in 20 L carboys at room temperature and were aerated by bubbling with air and by stirring.

The cell density of wild type cultures grown for the purpose of chloroplast transformation was measured using a compound microscope and a standard haemocytometer, as described by Harris (1989).

All the strains were routinely maintained on TAP plates at room temperature by restreaking on fresh medium once a month. Strains obtained after chloroplast transformation were kept on TAP medium supplemented with spectinomycin (at 100 μg/ml), ampicillin (at 50 μg/ml) and DCMU (at 10 μM). Ampicillin was added in order to reduce the possibility of bacterial contamination (Harris, 1989). The addition of DCMU at 10 μM should inhibit PS II activity and prevent competition from wild type copies of the *psbD* gene and from PS II revertants. Prior to inoculation of liquid cultures, cells were restreaked on fresh TAP and used after 5-10 days.

2.3 Plasmids and recombinant DNA techniques

2.3.1 Vectors and recombinant plasmids

All the vectors and recombinant plasmids used in this work are shown in Table 2.3. Plasmid pH3 (Erickson *et al.*, 1986), which was kindly provided by J.-D. Rochaix (Departments of Molecular Biology and Plant Biology, University of Geneva, Switzerland), contains a 7.5 kb *Hind* III fragment (carrying the *psbD* gene) cloned in pUC9.

Plasmid pUC-atpX-AAD (kindly provided by M. Goldschmidt-Clermont, Departments of Molecular Biology and Plant Biology, University of Geneva, Switzerland) is a pUC18 derivative that contains a 1.9 kb expression cassette (the *aadA* cassette, see section 3.1.1.2), surrounded by unique restriction sites, which confers resistance to spectinomycin and streptomycin (Goldschmidt-Clermont, 1991).

Plasmid p17 contains a 7.6 kb *Bam* HI fragment carrying the *atpB* gene and its flanking regions, cloned into pBR313 (Boynton *et al.*, 1988).

Table 2.3: Vectors and recombinant plasmids

Plasmids	Description	Reference
pTZ19U	derivative of pUC19 into which the single-stranded replication origin of phage f1 has been incorporated	Bio-Rad
pBluescript KS	used as a general cloning vector; derivative of pUC19 into which the single-stranded replication origin of the phage f1 has been incorporated	Stratagene Ltd.
pH3	has a 7.5 kb *Hind* III fragment containing the *psbD* gene and its flanking regions, cloned into the *Hind* III site of pUC9	Erickson *et al.*, 1986
pUC-atpX-AAD	pUC18 derivative containing a 1.9 kb selectable marker which confers resistance to spectinomycin	Goldschmidt-Clermont, 1991
p17	has a 7.6 kb *Bam* HI fragment containing the *atpB* gene and its flanking regions, cloned into pBR313	Boynton *et al.*, 1988
pCA1	has an approximate 4.7 kb *Hpa* I/*Hind* III fragment containing the *psbD* gene and its flanking regions, cloned into pTZ19U	This work
pNsi16	is a derivative of pCA1 and contains a 1.9 kb $spec^R$ cassette inserted at an *Nsi* I restriction site, 261 bp upstream of the 5′ end of *psbD*	This work
pXba9	is a derivative of pCA1 and contains a 1.9 kb $spec^R$ cassette inserted at an *Xba* I restriction site, 295 bp downstream of the 3′ end of *psbD*	This work

2.3.2 Small and large scale plasmid purification from *E. coli*

Plasmid DNA was isolated from cultures up to 5 ml (miniprep) using the alkaline lysis procedure (Sambrook *et al.*, 1989). The DNA was extracted with phenol/chloroform (1:1) and then chloroform and the double stranded plasmid DNA precipitated using isopropanol. For the isolation of highly pure plasmid DNA (for sequencing purposes) the Wizard Miniprep Purification system from Promega was used.

For large-scale plasmid DNA isolation (maxiprep) the alkaline lysis method was also used (Sambrook *et al.*, 1989). A 300 ml culture, grown overnight in TB medium, was lysed using essentially the same solutions as for the small scale preparation apart from the addition of lysozyme to aid cell wall breakage. Plasmid DNA was then purified using either a CsCl-Ethidium bromide (EtBr) gradient or after precipitation with polyethylene glycol

(PEG). In the first method, the nucleic acid solution was initially mixed with CsCl (1 g for 1 ml of the DNA suspension) and after the latter had been dissolved, EtBr was added to a concentration of 1 mg /ml. The suspension was loaded into 25 x 89 mm Beckman "Quick-Seal" tubes. The tubes were then filled completely with mineral oil (Sigma), heat sealed and centrifuged for 24-48 hours at 45000 rpm in a Ti70 rotor (Beckman) at 20°C. After centrifugation the DNA bands were visualised under long wavelength UV light. The lower band containing covalently closed circular DNA was collected using a 10 ml syringe. Ethidium bromide was removed using CsCl-saturated isopropanol. CsCl was removed by dialysis against TE.

In the method employing PEG, the covalently closed circular DNA was purified after precipitation with a 20% PEG 6000/ 2.5 M NaCl solution.

2.3.3 Transformation of *E. coli*

2.3.3.1 Preparation of competent cells

DH5-α and CJ236 competent cells were prepared according to the method of Hanahan (1983) (as modified by C. Flores, personal communication). Several colonies were picked from a freshly grown plate and dispersed into 1 ml of Ψ medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 0.4% $MgSO_4$, 10 mM KCl pH 7.6). This was used to inoculate 100 ml of prewarmed Ψ medium in a 1 L flask. The culture was grown at 37°C with vigorous shaking until the OD_{550} was 0.48. Cells were then chilled on ice, transferred to two 50 ml Falcon tubes and pelleted by centrifugation in a Chillspin benchtop centrifuge at 2500 rpm for 5 minutes at 4°C. Pelleted cells were drained thoroughly and all work was continued in the cold room (at 4°C). Cells were resuspended in 30 ml of ice-cold RF1 solution (100 mM RbCl, 50 mM MnCl, 30 mM potassium acetate, 10 mM $CaCl_2$, 15% (v/v) glycerol, pH 5.8 with 0.2 M acetic acid and filter-sterilised) and either left on ice for 20 minutes (in the case of CJ236) or pelleted immediately (in the case of DH5-α) at 2500 rpm for 5 minutes at 4°C. The pellet was then resuspended gently in 4 ml of ice-cold RF2 solution (10 mM morpholinopropane-sulphonic acid (MOPS) pH 7.0, 10 mM RbCl, 75 mM $CaCl_2$, 15% (v/v) glycerol). The competent cells were dispersed in 0.2 ml aliquots and stored at -80°C.

2.3.3.2 Transformation procedure

Competent *E. coli* cells were transformed using the procedure described in the MUTAGENE phagemid *in vitro* mutagenesis Instruction Manual (Bio-Rad). 40-100 µl of competent cells were thawed on ice until just liquid. Plasmid DNA (10-100 ng) was then added and the mixture was incubated on ice for 1 hour. Cells were then heat-shocked at 42°C for 3 minutes and immediately placed on ice for 5 minutes. 4 volumes of 2xYT medium was then added and

the cells were incubated at 37°C for 1 hour to allow for the antibiotic resistance gene carried by the plasmid, to be expressed. The culture was then spread onto 2xYT plates containing the appropriate antibiotic and the plates incubated at 37°C overnight.

2.3.4 Restriction enzyme digestions

Restriction enzyme digestions were carried out at 37°C for 1-3 hours and the reaction stopped (if necessary) by placing the mixture at 65°C for 10 minutes. Enzymes and buffers were purchased from New England Biolabs (NEB).

2.3.5 Agarose gel electrophoresis

DNA fragments were routinely separated by electrophoresis through horizontal agarose gels. The gels were prepared by dissolving ultrapure agarose (BRL) in TAE buffer (40 mM Tris acetate, 1 mM EDTA pH 8.0) at 100°C, followed by the addition of 0.1 μg/ml of the intercalating dye EtBr. The running buffer was TAE. In order to aid the visualisation of the low molecular weight bands, 0.5 μg/ml EtBr was also added in the part of the electrophoresis tank containing the anode. Prior to electrophoresis DNA samples were mixed at a ratio of 2:1 - 5:1 with loading buffer containing 0.05% (w/v) bromophenol blue, 40% (w/v) sucrose, 0.1 M EDTA pH 8.0 and 0.5% (w/v) SDS (purchased from Sigma). Electrophoresis was carried out in a 58x75 mm or a 128x110 mm apparatus (NBL) at 70-100 Volts. Large gels for Southern analysis were run in a Bio-Rad Sub-cell apparatus (150x250 mm) at 20 Volts overnight until the desired separation had been achieved. The standard marker was the 1 kb DNA ladder from BRL (with fragments of 12216 bp, 11198 bp, 10180 bp, 9162 bp, 8144 bp, 7126 bp, 6108 bp, 5090 bp, 4072 bp, 3054 bp, 2036 bp, 1636 bp, 1018 bp, 517 bp, 506 bp, 396 bp, 344 bp, 298 bp, 220 bp, 201 bp, 154 bp, 134 bp and 75 bp). DNA bands were visualised using a short wave UV transilluminator and photographed using a Polaroid CU-5 camera equipped with filters (Wratten 2A clear and 2A red) and loaded with a Polaroid 667 film.

2.3.5.1 Recovery of DNA from agarose gels

DNA samples were recovered from agarose gels by excising the band of interest from the gel and then purifying it using either the GeneClean II kit (BIO-101 Inc.) or by electroelution in a dialysis bag (Sambrook *et al.*, 1989).

2.3.6 Ligation reactions

The ligation mixture typically contained 10-100 ng of digested vector DNA, insert DNA in either equimolar amounts (for sticky-end ligations) or in 5x excess (for blunt-end

ligations), 1x T4 DNA ligation buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 mg/ml BSA, Integra Biosciences) and 2-5 units of T4 DNA ligase (NEB) in a volume of 10 µl. In the case of blunt-end ligations, the ligation buffer also contained 50% (w/v) PEG. The reaction mixture was incubated either at room temperature for 1 hour or at 4°C overnight. 4-5 µl of the reaction was used transformed into *E. coli* competent cells. Recombinants were identified on the basis of antibiotic resistance expressed by a gene carried in the insert DNA or by their inability to form blue colonies on plates containing the inducer IPTG (isopropylthio-β-D-galactoside: 1 µl/ml from a 100 mM stock solution, filter sterilised) and the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside: 2.5 µl/ml from a 2% (w/v) stock solution in dimethylformamide) (Sambrook *et al.*, 1989). The structure of the recombinant plasmids was verified by restriction analysis of minipreparations of plasmid DNA.

2.3.7 Quantitation of DNA

DNA prepared from maxipreps was quantified spectrophotometrically in a Shimadzu MPS 2000 spectrophotometer by measuring the absorbance of a DNA solution (in H_2O or TE) at 260 and 280 nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid in the sample (an OD_{260} of 1.0 corresponds to approximately 50 µg/ml of double stranded DNA). The ratio of the OD_{260}/OD_{280} measurements provided an estimation of the purity of the DNA preparation. This ratio should be between 1.8 and 2.0. Ratios lower than 1.6 indicated significant contamination with protein or phenol whereas ratios equal to 2.0 indicated the presence of RNA.

The amount of DNA obtained from minipreparations was estimated visually by running an aliquot in an agarose gel and comparing the band of interest with known amounts of DNA.

2.4 Site-directed mutagenesis

Site-directed mutagenesis was carried out using Bio-Rad's "Muta-gene" phagemid *in vitro* mutagenesis kit. This kit is based on a method described by Kunkel *et al.* (1987), which provides a very strong selection against the non-mutagenised strand of a double-stranded plasmid DNA. A phagemid is a plasmid into which the single-stranded replication origin of a filamentous bacteriophage has been incorporated. The phagemid replicates as an ordinary double-stranded circular plasmid until its host is superinfected with a helper phage derived from f1 or its close relative M13. The proteins coded for by the superinfecting phage act at the single strand origin and cause the phagemid DNA to be replicated, packaged and extruded from the cell as if it were a single-stranded phage.

The approach used in the "Muta-gene" phagemid kit is to clone the DNA to be mutated into the phagemid pTZ19U and introduce this recombinant plasmid into an *E. coli* *dut*$^-$ *ung*$^-$ strain (CJ236). Phagemid particles whose single strand of DNA contains some uracils are produced from these cells by superinfection with the helper phage M13KO7. The uracil-containing DNA is purified, used as template in an *in vitro* mutagenesis reaction and transformed into a *dut*$^+$ *ung*$^+$ strain (e.g., DH5-α), thus selecting against the parental (uracil-containing) strand. This method has been reported to result in a high efficiency of mutant production (>50%) (Kunkel *et al.*, 1987).

Single-stranded DNA was prepared from phagemids according to the manufacturer's protocol and was annealed to one of the synthetic oligonucleotides shown in Table 2.4. In the case of the Leu205Tyr mutant, pCA1 was used as a template. *Chlamydomonas* mutant strains, Thr2Ala and Thr2Ser, were prepared using plasmid pNsi16 as template. The mutagenic oligonucleotides were designed to create (or delete) a restriction site along with the mutation.

The annealing and second strand synthesis reactions contained the following reagents:

Annealing reaction: 2 µl (200 ng) of ssDNA template, 1 µl (6 pmol) of the mutagenic oligonucleotide, 1 µl of 10x annealing buffer (200 mM Tris pH 7.5, 20 mM $MgCl_2$, 500 mM NaCl) and 6 µl of H_2O.

Second strand synthesis reaction: 1 µl of 10x synthesis buffer (5 mM dNTPs, 10 mM ATP, 100 mM Tris pH 7.9, 50 mM $MgCl_2$, 20 mM DTT), 1U of T7 DNA polymerase and 4U of T4 DNA ligase.

The second strand synthesis reaction was stopped by the addition of 50 µl TE buffer. 10 µl of this reaction mixture were used to transform *E. coli* DH5-α cells. Clones carrying the mutations were isolated and the mutations verified by restriction analysis and DNA sequencing.

Plasmids carrying the altered amino acid sequences were used to transform *Chlamydomonas* cells.

Table 2.4: Mutagenic oligonucleotides. Altered nucleotides are indicated in bold. New restriction sites are underlined.

Oligonucleotides	Sequence 5'→3'	Restriction sites
D2-L205Y	GTT GCT GGT **GTA TAC** GGT GCT GCT TTA	new *Acc* I site
D2-T2A	ATA CAC GCA ATG **GCT** ATT GCG ATC GGT	Loss of a *Mun* I site
D2-T2S	ATA CAC GCA ATG **TCT** ATT GCG ATC CGT	Loss of a *Mun* I site

2.5 *Chlamydomonas* chloroplast transformation

Chloroplast transformation was performed essentially as described by Boynton and Gillham (1993). Wild type (CC125) cells were grown to a density of 2-4 x 10^6 cells/ml, pelleted by centrifugation and resuspended to a density of approximately 1.2 x 10^8 cells/ml in TAP. 1-ml aliquots were diluted 1:1 with 0.2% soft agar in TAP medium at 42^{0}C, and two 0.7 ml aliquots containing approximately 4 x10^7 cells each were dispersed on the surfaces of 10-cm diameter petri plates of solid TAP medium. The plates were gently swirled on the bench surface to disperse soft agar evenly, then covered with aluminium foil for 2 hr to prevent the cells from swimming towards the light while the agar solidifies, and incubated for 16-48 hr in dim light.

Plasmid DNA was precipitated onto either tungsten or gold particles (Bio-Rad) according to the following procedures.

Precipitation onto tungsten particles (Sanford *et al.*, 1993): 60 mg of tungsten M10 microprojectiles were resuspended in 1 ml of 100% ethanol, left to soak for 15 min and then pelleted by centrifugation for 3-5 min. Particles were then washed 3 times in sterile distilled water and finally resuspended in 1 ml of 50% glycerol. 25 µl of resuspended tungsten particles were aliquoted into a fresh tube (whilst vortexing continuously), immediately followed by 2.5 µl of plasmid DNA (at 1 mg/ml), 25 µl of 2.5 M $CaCl_2$ and 10 µl of 0.1 M spermidine (free base). Vortexing was continued for several minutes and then the tubes were pulse centrifuged. 50 µl of supernatant was discarded (leaving approximately 6 µl - enough for three shots). 2 µl were then immediately loaded onto the centre of each macroprojectile.

Precipitation onto gold particles (1.0 µm diameter) (Boynton and Gillham, 1993): 6 mg gold particles (enough for 10 shots) were added to a 1.5 ml microfuge tube with 10 µl absolute ethanol and vortexed for 1-2 minutes, spun in a microfuge for 1 minute and the pellet washed twice with 1 ml sterile distilled water. The pellet was uniformly resuspended in 100 µl sterile water and 50 µl aliquots were removed to individual 1.5 ml microfuge tubes. To each tube, 5 µl donor DNA at 1 µg/ml, 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine (free base) were added in sequence. The tubes were vortexed for 3 minutes, spun in the microfuge for 10 seconds and as much supernatant as possible was removed. The pelleted particles were washed with 250 µl of absolute ethanol, vortexed briefly, spun again and the pellet resuspended in 60 µl of absolute ethanol. A 10 µl aliquot of the resuspended particles was loaded onto the centre of each macroprojectile and allowed to dry before use.

0.22 blank cartridges (Shearline) were used as accelerators for bombardment in the Particle Accelerator (Shearline).

One to three hours after bombardment, the cells were respread on selective TAP media containing 100 µg/ml spectinomycin. 50 µg/ml ampicillin was also added to reduce the possibility of bacterial contamination (Harris, 1989).

2.6 Isolation of DNA from *Chlamydomonas*

Total cellular DNA was initially isolated using a slight modification of the method of Newman *et al.* (1990). A patch of cells, growing on solid media, was scraped up using a sterile toothpick and resuspended in 1 ml of cold TEN buffer (150 mM NaCl, 10 mM Na_2EDTA, 10 mM Tris pH 8.0), pelleted at room temperature and resuspended in 0.4 ml TEN to which 40 µl 20% SDS, 40 µl 20% Sarkosyl and 70 µl of 25 mg/ml Pronase were added. Samples were gently vortexed, rotated for 5 min at 4°C and then extracted with 0.65 ml TE-saturated phenol/chloroform for 10 min at room temperature with gentle intermittent vortexing. The top aqueous phase was recovered after a 10 min centrifugation in a microfuge at room temperature and the DNA precipitated with 1 ml ice-cold 95% ethanol and 20 µl of 5M NaCl at -20°C for 30 min. The precipitate was collected by centrifugation for 10 min at room temperature, washed with cold 70% ethanol, and resuspended in 50 µl TE.

During later stages of the project, the above protocol was further modified: Instead of precipitating the nucleic acids after phenol/chloroform extraction, the top aqueous phase was mixed with 1 ml of Magic™ resin (Promega), and the DNA recovered using the Magic™ miniprep DNA purification system (Promega).

Chloroplast DNA (ctDNA) was prepared according to the procedure described by Roffey *et al.* (1991). 1 L of cells, grown to late logarithmic phase, was harvested by centrifugation for 5 minutes at 3,000 rpm, in an A6.9 rotor (KONTRON) at 4°C. The pellets were washed and resuspended in 20 ml (in total) of 50 mM Tris pH 8.0/50 mM EDTA, frozen in liquid nitrogen and ground to a fine powder. 20% SDS was added to a final concentration of 1% and the mixture was incubated at 60°C for 30 minutes. Caesium chloride was added (at 1.1 g/ml) and the mixture was incubated at 60°C for 15 minutes. The suspension was then centrifuged at 4,000xg for 10 min to remove cell debris. The supernatant was removed from below the pellet and bisbenzimide (Sigma) was added to a final concentration of 0.1 mg/ml. The solution was then centrifuged at 44,000 rpm in a Ti70 rotor (Beckman) for 24 hours. The chloroplast DNA (upper band) was removed and dialysed vs. 2 litres of TE at 4°C overnight. The ctDNA was precipitated once with 2 M ammonium acetate and 2.5 volumes ethanol, resuspended in TE and extracted with phenol/chloroform, before being re-precipitated (with ammonium acetate and ethanol) and resuspended in 100 µl of TE.

2.7 Southern blotting

Southern blot hybridisations of C. *reinhardtii* DNA were performed following standard protocols (Sambrook *et al.*, 1989). 100-300 ng of ctDNA or 10-20µl of total DNA (obtained from a miniprep), digested with the appropriate restriction enzyme, were fractionated on a 1.0% agarose gel and then transferred to a Hybond N^+ membrane (Amersham) using a bidirectional method (Sambrook *et al.*, 1989). The membrane was air-

dried and either used for hybridisation with a radiolabelled DNA probe or stored at room temperature until required.

Radiolabelled probes were prepared using the Random Primed Labelling kit from Boehringer-Manheim. This system is based on the method of Feinberg and Vogelstein (1983). 25-50 ng of double stranded DNA were labelled for 30 minutes at 37°C, using 50 µCi [α-^{32}P] dCTP and the reaction was stopped by the addition of 1.6 µl of a 0.25 M EDTA solution. Unincorporated deoxyribonucleoside triphosphates were removed by passing the reaction mixture through a NICK column (Pharmacia).

Hybridisation was performed at 65°C for 16-20 hours in a Hybaid hybridisation oven. The hybridisation solution contained 5 x SSC (0.75 M NaCl and 0.075 M Na_3citrate), 5 x Denhardt's solution (0.5 g Ficoll, 0.5 g Polyvinylpyrrolidone, 0.5 g bovine serum albumin), 0.5 % SDS and 30 µg/ml calf thymus DNA (denatured by heating at 100°C for 5 minutes before use). The radiolabelled probe was denatured by heating at 100°C before addition to the hybridisation solution. Before hybridisation a pre-treatment step (pre-hybridisation) of 2.5 hours was also carried out. Filters were washed at 65°C with 2 x SSC, 0.1% SDS for 10 minutes (twice), then with 1 x SSC, 0.1% SDS for 20 minutes and finally with 0.1 x SSC, 0.1% SDS for 15 minutes. Autoradiograms were obtained using FUJI RX film.

2.8 Polymerase Chain Reaction (PCR)

PCR was routinely used to amplify DNA isolated from *Chlamydomonas*. The reactions were performed in a Techne PHC-3 thermocycler using the components of the Gene-Amp kit (Perkin Elmer Cetus). Typical reactions contained 5 µl of 10x reaction buffer (100 mM Tris pH 8.3, 500 mM KCl and 0.01% gelatin), 2-8 mM of $MgCl_2$ (according to the nucleotide sequence of the primers), 8 µl of dNTP mixture (final concentration: 200µM in each dNTP), 1µl (50 pmol) of each primer, 1 µl of either chloroplast or total DNA and 0.5 µl (2.5 U) of *Taq* DNA polymerase (Perkin Elmer Cetus). Amplified DNA for sequencing purposes was prepared using a mixture of *Taq* and *Pfu* (Stratagene) DNA polymerases at a ratio of 16:1 (Barnes, 1994). All PCR primers (Table 2.5) were custom-made by Oswell DNA Services (Edinburgh). The DNA samples were denatured at 95°C for 5 minutes. This step was followed by 35 cycles of 95°C for 30 seconds, 42-56°C (according to the primers used) for 1 minute and 72°C for 2 minutes.

2.9 DNA sequencing

Sequencing reactions were performed using either the Sequenase 2.0 kit (United States Biochemicals) or the T7 sequencing kit from Pharmacia and according to the manufacturer's instructions. Primers used for sequencing purposes (shown in Table 2.5) were

either prepared by C. Lichtenstein using a Pharmacia LKB Gene Assembler Plus oligonucleotide synthesiser or were obtained from Oswell DNA Services (Edinburgh).

Sequencing of algal DNA was performed using the following procedure: total DNA obtained from a miniprep was amplified using primers C10 and C70 (Table 2.5) which also incorporate into the PCR product restriction sites for the enzymes *Bam* HI and *Hind* III, respectively. For this particular PCR, a combination of *Taq* and *Pfu* DNA polymerases (at a ratio of 16:1, unit-wise) (Barnes, 1994) was used. *Pfu* DNA polymerase was used because of its ability to reduce errors during polymerisation (as it has a 3' to 5' proofreading exonuclease activity). The amplified DNA was then purified from the other components of the PCR using the "Wizard™ PCR preps" method (Promega), digested with *Bam* HI and *Hind* III and then cloned into the corresponding restriction sites of a pBluescript KS vector. After screening by restriction analysis for the presence of recombinant plasmids, overnight cultures were grown and sufficient plasmid DNA for a few sequencing reactions was isolated using the Wizard™ miniprep method (Promega). This method yielded plasmid DNA of high purity obviating the need of any extra purification steps.

Table 2.5: Oligonucleotides used as primers for PCR and sequencing, listed in 5' -3' orientation with bases matching the template in uppercase and linkers creating new restriction sites in lower case. Position is referring to the initiation codon of *psbD*

Oligo	Sequence	Position	Use
C1	AAATACACAATGATTAAAAT	-79 to -60	PCR/Seq.
C2	GGTACTACTTTCGTTACT	150 to 168	Seq.
C3	CACGGTGCATTTGGTTTA	348 to 364	Seq.
C4	CAAGGTTTCCACAACTGG	556 to 573	Seq.
C5	CGTTTCTGGTCACAAATC	751 to 768	Seq.
C6	CAAGAGATTCGTGCTGCT	901 to 918	Seq.
C7	AAAAATATATTATAGAGCGT	1068 to 1048	PCR/Seq.
C8	CAAGGGAATAGTAATAAACC	119 to 99	Seq.
C9	CAGGTTGGTTCTTTGC	493 to 509	Seq.
C10	ctcggatccAAATACACAATGATTAAAAT	-79 to -60	PCR
C70	tggaagcttAAAAATATATTATAGAGCGT	1068 to 1048	PCR
PCR A	TGGATATTTGGTACATTTAATTCC	-411 to -388	PCR
PCR B	CACTGCCTCTAATAAAGTC	on 5' end of *aadA* cassette	PCR

The sequencing reactions were electrophoresed through a 0.4 mm normal gel (6% acrylamide, 7M Urea) or a 0.2-0.4 mm wedge gel, in 1 x TBE buffer (100 mM Tris, 1 mM EDTA and boric acid to pH 8.9) using an IBI STS 45 DNA sequencing apparatus. The gel was run for 2-6 hours, depending on the distance from the primer to the sequence to be read. Following electrophoresis, the DNA was fixed in the gel and urea removed by soaking in 10% (v/v) methanol, 10% (v/v) acetic acid for 15 minutes to 1 hour. Subsequently, the gel was transferred to a sheet of Whatman 3MM paper and dried at 80°C for 1-1.5 hours in a Hoefer Slab gel dryer. Finally, a sheet of KODAK X-OMAT AR film was placed in direct contact with the dried gel and exposed at room temperature.

2.10 Growth measurements

Growth of the transformants was studied by growing liquid cultures photoautotrophically in 100 ml of HSM medium (in a 500 ml Erlenmeyer flask) at 25°C and at an incident light intensity of 50-70 $\mu E.m^{-2}.s^{-1}$. The cultures were bubbled with air and stirred continuously. The optical density (OD) at 750 nm was monitored in a Shimadzu MPS-2000 spectrophotometer, at regular intervals until the cultures had reached the stationary phase. In order to avoid errors in the measurements due to motility or settling, cells had to be thoroughly mixed and measurements taken rapidly. The growth pattern was described by the logarithmic value of the optical density versus time. The formula:

$$\frac{\log_{10} \text{final cell concentration} - \log_{10} \text{initial cell concentration}}{\log_{10} 2}$$

was used to calculate the number of doublings (Harris, 1989). This number was used for the determination of the growth rate (number of doublings over time) and of the doubling time.

2.11 Determination of chlorophyll content

The chlorophyll content of *Chlamydomonas* cells was measured using the equations of Arnon (1949):

$[\text{chl } a]\ (\text{mg/ml}) = [(A_{663} \times 0.0127) - (0.00269 \times A_{645})] \times \text{dilution}$

$[\text{chl } b]\ (\text{mg/ml}) = [(A_{645} \times 0.0229) - (0.00468 \times A_{663})] \times \text{dilution}$

$[\text{total chl}]\ (\text{mg/ml}) = [\text{chl } a] + [\text{chl } b]$

0.25-1 ml of cells were centrifuged for 3 minutes in a microfuge tube. The supernatant was discarded and 1 ml of 80% acetone was added and mixed thoroughly. The tubes were left for 10-30 minutes in the dark at room temperature, with occasional agitation. The samples were finally centrifuged for another 3 minutes in order to remove any insoluble material, and the absorbance at 663 and 645 nm was measured.

2.12 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein composition of samples was routinely analysed on polyacrylamide gels containing urea, using a discontinuous buffer system. For this purpose, a Hoefer SE 600 vertical electrophoresis apparatus was used. The running buffer (0.025 M Tris, 0.19 M glycine, 0.1% SDS, pH 8.3) was a variation of the Laemmli buffer system (Laemmli, 1970). The thickness of the gel was either 0.75 mm or 1.5 mm. Electrophoresis was carried out at 30 mA per 1.5 mm thick gel, or overnight at 10 mA per 1.5 mm thick gel, until the dye (bromophenol blue) front reached the bottom of the separation gel. When necessary, the electrophoresis tank was kept at 12°C by a thermostatic circulator (LKB 2219). Molecular weight standards from Pharmacia (94-14.4 kDa range) or Bio-Rad (prestained LMW marker, 112-20.5 kDa range) were run alongside samples. The monomer concentration (%T) of gels used throughout this work as well as the conditions of the run are shown in Table 2.6.

Samples were solubilised for 5 minutes-2 hours (depending on the sample) in the dark, prior to loading, by addition of an equal volume of solubilisation buffer (187 mM Tris pH 6.8, 30% glycerol, 9% SDS, 15% β-mercaptoethanol). Proteins from whole cells were solubilised by boiling the cell suspension (at a chlorophyll concentration of 0.5 μg/μl) for 1 minute in an equal volume of solubilisation buffer. Pigmented samples were loaded on the gel on an equal chlorophyll basis.

SDS-PAGE separated proteins were stained with Coomassie Brilliant Blue (CBB) G, Coomassie R250 or silver. For Coomassie staining, proteins were fixed by placing the gel either in 10% TCA for 10 minutes or in 25% methanol, 10% acetic acid for 30 minutes. The gel was then stained with Coomassie G (0.1% CBB G in 45% methanol, 10% acetic acid), or Coomassie R250 (0.5% CBB R250, 25% isopropanol, 10% acetic acid) for 2 hours to overnight and then destained by several washes in 25% isopropanol, 10% acetic acid. For silver staining, a modified version (N. Zelev, personal communication) of the rapid silver staining protocol presented in Ausubel *et al.* (1992), was used. The gel was initially placed in 100 ml fixing solution (13.5% formaldehyde, 40% methanol) for 10 minutes (for a 0.75 mm thick gel) and then washed twice with dH_2O for 5 minutes. Subsequently, the gel was soaked for 1 minute in 100 ml aqueous sodium dithionite (0.02%), rinsed rapidly (2x20 seconds) with dH_2O and incubated in 100 ml of 0.1% silver nitrate solution for 10 minutes. The gel was then rinsed rapidly with a small volume of dH_2O followed by a small volume of developing solution (3% Na_2CO_3, 0.05% formaldehyde, 0.0004% sodium dithionite). Then, the gel was soaked in 100 ml of developing solution until the desired level of staining had been reached. Staining was stopped by removing the developing solution and soaking the gel for a few minutes in destaining solution (25% isopropanol, 10% acetic acid).

Table 2.6: Acrylamide and urea concentration of gels run throughout this work. Acrylamide concentration in the stacking gel (0.104 M Tris pH 6.8) was 5%. Gels containing 8 M urea were run at room temperature within 4-6 hours to avoid urea precipitation.

Acrylamide concentration (%T) of the separating gel (0.75 M Tris pH 8.9)	urea
12%	6M
14%	8M
17%	6M
10-17%	6M
12-18%	8M

Gels were either stored in 7% acetic acid or dried. Before drying, the gel was incubated overnight in a solution containing 5% glycerol and 40% methanol. The gel was then transferred to a sheet of Whatman paper and dried for 3-4 hours at 60-70°C in a slab gel drier (Hoefer SE1160), under vacuum.

2.13 Western blotting

Protein samples resolved by SDS-PAGE were transferred to a nitrocellulose membrane according to the method of Dunn (1986). After electrophoresis the gel was incubated for 30 minutes in transfer buffer (3 mM Na_2CO_3, 10 mM $NaHCO_3$, 20% methanol). Transfer of proteins to the membrane (Schleicher and Schuell, 0.2 µm pore size) was carried out for 1.5 to 2 hours at 4°C in transfer buffer using a Trans-Blot Cell system from Bio-Rad, set at 40 Volts. After transfer, the nitrocellulose membrane was blocked against non-specific binding of antibodies by incubating in TBS buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween 20) for at least 30 minutes. The primary antibody was then added at a suitable concentration in 10-12 ml of TBS buffer and the membrane was incubated either overnight at 4°C or for 2 hours at room temperature. Following incubation with the primary antibody, the blot was washed 6x5 minutes with TBS buffer before the addition of the secondary antibody (anti-rabbit IgG-alkaline phosphatase conjugate, Sigma) at a dilution of 1:1000 in TBS buffer. The membrane was then incubated for 1 hour with the conjugate, washed 6x5 minutes with TBS and transferred into alkaline phosphatase (AP) buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 5 minutes. Blots were developed by the addition of 33 µl of a stock solution of 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt, (50 mg/ml in 100% dimethyl formamide) (Sigma) and 44 µl of a stock solution of nitroblue tetrazolium chloride, (75 mg/ml in 70% dimethyl formamide) (Sigma), in 10 ml of AP buffer. Development of the signal was stopped by adding H_2O to the membrane. The blot was placed on Whatman paper

to dry and stored at room temperature. Polyclonal antibodies against D1 (304-F, DuPont), D2 (701, DuPont), CP43, the 33 kDa extrinsic protein and LHCII polypeptides of spinach were supplied by Dr. P. Nixon and used at dilutions of 1:3000, 1:5000 1:20000, 1:5000 and 1:700, respectively. The polyclonal antibody against the PsaD subunit of photosystem I of higher plants, was kindly provided by Prof. R. Nechushtai (Department of Botany, The Hebrew University of Jerusalem, Israel) and used at a dilution of 1:500. The monoclonal antibody against phosphothreonine (#B-7661, monoclonal anti-phosphothreonine biotin conjugate, Sigma) was used at a dilution of 1:300.

2.14 Oxygen evolution measurements of whole cells

Oxygen evolution of whole cells was measured with a Hansatech DW Oxygen electrode linked to a Hansatech CB1 control box. The measurements were performed in HSM medium at 25°C and at saturating light intensities (4000-6000 $\mu E.m^{-2}.s^{-1}$) using a Gallex LS15 lamp. The oxygen electrode was calibrated before and after the end of the measurements using sodium dithionite to remove oxygen from air-saturated water. Samples were added in a volume of 1 ml and 1mM $NaHCO_3$ was added as a substrate for photosynthesis. In order to assay PS II function, 1mM potassium ferricyanide and either 1mM 2,5-dimethyl-*p*-benzoquinone (DMBQ) or 1mM 2,6-dichloro-*p*-benzoquinone (DCBQ) were added. The rates of O_2 evolution were expressed as μmoles $O_2 \cdot$ mg $chl^{-1} \cdot hr^{-1}$.

2.15 Light treatments of whole cells

Chlamydomonas cells grown with air bubbling and stirring in HSM medium until their middle or late exponential phase (OD_{750} of 0.5-1.5) were harvested by centrifugation at 2000 rpm for 10 minutes at room temperature in a Chillspin benchtop centrifuge. Cells were washed once with fresh HSM medium and resuspended in HSM to a chlorophyll concentration of 25 μg/ml. The cultures were then placed in flat glass dishes at 25°C, stirred and subjected to heat-filtered low light of 200 $\mu E.m^{-2}.s^{-1}$ or high light illumination of either 1000 or 2000 $\mu E.m^{-2}.s^{-1}$ (provided by an apparatus equipped with a 1 kW halogen lamp). Chloramphenicol (CAP, Sigma) at 200 μg/ml was then added to some of the samples to inhibit chloroplast protein synthesis. 1-2 ml samples were taken at set intervals during a time course of 4-5 hours and light-saturated oxygen evolution was measured (see section 2.14).

2.16 Fluorescence measurements of whole cells

The fluorescence induction characteristics of whole cells were measured in the laboratory of I. Vass at the Biological Research Centre, Szeged, Hungary. Cells were grown

and prepared for the measurements as described in section 2.17. Fluorescence induction was measured with a pulse amplitude modulated fluorometer (PAM 101, Walz) for up to 3 s, in the absence or presence of 10 μM DCMU.

Fluorescence changes related to state transitions were monitored at Imperial College according to the following procedure: *Chlamydomonas* cells grown with air bubbling and stirring in HSM medium until late exponential phase, were harvested by centrifugation at 2000 rpm for 10 minutes in a Chillspin benchtop centrifuge, at 20°C, and resuspended in the same medium at a chlorophyll concentration of 50 μg/ml. Cells were kept with gentle agitation under room lights (approximately 5 $\mu E.m^{-2}.s^{-1}$) before the fluorescence measurements. Chlorophyll fluorescence yield was measured with a pulse amplitude modulated fluorometer (PAM 101, Walz). Samples were enclosed, at a volume of 0.75 ml, in a KS-101 cuvette assembly and stirred by a mirrored stirring magnet. Before measuring the fluorescence yield, cells were incubated for 10 minutes under one of the four following conditions: (a) blue light (with an incident light intensity of ~79 $\mu E.m^{-2}.s^{-1}$) provided by a Flexilux 650 lamp (position I) equipped with a Corning 4-96 blue filter and a 25% neutral density (ND) filter; (b) red light (with an incident light intensity of ~4 $\mu E.m^{-2}.s^{-1}$) provided by a Flexilux 150HL lamp (position I) equipped with a red (706) interference filter; (c) in the dark and (d) dark, anaerobic obtained by the addition of 13 mM glucose and 7 μg/ml glucose oxidase. After this 10 minute preincubation period, the measuring pulse was turned on (100 kHz frequency) in order to measure the F_0 value. To measure the F_m value, pulses of saturating white light (with a duration of 500 ms) were given every 20 s. This light was provided by an Intralux (150 W) light source equipped with a Uniblitz SD-10 shutter drive timer.

Measurements of the decay of variable fluorescence in whole cells were carried out in the laboratory of B. Diner, at E. I. du Pont de Nemours, USA. These measurements were performed according to Nixon and Diner (1992). Whole cells were preincubated for 10 minutes in TAP medium containing 0.3 mM $K_3Fe(CN)_6$, 0.3 mM *p*-benzoquinone and 50 mM HEPES-NaOH, pH 7.5 before the beginning of the measurements.

2.17 Thermoluminescence measurements

Measurements of the thermoluminescence characteristics in whole cells of wild type and transformant strains were performed in the laboratory of I. Vass at the Biological Research Centre, Szeged, Hungary, using a home-built apparatus (Vass *et al.*, 1981). Cells, grown in TAP medium at 25°C and at an incident light intensity of 10 $\mu E.m^{-2}.s^{-1}$, were harvested by gentle filtration through glass microfibre filters (Whatman, GF/C, 25 mm diameter) to a chlorophyll content of 50 μg/filter. 400 μl of fresh TAP medium were added onto the layer of the filtered cells and samples were dark adapted for 3 minutes at 20°C in

the absence or presence of 10μM DCMU. The samples were then excited by a single saturating flash at -10°C which was followed by a fast cooling to -40°C.

2.18 Pulse-chase labelling of whole cells using [^{14}C]-acetate

Cells grown in TAP medium with air bubbling and stirring (either in the light or in the dark) until the early or middle exponential phase (OD_{750} of 0.2-0.5), were harvested by low speed centrifugation at 1500 rpm for 10 minutes at room temperature, in a Chillspin benchtop centrifuge. Cells were then washed twice in HSM medium and resuspended in HSM to a chlorophyll concentration of 25 μg/ml. As cells had been grown in an acetate-rich (TAP) medium, they were depleted of acetate by incubating them in HSM for 1 hour at an incident light intensity of 100 $\mu E.m^{-2}.s^{-1}$ and at 25°C with continuous stirring, prior to the labelling. 10 minutes before the addition of the label, the cytoplasmic protein synthesis inhibitor cycloheximide was added (at 10 μg/ml). [1-^{14}C]-sodium acetate (56 mCi/mmol specific activity, Amersham) was then added, at either 5 μCi/ml or at 2 μCi/ml and the incubation was continued for 5 minutes or 45 minutes, respectively. The reaction was stopped by the addition to the cell suspension of an equal volume of 50 mM cold sodium acetate.

For the chase, the labelled cells were washed twice in HSM containing 25 mM cold sodium acetate, to deplete them of the radioactive label and then resuspended in the same medium, also containing 10 μg/ml cycloheximide. The cell suspension was then incubated under the same conditions (100 $\mu E.m^{-2}.s^{-1}$, 25°C) for 90 more minutes. 10 ml aliquots were withdrawn at 0, 45 and 90 minutes and were either frozen in liquid nitrogen or were further processed for the preparation of crude thylakoid membranes (section 2.21.1).

2.19 *in vivo* [^{32}P]-labelling of thylakoid membrane proteins

[^{32}P]-labelling of *Chlamydomonas* whole cells was performed according to the method of de Vitry *et al.* (1991). 5 litres of cells were grown in TAP medium at room temperature, with continuous air bubbling and stirring, until late exponential phase. The culture was then concentrated by centrifugation at 700xg (KONTRON A 6.9 rotor) for 5 minutes at 4°C. The pelleted cells were resuspended in 50 ml of phosphate-depleted TAP and 1 mCi of [^{32}P]-orthophosphate in a volume of 100 μl was added. The culture was incubated with the label for 16 hours at room temperature under low light.

Thylakoid membranes were isolated according to the method described in section 2.21.2 except that all buffers contained 20 mM NaF, to inhibit phosphatases. PS II reaction centre (RC) particles were prepared as described in section 2.24. All buffers until the stage of the column wash, contained 20 mM NaF. Thylakoids and PS II RCs were flash frozen in liquid N_2 and kept at -80°C. Proteins were fractionated and gels stained as described in

section 2.12. Gels and blots were autoradiographed using a Phosphor Imager from Molecular Dynamics connected to a PC equipped with the Image Quant™ software.

2.20 *in vitro* [^{32}P]-labelling of thylakoid membrane proteins

Thylakoid membranes prepared as described in section 2.21.2, were spun down at 9000 rpm in a JA20 rotor (Beckman) for 10 minutes at 4°C. The pelleted membranes were resuspended in labelling buffer (100 mM sucrose, 50 mM HEPES/KOH pH 8.0, 10 mM $MgCl_2$, 0.2 mM ATP, 10 mM NaF) at a chlorophyll concentration of 0.2 mg/mL. 10 µCi of [γ-^{32}P]-ATP (specific activity ~3000 Ci/mmol, Amersham) for every 100 µg of chlorophyll were added and the suspension was incubated for 15 minutes at 50-70 $\mu E.m^{-2}.s^{-1}$ of heat-filtered light, at 25°C. The reaction was stopped by centrifugation at 9000 rpm for 10 minutes at 4°C. The labelled membranes were resuspended in MAGIC buffer (see section 2.21.2) containing 10 mM NaF, flash-frozen in liquid nitrogen and stored at -80°C.

2.21 Preparation of thylakoid membranes

2.21.1 Crude thylakoid membrane preparation

Thylakoid membranes were isolated from *Chlamydomonas* using a slight modification of a method developed by J. Komenda for *Synechocystis* sp. PCC 6803 cells (Komenda and Barber, 1995). The isolation procedure was carried out on ice in the cold room. Cells containing 250 µg of chlorophyll in a volume of 10 ml were pelleted by centrifugation at 3500 rpm for 10 minutes at 4°C in a Chillspin benchtop centrifuge. The cells were then washed once in 2 ml of buffer A (50 mM MES/NaOH pH 6.5, containing 5 mM EDTA, 1 mM phenylmethanesulphonyl fluoride (PMSF), 2 mM aminocaproic acid and 1 mM benzamidine), centrifuged again in a microfuge and resuspended in 0.5 ml of buffer A. The sample was then transferred to a 2.0 ml microfuge tube (cat. no. 15610, Bioquote Ltd.) containing 0.5 ml of glass beads (710-1180 microns, Sigma) and the cells were broken by vortexing (using a Whirlimixer, Fisons) twice for 2 minutes with 1 minute interruption for cooling on ice and to allow glass beads to settle out. Unbroken cells, cell debris and thylakoid membranes were transferred to a 2.0 ml eppendorf tube and the glass beads were washed three times with 0.5 ml of buffer A. The decanted material was then centrifuged at low speed in a microfuge for 30 seconds to pellet unbroken cells, cell debris and remaining glass beads. The supernantant was transferred to another 2.0 ml eppendorf tube and centrifuged in a microfuge at high speed for 10 minutes in order to pellet the thylakoid membranes. The latter were finally resuspended in 50 µl of MAGIC buffer (see section 2.21.2), flash frozen in liquid nitrogen and stored at -80°C.

2.21.2 Pure thylakoid membrane preparation

Pure thylakoid membranes (used for the isolation of PS II particles, PS II RCs and for *in vitro* [^{32}P]-labelling experiments) were prepared from *Chlamydomonas* cells according to the method of Diner and Wollman (1980). The amounts of buffers in the procedure described below are for 20 litres of cells.

Chlamydomonas cells (in a volume of 20 litres) were grown in TAP medium at room temperature with air bubbling, until late exponential phase. The culture was concentrated to 1.5 litres in a Millipore Cell Harvester and then centrifuged at 700xg (KONTRON A 6.9 rotor) for 5 minutes at 4^{0}C. The pelleted cells were then washed once in 250 ml of buffer A (25 mM HEPES/KOH pH 7.5, 1mM $MgCl_2$, 0.3 M sucrose), resuspended in another 120 ml of buffer A and broken in a French Press (Aminco) at 27.56 mPa (4000 lb/in^2). The suspension was centrifuged at 48000xg for 45 minutes at 4^{0}C, the pellets resuspended in 240 ml of buffer B (5 mM HEPES/KOH pH 7.5, 10 mM EDTA, 0.3 M sucrose) and centrifuged again at 48000xg for another 45 minutes. The pellets were then resuspended by homogenisation in a volume of Buffer C (5 mM HEPES/KOH pH 7.5, 10 mM EDTA, 2.2 M sucrose) sufficient to bring the concentration of sucrose to 1.75 M. A discontinuous sucrose gradient was then prepared by half-filling the centrifuge tubes with the suspension and then overlaying with 10 ml buffer D (5 mM HEPES/KOH pH 7.5, 0.5 M sucrose). The gradient was centrifuged at 100000xg for 1 hour at 4^{0}C in a KONTRON TST 28.38/17 rotor. After this centrifugation, thylakoid membranes floated on the 1.75 M sucrose layer, whereas unbroken cells, nuclei, cell wall materials, pyrenoids and starch granules were pelleted at the bottom. The 0.5 M sucrose layer was removed and the photosynthetic membranes were collected by aspiration, diluted with 5 volumes of buffer E (20 mM MES/pH 6.3, 5 mM $MgCl_2$, 15 mM NaCl, 10% glycerol) and pelleted by centrifugation at 48000xg for 20 minutes. The pelleted membranes were resuspended in MAGIC buffer (20 mM MES/pH 6.3, 5 mM $MgCl_2$, 15 mM NaCl and 10% glycerol) and stored at -80^{0}C.

2.22 Immunoprecipitations

30 µl of Protein A covalently bound to a Sepharose matrix (Protein A-Sepharose beads CL-4B, Pharmacia) were mixed with 1 µl of antiserum in a total volume of 100 µl in binding buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet-P40) (Mullet *et al.*, 1990). The entire procedure was performed in 0.5 ml eppendorf tubes. The mixture was incubated for 45 minutes at room temperature in order to allow the F_c region of the antibodies to bind to Protein A. After this incubation period, excess antiserum not bound to the Protein A matrix was removed by low speed centrifugation in a microfuge for 2 minutes and decanting off the supernatant. An equal volume of binding buffer was then added and this step was

repeated three times. After the third wash the supernatant was drawn off using a 2 ml syringe and needle.

SDS to a concentration of 2% was added to thylakoid membranes containing 20-25 µg chlorophyll (prepared as described in section 2.21.2 and labelled with [γ-^{32}P]-ATP) and proteins were solubilised by heating at 100°C for 2 minutes. The suspension was then diluted 10-fold with binding buffer and unsolubilised material was removed by high speed centrifugation in a microfuge for 5 minutes. The supernatant was then added to the antibody-Protein A complexes and the mixture was incubated at 4°C overnight on a rotating wheel. Thylakoid proteins not bound to antibodies were washed off by adding 300 µl of binding buffer, leaving the Protein A-antibody-thylakoid protein complexes to move to the bottom of the tube and decanting the supernatant. This procedure was repeated three times and after the third wash the supernatant was removed by a 2 ml syringe and needle. Proteins were detached from the matrix and solubilised by adding 20 µl of gel solubilisation buffer (section 2.12) and heating the mixture for 10 minutes at 85°C.

2.23 Preparation of PS II core complexes

The procedure for the preparation of photosystem II core complexes was based on the method of Bumann and Oesterhelt (1994). *Chlamydomonas* cells grown in 20 L of TAP medium with air bubbling at room temperature until late exponential phase, were harvested using a Millipore Cell Harvester. Cells were centrifuged at 700xg (KONTRON A 6.9 rotor) for 5 minutes at 4°C. The pelleted cells were then washed once in 240 ml of buffer AC (20 mM MES, 20 mM NaCl, pH 6.0), resuspended in another 100 ml of buffer AC and broken in a French Press (Aminco) at 27.56 mPa (4000 lb/in^2). The suspension was diluted to 240 ml with buffer AC and centrifuged for 1 hr at 130000xg (Beckman Ti70 rotor) and at 4°C. The resulting pellet was resuspended in buffer AC to a chlorophyll concentration of approximately 1 mg/ml. The suspension could be flash-frozen in liquid nitrogen and stored at -80°C at that stage. The thylakoid membranes were solubilised by adding n-dodecyl-β-D-maltoside (DM) at a detergent to chlorophyll ratio of 9:1, and stirring the suspension for 15 minutes at 4°C. The sample was then centrifuged for 10 minutes in a JA20 rotor (Beckman) at 12100xg (10000 rpm) and at 4°C. 0.235 volumes of a 0.5 M DM stock was added to the supernatant and mixed thoroughly by homogenisation at 4°C. The suspension was then overlaid on sucrose gradients, in aliquots containing approximately 1 mg of chlorophyll. The gradients consisted of 27 ml of 20-25% sucrose (w/w) in buffer BC (20 mM MES, 10 mM NaCl pH 6.0 and 0.03% DM). At the bottom of the tube, 3 ml of 60% sucrose (w/w) were added, to prevent sedimenting material from adhering to the tube wall. The gradients were centrifuged for at least 15 hours at 110000xg at 4°C in a fixed angle rotor (Ti70, Beckman). Bands corresponding to different PS II-

containing fractions were collected using a syringe and long needle, flash-frozen in liquid nitrogen and stored at -80°C.

2.24 Isolation of PS II reaction centre (RC) particles

For the isolation of PS II reaction centre (RC) particles, the method of S. Alizadeh (Alizadeh *et al.*, 1995) was followed:

Thylakoid membranes, prepared as described in section 2.21.2, were diluted in 2 volumes of 50 mM Tris pH 9.5, homogenised, and incubated on ice for 15 minutes. After a centrifugation step of 20 min at 48000xg, membranes were resuspended in 50 mM Tris pH 7.2 containing the detergent Triton X-100 (at concentrations ranging from 1.5 to 2.3 % (w/w)) and at a ratio of Triton X-100 to chlorophyll of 50. The suspension was left stirring at 4°C in the dark for 2 to 2.5 hours and then centrifuged at 48000xg for 1.5 hours. After this step, the supernatant was loaded on a Fractogel TSK DEAE-650 (S) (MERCK-BDH) column which had been previously equilibrated with a buffer containing 0.2% Triton X-100, 50 mM Tris pH 7.2 and 1mM n-dodecyl-β-D-maltoside (DM). The size of the column was either 20 cm x 1.5 cm (for suspensions containing >15 mg of chlorophyll) or 10 cm x 1 cm (for suspensions containing <15 mg of chlorophyll). After loading the sample (at a flow rate of 1 ml. min^{-1}), the column was washed with 20 mM of NaCl in the running buffer, until the absorbance of the eluate at 670 nm was < 0.1. A linear gradient of 20-150 mM NaCl was then applied, with a flow rate of 1 $ml.min^{-1}$, and 1 ml fractions were collected. Absorption spectra of the different fractions from 750 nm to 370 nm were taken at 10°C in a Shimadzu MPS-2000 spectrophotometer and analysed for the maximum absorbance at the red peak, the ratio of absorbance at 417 and 435 nm and the wavelength of the maximum absorbance in the red region. Fractions containing particles resembling a RC were pooled together, diluted in 4 volumes of 50 mM Tris pH 7.2 and 1.4 mM DM, and loaded (flow rate of 1 ml. min^{-1}) on a second Fractogel DEAE column (10 cm x 1 cm) which had been previously equilibrated with the running buffer. That way the sample would be concentrated and also exchanged into a milder detergent, such as DM. The latter step was necessary since Triton X-100 influences the spectral properties of PS II RCs (Seibert *et al.*, 1988; Seibert, 1993) as well as their stability (Chapman *et al.*, 1988; McTavish *et al.*, 1989). The sample was then eluted from the column directly using a step gradient of 200 mM NaCl (flow rate of 0.5 ml. min^{-1}) in running buffer or an intermediate wash with 30 mM NaCl (in running buffer) was carried out, in cases where further purification was necessary.

All steps were carried out at 4°C in the dark and the sample was stored at -80°C until use. The conditions used in trial experiments are shown in Table 4.2.

2.25 Quantitation of the relative amounts of Chl *a*, Pheo *a* and Cyt b_{559}

The ratio of Chl *a* to Pheo *a* was estimated spectrophotometrically in a Shimadzu MPS 2000 spectrophotometer using a modification of the method of Montoya *et al.* (1991). An aliquot of the sample obtained from the RC preparation, equivalent to 2-3 μg of chlorophyll, was mixed with 80% acetone and total pigment was extracted after 3 minutes centrifugation. The region between 580 nm and 500 nm was scanned 4 times consecutively and the absorption peak at 535 nm was measured. 5 μl of 1 M HCl were then added and the spectrum of the pheophytinised extract from 580 nm to 500 nm was taken, after 2 and 5 minutes. This procedure was repeated 2-3 times and again the absorption peak at 535 nm was measured. The values of the absorption maxima at 535 nm, before and after pheophytinisation, were compared in order to determine the ratio between Chl *a* and Pheo *a*, in the sample.

The relative amounts of Cyt b_{559} in the RC preparations were estimated as follows:

A spectrum of the sample, containing ≤2 μg of chlorophyll, was initially taken in order to calculate the concentration of chlorophylls. The region between 580nm and 500nm was then taken as a baseline followed by the addition of 1-2 mM ferricyanide to further oxidise the Cyt b_{559} present in the sample. Then, a few mg of dithionite were added, and a spectrum between 580 nm and 500 nm was taken. The amount of Cyt b_{559} per Chl *a* was calculated from dithionite-reduced minus ferricyanide-oxidised difference absorption spectra, using an extinction coefficient of 23.4 $mM^{-1}.cm^{-1}$ at 559 nm (Miyazaki *et al.*, 1989) and an extinction coefficient of 103 $mM^{-1}.cm^{-1}$ for Chl *a* at the red peak of the isolated PS II RC (De Las Rivas *et al.*, 1993).

2.26 Measurements of $Pheo^-$ and $P680^+$ photoaccumulation

Light-induced absorbance changes in RC particles were measured according to Barber *et al.* (1987) and Telfer *et al.* (1990), in a Perkin-Elmer 554 spectrophotometer equipped with a 150 W tungsten lamp for side illumination and set with a band width of 2 nm. For wavelengths below 600 nm the light was passed through a Schott RG 665 glass cut-off filter and above 650 nm through a Schott BG 18 filter. The light intensities at the surface of the cuvette were 1150 $\mu E.m^{-2}.s^{-1}$ in the case of the red filter and 750 $\mu E.m^{-2}.s^{-1}$ for the blue filter. The photomultiplier was protected from scattered actinic light by suitable cut-off filters. The optical pathlength was 10 mm and the cuvette volume was 1 ml. All measurements were carried out at 9°C in the presence of DM, to maintain maximum stability of the samples during the treatments and to prevent protein aggregation (De Las Rivas *et al.*, 1993). Chlorophyll concentrations of the samples were as described in the figure legends.

The photoaccumulation of reduced pheophytin was measured in the presence of sodium dithionite (Sigma) in 50 mM MES pH 6.5 and 2 mM DM. In order to account for any loss of activity of the sample during the course of the measurements, signals observed at any

wavelength were normalised to the signal size at 450 nm, which was measured every 4-5 flashes. By using an extinction coefficient of 24.2 $mM^{-1}.cm^{-1}$ for reduced pheophytin at 450 nm and an extinction coefficient of 103 $mM^{-1}.cm^{-1}$ for Chl *a* at the red peak (for a 6 chlorophyll RC, De Las Rivas *et al.*, 1993), the ratio of chlorophyll to $Pheo^-$ was calculated.

The photoaccumulation of $P680^+$ was measured in the presence of 400-1000 $\mu g.ml^{-1}$ silicomolybdate (PFALTZ & BAUER) in 50 mM Borate pH 8.0 and 2 mM DM. Anaerobic conditions were obtained by adding 0.1 $mg.ml^{-1}$ each of glucose oxidase and catalase plus 5 mM glucose and incubating for 5 minutes before measurements (De Las Rivas *et al.*, 1993). The sample was initially preilluminated for 1 minute in order to bleach β-carotene (Telfer *et al.*, 1990). The light-minus-dark absorbance spectra were taken using 4 second flashes of blue light. A fresh aliquot of silicomolybdate was added every 5 flashes to compensate for the instability of this compound at neutral pH (Schansker and van Rensen, 1992). The signal size at each wavelength was normalised to the signal observed at 680 nm which was measured every 5 flashes. The extinction coefficient used for $P680^+$-P680 was 75 $mM^{-1}.cm^{-1}$ (van Gorkom *et al.*, 1974) at 680 nm.

2.27 Femtosecond measurements

All femtosecond transient absorption measurements and data analyses were carried out according to Hastings *et al.* (1992), Rech *et al.* (1994) and Giorgi *et al.* (1994). These measurements were conducted by members of the Molecular Dynamics Group, Imperial College.

For the purpose of these experiments, the PS II RC particles were resuspended in a buffer containing 20 mM Bis-Tris pH 7.2, 20 mM NaCl, 10 mM $MgCl_2$ and 1.4 mM dodecyl maltoside. Measurements were conducted at 25°C, under anaerobic conditions by adding 5 mM glucose, 0.1 $mg.ml^{-1}$ glucose oxidase and 0.5 $mg.ml^{-1}$ catalase. Samples contained approximately 30 μg of chlorophyll and were mounted in a cylindrical cuvette (path length 2.5 mm) which was rotated in order to exchange the excited sample volume between excitation pulses. The time resolution of the spectrometer was approximately 150 fs. Data were collected on either three timescales (0-12 ps, 0-70 ps and 0-300 ps), for measurements in the Q_x absorbance region or on a 0-70 ps time scale for measurements in the Q_y region and using 694 nm excitation pulses to excite P680 preferentially.

3. Construction and Genetic Analysis of Site-Directed Mutants

3.1 Introduction

Site-directed mutagenesis is being extensively used for the study of structure-function relationships in PS II (Diner *et al.*, 1991; Nixon *et al.*, 1992a; Pakrasi, 1995; Pakrasi and Vermaas, 1992; Vermaas, 1993; Vermaas *et al.*, 1993; Webber *et al.*, 1995). In this method, a defined mutation, such as the addition, deletion or substitution of specific nucleotides, is incorporated into the target gene *in vitro*. The effect of the mutation is usually analysed by returning the modified gene to the organism from which it was obtained.

This technique has been used for the creation of the D2 mutants described in this work. The basic steps followed for the construction of these mutants can be summarised as follows: First, the segment of DNA to be mutagenised (namely, the *psbD* gene and flanking regions), was cloned in a plasmid vector suitable for *in vitro* mutagenesis. Then, specific changes of interest were introduced into the cloned gene by means of an *in vitro* mutagenesis procedure. The next step was the introduction of the modified DNA segment into the chloroplast of *Chlamydomonas reinhardtii* and its integration into the chloroplast genome by homologous recombination. The final step included the selection and identification of transformant lines that had the mutated gene incorporated into their chloroplast genomes.

3.1.1 Nuclear and chloroplast transformation in Chlamydomonas reinhardtii

3.1.1.1 Nuclear transformation

Transformation of the nuclear genome was first reported by Rochaix and van Dillewijn (1982) and involved complementation of the *arg-7* mutant of *C. reinhardtii* with the *ARG4* gene from yeast. However, this result did not prove to be reproducible. Moreover, this report did not demonstrate that the introduced DNA was stably expressed in the transformed cells. Consistent results were only obtained when *Chlamydomonas* genes were used as selectable markers and new and reliable methods for an efficient nuclear transformation system were developed (reviewed in Kindle and Sodeinde, 1994; Rochaix, 1995). Thus, Debuchy *et al.* (1989) and Mayfield and Kindle (1990) reported the nuclear transformation of *C. reinhardtii* using a particle gun. Debuchy *et al.* (1989) used the mutant *arg-7* as recipient strain and the *ARG7* gene, which encodes the enzyme argininosuccinate

lyase (ASL), as a selectable marker. Transformants were able to grow on medium without added arginine. Mayfield and Kindle (1990) introduced the *psbO* gene, which encodes the oxygen-evolving enhancer protein 1 (OEE1 or the 33 kDa extrinsic protein), to an OEE1-deficient, non-photosynthetic, acetate-requiring mutant. Transformants recovered photosynthetic competence and were able to grow in the absence of acetate. Kindle (1990) reported the nuclear transformation of *C. reinhardtii* by agitating the recipient cells with glass beads. The author transformed mutants containing the *nit1-305* mutation with two plasmids: one containing the wild-type *nit1* gene (which encodes the enzyme nitrate reductase) and the second containing an unselected gene. Transformants were selected by growth with nitrate as the sole nitrogen source. The unselected gene was found to be present in 10-50% of these nit^+ transformants.

DNA has also been introduced into the nucleus of *C. reinhardtii* using electroporation and agitation with silicon carbide whiskers (reviewed in Kindle and Sodeinde, 1994). Of all these methods, glass beads transformation has become the most widespread because of its simplicity and efficiency. In this method, transformation is achieved by vortexing cells in the presence of glass beads, polyethylene glycol and DNA. However, one major drawback of this method is the fact that cell wall-deficient strains have to be used as recipients (Kindle and Sodeinde, 1994).

The development of methods for nuclear transformation has been delayed by the finding that most transforming DNA integrates randomly at non-homologous sites in the nuclear genome. Transformants generated by particle bombardment may contain 5 to more than 20 copies of the introduced DNA whereas the copy number of transforming DNA in the glass beads method is usually significantly lower (1-3 copies) (Kindle, 1990). However, recently two groups demonstrated the occurence of homologous recombination during nuclear transformation. Sodeinde and Kindle (1993) succeeded in modifying the endogenous *nit1* gene by gene targeting. The authors also found that the nature and frequency of the homologous recombination events depended on the method of transformation. Thus, the glass beads method resulted in a very low number of homologous recombination/gene conversion events relative to non-homologous events. On the other hand, transformation using a particle gun was less efficient in producing nit^+ transformants than the glass beads method, but the number of homologous recombination events compared to random insertions was higher. Gumpel *et al.* (1994) using the glass bead transformation method, reported homologous recombination between two introduced plasmids sharing common *ARG7* sequences and also that the amount of common sequence required for recombination between the two molecules is not more than 230 bp. However, they did not succeed in demonstrating conclusive evidence of recombination between the introduced DNA and the endogenous *ARG7* gene. Using a similar transformation system, Farah *et al.* (1995) isolated a *psaF*-deficient mutant of *C. reinhardtii* by transforming a cw15-arg7A strain with two plasmids, one harboring a mutated version of the *psaF* gene and the other containing the *ARG7* gene as the selectable marker. Finally,

Nelson and Lefebvre (1995) reported the targeted gene disruption of the *NIT8* gene (which encodes a protein necessary for nitrate and nitrite assimilation by *C. reinhardtii*) by placing the *CRY1-1* selectable marker gene (which confers resistance to emetine) within the *NIT8* coding region.

3.1.1.2 Chloroplast transformation

The development of chloroplast transformation and its use in the study of chloroplast gene expression and function of chloroplast proteins, is reviewed in Boynton and Gillham (1993), Kindle and Sodeinde (1994), Webber *et al.* (1995) and Rochaix (1995). The first report of chloroplast transformation in *C. reinhardtii* was by Boynton *et al.* (1988). The recipient strain in this system was the non-photosynthetic mutant, *ac-u-c-2-21* (CC373), which had a 2.5 kb deletion affecting the 3' half of the chloroplast *atpB* gene. The donor DNA was a 7.6 kb chloroplast fragment cloned in pBR313 that carried the wild type *atpB* gene and had homology to sequences located on either side of the deletion in the recipient genome. In this procedure, DNA containing the wild type *atpB* gene was precipitated onto the surface of tungsten particles and introduced into *Chlamydomonas* cells by a gun powder discharge. Transformants were selected for restored photosynthesis by plating on HSM (minimal) medium.

DNA introduced in this way into the chloroplast of *C. reinhardtii* integrates into the chloroplast genome by homologous recombination, by a mechanism that probably involves either a double-exchange or a gene conversion event between the chloroplast sequences in the donor DNA and corresponding sequences in the recipient chloroplast genome (Boynton *et al.*, 1988; Newman *et al.*, 1990). Newman *et al.* (1990) have also reported that these exchange events almost always occur near the ends of the chloroplast donor insert, with about 90% of the events occuring within 850 bp of the vector-insert junctions.

Agitation with glass beads has been used for chloroplast transformation of a cell wall-less mutant of *C. reinhardtii*. However, in contrast to the situation for nuclear transformation, this technique was substantially less efficient than the bombardment method (Kindle *et al.*, 1991).

Two types of selectable markers are mainly been used for the selection of putative transformants. The first one is the 7.0 kb Bam11 chloroplast fragment from *C. reinhardtii* strain CC227 carrying the entire 16S rRNA gene with mutations conferring resistance to streptomycin and spectinomycin and the 5' half of the 23S rRNA with a mutation conferring resistance to erythromycin (Newman *et al.*, 1990). The second one is the *aadA* expression cassette which when expressed into the chloroplast of *C. reinhardtii* confers resistance to spectinomycin (Goldschmidt-Clermont, 1991). The first marker is usually used in conjuction with a cotransformation strategy for the introduction of genes with an unselectable phenotype. Thus, the gene of interest is introduced into the chloroplast together with a

separate plasmid containing the mutated rDNA genes. The success of this procedure relies on both fragments integrating into the chloroplast genome of the same cell. In order to increase the frequency of cotransformation, cells are usually grown in the presence of the chloroplast DNA synthesis inhibitor 5-fluorodeoxyuridine before transformation (Boynton *et al.*, 1988; Newman *et al.*, 1990). The latter reduces the chloroplast genome copy number by 8-10 fold (Hosler, 1989) and therefore, increases the probability of cointegration of the two transforming fragments into the same copy of the chloroplast genome (Newman *et al.*, 1990). However, there are two potential drawbacks associated with this method. Because of its ability to inhibit chloroplast DNA synthesis, 5-fluorodeoxyuridine has been used in the past as a chloroplast DNA mutagen (Harris, 1989). Therefore, transformants arising from this transformation system should always be analysed carefully for the existence of secondary mutations induced by 5-fluorodeoxyuridine. Moreover, the mutations in the rDNA genes leading to antibiotic resistance, have also been reported to impair the rate of chloroplast protein synthesis (Heifetz *et al.*, 1992). Photosystem II has been considered to rely on high rates of synthesis of the D1 protein to repair photodamage (reviewed in Prasil *et al.*, 1992; Aro *et al.*, 1993; Ohad *et al.*, 1994). Therefore, the ribosomal mutations introduced into the chloroplast genome of *C. reinhardtii* could render the cells more sensitive to photoinhibition, making the analysis of the photosynthetic phenotype of transformants more difficult (Heifetz *et al.*, 1992).

The second system used for selecting putative transformants after chloroplast transformation, is a chimeric construct comprising the bacterial *aadA* gene, encoding resistance to aminoglucosides such as spectinomycin and streptomycin, flanked by 5′ *atpA* and 3′ *rbcL* regulatory sequences from *C. reinhardtii*. This construct has been engineered as a convenient expression cassette with a set of unique restriction sites at each end (Goldschmidt-Clermont, 1991) (Figure 3.2a). The 5′ *atpA* fragment contains the promoter, the 5′ untranslated region and the sequence encoding the first 15 amino acids of *atpA*. The *rbcL* sequence contains the 3′ untranslated region of *rbcL* that contains an inverted repeat sequence thought to be important for mRNA stability. The *aadA* cassette has been used to disrupt or delete chloroplast genes (Takahashi *et al.*, 1991, 1994; Monod *et al.*, 1994; Künster *et al.*, 1995) and to select for transformants containing site-directed mutations (Takahashi *et al.*, 1992; Cui *et al.*, 1995).

Other endogenous chloroplast genes used as selectable markers include herbicide resistance encoded by a mutant *psbA* gene (Przibilla *et al.*, 1991) and a trans-splicing function encoded by the *tscA* gene (Goldschmidt-Clermont *et al.*, 1991).

The initial chloroplast transformation experiments were carried out using either the particle gun designed by J. Sanford and co-workers (Klein *et al.*, 1987) or devices based on that design. This type of gun uses 22-calibre blank gunpowder charges as an acceleration system and was initially marketed by DuPont as the PDS-1000 particle delivery system. This model has been fitted with a helium accelerator to replace the gunpowder. Moreover,

the tungsten microprojectiles used for DNA delivery have been substituted with gold particles. These design improvements have generally increased the transformation frequencies for chloroplast genes (reviewed in Boynton and Gillham, 1993).

The following sections in this chapter describe the creation and genotypic analysis of the site-directed mutants D2-Leu205Tyr, D2-Thr2Ala and D2-Thr2Ser.

3.2 Results

3.2.1 Creation of site-directed mutants

3.2.1.1 Construction of donor plasmids

The vectors and recombinant plasmids used throughout this work are outlined in Table 2.3. Plasmid pH3 was digested with *Hin*d III (to yield a 7.5 kb fragment containing the *psbD* gene, see Figure 3.1), then partially digested with *Hpa* I to give a 4.7 kb *Hpa* I/*Hin*d III fragment. The plasmid vector pTZ19U (Bio-Rad) was digested with *Hin*d III and *Sma* I and ligated with the 4.7 kb *Hpa* I/*Hin*d III fragment to yield the plasmid pCA1. The purpose of this subcloning step was to reduce the size of the *psbD*-containing fragment so that all the experimental manipulations would be less tedious. Moreover, this would help increase the efficiency of the second strand synthesis reaction, performed during the *in vitro* mutagenesis procedure. pTZ19U was chosen as a vector because it can be used to generate a single-stranded template in the form of a phagemid (see section 2.4).

In order to select for transformed cells, the *aadA* selectable marker was inserted into pCA1, into either a *Nsi* I restriction site 261 bp upstream of the translation initiation codon of *psbD* (so that mutants in a wild type background could be created) or into an *Xba* I restriction site located approximately 300 bp downstream of the stop codon of *psbD* (enabling the creation of mutants with a PS I$^-$ background, which would be useful later for the purification of the PS II reaction centre complex). As can be seen from Figure 3.1, there are two *Xba* I sites downstream of *psbD*, located 295 bp and 308 bp respectively, downstream of the stop codon of *psbD*. Insertion of the *aadA* cassette into these sites (see section 3.2.1.2), should lead to deletion of the 13 bp *Xba* I fragment.

The *aadA* cassette was kindly provided by M. Goldschmidt-Clermont, cloned in the plasmid pUC-atpX-AAD. The latter was digested with *Eco* RV and *Sma* I to yield a 1.9 kb blunt-end fragment containing the spectinomycin-resistance cassette. pCA1 was digested partially with *Nsi* I or to completion with *Xba* I. Ends were made blunt-end with T4 DNA polymerase and ligated with the 1.9 kb *Eco* RV/*Sma* I fragment in order to obtain plasmids pNsi16 and pXba9, respectively (Figure 3.2)

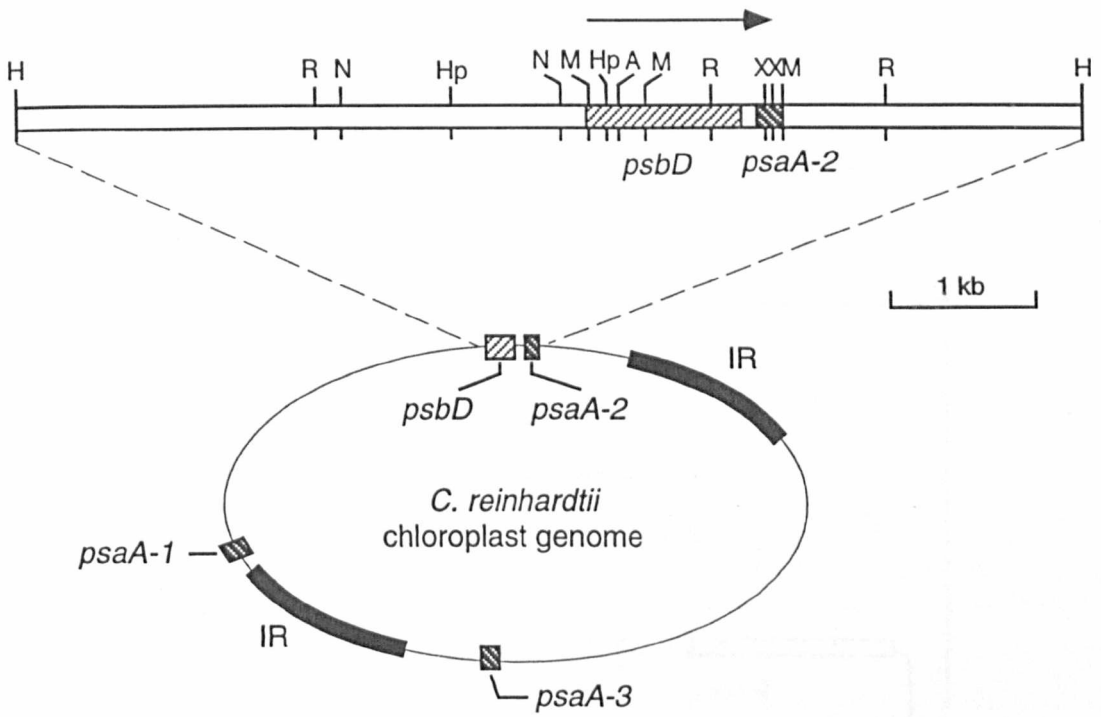


Figure 3.1: Restriction map of the 7.5 kb *Hin*d III fragment containing the *psbD* gene and flanking regions. Relative positions of restriction sites are indicated. The arrow above *psbD* indicates the direction of transcription. Restriction sites are: *Hin*d III (H), *Hpa* I (Hp), *Eco* RI (R), *Nsi* I (N), *Acc* I (A), *Mun* I (M) and *Xba* I (X)

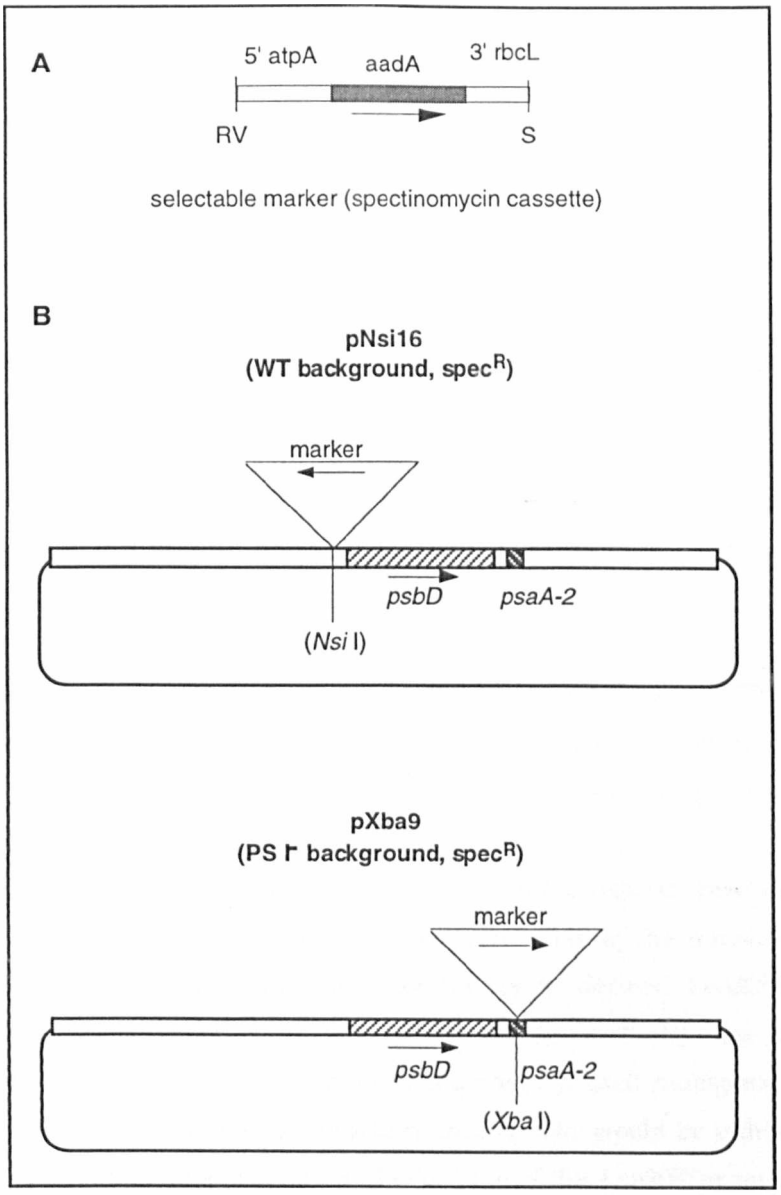


Figure 3.2: Panel A: the *aadA* cassette used as a selectable marker in the transformation experiments. RV: *Eco* RV; S: *Sma* I. Panel B: structure of the plasmids used for chloroplast transformation in *Chlamydomonas* (mutations on *psbD* are not shown). The *Nsi* I and *Xba* I restriction sites are in brackets to indicate that they have been blunt-ended.

3.2.1.2 Site-directed mutagenesis

The *psbD* gene was mutated using Bio-Rad's "Muta-gene" phagemid *in vitro* mutagenesis kit, which is based on a system described by Kunkel *et al.* (1987). This method requires the transformation of an *E. coli* host (CJ236) carrying the *dut⁻ ung⁻* mutations by a plasmid containing the target gene. When DNA is synthesised in such a mutant host, the nascent DNA carries a number of uracils in thymine positions. The *dut⁻* mutation inactivates

the enzyme dUTPase which results in high intracellular levels of dUTP. The *ung*⁻ mutation inactivates uracil N-glycosylase and as a result, incorporated uracil is allowed to remain in the DNA. A uracil-containing strand is then used as the template for the synthesis of a complementary strand (primed by the mutagenic oligo). When the resulting double-stranded DNA is transformed into a cell which does not carry the *ung*⁻ mutation, the uracil-containing strand is inactivated with high efficiency, leaving the non-uracil-containing survivor to replicate. The efficiency of the method is judged by analysis of an aliquot of the second-strand synthesis reaction on an agarose gel and by the appearance of colonies on plates, after transformation into cells that do not carry the *ung*⁻ mutation.

All three different mutations were constructed using this method. The mutagenic oligonucleotides are shown in Table 2.4. Initially, attempts were made to introduce the nucleotide changes specific for the three mutations using both pCA1 and pNsi16 as templates. However, the creation of the Leu205Tyr mutation using pNsi16 as a template has so far not been possible. The inefficiency of the method for this particular combination of template-oligonucleotide could be due to the large size of pNsi16 (approx. 9.4 kb). Thus, the Leu205Tyr mutation was created using pCA1 as template and the selectable marker was inserted into the *Nsi* I and *Xba* I restriction sites at a later stage, before chloroplast transformation.

A portion of the DNA from the second-strand synthesis reaction was used to transform *E. coli* DH5-α cells. Colonies appearing after plating the transformation mixture on selective media (containing ampicillin for the pCA1-derived Leu205Tyr mutant and spectinomycin for the pNsi16-derived Thr2Ala and Thr2Ser mutants) were analysed for the presence of the mutation by restriction analysis (Figure 3.3). Each mutagenic oligonucleotide (Table 2.4) was designed so that a restriction enzyme site would be either introduced or destroyed along with the codon alteration. In the case of the Leu205Tyr mutation, plasmids carrying the desired changes were identified by the presence of a ~480 bp fragment derived after digestion with *Acc* I (Figure 3.3, panel A, lanes 8, 9, 10). This fragment, which has been created by the presence of a new *Acc* I restriction site, is not present in the wild type pCA1 digest (lane 3). Plasmids carrying the specific changes for the Thr2Ala and Thr2Ser mutations were identified by the loss of a 423 bp fragment and the concomitant creation of a ~2.9 kb fragment, after digestion with *Mun* I (Figure 3.3, panel B, lanes 6, 7 and panel C, lanes 1, 3, 4, respectively). The absence of the 423 bp fragment in the mutant plasmids is due to the destruction of the *Mun* I restriction site present in the wild type pNsi16 (panel B, lane 3). Thus, the introduction of the mutation was identified by a restriction pattern which was different from the one obtained by digesting the parental pCA1 and pNsi16 plasmids with the same enzymes.

Clones exhibiting a restriction pattern indicative of the presence of the desired mutation were subjected to sequence analysis using a double-stranded DNA sequencing protocol (Sequenase 2.0, USB). Oligonucleotides used are shown in Table 2.5. For the

Leu205Tyr mutation, oligos C3 and C4 were used to identify the desired nucleotide sequence. Thr2Ala and Thr2Ser mutations were detected using oligo C1. For each mutation, one clone with the correct nucleotide sequence was chosen, and large scale DNA preparations were carried out. As a final confirmation, the whole *psbD* gene was sequenced in these clones, using primers C1-C7, to ensure that unwanted sequence alterations were not present in the gene. After the sequence of the generated mutant plasmids was confirmed, and the selectable marker inserted in the *Nsi* I and *Xba* I restriction sites of the Leu205Tyr-carrying plasmids, the DNA was transformed into the chloroplast genome of *Chlamydomonas*.

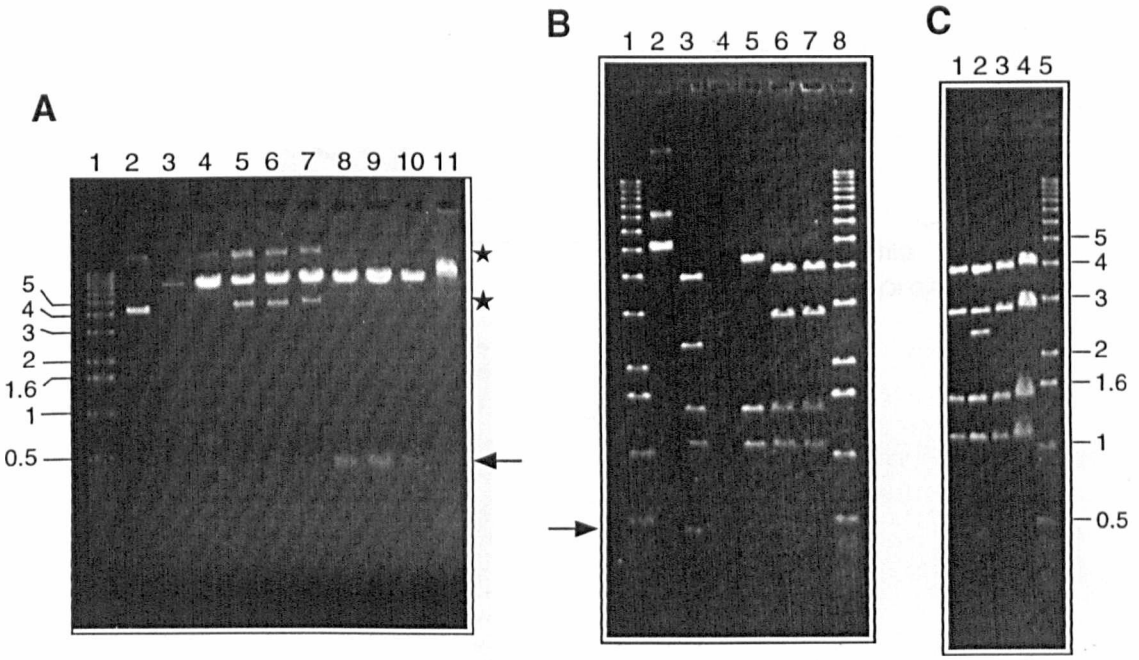


Figure 3.3: Restriction digestion patterns of mutant plasmids. Panel A: *Acc* I digests of pCA1 and pCA1/Leu205Tyr. Lane 1: marker; lane 2: uncut pCA1; lane 3: pCA1 digested with *Acc* I; lanes 4-11: candidate clones of pCA1/Leu205Tyr digested with *Acc* I. Panel B: *Mun* I digests of pNsi16 and pNsi16/Thr2Ala. Lanes 1, 8: marker; lane 2: uncut pNsi16; lane 3: pNsi16 digested with *Mun* I; lanes 5-7: candidate clones of pNsi16/Thr2Ala digested with *Mun* I. Panel C: *Mun* I digests of pNsi16/Thr2Ser. Lanes 1-4: candidate clones of pNsi16/Thr2Ser digested with *Mun* I; lane 5: marker. Arrows indicate either the band appearing in the Leu205Tyr mutants because of the new *Acc* I site (panel A) or the band absent in the Thr2Ala and Thr2Ser mutants because of the loss of the *Mun* I site (panels B and C). Bands indicated with asterisks in lanes 5, 6 and 7 of panel A are conformers of uncut plasmid. The size of some of the bands of the marker are indicated (in kb) in the far left and right of the figure.

3.2.1.3 Transformation of the chloroplast of Chlamydomonas

The experimental system chosen for the introduction of DNA into the chloroplast of *C. reinhardtii* was the biolistic method developed by J. Sanford and co-workers (Klein *et al.*, 1987). In this method, DNA is introduced into the chloroplast by means of a particle gun that delivers DNA-coated microprojectiles into the cell at high velocity.

The particle delivery instrument used in this work, was the gunpowder-driven model Mk2 from Shearline (Cambridge, UK). A schematic representation of this device is shown in Figure 3.4.

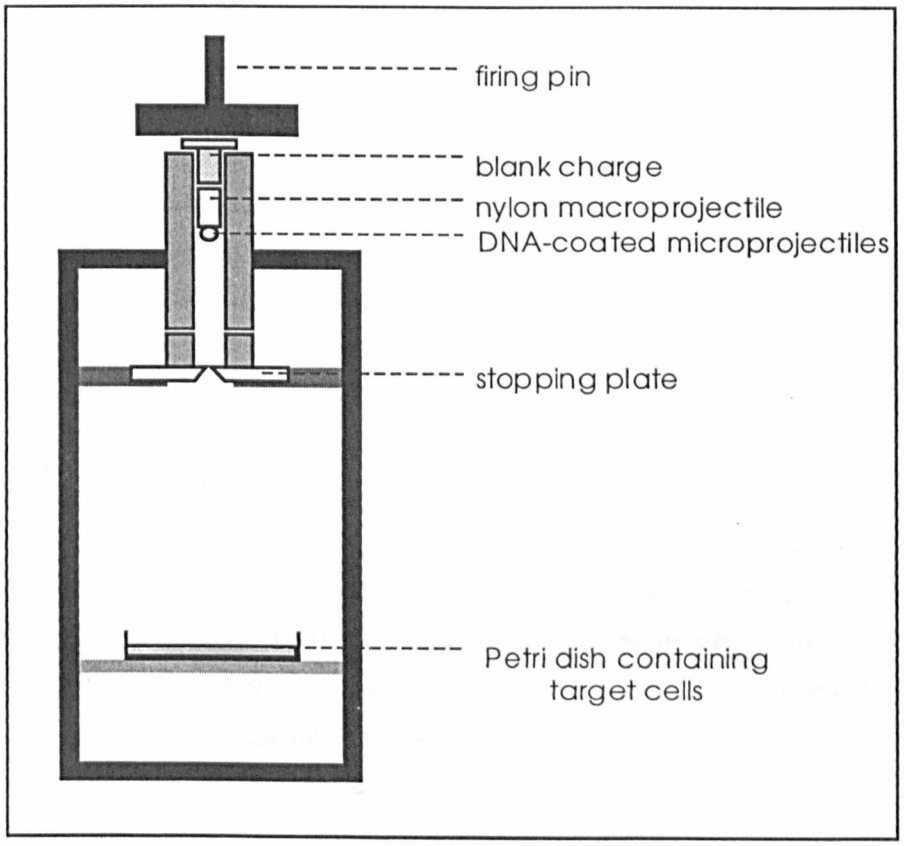


Figure 3.4: Schematic representation of the particle gun used in the transformation experiments

Plasmid DNA carrying the mutated *psbD* gene was precipitated onto the surface of tungsten or gold particles (microprojectiles). DNA-coated microprojectiles were then immediately loaded on the centre of a macroscopic carrier (macroprojectile). *C. reinhardtii* cells (on a petri dish) were placed in the chamber of the gun and finally the cells were bombarded.

After bombardment, cells were transferred onto TAP plates supplemented with spectinomycin and ampicillin until colonies appeared. These colonies where then transferred on plates containing spectinomycin, ampicillin and DCMU (see also section 2.2.2).

Initially, in order to optimise the technique, experiments were carried out using the system described by Boynton *et al.* (1988). This system included the ATP synthase deletion mutant, CC373, as the recipient strain and plasmid p17, which carries a wild type copy of the *atpB* gene, as donor DNA (see section 3.1.1.2). The parameters that were varied between experiments included the concentration at which cells were spread on the plates that were going to be bombarded (approximately 2-13x10^7 cells per plate), the type of plate on which these cells were spread (ordinary TAP plate or supplemented with 0.2 % soft agar), the distance between the stopping plate and the position of the cells in the chamber of the gun (7.4 cm to 16.4 cm) and the time between the bombardment and the respreading of cells on selective medium (1 hour to overnight).

The conditions that were finally chosen for the subsequent transformation experiments were the following: approximately 4-5x10^7 cells in 0.2% soft agar were spread on each target plate 16-20 hours before bombardment. Cells were placed in the chamber of the gun, at a distance of 10.4 cm from the stopping plate. Finally, approximately 3 hours after bombardment, 1.5 ml of TAP was added on each bombarded plate, cells were loosened from soft agar by rubbing the surface of the plate with a glass spreader and then transferred on selective media. Plates were then incubated at room temperature. Colonies usually appeared after 10-15 days.

It should be noted, however, that alteration of the above parameters did not improve noticeably the initial transformation frequencies. Although colonies appeared after almost all experiments, the transformation frequencies were very low (1-3 cells per bombarded plate) compared to the ones reported in the literature. Experiments using gold particles as microprojectiles, carried out during later stages of the project, did not improve the number of transformants obtained.

Before attempting to transform *Chlamydomonas* with plasmids carrying the mutated *psbD* gene, transformation experiments were carried out using pNsi16, as donor DNA, and wild type (CC125) *C. reinhardtii* as the recipient strain. This was done in order to test the efficiency of the technique when using this type of construct and also to examine the effect of the insertion of the *aadA* cassette itself on the expression of the *psbD* gene.

After this intermediate step, wild type *Chlamydomonas* cells were transformed with pXba9 (wild type *psbD*, *aadA* cassette inserted into *psaA-2*; see Figure 3.2) and with the mutant plasmids. Strains that resulted from transformation with pNsi16 and pXba9 were called Nsi16 and Xba9, respectively.

3.2.2 Genetic characterisation

After a few cycles of restreaking on selective media, to allow the introduced changes to be established in all the copies of the chloroplast genome, DNA was isolated for genetic characterisation.

3.2.2.1 Southern blotting of Nsi16

All transformants (mutants plus Nsi16 and Xba9) were initially characterised by Southern blotting. This analysis indicated that the resistance to spectinomycin was derived by the presence of the *aadA* cassette and also that transformants had reached a homoplasmic state. A 610 bp *Hinc* II/*Eco* RI fragment containing codons 107 to 310 of *psbD* was used as a *psbD*-specific probe. The spectinomycin cassette was detected using either the *Sma* I-digested Ω fragment (Prentki and Kirsch, 1984) (provided by Dr. P. Nixon) or an *Nco* I/*Hin*d III fragment containing the *aadA* gene from plasmid pUC-atpX-AAD (Goldschmidt-Clermont, 1991).

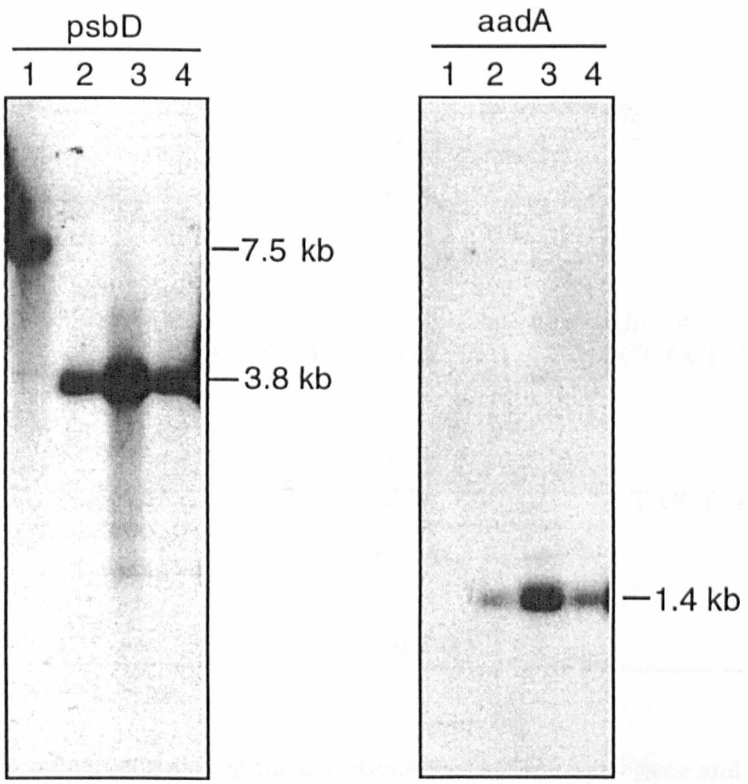


Figure 3.5: Southern blots of total DNA from wild type (lane 1) and three independent clones of transformant Nsi16 (wild type, spec^R) (lanes 2, 3, 4) digested with *Hin*d III and hybridised to either a 0.6 kb *psbD* probe (panel A) or to an *aadA* probe (panel B)

Both chloroplast and total DNA were used for Southern blottings. A variety of different mini-prep methods (eg. Newman *et al.*, 1990; Rochaix *et al.*, 1988) were employed for the isolation of total DNA. However, the DNA obtained could not be digested completely with most restriction endonucleases. The reason for this inefficiency was thought to be the presence of polysaccharides in the DNA preparation. These problems were alleviated when DNA was passed through a Wizard™ miniprep column (Promega) (see also section 2.6).

A southern blot of three clones of Nsi16 (wild type; $spec^R$) transformant is shown in Figure 3.5. The presence of a single hybridising band, around 3.8 kb in panel A, indicates the homoplasmic state of the transformants. The band at 1.4 kb in panel B, shows the presence of the $spec^R$ cassette in all three clones (see restriction map in Figure 3.6, for details).

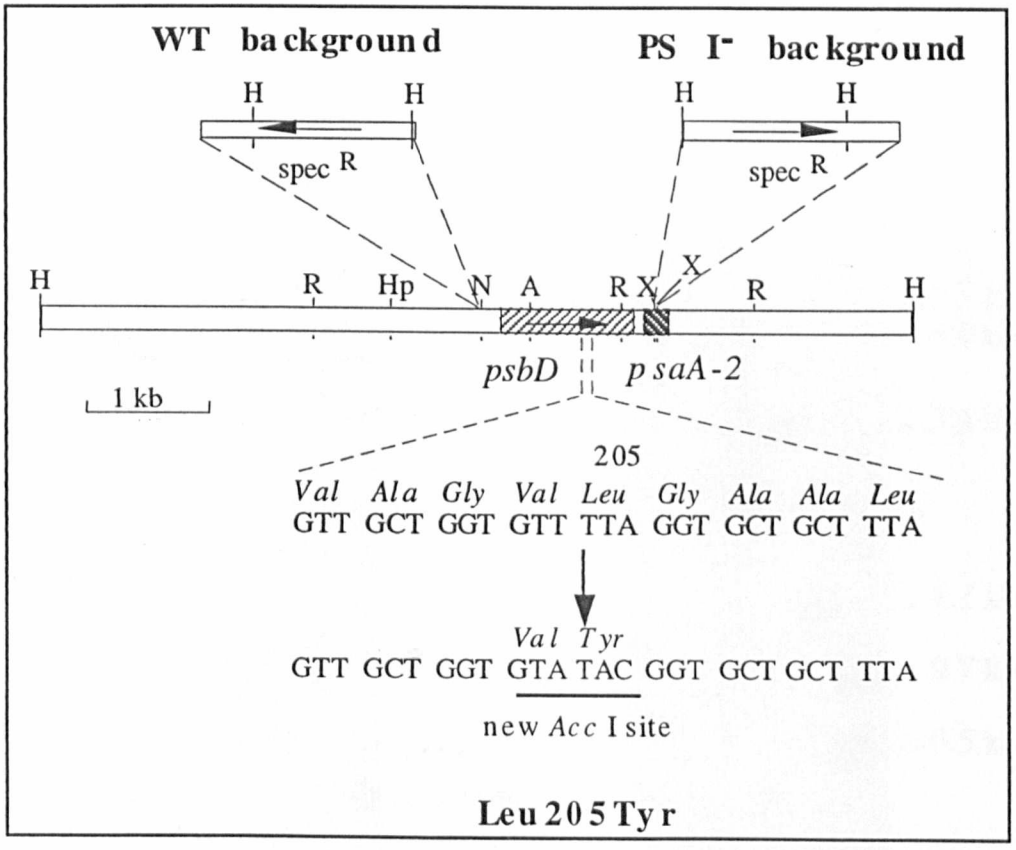


Figure 3.6: Composite map showing the mutations made to the *psbD* gene and flanking regions in the chloroplast genome of *C. reinhardtii*. The positions and orientations of *psbD*, *psaA*-2 and the $spec^R$ cassette in the various mutants are indicated. Restriction sites are: *Hind* III (H), *Eco* RI (R), *Nsi* I (N), *Acc* I (A), *Hinc* II (Hc), *Hpa* I (Hp) and *Xba* I (X).

3.2.2.2 Southern blotting of the Leu205Tyr mutant

The Leu205Tyr muation was created either in a pNsi16 background (wild type, spec^R) or in a pXba9 background (PS I$^-$, spec^R). Figure 3.7 shows a typical Southern analysis of DNA obtained from wild type and transformants. All the transformants were homoplasmic for the introduced changes. This can be seen by the absence of the wild type band (2.8 kb in panel A and 7.5 kb in panel B) in the transformants. Moreover, all transformants carrying the Leu205Tyr mutation contained the new *Acc* I restriction site associated with that mutation. This is indicated by the presence of two new bands when the DNA was digested with *Eco* RI/*Acc* I (0.45 and 0.3 kb, panel A, lane 4), and *Hin*d III/*Acc* I (0.7 and 0.5 kb panel B, lane 3). The presence of the *aadA* cassette in the transformants was confirmed by probing with an *aadA*-specific probe (not shown).

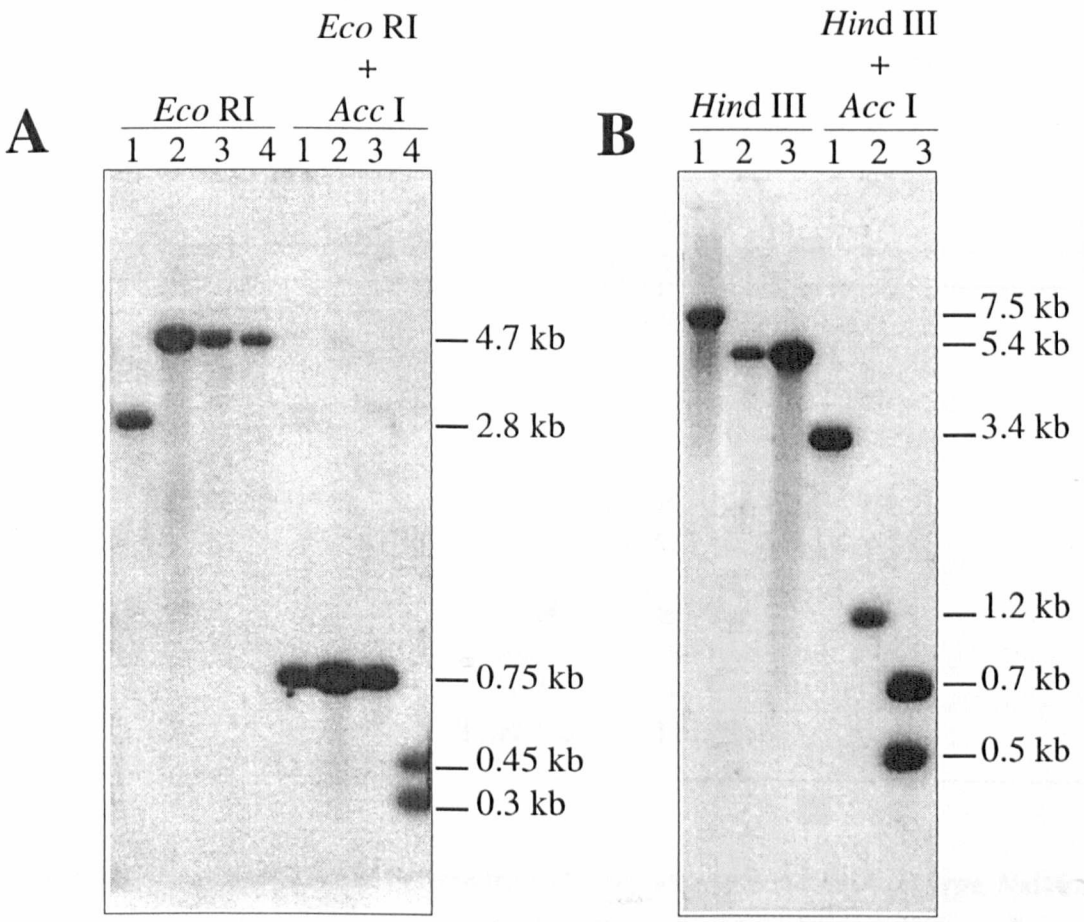


Figure 3.7: Southern blot hybridisation of DNA obtained from: (A) wild type (lanes 1); Nsi16 (WT background, spec^R) (lanes 2 and 3) and Leu205Tyr mutant (lanes 4), following digestion with *Eco* RI, *Eco* RI and *Acc* I, and (B) wild type (lanes 1); Xba9 (PS I$^-$, spec^R) (lanes 2); Leu205Tyr/PS I$^-$ mutant (lanes 3), following digestion with *Hin*d III, *Hin*d III and *Acc* I. Blots were probed with a 0.6 kb *psbD*-specific probe.

3.2.2.3 Southern blotting of the Thr2Ala and Thr2Ser mutants

Thr2Ala and Thr2Ser mutants were constructed in a pNsi16 background (Figure 3.8). Figure 3.9 shows a Southern analysis of chloroplast DNA isolated from wild type, transformant Nsi16 and the Thr2Ala and Thr2Ser mutants following hybridisation to a 0.6 kb *psbD* probe. The absence of a wild type band (7.5 kb) in the transformants (lanes 2, 3 and 4) indicates the homoplasmicity of the latter. The presence of a band with a size of 2.8 kb in the mutants (lanes 7, 8) indicate the loss of the *Mun* I site as a result of the introduced nucleotide changes associated with the mutations (see restriction map in Figure 3.8, for details). The presence of the *aadA* cassette in these transformants was confirmed by probing with an *aadA*-specific probe (not shown).

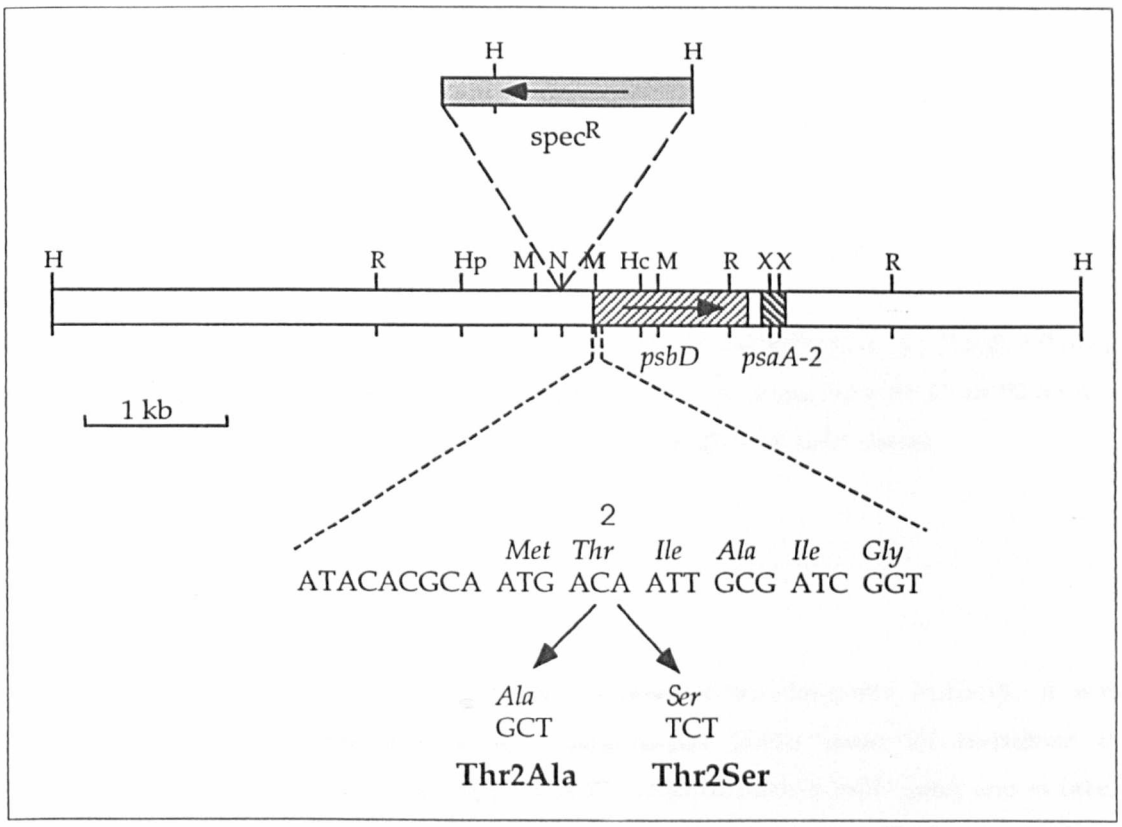


Figure 3.8: Restriction maps of *C. reinhardtii* chloroplast genome from wild type, Nsi16 and Thr2Ala, Thr2Ser mutants. Positions and orientations of *psbD*, *psaA*-2 and the spec^R cassette are shown. Restriction sites are: *Hind* III (H), *Eco* RI (R), *Nsi* I (N), *Acc* I (A), *Hinc* II (Hc), *Hpa* I (Hp) and *Xba* I (X), *Mun* I (M). The *Mun* I site eliminated along with the creation of the Thr2 mutations, is shown in bold.

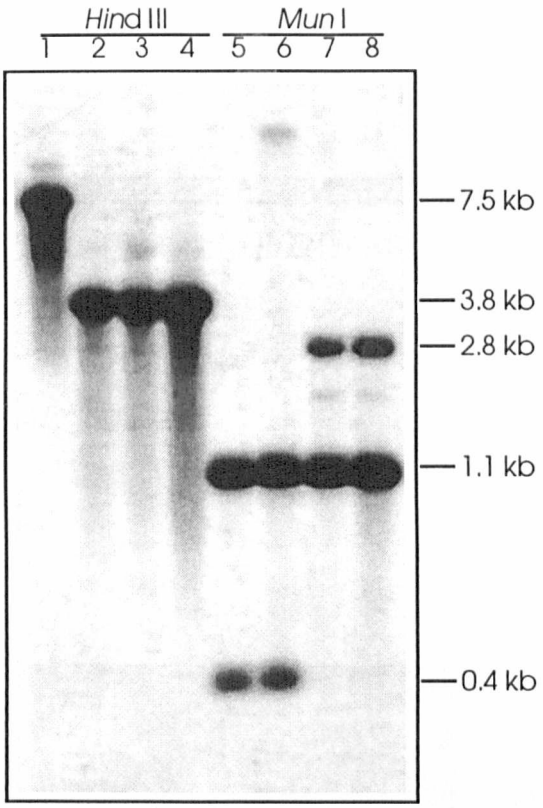
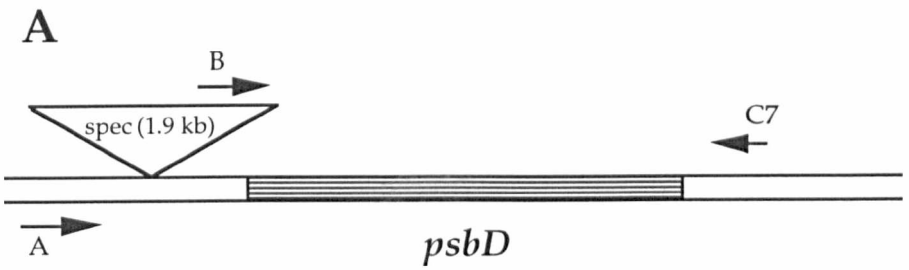


Figure 3.9: Southern blot analysis of wild type (lanes 1, 5), Nsi16 (lanes 2, 6), Thr2Ser (lanes 3, 7) and Thr2Ala (lanes 4, 8) mutants. Chloroplast DNA was digested with *Hin*d III (lanes 1-4) and *Mun* I (lanes 5-8) and hybridised to a 0.6 kb *psbD* probe.

3.2.2.4 PCR analysis

PCR was routinely used to analyse the genotype of transformants. Initially, it was used as a further confirmation that the transformant Nsi16 owed its resistance to spectinomycin to the presence of the *aadA* cassette upstream of the *psbD* gene, and in later stages of the project, as a quick way to analyse the genotype of mutants growing without spectinomycin in liquid cultures.

In the case of Nsi16, the PCR experiment was performed by using one primer that hybridises to the 5' end of the selectable marker (primer PCR B, Table 2.5) and a second primer that hybridises to the 3' end of *psbD*. In addition, a primer that hybridises to a region of the *psbD* locus, outside of the cassette, (primer PCR A, Table 2.5) was used as a control (Figure 3.10). Total DNA was used as a template for both wild type and Nsi16 strains.



A + C7 = 1430 bp
B + C7 = 1350 bp

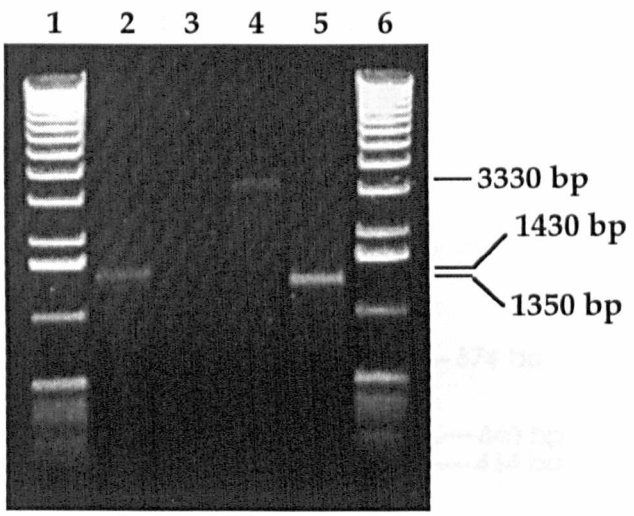


Figure 3.10: PCR analysis of wild type (lanes 2, 3) and transformant Nsi16 (lanes 4, 5). Panel A: Primers (A, B, C7) are indicated with arrows. Approximate sizes of amplified products are indicated. Panel B: Agarose gel showing the result of the analysis. Lanes 2, 3: wild type amplified with A+C7 or B+C7, respectively. Lanes 4, 5: Nsi16 amplified with A+C7 or B+C7, respectively. Lanes 1, 6: 1 kb DNA ladder

As expected, Nsi16 gave a band of 1350 bp, when amplified with B+C7 (Figure 3.10, lane 5), whereas no amplification product was observed when wild type DNA was amplified with the same primers. Amplification of DNA from Nsi16, with primers A+C7 gave a single fragment with a total size of approximately 3.3 kb (Figure 3.10, lane 4), (resulting from the addition of the selectable marker to the A+C7 fragment) indicating the homoplasmic state of the transformant.

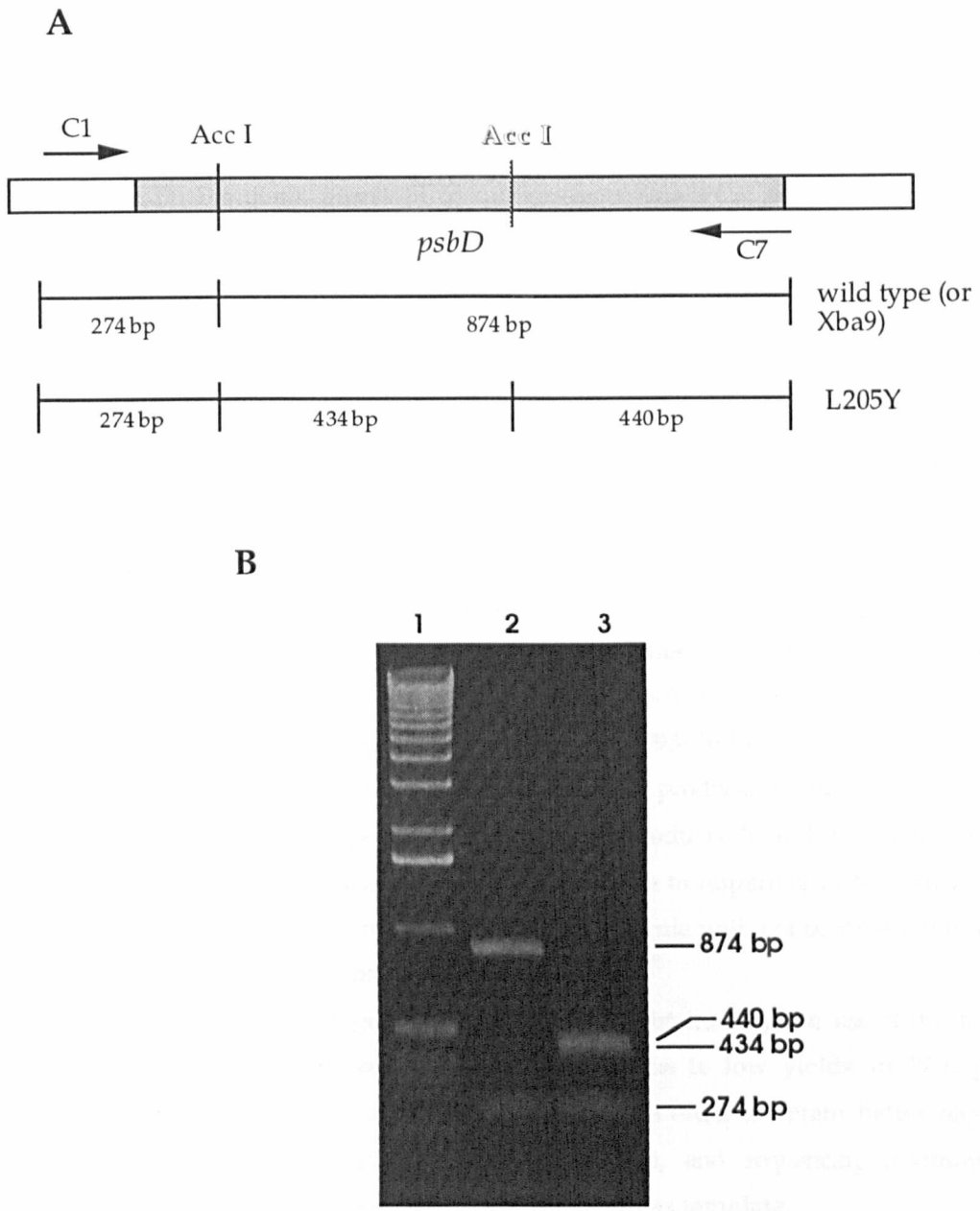


Figure 3.11: PCR analysis of Xba9 (PS I$^-$, spec^R) and mutant Leu205Tyr/PS I$^-$. Panel A: restriction map and digestion patterns of Xba9 and Leu205Tyr/PS I$^-$ with *Acc* I. Primers C1 and C7 are indicated with arrows. Panel B: agarose gel showing the results of the analysis. Lane 2, Xba9; Lane 3, Leu205Tyr/PS I$^-$; Lane 1, 1 kb DNA ladder

PCR analysis was mainly carried out in order to analyse the genotype of the Leu205Tyr/PS I$^-$ mutant, when it was growing without spectinomycin in 20 litre carboys that were going to be used for the preparation of thylakoid membranes (and subsequently, reaction centre particles). In order to make sure that the chloroplast genome of these cells contained the Leu205Tyr mutation, DNA was amplified with primers C1 and C7 (see Table 2.5), that reside on the 5' and 3' ends of the *psbD* gene and the PCR products digested with *Acc* I.

Figure 3.11 shows a typical PCR analysis performed on total DNA isolated from transformant Xba9 (PS I$^-$, spec^R) and from the Leu205Tyr/PS I$^-$ mutant growing in a 20 litre carboy. Digestion of Xba9 DNA with *Acc* I, amplified with the above combination of primers, yielded two fragments with sizes of 874 bp and 274 bp (Figure 3.11B). In the case of the Leu205Tyr/PS I$^-$ mutant, digestion of the corresponding PCR product with the same enzyme, always yielded 3 fragments, due to the presence of the new *Acc* I site (outlined in Figure 3.11A), indicating that the mutant was homoplasmic for the Leu205Tyr mutation.

3.2.2.5 DNA sequencing

To verify that the desired point mutations were present in the chloroplast genome of the various *C. reinhardtii* mutants, DNA obtained from transformants (including Nsi16 and Xba9) was sequenced.

Initially, two methods involving the direct sequencing of PCR products were employed. The first method was based on the production of single-stranded DNA by asymmetric PCR (McCabe, 1990). In the second method, DNA obtained from a standard PCR was denatured using a snap-cooling method (Ashworth, 1993) followed by ordinary, double-stranded sequencing reactions. Directly sequencing PCR products without an additional cloning step is generally preferable to sequencing cloned products. In addition to the benefit of simplicity, this greatly reduces the potential for errors due to imperfect PCR fidelity, as any random misincorporations in an individual template molecule will not be detectable against the much greater signals of the consensus sequence.

In spite of these advantages, the sequencing data obtained by the use of the methods mentioned above were of very low quality. This was due to low yields of PCR product obtained, especially in the case of the assymetric PCR. In order to obtain better sequencing data, a standard PCR was followed by a cloning step, and sequencing reactions were performed using a plasmid (instead of the PCR fragment) as template.

In order to make sure that the *psbD* gene contained only the desired mutation, the whole gene was sequenced in all the transformants using primers C1-C9 (Table 2.5). Representative sequencing data from the mutants are shown in Figures 3.12 and 3.13.

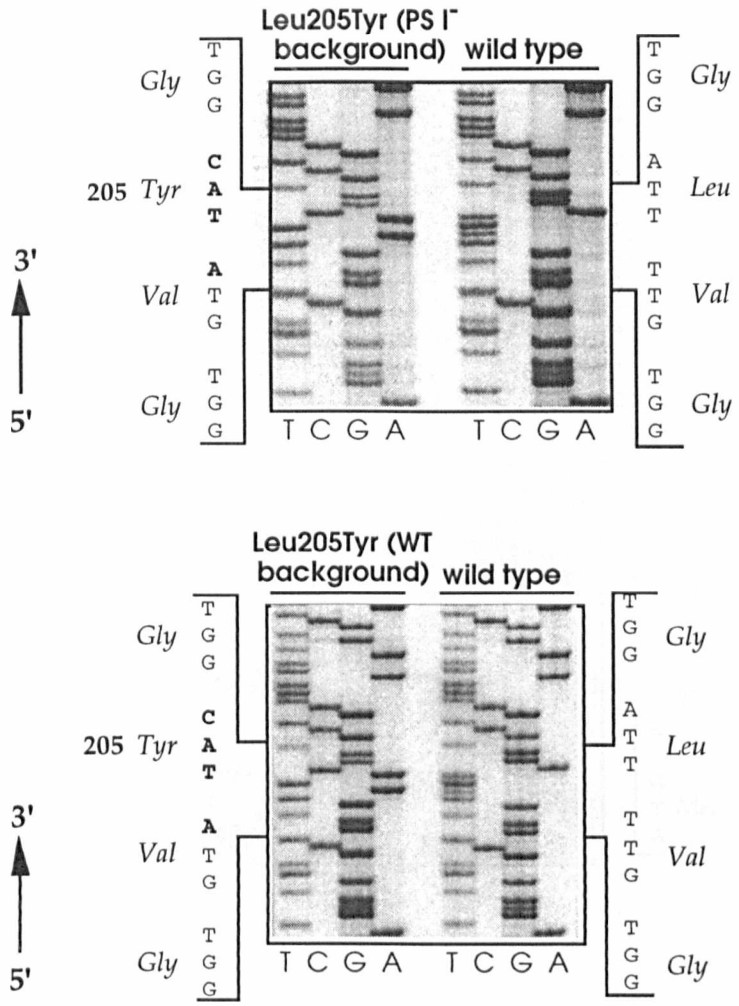


Figure 3.12: Sequencing autoradiograms of mutants Leu205Tyr/PS I⁻ and Leu205Tyr. Modified codons in the mutants are numbered

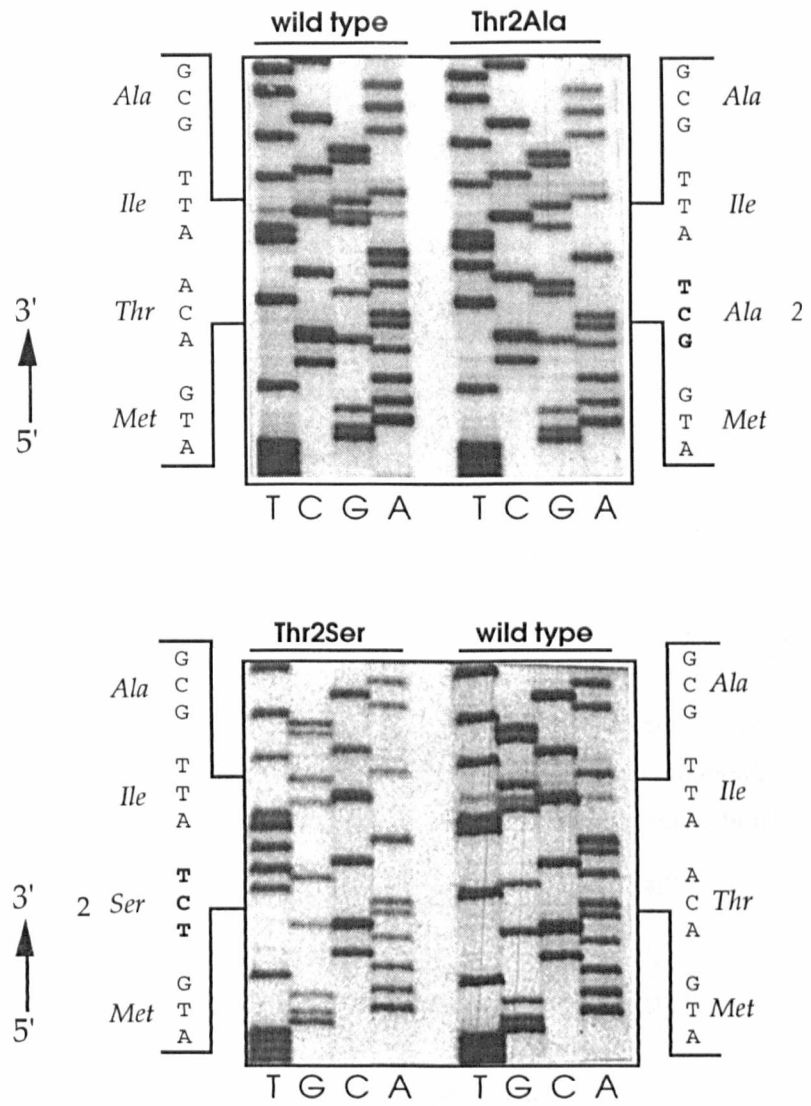


Figure 3.13: Sequencing autoradiograms of mutantsThr2Ala and Thr2Ser. In both cases, the the antisense strand was sequenced. Modified codons in the mutants are numbered

3.3 Discussion

In this chapter the construction and genetic characterisation of site-directed mutants of the D2 polypeptide in *Chlamydomonas reinhardtii* has been described. Mutant D2-Leu205Tyr was created in either a wild type or a PS I$^-$ background. Mutants D2-Thr2Ala and D2-Thr2Ser were created in a wild type background only.

The *psbD* gene was initially mutated *in vitro* by oligonucleotide-directed mutagenesis and then introduced into the chloroplast of *C. reinhardtii*. The mutagenic oligos

also introduced restriction fragment length polymorphisms (RFLPs) to the coding sequence of *psbD*, which were used to identify (by Southern blotting and PCR analysis) whether transformants carried the desired mutations in the *psbD* gene and whether the latter had been integrated at the correct position on the chloroplast genome. In order to select for the integration of the mutated *psbD* gene into the chloroplast, a selectable marker (the *aadA* cassette) was placed upstream or downstream of the gene prior to the transformation experiments.

Sequencing of the whole *psbD* gene in every plasmid construct used for transformation ruled out the possibility that unwanted mutations had been inadvertently introduced into the gene during the *in vitro* mutagenesis process. Moreover, extensive molecular characterisation (Southern analysis, DNA sequencing, PCR analysis) of DNA isolated from each algal transformant cell line confirmed the presence of the site-directed mutations at the desired *psbD* codon and revealed no differences in any other region of *psbD*. In this way, any differences observed in the mutant PS II phenotype could be directly linked to the mutation and not to any other factors. In order to reduce the possibility that the observed phenotype of the mutants was linked to secondary mutations in the chloroplast or nuclear genome (that could not be detected by the genotypic characterisation), two independent clones of each transformant bearing the same *psbD* mutation were used in some biochemical analyses (following chapters).

3.3.1 The chloroplast transformation experiments

3.3.1.1 Factors affecting the efficiency of transformation

Transformation of the chloroplast of *C. reinhardtii* was performed according to Boynton and Gillham (1993) using a particle gun. As mentioned in section 3.2.1.3, transformation frequencies were very low compared to the ones reported in the literature. Attempts to optimise the technique by changing various parameters of the procedure did not improve significantly the outcome of each transformation experiment.

There are several factors affecting the efficiency of a transformation experiment (discussed in Boynton and Gillham, 1993; Sanford *et al.*, 1993). These factors include the device used for the introduction of DNA into the chloroplast as well as the power source, the type and velocity of the DNA-coated particles (microprojectiles) and the distance between the microprojectile launch site and the target cells. In addition, biological parameters such as the DNA construct and the age and cell density of the recipient cells, have to be considered. The two most commonly used designs of particle guns are the ones driven by gunpowder and helium. The latter is considered to be superior in terms of the transformation frequencies achieved for both *Chlamydomonas* and higher plants (Sanford *et al.*, 1993). The type of the power source is directly related to the type and design of the particle gun. The

device used in this work (from Shearline) is driven by gunpowder. Although, the velocities achieved by the gunpowder and helium sources are similar, the former seems to have a higher velocity in the "epicentre" of the blast creating a central zone associated with cell death (and consequently without transformation) (Russel *et al.*, 1992). Cell death is probably caused by physical trauma to the cells from the gas blast and acoustic shock generated by the device (Russel *et al.*, 1992). Tungsten and gold particles have been employed as microprojectiles in chloroplast transformation. Tungsten particles are potentially toxic to certain cell types, are subject to surface oxidation that can alter DNA binding, can degrade catalytically DNA bound to it over time and are highly heterogeneous in shape and size, preventing optimisation of size for a particular cell type (Sanford *et al.*, 1993). Gold particles are biologically inert and are more uniform in shape and size than tungsten. However, DNA-coating of gold can be more variable because of perturbations of precipitation conditions. According to Sanford *et al.* (1993) DNA-coating of microprojectiles is one of the most important sources of variation affecting transformation efficiency, as the pattern of DNA precipitation and aggregation of the particles is unique and nonreproducible. The size of microprojectiles used in a transformation experiment depends on the cell type. Usually particles are chosen to be roughly one-tenth of the cell diameter (Sanford *et al.*, 1993). As the size of *C. reinhardtii* cells is around 10μm in diameter (Harris, 1989), M10 tungsten particles (having a 0.2-1.5 μm diameter) and 1 μm gold particles were used for the transformation experiments in this work.

Cell age and density are two important parameters for the efficiency of transformation experiments (Boynton and Gillham, 1993; Sanford *et al.*, 1993). The optimum targets for transformation are "young", actively dividing cells that can withstand the stresses of the bombardment process. On the other hand, a uniform monolayer of cells is optimal when cell density is concerned. This provides the greatest number of targets, without extraneous cells that can interfere with plating or selection.

It is not possible to identify the reason(s) for the inefficiency of the transformation experiments carried out in this work. However, possible reasons could include the quality of the tungsten particles used as microprojectiles. These particles were not specifically made for this type of experiment (tungsten particles specifically produced for chloroplast transformation are now commercially available from Bio-Rad) and because they were ageing they were probably oxidised. As mentioned above, surface oxidation of tungsten particles can alter DNA binding (Sanford *et al.*, 1993). Recent trial transformation experiments using tungsten particles obtained from Bio-Rad, were considerably more efficient. A transformation experiment using gold particles did not improve the transformation frequencies. Moreover, gold particles were found to be more difficult in handling, especially in the precipitation of DNA and loading of the DNA-coated particles onto the macroprojectiles.

3.3.1.2 Integration of the donor DNA into the chloroplast genome

Chloroplast transformation occurs by homologous gene replacement, apparently involving a double-exchange event between chloroplast sequences in the donor DNA and corresponding sequences in the recipient chloroplast genome (Boynton *et al.*, 1988; Newman *et al.*, 1990). The sites of exchange employed during integration of the donor DNA into the recipient genome were localised by Newman *et al.* (1990) using a combination of antibiotic resistance mutations in the 16S and 23S rRNA genes and RFLPs flanking these genes. Thus, exchange events were found to occur preferentially near the vector:insert junctions of the donor DNA. These exchange events did not include any of the vector sequences. Moreover, the authors reported that increased number of transformants were obtained by decreasing the heterology between the donor and recipient DNA sequences. However, 200 bp of homology between the selectable marker (or the area of heterology) and the donor insert:vector junction appears to be adequate to promote exchange events (Newman *et al.*, 1990, 1992). As the 16S and 23S rRNA genes are located within the inverted repeats of the chloroplast genome, the inital integration of the donor DNA into one copy of the inverted repeat results in the formation of transient heteroplasmic genomes. However, this event is followed by intramolecular copy correction (Harris, 1989) that finally yields homoplasmic molecules (Newman *et al.*, 1990). Newman *et al.* (1990) also found that bombardment of cells with tungsten microprojectiles can be mutagenic. Thus, a significant increase in the frequency of nuclear mutations to streptomycin resistance (1000-fold compared to the unbombarded control) and more modest increases in chloroplast mutations to streptomycin, spectinomycin and erythromycin resistance were seen in *C. reinhardtii* cells bombarded with tungsten particles devoid of donor DNA.

Following the initial reports of chloroplast transformation in *C. reinhardtii* by Boynton and co-workers (Boynton *et al.*, 1988), Blowers *et al.* (1989) showed that the *atpB* deletion mutant *ac-u-c-2-21* (CC373) can be transformed to photosynthetic competency, not only by double-stranded DNA plasmids, but also by single-strand DNA circles, or linear, duplex DNA molecules carrying the wild type *atpB* gene. The effectiveness of linearised duplex DNA molecules for chloroplast transformation appears to depend on the position of the donor gene relative to the ends of the linearised plasmid vector (Blowers *et al.*, 1989; Boynton and Gillham, 1993). In the case of the CC373 mutant, single-strand circular DNA of a given donor plasmid was only 25-50% as effective as the double-stranded DNA of that plasmid (Blowers *et al.*, 1989).

Boynton and Gillham (1993) also raise the possibility that chloroplast DNA sequences may become integrated into the nuclear genome as a consequence of chloroplast transformation. As mentioned in section 3.1.1.1, integration of donor genes in nuclear transformants of *C. reinhardtii* appears to occur randomly (Kindle, 1990). In the present

study, Southern blot analysis of chloroplast and nuclear DNA obtained from all the transformants after 2-3 rounds of restreaking did not indicate the presence of donor sequences in the nuclear DNA (not shown).

The methodology developed for chloroplast transformation in *C. reinhardtii* has successfully been adapted to the plastids of tobacco leaves, using similar antibiotic resistance chloroplast markers to produce transformed calli and ultimately whole plants (Maliga, 1993). Initially, transgenic lines were selected on the basis of resistance to spectinomycin and streptomycin, carried on a mutant 16S rRNA gene (Svab *et al.*, 1990). However, the process was 2-3 orders of magnitude less efficient than the biolistic transformation of nuclear genes. The use of a chimeric *aadA* expression cassette (analogous to the one used for *Chlamydomonas*), has increased dramatically transformation frequencies of tobacco plastids (Svab and Maliga, 1993). The transforming DNA is incoroporated into the plastid genome by two homologous recombination events. In the case of gene replacement, the 16S rRNA gene and flanking regions (which are part of the inverted repeat regions) are incorporated into the recipient plastid genome, resulting in complete or nearly complete replacement of the homologous plastid region (Staub and Maliga, 1992). This step is then followed by intramolecular correction of the copy on the second inverted repeat according to the transgenic template (Svab *et al.*, 1990; Staub and Maliga, 1992). Insertion of foreign genes (such as the *aadA* gene) is similarly obtained after two homologous recombination events via flanking plastid DNA sequences (Svab and Maliga, 1993).

Chloroplast transformation in plants is a relatively time consuming process. Approximately 3-5 months are required to obtain homopasmic transgenic seedlings (Svab and Maliga, 1993). Moreover, non-photosynthetic mutants cannot survive. Despite these disadvantages, the ability to transform the plastid genome of plants should lead to a rapid progress in understanding plastid biology of higher plants and may soon offer the possibility to engineer the photosynthetic apparatus of agronomically important crops (Maliga, 1993).

4. Biochemical and Biophysical Characterisation of the D2-Leu205Tyr Mutant

4.1 Introduction

The Leu205Tyr mutant was constructed in order to investigate whether the difference found between the purple bacterial reaction centre (RC) and the PS II RC in terms of the rate of primary charge separation, could be solely attributed to structural differences of only one amino acid residue: a tyrosine at position 210 of the M subunit in the RC of *Rhodobacter sphaeroides* is replaced by a leucine at position 205 of D2 (*Chlamydomonas* numbering, Leu206 in higher plants) in PS II (Michel and Deisenhofer, 1988). Figure 4.1 shows the proposed position of D2-Leu205 within the PS II RC [model from Ruffle *et al.* (1992)].The biochemical and biophysical characterisation of the D2-Leu205Tyr mutant is presented in the following sections of this chapter.

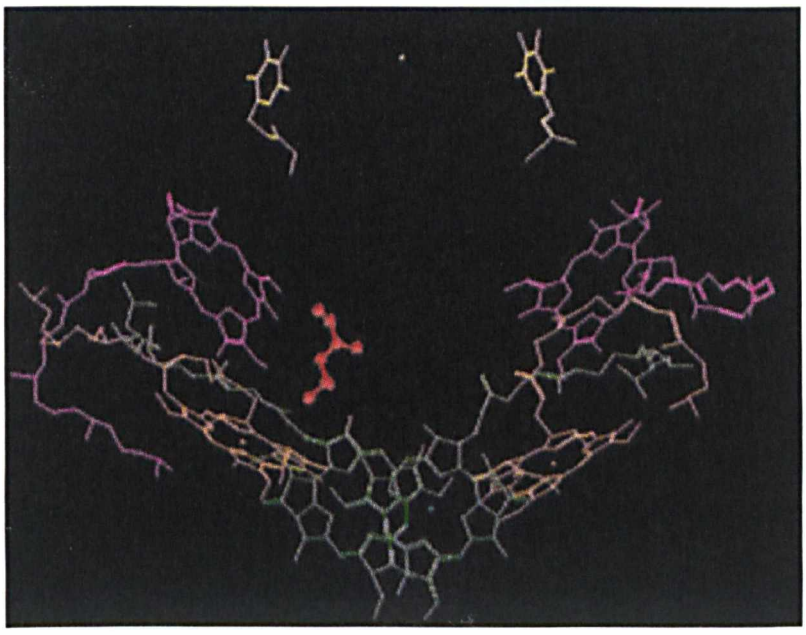


Figure 4.1: Proposed position of D2-Leu205 (in red) within the PS II reaction centre. The chlorophyll molecules that constitute P680 are shown in green; the accessory chlorophylls are shown in orange; the pheophytins are shown in purple and the quinones in yellow (model from Ruffle *et al.*, 1992). The side chain of leucine points towards the accessory chlorophyll.

4.2 Results

4.2.1 Photoautotrophic growth experiments

All transformants were initially analysed by carrying out growth and oxygen evolution experiments. Transformants were grown for approximately one week on TAP plates without spectinomycin and DCMU, before being transferred to minimal medium (HSM). The liquid cultures were then grown for 3 to 4 days to stationary phase. During the middle and late exponential phases, oxygen evolution measurements were carried out.

Figure 4.2 shows the growth curves of the wild type (strain CC125), transformant Nsi16 (wild type, $spec^R$) and the mutant Leu205Tyr. For the photoautotrophic growth experiments, only the version of the mutant created in a pNsi16 background was used, since the form created in a pXba9 background (PS I^-, $spec^R$) (Figure 3.2) could not grow photoautotrophically on HSM plates. As can be seen from Figure 4.2, both the Nsi16 and Leu205Tyr stains were able to grow photoautotrophically at similar growth rates to the wild type. The doubling time (measured from the initial 20 hours of growth) was approximately 11-12 hours for all strains.

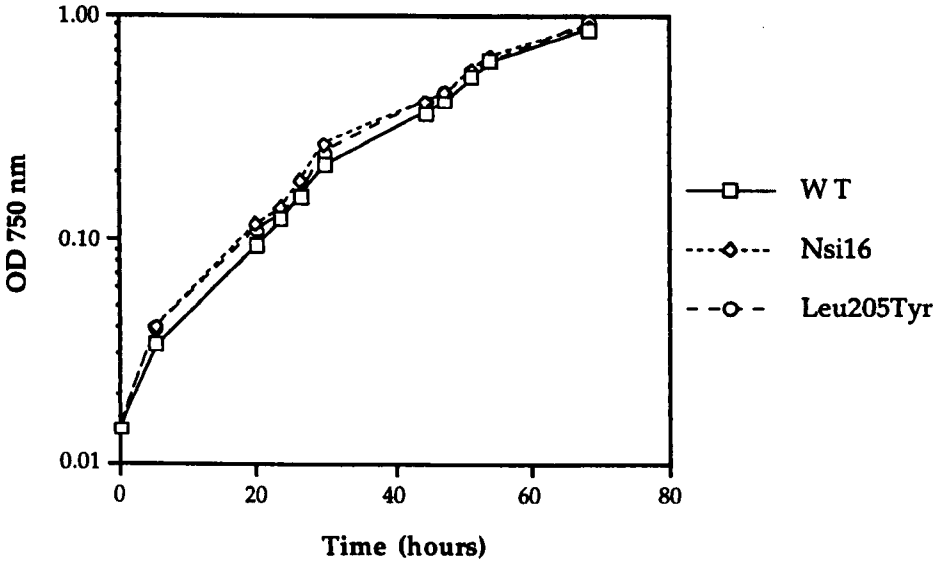


Figure 4.2: Photosynthetic growth curves of wild type (WT), transformant Nsi16 and mutant Leu205Tyr in HSM medium. Cultures were grown at 25°C at a light intensity of 50-70 $\mu E.m^{-2}.s^{-1}$ and were bubbled with air

After the end of the growth experiments, cells were spread on TAP plates containing spectinomycin and on HSM plates. This was done as a test of the genotype of the cells in each culture. The idea behind this experiment was that if more colonies appeared on HSM plates than on spectinomycin plates, that would mean that some of the cells in the transformant

cultures were lacking the spec^R cassette, possibly because of the presence of wild type copies of the chloroplast genome. However, in all cases, both types of plates contained similar numbers of colonies indicating that the transformant cultures did not contain cells lacking the spec^R cassette.

4.2.2 Oxygen evolution measurements of whole cells

Measurements of steady-state oxygen evolution of whole cells were carried out using a Clark-type oxygen electrode. The light intensity was at saturating levels (4000-6000 $\mu E.m^{-2}.s^{-1}$). DCBQ was preferred to DMBQ for the assay of PS II function, since it gave slightly higher rates of oxygen evolution (by approximately 10%).

This type of experiment was used initially with transformant Nsi16 (wild type, spec^R) in order to assess the effect of the presence of the *aadA* cassette alone on the function of PS II (and consequently on the expression of the *psbD* gene). Measurements were carried out in both TAP and HSM media. Nsi16 displayed similar rates of oxygen evolution to the wild type both in the presence of $NaHCO_3$ or artificial electron acceptors.

In order to determine the effect of the Leu205Tyr mutation on PS II electron transfer, oxygen evolution under saturating light conditions was measured in the presence of $NaHCO_3$ and artificial electron acceptors (DCBQ). Measurements using the Leu205Tyr mutant were carried out in HSM medium, therefore, the rates obtained reflected cultures growing photosynthetically. The oxygen evolution rates measured for the mutant, grown at 30-50 $\mu E.m^{-2}.s^{-1}$, in the presence of DCBQ were approximately 62% of the wild type rates (Table 4.1). These data indicate either a perturbed function of PS II or decreased levels of functional PS II centres.

Oxygen evolution measurements on whole cells were also conducted using transformant Xba9 (PS I$^-$, spec^R) and the version of the mutant which was deficient in PS I (Leu205Tyr/PS I$^-$). Cultures were grown in TAP medium at room temperature. In both strains, oxygen evolution could only be observed in the presence of artificial electron acceptors (potassium ferricyanide and DCBQ) (Table 4.1). In the absence of electron acceptors no oxygen evolution could be detected. This result confirmed the absence of PS I activity, created by the insertion of the spectinomycin cassette inside *psaA*-2. As can be seen from Table 4.1, the absence of PS I in Xba9 and Leu205Tyr/PS I$^-$ results in significantly reduced levels (at approximately 32-33% of the wild type) of oxygen evolution in these strains compared to wild type. This is consistent with the ~65% reduction in the oxygen evolving activity of the nuclear mutant F14, which lacks PS I, described by Chua *et al.* (1975).

No difference can be observed between the two PS I$^-$ strains in their respective rates of oxygen evolution. This contrasts with the reduced oxygen evolution rate observed in the Leu205Tyr mutant relative to its respective wild type control (Nsi16). The decreased oxygen-evolving activity in the Leu205Tyr mutant compared to its PS I$^-$ counterpart could be because

the former is more sensitive to photoinhibition. Indeed, Gong and Ohad (1991) have previously shown that a PS I-deficient mutant of *C. reinhardtii* is partially resistant to PS II photoinactivation. In order to address this question, the light-sensitivity of the Leu205Tyr mutant was studied in photoinhibition experiments (described in section 4.2.5).

Table 4.1: Oxygen evolution rates of wild type, Nsi16 and mutant Leu205Tyr grown in HSM and of Xba9 and Leu205Tyr/PS I$^-$ grown inTAP. Rates shown are after addition of either 1 mM $NaHCO_3$ or 1 mM potassium ferricyanide and 1 mM DCBQ. The data shown are based on at least three to four independent experiments

	+ $NaHCO_3$		+potassium ferricyanide, DCBQ	
strains	µmoles O_2.mg $Chl^{-1}.hr^{-1}$	% of wild type	µmoles O_2.mg $Chl^{-1}.hr^{-1}$	% of wild type
wild type	75±15	100%	167±6	100%
Nsi16	82±19	109±10%	171±12	102±5%
Leu205Tyr	71±5	93±29%	104±6	62±4%
Xba9	nd	nd	53[a]	32%
Leu205Tyr/PS I$^-$	nd	nd	55[a]	33%

[a]: rates for the PS I$^-$ strains are the average of two independent experiments. Standard errors are not shown; nd: not detected.

4.2.3 Fluorescence decay measurements of whole cells

To determine whether there were any perturbations to electron transfer in the Leu205Tyr mutant, the decay of variable fluorescence was measured according to Nixon and Diner (1992). These experiments were performed in the laboratory of B. Diner at E. I. du Pont de Nemours, USA.

The fluorescence yield is a sensitive measure of the oxidation state of redox components associated with the PS II reaction centre (reviewed in Papageorgiou, 1975; Nixon *et al.*, 1992a). Fluorescence yield is proportional to $[Q_A^-]$. The initial state $ZP680PheoQ_A$ gives a low quantum yield of fluorescence (F_0) and that of $ZP680PheoQ_A^-$ the highest fluorescence yield (F_m). The charge separated state $ZP680^+PheoQ_A^-$, generated within 300 ps of excitation of P680, is in a low fluorescence state (F_0) despite reduction of Q_A, because $P680^+$ quenches fluorescence (Butler *et al.*, 1973). Fluorescence yield then increases to F_m with the reduction of $P680^+$ by donor Z to form the state $Z^+P680PheoQ_A^-$. Oxidation of Q_A^- by electron transfer to Q_B lowers the fluorescence yield again with the formation of the state $ZP680PheoQ_AQ_B^-$.

The existence of these different fluorescence states provides a means to detect differences in the electron transfer process between the wild type and mutant Leu205Tyr, with regard to both the donor and acceptor sides. For this purpose, two types of assay were employed.

In the first assay, the relaxation of the relative quantum yield of variable fluorescence, F_v (which equals $F-F_o$) was measured using a train of weak probe flashes given after each of five saturating actinic flashes, spaced 0.6 seconds apart (Nixon and Diner, 1992; Whitelegge *et al.*, 1995). The measurements were carried out over a 5 ms range starting at 50 µs, when the high-fluorescence state was apparent. The cells were incubated for 10 minutes in the dark in the presence of 0.3 mM ferricyanide and 0.3 mM p-benzoquinone in TAP medium plus 50 mM HEPES-NaOH, pH 7.5. The 50 µs values of the variable fluorescence were normalised to F_0 $[F_v/F_0]$. As can be seen from Figure 4.3, all three strains used in this experiment (wild type, Nsi16, Leu205Tyr) showed repeatedly a high fluorescence state at 50 µs after each flash, as previously shown by Whitelegge *et al.* (1995) for wild type *C. reinhardtii*. If there was any disruption on the donor side of PS II, this would be indicated by a dramatic quenching of fluorescence at 50 µs on the second and subsequent flashes, as shown by Nixon and Diner (1992) for a mutant incapable of assembling the Mn cluster.

A comparison of the fluorescence yield at ~200 µs (which can be seen at approximately the second time point in Figure 4.3) after each of the actinic flashes is particularly revealing with regard to the rate of oxidation of Q_A^- by Q_B. The reduction of the quinone Q_B to the quinol is a two-step process. Initially, the reaction $Q_A^-Q_B \rightarrow Q_AQ_B^-$ (after one flash) takes place. After a second flash, the quinol Q_BH_2 is generated according to the reactions $Q_A^-Q_B^- \rightarrow Q_AQ_B^{2-}$ and $Q_AQ_B^{2-} + 2H^+ \rightarrow Q_AQ_BH_2$ (reviewed by Hansson and Wydrzynski, 1990). The formation of the Q_B quinol is slower than the formation of the Q_B semiquinone (Robinson and Crofts, 1983). The initial state is regenerated by the replacement of the quinol Q_BH_2 by Q_B. Thus, on every other flash the system finds itself in the same state. Consequently, the rate of oxidation of Q_A^- should oscillate with a period of two flashes. This oscillation of period two at 200 µs, could be observed in the wild type and all the transformants, indicating the there were no perturbations in the function of the acceptor side (oxidation of Q_A^- by Q_B), in the latter.

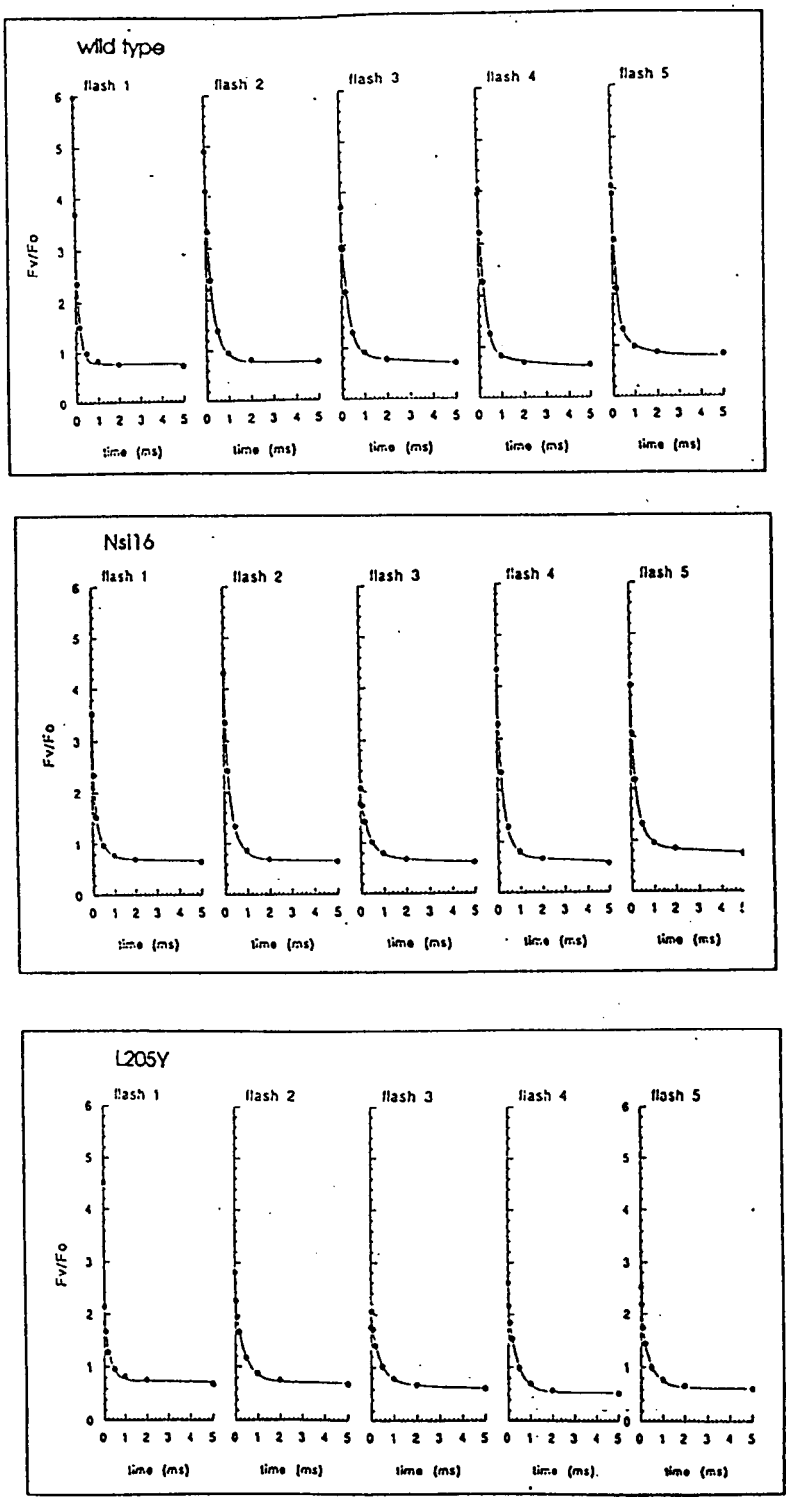


Figure 4.3: Relaxation of the variable fluorescence, F_v ($F-F_0$), after each of a series (1.67 Hz) of five saturating 2-μs flashes in whole cells of *C. reinhardtii* wild type, Nsi16 and Leu205Tyr strains. Shown is the 5-ms range following each flash starting at 50 μs. The 50 μs values were normalised to F_0 (F_v/F_0)

The second assay using fluorescence was the measurement of the relaxation of variable fluorescence resulting from charge recombination between Q_A^- and the PS II donor side (Figure 4.4). This was done by giving a single 2-μs saturating flash to intact cells in the presence of 30 μM DCMU, which blocks electron transfer from Q_A^- to Q_B. DCMU was added at least 1 minute before the beginning of the measurement. The relaxation of the fluorescence yield was then followed using weak probe flashes. In this case recombination should occur between Q_A^- and the oxidised donor side. The higher the equilibrated concentration of $P680^+$ (the less stabilised the oxidising equivalent) the faster the charge recombination (Nixon *et al.*, 1992b).

Figure 4.4 shows the results of this measurement using wild type, Nsi16 and Leu205Tyr strains. The pattern of fluorescence decay is similar between the wild type and Nsi16 strains and the Leu205Tyr mutant. However, the F_0 value of the Leu205Tyr mutant is higher compared to the wild type and transformant Nsi16, indicating a decreased photochemical quenching of fluorescence in the mutant.

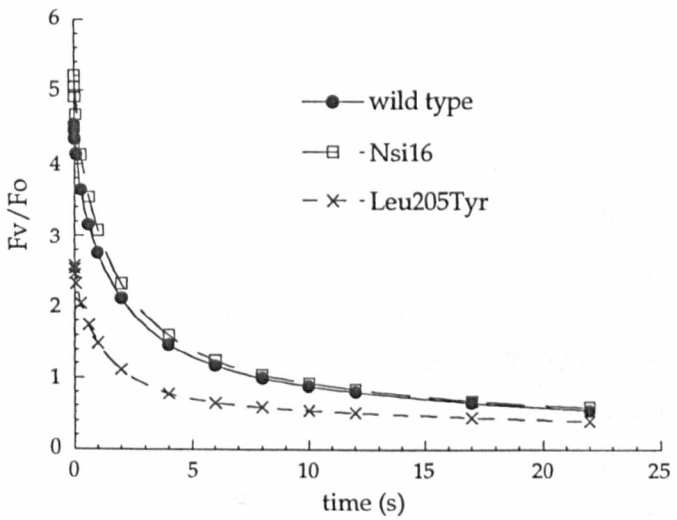


Figure 4.4: Relaxation of variable fluorescence, F_v ($F-F_0$), resulting from charge recombination between Q_A^- and the PS II donor side following a single saturating 2-μs flash excitation of whole cells of *C. reinhardtii*. Wild type is compared to transformant Nsi16 and to mutant Leu205Tyr. The variable fluorescence is normalised to F_0 (F_v/F_0). The F_0 values at 50 μs (the earliest time point), are as follows: wild type, 7156; Nsi16, 6983 and Leu205Tyr, 11012.

4.2.4 Thermoluminescence characteristics of transformant cells

When preilluminated photosynthetic material is warmed from low temperatures in the dark, light is emitted at characteristic temperatures. This phenomenon is known as thermoluminescence (TL) and can be observed in photosynthetic bacteria, cyanobacteria, algae and various higher plant preparations (reviewed by Vass and Inoue, 1992). TL is mainly associated with PS II. Since TL characteristics are very sensitive to subtle changes in redox properties of the involved electron transport components, the TL method has become a useful tool in probing a wide range of PS II redox reactions. The most important photosynthetic TL components arise from the thermally radiative recombination of positive charges stored in the S_2 and S_3 redox states of the water-oxidising complex, with electrons stabilised on the reduced electron acceptors Q_A and Q_B. The intensity of thermoluminescence reflects the amount of recombining charges, whereas the temperature of the peak is indicative of the energetic stabilisation of the charge separated pair. Thus, the higher the peak temperature of TL, the greater the stabilisation (Vass *et al.*, 1981).

When dark-adapted cells are illuminated with a flash of light, a single TL band is induced, called the B band, at around 30-40°C, which arises from the $S_2Q_B^-$ recombination (Rutherford *et al.*, 1982; Demeter and Vass, 1984). If electron transfer between Q_A and Q_B is blocked by DCMU, the B band is replaced by the so-called Q band at around 10-15°C, which arises from the $S_2Q_A^-$ recombination (Rutherford *et al.*, 1982; Demeter *et al.*, 1982).

The thermoluminescence measurements presented in this work were carried out in the laboratory of I. Vass at the Institute of Plant Physiology, Szeged, Hungary. Figure 4.5 shows the results from TL measurements from wild type, transformant Nsi16 and mutant Leu205Tyr. In the wild type and Nsi16 the peak position of the single flash-induced B band (from the $S_2Q_B^-$ recombination) (solid line in Figure 4.5) was found at 30-32°C. In the presence of 10 μM DCMU the single flash-induced Q band (from the $S_2Q_A^-$ recombination) (dashed line in Figure 4.5) appeared at 14-16°C. Moreover, the intensity of the Q band was two times higher than that of the B band. These data are in accordance with previous TL studies using *C. reinhardtii* whole cells (Ohad *et al.*, 1988; Etienne *et al.*, 1990; Demeter *et al.*, 1995).

The thermoluminescence intensity for the Leu205Tyr mutant was only about 20-25% of the wild type. This indicates a significantly lower amount of recombining charges in the mutant. The peak position of the Q band was the same as for the wild type. The position of the B band was also similar to the wild type although a slight shift towards lower temperatures (about 2°C) could be observed in the mutant.

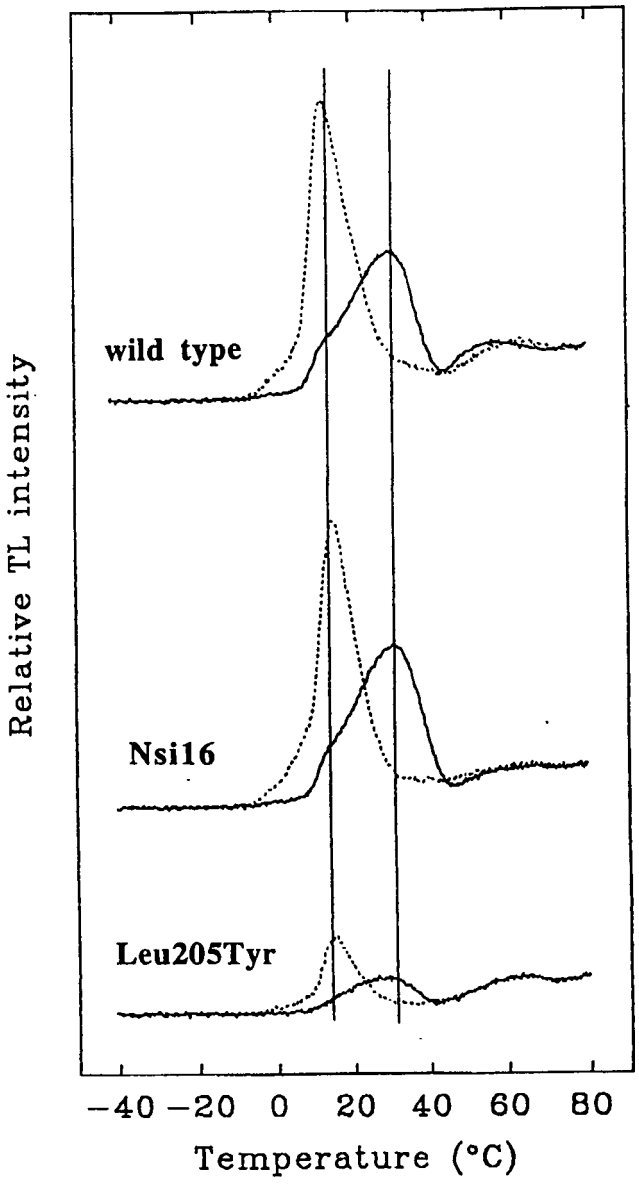


Figure 4.5: Single flash-induced TL curves from whole cells of wild type, Nsi16 and Leu205Tyr. Excitation was at -10°C in the absence (solid line) or presence (dashed line) of 10 μM DCMU. The chlorophyll content in all samples was 50 μg.

4.2.5 Photoinhibition experiments in whole cells

The loss of photosynthetic activity induced by an excess of light energisation of the photosynthetic apparatus is usually referred to as "photoinhibition" (Kyle *et al.*, 1984; reviewed in Prásil *et al.*, 1992; Aro *et al.*, 1993; Ohad *et al.*, 1994). Photoinhibition is a multistep process which targets mainly PS II at the molecular level and includes photoinactivation of the electron transport and breakdown of the D1 and D2 proteins. Protection from photoinhibition can be obtained only *in vivo* when there is *de novo* synthesis

and replacement of PS II RC polypeptides degraded during the damaging process. Photoinhibition has been extensively studied in *C. reinhardtii* (e.g., Kyle *et al.*, 1984; Ohad *et al.*, 1984; Schuster *et al.*, 1988; Ohad *et al.*, 1990; Bracht and Trebst, 1994; Zer and Ohad, 1995).

To monitor the rate of PS II photoinactivation in whole cells of *C. reinhardtii*, cells are exposed to photoinhibitory conditions (such as high light) and the loss of PS II activity is measured as the decrease of variable fluorescence or of the rate of oxygen evolution with time (e.g., Ohad *et al.*, 1984; Schuster *et al.*, 1988). The latter of these methods was used in this work to monitor the loss of PS II activity in wild type and transformant cells.

Thus, cultures grown in HSM medium with air bubbling until middle or late exponential phase were stirred in flat dishes at 25°C and subjected to high light illumination (1000 $\mu E.m^{-2}.s^{-1}$) for a period of 4-5 hours during which oxygen evolution was measured (see section 2.15).

Before using cells from transformant strains, the rate of PS II photoinactivation was studied in wild type cells exposed to low light (200 $\mu E.m^{-2}.s^{-1}$) and high light (1000 and 2000 $\mu E.m^{-2}.s^{-1}$) intensities (Figure 4.6). In these experiments cultures used were harvested in late exponential phase (OD_{750} >1).

At low light, a biphasic behaviour of PS II activity could be observed (Figure 4.6). An initial decline (for approximately one hour) of PS II activity was followed by a subsequent rise in the rates of oxygen evolution. This profile was obtained both in the absence (Figure 4.6A) and presence (Figure 4.6B) of the chloroplast protein synthesis inhibitor, chloramphenicol (CAP). The initial loss of PS II activity was more profound in the presence of CAP. The stimulation of PS II activity, detected after 1 hour of irradiance, could not be attributed to evaporation or an increase in cell density since the chlorophyll content of the culture was monitored during the course and at the end of the measurements. A slight stimulation of PS II activity in the absence of CAP, has also been observed by Nixon *et al.* (1995) for *Synechocystis* 6803 whole cells.

At high light intensities, in the absence of CAP, a similar loss of PS II activity was observed at both 1000 and 2000 $\mu E.m^{-2}.s^{-1}$ (Figure 4.6A). A slight difference in the extent of PS II activity remaining in the samples at the end of these experiments (between 30 and 40%) with that reported elsewhere (~20%) (Ohad *et al.*, 1984; Schuster *et al.*, 1988; both using the strain y-1 of *C. reinhardtii*) could be attributed to the use of higher light intensities (>2000 $\mu E.m^{-2}.s^{-1}$) compared to that used in the present work. The decline of PS II activity in the presence of CAP at high light intensities was more pronounced than that observed in the absence of inhibitors (Figure 4.6B). For the photoinhibition experiments using Nsi16 and Leu205Tyr cells, it was decided to use a light intensity of 1000 $\mu E.m^{-2}.s^{-1}$ as its effects on the loss of PS II activity were less prominent (as seen in Figure 4.6B) compared to 2000 $\mu E.m^{-2}.s^{-1}$. Therefore, distinction of subtle differences in the measurements between different samples would be easier.

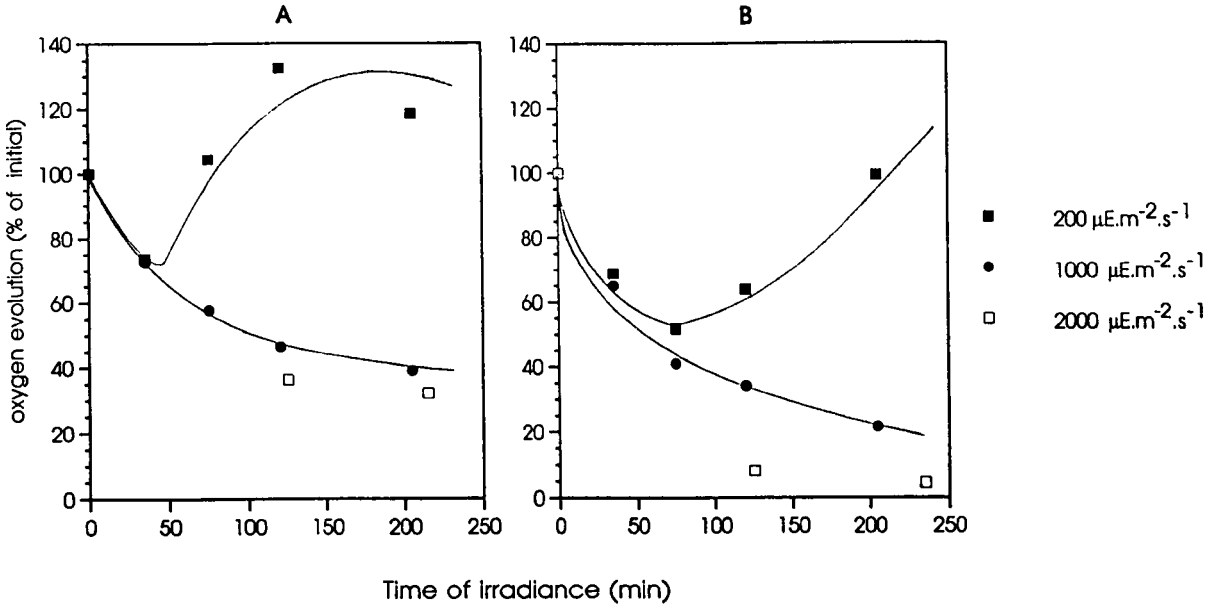


Figure 4.6: Effect of light treatment of different intensities, on the rate of oxygen evolution of wild type (CC125) cells in the absence (A) or presence (B) of 200 μg/ml chloramphenicol (CAP). Cells were suspended in HSM at 25°C, to a chlorophyll concentration of 25 μg/ml. Oxygen evolution was measured using 1 mM DCBQ and 1mM potassium ferricyanide. The 100% rates of oxygen evolution are approximately 337 μmoles O_2· mg chl^{-1}· hr^{-1} for the culture exposed to 200 $\mu E.m^{-2}.s^{-1}$, 320 μmoles O_2· mg chl^{-1}· hr^{-1} for the culture exposed to 1000 $\mu E.m^{-2}.s^{-1}$ and 335 μmoles O_2· mg chl^{-1}· hr^{-1} for the culture exposed to 2000 $\mu E.m^{-2}.s^{-1}$.

The effect of chloramphenicol and lincomycin (both chloroplast protein synthesis inhibitors) in the decline of PS II activity was also tested, by incubating wild type cells at 2000 $\mu E.m^{-2}.s^{-1}$. Although, chloramphenicol is widely used in such experiments, it has been reported that it is a good electron acceptor of photosystem I (PS I) and once reduced, transfers its electron to oxygen (to generate O_2^-) and also that it serves as an inhibitor of photosynthesis (Okada *et al.*, 1991). Because of these side effects, lincomycin (Rintamäki *et al.*, 1994; Zer *et al.*, 1994) and streptomycin (Schnettger *et al.*, 1992) have also been used as chloroplast synthesis inhibitors. The high-light treatment of wild type *C. reinhardtii* cells in the presence of chloroplast protein synthesis inhibitors, carried out in the present work, showed that after 4 hours of illumination, only 5% of PS II activity remained in the cultures containing CAP compared to 25% in the cultures containing lincomycin (not shown). However, despite the possible disadvantages of CAP, it was decided to use this antibiotic as it has been used most in many of the analogous experiments in the literature.

Figure 4.7 shows the results of high light treatments of whole cells from wild type and transformant Nsi16. The two strains showed similar rates of PS II photoinactivation either in the absence (Figure 4.7A) or presence (Figure 4.7B) of CAP. In the absence of CAP,

both strains showed a higher amount of PS II activity remaining (~95%) at the end of the experiment compared with the previous measurements (Figure 4.6). This difference could be attributed to the age of the cultures as cells in the initial experiments were harvested in their late exponential phase (OD_{750} >1) whereas in the subsequent experiments they were harvested while in the initial or middle exponential phase (OD_{750} 0.2-0.6).

When wild type and Leu205Tyr cells were incubated with light of 1000 $\mu E.m^{-2}.s^{-1}$ in the absence of CAP, both strains showed similar photoinactivation patterns (Figure 4.8A). However, different rates of PS II photoinactivation could be observed in the presence of CAP (Figure 4.8B), although the extent of the loss of activity was the same in the two strains. This data indicates that the Leu205Tyr mutant is relatively more susceptible to photoinhibition than the wild type since the initial rate of photoinhibition (after 50 minutes of irradiance) is almost double the rate of the wild type.

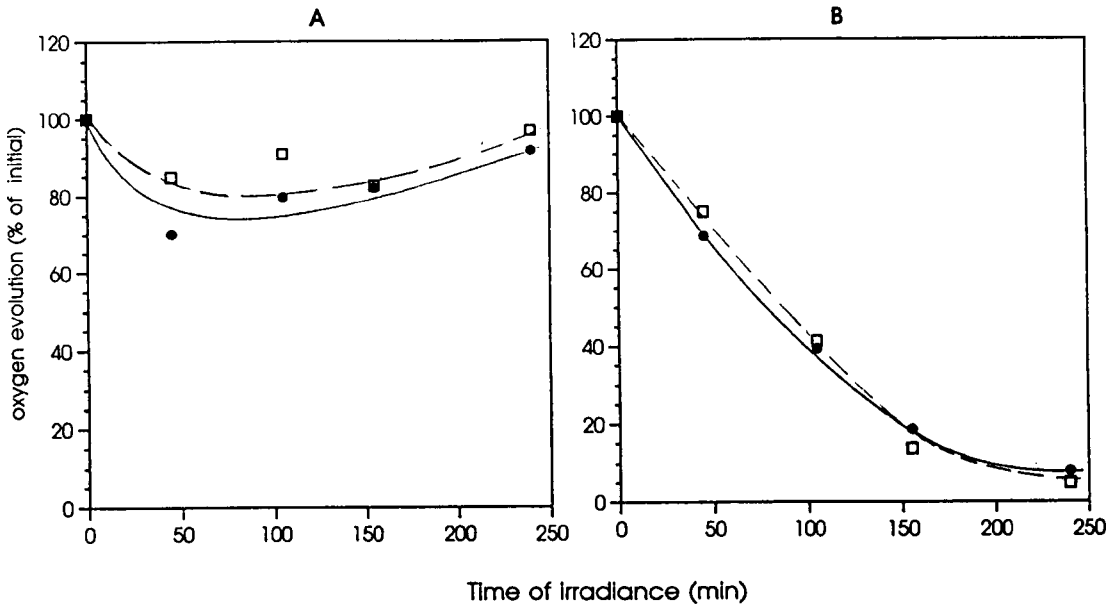


Figure 4.7: Effect of high light treatment (1000 μE.m^{-2}.s^{-1}) on the rate of oxygen evolution from whole cells of wild type (circles) and transformant Nsi16 (wild type, spec^{R}) (squares) in the absence (A) or presence (B) of 200 μg/ml chloramphenicol. Light treatments and oxygen evolution measurements were performed as in Figure 4.6. The 100% rates of oxygen evolution were approximately 219 μmoles O_2· mg chl^{-1}· hr $^{-1}$for the wild type and 356 μmoles O_2· mg chl^{-1}· hr $^{-1}$for Nsi16.

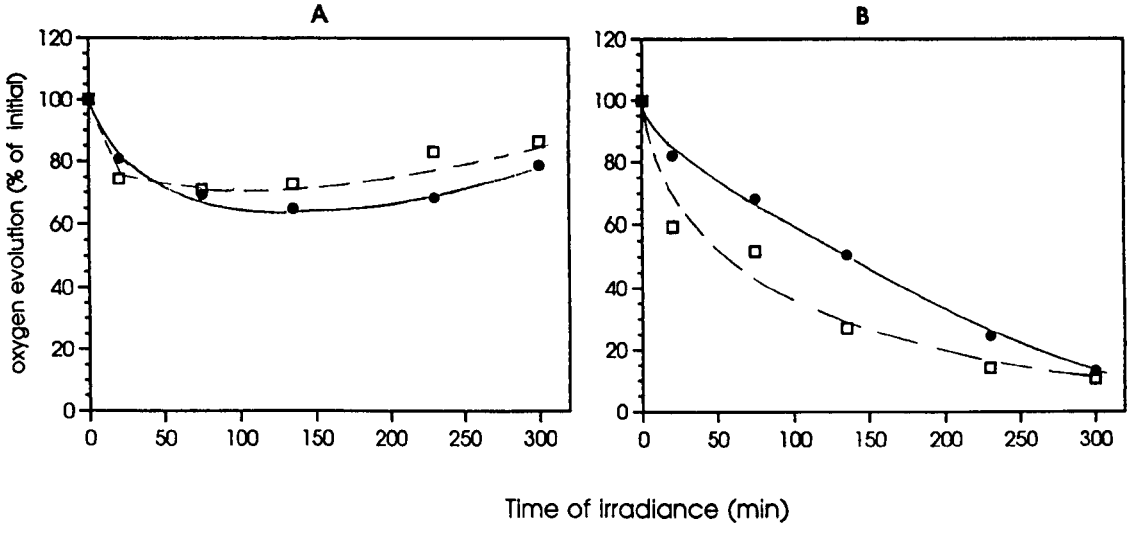


Figure 4.8: Effect of high light treatment (1000 μE.m^{-2}.s^{-1}) on the rate of oxygen evolution from whole cells of wild type (circles) and mutant Leu205Tyr (squares) in the absence (A) or presence (B) of 200 μg/ml chloramphenicol. Light treatments and oxygen evolution measurements were performed as in Figure 4.6. The 100% rates of oxygen evolution were approximately 329 μmoles O_2· mg chl^{-1}· hr $^{-1}$for the wild type and 161 μmoles O_2· mg chl^{-1}· hr $^{-1}$ for Leu205Tyr.

4.2.6 Determination of levels of PS I and PS II proteins in the mutant D2-Leu205Tyr/PS I⁻

Before using the transformants created in a pXba9 background (Xba9 and Leu205Tyr/PS I⁻, see section 3.2.1) for the isolation of PS II RCs, the steady-state levels of D1 and D2 proteins as well as of polypeptides of photosystem I, were determined by SDS-PAGE and Western blotting. Figure 4.9 shows an SDS-PAGE of thylakoid membrane proteins isolated from the wild type, the transformant Xba9 and the Leu205Tyr/PS I⁻ mutant. As controls, thylakoids isolated from the PS I-deficient mutant F54-14 (CC2655), that lacks PS I and ATPase, and pea thylakoids, were also included in the analysis.

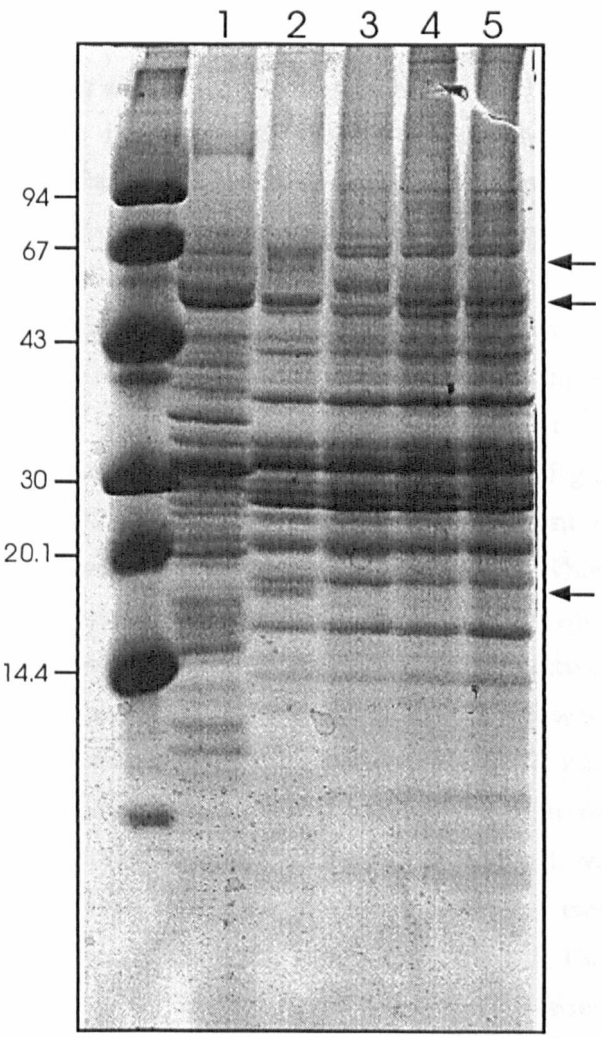


Figure 4.9: Coomassie blue-stained 10-17%, 6 M urea-SDS gel, of thylakoid membrane polypeptides, isolated from peas (lane 1) and from *C. reinhardtii* strains CC125 (wild type) (lane 2), CC2655 (PS I⁻, lacks ATPase) (lane 3), Xba9 (PS I⁻) (lane 4) and Leu205Tyr (Leu205Tyr, PS I⁻) (lane 5). The amount of chlorophyll loaded per lane is 10 μg. The bands of the molecular weight marker are indicated. Areas of difference between lanes, are shown with arrows

The polypeptide analysis of a number of nuclear and chloroplast mutants deficient in PS I from C. *reinhardtii* has shown that all these mutants lack a specific set of PS I polypeptides (Girard *et al.*, 1980; Girard-Bascou *et al.*, 1987; Takahashi *et al.*, 1991). The differences between the three PS I-deficient strains (CC2655, Xba9 and Leu205Tyr/PS I$^-$) and wild type are prominent in two areas: the first is at approximately 62 kDa, where the two subunits of the reaction centre of PS I (PsaA and PsaB) migrate (Chua *et al.* 1975): the photosystem I reaction centre proteins, seen as a diffuse band in the wild type, are not detected in the transformants. The second area is at approximately 18 kDa (indicated by an arrow) where the products of the *psaD* and *psaF* genes (also designated as polypeptides 20 and 21 in C. *reinhardtii*), migrate (Takahashi *et al.*, 1991). This band is absent from the two transformants and strain CC2655. The same polypeptide profile is observed in a PS I$^-$ mutant constructed by Takahashi *et al.* (1991) where the *aadA* cassette has been used to disrupt the *psaC* gene of photosystem I (Takahashi *et al.*, 1991). Other differences (mainly in polypeptides of low molecular weight) between the wild type and the transformants arising from the absence of PS I in the latter cannot be observed in this gel, due to its resolution and the type of staining employed (Coomassie blue G).

The band at approximately 56 kDa (indicated with an arrow), which is absent from CC2655, probably belongs to the α and β subunits of the ATPase complex.

The presence of D1, D2 and the absence of PS I in the transformants, was further confirmed using Western blotting experiments. As can be seen from Figure 4.10, approximately the same levels of the D1 and D2 polypeptides were present in Xba9 (lane 4) and Leu205Tyr/PS I$^-$ (lane 5). The band at approximately 18-19 kDa, which is absent in CC2655, Xba9 and Leu205Tyr/PS I$^-$, has been reported to be made of polypeptides PsaD and PsaF (designated as polypeptides 20 and 21 in C. *reinhardtii*, respectively, Takahashi *et al.*, 1991). The absence of PsaD in the two transformants and CC2655 was confirmed by Western blotting (Figure 4.10) using an antiserum raised against the PsaD polypeptide from higher plants. The apparent molecular masses of the PsaD polypeptides from higher plants and cyanobacteria have been reported to be in the range of 15-22 kDa (reviewed in Bryant, 1992; Golbeck, 1993). There are no reports in the literature about the molecular weight of this subunit in C. *reinhardtii*. However, as can be seen from Figure 4.10, PsaD has a smaller size in *Chlamydomonas* compared to its higher plant counterpart. Western blottings were also performed using antisera raised against the reaction centre proteins and the PsaE subunit of PS I from higher plants. However, these antibodies failed to cross-react with the corresponding polypeptides from *Chlamydomonas*.

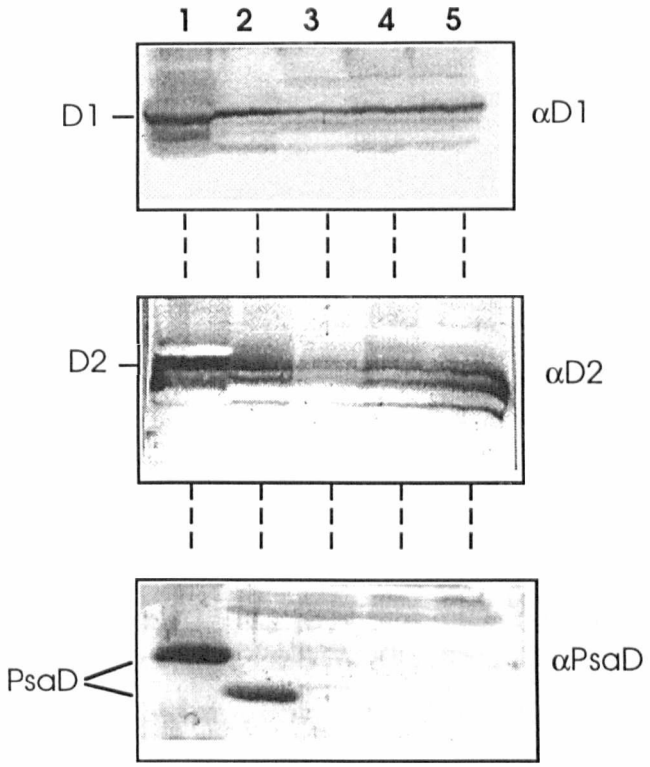


Figure 4.10: Western blots of thylakoid membrane polypeptides, isolated from peas (lane 1) and from *C. reinhardtii* strains CC125 (wild type) (lane 2), CC2655 (PS I⁻, lacks ATPase) (lane 3), Xba9 (PS I⁻) (lane 4) and Leu205Tyr (Leu205Tyr, PS I⁻) (lane 5). Antibodies used are shown next to the blots

4.2.7 Photosystem II reaction centre (RC) preparations

In order to measure the rate of primary electron transfer in the Leu205Tyr mutant, PS II reaction centre (RC) particles were prepared from thylakoid membranes isolated from 20 L cultures.

For the isolation of photosystem II reaction centre particles, the form of the Leu205Tyr mutant that carried the *aadA* cassette downstream of the *psbD* gene, was used (Leu205Tyr/PS I⁻). The rationale was that PS II RC particles could be isolated from the Leu205Tyr/PS I⁻ strain by applying the method of Alizadeh *et al.* (1995). This method employed strain CC2655, which lacks PS I and ATP synthase, for the isolation of PS II RCs.

Starter cultures of 50 ml and 1 litre were grown in TAP containing spectinomycin, before inoculating a 20 litre carboy. The 20 litre culture was grown in TAP medium without spectinomycin for 4-7 days, at room temperature, until it had reached an OD_{750} of 0.4-0.6. After the second day of growth, the optical density (OD) as well as the chlorophyll content of the culture were continuously monitored. Oxygen evolution measurements on whole cells

and plating on minimal medium (HSM) were also conducted before harvesting the culture, in order to confirm the lack of PS I. Moreover, total DNA was isolated from the culture, followed by PCR analysis (and in some cases DNA sequencing) to verify the presence of the mutation.

4.2.7.1 Isolation of reaction centre particles from transformant Xba9

Before attempting to isolate a PS II RC particle from the Leu205Tyr/PS I$^-$ mutant, RCs were isolated from the Xba9 transformant. This was done in order to test the applicability of the method of Alizadeh *et al.* (1995), to a strain with the above characteristics (i.e., lacking PS I but containing the ATPase complex) and also to have a positive control for the biophysical measurements.

A 20 litre culture of Xba9 cells was grown for 6 days at room temperature, until it had reached and OD_{750} of 0.5. Oxygen evolution measurements carried out on whole cells, before harvesting the culture, confirmed the absence of PS I (see section 4.2.2). Unlike wild type, cells from Xba9 could only evolve oxygen when supplied with artificial electron acceptors (DCBQ and ferricyanide). Thylakoid membranes were produced according to the method of Diner and Wollman (1980) (see section 2.21.2). The low chlorophyll *a/b* ratio of 1.8 in the Xba9 thylakoids (compared with a value of approximately 2.1 in the wild type), indicated that this transformant lacked PS I, since this complex binds only chlorophyll *a*. The same ratio has been reported for whole cells and thylakoids from *C. reinhardtii* CC2655 (which also lacks PS I) (Diner and Wollman, 1980) and for the PS I-deficient mutant of Takahashi *et al.* (1991).

Spectroscopically pure PS II RCs from transformant Xba9 were isolated according to the method of Alizadeh *et al.* (1995), with the exception that 1.7% Triton X-100 was used to solubilise the thylakoid membranes. Under the conditions used (see section 2.24) the yield of chlorophyll was approximately 0.18% (relative to the total amount of chlorophyll contained in the thylakoids used as starting material).

The conditions and final yield of this PS II RC preparation are very similar to the ones reported by Alizadeh *et al.* (1995) for the corresponding preparation from *C. reinhardtii* CC2655. The conditions used in that method employed 1.5% Triton X-100 for the solubilisation of thylakoid membranes and the final yield of the preparation was approximately 0.16% (Alizadeh *et al.*, 1995).

The isolated PS II RC complex from transformant Xba9 (Figure 4.11) was spectroscopically identical to the preparation of Alizadeh *et al.* (1995) and also to the isolated PS II RC from peas (Figure 4.13). In all *Chlamydomonas* samples the absorption maximum of the red peak was at 676 nm. The ratio of Chl *a* per Pheo *a* was found to be 6.1 Chl *a* per 2 Pheo *a*. Oxidised minus reduced difference spectra indicated that there were

approximately 1.17 cytochrome b_{559} per 2 Pheo *a*. The isolated PS II RC from pea contains approximately 6 chl *a* per 2 pheo *a* per 1 cyt b_{559} (reviewed in Satoh, 1993).

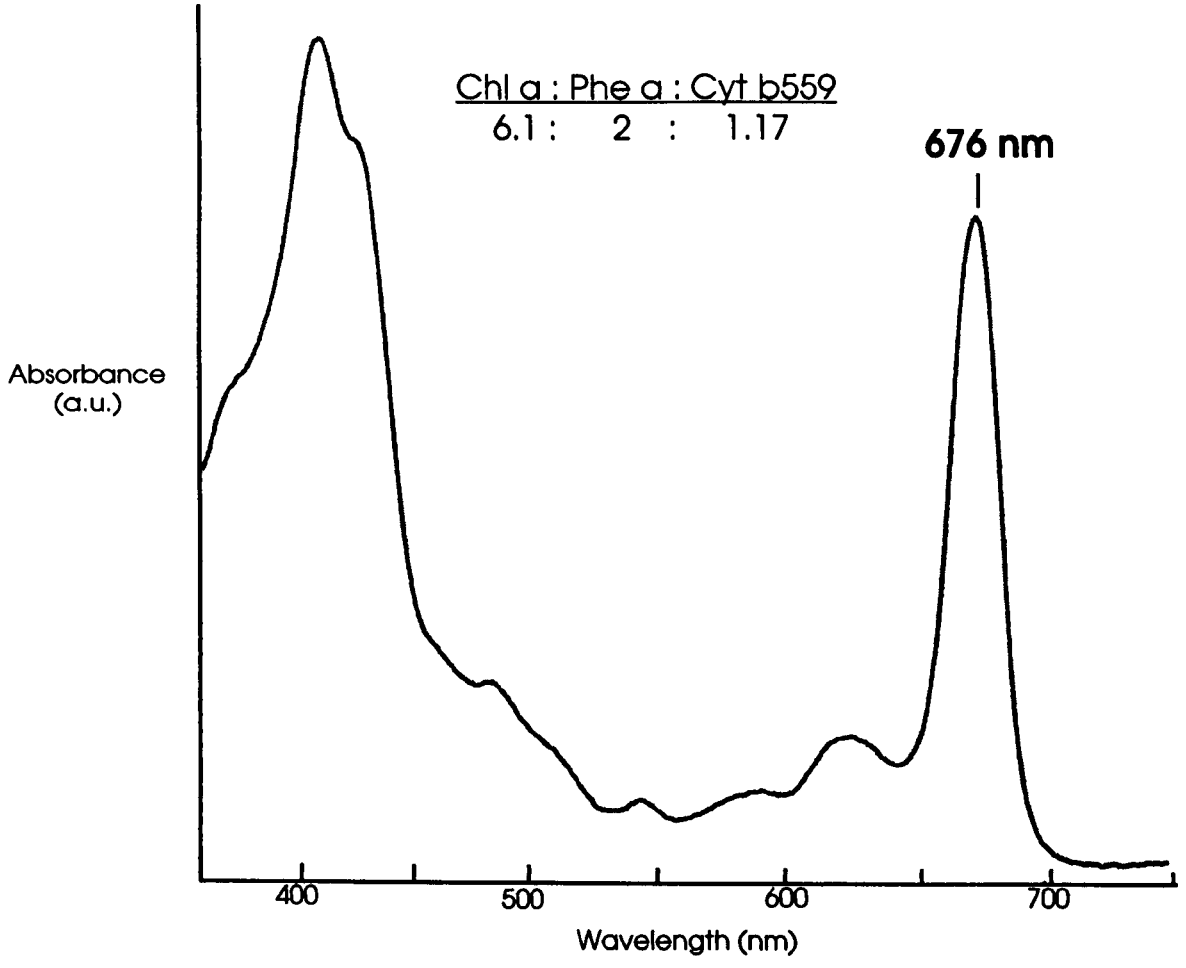


Figure 4.11: Absorbance spectrum of the isolated PS II RC from transformant Xba9 at 10°C

Figure 4.12 shows a comparison of the polypeptide composition of PS II RCs isolated from peas (lane 1), *C. reinhardtii* CC2655 (lane 2) and transformant Xba9 (lane 3). Apart from the five polypeptides constituting the PS II RC (D1, D2, α and β subunits of cytochrome b_{559} and PsbI polypeptide), a relatively high contamination with other polypeptides can be seen in the lane of Xba9 (although, the amount of Xba9 PS II RC loaded in the gel is 5 times more, on a chlorophyll basis, compared to the pea and *C. reinhardtii* CC2655). It is not possible to assign these bands to specific polypeptides directly from the gel, although presumably several may arise from subunits of the ATP synthase complex. For this purpose, a Western blot using various antibodies would be more appropriate. However, since these polypeptides did not seem to bind any chlorophylls, it was decided that identification of these bands at this stage was not necessary.

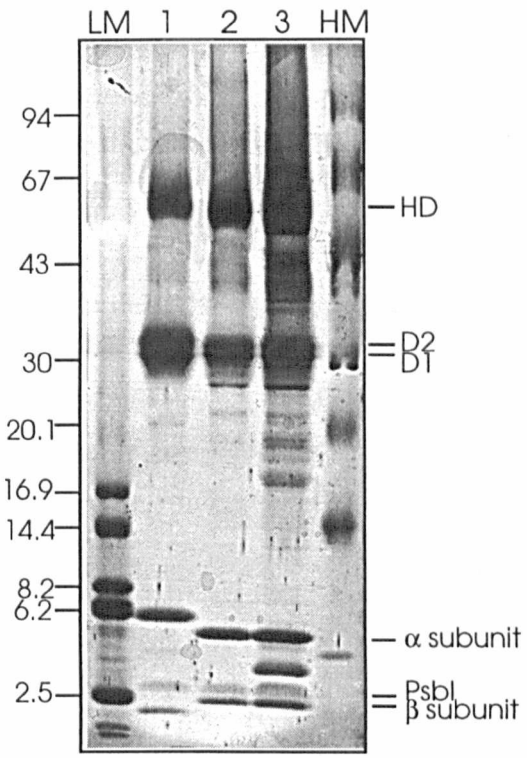


Figure 4.12: Silver-stained SDS-PAGE of isolated PS II RCs from pea (lane 1), *C. reinhardtii* strain CC2655 (lane 2) and transformant Xba9 (lane 3). The amount of chlorophyll was 0.2 μg for pea and CC2655 and 1 μg for Xba9. Lanes LM and HM represent the low and high molecular weight markers, respectively. HD indicates the heterodimer of D1 and D2. α and β subunits refer to cytochrome b_{559}.

4.2.7.2 Isolation of reaction centre particles from the D2-Leu205Tyr/PSI⁻ mutant

For the preparation of PS II RC particles from the Leu205Tyr/PS I⁻ mutant, 20 litre cultures were grown in TAP medium to late exponential phase (OD_{750} of 0.4-0.6) and thylakoids produced in a similar manner to the Xba9 transformant.

Similarly to Xba9, oxygen evolution measurements of the cultures just before harvesting, confirmed the absence of PS I, as cells could evolve oxygen only in the presence of artificial acceptors (DCBQ and ferricyanide) (section 4.2.2).

In addition, PCR analysis (section 3.2.2.4) and sequencing (data not shown) of DNA isolated from the culture, revealed the presence of the Leu205Tyr mutation.

Despite these results, the chlorophyll *a/b* ratio was found to be relatively high, between 2.5 and 2.7, for a PS I-deficient mutant.

Attempts to isolate a photosystem II reaction centre complex from the Leu205Tyr/PS I⁻ mutant, were based on the conditions outlined in the previous section for transformant Xba9. Trial experiments were carried out in order to establish the optimum protocol for purification of PS II RCs. The various preparations obtained were judged for their purity by their absorbance spectra. The conditions that were modified compared to the protocol used for Xba9, are shown in Table 4.2.

Table 4.2: Conditions changed from the original protocol, during trial preparations (starting from 6-10 mg chlorophyll) of PS II RCs from the Leu205Tyr/PS I⁻ mutant. Buffer A contained 20 mM NaCl in 0.2% Triton X-100, 50 mM Tris pH 7.2 and 1 mM DM. Buffer B was 0.2% Triton X-100, 50 mM Tris pH 7.2 and 1 mM DM.

%Triton X-100	Solubilisation period (hours)	Column wash buffer	NaCl gradient (mM)	Result[1]
2.3	2	A	20-150	CP
2.1	2	B	0-150	CP
1.9	2	A	20-150	RC
1.7	2	A	20-150	CP

[1] Reaction centre (RC) complexes had an A417:A435>1, whereas core particles (CP) had an A417:A435<1.

These attempts did not result in the isolation of a pure PS II RC complex. The absorbance spectrum of the best PS II RC preparation achieved is shown in Figure 4.13. This RC preparation has been isolated using essentially the same procedure as for Xba9 with the exception of the higher amount of Triton X-100 (1.9%) added to the solubilisation buffer (instead of 1.7% used for Xba9). The average chlorophyll yield (approximately 0.10%) obtained using these conditions was lower compared to Xba9 and CC2655 PS II RC preparations (see section 4.2.7.1).

The absorption maximum at the red peak of that RC preparation was at 673 nm. The ratio of absorbance at 417 to 435 nm was low (approximately 1.06) compared to the pea RC (1.14) and to the RC from Xba9 (1.15) (Figure 4.13). The A417:A435 ratio is a measure of the quality of a preparation in terms of presence of extra chlorophylls. Chlorophyll *a* absorbs more at 435 nm than at 417 nm (Lichtenthaler, 1987). On the other hand, pheophytins absorb at 410 nm and cytochrome b_{559} in its oxidised form, which represents more than 70% of cytochrome present in the RC (Ahmad *et al.*, 1993), absorbs at 415 nm (Wasserman, 1980). The presence of some residual chlorophyll *b* in the absorbance spectrum of the Leu205Tyr/PS I⁻ mutant can also be seen at 450 nm and 640 nm (Figure 4.13).

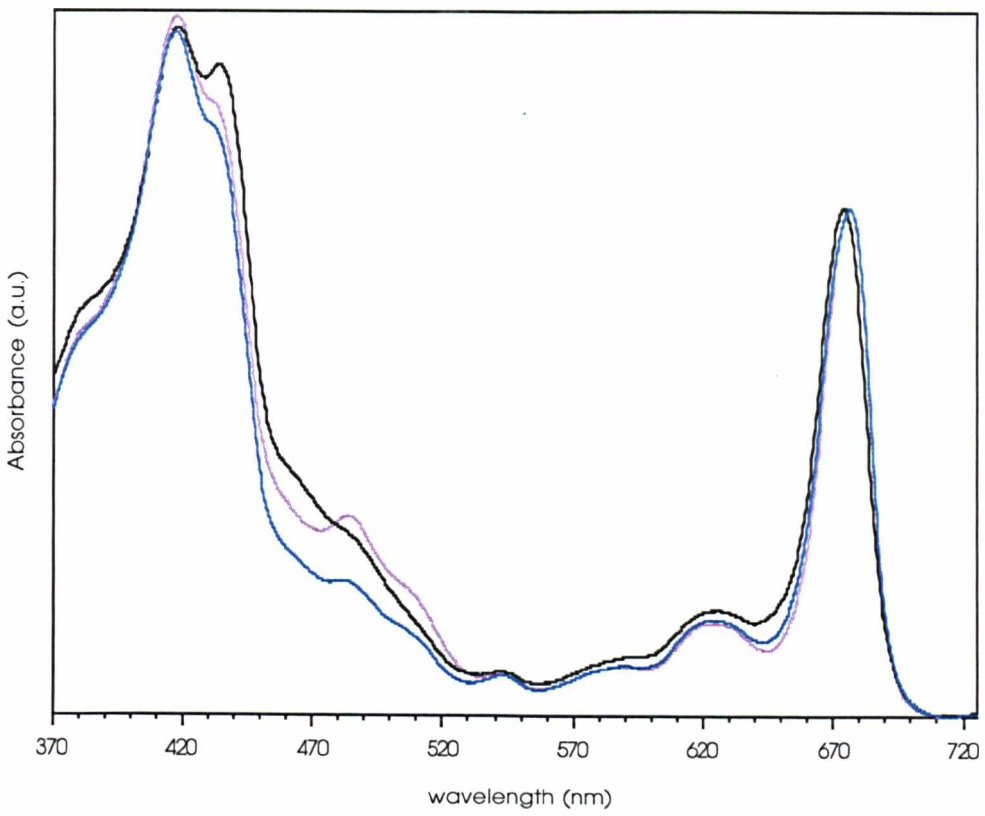


Figure 4.13: Absorption spectra of a PS II RC preparation from the Leu205Tyr/PS I⁻ mutant (black trace), and of RCs isolated from transformant Xba9 (blue trace) and from pea (purple trace)

The ratio of Chl *a* per Pheo *a* was found to be 7.4 Chl *a* per 2 Pheo *a*. Oxidised minus reduced difference spectra indicated that there were approximately 8.7 Chl *a* per 1 cytochrome b_{559}.

4.2.8 Measurements of Pheo⁻ and P680⁺ photoaccumulation

As mentioned in section 1.2.2.2, the photochemical activity of the isolated PS II RC is restricted to primary charge separation which is quickly reversed by a charge recombination reaction (reviewed in Seibert, 1993). However, charge stabilisation and light-induced electron transfer are possible if suitable electron donors and acceptors are added. In the presence of an efficient electron donor, such as sodium dithionite, it is possible to photoaccumulate reduced pheophytin (Pheo⁻) (Nanba and Satoh, 1987; Barber *et al.*, 1987). On the other hand, when isolated PS II RCs are incubated with an electron acceptor, such as silicomolybdate, light-induced absorbance changes can be observed which are attributed to the photoaccumulation of P680⁺ (Barber *et al.*, 1987). In order to test whether the absorption properties of Pheo⁻ and P680⁺ were modified in the isolated PS II RCs from mutant Leu205Tyr/PS I⁻, PS II RC complexes isolated from Xba9 (wild type control) and Leu205Tyr/PS I⁻ were illuminated in the presence of either sodium dithionite or

silicomolybdate. The absorbance difference spectra obtained from the two strains were then compared to each other and to corresponding spectra from PS II RCs isolated from pea and *C. reinhardtii* CC2655. The ground state absorbance spectra of the PS II RC complexes used in these measurements are shown in Figure 4.13.

The light-dark absorbance spectra for the Xba9 and Leu205Tyr/PS I$^-$ strains, obtained after illumination of isolated PS II RC complexes in the presence of sodium dithionite, were typical of pheophytin reduction (Figure 4.14). All the spectra were similar to analogous spectra from pea (Barber *et al.*, 1987) and *C. reinhardtii* CC2655 (Alizadeh *et al.*, 1995) RCs. In the case of measurements in the blue region (350 to 600 nm, Figure 4.14A, B), the absorbance difference spectrum for the isolated PS II RC from Xba9 showed characteristic negative peaks at 422, 450, 515 and 544 nm (Figure 4.14A). The equivalent negative peaks for the PS II RC from Leu205Tyr/PS I$^-$ were at 420, 445, 515 and 544 nm. Phe$^-$ photoaccumulation measurements in the red region (650 to 850 nm) were performed only for the Leu205Tyr/PS I$^-$ mutant (because of lack of available material from Xba9). The light-dark difference spectrum was obtained with a minimum at 678 nm. The corresponding spectrum for a wild type PS II RC from *C. reinhardtii* CC2655 shows a maximum bleach at 682 nm (Alizadeh *et al.*, 1995). The activity of the samples used in these measurements were compared by monitoring the absorbance changes at 450 nm which is indicative of the Pheo anion production (Klimov *et al.*, 1977). Thus, Xba9 PS II RCs were found to accumulate a similar amount of Pheo$^-$, on a chlorophyll basis, as the PS II RCs from peas and *C. reinhardtii* CC2655. On the other hand, the isolated PS II RCs from the Leu205Tyr/PS I$^-$ mutant showed reduced activity by ~14% compared to Xba9 (after taking into account the extra chlorophyll content in the PS II RC of the Leu205Tyr/PS I$^-$ mutant).

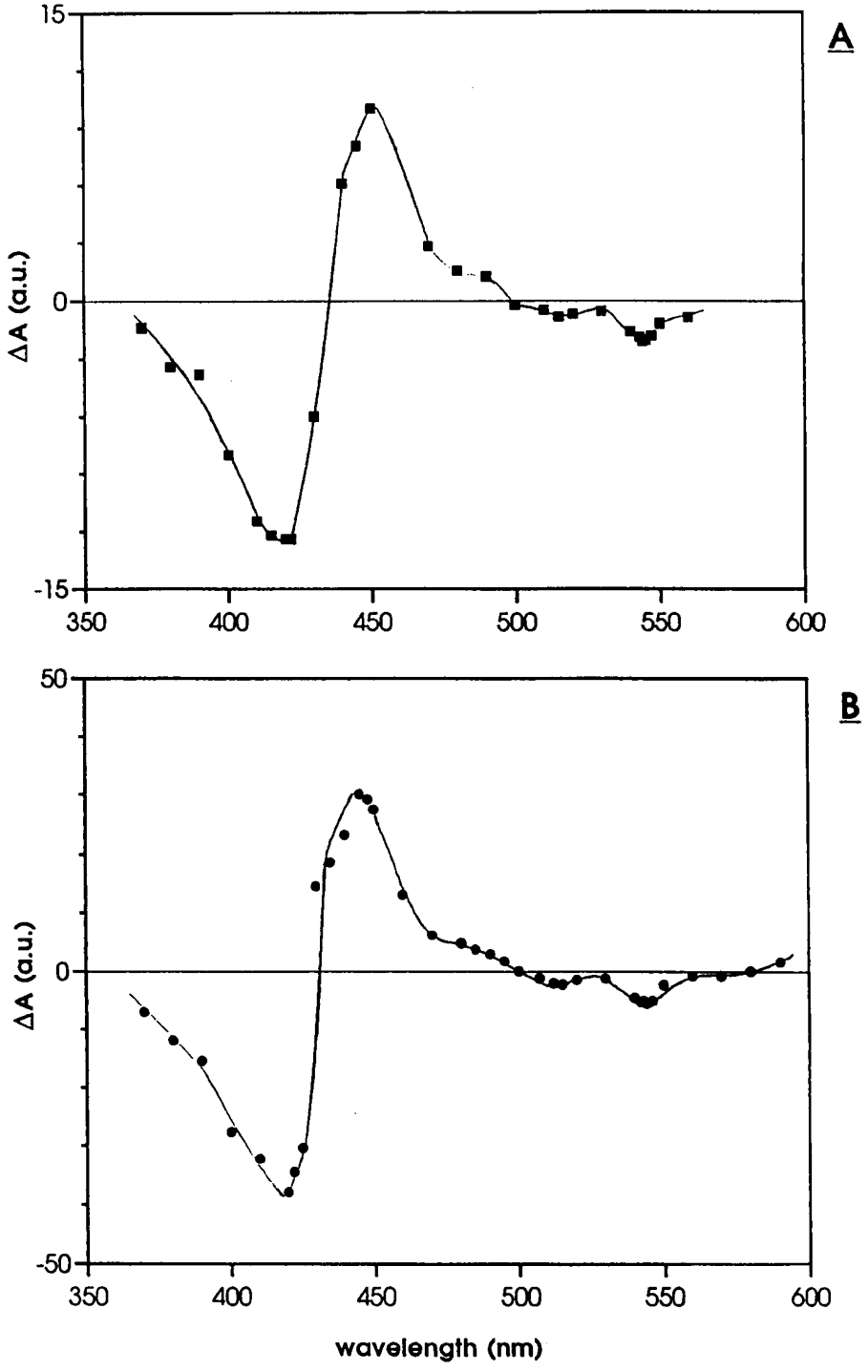


Figure 4.14: Light-dark difference spectra in the blue region (350-600 nm) of PS II RCs isolated from transformant Xba9 (A) and mutant Leu205Tyr/PS I⁻ (B). Spectra were obtained at 9°C in the presence of sodium dithionite in 50 mM MES/2 mM DM. Chlorophyll content was 1.8 μg for Xba9 and 5.5 μg for Leu205Tyr/PS I⁻.

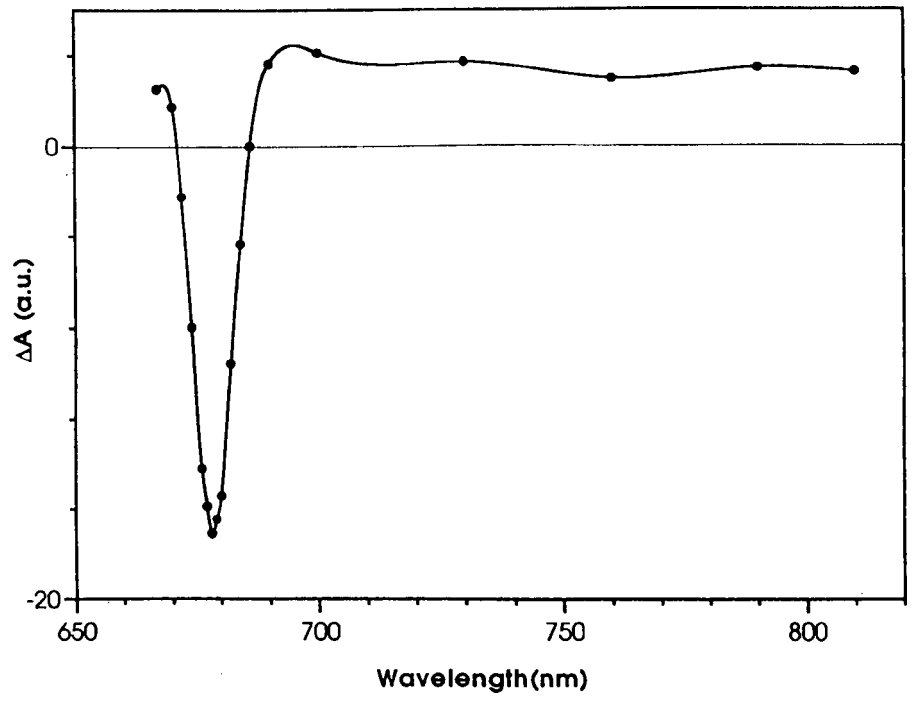


Figure 4.15: Light-dark difference spectrum in the red region (650-800 nm) of a PS II RC complex isolated from the Leu205Tyr/PS I⁻ mutant. Spectrum was obtained at 9°C in the presence of sodium dithionite in 50 mM MES/2 mM DM. Chlorophyll content was 5.5 μg.

The $P680^+$ photoaccumulation measurements were carried out under anaerobic conditions using an enzymatic oxygen trap (De Las Rivas *et al.*, 1993) to protect P680 against photodamage. When the PS II RC complexes from transformant Xba9 and mutant Leu205Tyr/PS I⁻ (see section 4.2.7.2) were illuminated in the presence of the electron acceptor silicomolybdate (SiMo), reversible light-induced changes, due to photoaccumulation of $P680^+$, were detected. Samples were preilluminated for 1 minute in order to bleach β-carotene and therefore, obtain the maximum reversible absorption change, before starting the measurements (Telfer *et al.*, 1990). The absorption difference spectrum obtained for the PS II RC isolated from Xba9, when illuminated in the presence of SiMo, was quite similar to analogous spectra from peas and *C. reinhardtii* CC2655 (Telfer *et al.*, 1990 and Alizadeh *et al.*, 1995, respectively). The reversible light-induced absorption changes were maximal at 680 nm and also showed a shoulder at approximately 670 nm (Figure 4.16A). This shoulder has been suggested by Telfer *et al.*, (1990) to arise from the oxidation of an accessory chlorophyll (Chl 670) due to donation of an electron to $P680^+$. The ratio of Chl/$[P680^+]$ was ~5.7, similar to pea and *C. reinhardtii* CC2655 PS II RCs.

The corresponding absorption difference spectrum from mutant Leu205Tyr/PS I⁻ showed a shift to the blue compared to Xba9. The maximum light-minus-dark absorption

change in the red was obtained at 678 nm (instead of 680 nm) suggesting that the optical properties of P680 might be modified in this mutant (Figures 4.16B and 4.17). Moreover, the shoulder at 670 nm was lost. By using an extinction coefficient of 75 $mM^{-1}.cm^{-1}$ at 680 nm, the ratio of Chl/[$P680^+$] in the PS II RCs isolated from the Leu205Tyr/PS I^- mutant was approximately 6.9, compared to ~5.7 for Xba9. In agreement with the $Pheo^-$ photoaccumulation assay, this value indicated a 20% reduction in the photochemical activity of the Leu205Tyr/PS I^- mutant PS II RCs compared to Xba9 (after taking into account the extra chlorophyll content in the PS II RC of the Leu205Tyr/PS I- mutant).

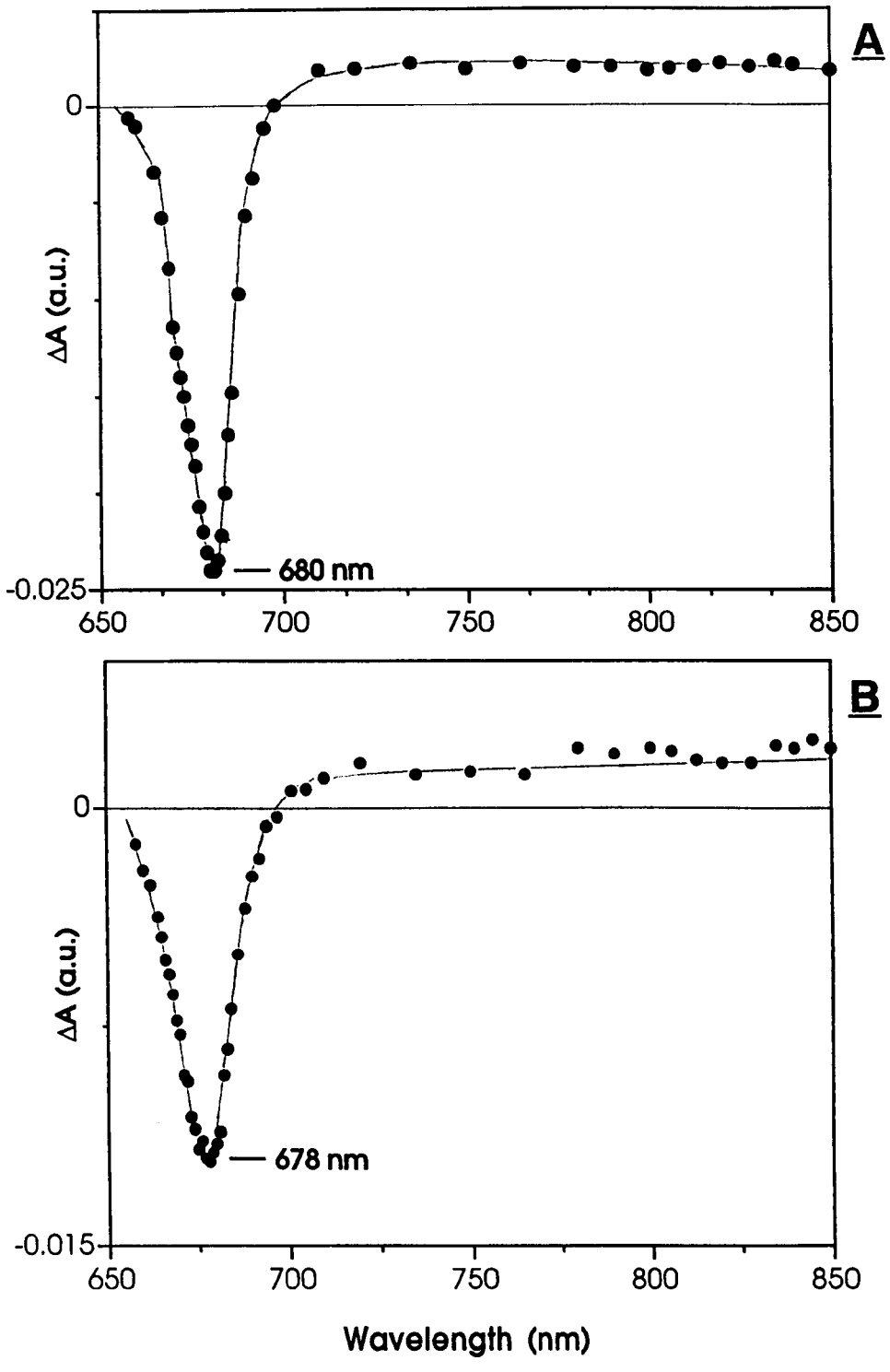


Figure 4.16: Light-dark difference spectra of isolated PS II RC preparations from transformant Xba9 (A) and mutant Leu205Tyr/PS I⁻ (B), obtained in the presence of SiMo at 9°C and under anaerobic conditions. The slit width was 2.0 nm. Samples were resuspended in Borate pH 8.0 /2 mM DM. Chlorophyll content was 2.25 μg for Xba9 and 1.85 μg for Leu205Tyr/PS I⁻.

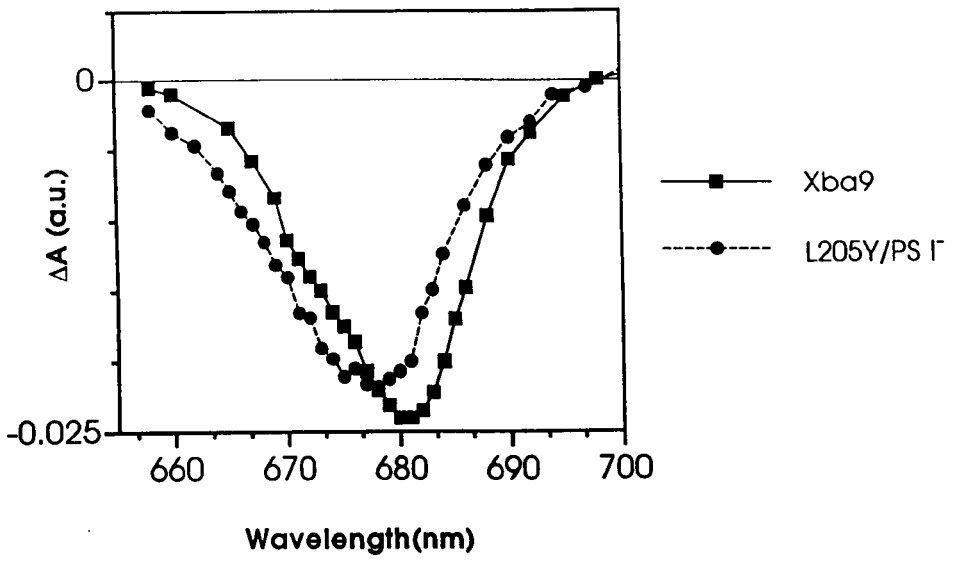


Figure 4.17: Comparison of spectra shown in Figure 4.16 in the area 655-700 nm (spectra are normalised to the same chlorophyll content).

4.2.9 Primary charge separation measurements

Femtosecond transient absorption measurements and data analysis were performed as described in section 2.28. The charge separation process was monitored in both the Q_x band of pheophytin (544 nm) and in the Q_y (680 nm) absorbance regions. Amongst the several different absorption changes that have been found to accompany charge separation in PS II, the bleaching of the pheophytin Q_x absorption band has been most unambiguously assigned to primary charge separation (Hastings *et al.*, 1992; Durrant *et al.*, 1993).

These measurements were carried out for Xba9 and the Leu205Tyr/PS I⁻ mutant using the PS II RC preparations described in section 4.2.7. Data were obtained using a multi-channel detector (Rech *et al.*, 1994). The data were collected with the polarisation of the probe beam rotated by 54.7^0 relative to the pump (the magic angle configuration) (Giorgi *et al.*, 1994). Figure 4.18 shows the transient absorption spectra obtained in the region of the Q_x absorption band. These spectra represent the amplitudes of two kinetic components resolved following excitation with 694 nm pulses of PS II RCs isolated from Xba9 and mutant Leu205Tyr/PS I⁻. The solid line represents the spectrum of a component which does not decay on a 0-70 ps time scale and which is assigned to the radical pair state $P680^+Pheo^-$. The dotted line shows the spectrum of a component which is assigned to the formation of the radical pair state. In the case of the isolated PS II RC from Xba9, this component had a lifetime of 21±2 ps. Bleaching of the pheophytin Q_x band was observed at 544±0.4 nm. The lifetime for charge separation as well as the spectral properties associated with that, are almost

identical to that obtained from PS II RCs isolated from peas and *C. reinhardtii* CC2655 (Giorgi *et al.*, 1994).

In the case of the PS II RC from the Leu205Tyr/PS I$^-$ mutant, the component associated with the formation of the radical pair (dashed line in Figure 4.18) had a lifetime of 38±5 ps. The amplitude and shape of this component appears to be identical to that of the 21±2 ps component as measured in the Xba9 PS II RCs. Therefore, the rate of primary charge separation in the mutant can be described by a 38 ps lifetime. Recently, Klug *et al.* (1995), using PS II RCs isolated from peas, observed a 3 ps component in addition to the 21 ps component associated with charge separation. Although, this component was found to contribute to charge separation, its amplitude was considerably lower than that of the 21 ps component (Klug *et al.*, 1995). The measurements using PS II RCs from Leu205Tyr/PS I$^-$ revealed that the amplitude of the 3 ps component was essentially zero across the whole of the Q_x absorption bands (Figure 4.18). Moreover, the amplitude of the non-decaying component assigned to the radical pair state P680$^+$Pheo$^-$ (solid line in Figure 4.18) showed a positive shift compared to the corresponding one from Xba9. The bleaching of the pheophytin Q_x band showed a negative peak at 543.5±0.4 nm.

The quantum yield of radical pair formation in the mutant PS II RC was estimated applying a simulation technique used by Giorgi *et al.* (1996) to estimate the radical pair yields of *Synechocystis* PCC 6803 wild type and mutant PS II RCs. The negative peak observed at 580 nm represents bleaching of the chlorophyll Q_x absorption band. The ratio between the features at 544 nm and 580 nm of the non-decaying component can be used to estimate the yield of radical pair formation. The estimated yield of radical pair formation at 60 ps in PS II RCs isolated from Xba9 was found to be similar to that observed for PS II RCs isolated from peas. However, the corresponding value for the PS II RC from the Leu205Tyr/PS I$^-$ mutant was consistent with a reduced yield (50%) of radical pair formation in the RC of the mutant relative to Xba9.

Primary charge separation measurements in the Q_y absorption region were only carried out for the Leu205Tyr/PS I$^-$ mutant, due to lack of available material from Xba9. These measurements showed that the component associated with the formation of the radical pair state P680$^+$Pheo$^-$ had a lifetime of 42±5 ps (data not shown). The bleaching of the radical pair in the Q_y band of the mutant was at 679.6±0.5 nm. The region which lies around 735 nm in the Q_y absorbance difference spectrum can be associated with charge separation (Klug *et al.*, 1995). The degree of formation of the radical pair is directly proportional to the magnitude of the -ΔOD in this region. In the isolated PS II RC from pea the ratio between the 3 and the 21 ps components is approximately 1:3 at 735 nm (Klug *et al.*, 1995). In the PS II RC from Leu205Tyr/PS I$^-$ the ratio between the 3 ps and the 42 ps components is approximately 1:7 at 735 nm. This result is consistent with the minimal amplitude of the 3 ps component observed in the Q_x region (Figure 4.18).

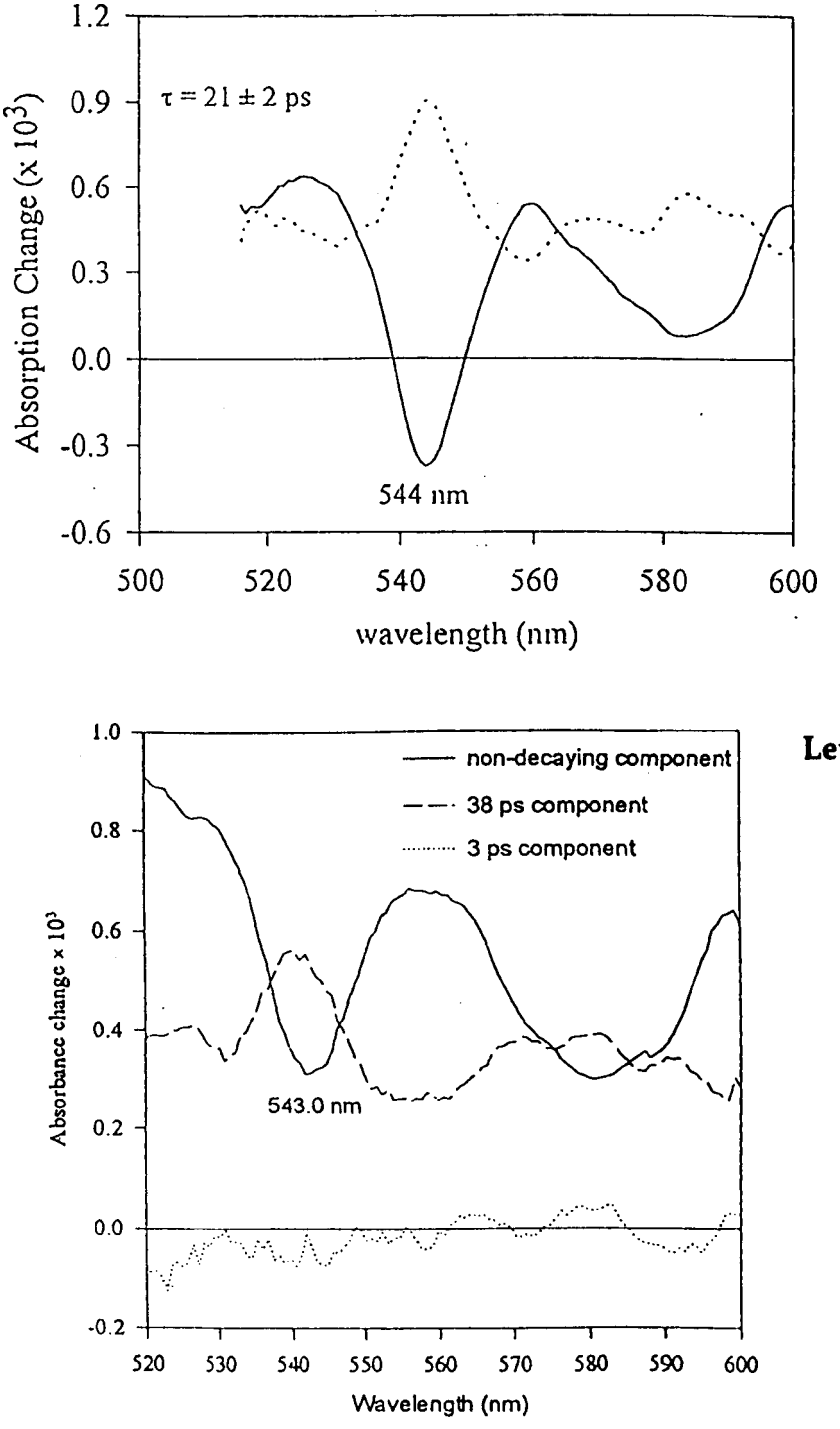


Figure 4.18: Spectra of the amplitudes of different kinetic components in the region of the Q_x absorption band resolved following excitation of PS II RCs isolated from transformant Xba9 and mutant Leu205Tyr/PS I⁻. The various kinetic components are described in detail in the text.

The measurements were carried out by members of the Molecular Dynamics Group, Imperial College

4.3 Discussion

Following their genetic characterisation, the two versions of the Leu205Tyr mutant were analysed using biochemical and biophysical techniques that would define their photosynthetic phenotype. The version of the mutant that was created in a pNsi16 (wild type, $spec^R$) background was used for physiological measurements whereas the mutant created in a pXba9 (PS I^-, $spec^R$) background was used for the isolation of PS II RC complexes and the measurement of primary electron transfer rates. "Wild type" control transformants (Nsi16 and Xba9) were also included in most of the measurements in order to ensure that the phenotype observed in the mutants could be directly attributed to the presence of the Leu205Tyr mutation only. The Nsi16 transformant, containing the *aadA* cassette upstream of the *psbD* gene, behaved very similarly to the wild type in all the assays carried out. Before being used in any experiments, cells were grown in liquid cultures without spectinomycin as the latter has been reported to partially inhibit chloroplast protein synthesis even in spectinomycin-resistant strains of *C. reinhardtii* (Monod *et al.*, 1994).

4.3.1.1 in vivo measurements

The Leu205Tyr mutant was able to grow photosynthetically at wild type rates, as shown by photoautotrophic growth experiments in liquid cultures. In the presence of artificial electron acceptors (DCBQ and ferricyanide), the mutant was able to evolve oxygen but at reduced rates (~60%) compared to wild type and transformant Nsi16. The reduction in oxygen evolution probably reflects a reduction in the number of PS II centres, although an effect on quantum yield cannot be excluded. The similarity in the growth rates between the wild type and the Leu205Tyr mutant could be because the light intensity during the growth experiment (50-70 $\mu E.m^{-2}.s^{-1}$) was not at limiting levels and therefore PS II activity was not the limiting factor in the growth of the mutant.

Oxygen evolution measurements of whole cells from the two strains lacking PS I (Xba9 and Leu205Tyr/PS I^-) showed that they could only evolve oxygen in the presence of artificial electron acceptors. This property was used as an indication of the absence of PS I from Xba9 and Leu205Tyr/PS I^-. Greenbaum *et al.* (1995) have reported photoevolution of oxygen from a PS I-deficient mutant of *C. reinhardtii* grown in liquid media under anaerobic conditions (either in pure helium or in helium containing CO_2). This mutant (B4) was also able to evolve hydrogen and photoassimilate CO_2 (when grown in CO_2-containing helium). However, when grown aerobically and at saturating light intensities, the mutant lost its photosynthetic activity within 24 hours. Thus, the authors concluded that PS I is required for stable oxygenic photosynthesis in aerobic environments but not under anaerobic conditions (Greenbaum *et al.*, 1995). The growth conditions of the two PS I-deficient strains used in this thesis were different to the ones used by Greenbaum *et al.* (1995), therefore, direct links

between the findings of this work and those of Greenbaum *et al.* (1995), concerning the photosynthetic capability of strains lacking PS I, cannot be made. Recently, Greenbaum and colleagues analysed the photoautotrophic growth of an additional PS I$^-$ mutant of *C. reinhardtii* (the F8 mutant) as well as B4 (Lee *et al.*, 1996). The authors presented data indicating photoautotrophic growth of both B4 and F8 mutants in minimal medium under both low and high light intensities (although, only for a few days under high light) and concluded that PS I is not required for photoautotrophic growth. However, both the B4 and F8 mutants are of nuclear origin and their exact genotype is not known. Indeed, Western blottings using antibodies against PsaA have recently revealed that there may be some of this protein still present in the two mutants (K. Redding, personal communication). Greenbaum's results are obviously in contrast with the inability of the Xba9 transformant to grow photoautotrophically in the absence of acetate. Considering that Xba9 carries a well-defined mutation in the chloroplast genome, our results indicate that PS I is an obligatory component for photoautotrophic growth of *Chlamydomonas reinhardtii.*

In contrast to the reduced oxygen evolving activity of the Leu205Tyr mutant relative to Nsi16, in the presence of acceptors, the PS I-deficient strain, Leu205Tyr/PS I$^-$, showed similar rates of oxygen evolution to Xba9. It has been reported in the literature (Gong and Ohad, 1991) that strains lacking PS I are partially protected against PS II photoinactivation. Therefore, photoinhibition measurements were carried out using mutant Leu205Tyr to test whether the apparent decrease in the oxygen-evolving capacity of this mutant could be linked with an enhanced susceptibility to photoinhibition. Thus, light treatments of whole cells were performed and the rate of the loss of PS II activity was followed by measuring the rates of electron transport from water to DCBQ. The Leu205Tyr mutant showed a very similar rate of light-induced PS II inactivation to the wild type and Nsi16 in the absence of a chloroplast protein synthesis inhibitor (chloramphenicol, CAP). In the presence of CAP a slightly higher rate of PS II photoinactivation could be observed in the mutant compared to Nsi16, although, the final extent of the loss of activity was similar to the wild type and Nsi16. CAP blocks chloroplast protein synthesis, therefore, the loss of PS II activity observed in the presence of this inhibitor, is a monitor of the damage to PS II that would otherwise be repaired by *de novo* protein synthesis (Komenda and Barber, 1995).

The kinetics on the acceptor side (oxidation of Q_A^- by Q_B) and the charge recombination with the donor side were studied using fluorescence decay measurements of whole cells. These measurements did not show any serious disturbances in the function of either the donor or acceptor side. However, using the variable fluorescence, F_v, as a probe for PS II activity (Gong and Ohad, 1991), a slightly decreased amount of functional PS II centres was estimated to be present in the mutant (by 15-20%). This result is in line with the reduced rate of oxygen evolution. The higher F_0 value in the Leu205Tyr mutant compared to the wild type, observed in the fluorescence measurements, indicates a decrease in the photochemical quenching of fluorescence (by PS II) in the mutant. A number of situations could give rise to an

increased F_0 value: (a) excitonic uncoupling of the antenna molecules from the reaction centre, (b) the presence of non-functional reaction centres associated with the antenna and (c) a slowdown in the primary charge separation process. The last possibility was examined in the femtosecond measurements on PS II RCs isolated from the Leu205Tyr/PS I$^-$ mutant.

The acceptor side of PS II was studied in more detail using thermoluminescence measurements of whole cells. The total intensity of thermoluminescence in the mutant was found to be considerably lower relative to the wild type and Nsi16. This lower TL intensity may reflect a lower amount of functional PS II centres in the mutant and also a reduction in the quantum yield of charge separation in PS II. This interpretation of the TL data is supported by the results obtained from the oxygen evolution and fluorescence measurements. The fact that the peak positions of the B and Q bands appeared at their normal characteristic temperatures in the mutant shows that the mutation did not cause any significant perturbations to the Q_A and Q_B sites. This is as expected as the residue at position 205 of D2 should point into the region occupied by P680, Pheo and the accessory chlorophyll located in the lumenal side of the thylakoid membrane (Ruffle *et al.*, 1992).

The chlorophyll *a/b* ratio in the L205Y/PS I$^-$ mutant (both in whole cells and thylakoid membranes) was found to be high for a PS I-deficient mutant (2.5-2.7 compared to 1.8 for Xba9). This result implied that the ratio between the PS II RCs (which bind only chlorophyll *a*) and LHCII (which bind both chlorophyll *a* and chlorophyll *b*) is probably altered in favour of the former and may indicate loss of LHCII from the mutant, although, a reduction in the levels of LHCI cannot be ruled out. The same ratio was obtained with another independent clone carrying the same mutation. The reason for the apparent reduction in LHCII content is unclear but may be related to effects on PS II photochemistry.

4.3.1.2 in vitro measurements

The primary electron transfer and the absorption characteristics of the PS I-deficient strains were analysed using isolated PS II RC complexes from the Leu205Tyr/PS I$^-$ mutant and from transformant Xba9 (as a PS II-"wild type" control).

The PS II RC particles isolated from transformant Xba9 had very similar spectroscopic characteristics to PS II RCs from peas and *C. reinhardtii* CC2655. Primary charge separation measurements using these RCs indicated that the radical pair $P680^+Pheo^-$ was formed in approximately 21 ps (the same as for PS II RCs isolated from peas). Therefore, the present work shows that the method of Alizadeh *et al.* (1995) for the isolation of PS II RCs from *C. reinhardtii*, can also be applied to strains that are only deficient in PS I (rather than in both PS I and the ATP synthase complex, such as CC2655).

The PS II RC particles isolated from the Ley205Tyr/PS I$^-$ mutant were not spectroscopically pure: they contained approximately 7.4 Chl *a* per 2 Pheo *a* and had an absorption maximum at the red peak of 673 nm. The only difference between the Xba9

transformant and the Leu205Tyr/PS I⁻ mutant is the replacement of Leu with Tyr at position 205 of D2, therefore, it can be concluded that the inability to produce pure PS II RCs is due to that mutation only and not to any other factors. The PS II RC particle isolated from the Leu205Tyr/PS I⁻ mutant contained a sufficiently low level of chlorophyll to allow photoselective excitation of P680 and proved to be quite stable under the conditions used in the photoaccumulation and ultrafast absorption measurements. Therefore, attempts to further purify this complex from the extra chlorophylls, were not carried out.

The light-dark absorbance spectra obtained after illumination of PS II RCs from Leu205Tyr/PS I⁻ in the presence of sodium dithionite and silicomolybdate were characteristic of the photoaccumulation of $Pheo^-$ and $P680^+$, respectively. The $Pheo^-$ absorbance difference spectrum in the blue region in the mutant showed a bleaching in the Q_x absorption band at 544 and 515 nm (the same as for the Xba9 PS II RC). These bands are both diagnostic of pheophytin photoreduction (Klimov *et al.*, 1977). However, the peaks at 450 and 420 nm showed a 2-5 nm shift to the blue compared to the Xba9 spectrum. The band at 450 nm is characteristic of the radical anion of pheophytin (Klimov *et al.*, 1977). The significance of this shift is unclear. These measurements also revealed that the peak of the pheophytin Q_y band in the mutant was at 678 nm, in contrast to the value reported for wild type PS II RCs from *C. reinhardtii* CC2655 of 682 nm. Measurements of $P680^+$ photoaccumulation in the red region indicated a blue shift in the maximum bleaching of the Q_y band of P680 in the mutant PS II RC of approximately 2 nm (678 nm), relative to Xba9 (680 nm). This shift could arise from enhanced contribution to the spectrum from an oxidised accessory chlorophyll, $Chl670^+$, due to donation of electrons to $P680^+$. Thus, the resulting negative peak of the light-dark spectrum could reflect a mixed population of $Chl670^+$ and $P680^+$. It is therefore possible that the mutation of Leu205 of D2 to tyrosine has enhanced the ability of $P680^+$ to oxidise other chlorophylls within the isolated PS II RC. That the optical properties of P680 were unaltered in this mutant, was shown by analysis of the radical pair spectrum.

In the RC of *Rhodobacter sphaeroides*, mutation of M-Tyr210 (the amino acid residue in the analogous position to D2-Leu205) has produced shifts in the Q_x and Q_y bands of P, BChl and BPheo. For the M-Tyr210Leu mutation, low temperature absorption spectra revealed red shifts of 3 nm in the Q_y band of P and of the monomer BChls (Gray *et al.*, 1990). Moreover, the Q_x band of the BPheo residing on the inactive branch, was found to be red-shifted by 2 nm (the Q_x band of the BPheo on the active branch was identical to the wild type). Red shifts of 3-5 nm in the Q_y band of P and the BChl of the active branch were also observed by Jones *et al.* (1994) upon mutation of M-Tyr210 to leucine, phenylalanine and histidine. The most marked changes were obtained in the M-Tyr210His RCs, where red shifts were observed in the position of both the BPheo Q_y and Q_x bands (Jones *et al.*, 1994). These authors suggested that this finding was an indication of an interaction between the histidine at the M-210 position and the BPheo on the active branch. Similar shifts to the ones reported in Gray *et al.* (1990), were also produced upon replacing M-Tyr210 with tryptophan (Shochat *et al.*, 1994). The

only difference was the shift to the blue by around 2 nm in the Q_x band of the BPheo of the active branch. These observations using purple bacterial mutant RCs show some similarities with the ones reported in this study.

The main aim in creating the D2-Leu205Tyr mutation in *C. reinhardtii* was to study the rate of primary charge separation in mutant PS II RCs. Ultrafast transient absorption measurements using isolated PS II RCs from transformant Xba9 ("wild type" control) showed that formation of the primary radical pair occurs with a lifetime of 21±2 ps, the same as for isolated PS II RCs from pea. The equivalent time constant in the mutant PS II RCs was 40±7 ps, a slowdown of almost 100% compared to Xba9. The mutation was not found to affect significantly the peak of the pheophytin Q_x transition band (at 544nm) or the Q_y band of the radical pair (at 680 nm). In the $P680^+$ photoaccumulation experiments of Leu205Tyr/PS I$^-$, a blue shift was observed in the maximum absorbance bleach in the red region of the spectrum (from 680 nm to 678 nm). The absence/inability to observe this bleach in the femtosecond measurements could be attributed to the different timescales used in the two experiments.

The yield of radical pair formation in the isolated PS II RCs of the mutant, during the femtosecond measurements was estimated to be reduced by around 50%. Therefore, replacement of the amino acid residue at position 205 of D2 (a leucine) with the one found in the analogous position in the purple bacterial RC (a tyrosine) not only did not result in a speed up of primary charge separation but also reduced the quantum yield of charge separation in the isolated PS II RC.

In the RC of *Rhodobacter sphaeroides*, the aromatic ring of M-Tyr210 has been reported to be within van der Waals distance of the macrocycles of P, BChl and BPheo on the active branch (Tiede *et al.*, 1988). Replacement of this residue with either leucine or phenylalanine has had significant effects on primary charge separation (Finkele *et al.*, 1990). The effect of the mutations was found to originate from the molecular properties of the exchanged amino acids and not from a secondary structural disorder (Gray *et al.*, 1990; Chirino *et al.*, 1994). According to Parson *et al.* (1990) the phenolic OH group of M-Tyr210 could interact electrostatically with the pigments in its vicinity. On the other hand, the phenyl ring possibly provides a pathway for the electron transfer from the special pair P to the bacteriopheophytin in the active branch (Nagarajan *et al.*, 1990). Jones *et al.* (1994) used low temperature absorbance and UV-Raman spectroscopy, in an effort to describe the effects in the electron transfer properties of the mutant RCs. The authors concluded that the effects of the mutations on the rate of primary electron transfer cannot be ascribed to a change in the electronic structure of the primary donor, P. An X-ray crystallographic structure does not exist for the PS II RC, therefore, it is very difficult to make any suggestions about the role of D2-Leu205 in primary electron transfer.

In conclusion, replacement of leucine at position 205 of the D2 protein in *C. reinhardtii* with a tyrosine, did not have any dramatic effects in terms of the ability of the organism to grow photosynthetically and to evolve oxygen. However, the Leu205Tyr

mutation caused a reduction in the number of active PS II RCs (as revealed by oxygen evolution and thermoluminescence measurements) and a decrease in the rate and quantum yield of primary electron transfer in the isolated PS II RC. Whether these effects on primary electron transfer also occur *in vivo* is an open question. However, the high F_0 value observed in fluorescence measurements and the reduced thermoluminescence signal (obtained after single flashes) are consistent with a reduced quantum yield *in vivo*.

5. Biochemical and Biophysical Characterisation of the D2-Thr2Ala and D2-Thr2Ser Mutants

5.1 Introduction

5.1.1 Phosphoproteins in the thylakoid membranes of higher plants

The existence of phosphoproteins in thylakoid membranes from higher plants was first reported by Bennett (1977) who described a number of polypeptides from pea, in the range of 7-70 kDa, which were reversibly phosphorylated in the presence of [^{32}P]-orthophosphate. The most conspicuously phosphorylated bands had molecular masses of around 26 and 9 kDa, respectively. The 26 kDa band was identified as being composed of polypeptides of the light-harvesting antenna of PS II (LHCII) (Bennett, 1977). The 9 kDa phosphoprotein was later shown (Farchaus and Dilley, 1986) to be a PS II polypeptide (the PsbH polypeptide). Phosphorylation of LHCII was found to occur on threonyl residues, located at the N-terminal segment of the protein, exposed to the stromal surface of the thylakoid membrane (Bennett, 1977, 1979, 1980). In addition to LHC II and the PsbH polypeptide, it is now known that CP43, D1 and D2 are also reversibly phosphorylated in pea and spinach (Ikeuchi *et al.*, 1987b; Telfer *et al.*, 1987; Michel and Bennett, 1987; Michel *et al.*, 1988). The phosphorylation site for CP43, D1, D2 and PsbH is located at the first threonine residue at the N-terminus of each protein and was determined by purifying and sequencing tryptic phosphopeptides derived from [^{32}P]-labelled PS II particles (Michel and Bennett, 1987; Michel *et al.*, 1988). The work of Michel *et al.* (1988) also established that the NH_2-terminal threonyl residue of CP43, D1 and D2 is located at position 1 of the amino acid sequence of the mature protein and that the α-amino group of this threonine is also acetylated. In the case of D1 and D2, this means that the initiating N-formylmethionine residue, predicted from the gene sequence (Zurawski *et al.*, 1982; Holschuch *et al.*, 1984), is post-translationally removed whereas in the case of CP43, the additional removal of the second NH_2-terminal residue (glutamate) would be necessary (Erickson and Rochaix, 1992). In addition to these proteins, Bergantino *et al.* (1995) recently reported phosphorylation of the minor chlorophyll *a/b* protein, CP29, from maize.

The reversible phosphorylation of thylakoid proteins is under the control of a protein kinase-phosphatase system (reviewed in Bennett, 1991; Allen, 1992). Early on it was suggested that the protein kinase system was regulated by the redox state of the plastoquinone (PQ) pool (Allen *et al.*, 1981). This initial view has been modified as evidence

has emerged for the presence of two kinases that are under different redox controls (Bennett, 1991; Allen, 1992). Different putative kinases have been purified with different molecular weights (25, 38, 64 kDa) (Bennett, 1991). Of these, a 64 kDa protein (designated tp64), purified by Coughlan and Hind (1986) had been suggested to be the genuine LHCII kinase (Allen, 1992). However, recent sequencing (Hind *et al.*, 1995) and biochemical data (Race *et al.*, 1995) have indicated that this protein is not a kinase.

The PQ antagonist DBMIB, which blocks electron flow from PQH_2 to the cytochrome *b/f* complex, gives preferential inhibition of LHCII phosphorylation compared with phosphorylation of the PS II proteins (Farchaus *et al.*, 1985). In addition, mutations affecting the cytochrome *b/f* complex abolished phosphorylation of LHCII only. These results have implicated reduction of the cytochrome *b/f* complex by reduced plastoquinone in activation of the LHCII kinase (Bennett, 1991). In accordance with the kinase system there appears to be more than one phosphatase responsible for dephosphorylation of LHCII and PS II phosphoproteins (Allen, 1992). Dephosphorylation of LHCII occurs with a half-time of 8-10 minutes, whereas that of the PsbH polypeptide may be as long as 40 minutes (Bennett, 1980). Moreover, the phosphorylation sites for PS II polypeptides are substrates for exogenous alkaline phosphatase activity, whereas those of LHCII are not (Harrison and Allen, 1991). It has often been assumed that the LHCII phosphatase is permanently active and functions independently of light or electron transport (Allen *et al.*, 1981). Allen (1992) has suggested that the LHCII phosphatase is light-activated by redox-controlled phosphorylation and that its activity persists in darkness for long enough for it to catalyse dephosphorylation of LHCII. However, Silverstein *et al.* (1993b) presented data supporting the idea that the phosphatase activity is redox-independent. Moreover, these authors could divide the phosphatase activity into four kinetic classes with the fastest class (I) including LHCII, class II including D1 and D2, class III including CP43 and PsbH and class IV a 19.5 kDa polypeptide that did not show any loss of [^{32}P]-labelling.

5.1.2 Phosphoproteins in the thylakoid membranes of *Chlamydomonas reinhardtii*

Phosphorylation of thylakoid membrane proteins in the chloroplast of *C. reinhardtii* was first shown by Owens and Ohad (1982). Intact cells or purified membranes were labelled *in vivo* with [^{32}P]-orthophosphate or *in vitro* with [γ-^{32}P]-ATP, respectively. In both cases the authors reported phosphorylation of at least six polypeptides which they attributed to protein components of LHCII (p11-p17), polypeptides p9/p10, D1 and the small subunits L5-L7 (polypeptide designation as in Chua, 1980) (Owens and Ohad, 1982). Later studies confirmed the phosphorylation of the LHCII components p11, p13 and p17 (migrating between 22 and 35 kDa, according to the gel system) and indicated the phosphorylation of polypeptide 6 (which corresponds to CP43) as well as D2 (Wollman and Delepelaire, 1984; Delepelaire and Wollman, 1985; Ikeuchi *et al.*, 1987a; de Vitry *et al.*, 1987; de Vitry and

Wollman, 1988). Polypeptides p9 and p10 were assigned to the minor chlorophyll *a/b* binding proteins CP29 and CP26 (Bassi and Wollman, 1991). Ikeuchi *et al.* (1987a) and de Vitry *et al.* (1991), using isolated PS II particles, showed that D1 is not phosphorylated, in disagreement with Owens and Ohad (1982). de Vitry *et al.* (1991) attributed bands L5 and L6, migrating with an apparent M_r of 8,500 in SDS-gels, as the two phosphorylated forms and band L7 the non-phosphorylated form of the PsbH polypeptide. de Vitry *et al.* (1987, 1991) and Ikeuchi *et al.* (1987a) also reported a phosphorylated band at around 5 kDa which has not been assigned to any known polypeptide. Recently, Allen and Staehelin (1994), in a very detailed examination of the phosphorylation pattern of the PS II antenna system in *C. reinhardtii*, reported that polypeptides p11, p13 and p17 (LHCII components) and CP29 (p9) are the main phosphorylated bands, after *in vitro* labelling of thylakoids with [γ-^{32}P]-ATP. CP26 (p10) was found to be only lightly labelled. Moreover, a broad, diffuse band labelling just above of CP29 in their gel system, was suggested to be a PS II component. The authors also reported labelling of the apoproteins of the reaction centre of PS I. Phosphorylation of the PS I RC apoproteins, which does not seem to occur in higher plants, has also been reported by Ikeuchi *et al.* (1987a).

5.1.2.1 Phosphorylation of D2 in Chlamydomonas reinhardtii

Phosphorylation of D2 in *C. reinhardtii* was first suggested by Delepelaire (1984) after carrying out pulse-chase labelling experiments with [^{14}C]-acetate in the presence of inhibitors of protein synthesis. After electrophoresis (using a urea-SDS gel system) and autoradiography, Delepelaire observed a doublet which he attributed to the phosphorylated and non-phosphorylated forms of D2 (D2.1 and D2.2, respectively). These experiments were carried out in order to identify the site of synthesis of various thylakoid membrane polypeptides. Cells were pulse-labelled with [^{14}C]-acetate in the presence of an inhibitor of cytoplasmic protein synthesis (anisomycin), and then chased in the presence of anisomycin and chloramphenicol (which blocks translation in the chloroplast). The main evidence for the identity of the two bands of the doublet as two forms of D2 came from Cleveland digests of D2.1 and D2.2 with papain which gave rise to identical patterns (although the data were not presented by the author). Evidence regarding the structural similarity between the two forms of D2, was presented by Jensen *et al.* (1986) who generated peptide maps of thylakoid membrane polypeptides, labelled *in vivo* with [^{35}S]-sulphate, by electrophoresis in the presence of *Staphylococcus aureus* V8 protease. Based on these initial results, Delepelaire and Wollman (1985), de Vitry *et al.* (1987, 1991), Ikeuchi *et al.* (1987a) and de Vitry and Wollman (1988), assigned a band with an apparent molecular weight of around 34 kDa, labelled with [^{32}P] in both *in vivo* and *in vitro* experiments and migrating between p9/p10 and p11/p13, as the phosphorylated form of D2 (D2.1). However, conclusive

evidence indicating phosphorylation of D2 (for example by immunoblotting, immunoprecipitation or amino acid sequencing) has not appeared in the literature to date.

5.1.3 Functional aspects of thylakoid protein phosphorylation

The phosphorylation of LHCII apoproteins plays an important role in the regulation of photosynthetic electron transport (Bennett, 1991; Allen, 1992). LHCII phosphorylation is involved in regulation of excitation energy distribution between photosystems I and II (Allen *et al.*, 1981; Bennett, 1991; Allen, 1992): Fluorescence experiments at 77 K (where the two photosystems fluoresce strongly and at different wavelengths) showed a decrease in emission from PS II and a roughly comparable increase emission from PS I, upon phosphorylation of LHCII (Bennett *et al.*, 1980). Moreover, at low light intensities, a phosphorylation-dependent decrease of electron flow through PS II can be observed which has been interpreted in terms of a reduction in absorption cross-section of PS II as a result of detachment of phosphorylated LHCII units (Bennett, 1991). The absorption cross-section changes upon phosphorylation of LHCII have been explained by the mobility of LHCII and the lateral heterogeneity of thylakoid membranes. Stacked membranes are highly enriched in PS II, while unstacked membranes are highly enriched in PS I (Andersson and Anderson, 1980). Phosphorylation of the LHCII units in stacked membranes leads to their detachment from PS II and consequently to a decrease in energy transfer to PS II. Some of the detached LHCII units migrate from the stacked membranes to the unstacked, where they can act as antennae for PS I (Bennett, 1991). Alternatively, LHCII phosphorylation could act on PS II heterogeneity, either to convert PS II-α centres into PS II-β centres or to cause complementary changes in the absorption cross-sections of PS II-α and PS II-β (Allen, 1992) [(where PS II-α centres are the PS II units that are found in stacked membranes and are highly connected for excitation energy transfer and PS II-β centres are in the unstacked membranes and function independently of each other with respect to energy transfer (Krause and Weis, 1991)].

The phosphorylation-dependent reversible migration of the mobile LHCII has been correlated with the phenomenon of state transitions (Bennett, 1977; Telfer *et al.*, 1983). At subsaturating light intensities it is advantageous to green plants to have a balanced distribution of excitation between PS II and PS I. Thus, overeduction or overexcitation of the electron transport chain is avoided and the most efficient utilisation of light is ensured. The operation of a mechanism serving to redistribute excitation energy has been shown in both green algae and higher plants (Bonaventura and Myers, 1969; Chow *et al.*, 1981). When PS II is overexcited relative to PS I (e.g., by illumination at 645 nm), chloroplasts are driven into state II. At the same time the plastoquinone (PQ) pool and the cytochrome *b/f* complex become reduced, the LHCII kinase is activated and LHCII becomes phosphorylated. The mobile LHCII then moves away from PS II, and supplies excitation energy to PS I. Conversely, the transition to state I occurs because light 1 (e.g., 710 nm) preferentially excites

PS I than PS II, the PQ pool and the cytochrome *b/f* complex become oxidised, the LHCII kinase is inactivated and the LHCII phosphatase dephosphorylates LHCII thereby returning excitation energy to PS II (Bennett, 1991; Allen, 1992). Recently, Allen (1995), based on data of Silverstein *et al.*, (1993a), suggested three sites of redox control as possible candidates for explaining state transitions: the first is the Q_i site of the cytochrome *b/f* complex, the second is the Q_B site of the PS II reaction centre and the third is the low-potential form of Cyt b_{559} of PS II. Therefore, according to the author, the redox control at the level of plastoquinone should only be used as an approximation. In addition, Allen (1995) suggested that a mechanism exists to control the rates of light utilisation by PS II and PS I at longer-time scales, by adjustment of the PS II/PS I stoichiometry and that this phenomenon is subject to control by the same redox sensors mentioned above for state transitions. Allen (1995) then postulated that these redox sensors initiate these different adaptations by control of gene expression at different levels, according to the time scale and amplitude of the response.

LHCII phosphorylation has also been implicated in the regulation of carbon assimilation, according to the demands for ATP and reducing power (Horton *et al.*, 1990, Bennett, 1991; Allen 1992). As the metabolic capacity for the utilisation of ATP and NAD(P)H varies according to various environmental constraints, the ratio of linear to cyclic electron flow is altered. An increase in PS I cyclic electron flow would require redistribution of excitation energy to PS I if maximum efficiency is to be obtained. This situation could result from either the reduction of the plastoquinone pool (Allen, 1992) or by relaxation of the pH gradient across the thylakoid membrane (Horton *et al.*, 1990, Bennett, 1991). LHCII phosphorylation is stimulated by the relaxation of the pH gradient (Bennett, 1991). Thus, factors increasing the demand for ATP and NAD(P)H would be expected to increase LHCII phosphorylation.

In contrast with LHCII phosphorylation, very little is known about the role of phosphorylation of the PS II core proteins. Possible roles that have been suggested include the spatial separation of the two populations of PS II centres found in grana and stroma lamellae (PS II-α and β) (Mattoo *et al.*, 1989), involvement in photoinhibition (discussed in section 5.4) and a role in biogenesis and assembly of PS II (reviewed in Allen, 1992). However, none of the effects observed after phosphorylation could be attributed to the N-terminal phosphorylation of a specific PS II core protein. In this respect, site-directed mutagenesis could prove to be a powerful tool as the N-terminal threonyl residues thought to be phosphorylated could be changed to others that cannot be phosphorylated and changes in the phenotype could be directly attributed to the loss of phosphorylation.

Thus, the D2-Thr2Ala and D2-Thr2Ser mutants were constructed, in order to study the role of the putative D2 phosphorylation in *C. reinhardtii*. Alanine is an aliphatic, non-polar amino acid that cannot be phosphorylated. Therefore, the Thr2Ala mutation should provide valuable information towards the understanding of the role of D2 phosphorylation. Serine, like threonine, contains a hydroxyl group and so may be able to functionally replace

threonine. Indeed, studies with mammalian cells and tissue extracts have shown that threonine can successfully replace serine in its ability to be phosphorylated by the native kinase (e.g., Hsieh *et al.*, 1993; Giannini *et al.*, 1995; Cheng *et al.*, 1996). Therefore, the Thr2Ser mutation will enable us to functionally evaluate the specificity of the PS II kinase towards this site i.e., the amino acid at position 2 of D2.

Functional analysis of these mutants could allow us to establish more conclusive roles for phosphorylation of PS II RC proteins, especially in relation to photoinhibition. This chapter describes the biochemical and biophysical properties of the two mutants and also attempts to identify phosphorylated D2 in autoradiograms of thylakoids and PS II RCs.

5.2 Results-(I): Phenotypic analysis

5.2.1 Photoautotrophic growth experiments

Photoautotrophic growth experiments indicated that mutants Thr2Ala and Thr2Ser were able to grow photoautotrophically and exhibit the same growth rate as the wild type and Nsi16 strains (Figure 5.1). Doubling times were approximately 10 hours for all strains.

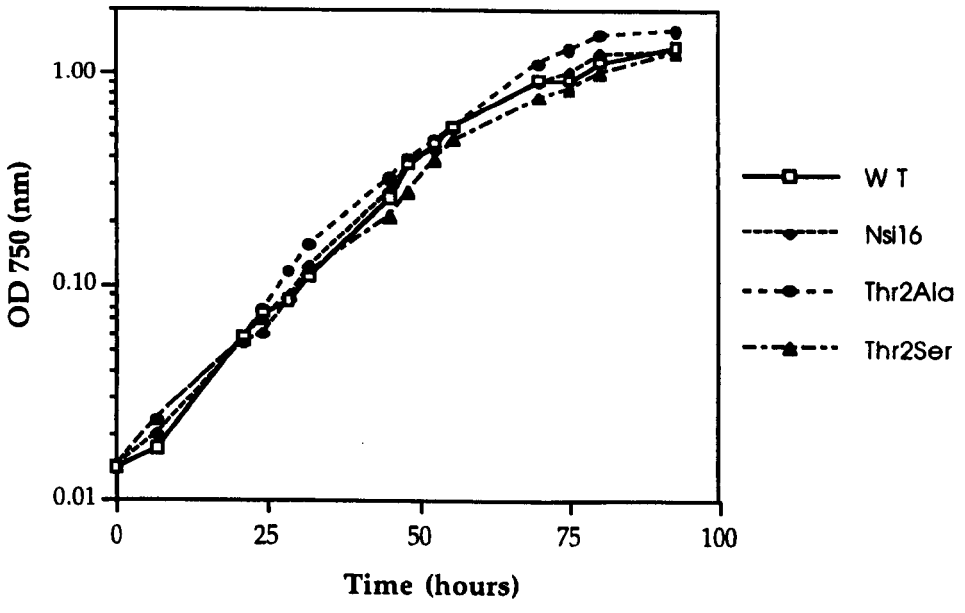


Figure 5.1: Photosynthetic growth patterns of wild type (WT), transformant Nsi16, and mutants Thr2Ala and Thr2Ser in HSM medium. Cultures were grown at 25°C at a light intensity of 50-70 $\mu E.m^{-2}.s^{-1}$ and were bubbled with air

After the end of the growth measurements, cells from the liquid cultures were spread on TAP plates containing spectinomycin and on HSM plates. The same number of colonies appeared on both types of plates indicating that the $spec^R$ cassette was stably maintained in the mutants.

5.2.2 Steady-state oxygen evolution measurements

In order to determine possible effects of the Thr2Ala and Thr2Ser mutations on photosynthetic electron transport, measurements of the light-induced steady-state rate of oxygen evolution were carried out. Cells were grown photoautotrophically in HSM (minimal) medium. Oxygen evolution measurements were carried out in HSM medium and the intensity of light was at saturating levels (4000-6000 $\mu E.m^{-2}.s^{-1}$). As can be seen from Table 5.1, mutant Thr2Ser showed wild type rates of oxygen evolution, both in the presence of bicarbonate and artificial electron acceptors (ferricyanide and DCBQ). The mutant Thr2Ala showed rates which were reduced on average by around 25% relative to the wild type. However, these values were quite variable and ranged from 55% to 100% of the wild type. The variability observed in the measurements of the Thr2Ala mutant cannot be attributed to differences between the wild type and mutant cells in terms of the age of the cultures, as the cell density (as judged by the OD at 750 nm) of both cultures was always at similar levels.

Table 5.1: Oxygen evolution measurements of the Thr2Ser and Thr2Ala mutants. Rates shown are after addition of 1 mM $NaHCO_3$ or 1 mM potassium ferricyanide and 1 mM DCBQ. Data presented are means of four independent experiments

	+ $NaHCO_3$		+potassium ferricyanide, DCBQ	
strains	μmoles O_2.mg $Chl^{-1}.hr^{-1}$	% of wild type	μmoles O_2.mg $Chl^{-1}.hr^{-1}$	% of wild type
wild type	89±12	100%	266±21	100%
Thr2Ser	98±23	104±23%	283±64	119±38%
Thr2Ala	67±9	75±15%	190±35	74±16%

5.2.3 Steady-state levels of thylakoid proteins in mutants Thr2Ser and Thr2Ala

The steady-state levels of the D1 and D2 proteins were determined by SDS-PAGE and Western blotting. Figure 5.2A shows an SDS-PAGE of thylakoid membrane polypeptides isolated from wild type, transformant Nsi16 and mutants Thr2Ser and Thr2Ala. The presence of D1 and D2 was assessed by Western blotting (Figure 5.2B, C). Both mutants showed similar steady-state levels of the D2 polypeptide with the wild type and Nsi16. Differences

appearing in the α-D1 blot (Figure 5.2, C) between strains may be due to incomplete transfer of protein to the nitrocellulose membrane.

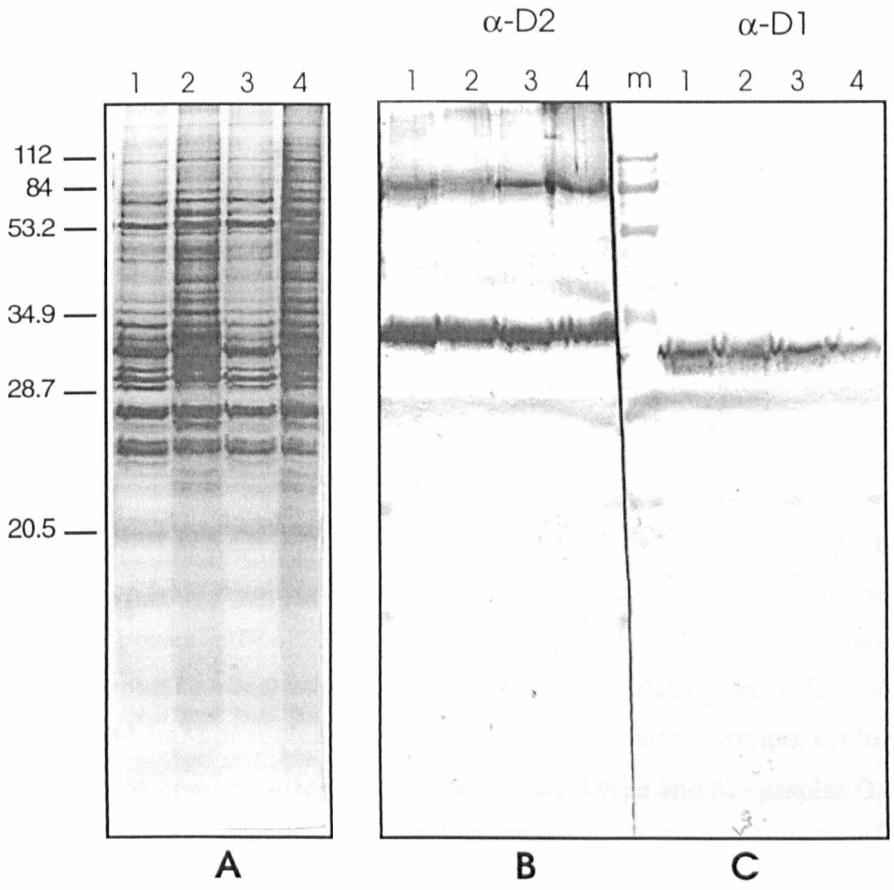


Figure 5.2: Silver-stained 14% gel containing 8 M urea (A) and Western blots using α-D2 (B) and α-D1 antibodies (C) of thylakoid membranes from wild type (lane 1), Nsi16 (lane 2), Thr2Ala (lane 3) and Thr2Ser (lane 4). The amount of chlorophyll loaded per lane is 1.5 μg for the gel and 5 μg for the blots. Numbers next to the gel indicate the bands of the molecular weight marker (lane m, prestained LMW, Bio-Rad)

5.2.4 Photoinhibition experiments in whole cells of mutants Thr2Ser and Thr2Ala

The loss of PS II activity under photoinhibitory conditions (high light) was studied in mutants Thr2Ala and Thr2Ser as outlined in section 4.2.5 for the Leu205Tyr mutant. Cells grown in HSM medium with air bubbling until middle or late exponential phase were stirred in flat dishes at 25°C, and subjected to an illumination of 1000 $\mu E.m^{-2}.s^{-1}$ for a period of 4-5 hours during which samples were taken for measurement of oxygen evolution. In order to measure the rate of PS II photoinactivation under conditions where there is no net synthesis and replacement of damaged PS II RC polypeptides, the chloroplast protein synthesis inhibitor chloramphenicol (CAP) was added at 200 μg/ml. As for the Leu205Tyr mutant, in

order to improve the accuracy of the measurements, the rates of oxygen evolution during the photoinhibitory treatment were measured in duplicate for each sample.

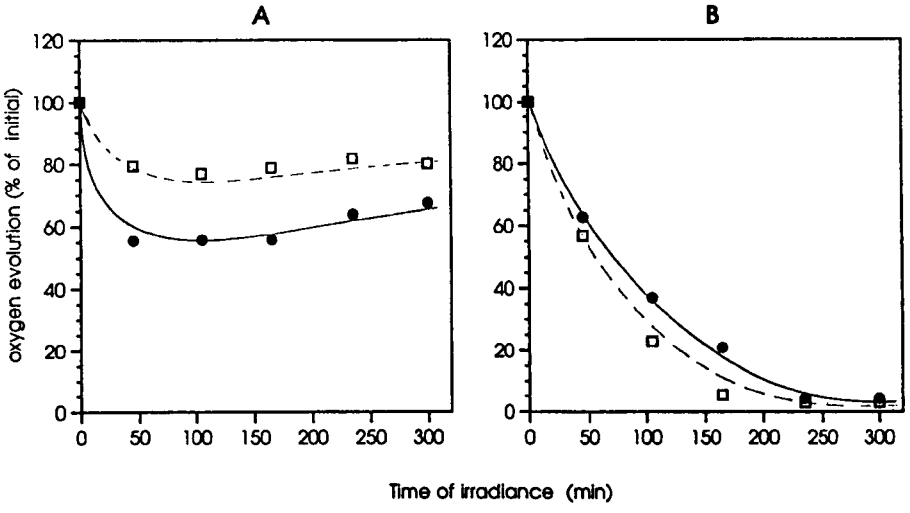


Figure 5.3: Effect of high light treatment (1000 μE.m^{-2}.s^{-1}) on the light-saturated rate of oxygen evolution from whole cells of wild type (circles) and mutant Thr2Ser (squares) in the absence (A) or presence (B) of 200 μg/ml chloramphenicol. Cells were suspended in HSM at 25°C, to a chlorophyll concentration of 25 μg/ml. Oxygen evolution was measured using 1 mM DCBQ and 1mM potassium ferricyanide. The 100% rates of oxygen evolution are approximately 251 μmoles O_2· mg chl^{-1}· hr^{-1} for the wild type and 333 μmoles O_2· mg chl^{-1}· hr^{-1} for Thr2Ser

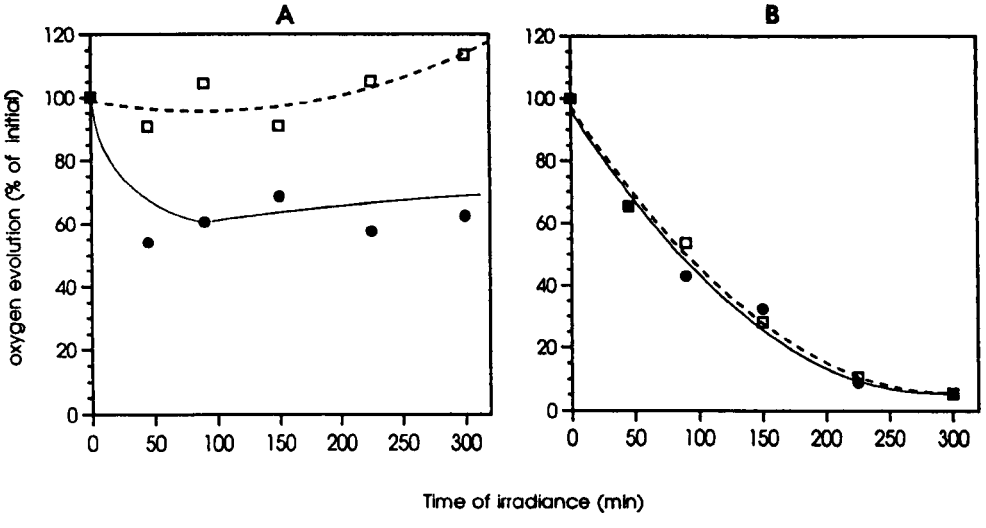


Figure 5.4: Effect of high light treatment (1000 μE.m^{-2}.s^{-1}) on the light-saturated rate of oxygen evolution from whole cells of wild type (circles) and mutant Thr2Ala (squares) in the absence (A) or presence (B) of 200 μg/ml chloramphenicol. Light treatments and oxygen evolution measurements were performed as in Figure 5.3. Data points are means of two independent experiments. The 100% rates of oxygen evolution are approximately 260 μmoles O_2· mg chl^{-1}· hr^{-1} for the wild type and 222 μmoles O_2· mg chl^{-1}· hr^{-1} for Thr2Ala

Figure 5.3 shows the results of high light treatments on whole cells from wild type and mutant Thr2Ser. The two strains showed similar rates of PS II photoinactivation in the presence (Figure 5.3B) of CAP ($t_{1/2}$ of ~80 minutes). In the absence of CAP, the experiment shown in Figure 5.3 A indicated that the mutant showed less PS II photoinactivation than the wild type. High-light treatments of the Thr2Ala mutant in the presence of CAP (Figure 5.4B) showed similar rates of PS II photoinactivation with the wild type strain ($t_{1/2}$ of ~80 minutes). However, when the wild type and the Thr2Ala mutant were incubated with light of 1000 $\mu E.m^{-2}.s^{-1}$ in the absence of CAP, there was no loss of PS II activity in the mutant (Figure 5.4A), even after more than 5 hours of illumination. Therefore, the Thr2Ala mutant did not display any apparent susceptibility to photoinhibition.

5.2.5 Fluorescence induction measurements in whole cells of mutants Thr2Ala and Thr2Ser

In the photosynthetic apparatus, excitation energy transferred to the reaction centres (RCs) of the two photosystems drives the primary photochemical reactions that initiate the photosynthetic energy conversion. In low light, under optimal conditions, the primary photochemistry occurs with high efficiency. A minor competing process of deactivation of excited pigments is the emission of fluorescence. At room temperature, most fluorescence is emitted by Chl *a* of PS II. The maximum fluorescence yield is around 3% and is obtained when all PS II RCs are inactive ("closed") for photochemistry. When all RCs are "open", the fluorescence yield is around 0.6%, owing to competition with photochemistry (reviewed in Krause and Weis, 1991). The Chl *a* fluorescence emission reflects photosynthetic activities in a complex manner. Thus, illumination of any photosynthetic system (ranging from leaves and whole cell organisms to various subcellular complexes such as thylakoids) with continuous light following a period of darkness, results in the rise of fluorescence from a minimal level (F_0) via an intermediate level (I) to a peak level (P or F_m), followed by a decay to a value close to F_0 (fluorescence induction curve or Kautsky curve). The decline of fluorescence reflects the light-activation of electron transport capacity at the PS I acceptor side, as well as the development of a number of overlapping fluorescence quenching processes (Schreiber *et al.*, 1986; Krause and Weis, 1991; Schreiber *et al.*, 1995). The maximum fluorescence level F_m corresponds to the situation when Q_A is fully reduced and therefore excitation of the primary donor P680, cannot result in stable charge separation (see also section 4.2.3).

A useful parameter derived from the kinetics of the fluorescence induction curve is the so-called variable fluorescence, F_v, which equals the fluorescence increase from F_0 to F_m. The F_v/F_m ratio has been found to remain within a very narrow range among leaves of different species and therefore, has become an important parameter of the physiological state of the photosynthetic apparatus. Thus, environmental stresses that affect PS II efficiency lead to a characteristic decrease in F_v/F_m.

In order to assess the filling-up of the electron acceptor pools (Q_A, Q_B and PQ) in the mutants Thr2Ala and Thr2Ser, the fluorescence induction characteristics of the wild type and the mutants were monitored in the absence and presence of DCMU. The latter compound blocks Q_A^- to Q_B electron transfer and ensures a fully reduced Q_A pool. As a result, the fluorescence rise from F_0 to F_m is much faster (Krause and Weis, 1991). In addition, the apparent F_0 level is slightly increased. As can be seen from Figure 5.5, the fluorescence induction curves of the mutants are similar to the wild type. All the induction curves are well structured and show all the characteristic phases of intact *C. reinhardtii* cells (e.g., Schreiber *et al.*, 1995; Srivastava *et al.*, 1995). The F_v/F_m values in the absence and presence of DCMU for both the wild type and the mutants were 0.66 and 0.69, respectively.

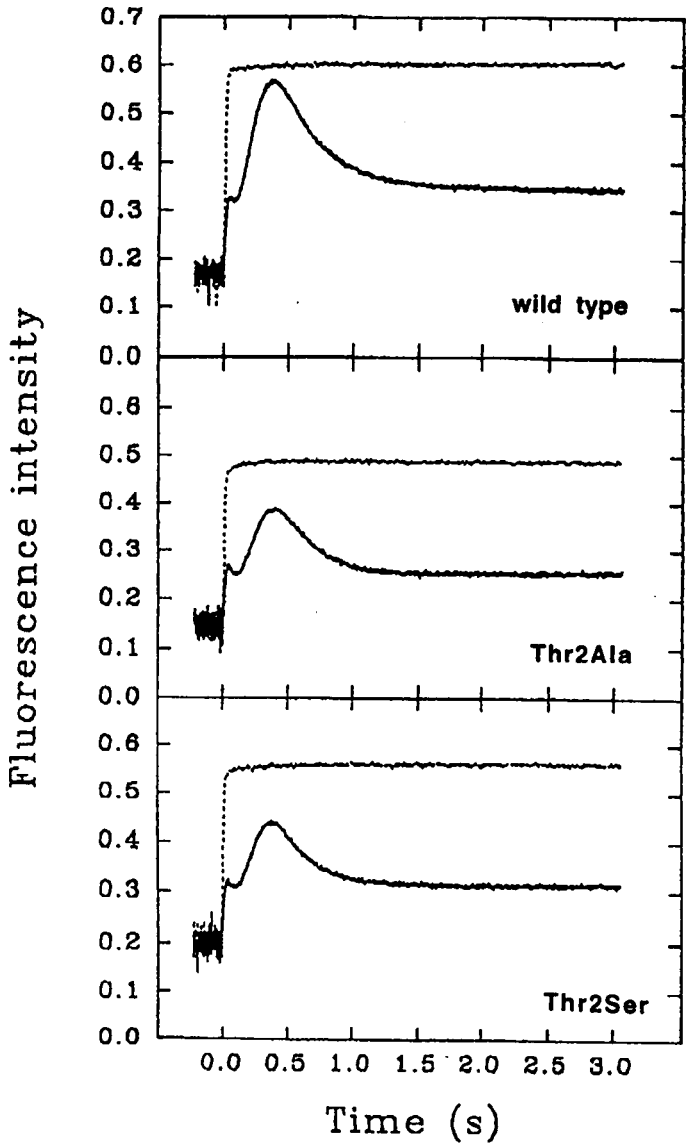


Figure 5.5: Fluorescence induction curves of wild type, and mutants Thr2Ala and Thr2Ser in the absence (solid line) or presence (dashed line) of 10 μM DCMU. Chlorophyll content in all samples was 50 μg.

5.2.5.1 State-transition measurements

The relationship between phosphorylation of LHCII and state transitions was outlined in section 5.1.3. Phosphorylation of LHCII as part of a state II transition, results in the reduction of the absorption cross-section of PS II (because of LHCII uncoupling) and a decrease of the electron flow through PS II, which can be measured as reduced fluorescence yield at room temperature (Chow *et al.*, 1981; Wollman and Delepelaire, 1984).

Based on this knowledge, an attempt was carried out to measure the yield of F_v (at room temperature) in the two threonine mutants, after incubation under conditions which should induce either state I or state II (see section 2.16). The methodology for this attempt was based on the previously published procedures of Wollman and Delepelaire (1984), Bulté *et al.* (1990) and Delphin *et al.* (1995). Thus, whole cells were incubated for 10 minutes under one of the four following conditions: light 2 (blue light), light 1 (far red light), in the dark and in the dark/anaerobic. After this 10 minute preincubation period, the F_0 and F_m values were measured by means of 100 kHz pulses obtained from the PAM 101 fluorometer and saturating light pulses provided by a high-intensity halogen lamp, respectively.

Table 5.2 shows the values of variable fluorescence (expressed as F_v/F_m) obtained for the wild type, transformant Nsi16 and mutants Thr2Ala and Thr2Ser. Incubation of wild type cells with blue light, resulted in a slightly lower F_v/F_m value than incubation with far red light. This is consistent with cells being in state II after incubation with light 2 and in state I after incubation with light 1. Incubation of the cells under dark/anaerobic conditions resulted in a fluorescence yield that resembled state II (Wollman and Delepelaire, 1984; Delphin *et al.*, 1995). The PQ pool in the thylakoid membranes of dark-adapted algae is a redox carrier common to two distinct electron transfer chains: the photosynthetic one, from H_2O to $NAD(P)^+$, and a respiratory-like one, from NAD(P)H to O_2. This second electron transfer chain, known as chlororespiration (Bennoun, 1982) can be inhibited under anaerobic conditions leading in the dark to complete reduction of the PQ pool and therefore, to state II (Wollman and Delepelaire, 1984). The fluorescence quenching observed with the wild type cells upon transition to state II was consistent throughout the course of the measurements. However, the analogous reduction of the fluorescence yield reported in the literature for state II-adapted *C. reinhardtii* cells, is much larger (around 40-50%, by comparing the F_m values in state II and state I) than the values obtained in the present work (only about 5%), (Wollman and Delepelaire, 1984, Delosme *et al.*, 1996).

Nsi16 showed very similar values to wild type after incubation with light 1 and under dark/anaerobic conditions. When Nsi16 cells were incubated with light 2, a lower value of F_v/F_m was obtained. However, this value lies within the experimental error. Lower values of F_v/F_m compared to the wild type, after induction of state II were observed in the two mutants. More significantly, the Thr2Ser mutant did not show almost any decrease in the fluorescence yield after adaptation under state II conditions (light 2 or dark\anaerobic)

compared to state I (light 1). The differences in the relative F_v/F_m yields between the wild type and the two mutants may indicate perturbations in state transitions in the latter. However, in order to verify these measurements, more experiments should be carried out.

Table 5.2: Relative values of variable fluorescence F_v, normalised to F_m $[(F_m-F_0)/F_m]$ from whole cells of wild type, transformant Nsi16 and mutants Thr2Ala and Thr2Ser, obtained after 500 ms saturating light pulses. Cells had been pre-incubated for 10 minutes with blue light (light 2, L2), far red light (light 1, L1), in the dark or in the dark/anaerobic before the start of the measurements. Chlorophyll content of the samples was 50 μg/ml

strains ↓ / incubation →	L2	L1	dark	dark/anaerobic
wild type	0.696±0.020	0.720±0.015	0.690±0.030	0.693±0.003
Nsi16	0.657±0.014	0.720±0.017	0.733±0.012	0.690±0.005
Thr2Ala	0.640±0.010	0.726±0.031	0.683±0.012	0.656±0.006
Thr2Ser	0.633±0.027	0.660±0.005	0.660±0.005	0.656±0.012

5.2.6 Fluorescence decay measurements in whole cells of mutants Thr2Ala and Thr2Ser

Fluorescence decay measurement analogous to the ones presented in section 4.2.3 for the Leu205Tyr mutant, were also performed for mutants Thr2Ala and Thr2Ser. These measurements were carried out in the laboratory of B. Diner, at E. I. du Pont de Nemours, USA. The correlation of fluorescence decay kinetics with the characteristics of electron transport in photosystem II is outlined in section 4.2.3.

Figure 5.6 shows the results from the 5-flash series experiment (see section 4.2.3 for conditions of the measurements) obtained with mutants Thr2Ala and Thr2Ser. These measurements showed that the donor side of the mutants operates normally. The same applies for the acceptor side, since at 200 μs a clear oscillation of period two was observed. These data were confirmed by the charge recombination measurements (see section 4.2.3 for conditions) where the wild type, Nsi16 and mutants Thr2Ala and Thr2Ser showed the same pattern of fluorescence decay (Figure 5.7).

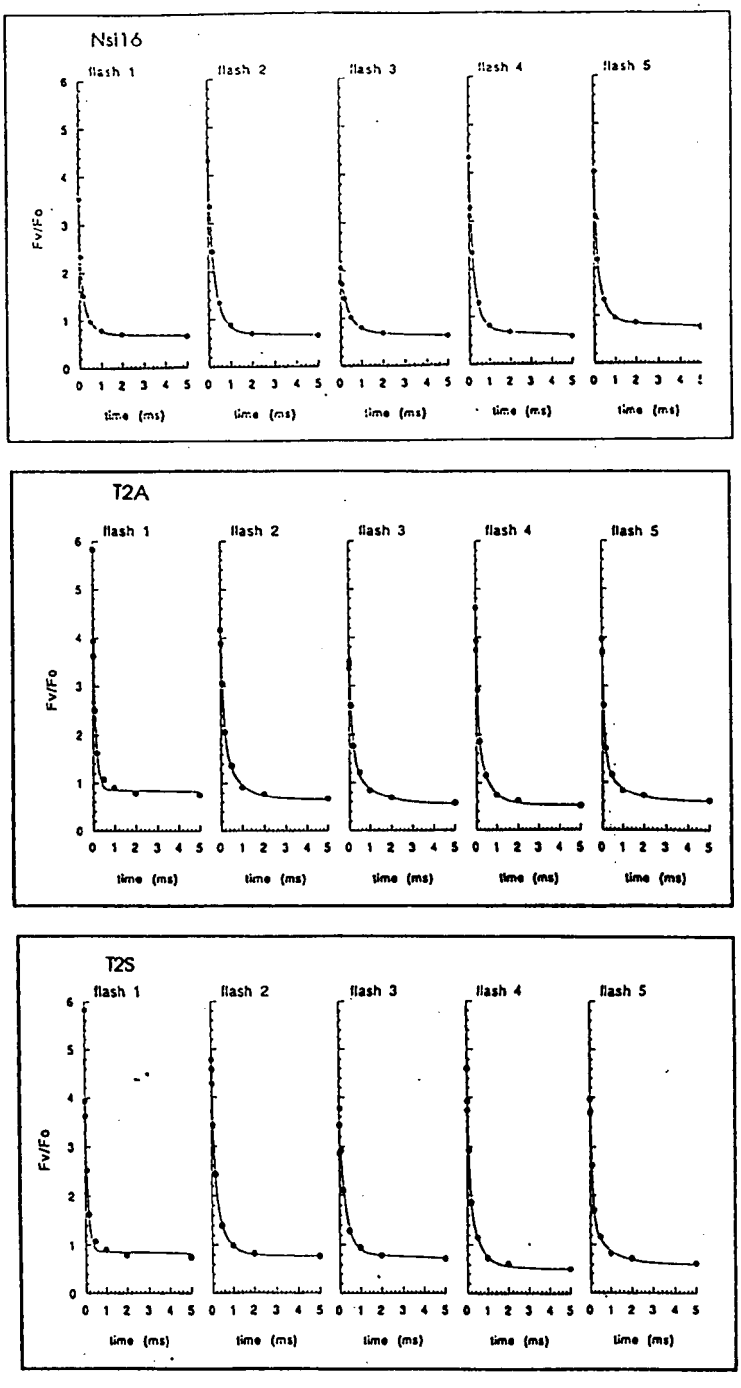


Figure 5.6: Relaxation of the variable fluorescence ($F-F_0$) after each of a series (1.67 Hz) of five saturating 2-μs flashes in whole cells of *C. reinhardtii* mutants Thr2Ala and Thr2Ser. Shown is the 5-ms range following each flash starting at 50 μs. The 50 μs values of the variable fluorescence are normalised to F_0 [$(F-F_0)/F_0$]

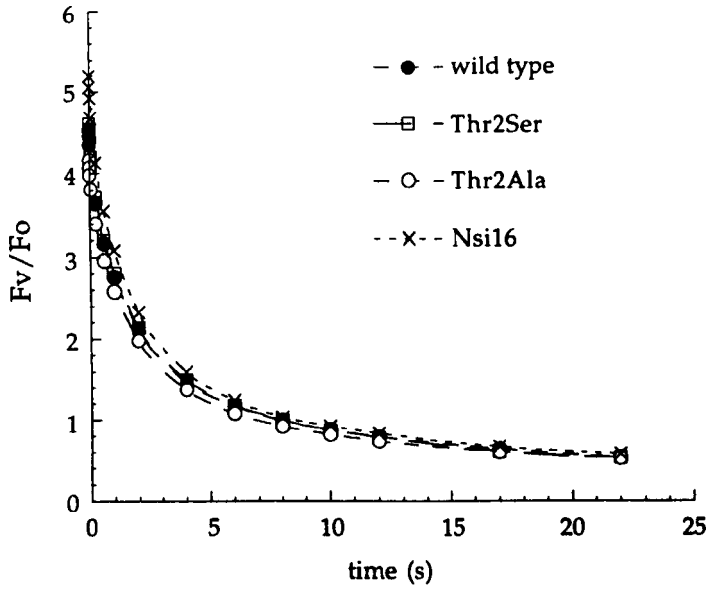


Figure 5.7: Relaxation of variable fluorescence ($F-F_0$) resulting from charge recombination between Q_A^- and the PS II donor side following a single saturating 2-μs flash excitation of whole cells of *C. reinhardtii*. The wild type is compared to transformant Nsi16 and to mutants Thr2Ala and Thr2Ser. The variable fluorescence is normalised to F_0 [$(F-F_0)/F_0$].

5.2.7 Thermoluminescence characteristics of mutants Thr2Ser and Thr2Ala

The acceptor side of PS II in mutants Thr2Ser and Thr2Ala was also studied using thermoluminescence measurements. The use of thermoluminescence (TL) for the study of electron transfer in the acceptor side of PS II is outlined in section 4.2.4. Figure 5.8 shows the TL curves from wild type and mutants Thr2Ser and Thr2Ala. In all strains the peak position of the single flash-induced B band (from the $S_2Q_B^-$ recombination) (solid line in Figure 5.8) was found at 30-32°C. In the presence of 10 μM DCMU the single flash-induced Q band (from the $S_2Q_A^-$ recombination) (dashed line in Figure 5.8) appeared at 14-16°C. Moreover, the intensity of the Q band was two times higher than that of the B band. Therefore, it seems that the replacement of threonine with serine or alanine does not affect PS II assembly and electron transport from the water-oxidising complex to Q_A and Q_B.

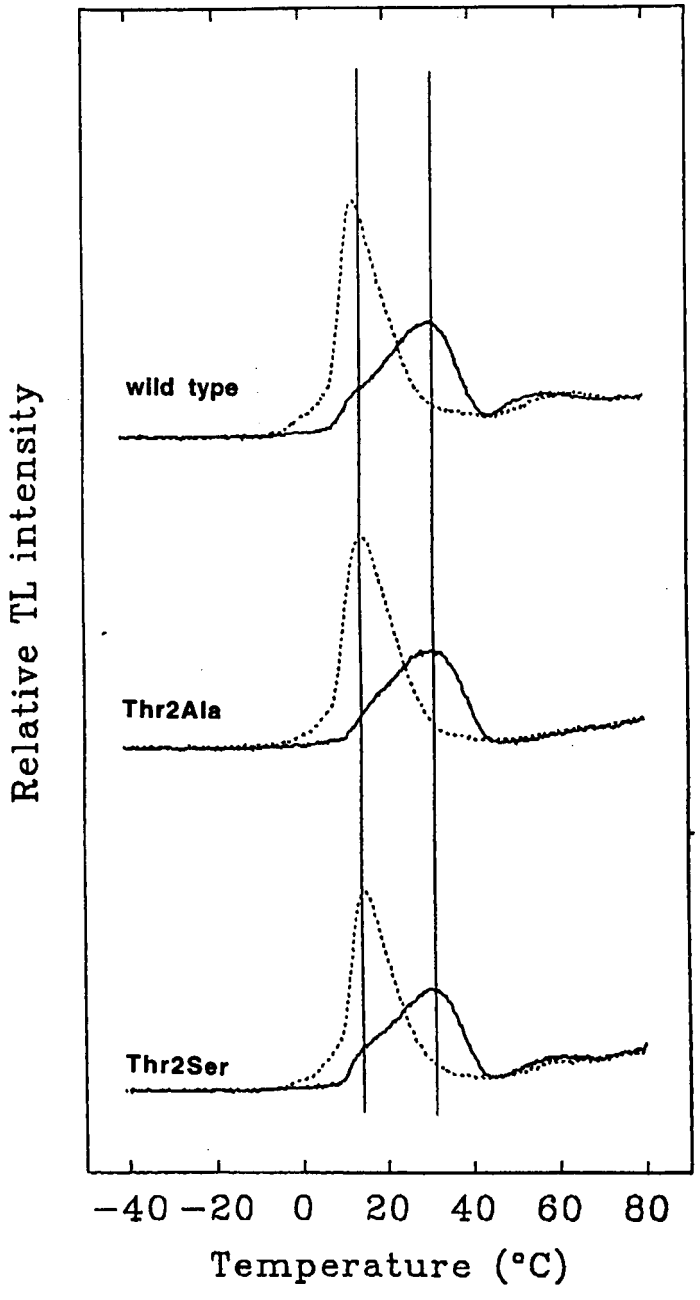


Figure 5.8: Single flash-induced TL curves from whole cells of wild type, Thr2Ala and Thr2Ser. Excitation was at -10°C in the absence (solid line) or presence (dashed line) of 10 μM DCMU. The chlorophyll content in all samples was 50 μg.

5.3 Results-(II): Attempts to identify phosphorylated D2

The D2 protein has not been shown conclusively to be phosphorylated in *C. reinhardtii* (see section 5.1.2.1). In order to confirm that D2 is indeed phosphorylated in wild type *C. reinhardtii*, thylakoids and cells were labelled *in vitro* and *in vivo* respectively, with radiolabelled phosphate and the presence of labelled D2 was sought using Western blotting. Moreover, attempts to identify D2 on urea-SDS gels and autoradiograms were carried out using immunoprecipitation, isolation of PS II core complexes, pulse-labelling of whole cells with [^{14}C]-acetate and immunoblotting using a monoclonal antibody against phosphothreonine. In these experiments, the Thr2Ala mutant was compared to the wild type as this strain should not be phosphorylated on Ala2.

5.3.1 *in vitro* phosphorylation of wild type thylakoids

Initial *in vitro* phosphorylation experiments were carried out using only the wild type strain in order to establish the conditions that would yield the best labelling of thylakoid membrane proteins. Cells were grown in the light or in the dark in TAP medium for 3-4 days until an OD_{750} of 0.5-0.8 had been reached. Thylakoids for *in vitro* phosphorylation experiments were obtained using the method of Diner and Wollman (1980) (section 2.21.2). The labelling reaction was carried out for 15 minutes, either in the light at 50-70 $\mu E.m^{-2}.s^{-1}$ (to reduce the PQ pool) or in the dark (to oxidise the PQ pool). Labelling was achieved using 10 μCi of [γ-^{32}P]-ATP (specific activity ~3000 Ci/mmol, Amersham) for every 100 μg of chlorophyll. The labelling buffer contained 10 mM sodium fluoride as an inhibitor of phosphatase activity (Larsson *et al.*, 1987) and 0.2 mM cold ATP as an inducer of the phosphorylation reaction. The results of such an experiment are shown in Figure 5.9. In broad detail, the pattern of phosphorylation is the same to ones previously described for this species (Owens and Ohad, 1982; Wollman and Delepelaire, 1984; Delepelaire and Wollman, 1985; Gans and Wollman, 1995). Moreover, the kinase was clearly stimulated in the light, although there is some labelling even in the sample derived from cells grown in the dark and labelled in the dark (under conditions where the PQ pool should be fully oxidised). *in vitro* labelling of thylakoid proteins in the dark is also observed in Wollman and Delepelaire (1984). However, labelling under illumination mostly stimulates phosphorylation of two LHCII bands (designated as p13 and p16) at around 25 kDa (Wollman and Delepelaire, 1984) whereas in this study, light stimulates labelling of all phosphorylated polypeptides (Figure 5.9 A). All the labelled bands can probably be attributed to antenna polypeptides, as shown by Western blotting using antibodies against the LHCII polypeptides of spinach (Figure 5.9 B). The uppermost, heavily labelled band can be assigned to CP29 (Bassi and Wollman, 1991; Allen and Staehelin, 1994). The labelled band migrating below CP29 is probably an LHCII polypeptide-one of p11 and p13 (Wollman and Delepelaire, 1984; Allen

and Staehelin, 1994). This band migrates in proximity to the upper part of D2 (compare panels B and C in Figure 5.9). Therefore, its identification as the phosphorylated form of D2 cannot be completely excluded.

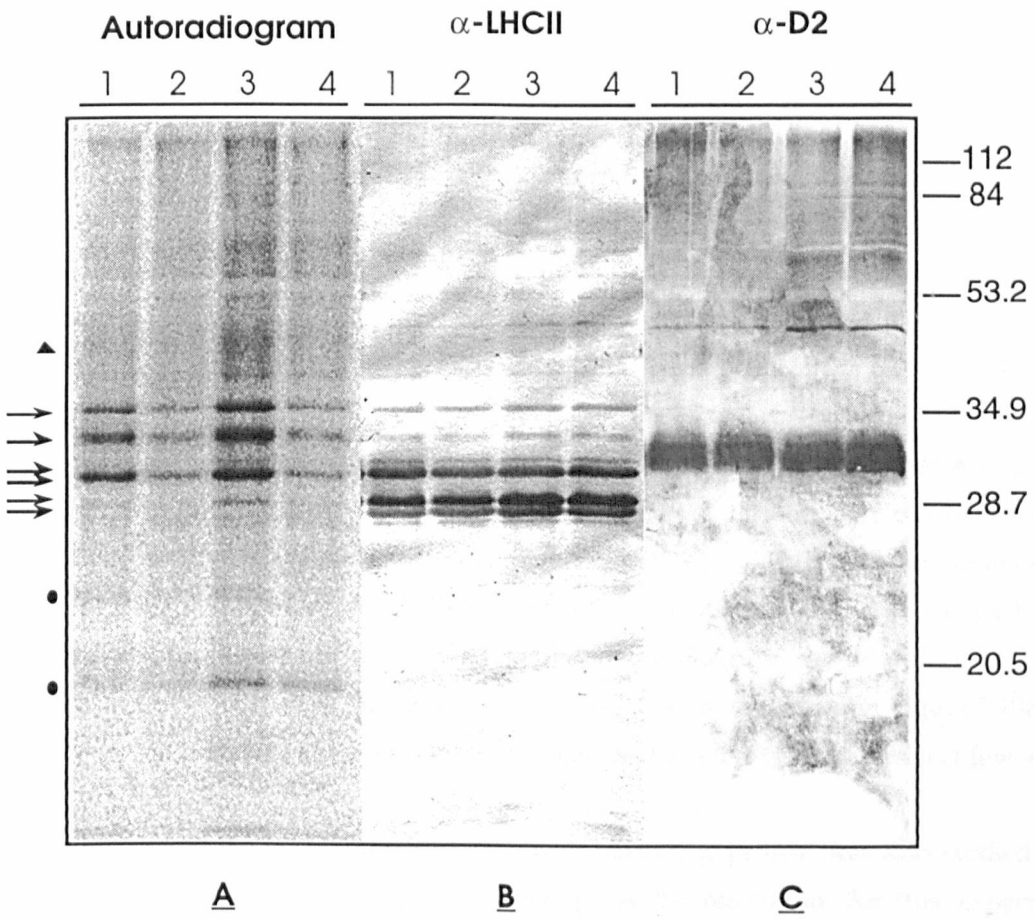


Figure 5.9: *in vitro* labelling of wild type *C. reinhardtii* thylakoid membranes with [γ-^{32}P]-ATP. Autoradiogram (A) of a 12%, 6 M urea-SDS gel and Western blotting with α-LHCII (B) and α-D2 (C) antibodies. Lanes: (1), cells grown in the light and thylakoids labelled in the light; (2), cells grown in the light and thylakoids labelled in the dark; (3), cells grown in the dark and thylakoids labelled in the light; (4), cells grown in the dark and thylakoids labelled in the dark. Arrows indicate positions of main LHCII bands picked up by the α-LHCII antibody. The triangle represents the position of CP43 and dots indicate unidentified phosphoproteins. Low molecular weight polypeptides have been run off the bottom of the gel. Numbers next to the α-D2 blot represent the bands of the molecular weight marker (prestained LMW, Bio-Rad)

5.3.2 *in vitro* phosphorylation of Thr2Ala thylakoids

Thr2Ala cells were grown and thylakoid membrane proteins labelled under the same conditions outlined in section 5.3.1 for the wild type. Thylakoids labelled with [γ-^{32}P]-ATP were fractionated on a 12%, 6 M urea-SDS gel and the position of the D2 protein was identified using Western blotting. As for the wild type thylakoids, labelling of the Thr2Ala thylakoid proteins is stimulated by light (Figure 5.10 A). The most intense labelling is observed in the sample representing mutant thylakoids isolated from dark-grown cells and labelled under light (Figure 5.10 A, lane 7). The difference between the amount of labelling between the mutant and the corresponding wild type sample (Figure 5.10 A, lane 3) cannot be attributed to differences in loading (as judged by the stained gel, not shown). In addition, higher level of labelling of CP29 (indicated by the top arrow at around 35 kDa in Figure 5.10A) relative to the other labelled polypeptides, can be observed in the mutant, relative to the wild type.

The most interesting difference, though, between the wild type and Thr2Ala samples was revealed in Western blotting experiments using an α-D2 antibody. As can be seen from Figure 5.10B, the Thr2Ala samples appear to lack the upper part of the immunodecorated D2 band seen in the wild type, regardless of the growth or labelling conditions. This part of D2 does not correspond to any labelled band in the autoradiogram and therefore cannot be attributed to the phosphorylated form of D2. In the experiment shown in Figure 5.10B, the reduced level of D2 in the Thr2Ala thylakoids compared to wild type may reflect fewer PS II centres.

The phosphorylation pattern of thylakoid membrane protein was also studied using a slightly different type of gel in an effort to improve the resolution. For this experiment, cells were grown in TAP in the light and thylakoids were labelled under illumination according to the conditions of section 5.3.1. Figure 5.11 shows the stained gel (14%, 8 M urea), the autoradiogram and blots with three different antibodies (α-D2, α-LHCII and α-CP43). The labelling pattern is very similar to the one presented in the previous figures. Under these conditions the upper part of D2 co-migrates with a labelled band, previously attributed to CP29 (see Figure 5.9A). However, as shown in Figures 5.9 and 5.10, this band should probably be assigned to this antenna polypeptide rather than D2. Moreover, as can be seen from Figure 5.11, the lightly labelled band migrating between the 53.2 and 34.9 markers in Figure 5.9 is most probably the phosphorylated form of CP43.

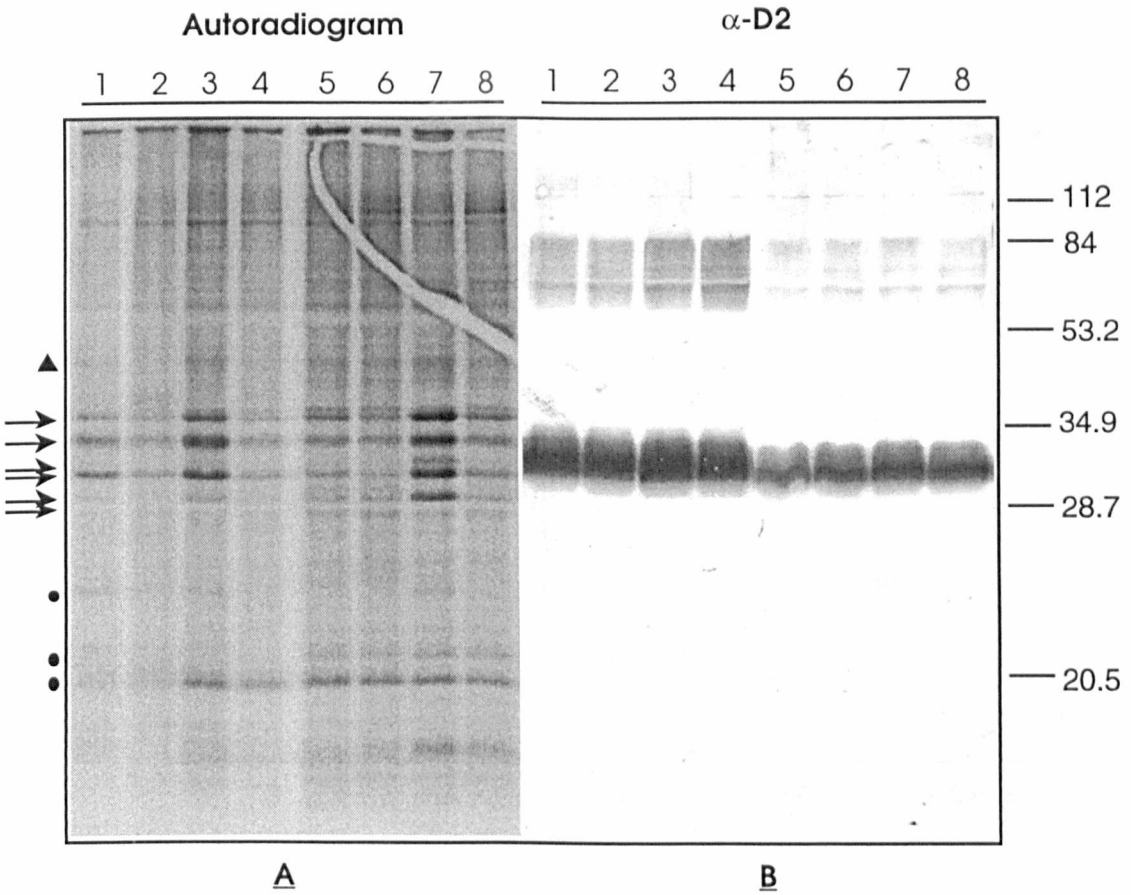


Figure 5.10: *in vitro* labelling of wild type (lanes 1-4) and Thr2Ala (lanes 5-8) *C. reinhardtii* thylakoid membranes with [γ-^{32}P]-ATP. Autoradiogram (A) of a 12%, 6 M urea-SDS gel and Western blotting (B) with α-D2 antibody. Lanes 1 and 5: cells grown in the light and thylakoids labelled in the light; lanes 2 and 6: cells grown in the light and thylakoids labelled in the dark; lanes 3 and 7: cells grown in the dark and thylakoids labelled in the light; lanes 4 and 8 cells grown in the dark and thylakoids labelled in the dark. Arrows indicate positions of main LHCII bands picked up by the α-LHCII antibody. The triangle represents the position of CP43 and dots indicate unidentified phosphoproteins. Numbers next to the α-D2 blot represent the bands of the molecular weight marker (prestained LMW, Bio-Rad)

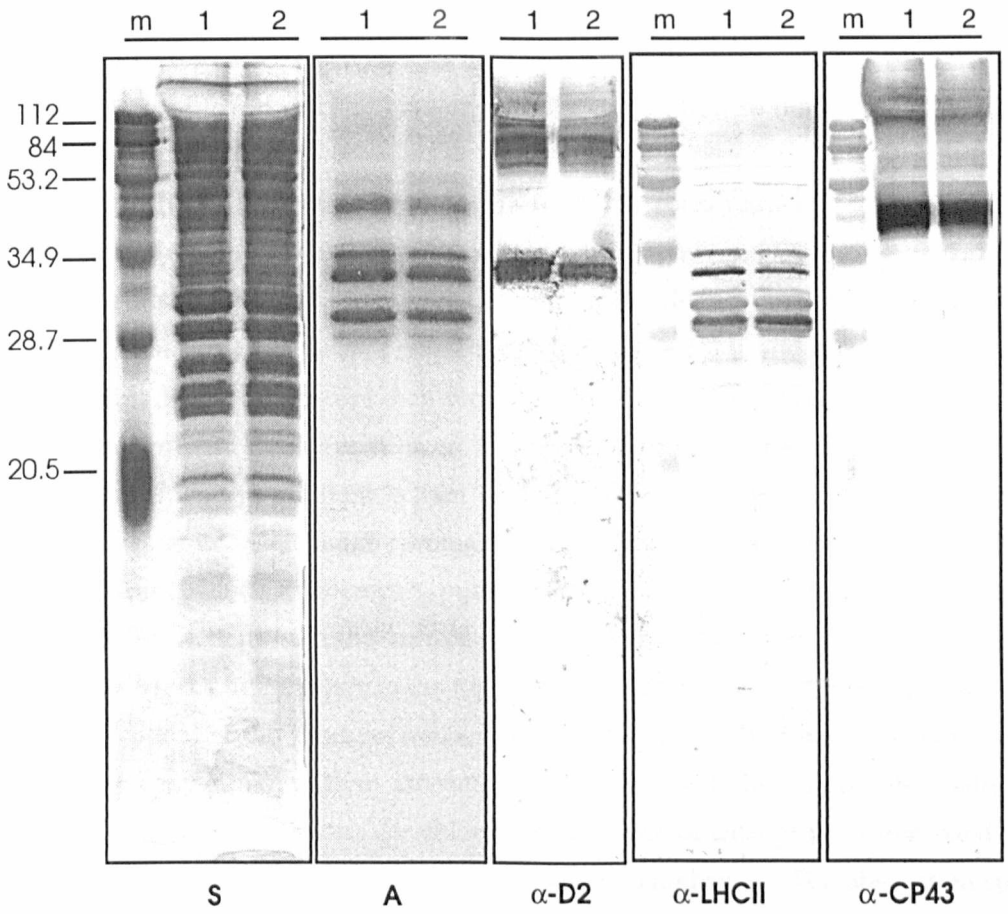


Figure 5.11: *in vitro* labelling of wild type and Thr2Ala *C. reinhardtii* thylakoid membranes with [γ-^{32}P]-ATP. Silver-stained urea-SDS gel (14%, 8 M urea) (S), autoradiogram (A), and α-D2, α-LHCII and α-CP43 western blots of wild type (lane 1) and Thr2Ala (lane 2) thylakoids. The molecular weight marker (prestained LMW, Bio-Rad) (lane m) and its bands are indicated.

5.3.3 Isolation of PS II core complexes

The main polypeptides being phosphorylated in *C. reinhardtii* belong to the light-harvesting antenna of PS II (Owens and Ohad, 1982; Wollman and Delepelaire, 1984). In order to study phosphorylation of PS II proteins, it would be advantageous to purify a complex which is devoid of LHCII, in a manner similar to studies carried out for higher plants (Telfer *et al.*, 1987; Michel *et al.*, 1988). Therefore, isolation of PS II RCs or PS II core complexes would resolve this ambiguity. However, PS II RCs from *C. reinhardtii* have only been purified from strains deficient in photosystem I (PS I) (Alizadeh *et al.*, 1995 and this work). PS II core complexes have been purified from higher plants (e.g., Satoh *et al.*, 1984; Yamada *et al.*, 1987; Fotinou and Ghanotakis, 1990), from *C. reinhardtii* (Diner and Wollman, 1980; de Vitry *et al.*, 1991; Bumann and Oesterhelt, 1994) and from cyanobacteria

(Tang and Diner, 1994). These particles are mostly deficient in light-harvesting complexes and usually contain CP43, CP47, one or more of the apoproteins of the oxygen-evolving complex (OEC) and low molecular weight subunits, in addition to the subunits found in the isolated PS II RC.

The method used in this work to isolate PS II cores particles from wild type *C. reinhardtii* was a simplified version of the method of Bumann and Oesterhelt (1994) (section 2.23). This method employs the use of the mild detergent, dodecyl maltoside (DM), at a low detergent-to-chlorophyll ratio to preferentially solubilise PS II and only small amounts of PS I. PS II-containing particles are then separated from light-harvesting complexes by means of sucrose gradient ultracentrifugation in the presence of additional detergent. The PS II-containing band from the gradient is then loaded on a Mono Q column and PS II cores can be purified following anion-exchange chromatography (Bumann and Oesterhelt, 1994)

The result of such an attempt to purify PS II cores is shown in Figure 5.12. The numbers corresponding to fractions from the sucrose gradient refer to the position of each band in the gradient, with band 1 being closer to the top of the ultracentrifugation tube and band 4 being closer to the bottom of the tube. As can be seen from Figure 5.12B, the PS II-containing band contains some contamination from antenna polypeptides and this limits its usefulness in identifying [^{32}P]-labelled bands. The chlorophyll *a/b* ratio of each of the three bands shown in Figure 5.12, was 1.57 for band 1, 2.41 for band 3 and 6.08 for band 4. The absorption spectrum of band 4 (not shown) was similar to previously published spectra of isolated PS II core complexes from spinach (Yamada *et al.*, 1987), *Synechocystis* PCC 6803 (Tang and Diner, 1994) and *C. reinhardtii* (Diner and Wollman, 1980 and Bumann and Oesterhelt, 1994). However the spectrum of band 4 indicated the presence of chlorophyll *b* (from LHCII polypeptides) which resulted in a red absorption maximum of 674.5 nm (instead of 676 nm observed in pure PS II core complexes).

The use of this technique proved useful for the isolation of LHCII particles which were devoid of PS II (as monitored by the absence of D2) (Figure 5.12 C). However, the yield of the PS II-containing band from the sucrose gradient was very low (<1% of the initial chlorophyll concentration in cells) compared to the one reported by Bumann and Oesterhelt (1994) (8-12%). Therefore, further purification of this band by chromatography was not attempted. In addition, the banding pattern of the sucrose gradient was quite heterogeneous between different experiments rendering very difficult the separation of the contents of the gradient into different fractions. Because of these disadvantages, it was decided not to pursue with this technique for the separation of [γ-^{32}P]-ATP labelled thylakoids.

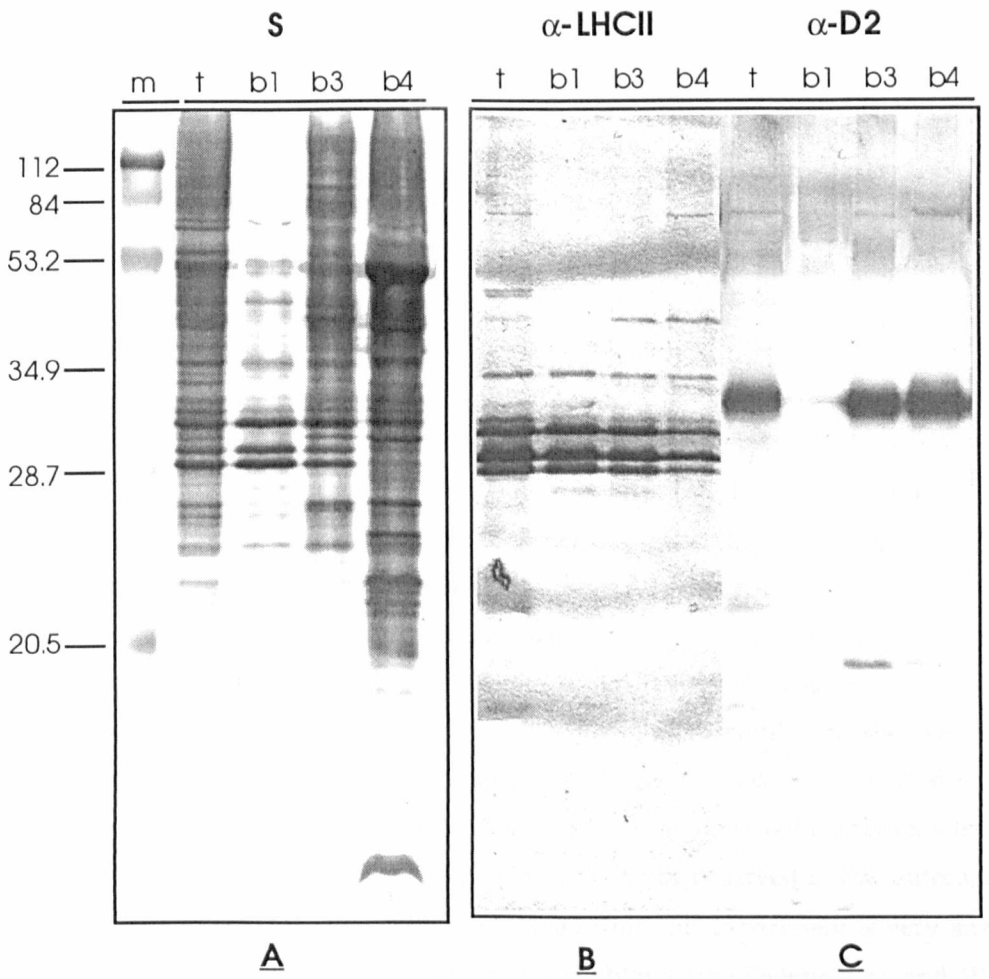


Figure 5.12: Silver-stained, 14%, 6 M urea-SDS gel (S, panel A) and Western blots with α-LHCII (B) and α-D2 (C) antibodies of fractions (b1, b3, b4) obtained after sucrose gradient centrifugation of thylakoid membranes (lane t) solubilised with DM (as described in text). The amount of chlorophyll was 3 μg for the thylakoid membranes and 1 μg for the different fractions. Lane m: molecular weight marker (prestained LMW, Bio-Rad)

5.3.4 *in vivo* phosphorylation of C. reinhardtii CC2655 whole cells

One of the main lines of evidence suggesting that D2 is phosphorylated in *C. reinhardtii* comes from the work of de Vitry *et al.* (1987, 1991) who used the double mutant F54-14 (strain CC2655) in *in vivo* [^{32}P]-labelling experiments. After labelling whole cells with [^{32}P]-orthophosphate, de Vitry and co-workers isolated PS II-containing particles which they used in order to show that the upper part of the D2 doublet (D2.1) corresponds to a phosphorylated band. The CC2655 strain lacks PS I and the ATPase complex. Therefore, it cannot be used as a control for the Thr2Ala and Thr2Ser mutants. However, this strain has been recently used successfully for the purification of PS II RCs (Alizadeh *et al.*, 1995).

Therefore, by labelling thylakoid membrane proteins with [^{32}P] and subsequently isolating PS II RCs, one should be able to positively identify whether D2 is phosphorylated or not. This strategy has been used successfully by Telfer et al. (1987) to show that D1 and D2 can be phosphorylated in vitro in peas.

The *in vivo* phosphorylation of *Chlamydomonas* cells with [^{32}P]-orthophosphate was performed according to the procedure described by de Vitry *et al.* (1991) (see section 2.19). Thylakoids and photosystem II reaction centre particles were prepared according to Alizadeh *et al.*, (1995) except that all buffers until the stage of column wash contained 20 mM NaF to inhibit dephosphorylation by phosphatases. Samples were analysed on a 10-17%, 6 M urea-SDS gel and then transferred to a nitrocellulose membrane. Radioactive bands were detected after exposure of either the dried gel or the blot to autoradiography using a PhosphorImager (Molecular Dynamics). The presence of D2 and the absence of most of LHCII from the isolated PS II RCs was verified by Western blotting (not shown).

Figure 5.13 shows the SDS-PAGE analysis (panel A) and the autoradiogram (panel B) of the blot, from the [^{32}P]-radiolabelled thylakoid proteins of *C. reinhardtii* strain CC2655. The labelling pattern of the thylakoid membranes is similar to the one observed after *in vitro* labelling of wild type thylakoids (Figure 5.9), as has been reported by Owens and Ohad (1982) and Wollman and Delepelaire (1984). Four bands with apparent molecular weights between approximately 25 kDa and 34.9 kDa can be observed in the autoradiogram (Figure 5.13B). The *in vivo* labelling pattern obtained from this experiment is very similar to corresponding *in vivo* phosphopolypeptide patterns obtained by Delepelaire and Wollman (1985), Gans and Wollman (1995) using wild type cells and by Delosme *et al.* (1996) using a PS I$^-$ mutant. The authors of these publications, assign these bands to p10, p9, p13/p11 and p17 (with descending order of molecular weight, starting from approximately 35 kDa). An additional labelled polypeptide in the low molecular weight region, has been designated as a "PS II" phosphoprotein in these publications (probably the phosphorylated form of the PsbH polypeptide). The latter does not appear in the autoradiogram of Figure 5.13, presumably because of the poor signal.

Labelled bands could not be observed in the lane with the PS II RC polypeptides (lane 2). From this result, one could conclude that D2 (as well as any other PS II RC protein) is not phosphorylated in this strain of C. *reinhardtii*. However, as can be seen from lane 1, the level of labelling of the thylakoids was very poor. The reason for this poor labelling of the thylakoids is not clear (possible reasons are discussed in section 5.4).

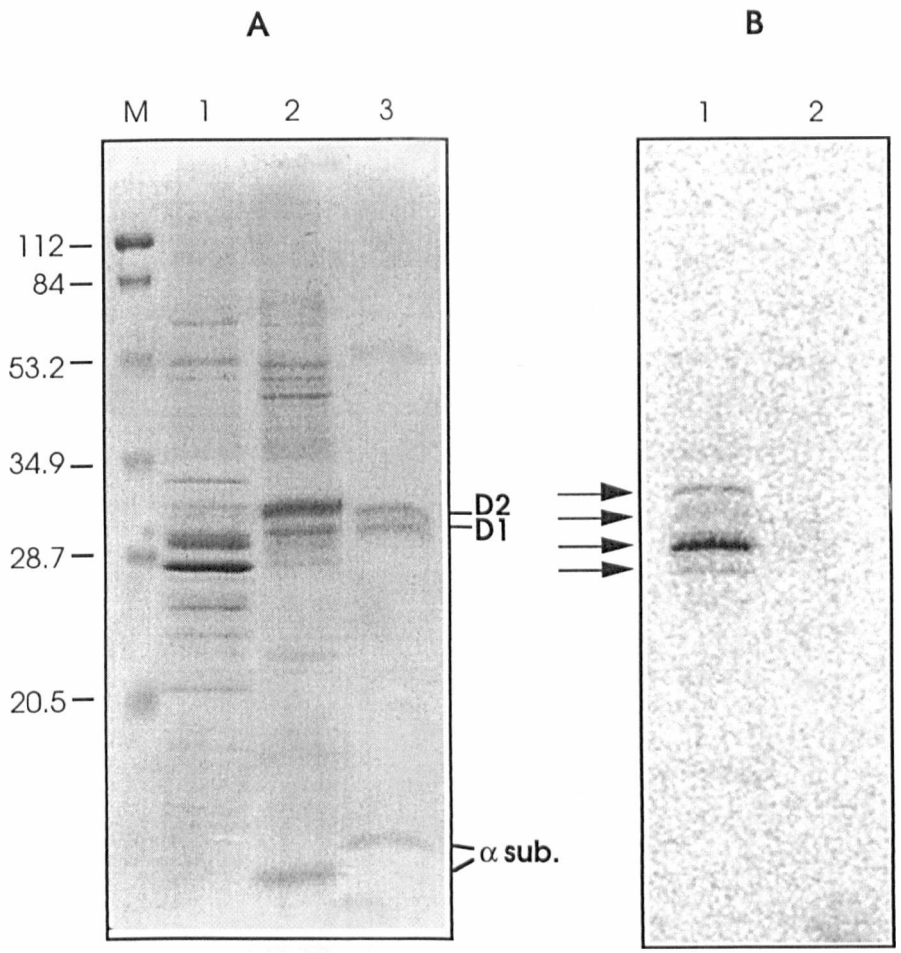


Figure 5.13: *in vivo* phosphorylation of *C. reinhardtii* CC2655 cells with [^{32}P]-orthophosphate. 10-17%, 6 M urea Coomassie blue-stained gel (A) and subsequent autoradiography (B) of thylakoid membranes (lane 1) and isolated PS II RCs (lane 2). Lane 3: PS II RCs from pea. Lane M: molecular weight marker (prestained LMW, Bio-Rad). The autoradiogram was obtained by exposing a blot to a PhosphorImager screen for 7 days. The amount of chlorophyll was 5 μg for the CC2655 thylakoids, 1 μg for the CC2655 PS II RCs and 0.2 μg for pea PS II RCs

5.3.5 Pulse-chase labelling of whole cells using [^{14}C]-acetate

The initial suggestions by Delepelaire (1984) about D2 phosphorylation were based on the observation that two polypeptides migrating very close to each other in urea-SDS gels, in the area of D2, show the same proteolytic digestion profiles. These bands can be more clearly observed in autoradiograms of thylakoids or whole cells after pulse labelling of whole cells with [^{14}C]-acetate. Those two bands have since then been called D2.1 (the phosphorylated form) and D2.2 (the non-phosphorylated form) (Delepelaire, 1984). The D2 doublet in urea-SDS gels or in autoradiograms from [^{14}C]-pulse labelled cells, has also been

shown by de Vitry *et al.* (1987, 1989, 1991), Bennoun *et al.* (1986), Jensen *et al.* (1986) and reported by Schuster *et al.* (1988) (although the D2 doublet cannot be seen in the respective autoradiogram).

In order to test whether the D2 doublet is a reproducible observation in autoradiograms of [^{14}C]-pulse labelled cells, wild type cells were grown in TAP at 26^{0}C for 2-3 days until an OD_{750} of 0.2-0.5. Cells were then pulse-labelled with [^{14}C]-acetate either at 2 µCi/ml for 45 minutes or at 5 µCi/ml for 5 minutes. Labelling was performed in the absence of protein synthesis inhibitors or in the presence of chloramphenicol (CAP) at 200 µg/ml or cycloheximide (CHI) at 10 µg/ml, as described in section 2.18. Thylakoid membranes were isolated according to the procedure of Komenda and Barber (1995) (section 2.21.1) and analysed on urea-SDS gels.

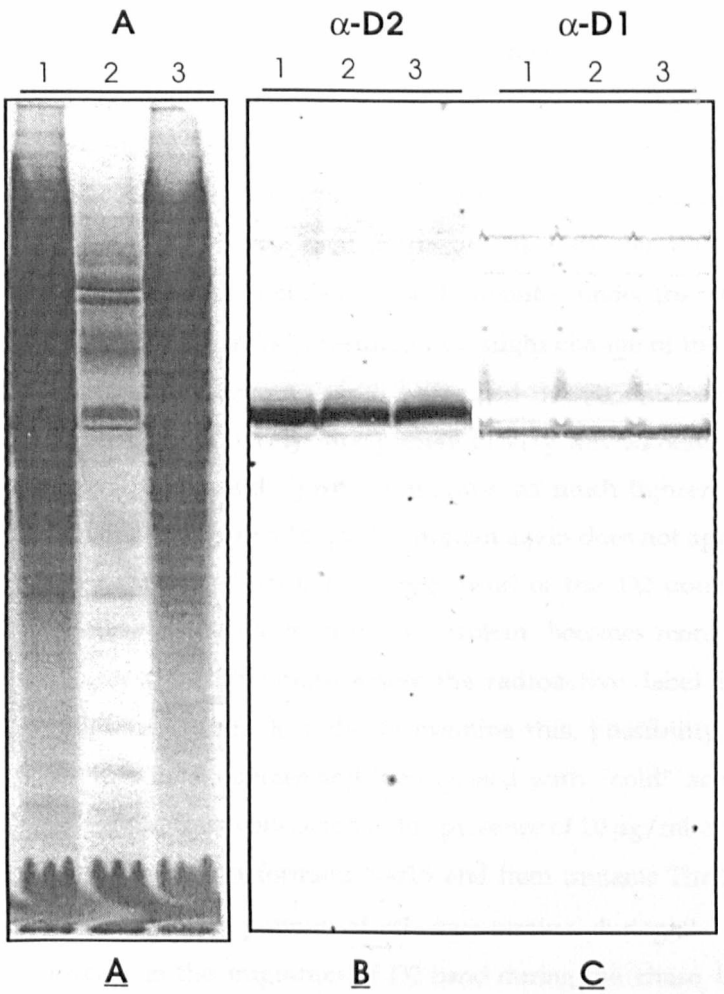


Figure 5.14: Autoradiogram (A) of a 12-18%, 8 M urea -SDS gel and Western blotting with α-D2 (B) and α-D1 (C) antibodies. Wild type cells were pulse-labelled for 5 minutes with [^{14}C]-acetate in the absence of inhibitors (lane 1) or in the presence of cycloheximide (lane 2) or chloramphenicol (lane 3).

Figure 5.14 shows the autoradiogram (of a 12-18%, 8 M urea-SDS gel) of thylakoid membranes isolated from cells labelled for 5 minutes. In the absence of any protein synthesis inhibitors it is difficult to assign the radiolabelled bands to particular protein subunits. Moreover as can be seen by comparing lanes 2 and 3, most of the polypeptides synthesised during this period were of cytoplasmic origin. Lane 2 (panel A) shows the labelling profile of chloroplast-encoded proteins (as cycloheximide inhibits cytoplasmic protein synthesis). This profile is very similar to the ones shown previously by other groups (Delepelaire, 1984; de Vitry *et al.*, 1989; van Wijk *et al.*, 1994; Kuras and Wollman, 1994) with the exception of the appearance of D2 (see below). The D1 and D2 polypeptides can be clearly distinguished by Western blotting using the corresponding antisera (panels B and C). As can be seen from lane 2 of the autoradiogram (panel A), D2 appears as a relatively thick band; however, a doublet cannot be observed.

When the products of the two different labelling times (5 minutes compared to 45 minutes) were analysed on a 14%, 8 M urea-SDS gel, differences in the labelling pattern could be observed. The two bands migrating in the area of D1 in the 5 minutes pulse (lane 1 of Figure 5.15) probably represent the precursor and the mature form of the protein. After 45 minutes of incubation with [^{14}C]-acetate, all label is incorporated into the mature form only. The heavily labelled band above D2 has been identified as CP43 by Western blotting (not shown). Of particular note is that incubation for 45 minutes under the conditions used for labelling (incident light of 100 $\mu E.m^{-2}.s^{-1}$), resulted in a slight change of the mobility of CP43. Moreover, two bands migrating between the 28.7 kDa and the 20.5 kDa markers and in the low molecular weight area, respectively, are present in very low amounts in the 45 minutes label (Figure 5.15, lane 2). Although, proteins migrate as much tighter bands in this gel system compared to the one of Figure 5.14, the D2 protein again does not appear as a doublet.

According to Delepelaire (1984), the upper band of the D2 doublet (D2.1), which should represent the phosphorylated form of the protein, becomes more apparent when a period of incubation follows the [^{14}C]-pulse where the radioactive label has been removed from the incubation mixture (chase). In order to examine this possibility, cells were pulse labelled with [^{14}C]-acetate for 5 minutes and then chased with "cold" acetate for 45 and 90 minutes. Both pulse and chase were conducted in the presence of 10 μg/ml cycloheximide. The experiment included cells from transformant Nsi16 and from mutants Thr2Ser and Thr2Ala. Figure 5.16A shows the labelling pattern of all four strains during the pulse and chase periods. A slight decrease in the migration of D2 band during the chase is apparent in all strains. In addition, an apparent decrease of the label on the lower part of D1 over the time of the chase, can also be observed. However, no difference in the labelling pattern between the wild type and the mutant strains can be observed. Differences between the total radioactivity incorporated during the pulse among the four strains can be attributed to differences in loading (as judged by the stained gel).

Figure 5.16 B shows a Western blot using antisera against D2. No differences can be observed between the mutants and the wild type in terms of the D2 content. The decrease in the migration of D2 during the chase, observed in the autoradiogram, can also be seen in the Western blot. This difference in migration is accompanied by a widening of this protein band, in all four strains.

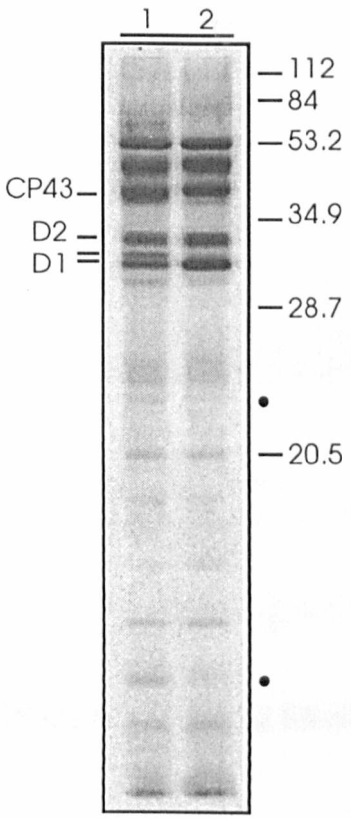


Figure 5.15: Autoradiogram of a 14%, 8 M urea -SDS gel of thylakoid membranes isolated from cells that have been pulse-labelled with [^{14}C]-acetate for either 5 minutes (lane 1) or 45 minutes (lane 2) in the presence of 10 µg/ml cycloheximide. The positions of D1, D2 and CP43 are indicated. (•) indicate bands present in considerably lower amounts in the 45 minutes label compared to the 5 minutes one. Bands of the molecular weight marker (prestained LMW, Bio-Rad) are indicated

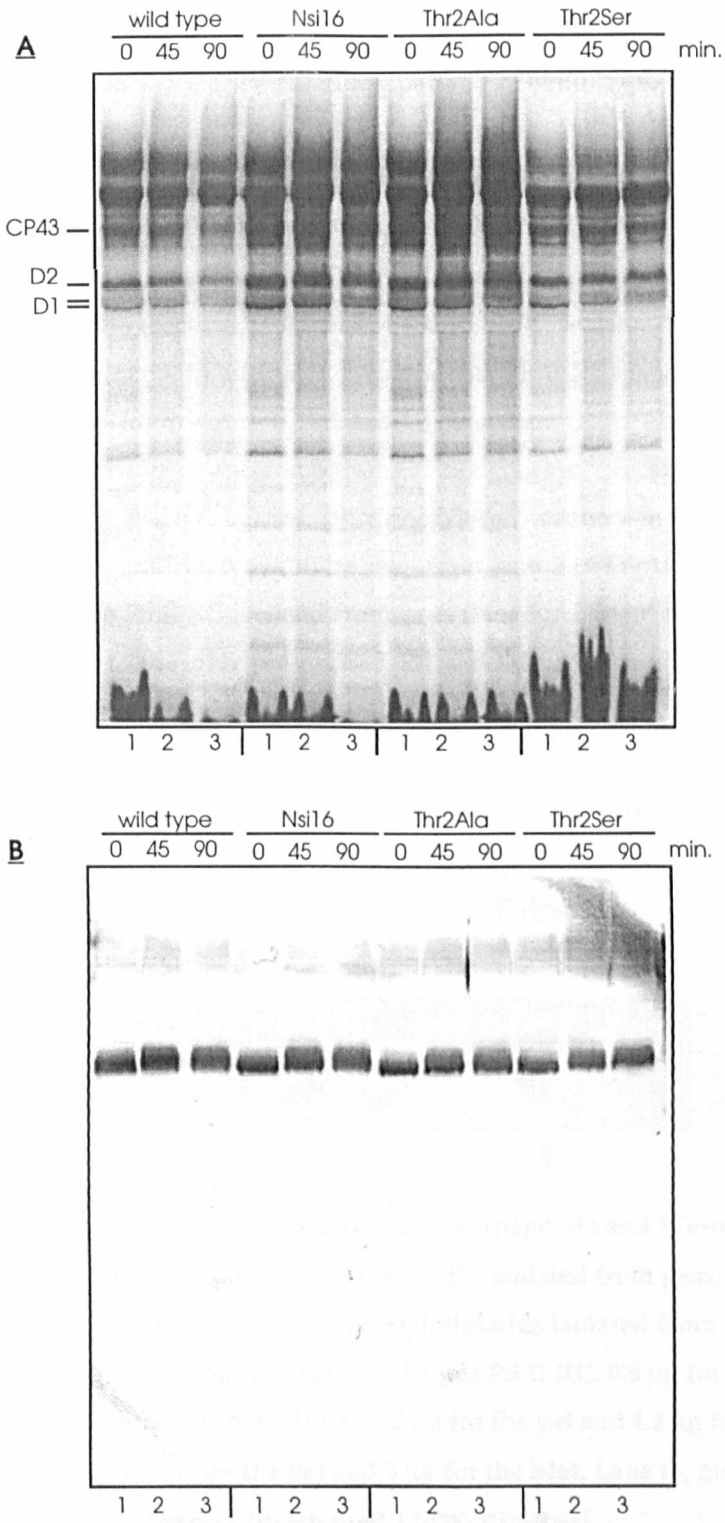


Figure 5.16: (A) Autoradiogram and (B) α-D2 Western blot of a 14%, 8 M urea -SDS gel of thylakoid membranes isolated from wild type, Nsi16, Thr2Ala and Thr2Ser cells pulse-labelled for 5 minutes with [^{14}C]-acetate, at an incident light intensity of 100 μE.m^{-2}.s^{-1} and then chased for 45 and 90 minutes (lanes 2 and 3, respectively) in the absence of label. Both pulse and chase were carried out in the presence of 10 μg/ml cycloheximide

5.3.6 Identification of labelled polypeptides using an α-phosphothreonine antibody

The *in vivo* and *in vitro* [^{32}P]-labelling pattern of wild type thylakoid membrane proteins was finally studied using a monoclonal antibody raised against phosphothreonine (Sigma). This antibody has been recently shown to recognise all the main phosphorylated polypeptides of the higher plant PS II core (CP43, D2, D1, PsbH polypeptide) (Giardi *et al.*, 1995). Figure 5.17 shows a silver-stained gel (A) and a Western blot (B) of wild type thylakoids labelled *in vitro* (section 5.3.2) and of the PS II RC isolated from the *in vivo* [^{32}P]-labelled cells of strain CC2655. In addition, non-labelled PS II RCs isolated from peas, were included. As can be seen from Figure 5.17B lane 3, the four main phosphorylated bands, observed in the previous autoradiograms, are being recognised as carrying phosphothreonines. The absence of any phosphorylated protein in the PS II RC of *C. reinhardtii* CC2655 is also confirmed as can be seen from lane 2 of Figure 5.17B. On the other hand, in the lane with the PS II RC isolated from peas (lane 1), a broad band can be observed, which can be attributed to the phosphorylated D1 and D2 proteins (Telfer *et al.*, 1987). As the PS II RC isolated from *C. reinhardtii* CC2655 is not as pure as the corresponding PS II RC from peas (see Figure 5.13), the amount of sample from the *Chlamydomonas* preparation, loaded for the Western blot, is twice as much as the one from peas (on a chlorophyll basis).

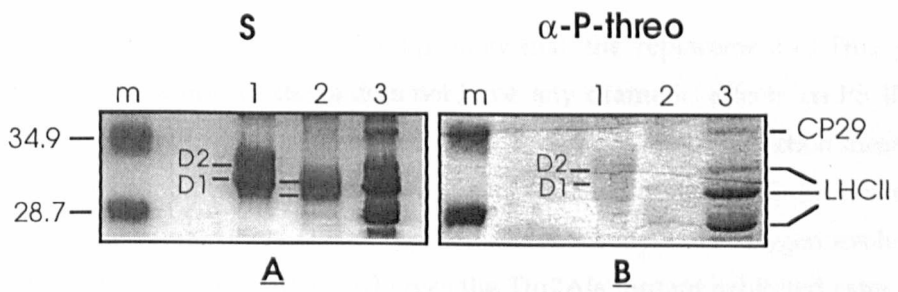


Figure 5.17: Silver-stained 10-17%, 6 M urea-SDS gel (panel A) and Western blot with α-phosphothreonine antibody (panel B). Lane 1, PS II RC isolated from peas; lane 2, PS II RCs isolated from *C. reinhardtii* CC2655; lane 3, thylakoids isolated from wild type *C. reinhardtii*. The amount of chlorophyll was: for the pea PS II RC, 0.3 μg for the gel and 0.6 μg for the blot; for the *Chlamydomonas* PS II RC, 0.2 μg for the gel and 1.2 μg for the blot and for the wild type thylakoids, 1μg for the gel and 3 μg for the blot. Lane m, molecular weight marker (prestained LMW, Bio-Rad).

Figure 5.18 shows a Western blot of thylakoids isolated from wild type and CC2655 strains, labelled *in vitro* and *in vivo*, respectively. The same bands are recognised by the α-phosphothreonine antibody as being phosphorylated in the two strains. Although, the phosphorylation profile looks very similar between the two strains, the band which has been attributed to CP29 in this chapter, shows a very strong cross reaction with the α-

phosphothreonine antibody. The reason for that, as well as the identity of the strongly labelled bands in the region between the 34.9 and 53.2 molecular weight markers, is not clear.

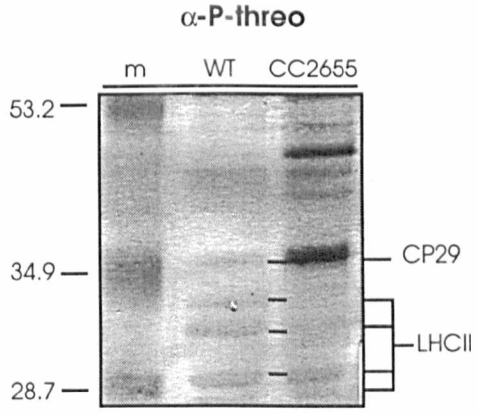


Figure 5.18: Western blot of thylakoids isolated from wild type and strain CC2655 with an α-phosphothreonine antibody. Lane m, molecular weight marker (prestained LMW, Bio-Rad).

5.4 Discussion

The results presented in this chapter show that the replacement of Thr2 of the D2 protein with either alanine or serine does not have any dramatic effects on PS II function. Photoautotrophic growth rates, fluorescence decay and fluorescence induction measurements, as well as thermoluminescence measurements have indicated that both Thr2Ala and Thr2Ser mutants are similar to wild type. Wild type levels of steady-state oxygen evolution were also obtained for the Thr2Ser mutant, whereas the Thr2Ala mutant exhibited rates of oxygen evolution from around 55% to 100% of the wild type, probably reflecting the inherent variability of this type of measurement.

One of the aims of the construction of the two threonine mutants, was to study the relationship between the phosphorylation of PS II proteins and photoinhibition (reviewed in Allen, 1992). Initial reports suggested that phosphorylation of thylakoid membrane proteins can afford a protective role against photoinhibiton (Horton and Lee, 1985). However, this report did not concern specifically the phosphorylation of PS II proteins. Michel *et al.* (1988) have suggested that phosphorylation of the NH_2 terminus of D1 or D2 leads to interaction between the phosphoryl group and the iron atom located between the Q_A and Q_B binding sites. Moreover, the authors, speculated that phosphorylation of PS II proteins might be important in providing pH buffering which in turn, would affect protonation of D1 and D2 and also Q_A to Q_B electron transfer (Michel *et al.*, 1988). According to Harrison and Allen (1991) either of these mechanisms could play a role in sustaining electron transport at high light intensities, therefore, a protective role against

photoinhibition was predicted for PS II phosphorylation. Recently, phosphorylation of PS II proteins and in particular of D1, have been related to the regulation of D1 degradation, as part of PS II photoinactivation during photoinhibition. The relation of light-induced D1 degradation in relation to photoinhibition *in vivo* has been reviewed by Ohad *et al.* (1994). It has been proposed that phosphorylation of D1 in higher plants is induced by a photoinhibitory damage to PS II (Aro *et al.*, 1993) and might regulate its rapid turnover by preventing its degradation (Aro *et al.*, 1992; Rintamäki *et al.*, 1995; Koivuniemi *et al.*, 1995; Ebbert and Godde, 1995). More specifically, Rintamäki *et al.* (1995) suggested that damaged D1 is protected from degradation under conditions where the repair cycle cannot cope with the rate of photoinhibition and that this protection is provided in the form of phosphorylation. As phosphorylated D1 is degraded under photoinhibitory illumination more slowly than the non-phosphorylated form of the protein (Aro *et al.*, 1992; Ebbert and Godde, 1995), this form could act as the storage form of photodamaged D1 when the rate of damage to PS II exceeds the capacity of the thylakoids to repair the PS II centres (Rintamäki *et al.*, 1995). The D2 degradation pattern during photoinhibition shares many of the properties ascribed to D1 degradation (Andersson *et al.*, 1994). Koivuniemi *et al.* (1995) reported that the degradation rate of the phosphorylated form of D2 in higher plants is also reduced under photoinhibitory illumination and suggested a similar regulation to its degradation to the one outlined for D1.

The pattern of PS II photoinactivation and D1 degradation in *C. reinhardtii* under photoinhibitory light is similar to the one reported for higher plants (Ohad *et al.*, 1984; Prasil *et al.*, 1992). However, D1 has not been shown to be phosphorylated in *C. reinhardtii* (Delepelaire and Wollman, 1985; de Vitry *et al.*, 1991) and the same applies for cyanobacteria (Bennett, 1991).

The possible role of D2 phosphorylation in relation to photoinhibition, in *C. reinhardtii*, was studied by incubating whole cells of the Thr2Ala and Thr2Ser mutants at high light and monitoring the rate of PS II photoinactivation. Giardi *et al.* (1994) using differentially phosphorylated PS II cores from spinach, reported that PS II cores that had only the D1 and D2 proteins phosphorylated were more sensitive to high light than cores having the CP43 and PsbH proteins only phosphorylated and suggested that phosphorylation of D1 and D2 increases the sensitivity of PS II to light. Therefore, assuming that D2 is phosphorylated in *C. reinhardtii* and that the Thr2Ala mutation abolishes D2 phosphorylation, one could predict that PS II phosphorylation should increase the rate of PS II photoinactivation. However, when cells from the wild type and Thr2Ala strains were treated with high light in the presence of chloroplast synthesis inhibitors (chloramphenicol) there was no difference in the rates of loss of PS II activity between the wild type and the mutant. Under these conditions the loss of PS II activity is a monitor of the damage to PS II that would normally be repaired by new protein synthesis. Similar results were obtained with mutant Thr2Ser.

The possible role of D2 phosphorylation in state transitions was tested by subjecting wild type and mutant cells to conditions that would adapt them into either state I or state II and then measuring the relative yield (F_v/F_m) of variable fluorescence. Using this technique, a lower F_v/F_m was obtained in the wild type when cells were adapted to state I than when they were adapted to state II. The same was observed for the Thr2Ala mutant, although a lower F_v/F_m after adaptation to state II, was observed in the latter compared to the wild type. Therefore, it could be concluded that state transitions were functioning in the Thr2Ala strain. When the same measurements were carried out using cells from mutant Thr2Ser, a relatively lower fluorescence yield was obtained, irrespective of the conditions of preincubation. This result could imply that the Thr2Ser cells could not undergo state transitions.

Attempts to interpret the results obtained from the phenotypic analysis of the mutants have been based on the assumption that D2 can be phosphorylated in wild type *C. reinhardtii*. The evidence for D2 phosphorylation in *C. reinhardtii* in the literature, are based on autoradiograms of [^{14}C]- and [^{32}P]-labelled cells and thylakoid membranes (Delepelaire and Wollman, 1985; de Vitry and Wollman, 1988; de Vitry *et al.*, 1987, 1989, 1991). These autoradiograms have not been accompanied by Western blots to identify D2. In an effort to obtain satisfactory data concerning the state of D2 phosphorylation in the wild type and mutant strains, different approaches were followed, including *in vitro* and *in vivo* [^{32}P]-labelling of thylakoid membrane proteins, identification of labelled bands using a monoclonal antibody against phosphothreonine and pulse-chase labelling of whole cells with [^{14}C]-acetate.

As has been discussed in section 5.3.1, the *in vitro* phosphorylation pattern of thylakoid membrane polypeptides from the wild type is similar to analogous results from the literature. The *in vitro* phosphorylation of thylakoids from the Thr2Ala mutant did not show any differences in the labelling pattern from the wild type. Thus, there was no band in the 30-35 kDa region that was present in the wild type and absent from the mutant. In both strains there was a stimulation in the activity of the kinase when the labelling was performed in the light. Moreover, the total amount of label incorporated, was much higher in the thylakoids isolated from dark-grown cells and labelled in the light than in the thylakoids isolated from light-grown cells labelled in the light. This implies that the PQ pool of the light grown cells was not completely oxidised before the beginning of labelling. Therefore, there was still a population of LHCII proteins phosphorylated after the thylakoid isolation procedure, although the latter was conducted entirely in the dark and had a duration of around 6 hours.

Western blotting with α-D2 antisera of the labelled thylakoids revealed that the upper part of D2 is missing in the Thr2Ala lanes. The loss of this part of D2 in the mutant, did not correspond to a loss of any labelled band. Therefore, it cannot be attributed to the absence of a phosphorylated form of D2. This result, together with the corresponding

autoradiogram present us with three possibilities concerning phosphorylation of D2: (i) D2 can be phosphorylated in the wild type but not in the Thr2Ala mutant. This is quite unlikely as there is no labelled band in the wild type that is missing from the mutant; (ii) D2 can be phosphorylated in both strains. In this case, Thr2Ala would have to be phosphorylated at other threonine residues, such as the ones present at positions 7 and 13 of the amino acid sequence (Erickson *et al.*, 1986). Moreover, the phosphorylated form of D2 would have to be located in the bottom part of the diffuse D2 band of the Western blot. Phosphorylation is known to result in the conversion of the protein to a slower migrating band (e.g., Elich *et al.*, 1992; Aro *et al.*, 1992, for the D1 protein of higher plants or de Vitry *et al.*, 1987, 1991, for CP43 of *Chlamydomonas*). However, one has to bear in mind that the replacement of just one amino acid residue (Thr to Ala) could alter the mobility of a protein on SDS-PAGE, as in the case of the Pro161Leu mutation of D2 in *Synechocystis* PCC 6803 (Tommos *et. al.*, 1994); (iii) D2 cannot be phosphorylated in *C. reinhardtii*. Although, the data from the use of autoradiography and Western blotting of wild type and Thr2Ala thylakoids could point towards this direction, the possibility that phosphorylated D2 migrates as a very diffuse band (Ikeuchi *et al.*, 1987a) which is not possible to detect in an autoradiogram of thylakoid membranes, cannot be excluded. Moreover, as only the non-phosphorylated form of D2 could be phosphorylated in *in vitro* labelling experiments, it is possible that the phosphorylated form of D2 is quite stable and difficult to dephosphorylate.

The results from the *in vitro* labelling experiments gave further evidence that the labelled band migrating just on top of the upper part of D2 cannot be attributed to the phosphorylated form of this protein, as it is present in both the wild type and Thr2Ala lanes. This band has most commonly been attributed to phosphorylated D2 in the literature (e.g., see autoradiograms in Lemaire *et al.*, 1987; de Vitry and Wollman, 1988; de Vitry *et al.*, 1991; Gans and Wollman, 1995).

As identification of labelled D2 in autoradiograms of labelled thylakoid membranes is an arduous task, attempts were carried out to "amplify" the presence of D2 either by immunoprecipitation or by the isolation of PS II-enriched particles (PS II cores and PS II RCs). Extensive immunoprecipitation trial experiments were not successful (results not shown). In addition, PS II-enriched core particles were produced but as they contained significant contamination from LHCII and minor antenna polypeptides, they were not used in [^{32}P]-labelling experiments. A more revealing experiment regarding D2 phosphorylation, was the *in vivo* labelling of whole cells with [^{32}P]-orthophosphate and the subsequent isolation of PS II RCs. Whole cells of the *C. reinhardtii* CC2655 strain, which is deficient in PS I and the ATPase complexes, were labelled with [^{32}P]-orthophosphate and PS II RCs were isolate according to Alizadeh *et al.* (1995). The labelling pattern of the thylakoid membranes was quite similar to the *in vitro* labelling profile. No labelled band could be observed in the PS II RC lane, implying that none of the PS II RC proteins in *C. reinhardtii*, is phosphorylated. However, the total labelling of the thylakoids was relatively poor, probably reflecting

inappropriate conditions during incubation of the cells with [^{32}P]-orthophosphate. Alternatively, the amount of NaF added in the buffers (20 mM) might be inadequate to inhibit the activity of phosphatases. Gans and Wollman (1995) have reported that they were only able to inhibit phosphatases when the NaF concentration was 100 mM and they suggested that with intact cells of *C. reinhardtii* this is because of the restricted diffusion of NaF through both the plasma membrane and the double chloroplast envelope. Regarding the absence of any label from the PS II RC, one should not exclude the possibility that D2 was phosphorylated before the depletion of the medium from phosphate and the incubation with [^{32}P]-orthophosphate (although cells were incubated with the label for 16 hours) and that the rate of exchange between the radioactive and non-radioactive phosphate was low. Alternatively, significant reserves of phosphate might be present in cells after washing and during incubation of the latter with the label. In this case, the chances of non-radioactive phosphate being incorporated into thylakoid proteins (rather than [^{32}P]), would still be high.

The results from the *in vivo* [^{32}P]-labelling were confirmed by the use of a monoclonal antibody that recognises phosphothreonine i.e., no protein cross-reacted with the antibody when the latter was used in Western blots containing labelled PS II RCs from *C. reinhardtii* CC2655. On the other hand the antibody could recognise all the main bands observed after in vitro labelling of wild type thylakoids. Moreover, the antibody cross-reacted with the D1 and D2 proteins from PS II RCs isolated from peas. Although, this sample had not previously been used in any labelling experiments, it still contained small amounts of phosphorylated polypeptides. This is in line with previous reports that some degree of phosphorylation of the PS II proteins is always present under physiological conditions (Elich *et al.*, 1992; Giardi *et al.*, 1994).

Attempts to identify the two bands comprising the D2 doublet (D2.1 and D2.2) (Delepelaire, 1984) were also carried out during the course of this study. Thus, cells were pulse-labelled with [^{14}C]-acetate and chased in the absence of radioactive label. In these experiments, D2 always appeared as one band. Moreover, no differences could be observed between the wild type and the Thr2Ala and Thr2Ser strains in terms of the labelling pattern. However, in all strains, D2 appeared to migrate slower during the chase, than at time 0 (just after the pulse). This pattern of migration after a light incubation resembles the migration pattern of D1 after photoinhibitory treatments of higher plants (e.g., Ponticos *et al.*, 1993). Moreover, a widening of D2 was observed during the chase, which is not mirrored in the autoradiogram. The latter observation could reflect the two populations of D2 within the chloroplast of *Chlamydomonas*. The vast majority of D2 is in the PS II complex where it can be damaged by photoinhibition (Prasil *et al.*, 1992) and therefore appear as a broadened band in a Western blot. On the other hand, the radiolabelled D2 observed in the autoradiogram corresponds to the newly synthesised protein which may not be incorporated into the PS II complex and therefore, is not affected by photoinhibitory damage.

In summary, replacement of Thr2 of D2 with either alanine or serine did not result in any dramatic changes in the PS II function. Differences between the wild type and the mutants were detected during photoinhibition and state transition measurements, however further analyses would have to be performed in order to understand and evaluate the significance of these results.

The data presented in this work did not provide us with conclusive evidence concerning D2 phosphorylation in either the wild type or the mutant strains. Most of the labelled bands observed after *in vitro* and *in vivo* labelling with [^{32}P] could be attributed to polypeptides belonging to the LHCII complex or to minor antenna polypeptides (CP29). In addition, data obtained from *in vivo* [^{32}P]-labelling of cells belonging to the *C. reinhardtii* CC2655 strain and subsequent isolation of PS II RCs did not reveal phosphorylation of any PS II RC protein. It is, therefore, possible that the band that has been reported in the literature as the phosphorylated form of D2 (designated as D2.1), is in fact a phosphorylated form of a LHCII polypeptide. This band has been shown by de Vitry *et al.* (1987, 1991) to disappear after treatment of PS II particles (labelled with [^{32}P]-orthophosphate) with alkaline phosphatase. However, this band could readily be an antenna polypeptide present as a "contaminant" in their preparation. Indeed, the chl *a/b* ratio (7±1) of the PS II preparation of de Vitry *et al.* (1991) does not differ substantially to the corresponding ratio of the PS II preparation shown in the present work (6.08 for band 4 in section 5.3.3). The latter was shown by Western blotting to contain some contamination from antenna polypeptides (see section 5.3.3). Indeed, de Vitry *et al.* (1991) noted the weak presence of polypeptides p10 (CP26) and p13 in the autoradiogram of their [^{32}P]-labelled PS II particles. The so-called D2.1 band has also appeared in autoradiograms of [^{14}C]-labelled cells (Delepelaire, 1984; Bennoun *et al.*, 1986; Jensen *et al.*, 1996; Kuras and Wollman, 1994). However, no definite identification has been made.

From the data presented here and in the literature, it is evident that more work would have to be carried out in order to conclude about the phosphorylation state of PS II proteins in *C. reinhardtii*. This work would have to combine the use of mutant strains deficient in either the PS II RC proteins or in the LHCII complex, with the use of wild type PS II RCs and methods such as the one used by Bennett and co-corkers for the identification of the phosphorylation site of PS II phosphoproteins in spinach (Michel et al., 1988).

6. General Conclusions

The work described in this thesis has demonstrated the use of the green alga *Chlamydomonas reinhardtii* as a model plant system for the dissection of structure-function relationships within the D2 protein of photosystem II. As described in chapter 1 (Introduction), *C. reinhardtii* possesses a plethora of characteristics that make it an attractive system to study photosynthesis. In connection with the D2 protein, *Chlamydomonas* has two particular advantages over higher plants and cyanobacteria. Both of them arise from the position of the gene encoding D2 (the *psbD* gene) on the chloroplast genome of the organism. Thus, unlike higher plants, the *psbD* gene is not co-transcribed with the *psbC* gene (which encodes the CP43 protein of PS II). This characteristic gives the opportunity to construct site-directed mutants of D2, particularly at the C-terminus, without affecting expression of the CP43 protein. In addition, the presence of the *psaA*-2 exon (which, together with the other two exons of the *psaA* gene, encodes the PsaA polypeptide of PS I) immediately downstream of *psbD* enables the construction of D2 mutants in a PS I$^-$ background. This was achieved in the present work by insertion of a selectable marker (the *aadA* expression cassette) inside the *psaA*-2 exon. This feature is very important, as it enables the isolation of PS II reaction centre complexes based on already published procedures that employ PS I$^-$ strains for this purpose.

The ability to construct D2 mutants in a PS I$^-$ background has been clearly demonstrated in this thesis in the form of the Leu205Tyr mutant. This mutation was constructed with the aim to study the role of this residue (D2-Leu205) in primary electron transfer in PS II. The Leu205Tyr mutant was created in both a wild type (by inserting the *aadA* selectable marker in a position within the 5'-UTR of *psbD* that does not interfere with *psbD* expression) and a PS I$^-$ background. The first version of the mutant was used for *in vivo* analyses whereas the Leu205Tyr/PS I$^-$ mutant was used for biophysical measurements on isolated PS II RCs. The data obtained from the characterisation of this mutant showed that the replacement of Leu205 with a Tyr resulted in a reduced quantum yield and a slowdown in the rate of primary charge separation. Therefore, one could conclude that Leu205 has a role in primary electron transfer. However, in the absence of an X-ray crystallographic structure of PS II, the nature of this role remains unknown. Thus, it is not possible to speculate whether the changes observed in the Leu205Tyr mutant relative to wild type originate from the chemical nature of tyrosine or from structural rearrangements due to the larger size of Tyr compared to Leu. This mutation provides an example of the inherent limitations of protein engineering as a means to study structure-function relationships alone, in the absence of crystallographic data.

The second set of mutants created in *C. reinhardtii*, as part of this project, where the D2-Thr2Ala and D2-Thr2Ser mutants. The N-terminal Thr2 has been shown (Michel *et al.*, 1988) to be the site of D2 phosphorylation in higher plants. D2 has also been suggested to be phosphorylated in *C. reinhardtii*. The role of this phosphorylation remains unknown. The Thr2Ala and Thr2Ser mutations were created in order to gain knowledge concerning the phosphorylation of PS II RC proteins. None of the two mutations resulted in any serious disturbances of PS II function. Therefore, one can conclude that Thr2 does not have any significant role in the function of PS II in *C. reinhardtii*. Attempts to identify phosphorylated D2 in wild type *C. reinhardtii* were also carried out. However, it has not been possible to observe the phosphorylated form of D2. In the contrary, evidence was obtained (by both *in vivo* and *in vitro* labelling with [^{32}P] and by the use of an α-phosphothreonine antibody) that D2 is probably not phosphorylated in *C. reinhardtii*. This suggestion would be in line with the minimal effects of the Thr2 mutations in PS II function.

References

Abdel-Mawgood A.L. and Dilley R.A. (1990) Cloning and nucleotide sequence of the *psbH* gene from the cyanobacterium *Synechocystis* 6803. *Plant Mol Biol* **13**: 469-479

Ahmad I., Giorgi L., Barber J., Porter G. and Klug D.R. (1993) Redox potential of cytochrome b_{559} in the D1/D2/cytochrome b_{559} reaction centre of photosystem II. *Biochim Biophys Acta* **1143**: 239-242

Alizadeh S., Nechushtai R., Barber J. and Nixon P. (1994) Nucleotide sequence of the *psbE*, *psbF* and *trnM* genes from the chloroplast genome of *Chlamydomonas reinhardtii*. *Biochim Biophys Acta* **1188**: 439-442

Alizadeh S., Nixon P.J., Telfer A. and Barber J. (1995) Isolation and characterisation of the Photosystem two reaction centre complex from a double mutant of *Chlamydomonas reinhardtii*. *Photosynth Res* **43**: 165-171

Allen J.F. (1992) Protein phosphorylation in regulation of photosynthesis. *Biochim Biophys Acta* **1098**: 275-335

Allen J.F. (1995) Thylakoid protein phosphorylation, state 1-state 2 transitions, and photosystem stoichiometry adjustment: redox control at multiple levels of gene expression. *Physiol Plantarum* **93**: 196-205

Allen J.F., Bennett J., Steinback K.E. and Arntzen C.J. (1981) Chloroplast protein phosphorylation couples plastoquinone redox state to distribution of excitation energy between photosystems. *Nature* **291**: 25-29

Allen J.P., Feher G., Yeates T.O., Komiya H. and Rees D.C. (1987a) Structure of the reaction centre from *Rhodobacter sphaeroides* R-26: The cofactors. *Proc Natl Acad Sci USA* **84**: 5730-5734

Allen J.P., Feher G., Yeates T.O., Komiya H. and Rees D.C. (1987b) Structure of the reaction centre from *Rhodobacter sphaeroides* R-26: The protein subunits. *Proc Natl Acad Sci USA* **84**: 6162-6166

Allen K.D. and Staehelin L.A. (1994) Polypeptide composition, assembly and phosphorylation patterns of the photosystem II antenna system of *Chlamydomonas reinhardtii*. *Planta* **194**: 42-54

Anbudurai P.R. and Pakrasi H.B. (1993) Mutational analysis of the PsbL protein of Photosystem II in the cyanobacterium *Synechocystis* sp. PCC 6803. *Z Naturforsch* **48c**: 267-274

Anderson J.M. (1992) Cytochrome b_6f complex: Dynamic molecular organisation, function and acclimation. *Photosynth Res* **34**: 341-357

Anderson J.M. and Andersson B. (1988) The dynamic photosynthetic membrane and regulation of solar energy conservation. *Trends Biochem Sci* **13**: 351-355

Anderson J.M. and Melis A. (1983) Localisation of different photosystems in separate regions of chloroplast membranes. *Proc Natl Acad Sci USA* **80**: 745-749

Andersson B. and Anderson J.M. (1980) Lateral heterogeneity in the distribution of chlorophyll-protein complexes of the thylakoid membranes of spinach chloroplasts. *Biochim Biophys Acta* **593**: 427-440

Andersson B. and Barber J. (1994) Composition, organisation, and dynamics of thylakoid membranes. In: Barber J (ed.), "Molecular Processes of Photosynthesis", Vol. 10, pp 1-53, JAI Press Inc., USA.

Andersson B., Anderson J.M. and Ryrie I.J. (1982) Transbilayer organisation of the chlorophyll-proteins of spinach thylakoids. *Eur J Biochem* **123**: 465-472

Amon D.I. (1949) Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. *Plant Physiol* **24**: 1-15

Aro E.-M., Kettunen R. and Tyystjärvi E. (1992) ATP and light regulate D1 protein modification and degradation. Role of D1* in photoinhibition. *FEBS Lett* **297**: 29-33

Aro E.-M., Virgin I. and Andersson B. (1993) Photoinhibition of Photosystem II. Inactivation, protein damage and turnover. *Biochim Biophys Acta* **1143**: 113-134

Ashworth A. (1993) Cloning transcription factors by homology. In: Latchman DS (ed), "Transcription factors-a practical approach", IRL Press, Oxford

Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A. and Struhl K. (1992) Short Protocols in Molecular Biology. Green Publishing Associates

Barber J. (1987) Photosynthetic reaction centres: a common link. *Trends Biochem Sci* **12**: 321-326

Barber J. (1989) Function and molecular biology of photosystem II. In: Miflin B.J. (ed) "Oxford Surveys of Plant Molecular and Cell Biology", Vol. 6, pp 115-162, Oxford University Press, Oxford

Barber J. (1993) Learning about photosystem II from analogies with purple photosynthetic bacteria. *Bioenergetics Group and British Photobiology Society Joint Colloquium, Sheffield, UK, 1993*, pp 981-986

Barber J. (1994) F_1-ATPase structure: getting our bearings for ATP synthesis. *Structure* **2**: 889-890

Barber J. and Andersson B. (1994) Revealing the blueprint of photosynthesis. *Nature* **370**: 31-34

Barber J. and De Las Rivas J. (1993) A functional model for the role of cytochrome b_{559} in the protection against photoinhibition. *Proc Natl Acad Sci USA* **90**: 10942-10946

Barber J., Chapman D.J. and Telfer A. (1987) Characterisation of a PS II reaction centre from chloroplasts of *Pisum sativum*. *FEBS Lett* **220**: 67-73

Barnes W.M. (1994) PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates. *Proc Natl Acad Sci USA* **91**: 2216-2220

Barry B.A. and Babcock G.T. (1987) Tyrosine radicals are involved in the photosynthetic oxygen-evolving system. *Proc Natl Acad Sci USA* **84**: 7099-7103

Barry B.A., El-Deeb M., Sithole I., Debus R., McIntosh L. and Babcock G.T. (1990) Structural studies of the stable tyrosine radical, YD^+, in photosystem II. In: Baltscheffsky M (ed) "Current Research in Photosynthesis", Vol. 1, pp 483-486, Kluwer Academic Publishers, Dordrecht

Bassi R. and Wollman F.-A. (1991) The chlorophyll-*a/b* proteins of photosystem II in *Chlamydomonas reinhardtii*. *Planta* **183**: 423-433

Becker M., Nagarajan V., Middendorf D., Parson W.W., Martin J.E. and Blankenship R.E. (1991) Temperature dependence of the initial electron transfer kinetics in photosynthetic reaction centres of *Chloroflexus aurantiacus*. *Biochim Biophys Acta* **1057**: 299-312

Bennett J. (1977) Phosphorylation of chloroplast membrane proteins. *Nature* **269**: 344-346

Bennett J. (1979) Chloroplast phosphoproteins. The protein kinase of thylakoid membranes is light-dependent. *FEBS Lett* **103**: 342-344

Bennett J. (1980) Chloroplast phosphoproteins. Evidence for a thylakoid-bound phosphoprotein phosphatase. *Eur J Biochem* **104**: 85-89

Bennett J. (1991) Protein phosphorylation in green plant chloroplasts. *Ann Rev Plant Physiol Plant Mol Biol* **42**: 281-311

Bennett J., Steinback K.E. and Arntzen C.J. (1980) Chloroplast phosphoproteins: Regulation of excitation energy transfer by phosphorylation of thylakoid membrane polypeptides. *Proc Natl Acad Sci USA* **77**: 5253-5257

Bennoun P. (1982) Evidence for a respiratory chain in the chloroplast. *Proc Natl Acad Sci USA* **79**: 4352-4356

Bennoun P., Spierer-Herz M., Erickson J., Girard-Bascou J., Pierre Y., Delosme M. and Rochaix J.-D. (1986) Characterisation of photosystem II mutants of *Chlamydomonas reinhardii* lacking the *psbA* gene. *Plant Mol Biol* **6**: 151-160

Bergantino E., Dainese P., Cerovic Z., Sechi S. and Bassi R. (1995) A post-translational modification of the Photosystem II subunit CP29 protects maize from cold stress. *J Biol Chem* **270**: 8474-8481

Blowers A.D., Bogorad L., Shark K.B. and Sanford J.C. (1989) Studies on *Chlamydomonas* chloroplast transformation: Foreign DNA can be stably maintained in the chromosome. *Plant Cell* **1**: 123-132

Blubaugh D.J. and Govindjee (1988) The molecular mechanism of the bicarbonate effect at the plastoquinone reductase site of photosynthesis. *Photosynth Res* **19**: 85-28

Boekema E.J., Hankamer B., Bald D., Kruip J., Nield J., Boonstra J.F., Barber J. and Rögner M. (1995) Supramolecular structure of the photosystem II complex from green plants and cyanobacteria. *Proc Natl Acad Sci USA* **92**: 175-179

Boerner R.J., Nguyen A.P., Barry B.A. and Debus R.J. (1992) Evidence from directed mutagenesis that aspartate 170 of the D1 polypeptide influences the assembly and/or stability of the manganese cluster in the photosynthetic water-splitting complex. *Biochemistry* **31**: 6660-6672

Boichenko V.A., Klimov V.V., Mayes S.R. and Barber J. (1993) Characterisation of the light-induced oxygen gas exchange from the IC2 deletion mutant of *Synechocystis* PCC 6803 lacking the photosystem II 33 kDa extrinsic protein. *Z Naturforsch* **48c**: 224-233

Bonaventura C. and Myers J. (1969) Fluorescence and oxygen evolution for *Chlorella pyrenoidosa*. *Biochim Biophys Acta* **189**: 366-383

Booth P.J., Crystall B., Ahmad I., Barber J., Porter G. and Klug D.R. (1991) Observation of multiple radical pair states in Photosystem II reaction centres. *Biochemistry* **30**: 7573-7586

Boudreau E., Otis C. and Turmel M. (1994) Conserved gene clusters in the highly rearranged chloroplast genomes of *Chlamydomonas moewusii* and *Chlamydomonas reinhardtii*. *Plant Mol Biol* **24**: 585-602

Boussac A., Zimmermann J.-L., Rutherford A.W. and Lavergne J. (1990) Histidine oxidation in the oxygen-evolving Photosystem-II enzyme. *Nature* **347**: 303-306

Boynton J.E. and Gillham N.W. (1993) Chloroplast transformation in *Chlamydomonas*. *Methods Enzymol* **217**: 510-536

Boynton J.E., Gillham N.W., Harris E.H., Hosler J.P., Jonhson A.M., Jones A.R., Randolph-Anderson B.L., Robertson D., Klein T.D., Shark K.B. and Sanford J.C. (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. *Science* **240**: 1534-1538

Bracht E. and Trebst A. (1994) Hypothesis on the control of D1 protein turnover by nuclear coded proteins in *Chlamydomonas reinhardtii*. *Z Naturforsch* **49c**: 439-446

Breyton C., Tribet C., Olive J., Recouvreur M. and Popot J.-L. (1995) Molecular weight and state of association of the purified b_6f complex from *Chlamydomonas reinhardtii*. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 2, pp 591-594, Kluwer Academic Publishers, Netherlands

Bricker T. (1990) Structure and function of CPa-1 and CPa-2 in Photosystem II. *Photosynth Res* **24**: 1-13

Bryant D.A. (1992) Molecular biology of Photosystem I. In: Barber J (ed) "The Photosystems: Structure, Function and Molecular Biology", Vol. 11, pp 501-549, Elsevier Science Publishers B.V., Netherlands

Bulté L., Gans P., Rébeillé F. and Wollman F.-A. (1990) ATP control on state transitions *in vivo* in *Chlamydomonas reinhardtii*. *Biochim Biophys Acta* **1020**: 72-80

Bumann D. and Oesterhelt D. (1994) Purification and characterisation of oxygen-evolving photosystem II core complexes from the green alga *Chlamydomonas reinhardtii*. *Biochemistry* **33**: 10906-10910

Burnap R.L. and Sherman L.A. (1991) Deletion mutagenesis in *Synechocystis* sp. PCC 6803 indicates that the Mn-stabilising protein of Photosystem II is not essential for O_2 evolution. *Biochemistry* **30**: 440-446

Butler W.L., Visser J.W.M. and Symons H.L. (1973) The kinetics of light-induced changes of C-550, cytochrome b_{559} and fluorescence yield in chloroplasts at low temperature. *Biochim Biophys Acta* **292**: 140-151

Bylina E.J. and Youvan D.C. (1988) Directed mutations affecting spectroscopic and electron-transfer properties of the primary donor in the photosynthetic reaction centre. *Proc Natl Acad Sci USA* **85**: 7226-7230

Cao J. and Govindjee (1990) Anion effects on the electron acceptor side of photosystem II in a transformable cyanobacterium *Synechocystis* 6803. In: Baltscheffsky M (ed) "Current Research in Photosynthesis", Vol. 1, pp 515-518, Kluwer Academic Publishers, Dordrecht

Cao J.C., Vermaas W.F.J. and Govindjee (1991) Arginine residues in the D2 polypeptide may stabilize bicarbonate binding in photosystem II of *Synechocystis* sp. 6803. *Biochim Biophys Acta* **1059**: 171-180

Carpenter S.D., Charité J., Eggers B. and Vermaas W.F. (1990) The *psbC* start codon in *Synechocystis* sp. PCC 6803. *FEBS Lett* **260**: 135-137

Chan C.K., Chen L.X.Q., DiMagno T.J., Hanson D.K., Nance S.L., Schiffer M., Norris J.R. and Fleming G.R. (1991) Initial electron transfer in photosynthetic reaction centres of *Rhodobacter capsulatus* mutants. *Chem Phys Lett* **176**: 366-372

Chang C.-H., Tiede D., Tang J., Smith U., Norris J. and Schiffer M. (1986) Structure of *Rhodopseudomonas sphaeroides* R-26 reaction centres. *FEBS Lett* **205**: 82-86

Chapman D.J., Gounaris K. and Barber J. (1988) Electron transport properties of the isolated D1-D2-cytochrome b_{559} photosystem II reaction centre. *Biochim Biophys Acta* **933**: 421-431

Cheater A.J., O'Connor H.E., Ruffle S.V., Nugent J.H.A. and Purton S. (1995) Elimination of PSII-H phosphorylation in *Chlamydomonas reinhardtii* does not affest PSII assembly or function. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 3, pp 865-868, Kluwer Academic Publishers, Netherlands

Chirino A.J., Lous E.J., Huber M., Allen J.P., Schenk C.C., Paddock M.L., Feher G. and Rees D.C. (1994) Crystallographic analysis of site-directed mutants of the photosynthetic reaction center from *Rhodobacter sphaeroides*. *Biochemistry* **33**: 4584-4593

Choquet Y., Goldschmidt-Clermont M., Girard-Bascou J., Kück U., Bennoun P. and Rochaix J.-D. (1988) Mutant phenotypes support a *trans*-splicing mechanism for the expression of the tripartite *psaA* gene in the *C. reinhardtii* chloroplast. *Cell* **52**: 903-913

Chow W.S., Telfer A., Chapman D.J. and Barber J. (1981) State 1-state 2 transition in leaves and its association with ATP-induced chlorophyll fluorescence quenching. *Biochim Biophys Acta* **638**: 60-68

Christopher D.A. and Mullet J.E. (1994) Separate photosensory pathways co-regulate blue light/ultraviolet-A-activated *psbD-psbC* transcription and light-induced D2 and CP43 degradation in barley (*Hordeum vulgare*) chloroplasts. *Plant Physiol* **104**: 1119-1129

Christopher D.A., Kim M. and Mullet J.E. (1992) A novel light-regulated promoter is conserved in cereal and dicot chloroplasts. *Plant Cell* **4**: 785-798

Chua N.-H. (1980) Electrophoretic analysis of chloroplast proteins. *Methods Enzymol.* **60c**: 434-446

Chua N.-H. and Gillham N.W. (1977) The sites of synthesis of principle thylakoid membrane polypeptides in *Chlamydomonas reinhardtii*. *Cell Biology* **74**: 441-452

Chua N.H., Matlin K. and Bennoun P. (1975) A chlorophyll-protein complex lacking in photosystem I mutants of *Chlamydomonas reinhardtii*. *J Cell Biol* **67**: 361-377

Cogdell R. and Malkin R. (1992) An introduction to plant and bacterial photosystems. In: Barber J (ed) "The photosystems: structure, function and molecular biology", Vol. 11, pp 1-15, Elsevier Science Publishers, Amsterdam

Coleman W.J. and Youvan D.C. (1990) Spectroscopic analysis of genetically modified photosynthetic reaction centres. *Annu Rev Biophys Chem* **19**: 333-367

Coughlan S.J. and Hind G. (1986) Purification and characterisation of a membrane-bound protein kinase from spinach thylakoids. *J Biol Chem* **261**: 11378-11385

Cramer W.A., Furbacher P.N., Szczepaniak A. and Tae G.-S. (1991) Electron transport between photosystem II and photosystem I. In: Lee CP (ed) "Current topics in Bioenergetics", Vol. 16, pp 179-222, Academic Press, New York

Cramer W.A., Martinez S.E., Furbacher P.N., Huang D. and Smith J.L. (1994) The cytochrome b_6f complex. *Curr. Opin. Struct. Biol.* **4**: 536-544

Cui L., Bingham S.E., Kuhn M., Kass H., Lubitz W. and Webber A.N. (1995) Site-directed mutagenesis of conserved histidines in the helix VIII domain of PsaB impairs assembly of the Photosystem I reaction centre without altering spectroscopic characteristics of P_{700}. *Biochemistry* **34**: 1549-1558

De Las Rivas J., Telfer A. and Barber J. (1993) Two coupled β-carotene molecules protect P680 from photodamage in isolated photosystem II reaction centres. *Biochim Biophys Acta* **1142**: 155-164

de Vitry C. and Wollman F.-A. (1988) Changes in phosphorylation of thylakoid membrane proteins in light-harvesting complex mutants from *Chlamydomonas reinhardtii*. *Biochim Biophys Acta* **933**: 444-449

de Vitry C., Breyton C., Pierre Y. and Popot J.-L. (1995) The 4-kDa chloroplast polypeptide of cytochrome b_6f complex encoded by the nuclear *petX* gene: nucleic and protein sequences, targeting signals, and membrane topology. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 2, pp 595-598, Kluwer Academic Publishers, Netherlands

de Vitry C., Diner B.A. and Lemoine Y. (1987) Chemical composition of Photosystem II reaction centres (PS II): phosphorylation of PS II polypeptides. In: Biggins J (ed), "Progress in Photosynthesis Research", Vol. II, pp 105-108, Martinus Nijhoff Publishers, Dordrecht, Netherland

de Vitry C., Diner B.A. and Popot J.-L. (1991) Photosystem II particles from *Chlamydomonas reinhardtii*. *J Biol Chem* **266**: 16614-16621

de Vitry C., Olive J., Drapier D., Recouvreur M. and Wollman F.-A. (1989) Posttranslational events leading to the assembly of Photosystem II protein complex: a study using photosynthesis mutants from *Chlamydomonas reinhardtii*. *J Cell Biol* **109**: 991-1006

de Vitry C., Wollman F.-A. and Delepelaire P. (1984) Function of the polypeptides of the Photosystem II reaction centre in *Chlamydomonas reinhardtii*, localisation of the primary reactants. *Biochim Biophys Acta* **767**: 415-422

Debuchy R., Purton S. and Rochaix J.-D. (1989) The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the ARG7 locus. *EMBO J* **5**: 1745-1754

Debus R.J. (1992) The manganese and calcium ions of photosynthetic oxygen evolution. *Biochim Biophys Acta* **1102**: 269-352

Debus R.J., Barry B.A., Babcock G.T. and McIntosh L. (1988a) Site-directed mutagenesis identifies a tyrosine radical involved in the photosynthetic oxygen-evolving system. *Proc Natl Acad Sci USA* **85**: 427-430

Debus R.J., Barry B.A., Sithole I., Babcock G.T. and McIntosh L. (1988b) Directed mutagenesis indicates that the donor to $P680^+$ in Photosystem II is tyrosine-161 of the D1 polypeptide. *Biochemistry* **27**: 9071-9074

Dedner N., Meyer H.E., Ashton C. and Wildner G.F. (1988) N-terminal sequence analysis of the 8 kDa protein in *Chlamydomonas reinhardtii* - localisation of the phosphothreonine. *FEBS Lett* **236**: 77-82

Deisenhofer J., Epp O., Miki K., Huber R. and Michel H. (1984) X-ray structure analysis of a membrane protein complex. Electron density map at 3 Å resolution and a model of the chromophores of the photosynthetic reaction centre from *Rhodopseudomonas viridis*. *J Mol Biol* **180**: 385-398

Deisenhofer J., Epp O., Miki K., Huber R. and Michel H. (1985) Structure of the protein subunits in the photosynthetic reaction centre of *Rhodopseudomonas viridis* at 3 Å resolution. *Nature* **318**: 618-624

Delepelaire P. (1984) Partial characterization of the biosynthesis and integration of the Photosystem II reaction centres in the thylakoid membrane of *Chlamydomonas reinhardtii*. *EMBO J* **3**: 701-706

Delepelaire P. and Wollman F.-A. (1985) Correlations between fluorescence and phosphorylation changes in thylakoid membranes of *Chlamydomonas reinhardtii* in vivo: a kinetic analysis. *Biochim Biophys Acta* **809**: 277-283

Delosme R., Olive J. and Wollman F.-A. (1996) Changes in light energy distribution upon state transitions: an in vivo photoacoustic study of the wild type and photosynthesis mutants from *Chlamydomonas reinhardtii*. *Biochim Biophys Acta* **1273**: 150-158

Delphin E., Duval J.-C. and Kirilovsky D. (1995) Comparison of state 1-state 2 transitions in the green alga *Chlamydomonas reinhardtii* and in the red alga *Rhodella violacea*: effect of kinase and phosphatase inhibitors. *Biochim Biophys Acta* **1232**: 91-95

Demeter S. and Vass I. (1984) Charge accumulation and recombination in Photosystem II studied by thermoluminescence. I Participation of the primary acceptor Q and secondary acceptor B in the generation of thermoluminescence of chloroplasts. *Biochim Biophys Acta* **764**: 24-32

Demeter S., Droppa M., Vass I. and Horvath G. (1982) The thermoluminescence of chloroplasts in the presence of Photosystem II herbicides. *Photobiochem Photobiophys* **4**: 163-168

Demeter S., Janda T., Kovacs L., Mende D. and Wiessner W. (1995) Effects of in vivo CO_2-depletion on electron transport and photoinhibition in the green algae, *Chlamydobotrys stellata* and *Chlamydomonas reinhardtii*. *Biochim Biophys Acta* **1229**: 166-174

Diner B.A. and Wollman F.-A. (1980) Isolation of highly active photosystem II particles from a mutant of *Chlamydomonas reinhardtii*. *Eur J Biochem* **110**: 521-526

Diner B.A., Nixon P.J. and Farchaus J.W. (1991) Site-directed mutagenesis of photosynthetic reaction centres. *Curr. Opin. Struct. Biol.* **1**: 546-554

Donovan B., Walker II L.A., Yocum C.F. and Sension R.J. (1995) Femtosecond studies of the primary charge separation in different preparations of photosystem II. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 1, pp 667-670, Kluwer Academic Publishers, Netherlands

Dreyfuss B.W. and Thornber J.P. (1994) Assembly of the light-harvesting complexes (LHCs) of photosystem II. *Plant Physiol* **106**: 829-839

Dunn S.D. (1986) Effect of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on Western blots by monoclonal antibodies. *Anal. Biochem.* **157**: 144-153

Durrant J.R., Hastings G., Joseph D.M., Barber J., Porter G. and Klug D.R. (1993) Rate of oxidation of P680 in isolated photosystem 2 reaction centres monitored by loss of chlorophyll stimulated emission. *Biochemistry* **32**: 8259-8267

Durrant J.R., Hastings G., Joseph D.M., Barber J., Porter G. and Klug D.R. (1992) Subpicosecond equilibration of excitation energy in isolated photosystem II reaction centres. *Proc Natl Acad Sci USA* **89**: 11632-11636

Eaton-Rye J.J. and Govindjee (1988) Electron transfer through the quinone acceptor complex of Photosystem II after one or two actinic flashes in bicarbonate-depleted spinach thylakoid membranes. *Biochim Biophys Acta* **935**: 248-257

Eaton-Rye J.J. and Murata N. (1989) Evidence that the amino-terminus of the 33 kDa extrinsic protein is required for binding to the Photosystem II complex. *Biochim Biophys Acta* **977**: 219-226

Ebbert V. and Godde D. (1995) Phosphorylation of PS II polypeptides prevents degradation of the D1 protein. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 1, pp 579-582, Kluwer Academic Publishers, Netherlands

Eggers B. and Vermaas W. (1993) Truncation of the D2 protein in *Synechocystis* sp. PCC 6803: A role of the C-terminal domain of D2 in photosystem II function and stability. *Biochemistry* **32**: 11419-11427

Elich T.D., Edelman M. and Mattoo A.K. (1992) Identification, characterization, and resolution of the *in vivo* phosphorylated form of the D1 photosystem II reaction centre protein. *J Biol Chem* **267**: 3253-3259

Enami I., Kaneko M., Kitamura N., Koike H. and Sonoike K. (1991) Total immobilisation of the extrinsic 33 kDa protein in spinach photosystem II membrane preparations. Protein stoichiometry and stabilisation of oxygen evolution. *Biochim Biophys Acta* **1060**: 224-232

Erickson J.M. and Rochaix J.-D. (1992) The molecular biology of photosystem II. In: Barber J (ed) "The photosystems: structure, function and molecular biology", Vol. 11, pp 101-178, Elsevier Science Publishers, Amsterdam

Erickson J.M., Delepelaire P. and Rochaix J.-D. (1985) Analysis of genes encoding two photosystem II proteins of the 30-34 kDa size class. In: Steinback KE, Bonitz S, Arntzen CJ and Bogorad L (eds) "Molecular biology of the photosynthetic apparatus", pp 53-65, Cold Spring Harbor Laboratory, Cold Spring Harbor

Erickson J.M., Rahire M. and Rochaix J.D. (1984) *Chlamydomonas reinhardtii* gene for the 32,000 mol. wt. protein of photosystem II contains four large introns and is located entirely within the chloroplast inverted repeat. *EMBO J* **3**: 2753-2762

Erickson J.M., Rahire M., Malnoé P., Girard-Bascou J., Pierre Y., Bennoun P. and Rochaix J.-D. (1986) Lack of the D2 protein in a *Chlamydomonas reinhardtii psbD* mutant affects photosystem II stability and D1 expression. *EMBO J* **5**: 1745-1754

Etienne A.-L., Ducruet J.-M., Ajlani G. and Vernotte C. (1990) Comparative studies on electron transfer in photosystem II of herbicide-resistant mutants from different organisms. *Biochimica Biophysica Acta* **1015**: 435-440

Farah J., Rappaport F., Choquet Y., Joliot P. and Rochaix J.-D. (1995) Isolation of a *psaF*-deficient mutant of *Chlamydomonas reinhardtii*-efficient interaction of plastocyanin with the Photosystem-I reaction-centre is mediated by the PsaF subunit. *EMBO J* **14**: 4976-4984

Farchaus J. and Dilley R.A. (1986) Purification and partial sequence of the M_r 10,000 phosphoprotein from spinach thylakoids. *Archiv. Biochem. Biophys.* **244**: 94-101

Farchaus J., Dilley R.A. and Cramer W.A. (1985) Selective inhibition of the spinach thylakoid LHCII protein kinase. *Biochim Biophys Acta* **809**: 17-26

Feinberg A.P. and Vogelstein B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**: 6-13

Finkele U., Lauterwasser C., Zinth W., Gray K.A. and Oesterhelt D. (1990) Role of tyrosine M210 in the initial charge separation of reaction centres of *Rhodobacter sphaeroides*. *Biochemistry* **29**: 8517-8521

Fong A. and Syrzycki S.J. (1992) Chloroplast RNA polymerase genes of *Chlamydomonas reinhardtii* exhibit an unusual structure and arrangement. *Curr Genet* **21**: 485-497

Ford R.C., Rosenberg M.F., Shepherd F.H., McPhie P. and Holzenburg A. (1995) Photosystem-II-3-D structure and the role of the extrinsic subunits in photosynthetic oxygen evolution. *Micron* **26**: 133-140

Fotinou C. and Ghanotakis D.F. (1990) A preparative method for the isolation of the 43 kDa, 47 kDa and the D1-D2-cyt b_{559} species directly from thylakoid membranes. *Photosynth Res* **25**: 141-145

Funk C., Schröder W.P., Green B.R., Renger G. and Andersson B. (1994) The intrinsic 22 kDa protein is a chlorophyll-binding subunit of photosystem II. *FEBS Lett* **342**: 261-266

Gans P. and Wollman F.-A. (1995) The effect of cyanide on state transitions in *Chlamydomonas reinhardtii*. *Biochim Biophys Acta* **1228**: 51-57

Giardi M.T., Komenda J. and Masojidek J. (1994) Involvement of protein phosphorylation in the sensitivity of photosystem II to strong illumination. *Physiol Plantarum* **92**: 181-187

Giardi M.T., Kucera T., Briantais J.-M. and Hodges M. (1995) Decreased photosystem II core phosphorylation in a yellow-green mutant of wheat showing monophasic fluorescence induction curve. *Plant Physiol* **109**: 1059-1068

Giorgi L.B., Durrant J.R., Alizadeh S., Nixon P.J., Joseph D.M., Rech T., Barber J., Porter G. and Klug D.R. (1994) Comparison of primary electron transfer in Photosystem II reaction centres isolated from the higher plant *Pisum sativum* and the green alga *Chlamydomonas reinhardtii*. *Biochim Biophys Acta* **1186**: 247-251

Giorgi L.B., Nixon P.J., Merry S.A.P., Joseph D.M., Durrant J.R., De Las Rivas J., Barber J., Porter G. and Klug D.R. (1996) Comparison of primary charge separation in the Photosystem II reaction centre complex isolated from wild-type and D1-130 mutants of the cyanobacterium *Synechocystis* PCC 6803. *J Biol Chem* **271**: 2093-2101

Girard J., Chua N.-H., Bennoun P., Schmidt G. and Delosme M. (1980) Studies on mutants deficient in the photosystem I reaction centres in *Chlamydomonas reinhardtii*. *Curr Genet* **2**: 215-221

Girard-Bascou J., Choquet Y., Schneider M., Delosme M. and Dron M. (1987) Characterisation of a chloroplast mutation in the *psaA2* gene of *Chlamydomonas reinhardtii*. *Curr Genet* **12**: 489-495

Glaser E. and Norling B. (1991) Chloroplast and plant mitochondrial ATP synthases. In: Lee CP (ed) "Current topics in Bioenergetics", Vol. 16, pp 223-263, Academic Press, New York

Golbeck J.H. (1992) Structure and function of Photosystem-I. *Ann Rev Plant Physiol Plant Mol Biol* **43**: 293-324

Golbeck J.H. (1993) The structure of photosystem I. *Curr. Opin. Struct. Biol.* **3**: 508-514

Golbeck J.H. and Bryant D.A. (1991) Photosystem I. In: Lee CP (ed) "Current topics in bioenergetics", pp 83-177, Academic Press, New York

Goldschmidt-Clermont M. (1991) Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker for site-directed transformation of *Chlamydomonas*. *Nucleic Acids Res* **19**: 4083-4089

Goldschmidt-Clermont M., Choquet Y., Girard-Bascou J., Michel F., Schrimer-Rahire M. and Rochaix J.-D. (1991) A Small chloroplast RNA may be required for *trans*-splicing in *Chlamydomonas reinhardtii*. *Cell* **65**: 135-143

Goldschmidt-Clermont M., Girard-Bascou J., Choquet Y. and Rochaix J.-D. (1990) Trans-splicing mutants of *Chlamydomonas reinhardtii*. *Mol Gen Genet* **223**: 417-425

Gong H. and Ohad I. (1991) The PQ/PQH_2 ratio and occupancy of photosystem II-Q_B site by plastoquinone control the degradation of D1 protein during photoinhibition *in vivo*. *J Biol Chem* **266**: 21293-21299

Gorman D.S. and Levine P. (1965) Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardi*. *Proc Natl Acad Sci USA* **54**: 1665-1669

Gounaris K., Chapman D.J., Booth P., Crystall B., Giorgi L.B., Klug D.R., Porter G. and Barber J. (1990) Comparison of the D1/D2/cytochrome b_{559} reaction centre complex of photosystem two isolated by two different methods. *FEBS Lett* **265**: 88-92

Govindjee and van Rensen J.J.S. (1993) Photosystem II reaction centre and bicarbonate. In: Deisenhofer J and Norris JR (eds) "The photosyntetic reaction centre", Vol. 1, pp 357-389, Academic Press, London

Gray K.A., Farchaus J.W., Wachtveitl J., Breton J. and Oesterhelt D. (1990) Initial characterisation of site-directed mutants of tyrosine M210 in the reaction centre of *Rhodobacter sphaeroides*. *EMBO J* **9**: 2061-2070

Green B.R., Pichersky E. and Kloppstech K. (1991) Chlorophyll *a/b*-binding proteins: An extended family. *Trends Biochem Sci* **16**: 181-186

Greenbaum E., Lee J.W., Tevault C.T., Blankiship S.L. and Mets L.J. (1995) CO_2 fixation and photoevolution of H_2 and O_2 in a mutant of *Chlamydomonas* lacking photosystem I. *Nature* **376**: 438-441

Greenfield S.R., Wasielewski M.R., Govindjee and Seibert M. (1995) Femtosecond spectroscopy of PSII reaction centres: new results. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 1, pp 663-666, Kluwer Academic Publishers, Netherlands

Grossman A.R., Bhaya D., Apt K.E. and Kehoe D.M. (1995) Light-harvesting complexes in oxygenic photosynthesis: Diversity, control, and evolution. *Ann Rev Genet* **29**: 231-288

Gumpel N.J., Rochaix J.-D. and Purton S. (1994) Studies on homologous recombination in the green alga *Chlamydomonas reinhardtii*. *Curr Genet* **26**: 438-442

Haley J. and Bogorad L. (1989) A 4-kDa maize chloroplast polypeptide associated with the cytochrome b_6-f complex: subunit 5, encoded by the chloroplast *petE* gene. *Proc Natl Acad Sci USA* **86**: 1534-1538

Hallahan B.J., Nugent J.H.A., Warden J.T. and Evans M.W. (1990) Investigation of the origin of the "S_3" EPR signal from the oxygen evolving complex of Photosystem 2: the role of tyrosine Z. *Biochemistry* **31**: 4562-4573

Hanahan D. (1983) Studies on transformation of *E. coli* plasmids. *J Mol Biol* **166**: 557-580

Hansson O. and Wydrzynski T. (1990) Current perceptions of Photosystem II. *Photosynth Res* **23**: 131-162

Harris E.H. (1989) The *Chlamydomonas* Sourcebook. Academic Press, New York

Harrison M.A. and Allen J.F. (1991) Light-dependent phosphorylation of Photosystem II polypeptides maintains electron transport at high light intensity: separation from effects of phosphorylation of LHC-II. *Biochim Biophys Acta* **1058**: 289-296

Hastings G., Durrant J.R., Hong Q., Barber J., Porter G. and Klug D.R. (1992) Observation of pheophytin reduction in photosystem II reaction centres using femtosecond absorption spectroscopy. *Biochemistry* **31**: 7638-7647

Heifetz P.B., Lers A., Boynton J.E., Gillham N.W. and Osmond C.B. (1992) Photosynthetic consequences of specific chloroplast gene mutations affecting synthesis and function of the Photosystem II D1 protein. In: Murata N (ed), "Research in Photosynthesis", Vol. 3, pp 417-420, Kluwer Academic Press, Netherlands

Heiss U. and Johanningmeier U. (1992) Analysis of a herbicide resistant mutant obtained by transformation of the *Chlamydomonas* chloroplast. *Photosynth Res* **34**: 311-317

Hind G., Marshak D.R. and Coughlan S.J. (1995) Spinach thylakoid polyphenol oxidase-cloning, characterisation, and relation to a putative protein-kinase. *Biochemistry* **34**: 8157-8164

Holschuh K., Bottomley W. and Whitfeld P.R. (1984) Structure of the spinach chloroplast genes for the D2 and 44 kd reaction-centre proteins of photosystem II and for tRNA-Ser(UGA). *Nucleic Acids Res* **12**: 8819-8834

Holzapfel W., Finkele U., Kaiser W., Oesterhelt D., Scheer H., Stilz H.U. and Zinth W. (1989) Observation of a bacteriochlorophyll anion radical during the primary charge separation in a reaction centre. *Chem Phys Lett* **160**: 1-7

Holzenburg A., Bewley M.C., Wilson F.H., Nicholson W.V. and Ford R.C. (1993) Three-dimensional structure of Photosystem II. *Nature* **363**: 470-472

Horton P. and Lee P. (1985) Phosphorylation of chloroplast membrane proteins partially protects against photoinhibition. *Planta* **165**: 37-42

Horton P., Lee P. and Fenryhough P. (1990) Emerson enhancement, photosynthetic control and protein phosphorylation in isolated maize mesophyll chloroplasts; dependence upon carbon metabolism. *Biochim Biophys Acta* **1017**: 160-166

Hosler J.P., Wurtz E.A., Harris E.H., Gillham N.W. and Boynton J.E. (1989) Relationship between gene dosage and gene expression in the chloroplast of *Chlamydomonas reinhardtii*. *Plant Physiol* **91**: 648-655

Hutchison R.S. and Sayre R.T. (1995) Site-specific mutagenesis at histidine 118 of the photosystem II D1 protein of *Chlamydomonas reinhardtii*. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 1, pp 471-474, Kluwer Academic Publishers, Netherlands

Ikeuchi M. and Inoue Y. (1988) A new 4.8 kDa-polypeptide intrinsic to the PS II reaction centre, as revealed by modified SDS-PAGE with improved resolution of low-molecular-weight proteins. *Plant Cell Physiol* **29**: 1233-1239

Ikeuchi M., Eggers B., Shen G., Webber A., Yu J., Hirano A., Inoue Y. and Vermaas W. (1991) Cloning of the *psbK* gene from *Synechocystis* sp. PCC 6803 and characterisation of photosystem II in mutants lacking PSII-K. *J Biol Chem* **266**: 11111-11115

Ikeuchi M., Koike H. and Inoue Y. (1989) N-terminal sequencing of low-molecular-mass components in cyanobacterial photosystem II core complex. Two components correspond to unidentified open reading frames of plant chloroplast DNA. *FEBS Lett* **253**: 178-182

Ikeuchi M., Plumley F.G., Inoue Y. and Schmidt G.W. (1987a) Identification of phosphorylated reaction centre polypeptides in thylakoids of *Chlamydomonas reinhardtii* and *Pisum sativum*. In: Biggens J (ed), "Progress in Photosynthesis Research", Vol. 2, pp 805-808, Martinus Nijhoff Publishers, Dordrecht

Ikeuchi M., Plumley F.G., Inoue Y. and Schmidt G.W. (1987b) Phosphorylation of Photosystem II components, CP43 apoprotein, D1, D2, and 10 to 11 kilodalton protein in chloroplast thylakoids of higher plants. *Plant Physiol* **85**: 638-642

Irrgang K.-D., Shi L.-X., Funk C. and Schröder W.P. (1995) A nuclear-encoded subunit of the photosystem II reaction centre. *J Biol Chem* **270**: 17588-17593

Jensen K.H., Herrin D.L., Plumley F.G. and Schmidt G.W. (1986) Biogenesis of photosystem II complexes: transcriptional, translational, and posttranslational regulation. *J Cell Biol* **103**: 1315-1325

Jones M.R., Heer-Dawson M., Mattioli T.A., Hunter C.N. and Robert B. (1994) Site-specific mutagenesis of the reaction centre from *Rhodobacter sphaeroides* studied by Fourier transform Raman spectroscopy: mutations at tyrosine M210 do not affect the electronic structure of the primary donor. *FEBS Lett* **339**: 18-24

Karabin G.D., Farley M. and Hallick R.B. (1984) Chloroplast gene for M_r 32,000 polypeptide of photosystem II in *Euglena gracilis* is interrupted by four introns with conserved boundary sequences. *Nucleic Acids Res* **12**: 5801-5812

Karlin-Neumann G.A., Kohorn B.D., Thornber J.P. and Tobin E.M. (1985) A chlorophyll *a/b*-protein encoded by a gene containing an intron with characteristics of a transposable element. *J Mol Appl Genet* **3**: 45-61

Kim S., Sandusky P., Powlby N.R., Aebersold B., Green B.R., Vlahakis S., Yocum C.F. and Pickersky E. (1992) Characterisation of a spinach *psbS* cDNA encoding the 22-kDa protein of photosystem II. *FEBS Lett* **314**: 67-71

Kindle K.L. (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA* **87**: 1228-1232

Kindle K.L. and Sodeinde O.A. (1994) Nuclear and chloroplast transformation in *Chlamydomonas reinhardtii*: strategies for genetic manipulation and gene expression. *J App Phycol* **6**: 231-238

Kindle K.L., Richards K.L. and Stern D.B. (1991) Engineering the chloroplast genome: Techniques and capabilities for chloroplast transformation in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA* **88**: 1721-1725

Kirmaier C. and Holten D. (1987) Primary photochemistry of reaction centres from the photosynthetic purple bacteria. *Photosynth Res* **13**: 225-260

Kirmaier C. and Holten D. (1993) Electron transfer and charge recombination reactions in wild type and mutant bacterial reaction centres. In: Deisenhofer J and Norris JR (eds) "The Photosynthetic Reaction Centre", Vol. 2, pp 49-70, Academic Press

Kitamura K., Ozawa S., Shiina T. and Toyoshima Y. (1994) L protein, encoded by *psbL*, restores normal functioning of the primary quinone acceptor, Q_A, in isolated D1/D2/CP47/Cytb-559/I photosystem II reaction centre complex. *FEBS Lett* **354**: 113-116

Klein T.M., Wolf E.D., Wu R. and Sanford J.C. (1987) High velocity microprojectiles for delivering nucleic acids into living cells. *Nature* **327**: 70-73

Kless H., Vermaas W.F.J. and Edelman M. (1992) Photosystem II function and integrity in spite of drastic protein changes in a conserved region of the D2 protein. *Biochemistry* **31**: 11065-11071

Klimov V.V., Allahkverdiev S.I., Demeter S. and Krasnovsky A.A. (1979) Pheophytin reduction in photosystem II of chloroplasts in relation to oxidation-reduction potential of medium. *Dokl Akad Nauk SSSR* **249**: 227-230

Klimov V.V., Klevanik A.V., Shuvalov V.A. and Krasnovsky A.A. (1977) Reduction of pheophytin in the primary light reaction of Photosystem II. *FEBS Lett* **82**: 183-186

Klug D.R., Rech T., Joseph D.M., Barber J., Durrant J.R. and Porter G. (1995) Primary processes in isolated Photosystem II reaction centres probed by magic angle transient absorption spectroscopy. *Chem Phys* **194**: 433-442

Kobayashi M., Maeda H., Watanabe T., Nakane H. and Satoh K. (1990) Chlorophyll *a* and β-carotene content in the D1/D2 cytochrome *b*-559 reaction centre complex from spinach. *FEBS Lett* **260**: 138-140

Koike H., Mamada K., Ikeuchi M. and Inoue Y. (1989) Low-molecular-mass proteins in cyanobacterial photosystem II: identification of *psbH* and *psbK* gene products by N-terminal sequencing. *FEBS Lett* **244**: 391-396

Koivuniemi A., Aro E.-M. and Andersson B. (1995) Degradation of the D1- and D2-proteins of photosystem II in higher plants is regulated by reversible phosphorylation. *Biochemistry* **34**: 16022-16029

Kok B., Forbush B. and McGloin M. (1970) Cooperation of charges in photosynthetic oxygen evolution: I. A linear four step mechanism. *Photochem Photobiol* **11**: 457-475

Komenda J. and Barber J. (1995) Comparison of *psbO* and *psbH* deletion mutants of *Synechocystis* PCC 6803 indicates that degradation of D1 protein Is regulated by the Q_B site and dependent on protein synthesis. *Biochemistry* **34**: 9625-9631

Kramer D.M., Roffey R.A., Govindjee and Sayre R.T. (1994) The A_T thermoluminescence band from *Chlamydomonas reinardtii* and the effects of mutagenesis of histidine residues on the donor side of the Photosystem II D1 polypeptide. *Biochim Biophys Acta* **1185**: 228-237

Krause G.H. and Weis E. (1991) Chlorophyll fluorescence and photosynthesis: The basics. *Ann Rev Plant Physiol Plant Mol Biol* **42**: 313-349

Krauss N., Hinrichs W., Witt I., Fromme P., Pritzkow W., Dauter Z., Wilson K.S., Witt H.T. and Saenger W. (1993) 3-dimensional structure of system-I of photosynthesis at 6 Ångstrom resolution. *Nature* **361**: 326-331

Kuchka M.R., Goldschmidt-Clermont M., van Dillewijn J. and Rochaix J.-D. (1989) Mutation at the *Chlamydomonas* nuclear *NAC2* locus specifically affects stability of the chloroplast *psbD* transcript encoding polypeptide D2 of PS II. *Cell* **58**: 869-876

Kuchka M.R., Mayfield S.P. and Rochaix J.-D. (1988) Nuclear mutations specifically affect the synthesis and/or degradation of the chloroplast-encoded D2 polypeptide of photosystem II in *Chlamydomonas reinhardtii*. *EMBO J* **7**: 319-324

Kumazaki S., Joseph D.M., Crystall B., Tachibana Y., Durrant J.R., Barber J., Porter G., Yoshihara K. and Klug D.R. (1995) Experimental observation of multiple trapping/charge separation steps in the isolated PS 2 reaction centre. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 1, pp 883-886, Kluwer Academic Publishers, Netherlands

Kunkel T.A., Roberts J.D. and Zakour R.A. (1987) Rapid and efficient site-directed mutagenesis without phenotypic selection. *Methods Enzymol* **154**: 367-382

Kuras R. and Wollman F.-A. (1994) The assembly of cytochrome b_6/f complexes: an approach using genetic transformation of the green alga *Chlamydomonas reinhardtii*. *EMBO J* **13**: 1019-1027

Kück U., Choquet Y., Schneider M., Dron M. and Bennoun P. (1987) Structural and transcription analysis of two homologous genes for the P700 chlorophyll α-apoproteins in *Chlamydomonas reinhardii*: evidence for *in vivo trans*-splicing. *EMBO J* **6**: 2185-2195

Kühlbrandt W. (1988) Structure of the light-harvesting chlorophyll *a/b*-protein complex from photosynthetic membranes. In: Scheer H and Schneider S (eds) "Photosynthetic light-harvesting systems", pp 211-216, Walter de Gruyter, Berlin

Kühlbrandt W. and Wang D.N. (1991) Three-dimensional structure of plant light-harvesting complex determined by electron crystallography. *Nature* **350**: 130-134

Kühlbrandt W., Wang D.N. and Fujiyoshi Y. (1994) Atomic model of plant light-harvesting complex by electron crystallography. *Nature* **367**: 614-621

Kyle D.J., Ohad I. and Arntzen C.J. (1984) Membrane protein damage and repair: selective loss of a quinone protein function in chloroplast membranes. *Proc Natl Acad Sci USA* **81**: 4070-4074

Künster P., Guardiola A., Takahashi Y. and Rochaix J.-D. (1995) Loss of the chloroplast *psbI* gene does not inactivate photosystem II in *Chlamydomonas reinhardtii*. *J Biol Chem* **270**: 9651-9654

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277**: 680-685

Larsson U.K., Sundby C. and Andersson B. (1987) Characterisation of 2 different subpopulations of spinach light-harvesting chlorophyll a/b-protein complex (LHC-II)-polypeptide composition, phosphorylation pattern and association with Photosystem-II. *Biochim Biophys Acta* **894**: 59-68

Lee J.W., Tevault C.V., Owens T.G. and Greenbaum E. (1996) Oxygenic photoautotrophic growth without photosystem I. *Science* **273**: 364-367

Lemaire C., Girard-Bascou J. and Wollman F.-A. (1987) Characterisation of the b6/f complex subunits and studies on the LHC-kinase in *Chlamydomonas reinhardtii* using mutant strains altered in the b6/f complex. In: Biggins J (ed), "Progress in Photosynthesis Research", Vol. 4, pp 655-658, Martinus Nijhoff, Dodrecht

Lers A., Heifetz P.B., Boynton J.E., Gilham N.W. and Osmond C.B. (1992) The carboxy terminal extension of the D1 protein of photosystem II is not required for optimal photosynthetic performance under CO_2 and light saturated conditions. *J Biol Chem* **267**: 17494-17497

Lichtenthaler H.K. (1987) Pigments of photosynthetic biomembranes. *Methods Enzymol* **148**: 350-400

Lind L.K., Shukla V.K., Nyhus K.J. and Pakrasi H.B. (1993) Genetic and immunological analyses of the cyanobacterium *Synechocystis* sp. PCC 6803 show that the protein encoded by the *psbJ* gene regulates the number of Photosystem II centres in thylakoid membranes. *J Biol Chem* **268**: 1575-1579

Ljunberg U., Åkerlund H.-E. and Andersson B. (1986) Isolation and characterisation of the 10-kDa and 22-kDa polypeptides of higher plant photosystem 2. *Eur J Biochem* **158**: 477-482

Lorkovic Z.J., Scroeder W.P., Pakrasi H.B., Irrgang K.-D., Herrmann R.G. and Oelmüller R. (1995) Molecular characterisation of PsbW, a novel and only nuclear encoded component of the photosystem II reaction centre complex in spinach. *Proc Natl Acad Sci USA* **92**: 8930-8934

Lyon M.K., Marr K.M. and Furcinitti P.S. (1993) Formation and characterisation of two-dimensional crystals of photosystem II. *J Structural Biol* **110**: 133-140

Maliga P. (1993) Towards plastid transformation in flowering plants. *Trends Biotechnol* **11**: 101-107

Marder J.B., Goloubinoff P. and Edelman M. (1984) Molecular architecture of the rapidly metabolised 32-kilo Dalton protein of photosystem II-indications for COOH-terminal processing of a chloroplast membrane polypeptide. *J Biol Chem* **106**: 609-616

Marr K.M., Mastronarde D.M. and Lyon M.K. (1996) 2-dimensional crystals of photosystem-II-biochemical characterisation, cryoelectron microscopy and localisation of the D1 and cytochrome b559 polypeptides. *J Cell Biol* **132**: 823-833

Mattioli T.A., Gray K.A., Lutz M., Oesterhelt D. and Robert B. (1991) Resonance Raman characterisation of *Rhodobacter sphaeroides* reaction centres bearing site-directed mutations at tyrosine M210. *Biochemistry* **30**: 1715-1722

Mattoo A.K. and Edelman M. (1987) Intramembrane translocation and posttranslational palmitoylation of the chloroplast 32-kDa herbicide-binding protein. *Proc Natl Acad Sci USA* **84**: 1497-1501

Mattoo A.K., Marder J.B. and Edelman M. (1989) Dynamics of the photosystem II reaction centre. *Cell* **56**: 241-246

Mayes S.R. and Barber J. (1991) Primary structure of the *psbN-psbH-petC-petA* gene cluster of cyanobacterium *Synechocystis* PCC 6803. *Plant Mol Biol* **17**: 289-293

Mayfield S.P. and Kindle K.L. (1990) Stable nuclear transformation of *Chlamydomonas reinhardtii* by using a *C. reinhardtii* gene as the selectable marker. *Proc Natl Acad Sci USA* **87**: 2087-2091

Mayfield S.P., Bennoun P. and Rochaix J.-D. (1987a) Expression of the nuclear-encoded OEE1 protein is required for oxygen evolution and stability of Photosystem II particles in *Chlamydomonas reinhardtii*. *EMBO J* **6**: 313-318

Mayfield S.P., Rahire M., Frank G., Zuber H. and Rochaix J.-D. (1987b) Expression of the nuclear gene encoding oxygen evolving enhancer protein 2 is required for high levels of photosynthetic oxygen evolution in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA* **84**: 749-753

Mayfield S.P., Yohn C.B., Cohen A. and Danon A. (1995) Regulation of chloroplast gene expression. *Ann Rev Plant Physiol Plant Mol Biol* **46**: 147-166

McCabe P.C. (1990) Production of single-stranded DNA by assymetric PCR. In: Innis MA, Gelfand DH, Snissky JJ and White TJ (eds) "PCR protocols. A guide to methods and applications", pp 76-83, Academic Press, San Diego

McTavish H., Picorel R. and Seibert M. (1989) Stabilisation of the isolated photosystem II reaction centre complex in the dark and in the light using polyethylene glycol and an oxygen-scrubbing system. *Plant Physiol* **89**: 452-456

Metz J.G., Nixon P.J., Rögner M., Brudvig G.W. and Diner B.A. (1989) Direct alteration of the D1 polypeptide of photosystem II: Evidence that tyrosine-161 is the redox component Z, connecting the oxygen evolving complex to the primary electron donor, P680. *Biochemistry* **28**: 6960-6969

Michel H. and Deisenhofer J. (1988) Relevance of the photosynthetic reaction centre from purple bacteria to the structure of photosystem II. *Biochemistry* **27**: 1-7

Michel H., Hunt D.F., Shabanowitz J. and Bennnett J. (1988) Tandem Mass Spectrometry reveals that three photosystem II proteins of spinach chloroplasts contain *N*-Acetyl-*O*-phopsphothreonine at their NH_2 termini. *J Biol Chem* **3**: 1123-1130

Michel H.P. and Bennett J. (1987) Identification of the phosphorylation site of an 8.3 kDa protein from photosystem II of spinach. *FEBS Lett* **212**: 103-108

Miller K.R. and Staehelin L.A. (1976) Analysis of the thylakoid outer surface. *J Cell Biol* **68**: 30-47

Mitchell P. (1975) A chemiosmotic molecular mechanism of the cytochrome b-c1 complex in the respiratory chain: proton motive ubiquinone cycle. *FEBS Lett* **56**: 1-6

Miyao M. and Murata N. (1984) Role of the 33 kDa polypeptide in preserving manganese in the photosynthetic oxygen-evolution system and its replacement by chloride ions. *FEBS Lett* **170**: 350-354

Miyazaki A., Shina T., Toyoshima Y., Gounaris K. and Barber J. (1989) Stoichiometry of cytochrome b_{559} in photosystem II. *Biochim Biophys Acta* **975**: 142-147

Monod C., Takahashi Y., Goldschmidt-Clermont M. and Rochaix J.-D. (1994) The chloroplast *ycf8* open reading frame encodes a photosystem II polypeptide which maintains photosynthetic activity under adverse growth conditions. *EMBO J* **13**: 2747-2754

Montoya G., Yurela I. and Picorel R. (1991) Pigment stoichiometry of a newly isolated D1-D2-cyt b_{559} complex from the higher plant *Beta vulgaris* L. *FEBS Lett* **283**: 255-258

Mor T.S., Ohad I., Hirschberg J. and Pakrasi H.B. (1995) An unusual organisation of the genes encoding cytochrome b_{559} in *Chlamydomonas reinhardtii*. *Mol Gen Genet* **246**: 600-604
Morais F., Barber J. and Nixon P.J. (1995) Insertional inactivation of the chloroplast *psbE* gene in the green alga *Chlamydomonas reinhardtii*. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 1, pp 791-794, Kluwer Academic Publishers, Netherlands

Mullet J.E., Gamble-Klein P. and Klein R.R. (1990) Chlorophyll regulates accumulation of the plastid-encoded chlorophyll apoproteins CP43 and D1 by increasing apoprotein stability. *Proc Natl Acad Sci USA* **87**: 4038-4042

Möenne-Loccoz P., Robert B. and Lutz M. (1989) A resonance Raman characterisation of the primary electron acceptor in photosystem II. *Biochemistry* **28**: 3641-3645

Nadebryk E., Andrianambinitsoa S., Berger G., Leonard M., Mantele W. and Breton J. (1990) Characterisation of bonding interactions of the intermediary electron acceptor in the reaction centre of photosystem II by FTIR spectroscopy. *Biochim Biophys Acta* **1016**: 49-54

Nagarajan V., Parson W.W., Gaul D. and Schenk C. (1990) Effect of specific mutations of tyrosine-(M)210 on the primary photosynthetic electron-transfer process in *Rhodobacter sphaeroides*. *Proc Natl Acad Sci USA* **87**: 7888-7892

Nakatani H.Y., Ke B., Dolan E. and Arntzen C.J. (1984) Identity of the photosystem II reaction centre polypeptide. *Biochim Biophys Acta* **765**: 347-352

Nanba O. and Satoh K. (1987) Isolation of a photosystem II reaction centre containing D1 and D2 polypeptide and cytochrome b_{559} *Proc Natl Acad Sci USA* **84**: 109-112

Nedbal L., Samson G. and Whitmarsh J. (1992) Redox state of a one-electron component controls the rate of photoinhibition of Photosystem II. *Proc Natl Acad Sci USA* **89**: 7929-7933

Nelson J.A.E. and Lefebvre P.A. (1995) Targeted disruption of the *NIT8* gene in *Chlamydomonas reinhardtii*. *Mol. Cell. Biol.* **15**: 5762-5769

Newman S.J., Boynton J.E., Gillham N.W., Randolph-Anderson B.L., Jonhson A.M. and Harris E.H. (1990) Transformation of chloroplast ribosomal RNA genes in *Chlamydomonas*: molecular and genetic characterisation of integration events. *Genetics* **126**: 875-888

Newman S.M., Harris E.H., Jonhson A.M., Boynton J.E. and Gillham N.W. (1992) Nonrandom distribution of chloroplast recombination events in *Chlamydomonas reinhardtii*: Evidence for a hotspot and an adjacent cold region. *Genetics* **132**: 413-429

Nickelsen J. and Rochaix J.-D. (1994) Regulation of the synthesis of D1 and D2 proteins of photosystem II. In: Baker NR and Bowyer JR (eds), "Photoinhibition of Photosynthesis", Vol. pp 179-194, BIOS, Oxford, U.K.

Nickelsen J., van Dillewijn J., Rahire M. and Rochaix J.-D. (1994) Determinants for stability of the chloroplast *psbD* RNA are located within its short leader region in *Chlamydomonas reinhardtii*. *EMBO J* **13**: 3182-3191

Nilsson F., Andersson B. and Jansson C. (1990) Photosystem II characteristics of a *Synechocystis* 6803 mutant lacking synthesis of the D1-polypeptide. *Plant Mol Biol* **14**: 1051-1054

Nixon P.J. and Diner B.A. (1992) Aspartate 170 of the photosystem II reaction center polypeptide D1 is involved in the assembly of the oxygen evolving manganese cluster. *Biochemistry* **31**: 942-948

Nixon P.J., Chisholm D.A. and Diner B.A. (1992a) Isolation and functional analysis of random and site-directed mutants of photosystem II. In: Shewry PR and Gutteridge S (eds) "Plant Protein Engineering", pp 93-141, Cambridge University Press, Cambridge
Nixon P.J., Komenda J., Barber J., Deak Z., Vass I. and Diner B.A. (1995) Deletion of the PEST-like region of photosystem two modifies the Q_B-binding pocket but does not prevent rapid turnover of D1. *J Biol Chem* **270**: 14919-14927

Nixon P.J., Trost J.T. and Diner B.A. (1992b) Role of the carboxy terminus of polypeptide D1 in the assembly of a functional water-oxidizing manganese cluster in photosystem II of the cyanobacterium *Synechocystis* sp. PCC 6803: Assembly requires a free carboxyl group at C-terminal position 344. *Biochemistry* **31**: 10859-10871

Nuijs A.M., van Gorkom H.J., Plitjer J.J. and Duysens L.N.M. (1986) Primary-charge separation and excitation of chlorophyll *a* in photosystem II particles from spinach as studied by picosecond absorption-difference spectroscopy. *Biochim Biophys Acta* **848**: 167-175

Nyhus K.J. and Pakrasi H.B. (1989) Possible role of *psbJ* in photosystem II: site-directed mutagenesis in the cyanobacterium, *Synechocystis* 6803. In: Barber J and Malkin R (eds) "Techniques and New Developments in Photosynthesis Research", pp 469-472, Plenum Publishing Corporation, New York

Odom W.R. and Bricker T.M. (1992) Interaction of CPa-1 with the manganese-stabilising protein of photosystem II: identification of domains cross-linked by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. *Biochemistry* **31**: 5616-5620

Oettmeier W. (1992) Herbicides of photosystem II. In: Barber J (ed) "The photosystems: structure, function and molecular biology", Vol. 11, pp 349-408, Elsevier Science Publishers, Amsterdam

Ohad I., Adir N., Koike H., Kyle D.J. and Inoue Y. (1990) Mechanism of photoinhibition *in vivo*. *J Biol Chem* **265**: 1972-1979

Ohad I., Keren N., Zer H., Gong H., Mor T.S., Gal A., Tal S. and Domovich Y. (1994) Light-induced degradation of photosystem II reaction centre D1 protein *in vivo*: an integrative approach. In: Baker N and Bowyer JR (eds) "Photoinhibition of Photosynthesis: from molecular mechanisms to the field", pp 161-177, Bios Scientific Publishers, Oxford

Ohad I., Koike H., Shochat S. and Inoue Y. (1988) Changes in the properties of reaction centre II during the initial stages of photoinhibition as revealed by thermoluminescence measurements. *Biochim Biophys Acta* **933**: 288-298

Ohad I., Kyle D.J. and Arntzen C.J. (1984) Membrane protein damage and repair: removal and replacement of inactivated 32-kilodalton polypeptides in chloroplast membranes. *J Cell Biol* **99**: 481-485

Okada K., Satoh K. and Katoh S. (1991) Chloramphenicol is an inhibitor of photosynthesis. *FEBS Lett* **295**: 155-158

Olive J. and Vallon O. (1991) Structural organisation of the thylakoid membrane: freeze-fracture and immunocytochemical analysis. *Journal of Electron Microscopy Technique* **18**: 360-374

Ono T.A. and Inoue Y. (1984) Ca^{2+} -dependent restoration of O_2-evolving activity in $CaCl_2$-washed PS II particles depleted of 33, 24 and 16 kDa polpypeptides. *FEBS Lett* **168**: 281-286

Owens G.C. and Ohad I. (1982) Phosphorylation of *Chlamydomonas reinhardi* chloroplast membrane proteins in vivo and in vitro. *J Cell Biol* **93**: 712-718

Packham N.K., Hodges M., Etienne A.L. and Briantais J.M. (1988) Changes in the flash-induced oxygen yield pattern by thylakoid membrane phosphorylation. *Photosynth Res* **10**: 221-232

Pakrasi H.B. (1995) Genetic analysis of the form and function of photosystem I and photosystem II. *Ann Rev Genet* **29**: 755-776

Pakrasi H.B. and Vermaas W.F.J. (1992) Protein Engineering of Photosystem II. In: Barber J (ed) "The Photosystems: Structure, Function and Molecular Biology", Vol. 11, pp 231-257, Elsevier Publishers, Amsterdam

Papageorgiou G. (1975) Chlorophyll fluorescence: an intrinsic probe of photosynthesis. In: Govindjee (ed) "Bioenergetics of Photosynthesis", pp 319-371, Academic Press, New York

Parson W.W., Chu Z.T. and Warshel A. (1990) Electrostatic control of charge separation in bacterial photosynthesis. *Biochim Biophys Acta* **1017**: 251-272

Philbrick J.B., Diner B.A. and Zilinskas B.A. (1991) Construction and characterisation of cyanobacterial mutants lacking the manganese-stabilising polypeptide of Photosystem II. *J Biol Chem* **266**: 13370-13376

Plato M., Michel-Beyerle M.E., Bixon M. and Jortner J. (1989) On the role of tryptophan as a superexchange mediator for quinone reduction in photosynthetic reaction centres. *FEBS Lett* **249**: 70-74

Ponticos M., Shipton C.A., De Las Rivas J. and Barber J. (1993) Two D1 protein degradation patterns in isolated photosystem 2 core and reaction centre complexes. *Photosynthetica* **28(2)**: 215-224

Prasil O., Adir N. and Ohad I. (1992) Dynamics of photosystem II: mechanism of photoinhibition and recovery processes. In: Barber J (ed) "The Photosystems: Structure, Function and Molecular Biology", Vol. 11, pp 295-348, Elsevier Science Publications, Amsterdam

Prentki P. and Kirsch H. (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29**: 303-313

Przibilla E., Heiss S., Johanningmeier U. and Trebst A. (1991) Site-specific mutagenesis of the D1 subunit of photosystem II in wild-type *Chlamydomonas*. *Plant Cell* **3**: 169-174

Putnam-Evans C. and Bricker T.M. (1994) Site-directed mutagenesis of the CP47 protein of photosystem II: alteration of the basic residue 448R to 448G prevents the assembly of functional photosystem II centres under chloride-limiting conditions. *Biochemistry* **33**: 10770-10776

Race H.L. and Gounaris K. (1993) Identification of the *psbH* gene product as 6 kDa phosphoprotein in the cyanobacterium *Synechocystis* 6803. *FEBS Lett* **323**: 35-39

Race H.L., Eaton-Rye J.J. and Hind G. (1995) A 64-kDa protein is a substrate for phosphorylation by a distinct thylakoid protein-kinase. *Photosynth Res* **43**: 231-239

Rech T., Durrant J.R., Joseph D.M., Barber J., Porter G. and Klug D.R. (1994) Does slow energy transfer limit the observed time constant for radical pair formation in photosystem two reaction centres? *Biochemistry* **33**: 14469-14474

Rintamäki E., Kettunen R., Tyystjärvi E. and Aro E.-M. (1995) Light-dependent phosphorylation of D1 reaction centre protein of photosystem II: hypothesis for the functional role in vivo. *Physiol Plantarum* **93**: 191-195

Rintamäki E., Salo R. and Aro E.-M. (1994) Rapid turnover of the D1 reaction-centre protein of photosystem II as a protection mechanism against photoinhibition in a moss, *Ceratodon purpureus* (Hedw.) Brid. *Planta* **193**: 520-529

Robinson H.H. and Crofts A.R. (1983) Kinetics of the oxidation-reduction reactions of the photosystem II quinone acceptor complex, and the pathway for deactivation. *FEBS Lett* **153**: 221-226

Rochaix J.-D. (1995) *Chlamydomonas reinhardtii* as the photosynthetic yeast. *Ann Rev Genet* **29**: 209-230

Rochaix J.-D. and van Dillewijn J. (1982) Transformation of the green alga *Chlamydomonas reinhardtii* with yeast DNA. *Nature* **296**: 70-73

Rochaix J.-D., Dron M., Rahire M. and Malnöe P. (1984) Sequence homology between the 32 kdalton and the D2 chloroplast membrane polypeptides of *Chlamydomonas reinhardii*. *Plant Mol Biol* **3**: 363-370

Rochaix J.-D., Mayfield S., Goldschimdt-Clermont M. and Erickson J. (1988) Molecular biology of *Chlamydomonas*. In: Shaw CH (ed) "Plant Molecular Biology", pp 253-275, IRL Press, Oxford

Roelofs T.A., Kwa S.L.S., van Grondelle R., Dekker J.P. and Holzwarth A.R. (1993) Primary processes and structure of the Photosystem-II reaction centre. 2. Low temperature picosecond fluorescence kinetics of a D1-D2-cyt b_{559} reaction centre complex isolated by short Triton exposure. *Biochim Biophys Acta* **1143**: 147-157

Roffey R.A., Golbeck J.H., Russ Hille C. and Sayre R.T. (1991) Photosynthetic electron transport in genetically altered photosystem II reaction centers of chloroplasts. *Proc Natl Acad Sci USA* **88**: 9122-9126

Roffey R.A., Kramer D.M., Govindjee and Sayre R.T. (1994a) Lumenal side histidine mutations in the D1 protein of photosystem II affect donor side electron transfer in *Chlamydomonas reinhardtii*. *Biochim Biophys Acta* **1185**: 257-270

Roffey R.A., van Wijk K.J., Sayre R.T. and Styring S. (1994b) Spectroscopic characterisation of Tyrosine-Z in histidine 190 mutants of the D1 protein in photosystem II (PSII) in *Chlamydomonas reinhardtii*. *J Biol Chem* **269**: 5115-5121

Rögner M., Boekema E.J. and Barber J. (1995) How does photosystem 2 split water? the structural basis of efficient energy conversion. *Trends Biochem Sci* **21**: 44-49

Ruffle S.V., Donnelly D., Blundell T.L. and Nugent J.H.A. (1992) A three-dimensional model of the photosystem II reaction centre of *Pisum sativum*. *Photosynth Res* **34**: 287-300

Ruffle S.V., O'Connor H.E., Cheater A.J., Purton S. and Nugent J.H.A. (1995) The construction and analysis of a disruption mutant of *psbH* in *Chlamydomonas reinhardtii*. In: Mathis P

(ed), "Photosynthesis: from Light to Biosphere", Vol. 3, pp 663-666, Kluwer Academic Publishers, Netherlands

Russell J.A., Roy M.K. and Sanford J.C. (1992) Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. *Plant Physiol* **98**: 1050-1056

Rutherford A.W., Crofts A.R. and Inoue Y. (1982) Thermoluminescence as a pobe of Photosystem II photochemistry. The origin of the flash-induced glow peaks. *Biochim Biophys Acta* **682**: 457-465

Rutherford A.W., Zimmermann J.-L. and Boussac A. (1992) Oxygen evolution. In: Barber J (ed) "The Photosystems: Structure, Function and Molecular Biology", Vol. 11, pp 179-229, Elsevier Science Publishers, Amsterdam

Sambrook J., Fritsch E.F. and Maniatis J. (1989) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, New York

Sanford J.C., Smith F.D. and Russell J.A. (1993) Optimizing the biolistic process for different biological applications. *Methods Enzymol* **217**: 483-509

Santini C., Tidu V., Tognon G., Ghiretti Magaldi A. and Bassi R. (1994) Three-dimensional structure of the higher plant photosystem II reaction cente and evidence for its dimeric organisation *in vivo*. *Eur J Biochem* **221**: 307-315

Satoh K. (1993) Isolation and properties of the photosystem II reaction center. In: Deisenhofer J and Norris JR (eds) "The Photosyntetic Reaction Centre", Vol. 1, pp 289-318, Academic Press, London

Satoh K., Ohno T. and Katoh S. (1984) An oxygen-evolving complex with a simple subunit structure a water-plastoquinone oxidoreductase from the thermophilic cyanobacterium *Synechococcus* sp. *FEBS Lett* **180**: 326-330

Sayre R.T. and Wrobel-Boerner E.A. (1994) Molecular topology of the Photosystem II chlorophyll *a* binding protein, CP43: Topology of a thylakoid membrane protein. *Photosynth Res* **40**: 11-19

Sayre R.T., Andersson B. and Bogorad L. (1986) The topology of a membrane protein: the orientation of the 32 kD Q_B-binding chloroplast thylakoid membrane protein. *Cell* **47**: 601-608

Schansker G. and van Rensen J.J.S. (1992) Evaluation of the use of silicomolybdate as an electron acceptor for photosystem-2 in photoinhibition research. *Photosynthetica* **27**: 145-157

Schansker W.F.J., Vermaas W.F.J. and van Rensen J.J.S. (1995) Effect of the substitution of glycine 215 by tryptophan in the D2 protein of *Synechocystis* sp. PCC 6803 on the characteristics of Photosystem II. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 4, pp 351-354, Kluwer Academic Publishers, Netherlands

Schiffer M. and Norris J.R. (1993) Structure and function of the photosynthetic reaction centre of *Rhodobacter sphaeroides*. In: Deisenhofer J and Norris JR (eds) "The Photosynthetic Reaction Centre", Vol. 1, pp 1-12, Academic Press, London

Schmidt S., Arlt P., Hamm H., Huber T., Nagele J., Wachtveitl M., Meyer H., Scheer H. and Zinth W. (1994) Energetics of the primary electron-transfer reaction revealed by ultrafast spectroscopy on modified bacterial reaction centres. *Chem Phys Lett* **223**: 116-120

Schnettger B., Santore U.J., Critchley C., Leitsch J. and Krause G.H. (1992) Light-induced turnover of the D1 protein of the photosystem II reaction centre and photoinhibition *in vivo*. In: Murata N (ed), "Research in Photosynthesis", Vol. 4, pp 423-426, Kluwer Academic Press, Netherlands

Schrader S. and Johanningmeier U. (1992) The carboxy-terminal extension of the D1-precursor protein is dispensable for a functional photosystem II complex in *Chlamydomonas reinhardtii*. *Plant Mol Biol* **10**: 251-256

Schreiber U., Endo T., Mi H. and Asada K. (1995) Quenching analysis of chlorophyll fluorescence by the saturation pulse method: particular aspects relating to the study of eukaryotic algae and cyanobacteria. *Plant Cell Physiol* **36(5)**: 873-882

Schreiber U., Schliwa U. and Bilger W. (1986) Continuous recording of photochemical and non-photochemical clorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynth Res* **10**: 51-62

Schubert W.D., Klukas O., Krauss N., Saenger W., Fromme P. and Witt H.T. (1995) Present state of the crystal structure analysis of Photosystem I at 4.5 Å resolution. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 2, pp 3-10, Kluwer Academic Publishers, Netherlands

Schuster G., Timberg R. and Ohad I. (1988) Turnover of thylakoid photosystem II proteins during photoinhibition of *Chlamydomonas reinhardtii*. *Eur J Biochem* **177**: 403-410

Seibert M. (1993) Biochemical, biophysical and structural characterisation of the isolated Photosystem II reaction centre complex. In: Deisenhofer J and Norris JR (eds) "The Photosynthetic Reaction Centre", Vol. 1, pp 319-356, Academic Press, London

Seibert M., Picorel R., Rubin A.B. and Conolly J.S. (1988) Spectral, photophysical and stability properties of isolated photosystem II reaction centres. *Plant Physiol* **87**: 303-306

Sexton T.B., Christopher D.A. and Mullet J.E. (1990) Light-induced switch in barley *psbD-psbC* promoter utilisation: a novel mechanism regulating chloroplast gene expression. *EMBO J* **9**: 4485-4494

Shen J.-R., Vermaas W. and Inoue Y. (1995) The role of cytochrome c-550 as studied through reverse genetics and mutant characterisation in Synechocystis sp. PCC 6803. *J Biol Chem* **270**: 6901-6907

Shen J.R. and Inoue Y. (1993) Binding and functional properties of two new extrinsic components, cytochrome *c*-550 and a 12 kDa protein, in cyanobacterial Photosystem II. *Biochemistry* **32**: 1825-1832

Shiozawa J.A., Lottspeich F., Oesterhelt D. and Feick R. (1989) The primary structure of the *Chloroflexus aurianticus* reaction-center polypeptides. *Eur J Biochem* **180**: 75-84

Shochat S., Arlt T., Francke C., Gast P., van Noort P.I., Otte S.C.M., Schelvis H.P.M., Schmidt S., Vijgenboom E., Vrieze J., Zinth W. and Hoff A.J. (1994) Spectroscopic characterization of reaction centers of the (M)Y210W mutant of the photosynthetic bacterium *Rhodobacter sphaeroides*. *Photosynth Res* **40**: 55-66

Silverstein T., Cheng I. and Allen J.F. (1993a) Redox titration of multiple protein phosphorylations in pea chloroplast thylakoids. *Biochim Biophys Acta* **1183**: 215-220

Silverstein T., Cheng I. and Allen J.F. (1993b) Chloroplast thylakoid protein phosphatase reactions are redox-independent and kinetically heterogeneous. *FEBS Lett* **334**: 101-105

Sodeinde O.A. and Kindle K.L. (1993) Homologous recombination in the nuclear genome of *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA* **90**: 9199-9203

Srivastava A., Strasser R.J. and Govindjee (1995) Polyphasic rise of chlorophyll *a* fluorescence in herbicide-resistant D1 mutants of *Chlamydomonas reinhardtii*. *Photosynth Res* **43**: 131-141

Staub J.M. and Maliga P. (1992) Long regions of homologous DNA are incorporated into the tobacco plastid genome by transformation. *Plant Cell* **4**: 39-45

Stiehl H.H. and Witt H.T. (1969) Quantitative treatment of the function of plastoquinone in photosynthesis. *Z Naturforsch* **24b**: 1588-1598

Stockhaus J., Höfer M., Renger G., Westhoff P., Wydrzynski T. and Willmitzer L. (1990) Anti-sense RNA efficiently inhibits formation of the 10 kDa polypeptide of photosystem II in transgenic potato plants: analysis of the role of 10 kD protein. *EMBO J* **9**: 3013-3021

Sueoka N. (1960) Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardi*. *Proc Natl Acad Sci USA* **46**: 83-91

Sugiura M. (1989) The chloroplast chromosomes in land plants. *Ann Rev Cell Biol* **5**: 51-70

Sundby C. (1990) Bicarbonate effects on photoinhibition - including an explanation for the sensitivity to photoinhibition under anaerobic conditions. *FEBS Lett* **274**: 77-81

Svab Z. and Maliga P. (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proc Natl Acad Sci USA* **90**: 913-917

Svab Z., Hadjukiewicz P. and Maliga P. (1990) Stable transformation of plastids in higher plants. *Proc Natl Acad Sci USA* **87**: 8526-8530

Svensson B. and Styring S. (1995) The structural environment of the tyrosyl radicals in Photosystem II. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 1, pp 647-650, Kluwer Academic Publishers, Netherlands

Svensson B., Vass I. and Styring S. (1991) Sequence analysis of the D1 and D2 reaction center proteins of photosystem II. *Z Naturforsch* **46c**: 765-776

Svensson B., Vass I., Cedergren E. and Styring S. (1990) Structure of the donor side components in photosystem II predicted by computer modelling. *EMBO J* **9**: 2051-2059

Svensson P., Andreasson E. and Albertsson P.-A. (1991) Heterogeneity among Photosystem I. *Biochim Biophys Acta* **1060**: 45-50

Tae G.-S. and Cramer W.A. (1989) Lumen side topography of the α-subunit of the chloroplast cytochrome *b*-559. *FEBS Lett* **259**: 161-164

Takahashi M., Shiraishi T. and Asada K. (1988) COOH-terminal residues of D1 and the 44 kDa CPa-2 of the spinach photosystem II core complex. *FEBS Lett* **240**: 6-8

Takahashi Y., Goldschmidt-Clermont M., Soen S.-Y., Franzén L.G. and Rochaix J.-D. (1991) Directed chloroplast transformation in *Chlamydomonas reinhardtii*: insertional inactivation of the *psaC* gene encoding the iron sulfur protein destabilizes photosystem I. *EMBO J* **10**: 2033-2040

Takahashi Y., Matsumoto H., Goldschmidt-Clermont M. and Rochaix J.-D. (1994) Directed disruption of the *Chlamydomonas* chloroplast *psbK* gene destabilizes the photosystem II reaction centre complex. *Plant Mol Biol* **24**: 779-788

Takahashi Y., Nakane H., Kojima H. and Satoh K. (1990) Chromatographic purification and determination of the carboxyl-terminal sequences of photosystem II reaction centre protein-D1 and protein-D2. *Plant Cell Physiol* **31**: 273-280

Tang X.-S. and Diner B.A. (1994) Biochemical and spectroscopic characterisation of a new oxygen-evolving Photosystem II core complex from the cyanobacterium *Synechocystis* PCC 6803. *Biochemistry* **33**: 4594-4603

Tang X.-S., Chisholm D.A., Dismukes G.C., Brudvig G.W. and Diner B.A. (1993) Spectroscopic evidence from site-directed mutants of *Synechocystis* PCC6803 in favor of a close interaction between histidine 189 and redox-active tyrosine 160, both of polypeptide D2 of the photosystem II reaction center. *Biochemistry* **32**: 13742-13748

Taylor M.A., Nixon P.J., Todd C.M., Barber J. and Bowyer J.R. (1988) Characterisation of the D1 protein in a photosystem II mutant (LF-1) of *Scenedesmus obliquus* blocked on the oxidising side. Evidence supporting non-processing of D1 as the cause of the lesion. *FEBS Lett* **235**: 109-116

Telfer A., Allen J.F., Barber J. and Bennett J. (1983) Thylakoid protein phosphorylation during state1-state 2 transitions in osmotically shocked pea chloroplasts. *Biochim Biophys Acta* **722**: 176-181

Telfer A., He W.-Z. and Barber J. (1990) Spectral resolution of more than one chlorophyll electron donor in the isolated photosystem II reaction centre complex. *Biochim Biophys Acta* **1017**: 143-151

Telfer A., Marder J.B. and Barber J. (1987) Photosystem II reaction centres isolated from phosphorylated pea thylakoids carry phosphate on the D1 and D2 polypeptide subunits. *Biochim Biophys Acta* **893**: 557-563

Thompson L.K. and Brudvig G.W. (1988) Cytochrome *b*-559 may function to protect Photosystem II from photoinhibition. *Biochemistry* **27**: 6653-6658

Thornber J.P., Cogdell R.J., Chitnis P., Morishige D.T., Peter G.F., Gómez S.M., Anandan S., Preiss S., Dreyfuss B.W., Lee A., Takeuchi T. and Kerfeld C. (1994) Antenna pigment-protein complexes of higher plants and purple bacteria. In: Barber J (ed) "Molecular Processes of Photosynthesis", Vol. 10, pp 55-118, JAI Press Inc., USA

Tiede D.M., Budil D.E., Tang J., El-Kabbani O., Norris J.R., Chang C.-H. and Schiffer M. (1988) Symmetry breaking structures involved in the docking of cytochrome c and primary electron transfer in reaction centres of *Rhodobacter sphaeroides*. In: Breton J and Vermeglio A (eds) "The Photosynthetic Bacterial Reaction Centre: Structure and Dynamics", pp 13-20, Plenum Press, New York

Tommos C., Davidsson L., Svensson B., Madsen C., Vermaas W.F.J. and Styring S. (1993) Modified spectra of the Tyrosine D radical in photosystem II in site-directed mutants of *Synechocystis* sp. PCC 6803: identification of side chains in the immediate vicinity of Tyrosine D on the D2 protein. *Biochemistry* **32**: 5436-5441

Trebst A. (1986) The topology of the plastoquinone and herbicide binding peptides of photosytem II in the thylakoid membrane. *Z Naturforsch* **41c**: 240-245

Tsiotis G., Walz T., Spyridaki A., Lustig A., Engel A. and Ghanotakis D. (1996) Tubular crystals of a photosystem-II core complex. *J Mol Biol* **259**: 241-248

van der Bolt F. and Vermaas W. (1992) Photoinactivation of Photosystem II as studied with site-directed D2 mutants of the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochim Biophys Acta* **1098**: 247-254

van Gorkom H.J., Tamminga J.J., Haveman J. and van der Linden I.K. (1974) Primary reactions, plastoquinone and fluorescence yield in subchloroplast fragments prepared with deoxycholate. *Biochim Biophys Acta* **347**: 417-438

van Niel C.B. (1962) The present state of the comparative study of photosynthesis. *Annu Rev Plant Physiol* **13**: 1-26

van Wijk K.J., Nilsson L.O. and Styring S. (1994) Synthesis of reaction centre proteins and reactivation of redox components during repair of Photosysem II after light-induced inactivation. *J Biol Chem* **269**: 28832-28392

Vass I. and Inoue Y. (1992) Thermoluminescence in the study of Photosystem II. In: Barber J (ed) "The Photosystems: Structure, Function and Molecular Biology", Vol. 11, pp 259-294, Elsevier, Amsterdam

Vass I., Cook K.M., Deak Z., Mayes S.R. and Barber J. (1992) Thermoluminescence and flash-oxygen characterisation of the IC2 deletion mutant of *Synechocystis* sp. PCC 6803 lacking the Photosystem II 33 kDa protein. *Biochim Biophys Acta* **1102**: 195-201

Vass I., Horváth G., Herczeg T. and Demeter S. (1981) Photosynthetic energy conservation investigated by thermoluminescence. Activation energies and half-lives of thermoluminescence bands of chloroplasts determined by mathematical resolution of glow curves. *Biochim Biophys Acta* **634**: 140-152

Vermaas W. (1993) Molecular-biological approaches to analyse Photosystem II structure and function. *Ann Rev Plant Physiol Plant Mol Biol* **44**: 457-481

Vermaas W.F.J., Charité J. and Shen G. (1990a) Q_A binding in D2 contributes to the functional and structural stability of photosystem II. *Z Naturforsch* **45C**: 359-365

Vermaas W., Charité J. and Shen G. (1990b) Glu-69 of the D2 protein in photosystem II is a potential ligand to Mn involved in photosynthetic oxygen evolution. *Biochemistry* **29**: 5325-5332

Vermaas W., Vass I., Eggers B. and Styring S. (1994) Mutation of a putative ligand to the non-haem iron in Photosystem II: implications for Q_A reactivity, electron transfer and herbicide binding. *Biochim Biophys Acta* **1184**: 263-272

Vermaas W.F.J., Ikeuchi M. and Inoue Y. (1988a) Protein composition of the photosystem II core complex in genetically engineered mutants of the cyanobacterium *Synechocystis* sp. PCC 6803. *Photosynth Res* **17**: 97-113

Vermaas W.F.J., Rutherford A.W. and Hansson O. (1988b) Site-directed mutagenesis in photosystem II of the cyanobacterium *Synechocystis* sp. PCC6803: donor D is a tyrosine residue in the D2 protein. *Proc Natl Acad Sci USA* **85**: 8477-8481

Vermaas W.F.J., Styring S., Schröder W.P. and Andersson B. (1993) Photosynthetic water oxidation: The protein framework. *Photosynth Res* **38**: 249-263

Vermaas W.F.J., Williams J.G.K. and Arntzen C.J. (1987a) Site-directed mutations of two histidine residues in the D2 protein inactivate and destabilise photosystem II in the cyanobacterium *Synechocystis* 6803. *Z Naturforsch* **42c**: 762-768

Wasielewski M.R., Johnson D.G., Seibert M. and Govindjee (1989) Determination of the primary charge separation rate in isolated photosystem II reaction centres with 500-fs time resolution. *Proc Natl Acad Sci USA* **86**: 524-528

Wasserman A.R. (1980) Chloroplast cytochromes f, b_{559}, and b_6. *Methods Enzymol* **69**: 181-202

Webber A.N., Bingham S.E. and Lee H. (1995) Genetic engineering of thylakoid protein complexes by chloroplast transformation in *Chlamydomonas reinhardtii*. *Photosynth Res* **44**: 191-205

Webber A.N., Packman L., Chapman D.J., Barber J. and Gray J.C. (1989) A fifth chloroplast-encoded polypeptide is present in the photosystem II reaction centre complex. *FEBS Lett* **242**: 259-262

Wedel N., Klein R., Ljungberg U., Andersson B. and Herrmann R.G. (1992) The single-copy gene *psbS* codes for a phyllogenetically intriguing 22-kDa polypeptide of photosystem II. *FEBS Lett* **314**: 61-66

Weeks D. (1992) *Chlamydomonas*: An increasingly powerful model plant cell system. *Plant Cell* **4**: 871-878

Whitelegge J.P., Koo D. and Erickson J.M. (1992) Site-directed mutagenesis of the chloroplast *psbA* gene encoding the D1 polypeptide of Photosystem II in *Chlamydomonas reinhardtii*: changes at aspartate 170 affect the assembly of a functional water-splitting manganese cluster. In: Murata N (ed), "Research in Photosynthesis", Vol. 2, pp 151-154, Kluwer Academic Press, Netherlands

Whitelegge J.P., Koo D., Diner B.A., Domian I. and Erickson J.M. (1995) Assembly of the photosystem II oxygen-evolving complex is inhibited in *psbA* site-directed mutants of *Chlamydomonas reinhardtii*. *J Biol Chem* **270**: 225-235

Williams J.C., Alden R.G., Murchison H.A., Peloquin J.M., Woodbury N.W. and Allen J.P. (1992) Effects of mutations near the Bacteriochlorophylls in reaction centres from *Rhodobacter sphaeroides*. *Biochemistry* **31**: 11029-11037

Williams J.C., Steiner L.A. and Feher G. (1986) Primary structure of the reaction centre from *Rhodopseudomonas sphaeroides*. *Proteins: Structure, Function and Genetics* **1**: 312-325

Williams J.C., Steiner L.A., Feher G. and Simon M.I. (1984) Primary structure of the L subunit of the reaction centre from *Rhodopseudomonas sphaeroides*. *Proc Natl Acad Sci USA* **81**: 7303-7307

Wollman F.-A. and Delepelaire P. (1984) Correlation between changes in light energy distribution and changes in thylakoid membrane polypeptide phosphorylation in *Chlamydomonas reinhardtii*. *J Cell Biol* **98**: 1-7

Woodbury N.W., Lin S., Lin X., Peloquin J.M., Taguchi A.K.W., Williams J.C. and Allen J.P. (1995) The role of reaction centre excited state evolution during charge separation in a *Rb. sphaeroides* mutant with an initial electron donor midpoint potential 260 mV above wild type. *Chem Phys* **197**: 405-421

Wu H.Y. and Kuchka M.R. (1995) A nuclear suppressor overcomes defects in the synthesis of the chloroplast *psbD* gene product caused by mutations in two distinct nuclear genes of *Chlamydomonas*. *Curr Genet* **27**: 263-269

Wynn R.M. and Malkin R. (1988) Interaction of plastocyanin with Photosystem I: A chemical cross-linking study of the polypeptide that binds plastocyanin. *Biochemistry* **27**: 5863-5869

Xiong J., Hutchison R., Sayre R. and Govindjee (1995) Characterisation of a site-directed mutant (D1-arginine 269-glycine) of *Chlamydomonas reinhardtii*. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 1, pp 575-578, Kluwer Academic Publishers, Netherlands

Xu Q., Hoppe D., Chitnis V.P., Odom W.R., Guikema J.A. and Chitnis P.R. (1995) Mutational analysis of Photosystem-I polypeptides in the cyanobacterium *Synechocystis* sp. PCC-6803-targeted inactivation of *psaI* reveals the function of PsaI in the structural organisation of PsaL. *J Biol Chem* **270**: 16243-16250

Xu Q., Odom W.R., Guikema J.A., Chitnis V.P. and Chitnis P.R. (1994) Targeted deletion of *psaJ* from the cyanobacterium *Synechocystis* sp. PCC-6803 indicates structural interactions between the PsaJ and PsaF subunits of Photosystem-I. *Plant Mol Biol* **26**: 291-302

Yamada Y., Tang X.-S., Itoh S. and Satoh K. (1987) Purification and properties of an oxygen-evolving Photosystem II reaction-centre complex from spinach. *Biochim Biophys Acta* **891**: 129-137

Zer H. and Ohad I. (1995) Photoinactivation of photosystem II induces changes in the photochemical reaction centre II abolishing the regulatory role of the Q_B site in the D1 protein degradation. *Eur J Biochem* **231**: 448-453

Zhang Z.-H., Mayes S.R., Vass I., Nagy L. and Barber J. (1993) Characterisation of the *psbK* locus of *Synechocystis* sp. PCC 6803 in terms of Photosystem II function. *Photosynth Res* **38**: 369-377

Zinth W., Arlt T., Penzkofer H., Hamm P., Bibikova M., Dohse B., Oesterhelt D., Meyer M. and Scheer H. (1995) Ultrafast infrared and visible spectroscopy of bacterial reaction centres. In: Mathis P (ed), "Photosynthesis: from Light to Biosphere", Vol. 1, pp 389-394, Kluwer Academic Publishers, Netherlands

Zurawski G., Bohnert H.J., Whitfeld P.R. and Bottomley W. (1982) Nucleotide sequence of the gene for the M_r 32,000 thylakoid membrane protein from *Spinacea oleracea* and *Nicotiana debneyi* predicts a totally conserved primary translation product of M_r 38,950. *Proc Natl Acad Sci USA* **79**: 7699-7703