Analysis of Photosystem I mutants in

Chlamydomonas reinhardtii.

A thesis submitted for the degree of

Doctor of Philosophy

by

Vaishali Patel  B.Sc. (Hons)

Department of Biology
University College London
ABSTRACT

F₅ is a [4Fe-4S] iron-sulphur centre that is believed to be bound by both PsaA and PsaB. This is a rather unusual arrangement, as [4Fe-4S] iron-sulphur centres are usually bound by one protein, rather than two. This may impose constraints on the interactions of the other subunits of PSI. The region believed to be involved in binding F₅ is highly conserved, and a possible role has not yet been identified for most of the amino acids within this region. In this study, the function of several amino acids of this region has been examined, by creating amino acid substitutions, and then examining the effect on PSI assembly, and electron transport.

Mutant D576L is a nonphotosynthetic mutant of C. reinhardtii. D576 is an amino acid present within the F₅ binding region, and is believed to be important in the coordination of PsaC, which in turn coordinates the terminal electron acceptors of PSI. The role of this conserved amino acid residue was examined by substituting it with a leucine residue. This substitution caused the PSI complex to be disrupted, and also altered electron transport to F₆/F₇. Second site suppressor mutants of the D576L mutation were also isolated. These suppressor mutants were effected in the same way as the original D576L mutants, in terms of electron transport to F₆/F₇, but were able to grow photoautotrophically. This unknown spontaneous mutation is identified as a nuclear mutation. The results show that D576 is important for the binding of PsaC, and that in the suppressor mutants electron transfer rates from A₅ to F₅ are altered, but restored to wild type values in the suppressor mutants.

A library of mutations was created by randomly mutating three amino acids, Arg572, Phe573 and Pro574, of the F₅ binding motif in PsaA. Three mutants of C. reinhardtii were produced. These mutants were selected for their ability to grow photoautotrophically, as the aim was to create minor disruptions in the photosystem, which would still allow PSI to assemble and function. It was hoped that this would provide information on the possible functions of these amino acids. However, all three mutants appear to assemble as much PSI as wild type C. reinhardtii, and the electron transport properties were also similar. Rates of electron transport to F₆/F₇ were not altered, compared to wild type C. reinhardtii.
The role of a small chloroplast encoded subunit, PsaJ, of PSI was also examined. The function of PsaJ, which is a membrane protein, is unknown, although it has been suggested that it may coordinate PsaF. The amino acid structure of PsaJ was examined, and revealed regions of hydrophobicity, which are consistent with a membrane protein. However, unlike most membrane proteins which are composed of \( \alpha \)-helices, the secondary structure of PsaJ is predicted to be composed of \( \beta \)-sheets. This may prove to have some bearing on its function. The \( psaJ \) gene was deleted, and \( aadA \) was inserted in its place. This construct was used to transform \( C. reinhardtii \), but transformants failed to emerge.
For Mum and Dad.
ACKNOWLEDGEMENTS

I would like to thank my supervisors Professor Mike Evans and Dr. Saul Purton for their help and advice during this project. Thanks also go to all the members of the group who have always been available for help and advice, especially Amanda, Laura and Sue, who were always there for a chat and who never minded me scrounging plates and solutions from them. I would also like to acknowledge the BBSRC who funded my studentship.

I would like to thank my brother Viral, and my Mum and Dad, who have always encouraged and believed in me and without whose support this would not have been possible. Finally, I would like to thank my husband, Prashant, for his endless patience on the subject of photosynthesis, and all the joy and laughter over the last year.
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ABBREVIATIONS.

A. Units.

A1. Units of Length

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<thead>
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<th>Unit</th>
<th>Abbreviation</th>
<th>Conversion</th>
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<tbody>
<tr>
<td>m</td>
<td>metre</td>
<td>(10^2 m)</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
<td>(10^-2 m)</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
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</tr>
<tr>
<td>μm</td>
<td>micrometre</td>
<td>(10^-6 m)</td>
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<td>nm</td>
<td>nanometre</td>
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A2. Units of Volume

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<td>ml</td>
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<tr>
<td>μl</td>
<td>microlitre</td>
<td>(10^-6 l)</td>
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A3. Units of Mass

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<tr>
<td>mg</td>
<td>milligram</td>
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<tr>
<td>μg</td>
<td>microgram</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<td>pg</td>
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A4. Units of Concentration

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<tbody>
<tr>
<td>M</td>
<td>molar</td>
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mM  millimolar  \((10^{-3} \text{ M})\)
\(\mu\text{M}\)  micromolar  \((10^{-6} \text{ M})\)
\(\text{nM}\)  nanomolar  \((10^{-9} \text{ M})\)
\(\text{pM}\)  picomolar  \((10^{-12} \text{ M})\)
g/l  gram per litre
mg/l  milligam per litre
mg/ml  milligram per millilitre
v/v  volume to volume
w/v  weight to volume
w/w  weight to weight

**A5. Other Units**

bp  base pair
C  degrees centigrade
Kbp  kilo base pair \((10^3 \text{ bp})\)
hr  hour
min  minute
mA  milliamp
sec  second
V  volt
B. Other Abbreviations

A
absorbance

ADP
adenosine diphosphate

ATP
adenosine triphosphate

BCIP
5-bromo-4-chloro-3-indolyl phosphate

cDNA
complementary deoxyribonucleic acid

Chl
chlorophyll

cw
cell wall

dATP (A)
2' deoxyadenosine 5'-triphosphate

dCTP (C)
2' deoxycytidine 5'-triphosphate

DEPC
diethylpyrocarbonate

dGTP (G)
2' deoxyguanosine 5'-triphosphate

dH2O
distilled water

dNTP
2' deoxynucleoside 5'-triphosphate

dTTP (T)
2' deoxythymidine 5'-triphosphate

ddATP
2',3' dideoxyadenosine 5'-triphosphate

ddCTP
2',3' dideoxycytidine 5'-triphosphate

ddH2O
double distilled water

ddGTP
2',3' dideoxyguanosine 5'-triphosphate

ddNTP
2',3' dideoxynucleoside 5'-triphosphate

ddTTP
2',3' dideoxythymidine 5'-triphosphate

DNA
deoxyribonucleic acid

DTT
dithiothreitol

ECL
enhanced chemiluminescence

EDTA
ethylenediaminetetraacetic acid

EPR
electron paramagnetic resonance

ER
endoplasmic reticulum

EtBr
ethidium bromide

GDP
guanosine diphosphate
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<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IR</td>
<td>inverted repeat</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D thiogalactoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth medium</td>
</tr>
<tr>
<td>LHC</td>
<td>light harvesting complex</td>
</tr>
<tr>
<td>LHCP</td>
<td>light harvesting complex protein</td>
</tr>
<tr>
<td>LMW</td>
<td>low molecular weight</td>
</tr>
<tr>
<td>M&lt;sub&gt;r&lt;/sub&gt;</td>
<td>relative molecular weight</td>
</tr>
<tr>
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</tr>
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<td>mating type</td>
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<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<td>NBT</td>
<td>nitro blue tetrazolium</td>
</tr>
<tr>
<td>OEC</td>
<td>oxygen evolving complex</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
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<td>PAGE</td>
<td>polyacrylamide electrophoresis</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PMSF</td>
<td>phenylmethanesulfonyl floride</td>
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<td>PSI</td>
<td>photosystem I</td>
</tr>
<tr>
<td>PSII</td>
<td>photosystem II</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RuBisCO</td>
<td>ribulose bisphosphate carboxylase oxygenase</td>
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<tr>
<td>s</td>
<td>sterile</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
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<td>SSC</td>
<td>salt sodium citrate</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>------------------------------------------------</td>
</tr>
<tr>
<td>SRP</td>
<td>signal recognition particle</td>
</tr>
<tr>
<td>TAP</td>
<td>tris acetate phosphate medium</td>
</tr>
<tr>
<td>TAE</td>
<td>tris acetate EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>tris borate EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>tris EDTA</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N' tetramethyl-ethylenediamine</td>
</tr>
<tr>
<td>TEN</td>
<td>Tris EDTA sodium chloride</td>
</tr>
<tr>
<td>TM</td>
<td>tris-minimal</td>
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<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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<tr>
<td>UV</td>
<td>ultra violet</td>
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<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
<tr>
<td>Amino Acid</td>
<td>Three letter abbreviation</td>
</tr>
<tr>
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</tr>
<tr>
<td>Alanine</td>
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D. Nucleic Acid Abbreviations.

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<tr>
<td>Thymine</td>
<td>T</td>
</tr>
<tr>
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CHAPTER 1: INTRODUCTION
CHAPTER 1: INTRODUCTION

1.1 Photosynthesis

Photosynthesis is the process by which higher plants, algae and certain bacteria convert light energy into chemical energy. This conversion of energy is a very complex biochemical process, which relies on the interactions of a number of pigment molecules and electron transfer proteins. Collectively, these are known as a photosynthetic unit.

The primary event of photosynthesis is the absorption of light by a receptor molecule, chlorophyll. Chlorophyll is composed of a chlorin molecule, coordinated by a Magnesium atom. A distinctive feature of chlorophyll is the presence of phytol, a hydrophobic 20-Carbon alcohol, esterified to an acid side chain. Chlorophylls are very effective photoreceptors because they have a network of alternating double and single bonds. They have very strong absorption bands in the visible region of the spectrum, where solar light reaching the Earth is maximal. Different types of chlorophylls exist, in which the side chains differ slightly. This means that disparate chlorophylls absorb light at different wavelengths.

After interception of light, the excitation energy passes from the first chlorophyll, located in a light harvesting complex (LHC), until it reaches a reaction centre. The efficiency of electron transfer from LHCs to reaction centres is very high, due to the orientation of the LHC with respect to the reaction centre. At the reaction centre, the excitation energy from the chlorophyll is converted into a separation of charge, followed by electron transfer. This set of reactions are known as the light reactions, because they require the participation of light to start the process. The end result of electron transfer is the reduction of NADPH or (NADH) and the synthesis of ATP. These are then used in the second part of photosynthesis: the dark reactions. This set of reactions are so called because they do not directly require light.

The NADPH and the ATP produced in the light reactions are used to convert CO$_2$ to carbohydrates. Carbon fixation is a cyclic process. Atmospheric CO$_2$ combines with
ribulose 1, 5 bisphosphate to form a transient 6-Carbon compound, which is rapidly hydrolysed to two molecules of 3-phosphoglycerate. This is a highly exergonic reaction, and is catalysed by the enzyme ribulose 1, 5 bisphosphate carboxylase (Rubisco). Rubisco is a large enzyme (approximately 500 kDa), and is composed of eight large and eight small subunits. It is located on the stromal surface of the thylakoids. As the enzyme works slowly, processing only three molecules of substrate per second, compared to about one thousand for a typical enzyme, many copies of the enzyme are needed. Indeed, Rubisco often represents more than 50% of the total cell weight, and is reputed to be the most abundant protein on Earth. The 3-phosphoglycerate is converted into 1,3-bisphosphoglycerate, using ATP. This compound is then converted into glyceraldehyde 3-phosphate. Subsequently, a complex series of reactions involving sugar intermediates occur, resulting in the regeneration of ribulose 1, 5 bisphosphate. This set of reactions is known as the Calvin Cycle. Each turn of the cycle 'fixes' one molecule of CO₂, with one molecule of glyceraldehyde 3-phosphate as the net gain. The majority of glyceraldehyde 3-phosphate thus produced is imported to the cytosol, where it serves as a central intermediate in biosynthetic pathways. The remainder of the glyceraldehyde 3-phosphate is converted into starch grains, and stored in the chloroplast to serve as a carbohydrate reserve.

1.2 Reaction Centres and Photosystems.
The photochemical reaction centre is classified as the minimal unit that can catalyse electron transfer processes leading to charge stabilisation (Barber, 1987). Reaction centres are pigment-protein complexes, containing electron donors and electron acceptors. Reaction centres are intimately associated with LHCs, which ‘funnel’ the excitation energy required to start their various reactions. The excitation energy that arrives from the antenna chlorophylls is used for the photochemical oxidation of a special pair of chlorophylls. Upon excitation, an electron is passed to the primary electron acceptor. Oxidation of the primary electron acceptors, via a chain of donors and acceptors, drives ATP synthesis. A photosystem therefore enables light to cause a net electron transfer from a weak electron donor to a molecule which is a strong electron donor in its reduced form. In this way, excitation energy that would otherwise be released as fluorescence or heat is used to raise
the energy of an electron and create a strong electron donor.

All photosynthetic reaction centres follow this basic chain of events, summarised in the equation below.

\[ \text{hv} \]

\[ P_D P_A \rightarrow P_D^+ P_A \rightarrow P_D^+ P_A^- \]

where \( P_D \) is the primary electron donor and \( P_A \) is the primary electron acceptor.

Reaction centres use a ‘special pair’ of chlorophyll molecules as the primary electron donor, and chlorophyll or pheophytin as the primary electron acceptor. When excited by light, the primary donor gives an electron to the primary acceptor. Secondary electron donors and acceptors function rapidly to stabilise the charge separation, and prevent back reactions.

There are two different types of reaction centres, which are found distributed between two distinct classes of anaerobic photosynthetic bacteria. Type I reaction centres are characterised by having low potential iron-sulphur centres as terminal electron acceptors, and are found in green sulphur bacteria and heliobacteria. Type II reaction centres use quinones as their terminal electron acceptors, and are found in purple sulphur and non-sulphur photosynthetic bacteria (and certain green bacteria).

Both types of reaction centre occur in higher plants, algae and aerobic cyanobacteria, where they are coupled in series. Together, they catalyse the oxidation of water and create
the low potential needed to reduce NADP^+. The oxidising potential required to split water is provided by a type II reaction centre known as Photosystem II (PSII). The iron-sulphur containing type I reaction centre of oxygenic photosynthesis is known as Photosystem I (PSI).

1.2.1 Type I Photosynthetic Bacteria.

Type I reaction centres are found in the green sulphur bacteria and the heliobacteria. They are characterised by a number of bound electron acceptors that have low redox potentials (Cogdell et al., 1992). These iron-sulphur centres have $E_m$ values more electronegative than the $E_m$ of NADPH (NADH). This allows for direct photoreduction of pyridine nucleotides in an energetically downhill electron transfer sequence, utilising a low potential stable electron acceptor generated during the charge separation event in the reaction centre complex (Cogdell et al., 1992).

Green sulphur bacteria grow in strongly reducing, usually hydrogen sulphide rich environments in dim light. The bacteria are adapted to grow under these dim light conditions by having a large antenna system called a chlorosome. These chlorosomes add about 3000-5000 bacteriochlorophyll pigments to the several hundred BChl $a$ molecules already present in the reaction centre (Lockau et al., 1993).

Heliobacteria are a recently discovered family of bacteria. Heliobacteria are unique among photosynthetic prokaryotes because they contain a new type of chlorophyll, bacteriochlorophyll $g$ (Bchl $g$). This chlorophyll is a combination of chlorophyll $a$ (Chl $a$) of higher plants and bacteriochlorophyll $b$ (BChl $b$) of some purple photosynthetic bacteria (Trost et al., 1992). Heliobacteria differ from green sulphur bacteria in that they only have a small antenna system of approximately 30-60 chlorophylls per primary electron donor P798.

EPR spectroscopy has established that the oxidised primary donor in Chlorobium limicola, and in Heliobacterium chlorum is probably a dimer. This technique has also shown that
isolated complexes contain at least three photoreducible iron-sulphur centres that have been designated F_x, F_A and F_B (Nitschke et al., 1990; Kjær et al., 1996). Indirect evidence also exists that these reaction centres have a quinone-type electron acceptor analogous to A_i in PS1 (Feiler et al., 1995; Nitschke et al., 1990). The absorption spectra of the primary electron acceptor in green sulphur bacteria and heliobacteria are very similar and comparable to A_o, a chlorophyll a molecule (Feiler et al., 1995).

Reaction centres have been isolated from green sulphur bacteria of the Chlorobium spp., and have been shown to contain several different subunits. It is dominated by two polypeptides, with an apparent molecular weight of 65 kDa, on a SDS-PAGE gel (Nitschke et al., 1991). In heliobacteria only one core subunit has been discovered (Büttner et al., 1992). There is relatively little conservation between sequences from Chlorobium limicola and Heliohabillus mobilis reaction centre core proteins and corresponding PS1 proteins. However 'hot spots' of conserved region can be identified and can be expected to be essential for photosystem structure and function. In particular, the sequence homology of the primary sequence of the dominant polypeptide of both Chlorobium and heliobacteria is particularly striking in the region of the F_x binding site (Lockau et al., 1993). Moreover, in green sulphur bacteria a gene has been identified that codes for a protein which is able to bind two [4Fe-4S] iron-sulphur centres (Büttner et al., 1992). The location of this protein, with respect to the dominant polypeptide, makes it a likely candidate for the apoprotein of the terminal iron-sulphur centres F_A and F_B (Lockau et al., 1993).

1.2.2 Type II Photosynthetic Bacteria.

Type II reaction centres are found in purple non sulphur and sulphur photosynthetic bacteria. They are characterised by their use of quinones as the terminal electron acceptor. The electron acceptor is only mildly reducing and so these organisms produce reduced pyridine nucleotides by energy-linked reversed electron transport (Cogdell et al., 1992).

The structures of the reaction centre complex of several species of purple bacteria have been elucidated. Purple bacteria contain two light harvesting antenna complexes, LH1 and
LH2, to which BChl and carotenoids are non covalently attached. Light energy is harvested by the antenna complexes and, within the reaction centre, is used to promote the oxidation of a special pair of BChls, which are the primary electron donor. This oxidation is coupled with the reduction of a quinone. When the quinone becomes doubly reduced, it migrates to the cytochrome $bc$ complex. This then cycles electrons from the quinone back to the reaction centre, via cytochrome $c_2$. The cytochrome $bc$ complex also translocates protons across the cytoplasmic membrane which results in the creation of a proton gradient, which is then used to generate ATP (Bauer et al., 1996).

Most purple bacterial reaction centres contain three subunits: H, M and L. Some species only contain M and L subunits, while others have a bound four haem, one polypeptide, c-type cytochrome. This acts as the immediate electron donor to the photooxidised special pair BChls. The two integral proteins of reaction centre Type II are the L and M subunits. Both of these proteins are integral membrane proteins, with five transmembrane helices. The folding patterns of both proteins are very similar. Together, both proteins form a ‘cage’, inside which the cofactors of the reaction centre are associated.

It is believed that the bacterial reaction centre L and M subunits and the D1 and D2 proteins of PSII are evolutionarily linked. Although sequence homology of L and M to D1 and D2 is very small (only about 10%), many conserved residues are the same. Strong homology is seen in the putative pigment binding regions (Cogdell et al., 1992). PS II carries out electron transfer reactions similar to the reaction centres of purple bacteria. Both systems have pheophytin and quinone molecules ($Q_A$ and $Q_B$) as electron acceptors. Although the chemical nature of the cofactors differs between the two systems, they possess approximately the same redox values and undergo electron transfer at the similar rates.

1.3 Oxygenic Photosynthesis.
Oxygenic photosynthesis occurs in all eukaryotic photosynthetic organisms, cyanobacteria
and prochlorophytes. Water is 'split', and is the source of electrons, while NADP⁺ is the terminal electron acceptor. The creation of an electrochemical proton gradient across the photosynthetic membrane is coupled to electron transport, which drives ATP synthesis. Electron transport is driven by PS1 and PS2, which are coupled, and located in specialised membranes called thylakoids. Although the organisation of the photosynthetic machinery of organisms that carry out this type of photosynthesis is different, the photosynthetic reactions are very similar between both the eukaryotes and the prokaryotes.

1.3.1 Oxygenic Prokaryotes.

(A) The Prochlorophytes.

Until recently, it was believed that the combination of the photosynthetic components chlorophyll a and chlorophyll b was common only to higher plants and the green algae. The prochlorophytes are a group of prokaryotic photosynthetic organisms, that have been discovered within the last two decades, and have been found to contain Chl a and chlorophyll b. Three genera have been identified thus far, each with 1 species: Prochlorothrix hollandica, Prochloron didemni and Prochlorococcus marinus (Post et al., 1994).

The thylakoids are located in the border areas of the cytoplasm, often running parallel to one another, and the cell wall. However, it is difficult to distinguish between well defined granal stacks and unstacked stromal membranes, that are characteristic of chloroplasts (See section 1.4). Instead the thylakoids appear to be locally appressed. This is probably due to the absence of phycobilisomes (see section 1.3.1 (B)), and the presence of Chl b containing antennae (Matthijs et al., 1994; Post et al., 1994). Within the cell cytoplasm, inclusion bodies known as carboxysomes are also found. These structures contain the enzymes required for efficient carbon fixation (Post et al., 1994).

PSI and PSII have been recovered from Prochlorothrix and Prochloron species, and have been found to exhibit photochemical activity. Immunological analysis of these
photosystems shows the presence of the main reaction centre proteins, as found in cyanobacteria and chloroplasts. PSI particles from *Prochloron* and *Prochlorothrix* show a polypeptide composition very similar to that of higher plants. the composition of PSII is less clear, but the major proteins have been identified (Post *et al.*, 1994). Virtually all the components involved in oxygenic electron transport have been identified in *Prochlorothrix* and *Prochloron*. Cytochrome *f* and cytochrome *b₆* have been identified in different species of *Prochlorothrix* and *Prochloron*. *petB* and *petD* genes encoding cytb apoprotein and subunit 4 of the cytochrome *b₆/f* complex have also been cloned and sequenced. In addition, soluble fractions from *Prochlorothrix* have been shown to contain the same primary electron donor as PSII, plastocyanin.

(B) The Cyanobacteria.

Cyanobacteria are a group of bacteria that can carry out oxygenic photosynthesis. They are unique among bacteria in that they possess both Type I and Type II reaction centres, that is, they have PSI and PSII, which are much the same as in eukaryotes. They also possess thylakoid membranes, in which the photosystems and the other complexes involved in photosynthesis are found.

In the light reactions of photosynthesis, red and blue wavelengths of visible light are mainly absorbed by cyclic tetrapyrroles (chlorophylls). Green, yellow and orange wavelengths are mostly absorbed by open chained tetrapyrroles, the phycobilins. Pigments harvest light energy, and trap excitation energy at the ‘special pair’, and finally transduce light energy into stable charge separation. Pigments alone cannot perform the primary steps of photosynthesis. Proteins are required to orientate the pigments, to give them the appropriate conformation and physical separation, and regulate the absorption properties.

Cyanobacteria do not contain LHCII. Their light harvesting complexes for PSII, and to a smaller extent PSI, are large multiprotein structures, located on the stromal side of the thylakoids. They are known as phycobilisomes, and absorb visible light at 450-665 nm.
These specialised light harvesting organelles are found only in cyanobacteria and red algae. The phycobilisome complex is primarily composed of phycobiliproteins. These proteins are a family of coloured, water soluble proteins, bearing covalently attached, open-chain tetapyrroles known as phycobilins. Phycobilisome complexes also contain smaller amounts of other polypeptides, which do not bear chromophores, but are absolutely required for correct assembly, and functional organization. Phycobilisomes have two structural elements: a core structure, and peripheral rods. The peripheral rods radiate from the lateral surfaces of the core which are not in contact with the thylakoid membrane. Light energy is absorbed mainly by the peripheral rods. Absorbed light energy is transferred by a very rapid downhill energy transfer from phycoerythrin or phycoerythrocyanin to C-phycocyanin (Sidler, 1994). From here, the energy is transferred to allophycocyanin, which acts as the final energy transmitters from the phycobilisome to Chl $\alpha$ associated with PSII. The light reactions of photosynthesis from this point occur in the same manner as in green algae and higher plants.

1.3.2 Oxygenic Eukaryotes.

All eukaryotes capable of photosynthesing are oxygenic. All the photosystems, the components for electron transport, cytochrome $b_{6}/f$ complex and the ATP synthase complex are bound to the thylakoid membranes. These membranes are located within specialised organelles called chloroplasts.

1.4 Chloroplasts.

It is generally believed that chloroplasts were once free living photosynthetic prokaryotes that were endocytosed by primitive eukaryotic cells. Once endocytosed, they developed a symbiotic relationship with the cell. Subsequent evolutionary events have resulted in the chloroplast becoming a highly complex, specialised organelle of photosynthetic eukaryotic cells (see figure 1.1).

Chloroplasts are encased in a double membrane, the outer of which is highly permeable
Figure 1.1. EM picture of a chloroplast. Chloroplasts are further discussed in section 1.4.
(the inner is less so). The inner membrane surrounds the stroma, within which are found the thylakoids. The thylakoids are the major photosynthetic component of the chloroplast.

The thylakoids (see figure 1.2) are flattened disc-like sacs, within which are located PSI, PSII, Cytochrome b$_6$f complex and the ATP synthase complex. The thylakoids are differentiated into two regions: stacked (appressed) and unstacked (nonappressed). The stacked region of chloroplasts is called the grana, and it is within this region that the majority of PSII is found. The stroma lamellae are not stacked, but connect the grana lamellae. This region is stroma exposed, and the majority of PSI and the ATP synthase complex is located here. The stroma also contains DNA, as chloroplasts possess an independent genome.

1.4.1 The Chloroplast Genome.

The chloroplast genome is a circular, double stranded entity. Although the majority of chloroplast proteins are encoded for by the nucleus, the chloroplast genome does encode some proteins. Chloroplasts contain all the components and enzymes required to replicate and express their DNA. Polymerases, translation factors and ribosomes are found in the stroma.

The chloroplast genome is approximately the same size in most species, being between 120-160 kbp in size. The green algae are the exception to this rule, in that they have a wide range of chloroplast genome sizes (85-292 kbp). One of the largest chloroplast genomes is possessed by the green alga Acetabularia which has a chloroplast genome of about 2000 kbp.

There are several striking differences between plastid and nuclear genomes. The chloroplast genome is polyploid, and is present as 100-10,000 copies per cell. The informational content of the chloroplast genome is reduced, compared to nuclear genome (by $10^3$-$10^4$). There are approximately 120 chloroplast encoded genes. Of these, roughly
Figure 1.2. Schematic representation showing the distribution of the photosynthetic complexes in the thylakoid membranes of the chloroplast (figure redrawn from Anderson (1992)).

- ATP synthase complex
- PSII complex
- PSI complex
- Cytb6f complex
half encode genes for plastid protein synthesis. About another 30 code for subunits of the various photosystems, photosynthetic complexes, and enzymes. A few other genes encode factors involved in respiration. The functions of the remainder are unknown.

The chloroplast genomes of several organisms have been completely sequenced, including liverwort (Ohyama et al., 1986), rice (Hiratsuka et al., 1989), and tobacco (Shinozaki et al., 1986). This has shown that plastid genomes are arranged conservatively, and contain several distinct features. The genome has five repeat families: one organised as a two-copy inverted repeat, two tandem repeats, two dispersed repeats. The most widespread is a large 10-76 kbp inverted duplication found in chloroplast DNA from almost all land plants and from several major lineages of algae. Contained within this inverted repeat is a complete set of tRNA genes. This inverted repeat is asymmetrically placed, so that the chloroplast genome is divided into small and large single copy regions (Palmer et al., 1985). Surprisingly, 11 genes encoding subunits of a putative respiratory chain NADH dehydrogenase complex (ndh) have been discovered (Ohyama et al., 1986; Shinozaki et al., 1986; Hiratsuka et al., 1989). These ndh genes are expressed in mature chloroplasts, and may be involved in chlororespiration or as a link between PSI and cyt b\_6/f in cyclic photophosphorylation (Allen, 1993).

### 1.4.2 Origin of Chloroplasts.

It is believed that chloroplasts developed from an endocytotic event. Free living organisms, capable of photosynthesis, became endocytosed by prehistoric prokaryotes, and a mutually beneficial relationship evolved.

When prochlorophytes were first discovered, it was believed that they were the progenitors of chloroplasts. However, if genome size, thylakoid structure and composition and gene homology are examined this idea can probably be discarded. The size of the prochlorophyte genome is larger than that of the chloroplast. It is possible, however, that a loss of gene size may have occurred during endocytosis, and gene transfer to the host
leading to loss of any redundant proteins. It must also be noted that organelle DNA is AT rich, while photosynthetic prokaryotes are, in comparison, GC rich. Gene homology studies on *Prochloron* have been performed, and partial 5S rRNA and 16S rRNA sequences have established the position of *Prochloron* among the cyanobacteria, distant from the chloroplast (Post *et al.*, 1994). Gene sequences, and their operon structure in the prochlorophytes are also more like the cyanobacteria. The *rbcLS* (Rubisco large and small subunit) genes are linked in cyanobacteria and *Prochlorothrix*, while in eukaryotes *rbcL* is a plastid encode gene, and *rbcS* is nuclear encoded (Post *et al.*, 1994; 

Taxonomic studies also place the prochlorophytes among the cyanobacteria, although the lack of phycobilisomes most distinguishes them.

All this evidence demonstrates that the Prochlorophytes are closer in evolutionary terms to the cyanobacteria than they are to chloroplasts. Indeed, the main discriminating features appear to be only the lack of phycobilisomes and the presence of chlorophyll b. Considering this data, it is likely that the prochlorophytes were descended from cyanobacteria, and did not give rise to chloroplasts. It is more probable that perhaps the cyanobacteria gave rise to the chloroplast, although more data from cyanabacterial species and chloroplast containing species needs to be examined to resolve this mystery.

1.4.3 Chloroplast Gene Regulation.

The expression of chloroplast genes involves the activation of plastid genes whose products are required in the chloroplast. It also requires the modification of gene expression within the developed chloroplast in response to changing environmental conditions. The expression of chloroplast genes is linked with the expression of nuclear encoded genes. These nuclear genes encode the structural and enzymatic elements required for chloroplast function, as well as the majority of the regulatory factors. Several processes are thought to be important in the regulation of chloroplast gene expression.

(I) *Transcription:* Examination of chloroplast mRNA accumulation in a number of
species has shown that mRNA levels fluctuate in response to both developmental and environmental signals (Mayfield et al., 1995). The transcription of chloroplast genes fluctuates in relation to plastid chloroplast development, chloroplast type and cell cycle. Transcription rates can differ relatively from one plant species to the next for a specific gene, for example the rate of psbA transcription (compared to that of 16S rRNA transcription) is 30% in spinach and only 15% in C. reinhardtii (Blowers et al., 1993). This indicates that there is some level of control of the rate of transcription at the level of individual genes. Transcription levels are also affected by light and dark growth phases. For example, in tomato plants, transcription of chloroplast genes increases during the night and early morning (Mayfield et al., 1995). However, the transcription of individual genes varies only slightly compared to the overall change in transcriptional activity. This seems to suggest that transcription is a general regulator of plastid gene expression, rather than of individual genes.

(II) mRNA Processing: This is not so well defined as to assign it a role in the regulation of chloroplast gene expression. Although there is little evidence to support this idea, differential processing could be a potential target for regulation. Many chloroplast genes contain introns, and splicing is an important, but not necessarily a regulatory, process in chloroplast gene expression. There are two major classes of introns, group I and group II; most higher plant chloroplast introns belong to group II, although group I introns are present in the chloroplast ribosomal RNA and psbA genes of several Chlamydomonas species. The psaA gene product of Chlamydomonas is encoded on three widely spaced exons, flanked by sequences characteristic of group II introns. The transcripts from the three exons require trans-splicing steps to produce the mature mRNA. The chloroplast tscA RNA is also necessary, maybe to complete the catalytic core of intron one and so promote efficient splicing (Rochaix, 1992). This trans-splicing of psaA mRNAs involves 14 nuclear loci (Goldschmidt-Clermont et al., 1991). The editing of RNA to produce a functional RNA molecule could play a direct role in regulation of chloroplast gene expression. RNA editing factors may also be
nuclear-encoded and their absence could prevent correct chloroplast gene expression. RNA editing has been described in the chloroplasts of higher plants, but has not yet been demonstrated in the *Chlamydomonas* chloroplast (Gray and Covello, 1993). In some instances, processing of RNA has been shown to effect chloroplast gene expression directly. For example, the methyl-jasmonate induced reduction in *rbcL* (Rubisco large subunit) mRNA translation. Altered processing that produces an *rbcL* transcript with a large 5'-untranslated region is responsible for a reduction in translation levels (Mayfield *et al.*, 1995).

(III) mRNA Stability: Direct measurement of mRNA stability has shown that chloroplast mRNAs have dramatically different lifetimes, depending on the developmental stage of the chloroplast, chloroplast type, and growth conditions. Most chloroplast encoded monocistronic and polycistronic mRNAs contain inverted repeats in their 3'-untranslated regions. Inverted repeats have the potential to form stem-loop structures. These stem loop structures may have a role to play in RNA processing, or as protective devices against nucleolytic degradation, by impeding processive 3'-5' (or 5'-3') exonuclease activity, thereby conferring mRNA stability. Deletions into the 3'-inverted repeat of the *atpB* coding region of *C. reinhardtii* show that although a complete inverted repeat is not required for accumulation of *atpB* mRNA, larger deletions eventually result in a reduction of *atpB* transcript (Stern *et al.*, 1991). Some 5'-untranslated regions also have sequences that have the potential to form stem loops, which may have a role to play in mRNA stability (Mayfield *et al.*, 1995). Deletions into the *petD* 5'-untranslated region show the entire region is required for transcript stability *in vivo* (Sakamoto *et al.*, 1994a).

1.5 The Photosynthetic Components Of The Chloroplast.

The light reactions of photosynthesis occur in the thylakoid membranes of chloroplasts. Within the thylakoids are contained three large, transmembrane, multi subunit complexes. These complexes carry out the electron transfer reactions of photosynthesis. The
complexes are: Photosystem II (PSII), Cytochrome $b_{6}/f$ (Cyt $b_{6}/f$), and Photosystem I (PSI). The relative stoichiometries of the complexes are thought to be highly regulated, although amounts can vary between species (Anderson, 1992). A fourth complex is also present in the thylakoids, in close proximity to PSII, Cyt $b_{6}/f$ and PSI. This is the ATP synthase complex, which utilises the proton gradient generated by the photosynthetic complexes to produce ATP. The location of these complexes is highly defined. PSI and the ATP synthase complexes are mainly located in the stroma exposed regions of the thylakoids, while PSII complexes are found mainly in the stacked (appressed) regions. The Cyt $b_{6}/f$ complexes are distributed throughout the thylakoids (see figure 1.2).

1.5.1 Photosystem II.

PSII is a multi subunit complex that is evolutionarily linked to the Type II bacterial reaction centres. It carries out the light catalysed oxidation of $H_{2}O$, and the reduction of a bound quinone, and is found in the appressed regions of the thylakoids (see figure 1.2). The photosystem is composed of both integral membrane proteins and extrinsic proteins. The core complex of PSII includes all the proteins, pigments and cofactors required for the light-driven transfer of electrons from $H_{2}O$ to oxidised plastoquinone. It contains three chlorophyll-protein complexes: the reaction centre, where initial charge separation occurs and two internal chlorophyll $a$ light harvesting antennae, CP47 and CP43.

CP47 and CP43 are the internal light harvesting proteins of PSII. They are encoded for by the $psbB$ and $psbC$ chloroplast genes. Each polypeptide binds 20 chlorophyll $a$, and 4-5 β-carotene molecules. As well as functioning as antennae, these proteins may also contribute to the protein environment of the water-splitting mechanism.

The reaction centre of PSII (see figure 1.3) contains 4-6 Chl $a$ molecules, two pheophytins, and two quinones, bound by a pair of hydrophobic proteins D1 and D2. These are the two major proteins of PSII and are encoded by the chloroplast genes $psbA$ and
Figure 1.3. Diagramatic representation of the PSII reaction centre.
Figure 1.4. Diagram showing the main cofactors of PSII and the pathway of electrons through the reaction centre. Electron flow occurs from water to Qb. Water donates electrons to P680* via YZ (D1Tyr161). The water oxidising complex involves Mn, possibly Ca2+ and Cl cofactors, oxygen evolution requiring 4 turnovers of the reaction centre. YD (D2Tyr161), chlorophyll Z (Chl Z), and cytochrome b559 (Cytb559) are alternative electron donors, under suitable conditions. A non-haem iron is located between QA and QB (both these are plastoquinone molecules). Bicarbonate also binds in this region. QB picks up 2 electrons and 2 protons and transfers these to the membrane plastoquinone (PQ) pool (Redrawn from Nugent (1996)).
psbD genes, respectively. In addition to these components, PSII reaction centres also contain three other proteins: PsbI and PsbW, and Cyt b_{559}, none of which are thought to bind chlorophyll. Cyt b_{559} is composed of alpha and beta subunits, coded for by psbE and psbF genes. These subunits are linked by a haem, and play an important role in the stabilization of PSII complexes (Rochaix, 1992). They may do this by acting as a photoprotective agent. In addition, Cyt b_{559} is thought to be involved in cyclic electron flow around PSII, because it has been shown to be photooxidised by both P680* and photoreduced by plastoquinone (Evans and Nugent, 1993). Other subunits of the PSII reaction centre include PsbH, and PsbJ, PsbK, PsbL, PsbM and PsbN proteins (de Vitry et al., 1991). The functions of these small, integral membrane proteins has not yet been determined, although it has been suggested that PsbL, PsbM and PsbN may be associated with the oxygen-evolving complex (Erickson and Rochaix, 1992). The primary electron donor of PSII, two core chlorophyll a molecules, absorb maximally at 680nm, and so are known as P680.

After light absorption, an electron is transferred from the excited state P680* to a pheophytin, which in turn reduces a bound plastoquinone, called Q_A. This first plastoquinone in turn reduces a second plastoquinone (Q_B). PSII is also associated with a H_2O splitting complex. On the donor side of PSII, the chlorophyll cation radical P680' oxidises a tyrosine residue, Z, which in turn oxidises a cluster of four Mn atoms, which form the catalytic site of H_2O oxidation. The Mn cluster is able to accumulate the four oxidising equivalents that are necessary to release O_2 from two molecules of H_2O (see figure 1.4).

The oxygen-evolving complex (OEC) of PSII is located on the luminal side of the thylakoids, and consists of three peripheral proteins, OEE1, OEE2 and OEE3. These proteins are nuclear encoded by the psbO, psbP and psbQ genes, respectively. Another protein, PsbR, is believed to be one of 3 regulatory OEC components, perhaps anchoring the OEE proteins to the PSII core, and regulating their function (Erickson and Rochaix, 1992). OEE1 is thought to stabilize the redox active tyrosine, Z, which is Tyr161 of the D1
protein, which becomes oxidised by P680\(^+\), and in turn oxidises a cluster of Mn ions. A second redox active residue, Tyr 160, on the D2 protein has been identified, but does not appear to play an essential role in electron transfer (Evans and Nugent, 1993). Four Mn ions are associated with the OEC, and become converted to higher oxidising states, by four separate photochemical events involving P680\(^+\) and Z. The end result is that two \(\text{H}_2\text{O}\) molecules are ‘split’, releasing four electrons, four protons, and one molecule of \(\text{O}_2\). Oxygen is released, the electrons are donated to reduce P680\(^+\), and the protons used to generate the proton motive force to drive ATP synthesis.

1.5.2 Cytochrome \(b/f\) Complex.

This complex occupies a central position in photosynthetic electron transport, and proton translocation, by linking PSII to PSI in linear electron flow from \(\text{H}_2\text{O}\) to NADP\(^+\), and around PSI in cyclic electron flow (Anderson, 1992). Cyt \(b/f\) complexes are found throughout the thylakoids, unlike PSI and PSII. There are four major proteins, making up this complex (see figure 1.5). These are Cyt \(f\) (petA gene product), Cyt \(b_6\) (petB gene product), Rieske [2Fe-2S] protein (petC gene product), and a protein called subunit IV. These are all chloroplast encoded, except the Rieske protein, which is nuclear encoded. Several smaller subunits have also been characterised recently, coded for by the petG chloroplast gene (Schmidt and Malkin, 1993), petM nuclear gene (de Vitry et al., 1996), and the petL gene (Pierre et al., 1995).

The Cyt \(b/f\) complex accepts electrons from the plastoquinone pool on the acceptor side of PSII. The electrons are then donated to PSI, via a plastocyanin molecule. During cyclic electron flow the Cyt \(b/f\) complex accepts electrons from ferredoxin, and donates them to plastocyanin. This results in the creation of a transmembrane electrochemical charge and a gradient of protons, which is then used for ATP synthesis.

The redox state of Cyt \(b/f\) controls the activity of an enzyme, LHCII kinase. This enzyme catalyses the phosphorylation or dephosphorylation of the mobile, peripheral Chl \(a/b\)
Figure 1.5. Proposed arrangement of the polypeptides of the cytochrome $b_{6}f$ complex. The structure and function of the complex is further discussed in section 1.5.2. The binding of FNR, as indicated in the diagram, is under debate.
proteins of the PSII light-harvesting complex (LHCII). When PSII becomes overexcited (relative to PSI), the redox state of Cyt b_{6/f} activates LHCII kinase. Detachment of the phosphorylated LHCII proteins then occurs. The proteins become laterally distributed from appressed to the non-appressed regions of the thylakoids. This favours cyclic photophosphorylation, and thus increases the supply of ATP. When PSI becomes overexcited (relative to PSII), LHCII kinase is deactivated. Dephosphorylation of LHCII proteins then occurs, which cause them to migrate back to the appressed region (Hope, 1993). The Cyt b_{6/f} complex is thus very important in photosynthesis because it not only connects PSII to PSI, but also ensures that light energy is distributed evenly across both photosystems.

1.5.3 ATP Synthase Complex.
The chloroplast ATP synthase (CF_{1}-CF_{0}) is a reversible proton translocating ATPase that catalyses ATP synthesis at the expense of the energy stored in the electrochemical proton gradient across the thylakoid membrane (see figure 1.6). The complex is composed of an intrinsic proton translocation subcomplex CF_{0}, and an extrinsic catalytic subcomplex, CF_{1}. Photosynthetic electron transport generates a proton gradient across the thylakoid membrane. The resulting proton flux through CF_{0} activates the plastidal ATP-synthases and induces the ATP-formation in the catalytic site of CF_{1}. The complex is located predominantly in the non-appressed stromal thylakoids.

CF_{1}, when isolated, is a peripheral, hydrophilic protein that is capable of ATP hydrolysis, but incapable of net ATP synthesis (Ponomarenko et al., 1995). It is composed of five polypeptides α-ε. Three copies of α and β subunits are present in the complex, but only one copy of the other subunits (McCarty and Hightower, 1995). Every α and β subunit contains a nucleotide binding site: three catalytic nucleotide binding sites on the β subunits, and three non catalytic sites on the α subunits (Possmayer et al., 1995). The other, smaller subunits are involved in thylakoid binding (δ), and regulation of membrane bound ATP synthesis and soluble ATP hydrolysis (γ and ε). CF_{1} also requires
Figure 1.6. Schematic representation of the ATP Synthase Complex. The structure and function of this complex is discussed in section 1.5.3 (Figure redrawn from Schumann and Bickel-Sandkotter (1995)).
a divalent metal cofactor for synthesis and hydrolysis of ATP. This is believed to be $\text{Mg}^{2+}$. A total of six metal binding sites are thought to exist on $\text{CF}_1$.

The $\text{CF}_0$ part of the synthase is a hydrophobic, integral protein that contains the proton binding sites. $\text{CF}_0$ is responsible for proton translocation, and consists of four subunits: I, II, III, and IV. Twelve copies of subunit III are present (the other subunits are present as 1 copy). The genes encoding the subunits of this complex are located in both the chloroplast and the nuclear genome. Five of the $\text{CF}_1$-$\text{CF}_0$ subunits are encoded for by the chloroplast. These are subunits I and II, and subunits $\alpha$, $\beta$, and $\epsilon$. Subunit II of $\text{CF}_0$, and subunits $\gamma$ and $\delta$ of $\text{CF}_1$ are nuclear encoded (Fiedler et al., 1995).

1.6 Photosystem I.

PSI is a large, multi-subunit protein-chlorophyll complex. It is found embedded in the non-appressed regions of the thylakoid membranes. In plants, PSI associates with multiple membrane-embedded LHCIs, which serve as accessory antennas to harvest light and funnel its energy to the PSI reaction centre. The polypeptides and cofactors of PSI absorb photons and use their energy to cause primary photochemical charge separation. These charges are then stabilised by the spatial displacement of an electron through a series of electron transfer centres, ending with the reduction of ferredoxin (Chitnis et al., 1995). PSI can therefore be said to catalyse the light induced transfer of electrons from plastocyanin and cytochrome $c_6$ to ferredoxin (Chitnis, 1996; Fromme, 1996). The reduced ferredoxin is used by ferredoxin-NADP$^+$ oxidoreductase to produce NADPH, which is used in various metabolic reactions of the chloroplast (Chitnis, 1996). PSI also participates in cyclic electron transport.

PSI can be isolated as monomers (approximately 340kDa) or as trimers. Each monomer of PSI (see figure 1.7) comprises at least 11 polypeptides in cyanobacteria and 13 in chloroplasts, as well as approximately 100 Chl $a$ molecules, 20 carotenoids 3 [4Fe-4S] centres, and 2 phylloquinones (Golbeck and Bryant, 1991; Chitnis, 1996; Fromme,
Figure 1.7. Diagramatic representation of PSI (figure redrawn from Bryant (1992)).
1.6.1 Electron Transport In PSI.

The PSI complex absorbs photons, and uses their energy to catalyse the photooxidation of plastocyanin and the photoreduction of ferredoxin, a [2Fe-2S] protein (see figure 1.8). Chl a molecules of light harvesting complex I (LHCI) and of the PSI antenna complex absorb photons. The excitation energy is transferred to the primary electron donor, P700. P700 is so known because it absorbs maximally at this wavelength. It is composed of a dimer of Chl a molecules. Electronic excitation of P700 changes its midpoint potential from +0.43V to approximately -1.3V. This then means that the excited form of P700+ can donate an electron to the electron acceptor Aao, which is a Chl a monomer. P700+ is then rereduced by plastocyanin. On the acceptor side, the electron is rapidly transferred through a series of electron donors and acceptors. Aao transfers the electron to one of the two phylloquinone molecules of PSI, A1. The next intermediate electron acceptor is Fx, a [4Fe-4S] iron-sulphur centre. This iron-sulphur centre is coordinated by two polypeptides of PSI, PsaA and PsaB. Fx then transfers the electron to the terminal electron acceptors, FA and FB. These are also [4Fe-4S] iron-sulphur centres. They are coordinated by the PSI protein, PsaC. The exact path of electron transport through FA and FB is not known, and this is further discussed in section 1.6.4 (I). From FA/FB, the electron is finally used to reduce soluble ferredoxin.

1.6.2 The Polypeptide Composition of PSI.

PSI is composed of at least fourteen polypeptides. Most of the proteins making up PSI are relatively small in size (less than 20 kDa), and only three are known to bind cofactors. The subunits are encoded by both the chloroplast and nuclear genomes. They are believed to be synthesised using membrane-bound ribosomes. It is likely that membrane insertion occurs cotranslationally, as does the incorporation of cofactors such as Chl a. The absence of certain cofactors and polypeptides causes the PSI complex to destabilise. Any nuclear encoded subunits have to be imported into the chloroplast, after synthesis on cytoplasmic
Figure 1.8. Diagram showing the main cofactors of PSI and the pathway of electrons through the reaction centre. Absorption of light energy by P700 leads to a photochemical charge separation. Electron flow from plastocyanin to ferredoxin. Electron transfer from P700 occurs across the thylakoid membrane to reduce the iron-sulphur centres $F_X$, $F_A$ and $F_B$.

P700, Primary electron acceptor; $P700^+$, excited state of P700; $A_o$, chlorophyll electron acceptor; $A_1$, Phylloquinone (Redrawn from Nugent (1996)).
ribosomes. Once in the chloroplast, final processing of the polypeptide occurs, before it is positioned into the complex (see figure 1.7 for polypeptide positions of PSI).

1.6.2.1 The Nuclear Encoded Subunits of PSI.

(I) PsaD

PsaD is approximately 17-20kDa in size, depending on the species. It is structurally conserved in higher plants, algae and cyanobacteria (Golbeck and Bryant, 1991). Hydropathy analysis of the amino acid sequence indicates that it is a highly hydrophilic protein, unlikely to have any membrane spanning regions. It is stromally exposed, and has been identified, together with PsaC and PsaE, as forming a ‘cap’ of proteins on the stromal surface, in the structure of PSI (Krauss et al., 1993).

The major function of PsaD is to act as docking protein for the soluble [2Fe-2S] ferredoxin. Nearest neighbour analyses using a zero length cross linker (EDC) found that ferredoxin from *Synechococcus* sp. PCC7002 cross links to PsaD from *Synechococcus* sp. PCC6301 (Wynn et al., 1989). Inactivation mutants of PsaD in *Synechocystis* sp. PCC 6803 were found to grow slowly under photoautotropic conditions. These mutants showed inhibition of ferredoxin-mediated NADP⁺ photoreduction. Addition of PsaD led to recovery of approximately 25% of the wild type rates (Golbeck, 1994). The reason that PsaD may be able to act as a docking protein for ferredoxin is probably connected to its overall charge. The overall net charge of the PsaD protein is positive, due to the large number of charged lysine and arginine residues in the centre portion of the protein. This may help in binding ferredoxin, which is negatively charged.

One of the other functions of PsaD is to bind PsaC. In its absence, PsaC binds loosely to PSI (Li et al., 1991). This loose binding of PsaC would then make possible a range of docking orientations, each of which would place either Fₐ or Fₜ in proximity with the preceding electron donor. The addition of PsaD may serve to orientate PsaC to the preferred orientation, resulting in a stable PSI complex (Golbeck, 1992).
(II) PsaE

PsaE is highly conserved, 8-10 kDa protein, which is approximately 73% identical in sequence between species (Golbeck and Bryant, 1991). It can be cross linked to PsaD and PsaC, and forms a protruding mass on the stromal surface of the thylakoids with these proteins (Cohen et al., 1993).

The protein stimulates the reduction of soluble electron acceptors, such as ferredoxin or flavodoxin (Golbeck, 1994). It has been shown that the rate of ferredoxin-mediated NADP$^+$ photoreduction declines in spinach PSI, as PsaE is removed with chaotropic agents. When no PsaE is present in the PSI, the rate of NADP$^+$ photoreduction is reduced by about half. Addition of PsaE results in recovery of approximately half the missing rate (Weber et al., 1993). When PsaE is added to PsaE depleted PSI complexes from Synechococcus sp. the interaction between F$_a$/F$_b$ and ferredoxin was restored after a single turnover flash (Sonoike et al., 1993). Loss of PsaE leads to loss of NADP$^+$ photoreduction (Lagoutte and Vallon, 1993). An antibody raised against PsaE was also found to inhibit electron transport to NADP$^+$ (Weber and Strotman, 1993; Sonoike et al., 1993). Presumably, the binding of the antibody sterically hinders the access of ferredoxin to either F$_a$/F$_b$ centres or the catalytic site of ferredoxin-NADP$^+$ oxidoreductase. Rousseau et al. also showed that PSI complexes lacking PsaE (due to deletion mutagenesis) had a 25-fold slower rate of ferredoxin reduction, relative to wild type, after a saturating flash. When PsaE was added to the deficient complexes, the original rate was recovered. These results indicate that PsaE facilitates the interaction between F$_a$/F$_b$ and ferredoxin. It may promote the interaction between PsaC and the soluble ferredoxin, thereby enhancing photoreduction rates of ferredoxin (Falzone et al., 1994).

PsaE is also thought to be the docking protein that binds ferredoxin-NADP$^+$ oxidoreductase. Cross linking studies show that PsaE and ferredoxin-NADP$^+$ oxidoreductase are located close together. PsaE probably ensures that ferredoxin-NADP$^+$ oxidoreductase is in the correct orientation so that electron transfer from ferredoxin to
NADP⁺ is efficient (Anderson et al., 1992; Rousseau et al., 1993). However, this cannot be the main role of PsaE. The protein has been inactivated by transposen mutagenesis in Synechococcus sp. 7002 (Zhao et al., 1993). Photoautotrophic growth, oxygen evolution, and non-cyclic electron transport were not affected in these mutants. However, Synechococcus sp. PCC 7002 mutants where PsaE was inactivated were unable to grow photoheterotropically, and exhibited impaired growth characteristics at low light and low CO₂ levels (Zhao et al., 1993). In conditions such as these, cyclic electron transport is very important. During cyclic electron transport, electrons from the reducing side of the PSI complex are returned to P700⁺ via the Cyt b₆/f complex by a cyclic mechanism. This cyclic flow of electrons allows photosynthesis to occur from energy stored in the ATP produced by the photochemical gradient that is established (Fork and Herbert, 1993; Falzone et al., 1994; Mannan et al., 1994). It is likely that PsaE is involved in cyclic electron transport. This suggestion is confirmed by electron transport studies in intact cyanobacterial cells (Yu et al., 1993). PsaE therefore has several roles to play. It facilities the interaction between F₆/F₈ and ferredoxin, and is involved in the binding of ferredoxin-NADP⁺ oxidoreductase. It also has a role to play mediating cyclic electron transport in PSI, which becomes important at low light or CO₂ levels.

(III) PsaF

The PsaF protein is approximately 17kDa in size. Some controversy exists as to its exact position and function in PSI. It appears to be the docking protein for plastocyanin, and as such, is thought to be located on the lumenal side of the thylakoid membrane (Farah et al., 1995). However, the amino acid sequence has regions of hydrophobicity. If the resistance of PsaF to chaotropic extraction is also taken into consideration, both facts suggest that PsaF may be an integral membrane protein (Chitnis et al., 1995; Nugent 1996). The function of PsaF is also unclear. Crosslinking experiments have proposed that the protein functions as the plastocyanin or Cyt c₆ docking site on the oxidising side of PSI (Chitnis et al., 1995; Farah et al., 1995). The amino acid sequence has regions which are highly conserved between species, and shows a protein with many positive charges. By analogy
to PsaD, its function could be to counteract the negative charge repulsion between plastocyanin and the PSI complex (Scheller and Möller, 1990), and to bind plastocyanin in the correct orientation for electron transfer to P700 (Hatanaka *et al.*, 1993). However, deletion of *psaF* in *Synechocystis* sp. PCC6803 did not affect the ability of mutants to grow photoautotrophically. Neither did the mutants show any deficiencies in Cyt c6 dependent NADP" photoreduction or P700" rereduction kinetics (Chitnis *et al.*, 1995; Farah *et al.*, 1995). The exact function of this protein is therefore unresolved.

(IV) PsaG

*PsaG* is a small protein of about 10kDa. This protein has only been detected in eukaryotic PSIs. Although hydropathy analyses has suggested that it is not an intrinsic membrane protein, its exact location within PSI is unknown (Golbeck and Bryant, 1991).

The function of *PsaG* is unknown. There is low amino acid homology between different species (Golbeck and Bryant, 1991; Chitnis *et al.*, 1995). However, it does show some sequence homology to *PsaK*. It has been suggested that it may have a role to play in the interaction of PSI with LHCl (Chitnis *et al.*, 1995).

(V) PsaH

Like *PsaG*, *PsaH* has only been detected in green algae and higher plants. It is a small subunit, of about 10-11kDa. The amino acid sequence is polar overall, and it is therefore believed to be an extrinsic subunit on the stromal side of PSI (Golbeck and Bryant, 1991; Chitnis *et al.*, 1995). Although its exact function is unknown, its absence from the cyanobacterial PSI suggests that it may perform a role in the light harvesting antenna complex, which differs considerably in the cyanobacteria. This role may be the mediation of binding of the LHCl antenna complex to the PSI core complex. The different organisation of the antenna system of cyanobacteria could explain its apparent absence there (Scheller and Möller, 1990).
(VI) PsaK

PsaK is an integral membrane protein of about 8.4kDa. Cross linking studies place its location near to PsaA and PsaB (Fromme, 1996). Comparisons with the primary sequences of PsaK and PsaG show a significant similarity, perhaps indicating that an ancestral gene was duplicated in a chloroplast progenitor, which resulted in the evolution of PsaK and PsaG in eukaryotes but remained as single gene in cyanobacteria (Chitnis et al., 1995). The function of this protein is unknown.

(VII) PsaL

This is an integral membrane protein, 16-17 kDa in size. PsaL is not required for photosynthetic growth. Mutants of *Synechococcus* sp. PCC7002 lacking PsaL are able to grow as per wild type cells (Schluchter et al, 1996). PsaL cross links to PsaD, and there is a close association between them. In mutants of *Synechocystis* sp. PCC6803 that lack PsaL, the susceptibility of PsaD to chaotropic removal is increased (Xu et al, 1994). A structural interaction between PsaD and PsaL may exist that plays a role in their association with PSI. PsaL is also closely associated with PsaI. Absence of PsaI results in vastly reduced amounts of PsaL. In mutants lacking PsaL (or those that have reduced amounts of PsaL), PSI trimers do not form, and only monomers can be isolated (Xu et al, 1994; Xu et al, 1995; Schluchter et al, 1996). It is clear that PsaL is required for the formation of PSI trimers. It has been suggested that it is a component of the connecting domain that links the 3 monomers to form a trimer (Chitnis et al, 1995).

(VIII) PsaN

PsaN is a protein about 9.5kDa in size. It has not yet been detected in cyanobacteria. It is an extrinsic luminal protein, bound to the thylakoid by electrostatic interaction (Chitnis et al, 1995; Knoetzel and Simpson, 1993). Although its function is unknown, it has been speculated that it may function in mediating binding of antenna complexes to the PSI reaction centre and core complex (Knoetzel and Simpson, 1993).
1.6.2.2 The Minor Chloroplast Encoded Polypeptides of PSI.

(I) Psal

Psal is a protein of approximately 4 kDa. As the protein is very hydropathic, and resistant to extraction by chaotropic agents, it is probably an integral membrane protein (Chitnis et al., 1995; Golbeck and Bryant, 1991). Examination of the amino acid sequence of Psal of several species reveals that there is a high degree of amino acid conservation between species (Ikeuchi et al., 1991).

Psal inactivation mutants have been produced (Xu et al., 1995; Nakamoto et al., 1995). These mutants were found to grow photoautotrophically as well as wild type cells. Comparison of the thylakoids of both wild type and mutants cells showed that they did not differ in the amount of P700 present and in the characteristics of electron transfer from P700 to F_A/F_B (Nakamoto et al., 1995). It is therefore likely that Psal is dispensable, and not necessary for photosynthetic growth. However, the mutant cells were found to contain less chlorophyll, and as much as an 80% reduction in the levels of Psal. No reduction in the levels of other subunits of PSI was observed (Xu et al., 1995). It is probable that a structural interaction between Psal and PsaL may exist, which serves to stabilise the association of Psal with PSI.

(II) PsaM

PsaM is a chloroplast encoded protein of about 3kDa. The protein has a region of hydrophobicity, flanked by regions of hydrophilicity. It also cannot be removed by chaotropic extraction, and so is predicted to be an integral membrane protein (Chitnis et al., 1995; Fromme 1996). PsaM is absent from Psal-less mutants of Synechococcus sp. PCC7002, so it is probably stabilised by Psal. (Schluchter et al., 1996). PsaM has not yet been detected in higher plant PSI preparations (Fromme, 1996). However, in the liverwort chloroplast genome, an open reading frame is present for a PsaM-like protein (Ohyama et al., 1994). This has not been found in chloroplast genomes of rice and tobacco (Hiratsuka et al., 1989; Shinozaki et al., 1986). The function of this protein is unknown.
(III) PsaJ

PsaJ is small, hydrophobic protein, about 4.5 kDa in size. The exact role of PsaJ is unknown. PsaJ has been identified in PSI complexes from cyanobacteria (Xu et al., 1994) and higher plants (Ikeuchi et al., 1990). The polypeptide is predicted to have one transmembrane α-helix, consisting of a highly conserved sequence of 22 amino acids. The stromally exposed N-terminal is very short (four to five amino acids long), and hydrophilic. The C-terminal, however, is longer (>15 amino acids), and has an overall negative charge (Golbeck, 1992). The psaJ gene is located downstream from the psaF gene. They are both cotranscribed in cyanobacteria as a bicistronic message (Xu et al., 1993). A double deletion mutant of PsaF-PsaJ has been produced in Synechocystis sp. PCC6803. Although the mutant had the same phenotype as wild type cells, it was observed that the accessibility of PsaE for chaotropic agents was increased (Xu et al., 1994). It is possible that interactions may exist between PsaF, PsaJ and PsaE. Deletion of PsaJ has also been shown to lead to a reduction in the steady state RNA levels of psaF. The amount of PsaF in the membranes of the mutant was also reduced. Other polypeptides of PSI were found to be present at wild type levels in the absence of PsaJ. In PsaJ deletion mutant, PsaF is also more easily removed (Xu et al., 1994). The role of PsaJ may therefore be to stabilise the shorter, less hydrophobic PsaF.

1.6.2.3 The Major Chloroplast Encoded Polypeptides of PSI.

(I) PsaC

PsaC is a relatively small protein, of about 9 kDa. It is highly conserved between different species, and the differences that are present are conservative (Andersson and Franzén, 1992). It is coordinated by PsaD, as well as by PsaA and PsaB. PsaC shows a high degree of sequence homology to soluble ferredoxins from bacteria, particularly to the Peptococcus aerogenes ferredoxin (Dunn and Gray, 1988; Golbeck, 1994). The only significant differences between the Peptococcus aerogenes ferredoxin and PsaC is that PsaC contains eight extra amino acids at the PsaC-terminal end of the protein, and an extra ten amino acids between the two iron-sulphur binding sites. They presumably enlarge the internal
loop structure as the polypeptide chain folds back on itself. Ferredoxins are soluble, whilst PsaC is a membrane bound protein. These extra amino acids may be responsible for the binding of PsaC to the P700-Fx core (Naver et al., 1996). Deletion mutants of PsaC were created where the extra amino acids were deleted. The $F_A/F_B$ back reaction was less efficiently restored than in wild type cells, when PSI complexes were reconstituted with the modified PsaC. Moreover, the loop-deleted PsaC was unable to bind to the PSI core in the absence of PsaD (Naver et al., 1996). These results show that the binding properties of PsaC were altered, and that the extra amino acids do have a role to play in the binding of PsaC.

PsaC coordinates the iron-sulphur centres $F_A$ and $F_B$. These function as the tertiary electron acceptors, moving electrons out of the hydrophobic reaction centre core and into the hydrophilic stromal phase, further stabilising charge separation, and permitting soluble ferredoxin to be reduced with a high quantum efficiency (Yu et al., 1993; Golbeck, 1994). The participation of $F_A$ and $F_B$ in electron transfer within PSI was demonstrated in cyanobacterial PSI complexes where PsaC, PsaD, and PsaE were removed. In these complexes, NADP$^+$ photoreduction was lost. Photoreduction was observed again when PsaC and PsaD were rebound to the PSI core (Golbeck, 1994).

$F_A$ and $F_B$ are [4Fe-4S] iron-sulphur centres. These type of iron-sulphur centres are coordinated by four cysteine residues. In PsaC, nine conserved cysteine residues are present in the protein. Of these nine, eight are in the [4Fe-4S] iron-sulphur centre binding configuration $CxxCxxCxxxCP$. The Peptococcus aerogenes ferredoxin, which also binds 2 [4Fe-4S] centres, also has this [4Fe-4S] binding motif. In this protein, the first three cysteines of the motif provide three of the four ligands to the [4Fe-4S] iron-sulphur centre. The fourth cysteine of the motif provides the final ligand to the other [4Fe-4S] iron-sulphur centre. This geometry of the protein means that the two half chains do not form separate globular iron-sulphur domains. Each half chain cooperates in the same manner to form an approximately diad axis. The protein can be visualized as a dimer of two
connected polypeptides, containing shared [4Fe-4S] iron-sulphur clusters (Golbeck, 1994). PsaC probably exhibits similar folding patterns overall. However, the symmetry of the PsaC protein and the binding patterns of F_A and F_B, means that the symmetry of the protein is imperfect. The two iron-sulphur centres therefore have slightly different spectroscopic and redox properties.

The pathway of electron transfer through PsaC is unknown. An option of linear and branched pathways exists. If the choice is made based solely on the equilibrium midpoints of F_A (-530mV) and F_B (-580mV), a serial, linear pathway of electron flow is favoured, from F_X to F_B to F_A. This is supported by the observation that, after selective chemical inactivation of F_B, photoreduction of F_A is inhibited at low temperatures (Malkin et al., 1984). However, evidence exists that indicates that electron transfer to F_A via F_B is not obligatory. It has been observed that when F_B is destroyed by HgCl_2, an eight fold decrease in the rate of NADP^+ photoreduction is seen (He and Malkin, 1994). Mutants have been created where one cysteine ligand for each iron-sulphur centre has been changed to an aspartate residue. In Anabaena variabilis ATCC 29413, the changes were C13D (this cysteine binds F_B) and C50D (this cysteine binds F_A) (Mannan et al., 1996). In Synechocystis, the changes were C14D (this cysteine binds F_B) and C51D (this cysteine binds F_A) (Zhao et al., 1992). In both species, the changes resulted in the appropriate iron-sulphur centre becoming dysfunctional. The other iron-sulphur centre associated with PsaC functioned normally. It was found that F_A could be reduced in the presence of a photochemically inactive F_B, and that no enhanced reduction of F_B was seen when F_A is rendered non functional. This data shows that F_B is not essential for electron transfer from PSI to ferredoxin. The question of the path of electron transfer through PsaC has therefore still to be resolved.

(II) The PSI Heterodimer
The core of PSI is composed of two proteins, PsaA and PsaB. These proteins form the catalytic core of PSI. They bind most of the 100 Chl α molecules that PSI possess, as well
as 12-15 β-carotene molecules (these have a role to play in light harvesting). PsaA and PsaB are both similar in size (83kDa) and are homologous to each other. Both proteins have been demonstrated to be light inducible in maize (Fish et al., 1985). PsaA and PsaB are encoded by the chloroplast genes psaA and psaB. These two genes have been isolated and completely sequenced from maize (Fish et al., 1985), tobacco (Shinozaki et al., 1986), Liverwort (Ohyama et al., 1986), and Chlamydomonas reinhardtii (Kück et al., 1987), as well as several other plants and algae. In all cases, except Chlamydomonas reinhardtii, psaA and psaB are adjacent to one another, and cotranscribed. Analysis of their sequence shows that they are closely related, and the similarity between species is high: 95% similarity in higher plants, and 80% similarity between higher plants and cyanobacteria. PsaA and PsaB share an approximately 45% amino acid sequence similarity (Golbeck and Bryant, 1991; Fromme et al., 1996; Chitnis et al., 1995). The fact that these two proteins are so closely related appears to indicate that they probably arose from a gene duplication event of a single ancestral gene. They are believed to have evolved from the reaction centre protein of green sulphur bacteria and heliobacteriaia.

PsaA and PsaB are absolutely required for PSI assembly and function. PsaB has been inactivated in Synechocystis PCC.6803. PSI was completely disrupted in the mutant, while no effect was observed on PSII. While the expression of psaA was not found to be effected, the PsaA protein was not detected (Smart and McIntosh, 1993). Similarly, when expression of psaA was disrupted, PSI failed to assemble (Smart et al., 1991).

Examination of the amino acid sequence shows the presence of short, hydrophobic regions, 19-25 residues long (Chitnis et al., 1995; Cui et al., 1994). These hydrophobic regions have the ability to integrate with the thylakoid membrane. Both PsaA and PsaB are both speculated to have 11 transmembrane helices, connected by hydrophilic loops. The 4.5Å model of PSI from Synechococcus elongatus (Schubert et al., 1996) predicts 29 membrane spanning helices for PSI. Of these, 22 are symmetrically related, and thought to arise from PsaA and PsaB. The majority of the charged residues of the two proteins are
found in the predicted extramembrane loops. These charged residues may be involved in interactions with smaller subunits, and diffusible electron carriers (Chitnis et al., 1995). A large number of histidine residues are also found in the transmembrane helices, and are proposed to be involved in the coordination of Chl molecules (Fish et al., 1985). A large number of conserved aspartate and glutamine residues are also present, and are likely to be involved in the binding of antenna Chl a molecules.

An interesting feature is seen in the amino acid sequence surrounding helices VIII and IX of both PsaA and PsaB. A ‘leucine zipper’ motif, similar to those found in DNA-binding proteins, is found in this region, where leucine residues are repeated at every eight (at least) residues. This arrangement of amino acids results in the protrusion of the leucine residues out of the helix. Because this arrangement is found in both PsaA (four conserved leucines) and PsaB (five conserved leucines), the protruding leucines are able to act as a ‘zip’ (Kössel et al., 1990). In PSI, it is likely that the mechanism exists to dimerize PsaA and PsaB, both of which are highly hydrophobic membrane proteins (Golbeck, 1994). However, conservative changes in PsaB of two of the leucine residues (L522V and L536M) involved in the zipper formation in Synechocystis PCC6803 had no effect on the growth characteristics of the organism or light induced charge separation of P700 to F_a/F_b (Smart et al., 1993).

Perhaps the most important and interesting region found in PsaA and PsaB is the extra stromal region connecting helices VIII and IX. This region contains a set of amino acids that are absolutely conserved in every species examined so far, and is thought to be involved in the binding of F_X.

1.6.3 The F_X Binding Centre.

F_X is a [4Fe-4S] iron-sulphur centre. [4Fe-4S] iron-sulphur centres require four cysteine residues for coordination. Usually, these residues are found within one polypeptide, as is the case with PsaC and F_a/F_b. However, when the polypeptide sequences of both PsaA and
PsaB are examined, only three conserved cysteine residues are found in PsaA, and two conserved cysteine residues in PsaB. Neither protein appears able to coordinate $F_X$. Therefore, it is probable that both proteins are involved in the coordination of $F_X$, with $F_X$ forming an interpolypeptide ‘bridge’, linking the two proteins. It is likely that this arrangement causes the unusual spectral properties of $F_X$. However, it is possible that the unusual spectral properties are due to the ligands other than cysteine. For example, aspartic acid residues are able to serve as ligands to at least one of the iron atoms in some [4Fe-4S] ferredoxins (Canover et al., 1990). Cysteine ligands of the Reiske protein of the Cyt $b_6/f$ have been replaced with histidine residues, with no adverse effects (Britt et al., 1991). Thus, not only have aspartic acid residues been shown to act as ligands for iron-sulphur centres, but histidine residues have also been able to replace cysteine residues as ligands. The resolution of crystallised PSI complexes is not, as yet sufficiently clear enough to assign specific ligands for $F_X$.

The $F_X$ binding region is a very highly conserved sequence. The region surrounding the $F_X$ binding site is remarkably similar in bacteria, algae and higher plants. Moreover, the proposed $F_X$ binding motif has been found to be absolutely conserved in every single species examined thus far. In fact, it is also conserved absolutely in those organisms that have a Type I reaction centre as well (see figure 1.9). It is identical in both PsaA and PsaB, and has the following motif of amino acids:

Phe-Pro-Cys-Asp-Gly-Pro-Gly-Arg-Gly-Gly-Thr-Cys

This sequence of amino acids is located in the stromal exposed extramembrane loop that connects helices VIII and IX (Fish et al., 1985).

The two cysteines predicted to bind $F_X$ are found at either end of the motif. The other amino acids between them are predominantly small, hydrophobic entities. Conserved proline residues are also found, one adjacent to a cysteine ligand, and one in the centre
*Heliobacillus mobilis*
amino acids 413-449

K.S..........K......FPCLGPAYGGTC..S..DQ

*Chlorobium limicola*
amino acids 506-542

H.S..........K......FPCLGPVYGGTC..S..DQ

*Spinacea oleracea psaA*
amino acids 556-589

R.S..........K......FPDCGPGRRGGTC..S..DQ

*Spinacea oleracea psaB*
amino acids 542-575

R.S..........K......FPDCGPGGRGGTC..S..DA

$F_X$ Binding region

**Figure 1.9.** Simplified amino acid sequence alignment of C-terminal regions of PsaA and PsaB polypeptides with those of the related green sulphur bacteria and the heliobacteria.
of the region. Highly charged residues such as aspartic acid and arginine are also present. There is a preponderance of glycine residues (three glycines in an eight residue loop). This means that a wide variety of conformations can be generated in the interhelical loops, as glycine residues are very flexible. The proline residue in the centre of the region may allow the protein to fold back on itself, perhaps to place the cysteines in the correct position to ligate the iron atoms of Fx. Alternatively, the folding back of the protein may allow the positively charged arginine to participate in the binding of PsaC. The arginine residue occupies a central position in the interhelical loop. It is believed to be present at the apices of the loops, and is thought to bind the negatively charged PsaC (Weber et al., 1993). This arginine (in PsaB) has been changed to glutamic acid in Synechocystis and C. reinhardtii (Rodday et al., 1994; Rodday et al., 1995). The Synechocystis mutant assembled only 40% PSI, compared to wild type values, and was still weakly photosynthetic. However, in C. reinhardtii no PSI proteins were detected. The proline neighbouring this arginine was changed with no effect on PSI assembly (Weber et al., 1993). This implies that the effect of the arginine to glutamic acid change in C. reinhardtii may be due to a change in the side chain charge, rather a large disturbance in the secondary structure (Rodday et al., 1995). The results observed for the arginine to glutamic acid substitution are similar to those seen when psaC is insertionally inactivated in Synechocystis and C. reinhardtii. Takahasi et al. (1991) observed that PSI failed to assemble in the absence of PsaC, and that the other subunits rapidly turned over when psaC was inactivated. PsaC is critical for the assembly of PSI reaction centres in Chlamydomonas. Therefore, if the arginine residue is involved in the binding of PsaC, then substituting that residue with glutamic acid may result in an impaired interaction with PsaC, and cause destabilization of PSI.

When central proline was changed to leucine or alanine in C. reinhardtii, the mutants were found to assemble PSI, and grow photoautotrophically. However, the stability of the reaction centre was reduced, and the interaction with PsaC was affected. The PSI reaction centre in the proline to leucine mutant was more unstable (Rodday et al., 1994). This
seems to suggest that the isopropyl side chain in leucine may cause an unfavourable interaction, either due to increased hydrophobicity, or modification of the binding site conformation. In both the mutants $F_X$ was unaffected, and wild type PSI reconstitution kinetics were observed between $P700^+$ and $F_X^-$ and $F_A^+/F_B^-$.

Although the cysteine residues at either end of the motif are predicted to bind $F_X$, the possibility of other ligands within this region cannot be ruled out. However, experimental evidence does seem to indicate that these cysteine residues are important. Cysteine 560 of PsaB in *C. reinhardtii* was changed to a histidine, and the resulting mutant lacked the ability to assemble PSI reaction centres ([Weber *et al.*, 1993]). When the equivalent cysteine in *Synechocystis* sp. PCC6803 is changed to a serine, the resulting mutant was found to have an altered EPR spectrum of $F_X$ ([Smart *et al.*, 1993; Warren *et al.*, 1993]). This appears to indicate that this cysteine is required for correct PSI to assembly, and electron transfer through $F_X$. However, when proline 559, which is believed to provide an essential fold in the region, is altered, PSI accumulation is not affected. This is a rather surprising result, as this proline is conserved in all PSI-containing organisms, as well as type I prokaryotes ([Golbeck and Bryant, 1991; Trost *et al.*, 1992]). The other conserved cysteine in the PsaA motif, cysteine 575 in *C. reinhardtii*, has also been altered to histidine and aspartic acid ([Hallahan *et al.*, 1995]). PSI failed to assemble in the cysteine to aspartic acid mutant, while the cysteine to histidine mutant lacked an electron acceptor complex. These results show that both the conserved cysteines are important, and do have a role to play in the assembly of PSI, and coordination of iron-sulphur $F_X$.

The amino acids in this motif are remarkably conserved, and, as yet, most have no direct role assigned to them. Site directed mutagenesis provides a powerful tool for examining the possible roles that these amino acids may play in the coordination of $F_X$. Mutagenesis of these amino acids, and subsequent biochemical and biophysical studies on the mutant PSI complexes can be performed in the model organism, *Chlamydomonas reinhardtii*. 

66
1.7 The Model Organism.

Eukaryotic green algae provide powerful model systems for studying photosynthesis. Of the green algae, the unicellular *Chlamydomonas reinhardtii* (see figure 1.10) is a particularly useful organism for several reasons. It is a simple biflagellate organism, whose photosynthetic apparatus is closely related to higher plants. *C. reinhardtii* is found naturally in soil environments where it grows photoautotrophically. However, it is able to dispense with photosynthetic function and grow heterotrophically, if it is provided with a source of reduced carbon, such as acetate. This means photosynthetic mutations that are lethal in higher plants, which rely absolutely on photosynthesis, are viable in *Chlamydomonas* cells, thus allowing a genetic study of photosynthetic function. *C. reinhardtii* cells are amenable to molecular and biochemical analysis. They can be easily and inexpensively cultivated in the laboratory, and can be grown in large volumes. The life cycle is short, and the mean generation time is only about eight hours. The cell cycle can be synchronized easily, by a variety of light and dark regimes. Stocks of wild-type and characterised mutants are also available from world wide culture collections, for example Harris, (1984).

1.7.1 The Life cycle of *C. reinhardtii*.

*C. reinhardtii* cells are haploid and either of mating type plus (mt+) or mating type minus (mt-). These cells propagate vegetatively via mitosis. When starved of nitrogen, vegetative cells undergo gametogenesis. Mixing gametes of opposite mating types triggers a series of complex reactions that ultimately lead to the fusion of the gametes to form a zygote. Under appropriate environmental conditions, the resulting zygote can be induced to undergo meiosis, and produce a tetrad. Each tetrad consists of four haploid progeny (see figure 1.11 for life cycle). This simple mating process is easily induced in the laboratory by plating cells of opposite mating types onto media with depleted reduced nitrogen. Analysis of the progeny has shown that the nuclear genes are inherited according to Mendelian rules, while the chloroplast genes are inherited from the mt+ parent. The third genetic system in *C. reinhardtii* is the mitochondrial
Figure 1.10. Diagram showing the cell structure of *Chlamydomonas reinhardtii*. (Drawing by Keith Roberts)
Figure 1.11. Diagram showing the life cycle of *Chlamydomonas reinhardtii*. Normally, reproduction occurs asexually, but when cells are environmentally stressed, they produce gametes. Gametes from opposite mating types fuse to form a zygote, which is capable of meiosis, to produce 4 progeny cells (Figure adapted from Harris (1989)).
1.7.2 The Chloroplast Genome of *C. reinhardtii*.

*C. reinhardtii* has a single cup shaped chloroplast, which occupies approximately 40% of the cell. The chloroplast DNA, which is AT rich, consists of 196 kbp circular molecules, which are present at approximately 80 copies per cell. The DNA is organized into 8-10 DNA-protein complexes, called nucleoids.

The genome (figure 1.12) has been extensively mapped using restriction endonucleases and the fragments have been cloned into plasmid vectors thereby constructing a clone bank. Sequencing of the genome is still in progress and approximately 80 genes have been identified so far. Of these genes, 26 photosynthetic chloroplast genes have been identified, all of which show a high degree of similarity with the higher plant homologues. This is true for most of the genes in the genome. Variation is seen at the level of gene organisation, with algal genes showing considerable rearrangements of gene order relative to plant genomes. There is also less clustering of genes into multicistronic operons. The chloroplast genome encodes 18 tRNA genes, 14 ribosomal protein genes, 5 ribosomal RNA genes, and 6 genes encoding subunits of RNA polymerase (Rochaix, 1995). Three additional genes, *chlB*, *chlL* and *chlN*, are also present. These genes code for factors involved in chlorophyllide (the precursor of Chlorophyll *a*) synthesis, and are usually only found in species capable of chlorophyll synthesis in the dark. However, the genome lacks most of the 13 *ndh* genes, which have been suggested to be components of a chlororespiratory complex (Rochaix, 1995; Purton, 1995). In addition, there are several open reading frames (ORFs) in the *Chlamydomonas* genome that probably encode chloroplast proteins of unknown function. Homologues of some of these ORFs have been found in higher plants, while others are unique to *Chlamydomonas*. The complete sequence of the genome is near completion and will enable characterisation of these ORFs.
Figure 1.12. Map of *Chlamydomonas reinhardtii* chloroplast DNA, showing the locations of genes encoding proteins of the photosynthetic complexes. The circle shows the *Eco* RI restriction enzyme map, numbered according to the nomenclature of Harris (1989). (Diagram obtained from Webber *et al.*, 1995).
1.7.3 *psaA* in *C. reinhardtii*.

In higher plants, both *psaA* and *psaB* are continuous and adjacent to each other in the chloroplast genome (Fish *et al.*, 1985). In the chloroplast genome of *C. reinhardtii* however, PsaA is encoded for by three widely separated exons. The first exon is 30 codons in size, and located 50 kbp away from the second exon (60 codons), which, in turn, is 90 kbp away from exon 3, the largest of the three exons at 661 codons (Suguira, 1992). The exons are flanked by sequences that are characteristic of group II introns. Each exon is independently transcribed as a precursor, and the three RNAs are spliced *in trans* to form a single mature mRNA. In addition, the splicing of exons 1 and 2 requires the involvement of another RNA molecule (encoded for by *tscA*), that forms part of the discontinuous intron structure (Goldschmidt-Clermont *et al.*, 1991). The trans-splicing of these *psaA* mRNAs requires the involvement of fourteen nuclear loci (Goldschmidt-Clermont *et al.*, 1991). Exon 3 (*psaA*-3) contains the coding sequence for the conserved Fx binding region. This exon 3 can be isolated from the chloroplast genome wholly or partly for mutational analysis. After subsequent transformation, the altered *psaA*-3 exon integrates back into the genome in the correct position.

1.8 Chloroplast Transformation.

Manipulation of the chloroplast genome is a relatively recent innovation. One of the reasons that chloroplast transformation is difficult is that simply getting the DNA inside the chloroplast is a difficult task. To get to the chloroplast genome DNA has to pass through the cell wall, then the cell membrane, and then the double membrane of the chloroplast. Mature plant cells have many chloroplasts, each containing several hundred copies of chloroplast DNA. It would be difficult to transform all of these copies, in all of the chloroplasts, in all of the cells. In this respect, the fact that *Chlamydomonas* has a single chloroplast with fewer copies of the genome makes it preferable for *in vivo* analysis of the function and expression of chloroplast genes. However, higher plant genomes have been successfully transformed.

A transformation system, the biolistic technique developed by John Sanford at Cornell
University, has been adapted for *Chlamydomonas* chloroplast transformation (Boynton *et al.*, 1993). This method overcomes the major problem of organelle transformation; namely how to get enough DNA across the double membrane in order to transform each copy of chloroplast DNA. Transforming DNA is delivered into the chloroplast precipitated onto microparticles of gold or tungsten. The DNA integrates into the chloroplast genome by homologous recombination events, that result in native DNA being replaced with transformed DNA. Initially, a heteroplasmic state exists in the chloroplast, in which some copies of the genome carry the introduced DNA, and the rest remain unchanged. After several rounds of culturing the transformants from single cells under selective pressure, a homoplasmic state is achieved, in which all copies of the genome are altered. One of the major advantages of chloroplast transformation is that DNA can be targeted into the genome very specifically, because of the homologous recombination events. This technique was initially used to rescue a *C. reinhardtii* photosynthetic mutant with a deletion in the *atpB* chloroplast gene; transformation of the mutant cells with the cloned *atpB* gene restored photosynthetic function (Boynton *et al.*, 1988).

Dominant markers such as the *aadA* cassette are now available which allow selection for chloroplast gene modification, (Goldschmit-Clermont, 1991). This marker contains the sequence for the bacterial antibiotic resistance gene, aminoglycoside 3'adenyl transferase, conferring spectinomycin and streptomycin resistance, under the control of the *C. reinhardtii atpA* gene promoter. The cassette can be inserted into the cloned wild type gene, thereby causing a gene disruption. In addition, the cassette acts as a selective marker when co-transformed with chloroplast genes containing site-directed changes.
CHAPTER 2: MATERIALS AND METHODS.
CHAPTER 2: MATERIALS AND METHODS.

2.1 Chemicals, Reagents and Enzymes.
All the chemicals used were of the highest analytical grade available. Solutions and media were sterilised by autoclaving at 121°C for 20 minutes, with 15 lbs in⁻¹ pressure. All enzymes were purchased from New England Biolabs (Hitchin), Stratagene (Cambridge), Promega (Southampton) and Boeringer Mannheim (Lewis).[^5S-α 2'deoxyadenosine whose specific activity was >1000 ci/mmol at the activity date triphosphate (dATP), was purchased from Amersham International (Amersham).]

2.2 Growth and Storage of Bacterial Stocks.
The strains of *Escherichia coli* (*E. coli*) used were XL1 blue, JM109 and BL21 (DE3). The genotypes are described in Table 2.1 (Sambrook *et al*., 1989). Media used to culture *E. coli* are detailed in Table 2.2.

For long term storage, *E. coli* cultures were maintained as frozen glycerol stocks at -70°C. Glycerol stocks were prepared by mixing 900 µl of an overnight culture with 100 µl of sterile 80% glycerol and stored at -70°C.

In the short term, *E. coli* strains were maintained on 2YT or LB plates solidified with 2% (w/v) bactoagar containing the appropriate antibiotics. When required, a loopful of overnight culture or frozen glycerol stock was streaked onto a plate and incubated at 37°C overnight. These plates were stored at 4°C for up to 4 weeks.

2.3 Growth and maintenance of *C. reinhardtii* strains.
The strains of *Chlamydomonas reinhardtii* used were wild type strains CC1021 mating type plus (mt⁺) and WT12 mating type minus (mt⁻). *C. reinhardtii* cells were maintained on TAP or TM plates solidified with 2% agar (see table 2.3). This was supplemented with 50 µg ml⁻¹ spectinomycin, for all cell types other than wild type.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109</td>
<td>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac F[proAB lacI lacZΔM15Tn1O(tet)]</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>hsdS gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)</td>
</tr>
</tbody>
</table>

**Table 2.1. Bacterial Strain Genotypes**

<table>
<thead>
<tr>
<th></th>
<th>LB</th>
<th>2YT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bactotryptone</td>
<td>10g</td>
<td>16g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5g</td>
<td>10g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10g</td>
<td>5g</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>to 1 litre</td>
<td>to 1 litre</td>
</tr>
</tbody>
</table>

**Table 2.2. E. coli growth media.**

Media supplemented with the following antibiotics where necessary:

- Ampicillin (Amp) 50 mg/ml in ddH$_2$O
- Tetracycline (Tc) 15 mg/ml in 50% ethanol

Stock solutions were filter-sterilised using a leur lock syringe and a 0.45 μm filter and stored in aliquots at -20°C. Stock solutions diluted 1000x in media to achieve working concentrations.
<table>
<thead>
<tr>
<th></th>
<th>TAP Medium</th>
<th>TM Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>975 ml</td>
<td>924 ml</td>
</tr>
<tr>
<td>Tris</td>
<td>2.42 g</td>
<td>-</td>
</tr>
<tr>
<td>4x Beijerink</td>
<td>25 ml</td>
<td>25 ml</td>
</tr>
<tr>
<td>1M (K)PO₄ ph7.0*</td>
<td>1 ml</td>
<td>-</td>
</tr>
<tr>
<td>Trace Elements†</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Glacial Acetic</td>
<td>~1 ml to pH 7.0</td>
<td>-</td>
</tr>
<tr>
<td>Concentrated HCl</td>
<td>-</td>
<td>~1 ml to pH 7.0</td>
</tr>
<tr>
<td>2x PO₄ for HSM§</td>
<td>-</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

Table 2.3. *C. reinhardtii* growth media.

* 4x Beijerink Salts
  16 g NH₄Cl
  2 g CaCl₂
  4 g MgSO₄
  dissolve in 1 litre distilled H₂O

# 1 M (K)PO₄
  250 ml 1 M K₂HPO₄
  170 ml 1 M KH₂PO₄ (titrate to pH 7.0)

§ 2x PO₄ for HSM.
  0.08 M K₂HPO₄
  0.05 M KH₂PO₄
  adjust to pH 6.9 with KOH.

† Trace Elements
(i) Dissolve in 550 ml distilled water in the order indicated below, then heat to 100°C
  11.4 g H₃BO₄
  22 g ZnSO₄·7H₂O
  5.06 g MnCl₂·4H₂O
  4.99 g FeSO₄·7H₂O
  1.61 g CoCl₂·6H₂O
  1.57 g CuSO₄·4H₂O
  1.1 g (NH₄)₆Mo₇O₂₄·4H₂O
(ii) Dissolve 50 g EDTA·Na₂ in 250 ml H₂O by heating and add to the above solution.
  Reheat to 100°C. Cool to 80 - 90°C and adjust to pH6.5 - 6.8 with 20% KOH.
(iii) Adjust to 1 litre. Incubate at room temperature for two weeks and allow a rust coloured
  precipitate to form. The solution will change from green to purple.
(iv) Filter through three layers of Whatman No.1 paper under suction until the solution is
  clear. Store at 4C.
Cells were stored at 17°C and restreaked to fresh plates every 6-8 weeks. Working stocks were restreaked weekly. Wild type cells were grown at 50 μEm⁻²s⁻¹ illumination while photosynthetic mutants were grown in dim conditions of 6-8 μEm⁻²s⁻¹. TAP liquid medium was used to grow cells in large volumes for molecular and biochemical analysis. Liquid cultures were grown in Erlenmeyer flasks containing the appropriate medium, in the appropriate light conditions, at 25°C and aerated by shaking at 150 rpm. A loopful of working stock cells was used to inoculate a 25 ml volume of TAP, which was grown to stationary phase (~2x10⁷ cells ml⁻¹). An appropriate amount of this starter culture was used to inoculate a larger volume of medium. This was then grown to the cell density required. Aseptic technique was used throughout.

2.4 Counting *C. reinhardtii* cells using the Haemocytometer.

A 1 ml sample of cells from a *C. reinhardtii* liquid culture was first killed by the addition of 10 μl of iodine solution (25 mg ml⁻¹ in ethanol). A haemocytometer (Weber Scientific International Ltd.) was used to count cells from both grids and the average was multiplied by 10⁴ to give the cell concentration per ml.

2.5. Determination of Chlorophyll Concentration.

This was carried out according to the method of Arnon (1949), using the extinction coefficients given by MacKinney (1941) for chlorophyll extracted in 80% (v/v) acetone (pathlength 1 cm) The amount of each chlorophyll at 663nm and 645nm is given by:

\[
A_{663} = 82.04 \text{ Chl } a + 9.27 \text{ Chl } b \\
A_{645} = 16.75 \text{ Chl } a + 45.6 \text{ Chl } b
\]

where \( A = \text{absorbance} \)

Chlorophyll = chlorophyll concentration in mg/ml
Solving the above equations simultaneously:

\[
\begin{align*}
\text{Chl} \ a &= 0.0127 \ A_{663} - 0.00259 \ A_{645} \\
\text{Chl} \ b &= 0.0229 \ A_{645} - 0.00467 \ A_{663}
\end{align*}
\]

A value for total chlorophyll concentration can then be obtained, using the absorbance at 652 nm (at this wavelength, the optical absorption spectra of Chl \ a and Chl \ b intersect)

\[
\text{Total Chlorophyll} = 0.029 \ A_{652}
\]

A baseline reading was obtained with 1 ml 80% acetone, in the range 640-668 nm, using quartz cuvette in a Philips PU 8740 uv/vis scanning spectrophotometer. 1 ml of \textit{C. reinhardtii} cells, or 50 \mu l of thylakoid membranes (see section 2.16), were added to 9 ml or 9.95 ml of 80% acetone, respectively. The solution was mixed thoroughly, and left at room temperature for 5 minutes. The solution was then filtered through Whatman filter paper, and the absorbency measured at 652 nm. Total chlorophyll was calculated, and adjusted for dilution factors, to give a value in \mu g chlorophyll ml\(^{-1}\).

2.6 DNA Techniques.

2.6.1 Restriction Analysis.

DNA digestion was carried out using 6-10 fold excess of restriction enzyme in the appropriate buffer. Sterile double distilled water (sddH\(_2\)O) was used to make up the total reaction volume. Plasmid DNA was digested at 37\(^\circ\)C for 1-2 hours. Additional enzyme was added to the reaction mixture approximately 30 minutes prior to the end of incubation to ensure complete digestion of the DNA. In each case, a fraction of the digest was run on a 1\% agarose gel to ensure the enzyme had cut to completion.

2.6.2 Agarose Gel Electrophoresis of DNA.

DNA fragments larger than 500 base pairs (bp) were separated on 1\% agarose gels. DNA
fragments smaller than 500 bp were separated on 2% gels. The gels were made with TAE buffer (40 mM Tris-acetate, 10 mM EDTA.Na₂, pH 8.0), and run in TAE buffer. Prior to loading, samples were mixed with loading buffer (final composition, 0.1 M EDTA, 40% Glycerol, 0.001% SDS, 0.02% Bromophenol blue). Samples were run with lambda DNA markers, either digested with HindIII or Aval and BglII (fragment sizes shown in Table 2.4). Mini-gels were run at 80V for 1.5-2 hours and large gels were run at 40V for 16-18 hours. The gels were then stained for 20 minutes in 0.01% ethidium bromide solution (EtBr), destained in distilled water for 2x 10 minutes and the DNA visualised on a UV transilluminator. Instant photographic records were obtained using a UVP Gel Documentation System (Ultra Violet Products).

2.6.3 Recovery of DNA from Agarose Gels.

(I) GeneClean
DNA was recovered from agarose gels using the GeneClean II kit as described in the manufacturers’ protocol (Biolabs 101).

(II) Chambon Elution
DNA was purified as per the method described in Dretzen et al. (1981). DNA was resuspended in H₂O or TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA.Na₂ pH 8.0) buffer, as appropriate.

(III) "Freeze Squeeze"
After separation on an agarose gel, and subsequent staining of marker lanes, the band corresponding to the DNA fragment to be purified was excised from the gel. The band was soaked in sterile distilled H₂O for 15 minutes to remove the electrophoresis buffer. The gel slice was then placed in a 0.5 ml sterile microfuge tube, containing a small plug of sterile glass wool. The tube was frozen at -70°C for at least 30 minutes. A small hole was punched into the bottom of the frozen tube with a sterile hypodermic needle. The tube was then placed inside a 1.5 ml microfuge tube. The tubes were centrifuged at 10,000 rpm in a
Table 2.4. Sizes of DNA markers (in bp) for agarose gel electrophoresis

<table>
<thead>
<tr>
<th>λ: Ava I, Bgl II</th>
<th>λ: Hind III</th>
</tr>
</thead>
<tbody>
<tr>
<td>14,866</td>
<td>23,130</td>
</tr>
<tr>
<td>8,778</td>
<td>9,416</td>
</tr>
<tr>
<td>5,432</td>
<td>6,557</td>
</tr>
<tr>
<td>4,333</td>
<td>4,361</td>
</tr>
<tr>
<td>3,780</td>
<td>2,322</td>
</tr>
<tr>
<td>2,425</td>
<td>2,027</td>
</tr>
<tr>
<td>2,215</td>
<td>564</td>
</tr>
<tr>
<td>1,917</td>
<td>125</td>
</tr>
<tr>
<td>1,629</td>
<td></td>
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<tr>
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<td></td>
</tr>
<tr>
<td>1,076</td>
<td></td>
</tr>
<tr>
<td>540</td>
<td></td>
</tr>
</tbody>
</table>
MSE bench top centrifuge for 10 minutes at room temperature. Liquid in the larger tube, which contains the DNA was then ethanol precipitated with 2 volumes of ethanol. DNA was resuspended in \( \text{H}_2\text{O} \) or TE buffer, as appropriate.

### 2.6.4 Production of Recombinant Plasmids.

Recombinant plasmids were prepared by ligating isolated DNA fragments into a compatible restriction site within the plasmid vector of choice. Insert DNA and vector DNA was prepared by restriction digestion using the appropriate enzyme or enzymes (as in 2.6.1.) and gel purified (as in 2.6.3.).

Vector DNA digested with a single enzyme was treated with calf intestinal alkaline phosphatase (Boeringer-Mannheim) to prevent religation of the staggered ends, as described in Sambrook et al. (1989). Proteins were removed by phenol/chloroform extraction. One volume of TE saturated phenol and one volume of chloroform:isoamyl alcohol (24:1; v/v) was added to the restriction mix, and the phases mixed by vortexing. Following centrifugation at 13,000 rpm for 1 minute in a MSE bench top centrifuge, the upper aqueous phase was removed to a fresh 1.5 ml microfuge tube. The vector DNA was precipitated with two volumes of ice cold ethanol, as described in Sambrook et al. (1989). Vectors digested with two incompatible enzymes or with a blunt cutting enzyme were not treated with alkaline phosphatase.

Insert DNA with staggered ends to be ligated into a blunt cut vector was treated with mung bean nuclease (MBN) or T4 DNA Polymerase as described in Sambrook et al. (1989) and then phenol/chloroform extracted and precipitated as described above.

Prior to setting up the ligation reaction, both the vector and the insert were run on an agarose gel to check the concentration of the DNA. The ligation reactions were set up as described in Sambrook et al. (1989). Where cohesive termini were being ligated, an equimolar ratio of vector:insert was used. Where DNA was being blunt cloned, the molar
ratio of vector:insert used was 1:3. Ligations of cohesive termini were incubated at 17C for 12-16 hours, while blunt ended ligations were incubated at 12C for 16-20 hours.

2.7 Transformation of *E. coli*.

Recombinant plasmids were introduced into the appropriate strain of competent *E. coli* using a modified method of Cohen *et al.* (1972), as described in Sambrook *et al.* (1989). 100 µl of a stationary phase overnight culture was used to inoculate 10 ml of LB medium (supplemented with antibiotics where appropriate) and was grown at 37C in an orbital shaker at 200 rpm for 2.5 hours. Cells were harvested by centrifugation in a Mistral 3000 centrifuge at 3,000 rpm for 10 minutes. Cells were made competent by resuspension in 10 ml of cold (4C) 50mM CaCl₂ and incubated on ice for 5 minutes. Cells were harvested as before and then resuspended in 400 µl of cold (4C) 50 mM CaCl₂. Cells were incubated on ice for a further 30 minutes. Aliquots of 200 µl of competent cells were transferred to 30 ml Sterilin sample bottles and to these were added the transforming plasmid (typically 5 µl of a ligation reaction or 0.1 µg of a parental plasmid). The competent cell/plasmid mixture was incubated on ice for 30 minutes and then heat shocked at 42C for 60 seconds. After cooling briefly on ice, 1ml of LB medium was added to the cells which were then incubated at 37C for 30 - 45 minutes to allow expression of plasmid encoded antibiotic resistance markers. A 200 µl aliquot of the transformed cell suspension was then plated to LB or 2YT 2% agar plates supplemented with appropriate antibiotic(s). For pUC based vectors (Vieira & Messing, 1982) agar plates were also pre-treated with 4 µl of a 200 mg/ml stock solution of the non-substrate *lac* inducer IPTG and 40 µl of a 20 mg ml⁻¹ in dimethylformamide stock solution of the chromagenic substrate X-gal as described in Sambrook *et al.* (1989) to allow blue/white selection. The remaining cell suspension was then concentrated by centrifuging and discarding most of the medium prior to gently resuspending the cells in the remaining medium and plating as above. The plates were incubated inverted at 37C overnight and resultant recombinants were picked to fresh LB or 2YT 2% agar plates supplemented with the appropriate antibiotic(s) and grown inverted at 37C overnight. A loopful of these cells was then used to inoculate 10 ml LB or 2YT,
which was grown at 37°C, 150 rpm, overnight for miniprep analysis as described in section 2.7.3. A sample of the culture was used to prepare a glycerol stock as detailed in section 2.1.

2.8 Isolation of Plasmid DNA from *E. coli*.

2.8.1 Qiagen Preps'.

Qiagen DNA preps’ were performed according to the manufacturers’ protocol. The DNA was then resuspended in an appropriate volume of TE pH 8.0. DNA concentration was determined by diluting 1 μl of the DNA sample into 999 μl ddH₂O and measuring the absorbency at 260nm (1 mg/ml DNA is 20 absorbency units at 260nm). DNA was stored at -20°C.

2.8.2 Wizard Minipreps'.

Wizard DNA minipreps were performed according to the manufacturers’ protocol. The DNA was then resuspended in an appropriate volume of TE pH 8.0.

2.8.3 Plasmid Mini Preps'.

Small scale preparations of plasmid DNA ("minipreps") were performed using a modified method of that described by Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981), as described in Sambrook *et al.* (1989).

Cells from a 1 ml of overnight culture were harvested by centrifugation at 13,000 rpm for 60 seconds. All of the supernatant was removed and the cells washed once in 10 mM Tris-HCl, pH8. The bacterial pellet was resuspended in 100 μl of 50 mM Tris-HCl, pH8 and 10 mM EDTA. 200 μl of 0.2 M NaOH and 1% SDS was added, the sample mixed by inversion, and then incubated at room temperature for 3 minutes. 150 μl of 3 M KAc (pH5.5) was added to the mix, which was then incubated on ice for 5 minutes. The sample mixture was centrifuged at 13,000 rpm for 5 minutes. The supernatant was transferred to a fresh 1.5 ml microfuge tube, and extracted with 400 μl
phenol:chloroform:indol acetic acid (25:24:1 ratio). The upper aqueous phase was transferred to a fresh tube, and the DNA precipitated with 800 µl of ethanol. After washing with another 400 µl of ethanol, the DNA pellet was resuspended in 30 µl TE buffer, containing 20 µg ml⁻¹ RNase A. The sample was incubated at 37°C for 15 minutes, and then checked by gel electrophoresis, prior to freezing.

2.9 Preparation of Plasmid DNA from C. reinhardtii.

Small scale preparation of total DNA from C. reinhardtii was carried out according to the 'miniprep' method of Rochaix et al. (1988). Cells from a 25 ml culture at a density of 1x10⁷ cells ml⁻¹ were harvested by centrifugation in a 30 ml Sterilin sample bottle, at 3000 rpm for 5 minutes in a Mistral 3000 centrifuge. The supernatant was discarded and the cell pellet resuspended in 1 ml of TAP medium, transferred to a 1.5 ml microfuge tube and repelleted by centrifugation in an MSE microcentrifuge. The supernatant was discarded and the cell pellet was resuspended in 0.35 ml of TEN buffer (50 mM EDTA.Na₂ pH 8.0, 20 mM Tris-HCl pH 8.0, 0.1 M NaCl). 50 µl of pronase at 10 mg ml⁻¹ and 25 µl of 20% SDS were added and the cells were incubated at 55°C for two hours. 2 µl of diethylpyrocarbonate (DEPC) were added and incubation continued for a further 15 minutes at 70°C in a fume hood. The tube was briefly cooled on ice and 50 µl of 5 M potassium acetate added. The contents of the tube were mixed by shaking thoroughly and the tubes were incubated on ice for a further 30 minutes. The precipitate which formed was removed by centrifuging the tubes for 15 minutes in an MSE microcentrifuge at 13,000 rpm and transferring the supernatant to a fresh microfuge tube. Contaminating proteins were removed by phenol extraction. One volume of TE saturated phenol was added and the phases were mixed by vortexing. Following centrifugation at 13,000 rpm for 2 minutes, the upper, aqueous phase was transferred to a fresh microfuge tube and the extraction repeated three times. Phenol was removed from the solution by a final extraction with one volume of chloroform. The DNA in the recovered aqueous phase was subsequently precipitated by adding 2.5 volumes of cold (-20°C) absolute ethanol and incubating at -20°C for 30 minutes. The DNA was recovered by centrifuging the tubes for
10 minutes in a microcentrifuge to pellet the DNA. The supernatant was discarded and the pellets were washed with 70% (v/v) ethanol and then dried in a Rotovac vacuum drier. DNA pellets were finally resuspended in 50 μl TE pH 8.0, 1 μg ml⁻¹ RNase A.

2.10 Polymerase Chain Reaction (PCR).
The amplification of DNA fragments by PCR (Mullis and Faloona, 1987) was carried out using Vent DNA polymerase (New England Biolabs). Template DNA was prepared as in section 2.7 and 1 ng was used per reaction. To this was added: 200 μM each dATP, dCTP, dGTP, and dTTP, 1 μM each oligonucleotide primer, 2.5U Vent DNA polymerase and 1x Vent reaction buffer (supplied with enzyme, New England Biolab), and the volume was made up to 100 μl with ddH₂O. Varying amounts of MgSO₄ were added, according to the different DNA amplification reactions. Reactions were overlaid with 50 μl mineral oil. Reaction cycles used are described in Results chapters.

2.10.1 Colony Screening By PCR
A single colony was picked from a LB or 2YT plate with a sterile toothpick, and resuspended in 50 μl sdH₂O. The microfuge tube was vortexed to disperse the colony, and then boiled in a water bath for 5 minutes. The tube was briefly cooled on ice, and then centrifuged at 13, 000 rpm in a MSE bench top microfuge for 1 minute. A 10 μl aliquot of the supernatant was removed and put into a 0.5 ml PCR tube. A 50 μl PCR reaction was set up as described in 2.10. Reaction cycles used were appropriate to the primers. 10 μl of the PCR reaction was then run on an agarose gel (section 2.6.2).

2.11 DNA Sequence Analysis.
2.11.1 Manual DNA Sequencing.
DNA sequencing was carried out using a method derived from the dideoxy chain termination method of Sanger et al. (1977), using a Sequenase II sequencing kit (USB). The double stranded DNA template was prepared for sequencing by alkaline denaturation. 5 μg of plasmid DNA template was denatured at 37°C for 5 minutes by the addition of 2 μl
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5'-3'</th>
<th>Tm in °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7</td>
<td>AATACGACTCACTATAG</td>
<td>46</td>
</tr>
<tr>
<td>PsaB-3'</td>
<td>GTAGTCACGTAACCAACC</td>
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</tr>
<tr>
<td>PsaB-5'</td>
<td>CCTGGTTGGTTAGATGC</td>
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<tr>
<td>PsaC-3'</td>
<td>AAGGGTTTTTTAAAACGCAAGTG</td>
<td>72.8</td>
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<tr>
<td>PsaC-5'</td>
<td>TGATATGGAGAATGACATATTTAG</td>
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<tr>
<td>RPS12-3'</td>
<td>GATTCCACATCATGATAG</td>
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<tr>
<td>RPS12-5'</td>
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</tr>
<tr>
<td>PsaA3-3'</td>
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<tr>
<td>PsaA3-5'</td>
<td>AGACCCTGGAATTCTGCAC</td>
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<tr>
<td>Nastylinker-5'</td>
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<td>66</td>
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<tr>
<td>AadA-(Sense)</td>
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<tr>
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<td>AGGTCCGTCACA(N)₅GAAAC</td>
<td>81</td>
</tr>
<tr>
<td>N9-Antisense2</td>
<td>CCACGACCACGGACGTCACA(N)₅GAAACCTAAGTTAG</td>
<td>136</td>
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<tr>
<td>N9 Sense</td>
<td>CTAGGTTTC(N)₅TGTGACGGACC</td>
<td>89</td>
</tr>
<tr>
<td>VP Blunt</td>
<td>CGGTACTTGCAGGTTCTGCTTGG</td>
<td>76</td>
</tr>
</tbody>
</table>

Table 2.5. PCR primers.
of denaturation solution (100 μl 10 M NaOH, 2 μl 0.5 M EDTA, 900μl H₂O), in a total volume of 20 μl with ddH₂O. The DNA was precipitated by adding 3 μl of 3 M sodium acetate pH 5.4, and 75 μl of absolute ethanol. The DNA was recovered by centrifuging for 15 minutes, and then washed with 70% ethanol. The DNA was then air dried and resuspended in 7μl ddH₂O. Annealing was performed by adding 1μl of oligonucleotide primer at 20 ng μl⁻¹ and 2 μl of 5x Sequenase reaction buffer to the resuspended, denatured DNA template. This was then incubated at 37°C for 15 minutes. To the annealed DNA template:primer mix 1μl 0.1 M DTT, 2 μl labelling mix (provided in manufacturers kit), 0.5μl [³⁵S]dATP and 2 μl Sequenase enzyme were added. The reaction mix was incubated at room temperature for 5 minutes. A 3.5 μl aliquot of this reaction mix was added to 2.5 μl of each of the termination mixes (ddATP, ddTTP, ddCTP, ddGTP). The samples were incubated at 37°C for 5 minutes, after which time 4 μl of STOP (1% (w/v) xylene cyanol FF, 1% (w/v) bromophenol blue, 98% formamide, 10 mM EDTA pH8) mix was added. The samples were heated at 75°C for 2 minutes, prior to 4 μl being loaded onto a 6 M urea, 6% acrylamide gel. Gels were made, and run in 1x TBE Buffer (90 mM Tris-borate, 1 mM EDTA). Sequencing gels were prerun for 30 minutes before samples were applied. Electrophoresis was performed at 20 Watts for 2-3 hours for a short run, or 4-5 hours for a longer run. Once the run was completed, the sequencing gel was fixed in 10% methanol, 10% acetic acid and dried onto Whatman 3MM paper on a gel drier at 80°C for 2-3 hours. It was then exposed to Fuji RX X-ray film overnight at room temperature. Primers used for sequence analysis are shown in table 2.6.

2.11.2 Automated DNA Sequencing.
Automated DNA sequencing of plasmid DNA and PCR products was carried out on an ABI-Prism 310 Genetic analyzer. All sequencing reactions were performed using a DNA sequencing mix (from Perkin Elmer). This sequencing mix contains fluorescent dye labels, incorporated onto 3'-dye labelled deoxyribonucleoside triphosphates. Each of the four ddNTPs(ddATP, ddTTP, ddCTP, ddGTP) carry labels which emit light at different wavelengths when excited by laser light, which allows them to be identified. Sequencing
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3</td>
<td>ATTAACCCCTCACTAAAG</td>
</tr>
<tr>
<td>T7</td>
<td>AATACGACTCACTATAG</td>
</tr>
<tr>
<td>PsaA3(NASTY) 3'</td>
<td>CCGTACCCCCAAACATCAG</td>
</tr>
<tr>
<td>Pur4</td>
<td>CAAAGGACAGTAGTAGACACCA</td>
</tr>
</tbody>
</table>

**Table 2.6.** Sequencing primers.
reactions were carried out by PCR on a Perkin Elmer GeneAmp PCR System 2400 machine, as detailed in the manufacturers’ protocol. For the sequencing of plasmid DNA (purified by Qiagen preps' or Wizard preps', as described in sections 2.7) 1.5 μg of template was used. PCR products for sequencing were purified either on a QIAquick PCR purification kit (Qiagen) or on Microcon miroconcentrators (Amicon). 90 ng of purified PCR product and 1 μM of PCR primer was used per reaction.

2.12 Protein Gel Electrophoresis.

2.12.1 Gradient-SDS-Polyacrylamide Gel Electrophoresis (PAGE) of Proteins.

This was performed according to the method of Rochaix et al. (1988). A 10-20% resolving polyacrylamide gel with a 3% stacking gel was poured using a Biorad 20x20cm slab gel apparatus. 5x protein loading buffer (312.5 mM Tris-HCl pH6.8, 50%w/v glycerol, 10%w/v SDS, 0.025%w/v bromophenol blue, 25%v/v 2-mercaptoethanol) was diluted to a 1x concentration by addition to the protein samples, which were loaded onto the gel with Sigma wide range prestained markers. Electrophoresis was carried out at 4C, 8mA, overnight.

2.12.2 Native "Green" Gel Electrophoresis.

Green gel electrophoresis was performed according to a modified method of Delepelaire et al. (1979). A 7.5%-15% gradient gel was prepared. Buffers used to prepare the gel were as for normal gradient gels, but where SDS was used, this was replaced with LDS. Membrane samples, produced by the method described in section 2.16 were used. Sample buffer (50 mM Na₂CO₃, 50 mM DTT, 12% sucrose, 2% LDS) was added to the membrane samples (corresponding to 70 μg of chlorophyll), such that the ratio of chlorophyll:LDS (wt:wt) was 1:20. All of the sample was loaded immediately, and the gel run in the dark at 8mA, 4C, for 12-15 hours.

2.13 Western Analysis.

2.13.1 Western Blotting of Proteins to Nitrocellulose Membranes.
The protein samples separated as described in section 2.12.1 were transferred to nitrocellulose membranes by the method described by Towbin et al. (1979) using a Bio-Rad semi-dry blotter, at 20V, according to the manufacturers’ protocol.

### 2.13.2 Protein Detection.

Nitrocellulose filters, containing the bound proteins, were probed with the relevant antibodies. Sources of the antibodies and the titres used are shown in Table 2.7. Antibody binding was visualised using Enhanced Chemi-Luminescence (ECL) (Amersham International). The ECL technique is based on horseradish peroxidase (HRP) conjugated to the secondary antibody. HRP reacts with hydrogen peroxide to catalyse the oxidation of luminol in alkaline conditions, which results in the emission of light. This reaction is enhanced by the presence of chemical enhancers, such as phenol, enabling the light emission to be detected by a short exposure to autoradiography film (Hyperfilm). In both cases, incubation and detection of the bound antibody was carried out according to the respective manufacturers’ protocol.

### 2.14 Genetic Analysis of *C. reinhardtii*.

Cells were streaked onto fresh TAP plates at 3-4 day intervals, for 3 times. A large quantity of cells was then transferred onto TAP 1/10-N2 plates. The plates were kept at 17C, under dim light conditions. After 3-4 days, all of the cells were resuspended in 10 mM phosphate buffer, pH7 to 1-2 x 10^7 cells ml^-1_. Equal volumes of cells from opposite mating types were mixed, and at 1, 2, 3 and 4 hour intervals, large drops from the cell mixture were placed onto TAP-3% agar plates, made using a high grade of agar (e.g. A-7921 from Sigma) The plates were left under bright light overnight, then wrapped in aluminium foil. The plates were then stored at 18C for at least one week for the zygotes to mature. A sterile razor blade was used to scrape green cells of the plate. Plates were then examined under a dissecting microscope for the presence of zygotes. Zygotes were scraped together with a flamed glass "hockey stick", and then transferred onto a maturation plate on a wad of agar. Maturation plates are TAP-1% agar, and very wet. Zygotes are separated to single
<table>
<thead>
<tr>
<th>Antibodies raised against</th>
<th>Titre used</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsaA/B from synthetic polypeptide</td>
<td>1:5000</td>
<td>JA Guikema, Kansas State University.</td>
</tr>
<tr>
<td>PsaC from <em>C.reinhardtii</em></td>
<td>1:5000</td>
<td>J-D Rochaix, University of Geneva.</td>
</tr>
<tr>
<td>PsaC from <em>Synechocystis</em> PCC7002</td>
<td>1:1000</td>
<td>Produced By V. Patel.</td>
</tr>
<tr>
<td>PsaD from <em>Synechocystis</em> PCC7002</td>
<td>1:3000</td>
<td>D Bryant, Penn State University.</td>
</tr>
<tr>
<td>PsaE from <em>Synechocystis</em> PCC7002</td>
<td>1:500</td>
<td>D Bryant, Penn State University.</td>
</tr>
<tr>
<td>PsaF from <em>C.reinhardtii</em></td>
<td>1:5000</td>
<td>J-D Rochaix, University of Geneva.</td>
</tr>
</tbody>
</table>

*Table 2.7. Antibodies used for Protein Detection.*
positions on the plate. The plate is held over chloroform for 30 seconds to kill vegetative cells. Plates were left at dim light until visible colonies appeared. These were transferred to fresh TAP plates, and grown prior to the performance of growth studies.

2.15 Growth Studies of C. reinhardtii transformants

A 25 ml culture of cells was grown to stationary phase, and then harvested by centrifugation at 3000 rpm, 10 minutes. Cells were resuspended in ml of TAP. 5 µl of this was placed onto the appropriate plates. Once the liquid was absorbed into the agar, plates were stored at 18°C under appropriate light conditions. After 1-2 weeks, plates were examined and photographed.

2.16 Chloroplast Transformation of C. reinhardtii cells.

Chloroplast transformation of C. reinhardtii cells was performed according to the method described by Boynton et al. (1988). This is shown in figure 2.1.

2.16.1 Preparation of Cells.

A 25 ml culture of wild type cells (CC 1021 mt+) was grown to early stationary phase, under bright light conditions, 25°C. A 0.5 ml aliquot of this culture was used to inoculate 20 ml TAP. This culture was again grown to stationary phase, under the same conditions as before. The culture was then transferred to sterile 30 ml Sterilin bottles, and the cells harvested by centrifugation in a MSE Mistral 1000 centrifuge at 3000 rpm, for 10 minutes. Cells were resuspended in 0.6 ml TAP, and 0.4 ml of TAP top agar (Tap, 0.2% agar), at 42°C, was added. 0.7 ml of this mixture was then quickly added to a TAP plate, and spread by swirling. The plates were left to set in the dark. Plates were store in dim light conditions overnight, and used within one day.

2.16.2 Preparation of the Tungsten.

60 mg of Tungsten powder was vigorously vortexed with ethanol. After soaking the tungsten in ethanol for 15 minutes, it was pelleted by a brief centrifugation. The tungsten
The Biolistic process

Figure 2.1. Diagram illustrating the biolistic method of chloroplast transformation. DNA is precipitated onto tungsten microparticles, which are then fired at high velocity onto a lawn of *C. reinhardtii* cells. Once inside the cell, the DNA is integrated into the chloroplast genome via homologous recombination.
was washed in sterile dH\(_2\)O three times. After the third wash, the tungsten was resuspended in sterile 50% glycerol, such that the final volume was 1 ml.

**2.16.3 Biolistic Transformation.**

A 25 µl aliquot of the tungsten suspension was transferred to a fresh microfuge tube. To this was added, in order, 2.5 µl DNA (at 1 mg ml\(^{-1}\)), 25 µl 2.5 M CaCl\(_2\), and 10 µl 0.1 M spermidine, with 10 seconds vortexing after the addition of each item. The DNA-tungsten mix was finally vortexed for 60 seconds. After a brief centrifugation to pellet the particles, 50 µl of supernatant was removed. The tungsten was resuspended in the remainder of the supernatant. 3 µl of this suspension was loaded onto the centre of a macroprojectile, loaded into the gun barrel, and fired onto a plate of cells. Plates were left at 18°C, in dim light, overnight. 1 ml of TAP was added to the plates, and the cells released by gently stroking with a flamed glass spreader. The TAP was then drawn off, and put into a Sterilin bottle containing 1 ml TAP top agar (TAP, 0.7% agar), at 42°C. All of this was then poured onto a TAP plate containing 100 µg ml\(^{-1}\) spectinomycin, and spread by swirling the plate.

Plates were grown in dim light at 18°C, until the appearance of colonies (approximately 3-4 weeks). Once colonies appeared, they were transferred to fresh TAP plates containing 100 µg ml\(^{-1}\) spectinomycin.

**2.16.4 Segregation of the Chloroplast Genome to obtain homoplasmic colonies.**

Once transformed colonies were large enough, a loopful was picked and resuspended in 25 ml TAP medium, supplemented with spectinomycin (25 µl ml\(^{-1}\)), and grown to stationary phase. 100 µl stationary phase culture was placed in 10 ml TAP medium. 10 µl of this was then plated onto TAP plates, containing spectinomycin, and left at 18°C for approximately 2 weeks until the appearance of single colonies. These colonies were transferred to fresh TAP plus spectinomycin plates. The whole procedure was repeated twice more, to ensure that the transformed cells were homoplasmic.
2.17 *C. reinhardtii* Thylakoid Membrane PSI Preparation for EPR Analysis.  

A modified method described by Diner and Wollman (1980) was used to obtain photosynthetic membranes from *C. reinhardtii*. Throughout the extraction procedure, the cells were kept at 4°C. A 1000 ml culture of *C. reinhardtii* cells were grown to early stationary phase in TAP medium. This culture was used to inoculate 9 litres of TAP, which was grown to stationary phase, with constant stirring and constant aeration through Millipore filters (pore size 0.22 μm) and the appropriate light conditions. The cultures were condensed to one litre of cells using a Millipore pump with a 0.22 micron membrane and a pressure level of 2-3. The 1 litre culture was pelleted in a Sorval GSA rotor at 3,000xg for 5 minutes at 4°C, and resuspended in an appropriate volume of HSM buffer (20 mM Hepes pH 7.5, 0.35 M sucrose, 2 mM MgCl₂) using a paintbrush. The cells were pelleted as before, and resuspended in a small volume of HSM buffer. The chlorophyll concentration was taken (see section 2.4), and the cells diluted to 1 mg ml⁻¹. The cells were then passed through a French press at 4000 lb in⁻¹. The French pressed cells were centrifuged for 40 minutes in a SS-34 rotor at 20, 000 rpm, 4°C. The supernatant removed and the pellet was resuspended in 2.2 M sucrose, 10 mM EDTA, 5 mM Hepes using a paint brush, such that the final volume of sucrose was 1.75M. The suspension of cells was placed in an ultracentrifuge tube and overlaid with 0.5 M sucrose, 5 mM Hepes and spun for 2 hours in a T647.5 rotor at 40, 000 rpm, 4°C. Using an aspirator, the phases above and below the dark green band of membranes were removed and the membranes poured off and resuspended in a minimal volume of 0.5 M sucrose, 5 mM Hepes. The chlorophyll concentration was determined (see section 2.4), and the samples stored at -70°C.

2.18. EPR Analysis.  

EPR analysis was performed by Mike Evans in a Jeol X-band spectrometer with 100 kHz field modulation and an Oxford instruments ESR 9 liquid helium cryostat. Samples were placed in calibrated quartz tubes with a 3 mm internal diameter. Each EPR sample contained approximately 0.3 ml of material. All redox manipulations were carried out under oxygen-free nitrogen or argon. Further information on specific preparations can be
found in the relevant results section.
CHAPTER 3: THE CHARACTERISATION OF D576L SUPPRESSOR MUTANTS OF C. REINHARDTII.
CHAPTER 3: THE CHARACTERISATION OF D576L SUPPRESSOR MUTANTS OF C. REINHARDTII.

3.1 Introduction

Although the two cysteine residues at either end of the conserved Fx binding motif are generally believed to bind the iron-sulphur centre Fx, the possibility of other ligands cannot be excluded. Of particular interest is the aspartic acid residue at position 576 in PsaA of C. reinhardtii. Aspartic acid residues are also able to serve as ligands to one or more iron atoms in some [4Fe-4S] iron-sulphur centres (Canover et al., 1990). In addition, this aspartic acid residue may also be involved in forming salt bridges with the neighbouring arginine residue, which may serve to stabilise the heterodimer.

The roles that particular amino acids may play within proteins can be examined by substituting that amino acid for another. In this case, aspartic acid residue 576 was altered to a leucine residue. The resulting effects on the stability of the photosystem and electron transport, particularly through Fx, were then examined. This aspartic acid to leucine substitution was found to have a dramatic effect on the photosynthetic ability of the mutant, which was rendered completely non photosynthetic. In addition, several second site suppressor mutants of the original mutation were isolated so that the mechanism of action of the original mutation could be studied further. These suppressor mutants are capable of photosynthetic growth. This chapter reports on the characterisation of these suppressor mutants, and attempts to identify the mutation that is capable of suppressing the D576L amino acid substitution.

3.2 Creation of the D576L Mutant.

The D576L mutant was created by PCR mutagenesis, by Beverley Hallahan. The gene psaA-3 (exon 3 of psaA), was isolated from chloroplast DNA, prepared from C. reinhardtii strain cw15. This gene is found on fragment III of a BamHI digest of the chloroplast genome. It was cloned into the XbaI and BamHI sites of the plasmid pSKΔE-.
This plasmid is a derivative of pBluescript SK-, in which part of the polylinker has been deleted (EcoRV-Smal fragment). The resulting plasmid was called pBa3-AX. The 1.9 kb EcoRV-Smal aadA cassette, conferring resistance to spectinomycin and streptomycin, was isolated from pUC-atpX-AAD (Goldschmidt-Clermont, 1991), and cloned into the EcoR1 site of pBa3-AX, to create pBev. The aadA cassette was inserted in the opposite orientation to psaA-3, about 320 bp downstream. PCR mutagenesis was performed using a mutating primer, carrying the amino acid substitution and a silent AvrII change, and a T7 primer (see figure 3.1). The PCR product was isolated, using the ‘Gene Clean’ procedure, and cut with AvrII and BamHI, and cloned into the corresponding sites in the plasmid, to create pD576L. Changes were confirmed by DNA sequencing. pD576L was used to transform wild type C. reinhardtii (strain 2137) by the biolistic method. Transformants were selected on media containing spectinomycin. The transformants were taken to homoplasticity, by repeated single colony isolation.

3.2.1 Analysis of the D576L Mutant

D576 is adjacent to one of the cysteine residues believed to be involved in the binding of iron-sulphur centre Fx. It is possible that changes to this aspartic acid residue may effect the cysteine residue, and so disrupt Fx and electron transfer through it. Disruption of Fx therefore leads to loss of photosynthetic ability. The ability of the D576L mutant to grow photosynthetically was investigated. The mutant was grown in liquid culture, which was then ‘spotted’ onto solid media, containing a source of reduced carbon in the form of acetate (and grown in the dark), and on minimal media (grown under bright light conditions). The mutant was unable to support photoautotrophic growth, and, even with a source of reduced carbon, grew more slowly than wild type cells.

The mutant was characterised using electron paramagnetic resonance (EPR). EPR enables the activity of the photosystem to be measured, and the main paramagnetic species in each complex to be identified by their characteristic signals. At room
Figure 3.1. Diagram showing how the D576L mutant was created. *psaA-3* is located on fragment 3 of a *Bam* HI digest of the chloroplast genome. This fragment was isolated and cloned into a pSK vector, and *aadA* cloned into an *Eco RI* site. The D576L change was created by PCR, using the primers shown (see table 2.5). PCR was performed as described in materials and methods, with the following cycles: denaturation at 94°C for 5 mins. (1 cycle), denaturation at 94°C for 1 min., annealing 45°C for 1 min., extension at 72°C for 2 mins. (20 cycles), and extension at 72°C for 5 mins. (1 cycle).
temperature, EPR allows the identification of P700 oxidation. At low temperatures, EPR can identify the individual components of the PSI reaction centre.

The ability of D576L mutant to catalyse the oxidation of P700 was measured (figure 3.2). A light induced signal with the properties of P700	extsuperscript{+}, the reaction centre chlorophyll, was detected in both the wild type and D576L membranes. A control mutant, 2137:aadA, was also included in the measurements. This control mutant contains the aadA cassette, conferring resistance to spectinomycin but lacks any changes to PsaA. In both wild type and 2137:aadA controls, a signal which has the characteristic g value and line width of P700	extsuperscript{+} is detected, with approximately the same intensity. Although this signal is detected in the D576L mutant, the intensity of the signal is reduced to about 40-50% wild type levels.

Irreversibility of the signal at 15 K indicates electron transfer to F_{A}/F_{B}. A wider scan EPR than that for P700 shows the spectra of F_{A}/F_{B} reduction (figure 3.3). The spectra for wild type and D576L are significantly different. The spectrum of the D576L mutant is simpler than that of wild type cells. In D576L, the signal intensity per chlorophyll is lower than wild type, and a change of line shape is seen. This is probably due to an change in the distribution of electrons between F_{A} and F_{B}. In wild type cells, the electron is mainly associated with F_{A}, although some reduction of F_{B} can also be seen in the EPR spectrum. However, in the D576L mutant the line shape is greatly altered, and is more like the spectrum for spinach, where, as it appears in the mutant, the electron is mainly associated with a single iron-sulphur centre. However, the g values and line width of the spectrum in the g=1.96-1.85 region is shifted and narrowed in a way that prevents assignment of the electron to F_{A} or F_{B}.

The EPR spectra show that electron transfer through F_{X} is not affected in the D576L mutant. However, electron transfer to F_{A}/F_{B} is affected in some way. It has been
Figure 3.2. EPR scan in the g=2.00 region of *C. reinhardtii* unfractionated thylakoid membrane fragments. Membrane fragments, in 1ml chlorophyll/ml in 20 mM Tris-HCl containing 100mM NaCl, were reduced with 10mM sodium ascorbate, in the dark for 30 mins, frozen in the dark, and EPR spectra recorded before and after illumination at 15 K. The spectra shown are light minus dark difference spectra. EPR conditions: Temperature 15 K, microwave power 100 μW, modulation width 0.2 mT.
Figure 3.3. Wide scan EPR spectra in the region $g=2.00$ of *C. reinhardtii* unfractionated thylakoid membrane fragments. Samples were prepared and EPR spectra recorded as described in figure 3.2. The spectra shown are light minus dark difference spectra. Chlorophyll concentration was at 6mg/ml. EPR conditions: Temperature 15K, microwave power 10mW, modulation width 1mT.
observed that the iron-sulphur centre spectrum of *C. reinhardtii* preparations to which was added PsAD from cyanobacteria, are modified. This suggests that these variations are due to changes in the binding of PsAC to the membrane surface. In the D576L, it is possible that changes in the binding of PsAC may result in inefficient electron transfer through PSI, and so affect the ability of the mutant to grow photosynthetically.

### 3.3 Selection of Suppressor Mutants

Traditionally, mutations of a particular function have been investigated by isolating second site suppressor mutations. Such mutants may be able to provide information about the original mutation, and its mode of action. As the D576L amino acid substitution results in a nonfunctioning PSI, mutants suppressing the D576L change should be able to grow photosynthetically. Suppressor mutants were therefore selected for their photosynthetic ability.

The D576L mutant was grown in liquid culture, and plated onto minimal media, containing spectinomycin. Suppressor mutants were selected under bright light conditions. Of the many suppressor colonies that appeared, four were picked for further characterisation and analysis. All four suppressor mutants were taken through several rounds of selection to single colonies, so that they were homoplastic for the suppressor mutation.

The relative photosynthetic ability of the suppressor mutants, compared to wild type cells, was determined by spotting a small volume of liquid culture onto solid media, with and without a source of reduced carbon. The results for this can be seen in figure 3.4. Although all the mutants were found to grow photosynthetically, they grew more slowly than wild type *C. reinhardtii*.

### 3.3.1 Characterisation of the Suppressor Mutants.

Non-denaturing ‘green’ gel electrophoresis of the thylakoid membranes of the four
Figure 3.4. Spot tests showing that the suppressor mutants are photosynthetic, and able to grow on minimal medium, unlike the D576L mutant. 5μl of liquid culture was placed on agar plates containing minimal medium and a source of reduced carbon. Cells were grown under bright light conditions until visible growth was discernable.
suppressor mutants was performed, to determine the quantitative amount of PSI that is present, relative to wild type values. This type of gel electrophoresis allows separation of the complexes from each other, and not the individual subunits from each complex. Moreover, chlorophyll that is attached to the photosystem remains attached, allowing visualisation of the complex without staining. The core of PSI is resolved as a single green band on the acrylamide gel, and is termed CP1. The intensity of the band is an indication of the amount of PSI complex that is present in the thylakoid membranes. A picture of the gel is shown in figure 3.5. A band corresponding to CP1 is seen in the D576L mutant, but is much reduced compared to the wild type membranes. This corresponds with its lack of photosynthetic ability. In the suppressor mutants, the intensity of the CP1 band is greater than in the D576L mutant. However, the intensity of the CP1 band is reduced, compared to wild type membranes. It is therefore clear that the amount of PSI in the suppressor mutants is less than that present in wild type cells. Again this corresponds with the growth rates of the suppressor mutants. The suppressor mutants grow more slowly than wild type cells, and this may be due to prepared from wild the reduced amount of their PSI, or perhaps its reduced stability.

3.3.2 Western Analysis of the Suppressors.
In order to determine if the core polypeptides of the PSI complex were affected in the suppressors, Western analysis was performed using *C. reinhardtii* wild type, D576L, and suppressor mutant thylakoid membranes. The proteins were separated by SDS-PAGE on a 10-20% gel, blotted onto nitrocellulose and probed with the antibodies available, listed in table 2.6. The results are shown in figures 3.6 to 3.9. As expected, the level of PsaA/B (probed with PsaA/B antibody, and shown in figure 3.6) in D576L is vastly reduced, compared to wild type levels. The amount of PsaA/PsaB protein in the suppressors is also reduced compared to wild type cells, but is increased compared to the original D576L mutant. Levels of PsaC, PsaD and PsaF are also reduced in all the mutants compared to wild type cells. However, as the amount of PSI in the suppressor mutants is reduced, it is expected that the relative amounts of the individual subunits

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Figure 3.5. Quantitative 'green' gel analysis of the Suppressor mutants. Unfractionated thylakoid membranes, equivalent to 70μg chlorophyll, were separated on a 10-20% polyacrylamide gradient gel. The PSI complex is termed CP1, and is indicated on the picture. Samples were compared against the original D576L mutant and wild type.

CP2- PSII, 1- suppressor mutant 1, 2- suppressor mutant 2, 3- suppressor mutant 3, 4- suppressor mutant 4.
Figure 3.6. Western blot showing the amount of PsaA/PsaB proteins in the suppressor mutants, compared with wild type and the D576L mutant. Total protein extracts (equivalent to 10μg chlorophyll) from each of the mutants and from wild type cells were separated on a 10-20% SDS-PAGE gel. The proteins were transferred onto nitrocellulose filters and probed with an antibody raised against PsaA/PsaB. Antibody binding was detected using a secondary antibody conjugated to horse radish peroxidase, and subsequent detection by chemiluminescence.

1-Wild type, 2-D576L mutant, 3-6-Suppressor mutants 1-4.
Figure 3.7. Western blot showing the amount of PsaC protein in suppressor mutants, compared with wild type and the D576L mutant. Total protein extracts (equivalent to 10μg chlorophyll) from each of the mutants and from wild type cells were separated on a 10-20% SDS-PAGE gel. The proteins were transferred onto nitrocellulose filters and probed with an antibody raised against PsaC. Antibody binding was detected using a secondary antibody conjugated to horseradish peroxidase, and subsequent detection by chemiluminescence.

1-Wild type, 2-D576L mutant, 3-6-Suppressor mutants 1-4.
Figure 3.8. Western blot showing the amount of PsaD protein in the suppressor mutants, compared with wild type and the D576L mutant.

Total protein extracts (equivalent to 10 μg chlorophyll) from each of the mutants and from wild type cells was separated on a 10-20% SDS-PAGE gel. The proteins were transferred onto nitrocellulose filters and probed with an antibody raised against PsaD. Antibody binding was detected using a secondary antibody conjugated to horse radish peroxidase, and subsequent detection by chemiluminescence.

1-Wild type cells, 2-D576L mutant, 3-6- Suppressor mutants 1-4
Figure 3.9. Western blot showing the amount of Psa F protein in the suppressor mutants, compared with wild type and the D576L mutant.

Total protein extracts (equivalent to 10 μg chlorophyll) from each of the mutants and from wild type cells was separated on a 10-20% SDS-PAGE gel. The proteins were transferred onto nitrocellulose filters and probed with an antibody raised against PsaF. Antibody binding was detected using a secondary antibody conjugated to horse radish peroxidase, and subsequent detection by chemiluminescence.

1 - Wild type cells, 2 - D576L mutant, 3-6 - Suppressor mutants 1-4
would also be reduced.

3.4 Sequencing of the Suppressor Mutants.

The EPR spectra of the D576L mutant suggests that changes may have occurred in the binding of PsaC to the membrane. Aspartic acid residue 576 was not originally thought to be important in the binding of PsaC. Indeed, it was believed that arginine 580 is the important residue for PsaC binding. If the two conserved cysteine electron residues do bind F\textsubscript{x}, the consequences of this would be that the rest of the conserved amino acids in the F\textsubscript{x} binding region would pushed out into a loop, which would be stroma exposed. As this arginine residue occupies a central position in the F\textsubscript{x} binding motif, it would then be present at the apex of the loop. Arginine is positively charged, and so is thought to associate closely with the negatively charged PsaC. The results for the D576L mutants clearly indicate that this amino acid is important for the binding of PsaC. A similar mutant has been created in PsaB of \textit{C. reinhardtii} by Rodday \textit{et al.} (1995). D562 (equivalent to D576 in PsaA) was changed to asparagine, and the resulting mutant was found not to assemble PSI. This confirms that the inter cysteine loop is involved in PsaC binding and that the aspartic acid residue is important for the stable assembly of PsaC, and electron transfer to F\textsubscript{A}/F\textsubscript{B}. The F\textsubscript{A}/F\textsubscript{B} reduction EPR spectra of the D576L mutant suggests that the binding of PsaC to the PSI complex is affected. It is possible that changes may have occurred in the suppressor mutants that compensate for the alteration of PsaC binding. The most probable places for these changes are firstly in the PsaA or PsaB, which are involved in binding PsaC, or within PsaC itself. Any of these proteins could have become altered in the suppressor mutants so that stability of PsaC is increased.

3.4.1 Sequencing of the F\textsubscript{x} binding region in \textit{psaA}.

D576 is located in the F\textsubscript{x} binding region, as is the arginine residue also believed to bind PsaC. As the D576L change is carried on \textit{psaA}, it is logical to assume that any changes in the suppressor mutants would perhaps also be found in the locality of this region.
Chloroplast DNA was extracted from the suppressor mutants, and then used in a PCR reaction (using primers pBa3-antisense and pBa3-sense) to amplify the relevant section. The PCR products were purified and cloned into a bluescript vector, and then used to transform E. coli strain JM109. Transformants were selected by blue/white selection, and then grown in liquid culture prior to plasmid extraction. The plasmids from all four suppressor were sequenced manually. The sequence obtained is shown in figure 3.10. Sequencing has shown that D576L change is still present in all the suppressor mutants. Apart from this one change, however, the sequence of psaA around the Fx binding motif was found to be exactly the same as wild type. No explanation could be found for the reinstatement of photosynthesis by examining this region.

3.4.2 Sequencing of the region surrounding the Fx binding motif in psaB.

As the Fx binding region in psaA is unchanged from that of wild type (apart from the D576L change), it is possible that a change may have occurred in the corresponding region of PsaB, that could compensate for the D576L change in PsaA. PsaB also contains two amino acids that have been shown to be important in the binding of PsaC. These are an arginine residue between the two conserved cysteines, and D562 (equivalent to D576 in PsaA). Changes to either of these amino acids, or to others around this region could effect PsaC, and may have occurred to counteract the change in PsaA.

The Fx binding region of PsaB was therefore examined for any changes that may have occurred, that could result in a more stable association of PsaC. Primers were designed to flank the Fx binding region in psaB, and are shown in table 2.5. Chloroplast DNA was extracted from the suppressor mutants and psaB amplified by a PCR reaction. The 5'-psaB primer was used to sequence the purified PCR product, on an automated sequencer. The sequence obtained is shown in figure 3.11. Comparison of the sequence with that of wild type psaB reveals that the psaB sequence of the suppressor mutants is unaltered.
Figure 3.10. Sequencing gel of *psa*1 in the suppressor mutants 1-4 (Sup 1-4).
Sequencing was performed with the pur4 primer (see table 2.5), and reads antisense. The anticodon leading to the D576L change is highlighted in red, and cysteine 575 is shown in bold. The sequence is unchanged from that of wild type.
Figure 3.11. The \( \text{psaB} \) sequences obtained from the suppressor mutants are shown. They are compared with wild type \( \text{psaB} \) sequence. As can be seen from the alignment, no changes in \( \text{psaB} \) have occurred in the suppressor mutants.

WT - wild type, Sup1-4 - suppressor mutants 1-4

All sequences were obtained by sequencing PCR products in an automated ABI sequencer. PCR conditions were as described in materials and methods, and PCR cycles were as follows: 95°C for 5 mins (1 cycle), 95°C denaturation for 1 min., 55°C annealing for 1 min., 72°C extension for 2 mins (25 cycles), and then 72°C extension for 5 mins. (1 cycle).
3.4.3 Sequencing of *psaC*.

The altered spectra of F$_A$/F$_B$ in the original D576L mutant suggests that changes have occurred in the binding of PsaC (which coordinates both F$_A$ and F$_B$) to the PSI complex. Examination of PsaA and PsaB in the region believed to be involved in the binding of PsaC has shown that the amino acid residues implicated in the binding of PsaC are unchanged. Nor are any other changes found in the F$_X$ binding region, or the surrounding area. It is therefore reasonable to assume that PsaC may have become modified in the suppressor mutants in such a way as to increase its interactions with other subunits and so its stability, and to restore normal electron flow through the photosystem. This increased stability may be due to changes in charge or conformation of the protein.

Primers that flanked the *psaC* gene were designed. These primers are shown in table 2.5. Chloroplast DNA was extracted from the suppressor mutants, and *psaC* was amplified by PCR. The sequences obtained are shown in figure 3.12. Sequence comparison of the suppressor mutants’ *psaC* sequence with that of wild type shows that PsaC has not been altered in the suppressor mutants.

3.4 Sequencing of *psaJ*

PSI contains many small subunits, the function of the majority of which is unknown. These small subunits may have roles in increasing the overall stability of PSI, and the major subunits, or aiding the interactions between various components of PSI. It seemed possible, therefore, that one of these smaller subunits could have been altered in the suppressor mutants, so that the stability of PsaC, or its interaction with PsaA and PsaB is increased.

PsaJ is a small chloroplast encoded protein, that is believed to be located in the membrane. Its function is unclear. As PsaA, PsaB and PsaC are all chloroplast encoded genes, it is possible that the suppressor mutation is also chloroplast encoded. Other small subunits that are coded for by the chloroplast are Psal and PsaM. Although both of these are
Figure 3.12. The \(psaC\) sequences obtained from the suppressor mutants are shown. They are compared with wild type \(psaC\) sequence. As can be seen from the alignment, no changes in \(psaC\) have occurred in the suppressor mutants.

WT- wild type, Sup1-4- suppressor mutants 1-4

All sequences were obtained by sequencing PCR products in an automated ABI sequencer. PCR conditions were as described in materials and methods, and PCR cycles were as follows: 95°C for 5 mins (1 cycle), 95°C denaturation for 1 min., 55°C annealing for 1 min., 72 °C extension for 2 mins (25 cycles), and then 72 °C extension for 5 mins. (1 cycle).
possible candidates, they were not selected for examination because it is thought that they associate with each other. In Psal-less mutants of *Synechococcus* sp. PCC7002, PsaM is absent (Schluchter *et al.*, 1996). This may indicate that PsaM and Psal stabilise each other. Although Psaj has been proposed as a possible stabilising factor for PsaF, it has also been implicated in the interaction of PsaE with PSI. PsaE is closely associated with PsaC, and it is possible that changes in PsaJ could effect PsaE, which would then effect PsaC.

Primers that flanked the *psaJ* gene were designed, and are shown in table 2.5. Chloroplast DNA was extracted from each of the suppressor mutant, and *psaJ* was amplified by PCR. Sequencing was performed using the 5'-*psaJ* primer in the automated sequencer. The sequence obtained is shown in figure 3.13. Sequence comparison with wild type *psaJ* shows that the PsaJ protein was unchanged in the suppressor mutants.

### 3.5 EPR Analysis of theSuppressor Mutants.

DNA analysis of the major genes of PSI has not been successful in identifying the suppressor mutation of D576L. EPR analysis of the original mutant suggests that PsaC and its binding properties are effected in some way. It was expected that in the suppressor mutants the stability of PsaC would have changed, due to changes in PsaA, PsaB or PsaC. Sequencing of the genes encoding these proteins has shown that they are unchanged from wild type, and are not responsible the changed photosynthetic competence of the suppressor mutants. While it is possible that changes may have occurred in other parts of *psaA* or *psaB*, which were only partially sequenced, it is very time consuming completely sequenced two such large genes. It would be beneficial to be able to identify the region of PSI that is effected in the D576L mutant and in the suppressor mutants. This could then provide information about which subunits (or part of subunits) are involved. Examination of electron flow through the suppressor PSI complexes was examined so that the effected areas could be identified.
Figure 3.13. The psal sequences obtained from the suppressor mutants are shown. They are compared with wild type psal sequence. As can be seen from the alignment, no changes in psal have occurred in the suppressor mutants.

WT- wild type, Sup 1-4- suppressor mutants 1-4.

All sequences were obtained by sequencing PCR products in an automated ABI sequencer. PCR conditions were as described in materials and methods, and PCR cycles were as follows: 95°C for 5 mins (1 cycle), 95°C denaturation for 1 min., 50°C annealing for 1 min., 72°C extension for 2 mins (25 cycles), and then 72°C extension for 5 mins. (1 cycle).
3.5.1 \(F_A/F_B\) reduction in the Suppressor Mutants

EPR spectra of \(F_A/F_B\) reduction was performed on the thylakoid membranes of all four suppressor mutants. The spectra obtained are shown in figure 3.14. It was expected that the spectra of the suppressor mutants would have reverted back to wild type values, but this clearly not the case. EPR spectra for the reduction of \(F_A/F_B\) in the suppressor mutants are essentially the same as the equivalent EPR spectra of the D576L mutant. They have not reverted back to wild type spectra. However, the suppressor mutants are able to grow photosynthetically (although at lower rates than wild type). A change in the binding of PsaC, as was originally assumed, cannot be the reason for the reinstatement of photosynthetic ability in the suppressor mutants.

3.5.2 Oxidation of \(A_1\) in the Suppressor Mutants.

Forward electron transfer kinetics in PSI are difficult to measure because of the high chlorophyll concentration associated with the reaction centre, and the small extinction coefficients of the quinone and the iron-sulphur centres. The oxidation of \(A_1\), however, can be determined, either by forward electron transfer to \(F_X\) or by back reaction with \(P700^+\). Forward electron transfer can only be measured at temperatures above 230K, while the back reaction is seen at all temperatures in the appropriate sample redox state. The rate of the forward reaction at room temperature has been found to be in the range of 2-300 ns in spinach by EPR and optical spectroscopic techniques at 293K. The back reaction rate varies in different preparations, and with the redox state of the iron-sulphur acceptors, and is measured to be around 10\(\mu\)s and 100-150\(\mu\)s.

Pulsed EPR was used to examine the oxidation of \(A_1\). Using this method, the spin polarised \(P700/A_1^+\) radical pair is detected and its decay measured. However, at times longer than a few microseconds, it is not possible to distinguish between changes in the concentration of the radicals and the decay of polarisation. Therefore, detection of the slower rate was not attempted.
Figure 3.14. Wide scan EPR spectra in the $g=2.00$ region of *C. reinhardtii* unfractionated thylakoid membrane fragments. Samples were prepared and EPR spectra recorded as described in figure 3.3. The spectra shown are light minus dark difference spectra. Chlorophyll concentration 6 mg/ml. EPR conditions: Temperature 15K, microwave power 10 mW, Modulation width 1 mT.
The rate of forward electron transfer in wild type *C. reinhardtii* chloroplast membranes was measured. Rates for *C. reinhardtii* spinach membranes are similar (see table 3.1). The rate of $A_{1}^{-}$ reoxidation in the D576L mutant is less than wild type, but greater than other mutants lacking PSI. In the suppressor mutants, rates of $A_{1}^{-}$ reoxidation are similar to that for wild type, in the range of 200-300ns.

3.5.3 Recombination Rates between $P700^{+}$ and $A_{1}^{-}$ in the Suppressor Mutants.
Electron transport in PSI is effected by temperature in a peculiar way. In the experiments described above, the temperatures were at room temperature and just below freezing. At these temperatures, forward electron transfer to the bound iron-sulphur centres is rapid and back reactions from $F_{A}^{+}/F_{B}$ to $P700^{+}$ occur in milliseconds. As temperature decreases, the back reactions are inhibited, and $P700$ photooxidation appears irreversible. As temperature falls below 200K, the efficiency of forward electron transfer also declines. If illumination is continuous, irreversible oxidation of $P700$ and the reduction of $F_{A}^{+}/F_{B}$ is detected, even at 4K. If, however, a single turnover flash is provided, few reaction centres possess this stable charge separated state. In the majority of the reaction centres, a recombination between $P700^{+}$ and $A_{1}^{-}$ is seen. The recombination rate at 100K in wild type and the suppressor mutants was measured. The results of this can be found in table 3.2. The recombination rate in wild type *C. reinhardtii* is faster than that of spinach, being 14-18 $\mu$s compared with 25-30 $\mu$s in spinach. This rate is unchanged in the D576L mutant, and in the suppressor mutants.

3.6 Genetic Analysis of the Suppressor Mutants.
The lack of any obvious changes in the core polypeptides of PS1 suggests that perhaps one of the smaller subunits has been altered in the suppressor mutant. It is very time consuming to sequence all of the subunits of PSI, and it would be beneficial if the suppressor mutation could be isolated to either the chloroplast or nuclear genome.
<table>
<thead>
<tr>
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<th>$A_1$ Oxidation rates at 260K</th>
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<tbody>
<tr>
<td>Spinach</td>
<td>$453 \pm 95$ ns</td>
</tr>
<tr>
<td><em>C. reinhardtii</em> wild type</td>
<td>$355 \pm 136$ ns</td>
</tr>
<tr>
<td>D576L mutant</td>
<td>$848 \pm 146$ ns</td>
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<tr>
<td>Suppressor mutant 1</td>
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<tr>
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<tr>
<td>Suppressor mutant 4</td>
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**Table 3.1.** Table showing the rates forward electron transfer the mutants compared with wild type. The rate of forward electron transfer in the D576L mutant is slower than in wild type, but in the suppressor mutants the rate has recovered to near wild type levels.
Table 3.2. Table showing the back reaction rates between $A_i$ and P700 at 100K. This rate is unchanged in the suppressor mutants, compared to wild type. (The suppressor mutants rate given is a combination of one measurement for each suppressor mutant).
Identifying nuclear or chloroplast mutations in *Chlamydomonas* sp. is relatively easy. *Chlamydomonas* sp. cells are either mt+ or mt-. When starved of nitrogen, cells can be induced to mate, and transfer of DNA occurs. All chloroplast DNA is inherited from mt+ cells, whereas all mitochondrial DNA is inherited from mt- cells. Nuclear DNA is inherited in a Mendelian fashion. Crossing of the two cell types results in four progeny cells. The D576L mutant, and therefore the suppressor mutants are all mt+. When the suppressor mutants are crossed with wild type cells that are mt-, if the suppressor mutation is chloroplast encoded, all the progeny should be photosynthetic. On the other hand, if the suppressor mutation is nuclear encoded, only 50% of the progeny cells will be photosynthetic, because only two of the four cells will have inherited their nuclear DNA from the suppressor mutant.

The backcross experiment was performed as described in materials and methods, and is illustrated in figure 3.15. The D576L change in PsaA, and spectinomycin resistance is encoded by the chloroplast DNA. Therefore, all the progeny of a wild type mt-/?D576L suppressor mutant mt+ will carry this change and grow on media containing spectinomycin. All daughter cells were found to be able to grow on spectinomycin. If the suppressor mutation is chloroplast encoded, then all the daughter cells should be able to grow on minimal media. However, if the suppressor mutation is nuclear encoded, only 50% of the daughter cells should survive on minimal media. After crossing the suppressor mutants (mt+) with wild type cells (mt-), daughter cells were not separated from the zygote. The zygote was grown as a whole, and then spotted onto minimal media to check for photosynthetic growth. The results obtained are shown in figure 3.16. If the suppressor mutation is chloroplast encoded, then all the progeny should be photosynthetic and grow as well as the suppressor mutant on minimal media. This was not the case. None of the zygote 'spots' grew as well as the original suppressor or wild type cells. Indeed, only a small amount of growth can be seen in the zygote spots on minimal media. Although the level of growth in the zygote spots is expected to be approximately 50% of original suppressor levels (because only two out of four daughter cells will have the suppressor
Figure 3.15. Diagram showing how the different organelle genomes are inherited in *Chlamydomonas*. When a cell of mating type plus (mt+) is crossed with a cell of mating type minus (mt-), all mitochondrial DNA is inherited from the mt- parent, chloroplast DNA is inherited from the mt+ parent, while nuclear DNA is inherited in a 1:1 ratio.
Figure 3.16. Photograph showing the results of the Backcross experiment for suppressor mutant 1. Four zygotes were isolated, and used for the spot tests. All the zygotes grow on spectinomycin plates, but show very reduced amounts of growth on minimal media. This shows that the suppressor mutation is not carried on a chloroplast gene, but is nuclear encoded.
mutation, if it is nuclear encoded), this was not seen. However, as not all daughter cells can be expected to survive, this is as expected. The failure of the zygotes to grow as well as the original suppressor mutants suggests that the suppressor mutation is nuclear encoded, and not carried on the chloroplast, as was originally assumed.

3.7 Discussion

Examination of the protein composition of PSI shows that the amounts of the various subunits in the D576L mutant and in the suppressor mutants, compared to wild type cells, is altered. The P700 antibodies show that in the D576L mutant PsaA/B is present in very reduced levels, whereas the suppressor mutants show an increased level of PsaA/B protein, compared to D576L, but a reduced level compared to wild type. This corresponds to the results of the green gel, and to the growth characteristics of the mutants. All the suppressors grow more slowly than wild type cells, and the D576L mutant grows even more slowly than the suppressors, when both are supplied with a source of reduced carbon. All the mutants, D576L and the suppressors, had approximately equal amounts of PsaD protein. The PsaF protein is reduced in all the mutants, compared to wild type levels of the protein. Although it is expected that the levels of PsaF would not be significantly altered in the mutant cells, the reduced amount of PSI in the mutant complexes could account for this.

In the D576L mutant, EPR spectra showing electron transfer to $F_A/F_B$ are significantly altered. In the mutant, the electron is transferred to a single iron-sulphur centre, either $F_A$ or $F_B$, instead of both centres as in wild type cells. The line width of the signals is also altered, and is narrower than that of wild type cells. This change in electron distribution and the narrowing of line width suggests changes have occurred in the binding of PsaC to the heterodimer of PSI. It was expected, therefore, that in the suppressor mutants, which are photosynthetic in spite of the D576L change, that the binding of PsaC would have been altered again in such a way as to restore normal electron transport. The most obvious candidates for this change are the genes responsible for binding PsaC, and
PsaC itself. Sequencing analysis of part of psaA and psaB, and all of psaC in the suppressor mutants, however, has shown that the suppressor mutation is not present on any of these genes. It was also possible that one of the small, minor subunits of PS1 may also have been altered to stabilise PsaC. To this end, psaJ, a chloroplast gene encoding a protein of unknown function was also sequenced. However, this revealed no changes either. The backcross experiment shows that the suppressor mutation is, in fact, carried on a nuclear gene. If the binding of PsaC had been altered, and a nuclear gene is responsible for it, the most likely candidates would be PsaD or PsaE, both of which are closely associated with PsaC. All three proteins form a 'cap' on the stromal surface of the thylakoid membrane, and are in very close association with each other. The presence of PsaD is an absolute requirement for the binding of PsaC to the PSI core. In reconstitution experiments, where recombinant PsaC was added to PSI core complexes depleted of PsaC, PsaD and PsaE, restoration of light induced electron transfer to \( F_A/F_B \) is not seen. However, when PsaC is added to the depleted complexes in the presence of PsaD, it was found that electron transfer to \( F_A/F_B \) is resumed, and that the greater the amount of PsaD added to the depleted complexes, the faster electron flow resumed (Zhao et al., 1990). However, if the binding of PsaC has been altered in the D576L suppressor mutants, then one would expect the EPR spectra of \( F_A/F_B \) reduction to be different in both the original D576L mutant and the suppressor mutants. This is not the case. The spectra are essentially the same in both cases. Clearly, the binding of PsaC, and its subsequent effects on electron transfer through \( F_A/F_B \), has not been altered again in the suppressor mutants.

As an alteration in the binding properties of PsaC cannot be accountable for photosynthetic ability in the suppressor mutants, electron transfer in other parts of the photosystem was examined. Although the measurement of forward electron transfer in PSI is difficult, the oxidation of \( A_1 \) can be determined. Measurement of \( A_1 \) oxidation can occur by one of two ways, either by forward electron transfer to \( F_X \) or by back reaction with P700'. When the back reactions of \( A_1 \) oxidation were examined in both the D576L mutant and the suppressor mutants, EPR measurements suggest that recombination rates
between P700+ and A₁ are essentially the same in wild type cells, D576L mutant and the suppressor mutants. However, when the rate of forward electron transfer between A₁ and Fₓ is examined, the D576L mutant was found to have a slower rate of electron transfer from A₁ to Fₓ than wild type. In the suppressor mutants, the rate recovers. This suggests that the effect on electron transfer rates at this step is the factor responsible for lack of photosynthetic ability in the D576L mutant.

It appears that although the binding of PsaC, and so electron transfer through Fₐ/Fₖ is affected in the D576L, this is not the reason that the mutant is unable to photosynthesise. The D576L change still exists in the suppressors, which are effected in the same way as regards electron transport through Fₐ/Fₖ. It is possible that substituting leucine for aspartic acid causes changes in the structure of PsaA. Leucine is uncharged, compared to the negatively charged aspartic acid, and so it is possible that changes may have occurred in the overall charge of PsaA, which may have an effect on A₁. Subtle changes elsewhere in one of the nuclear encoded subunits could counteract this effect. PSI contains many small subunits, whose function is unknown. Although the suppressor mutation has been identified as a nuclear mutation, and this does narrow the field of investigation somewhat, identification of the exact subunits responsible for the suppressor mutation will be difficult.
CHAPTER 4: ‘NASTY’ MUTAGENESIS OF THE CONSERVED $F_x$ BINDING MOTIF IN PsaA OF C. REINHARDTII.
CHAPTER 4: 'NASTY' MUTAGENESIS OF THE CONSERVED F\textsubscript{X} BINDING MOTIF IN PsaA OF \textit{C. REINHARDTII}.

4.1 Introduction.

One of the most interesting and important regions in both PsaA and PsaB is the proposed F\textsubscript{X} binding region. This region contains a motif of amino acids that have been found to be absolutely conserved in every species of photosynthetic bacteria, algae and plant examined. Two cysteine residues are present at either end of the motif, and are believed to be involved in binding F\textsubscript{X}. However, this motif also contains ten other amino acids, which, as yet, have no known functions. Several of the amino acids are postulated to have functions relating to the structure of PsaA. These include the three glycine residues, which predominate the region, and the two proline residues. Proline residues are usually found at folds and 'kinks' within a protein. Several of the other amino acids, for example arginine, have been shown to be involved in binding PsaC. The binding of F\textsubscript{X} by the two cysteine residues pushes the motif into a stromally exposed loop, which would then place this arginine at the apex. This positioning of the positively charged arginine at the apex would then allow it to bind the negatively charged PsaC. The results presented in chapter 3 show that the aspartic acid residue adjacent to cysteine 575 in PsaA is also important for the coordination of PsaC.

The unusual spectral properties of F\textsubscript{X} may be due to the unique protein environment (F\textsubscript{X} is coordinated by two proteins, rather than one), but the possibility of ligands other than the two cysteine residues cannot be discounted. All of the amino acids in this motif are remarkably conserved, and most, as yet, most have no clearly defined role. Mutagenesis provides a valuable tool for investigating the function of these amino acids.

Site-directed mutagenesis can be used to introduce mutations at a predetermined site in DNA. While this has proved very useful in assigning possible functions for some amino acids in this region, it is time consuming and labourious to alter all of the residues in the
motif. In addition, it is impossible at present to predict with any degree of accuracy the
effect of substituting one amino acid for another. However, when mutagenesis is performed
using a pool of degenerate oligonucleotides, large populations of mutations can be
produced in one round of mutagenesis, and a library of mutants can be produced. This type
of random mutagenesis can only be used if the target amino acids are clustered, and so is
ideal for the $F_x$ binding region. Detailed structural information about PsaA is not
available, and it would be useful to be able to identify how the amino acids in this region
interact with the other components of PSI, and with $F_x$.

This chapter describes the random mutagenesis of three amino acids directly upstream of
the first cysteine (cysteine 575) in the $F_x$ binding region. A NASTY (Nature Should Tell
You) mutagenesis strategy was used. This relies on the same principle as nature, whereby
mutants containing mutations that are not viable are selected out. In this case, this involved
selecting mutants for the ability to grow photoautotrophically, so that all those changes that
result in an impaired PSI are selected out. The aim was not to completely disrupt PSI, but
to create subtle effects on the $F_x$ binding region,. The effects of these changes on the
stability of PSI and on electron transfer, particularly through $F_x$ can then be examined.

4.2 Random PCR Based Mutagenesis.
The mutagenesis of selected sites within a protein is routine, but is hampered by the need
to know which sites within a molecule should be investigated. Random mutagenesis,
however, can be used to screen proteins for structurally important sites and residues,
without a selected bias which a directed approach introduces. The three amino acids
upstream of cysteine 575, arginine, phenylalanine and proline, were selected for random
mutagenesis. After mutagenesis of $psaA$-3, the mutated gene was used to transform
$C. reinhardtii$, the model organism. Effects on electron transfer within PSI were observed.

4.2.1 Direct Cloning of Annealed Oligonucleotides.
It was originally assumed that it would be possible to anneal two pools of random
oligonucleotides, both containing the random changes to the nine nucleotides coding for Arg562, Phe563, and Pro564. The corresponding region could then be removed from a plasmid that contained enough flanking sequence to allow for homologous recombination in C. reinhardtii, and the annealed oligonucleotides cloned into its place.

Two sets of degenerate oligonucleotides, N9-sense and N9-antisense, were designed (see table 2.5, chapter 2). These oligonucleotides spanned the Fx binding region. 100 pmols of both sets of degenerate oligonucleotides were mixed together and denatured at 70C for 10 minutes, and then incubated in 0.3 M NaCl at 37C overnight, in order to anneal to each other. Each set of oligonucleotides was designed with AvrII and TthIII restriction enzyme sites at each end, so that they could be cloned directly into a vector. After annealing, the oligonucleotides were precipitated with ethanol and resuspended in TE buffer to a concentration of 150 ng/ul. A ligation reaction was performed, using the annealed oligonucleotides and pBHTth (cut with AvrII and TthIII) as the transforming plasmid, as described in materials and methods. The ligation mix was used to transform E. coli strain JM109. AvrII and TthIII are not compatible sites, and so the cut plasmid should not religate. Transformants were therefore selected for resistance to spectinomycin. Few transformants emerged, but all those that did were sequenced.

Sequencing revealed that none of the transformants contained the random changes incorporated into the oligonucleotides, and were parental pBHTth plasmid. It is possible that one of the enzymes may not have been cutting the parental plasmid to completion (or not at all), and so the plasmid would have religated to itself. When the restriction ability of both AvrII and TthIII was examined, it was found that TthIII was not cutting to completion. The distance between the two sites in pBHTth is only 17 base pairs, and so it is possible that the close proximity of both sites impairs the ability of TthIII. Digested with one enzyme at a time, rather than both together was also not successful.
4.2.2 PCR and Overlap Extension.

The process of overlap extension relies on the fact that sequences added to the 5'-end of a PCR primer become incorporated onto the end of the product molecule. By adding the appropriate sequences, a segment amplified by PCR can be made to ‘overlap’ sequences with another segment. In the subsequent reaction, the overlap is able to serve as a template. Two different PCR reactions were used to generate fragments with sequences in common at the ends to be joined. When both sets of PCR products are mixed, denatured and annealed, one strand of each fragment overlaps with the complimentary strand from the other fragment. One pair of strands overlap at their 5'-ends, and so are unable to serve as templates for PCR. The other set of strands provides free 3'-ends, which can serve as primers for DNA polymerase, which then extends the overlap in each direction. Flanking primers are also included in the reaction, and so a concurrent PCR reaction causes the recombinant PCR product to be amplified. The primers used, and the method is illustrated in figure 4.1.

This approach was also not successful. The first stage of the reaction, using N9 sense and T7 primers, and N9-antisense and T3 primers (see table 2.5, chapter 2), employed the following PCR cycles: denaturation at 94°C for 4 minutes (1 cycle), denaturation at 94°C for 1 minute, annealing at 35°C for 1 minute, extension at 72°C for 2 minutes (25 cycles), with a final extension at 72°C for 5 minutes. Products of the expected size were produced, but the overall efficiency of the reaction was low (see figure 4.2). The correctly sized products were purified, using the GeneClean procedure, as described in chapter 2. The two sets of PCR products were mixed together in approximately equal amounts (as determined on an agarose gel), and the second stage of the reaction was performed, using the PCR cycles described above. This second PCR reaction was unsuccessful. Increasing the Mg²⁺ concentration and increasing or decreasing the annealing temperature was not successful.

The N9-sense and N9-antisense primers that were used in this attempt were not designed
Stage 1. PCR produces two separate PCR products.

Stage 2. Both PCR products are purified and used as templates in a second PCR reaction.

Full length PCR product is formed.

Figure 4.1. Diagram illustrating the PCR and overlap extension approach. The diagram shows how a full length PCR product may be formed from two overlapping products. The method is discussed in section 4.2.2.
Figure 4.2. The products of the first stage PCR of the overlap extension approach using T3 and N9-antisense primers (A) and T7 and N9-sense primers (B). Products which were purified and used in the second stage are indicated with arrows. M- DNA AvaII/BglII digest (see table 2.4 for sizes).
as PCR primers, and it was thought that these may be at fault. It is important that primers
deigned with mismatches to the original sequence have enough sequence either side of the
mismatch that is true to the original sequence. This ensures that the primer is able to anneal
to the target plasmid. To overcome this possible problem, complete set of new
oligonucleotides were designed. These primers also reduced the size of the final product
by almost 2kb (see figure 4.3). Using these primers, the first stage PCR reactions were
successful. However, the second stage PCR reaction was still unsuccessful.

The overlap between the two PCR products from the first stage is only 16 base pairs.
Therefore it seemed likely that even if two complimentary strands were managing to
anneal, the other superfluous strand may also be annealing from the other end. This strand
will be much more strongly attached than the overlap strand, and so may be able to
displace it. In order to completely eliminate the superfluous bands from the second PCR
reaction, several methods were employed. The first stage PCR products can be denatured,
the denatured strands separated, and purified as single stranded DNA prior to use in the
second stage. In the first instance, the PCR products were separated on a normal agarose
gel, and the relevant band containing the PCR product was excised from the gel (prior to
staining). The PCR product was denatured by incubating the excised band in 0.3 M
NaOH, at room temperature for 30 minutes. The band was placed in the well of an agarose
gel made with 50 mM NaOH and 0.1 mM EDTA. After electrophoresis, part of the gel was
stained with EtBr, as described in materials and methods, and those bands that were poorly
stained were excised (three bands are seen: one is the double stranded product, and two are
the two single bands (these stain poorly)), and purified. As it is impossible to know which
bands will anneal to form free 3'-ends, both strands from both PCR products were used
in the second stage PCR reaction, in separate combinations. This method was also
unsuccessful in producing a second stage product.

It is also possible to generate single stranded DNA by asymmetric PCR. This involves
Stage 1. Two separate PCR reactions produce 1.36kbp and 3kbp products.

Stage 2. Both PCR products are purified and used as templates in a second PCR reaction.

Full length PCR product is formed, containing the random changes.

**Figure 4.3.** Diagram illustrating the second PCR and overlap extension approach, and showing the positions of the new primers. The method is discussed in section 4.2.2.
using an excess of one primer, and a limiting amount of the other primer. For the first few rounds of PCR, a double stranded product is produced, as both primers participate in the reaction. However, the limiting primer is rapidly depleted. The other primer is still able to participate in the reaction, and continues to prime the synthesis of single-stranded DNA. As there are only a limited amount of complimentary strands available, the majority of the strands produced by the excess primer remain as single strands. To generate strands that have free 3'-ends after annealing, single stranded products made with N9-sense and N9-antisense primers are required. The PCR reaction mix was as described in materials and method, except that the limiting primer was present as 1 pmol (usually used 100pmol). The PCR cycles were as described earlier. This was not successful.

4.2.3 PCR-Ligation-PCR.

This approach also relies on the use of two separate PCR reactions to generate the final product. The first stage is PCR amplification of the two gene fragments. Both PCR products are then purified and phosphorylated, and then ligated together. This ligated product is used as the template of a second PCR reaction, using the 5' primer from the upstream gene and the 3' primer from the downstream gene, which yields the final product. Ligation of the two PCR products generates many combinations of joined DNA fragments. However, in the second PCR reaction, only those that are ligated in the correct orientation will be able to participate in the reaction, and the major product will be the required fragment. The method, and the positions of the primers used is illustrated in figure 4.4.

The PCR cycles for both sets of PCR were as follows: denaturation at 95C for 5 minutes (1 cycle), denaturation at 95C for 1 minute, annealing at 60C for 1 minute, extension at 72C for 2 minutes (25 cycles), with a final extension at 72C for 5 minutes. Both sets of products were purified by GeneClean, as described in materials and methods, and resuspended to 50ngμl⁻¹ in TE buffer. 250ng of this was kinased and used in the ligation
Stage 1. Two separate PCR reactions produce products that are contiguous to each other.

Stage 2. Both PCR products are purified, kinased and ligated together. The ligated product is then used as a template in a PCR reaction involving the two flanking primers.

The final full length product is formed.

Figure 4.4. Diagram illustrating the PCR-Ligation-PCR approach. Primer VP-blunt was designed to begin priming at the next nucleotide to where N9-antisense2 stops. This means that the two PCR products should join together without any deleted or additional nucleotides. The approach is discussed in section 4.2.3.
reaction, and 1μl of the ligation mix was used in the second stage PCR reaction. Again although no difficulties were encountered with the first stage of the experiment, when the two products were ligated together, the second stage PCR using this as a template did not work.

4.2.4 The ‘Megaprimer’ Method.

This approach again relies on using a two stage PCR. It is similar to overlap extension, but uses one less primer to achieve essentially the same results. In the first stage, only one PCR reaction is performed, yielding product, which is then used as a long primer (in conjunction with a third, flanking primer) in a second PCR reaction.

For the first stage, primers psaA3-start and N9-antisense (see table 2.5, chapter 2) were used to generate a 1.36kbp product. The PCR reactions were as follows: denaturation at 94°C for 4 minutes (1 cycle), denaturation at 94°C for 0.5 minutes, annealing at 55°C for 1 minute, extension at 72°C for 2 minutes (20 cycles), with a final extension at 72°C for 5 minutes. The PCR product was purified by Freezesqueeze. This was then used as a primer in the second stage with aadA-start as the flanking primer. This is illustrated in fig 4.5. The second stage PCR was not successful. The success of PCR is effected by many things, and the addition of various compounds can sometimes be necessary. To this end, the PCR reaction mix was altered with the addition of several compounds, which included the use of different concentrations of MgSO₄ in the reaction (2, 4, 6 and 10mM). DMSO (1% and 10%), 12% glycerol, 10% formamide, plasmid denatured by boiling for 5 minutes, megaprimer boiled for 5 minutes, 100μM TMAC, and various combinations of these treatments. A possible cause of no amplification can be strong secondary structure of the template. The addition of DMSO, glycerol and formamide serve to ensure complete denaturation of the DNA, and possibly to reduce inter- and intra-strand reannealing. TMAC is a specificity enhancer that is able to reduce potential DNA mismatches during PCR, and to increase the stringency. Different concentrations of the 1.36kbp megaprimer were also used, ranging from 0.5μM-2μM, in conjunction with the other treatments.
Stage 1. 1.36kbp PCR product formed.

Stage 2. This PCR product is purified and used as a long primer in a second PCR reaction.

Full length PCR product is formed.

Figure 4.5. Diagram showing the 'Megaprimer' approach. The PCR product from the first reaction contains the random changes, and is then used as a long primer in the second stage (with a flanking primer), producing a full length product containing all the random changes.
None of these strategies were successful in producing a full length final product. Reducing
the annealing temperature failed to yield a product.

4.2.5 Possible Reasons for Failure.

One of the major drawbacks of PCR is the size of product. The size limit of PCR is
approximately 4-5kbp, although amplification up to 35kbp has been reported (Barnes,
1994). Originally the size of the final product was approximately 6.3kbp, although this
was reduced to 4.36kbp. However, it is likely that this reduction in size was not sufficient,
and the size of the product is responsible for the failure of the second stage PCR reaction.
This size of product is desirable because it not only contains most of the coding sequence
for psaA-3, but also contains the aadA cassette, which allows positive transformants to
be selected. If this method had been successful, then the final 4.36kbp PCR product could
have been used to directly transform C. reinhardtii, without the need for a vector. The
whole PCR product was designed to contain enough flanking sequence to eliminate the
need for a vector.

Although VENT DNA polymerase is the preferred DNA polymerase used in PCR
reactions, the use of a N-terminal deletion mutant of Taq, KlentaqI DNA polymerase,
一起 with pfu polymerase has been reported to produce longer length products (Barnes,
1994). However, when this method was employed in the overlap extension and PCR-
ligation-PCR methods, it did not prove to be successful.

The megaprimer method may also have failed because of a size limitation. Although the
size of the megaprimer has been restricted to below 0.4kbp, and the size of the final,
product to 1 kbp, the use of larger megaprimers (upto1.3kbp), and larger final products
(2kbp and 3.2kbp) has been reported (Ling and Robinson, 1995). Again, this employed
the use of pfu DNA polymerase or a Taq DNA polymerase extender. Both of these were
used in the megaprimer method, but failed to yield any product.
It became clear that a reduction in the size of the final product would be the best solution. It also appeared that simplifying the method would ensure success. Rather than using the final PCR product, containing the altered \( psaA-3 \) gene and selection in the form of \( aadK \), to directly transform \( C. reinhardtii \), without the need for a transforming vector, a system of cassette mutagenesis was chosen.

### 4.3 Random PCR and Cassette Mutagenesis.

This strategy involved the amplification of the \( aadA \) cassette, and part of \( psaA-3 \) using one pool of degenerate primers. The product that is created contains part of \( psaA-3 \) and the randomly changed nucleotides. After purification, the product is cloned into pBluescript, and the random changes identified by sequencing. The wild type sequence is then replaced with the altered sequence. This is then used to transform \( C. reinhardtii \). This approach is illustrated in figure 4.6.

One of the drawbacks of this method is that unique restriction sites are required at both ends of the cassette so that the cassette can be inserted in the correct position in the plasmid. If the cassette is to be correctly orientated in the plasmid, then both restriction sites must also be different. As such, naturally occurring sites are rare, they usually have to be engineered into the plasmid before mutagenesis can occur. Once a plasmid is available with the appropriate section of DNA flanked by suitable sites, mutagenesis can proceed.

#### 4.3.1 Creating the Plasmid Template for PCR-Mediated Mutagenesis.

The gene \( psaA-3 \) (exon 3 of \( psaA \)), was isolated from chloroplast DNA, prepared from \( C. reinhardtii \) strain \( cw15 \). This gene is found on fragment III of a \( BamHI \) digest of the chloroplast genome. It was cloned into the \( XbaI \) and \( BamHI \) sites of the plasmid pSKAE-. This plasmid is a derivative of pBluescript SK, in which part of the polylinker has been deleted (\( EcoRV-Smal \) fragment). The resulting plasmid was called pBa3-AX. The 1.9 kb \( EcoRV-Smal \) \( aadA \) cassette, conferring resistance to spectinomycin and streptomycin,
Stage 1. PCR using a pool of degenerate oligonucleotides.

Stage 2. Blunt clone PCR product into pBluescript cut with Sma I.

Stage 3. Cut cloned product with Avr II and Eco 47III to release fragment with random changes.

Stage 4. Clone this fragment into pBHTth which has had the corresponding fragment removed.

Figure 4.6. Diagram illustrating the Random PCR-Cassette mutagenesis strategy. The region of psaA-3 containing the nucleotides to be altered is amplified with a set of degenerate primers. This PCR product is then purified and bluntcloned into pBluescript. The recombinant plasmid is used to transform E. coli. Plasmid DNA is extracted from positive transformants and cut with Avr II and Eco 47III. This fragment is then cloned into pBHTth that has had the corresponding fragment removed.
was isolated from pUC-atpX-AAD (Goldschmidt-Clermont, 1991), and cloned into the 
EcorRI site of pBa3-AX, to create pBev, in the opposite orientation to psaA-3, about 320 
bp downstream. Two unique restriction sites were introduced into pBev by PCR-mediated 
site directed mutagenesis. These changes were 'silent', and did not effect the structure or 
function of PsaA. These unique sites were AvrII and TthIII, and the resulting plasmid was 
called pBHTth.

4.3.2 PCR-Mediated Random Mutagenesis.

One set of degenerate oligonucleotides that contains all of the random changes to the 
nine chosen nucleotides (primer N9 sense), and a set of primers that is exactly 
complimentary to the wild type target DNA (primer pBa3-antisense) were used in this 
reaction. The positions of the primers relative to the plasmid template are shown in figure 
4.6. PCR was performed using pBHTth as the template plasmid and the PCR reaction mix 
was as described in materials and methods, except that the buffer was supplemented with 
3mM MgSO4. The PCR cycles were denaturation at 94C for 5 minutes (1 cycle), 
denaturation at 94C for 1 minute, annealing at 50C for 1 minute, extension at 72C for 2 
minutes (25 cycles), with a final extension at 72C for 5 minutes.

The PCR product (see figure 4.7) was isolated, using the 'Gene Clean' procedure, and 
blunt cloned into pBluescript cut with Smal, as detailed in chapter 2. The plasmid was then 
used to transform E. coli strain JM109, as described in materials and methods. When 
pBluescript is used to clone DNA, selection is based on a 'blue/white' assay. The 
polycloning site of pBluescript (and therefore Smal) is contained within part of the lacZ 
gene. The host E. coli cells produce the carboxy-terminal fragment of β-galactosidase. 
While neither the host encoded or the plasmid encoded fragments are active, they can 
associate to form an enzymatically active protein. This protein cleaves the substrate X-gal, 
which results in blue colonies. However, insertion of a fragment of DNA into the 
polycloning site disrupts the lacZ gene, and so in these colonies X-gal is not cleaved, and
Figure 4.7. The PCR product obtained from PCR-mediated random mutagenesis (see section 4.3.2) is shown. 10 μl of PCR reaction mix was mixed with loading buffer, and separated by electrophoresis on an agarose gel. The gel was stained with EtBr, and DNA bands visualised under UV light. M- *Ava* I- *Bgl* II λ. DNA digest.
white colonies are formed. While the blue/white assay is very useful for quick selection, a relatively high number of false positives can be present. It is therefore advisable to confirm the presence of the insert in positive transformants. All transformants were screened by PCR. A crude whole cell extract was used as a template for PCR, using the original mutating primers, N9 sense and Ba3 antisense. A single colony was resuspended in sterile water, and then incubated at 100°C for 5 minutes. After briefly centrifuging, 10μl of the supernatant was used as a template in a PCR reaction. The amounts of primers and conditions of PCR were as described previously. The results for the PCR screening are shown in figure 4.8. A positive control with pBHTth as the template was also included, as well as a negative pBluescript control. True transformants are shown by the presence of a band on an agarose gel, the same size as the positive control.

4.3.3 Sequencing Positive Transformants.
Several of the positive transformants were chosen at random for sequencing. DNA was extracted by the Wizard mini prep procedure, as detailed in materials and methods, and sequenced using an ABI automated sequencer. The sequences obtained are shown in figure table 4.1. This type of random mutagenesis is slightly biased towards amino acids that are coded for by several different codons, and certain amino acids, such as leucine, alanine, glycine, arginine, serine and threonine appear more often than others. All the changes in the NASTY mutants are summarised in table 4.2.

4.3.4 The Final Construct.
Four positive pBluescript transformants (N6, N8, N9, N22) were cut with AvrII and Eco47III, as was pBHTth. The cleaved PCR product was purified from the restriction digest by the 'Freeze-squeeze' procedure, as detailed in materials and methods. Although the ends generated by AvrII and Eco47II are not compatible, to prevent religation of parental plasmid that has been cut only once, pBHTth cut with AvrII and Eco47III was isolated from any plasmid that was cut only once. The purified PCR product was ligated into appropriately cut pBHTth plasmid. Transformants were selected for spectinomycin
Figure 4.8. Screening of positive transformants by PCR. True transformants (3, 7, 8, 9, 12) have a band the same size as the positive control (pBHTth, 1). In the false positives (4, 5, 6, 10, 11) and the negative control (pbluescript, 2), no band is seen.

M - AvaI  BglII  λ  DNA digest.
Table 4.1. DNA sequences of the positive transformants. Transformants were sequenced with the psaA(NASTY) primer (see table 2.5). Only the sequence corresponding to Cys575 and the random changes is shown. Sequencing was performed with an antisense primer.
<table>
<thead>
<tr>
<th>Name</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>Arg Phe Pro Cys</td>
</tr>
<tr>
<td>Nasty 1</td>
<td>Gln Ile Thr Cys</td>
</tr>
<tr>
<td>Nasty 4</td>
<td>Asp Arg Asn Cys</td>
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<tr>
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<td>Glu Arg Gly Cys</td>
</tr>
<tr>
<td>Nasty 21</td>
<td><strong>Tyr STOP</strong> Try Cys</td>
</tr>
<tr>
<td>Nasty 22</td>
<td>Ser Tyr Asn Cys</td>
</tr>
<tr>
<td>Nasty 25</td>
<td>Gln Glu Ser Cys</td>
</tr>
<tr>
<td>Nasty 26</td>
<td>Glu Leu Tyr Cys</td>
</tr>
<tr>
<td>Nasty 27</td>
<td><strong>Glu</strong> Leu Tyr Cys</td>
</tr>
<tr>
<td>Nasty 28</td>
<td>Arg Gln <strong>Asp</strong> Cys</td>
</tr>
</tbody>
</table>

**Table 4.2.** Summary of the random changes to Arg562, Phe563 and Pro564.
resistance. Transformants were sequenced to confirm that the mutated cassette was cloned into the parental plasmid ‘in frame’. All four of the constructs were found to be in frame, although only the sequence for N6 is shown in figure 4.9.

4.3.5 Transformation of C. reinhardtii.

After confirming that the sequences were in frame, wild type C. reinhardtii strain CC1021 was transformed with the plasmids, by the biolistic method. This is illustrated in figure 2.1, chapter 2. After transformation, the cells were left under dim light overnight. They were then washed off the agar plates with 1ml of TAP, and transferred to minimal medium plates. These were exposed to bright light conditions. Colonies began to appear after 3-4 weeks. These were transferred to TAP plates containing 100μg/ml spectinomycin. Each mutant colony was taken through several rounds of selection.

4.4 Analysis of the Mutants.

The three mutants that produced transformants were mutants 8, 9 and 22, and these were named NASTY 8 (N8), NASTY 9 (N9) and NASTY 22 (N22). All three were taken through several rounds of selection to single colonies, prior to analysis. The growth of each mutant against wild type cells and a PSI- mutant was examined by plating 5μl of liquid culture onto a minimal medium agar plate. The plates were grown under bright light conditions until growth was visible (see figure 4.10).

4.4.1 aadA Resistance.

The aadA cassette provides resistance to streptomycin, as well as spectinomycin, which was used to select the transformants. As resistance to spectinomycin can sometimes develop in wild type cells, it was necessary to check for resistance to streptomycin. Cells were grown in liquid culture, under dim light conditions, for several days. 5μl of liquid culture was placed on solid agar plates containing both spectinomycin and streptomycin. Plates were grown under dim light for several days, and growth rates for both plates compared. The results for this can be seen in figure 4.11-4.13. The growth rates of the
Figure 4.9. Sequencing gel of the NASTY6 fragment in pBHTth. Cys575 is shown in green, random changes are shown in red, and the *Avr*II site is shown in blue. Comparison of the sequence with wild type shows that the construct is in frame.
Figure 4.10. Comparison of the growth of the NASTY mutants against wild type cells (WT) and a PSI minus mutant (D576L).
N8-NASTY8, N9-NASTY9, N22-NASTY22.
Figure 4.11. Comparison of the growth of NASTY8 (N8) on spectinomycin and streptomycin. Approximately equal amounts of growth on both antibiotics shows that N8 is a true mutant.
Figure 4.12. Comparison of the growth of NASTY9 (N9) on spectinomycin and streptomycin. Approximately equal amounts of growth on both antibiotics shows that N9 is a true mutant.
Figure 4.13. Comparison of the growth rate of NASTY22 (N22) on spectinomycin and streptomycin. Approximately equal amounts of growth on both antibiotics shows that N22 is a true mutant.
It is possible that only the spectinomycin resistance gene was integrated into the genome, and the NASTY mutants contain wild type DNA. The AvrII site that was introduced into pBHTth is a unique site that is not present in wild type *C. reinhardtii* chloroplast DNA. It can therefore be used to determine whether the mutated DNA has integrated with the wild type genome. Chloroplast DNA was extracted from wild type *C. reinhardtii* cells and from the nasty mutants. This was amplified by PCR with PsaA3-3' and PsaA3-5' primers (see table 2.5). The amplified DNA was purified and cut with AvrII, as detailed in chapter 2. In the wild type cells, the amplified DNA was not cut, and only 1 band appeared when cut bands were run on an agarose gel. However, in the NASTY mutants, 2 bands were seen, which shows that the mutated DNA was integrated into the wild type chloroplast genome.
mutants on both antibiotics was approximately the same, and so it can be concluded that these are true transformants, and not wild type cells that are resistant to spectinomycin (see opposite page).

### 4.4.2 Quantitative Comparison of PSI.

Non-denaturing 'Green' gel electrophoresis was performed on each of the NASTY mutants. This allow a crude comparison of the amount of PSI in each of the mutants, compared to a wild type and a PSI strain. The gel used in this procedure contains lithium dodecyl sulphate (LDS) instead of SDS, and, as LDS is a milder detergent than SDS, it allows the separation of complexes as a whole. In addition, chlorophyll associated with the complex remains bound to it, and so the bands do not require staining to be visualised.

Thylakoid membranes were purified from each mutant, as described in materials and methods, and used to load a 10-20% gradient gel. Electrophoresis was performed in the dark, at 4C for 12-16 hours at 8mA. The core of PSI is resolved as the topmost green band on the acrylamide gel, and is termed CP1. As it is the bound chlorophyll that gives CP1 its colour, fewer PSI complexes (and thus fewer chlorophyll molecules) result in paler bands. The intensity of colour of CP1 is therefore an indication of the amount of PSI complex that is present in the thylakoid membranes. A picture of the gel is shown in figure 4.14. Three controls were included in the gel. These were wild type, D576L (reduced PSI), and C575S (no PSI). The intensity of CP1 is approximately the same in all of the NASTY mutants, and in wild type, indicating that levels of PSI in the NASTY mutants were not much different from wild type cells. As the mutants were selected for their ability to grow photoautotrophically, this is not surprising.

### 4.5 EPR on the NASTY Mutants.

The mutants were further characterised using EPR. This enables the effect of the random changes in each of the NASTY mutants to be observed. The results of the
Figure 4.14. Quantitative ‘green’ gel analysis of the NASTY mutants. Unfractionated thylakoid membranes, equivalent to 70µg chlorophyll, were separated on a 10-20% polyacrylamide gradient gel. The PSI complex is termed CP1, and is indicated on the picture. Samples were compared against wild type, a mutant with reduced amounts of PSI (D576L), and a mutant with no PSI (C575S).

CP2-LHCII, N8 -NASTY mutant 8, N9 -NASTY mutant 9, N22 -NASTY mutant 22.
EPR experiments were not expected to be differ very much from those of wild type membranes, as selection was based on the ability to survive photoautotrophically.

4.5.1 $F_A/F_B$ Reduction in the NASTY Mutants.
The reduction of $F_A/F_B$ was measured in each of the NASTY mutants, and compared against wild type. EPR spectra measurements were taken from thylakoid membrane samples, produced as described in materials and methods. The spectra obtained are shown in figure 4.15. The spectra of all three of the mutants are similar to wild type values, as expected. However, there are slight differences in the distribution of electrons between iron-sulphur centres $F_A$ and $F_B$, and in line widths. In N22, there appears to be more $F_B$ than $F_A$.

4.6 Discussion.
This chapter detailed attempts to produce a library of random mutants in $C. reinhardtii$, so that functions could be assigned to three amino acids, arginine, phenylalanine and proline, of the conserved $F_X$ binding region. The first methods were designed to produce products that not only contained enough flanking sequence to allow for homologous recombination, but also contained an antibiotic resistance gene. Cloning of the PCR products was therefore not required, as the PCR product could be used to transform $C. reinhardtii$. However, this meant that the final size of the product was very large, as it incorporated almost all of $psaA$ and the $aadA$ cassette. Amplification of DNA by PCR is limited by the size of the product (the size limit is 4-5kbp), although amplification of larger products (up to 35kbp) has been reported by Barnes (1994), using a combination of polymerases. The size of the product in the first protocol is approximately 6.3kbp, although this was reduced to 4.36kbp in a later attempt. This is at the limits of PCR. The method employed by Barnes (1994), which used combinations of a N-terminal deletion mutant of $Taq$, KlentaqI DNA polymerase, together with $pfu$ polymerase, was used when normal PCR failed to produce a product. However, it did not prove to be successful. It is likely that the megaprimer method
Figure 4.15. Wide scan EPR spectra in the \( g = 2.00 \) region of *C. reinhardtii* unfractionated thylakoid membrane fragments. Membrane fragments, in 1 mg chlorophyll/ml, in 20 mM Tris-HCl containing 100 mM NaCl, were reduced with 10 mM sodium ascorbate, in the dark for 30 minutes, frozen in the dark, and EPR spectra recorded before and after illumination at 15K. The spectra shown are light minus dark difference spectra.

Chl concentration 6 mg/ml. EPR conditions: Temperature 15K, microwave power 10 mW, modulation width 1 mT.
(Kamman et al., 1989) may also have failed because of a size limitation. Although the size of the megaprimer has been reported to be restricted to below 0.4 kbp, and the size of the final product to approximately 1 kbp, the use of larger megaprimers, up to 1.3 kbp, and the production of larger final products (2 kbp and 3.2 kbp) has been reported (Ling and Robinson, 1995). Again, this employed the use of pfu DNA polymerase or a Taq DNA polymerase extender. Both of these were used in the megaprimer method, but failed to yield any product.

It became obvious that the way to succeed was to reduce the size of the product. To this end, a simple strategy of PCR combined with cassette mutagenesis was employed. A method was used which involved the amplification part of psaA-3 using one pool of degenerate primers. The product that was created contained part of psaA-3 and the randomly changed nucleotides, and was cloned into pBluescript. A small section of DNA containing the random changes was cut form the plasmid, and used to replace the corresponding section of DNA in a vector containing wild type psaA-3 and aadA. This recombinant plasmid was then used to transform C. reinhardtii. This strategy did work, and three C. reinhardtii transformants were produced. The aim of this project was to create subtle changes to the F$_x$ binding region. Mutants were therefore selected for their photosynthetic ability. This should ensure that mutants had fully operational PSI complexes, and that the introduced amino acid substitutions caused minor changes to the complex. Any substitutions causing PSI to be completely disrupted are selected out.

Three mutants of C. reinhardtii were obtained using this method. All three amino acids chosen to be altered are very highly conserved among photosynthetic species. As such, it was expected that substituting these for other amino acids would effect the photosystem, and that although these changes would be minor, they would be detectable. However, this was not the case. All three mutants were found to have similar rates of growth to wild type cells. EPR spectroscopy revealed that the rates of electron transfer were little different in the mutants, compared to wild type C. reinhardtii. However, as the mutants were
selected for their ability to grow photoautotrophically, perhaps this is not surprising.

Proline residues are usually found at turns, or ‘kinks’ in a protein, and it was assumed that the high degree of conservation meant that altering this proline residue would effect the photosystem. The proline residue was substituted with glycine (N8), threonine (N9) and asparagine (N22). While it was expected that the proline to glycine change in N8 would not have too great an effect, as glycine is a small residue, and is also found at folds within a protein, the changes in N9 and N22 were expected to be slightly more dramatic. Threonine is a hydrophilic amino acid, while asparagine is uncharged. Both these amino acid are also expected to take up more (or different) amounts of space, than proline, and so it was expected that they would effect the photosystem. Mutants of the corresponding proline in PsaB have been produced (Rodday et al., 1995), and found to be capable of PSI assembly and normal electron transfer. However, the stability of the photosystem was impaired, as was its interaction with PsaC.

The second residue altered was phenylalanine. The aromatic side chains of phenylalanine have significant potential for electrostatic interactions. This is because their pi-electrons are concentrated on the faces of the aromatic rings, with an electron deficit on the ring hydrogen atoms. These delocalised pi-electrons are able to interact with other pi-electrons, and to transfer electrons. As such, it is conceivable that perhaps this residue may exert some influence over $F_X$, its binding to the heterodimer, and perhaps over electron transfer reactions. This phenylalanine was replaced with lysine (N8), isoleucine (N9) and tyrosine (N22). In the case of the phenylalanine to tyrosine change, little change is expected as both are aromatic, highly hydrophobic amino acids. Lysine and arginine, on the other hand, have very polar side chains.

The third residue altered was arginine. This polar, positively charged amino acid was changed to tyrosine (N8), glutamine (N9), and serine (N22). Glutamine is an uncharged amino acid, possessing acidic side chains, while serine is a hydrophilic residue. While
tyrosine is often regarded as hydrophobic, its hydroxyl group does give it some polar properties. In addition, it contains pi-electrons, which behave like the pi-electrons of phenylalanine (see previous page).

In the case of Nasty8, a charged amino acid (arginine) and an aromatic amino acid (phenylalanine) have swapped place, with arginine being replaced with tyrosine (aromatic) and phenylalanine being replaced with lysine. The proline residue was replaced with glycine, which is also able to participate in folds. Overall, the changes in this mutant therefore do not appear to be as drastic as those of the other mutants. It is perhaps not surprising that this mutant behaves much like wild type *C. reinhardtii*. In Nasty22, little change is again expected, if the amino acids are examined on the basis of their charge and structure. Phenylalanine was replaced with tyrosine, another aromatic amino acid. However, the aromatic ring of tyrosine contains a hydroxyl group, which makes it less hydrophobic than phenylalanine. In N9 all three amino acids were replaced with those of a similar size. However, considerable changes have occurred in terms of charge and hydrophobicity, and it is surprising that this is not reflected in the behaviour of the photosystems of these mutants.

The EPR data for these mutants shows that they behave much like wild type *C. reinhardtii*. The results showing the reduction of $F_A/F_B$ are very similar to the wild type results. The only difference appears to be that the distribution of electrons between iron-sulphur centres $F_A$ and $F_B$ is different, in that in N22 there appears to be more $F_B$ than $F_A$. Slight differences of line width also exist in the mutants compared to wild type cells.

While it is disappointing that greater differences could not be seen between wild type cells and the NASTY mutants, this does raise the question as to why these amino acid are so highly conserved, if changes that are quite radical do not appear to affect the photosystem in any way. While the mutants were selected for their ability to grow photoautotrophically, it was expected that differences against wild type cells would be seen. Some of the amino
acids are considerably different in size and, perhaps more importantly, in charge, to the amino acid they replaced. It has been frequently suggested that the structure and function of PSI is dependent on interactions that hinge on the overall charges of the subunits. It was therefore surprising to find that PSI in the NASTY mutants, which differ considerably in overall charge to wild type, functioned normally. It is also surprising that the difference in size of the altered amino acids did not affect cys575, and consequently $F_X$, in spite of their close proximity. The results presented in this chapter clearly show that charges in this region do not appear to be important. The function of these amino acids is therefore still unresolved. However, a method has been devised which can be used to generate a large number of random mutants rapidly.
CHAPTER 5: EXAMINING THE ROLE OF PSAJ IN PS1.
CHAPTER 5: EXAMINING THE ROLE OF PSAJ IN PSI.

5.1 Introduction
The function of a particular protein can also be examined by creating a mutant in which that protein is inactivated or deleted. The functions of several subunits of PSI have been identified in this way. The resulting effects on PSI structure and assembly, and on electron transport within the photosystem are then observed to identify possible roles that the protein may have. This chapter also describes attempts to produce a null mutant of PsaJ, a small PSI subunit of unknown function, so that a possible role may be assigned.

PsaJ is one of the smaller subunits of PSI, being only about 4.5 kDa in size. Although its exact function is unknown, it has been predicted to stabilise PsaF. PsaJ is a hydrophobic, transmembrane subunit. In cyanobacteria, psaJ has been inactivated, and it was found that PsaE became more vulnerable to chaotropic attack, and that the amount of PsaF was reduced.

This section describes attempts to produce a null mutant of PsaJ in *C. reinhardtii*, so that a possible role may be assigned. Protein structure was also analysed so that possible functions could be determined.

5.2 Sequencing of *psaJ.*
In *C. reinhardtii*, *psaJ* is a chloroplast encoded gene, that is located upstream of the *rps12* gene. Both genes are found on fragment 17 of an EcoRI digest of the chloroplast genome. This fragment was originally isolated and cloned (to make p70-A), to sequence the *rps12* gene by Lui et al. (1989). Therefore, as *psaJ* was not intentionally sequenced, it is possible that errors may have been made reading the sequence. To confirm the published sequence, 5' and 3' upstream and downstream primers were designed for the predicted *psaJ* sequenced. The 5' primer for *psaJ* was used to sequence this gene in p70-A. The sequence obtained is shown in figure 5.1. Sequence comparison of the published sequence
Figure 5.1. Sequence of *psaJ*. p70A was sequenced in an ABI automated sequencer with the *psaJ*-5' primer (see table 2.5). The sequence of *psaJ* is shown in red, and that of p70A in black.
showed that both were identical, and no errors had been made.

5.3 Sequence Comparison of PsaJ Proteins From Different Species.
Sequence comparison of the same protein from different species can enable important regions to be identified. Conserved regions of the protein are likely to be important for overall function. An example of this can be seen in the conserved F$_\mathrm{X}$ binding region of PsaA. This region is absolutely conserved in every photosynthetic species examined, possessing a Type I reaction centre. It is possible that comparison of various PsaJ protein sequences may reveal conserved areas.

PsaJ protein sequences from several species were compared (see figure 5.2). The protein was found to be conserved among the different species examined. The PsaJ proteins of rice and maize were found to have the greatest similarity, being 95.2% similar. The C. reinhardtii PsaJ protein was also quite highly conserved among the different species examined, being between 64.3% (tobacco) and 78.4% (Synechocystis) similar.

5.4 Protein Analysis.
The amino acids were also examined in terms of their hydropathic properties and charge (see figure 5.3). The majority of the amino acids are hydrophobic. The bulk of these, approximately half, are located in the centre of the protein, and probably represent the membrane spanning region. Adjacent to this region are 4 hydrophilic, polar amino acids, that were found to be absolutely conserved among the species examined. On the other side (the carboxyl side), the hydrophobic region is also flanked by conserved, polar amino acids. A pair of acidic residues adjacent to these are also conserved. The amino acids at the carboxyl end are all conserved to some degree, and are all hydrophobic. It is possible that they may interact with another PS1 protein.

It has been shown that a “positive-inside rule” applies to thylakoid membrane proteins,
<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydomonas</td>
<td>MKDFTTYLSTAPVIAITIWFTTAGLLIEINRYFPDPLVF</td>
</tr>
<tr>
<td>Euglena</td>
<td>MKYFTTYLSTAPVVAVLWFTLTASLLIEINRFPDIL</td>
</tr>
<tr>
<td>Maize</td>
<td>MRDIKTYLSVAPVLSTLWFGALAGLLIEINRLFPDALSF</td>
</tr>
<tr>
<td>Liverwort</td>
<td>MQDVKTLYSTAPVLATLWFGFLAGLLIEINRFFPDALVP</td>
</tr>
<tr>
<td>Rice</td>
<td>MRDKTYLSVAPVSTLWFGALRGLLIEINRLFPDALSFPP</td>
</tr>
<tr>
<td>Synechocystis</td>
<td>MKHFLTLYSTAPVLAAIWMTITAGILIEFNRFPDLLFHPL</td>
</tr>
<tr>
<td>Tobacco</td>
<td>MRDLKTYLSVAPVLSTLWFGALAGLLIEINRFFPDALTFPP</td>
</tr>
</tbody>
</table>

**Figure 5.2.** Protein sequence comparison of PsaJ sequences. Residues that are absolutely conserved among the species examined are shown in red. Residues that are different in only one species are shown in blue, and residues that are different in two species are shown in green. Residues that are different in two or more species are shown in black.
Figure 5.3. PsaJ protein sequences with polar (red), and hydrophobic (blue) residues highlighted. The hydrophobic membrane spanning region can be seen in the centre of the protein.
where positively charged residues (i.e. lysine and arginine) tend to be cytoplasmically exposed (Gavel et al., 1991). This allows predictions to be made about the transmembrane orientation of a protein, once membrane spanning regions have been identified. However, as PsaJ is a very small protein, and is composed of only 37-44 amino acids (depending on the species), it is difficult to accurately predict the orientation. However, in the PsaJ proteins of *C. reinhardtii, Euglena, Maize, Rice, Synechocystis* and Tobacco, the second amino acid residue at the amino terminal is either lysine or arginine. At the carboxyl end of the protein, neither of these charged residues are present. This may suggest that the PsaJ protein is positioned with its amino terminal stromally exposed. The carboxyl end of the protein is hydrophobic, and so may not extend out of the membrane, or may be involved in interactions with other proteins. This end is likely to face the lumen, if the “positive-inside rule” is followed. It has been suggested that PsaJ interacts with PsaF, and helps to stabilise it. PsaF is located in the lumen, and perhaps the hydrophobic carboxyl end of PsaJ interacts with it.

The secondary structure of the protein was also examined. Examination of a variety of proteins of known structure allows each amino acid residue to be assigned as an α-helical, β-sheet, turn or coil moiety. Prediction of protein secondary structure in this way is approximately 60% accurate (Chou and Fasman, 1978). The sequence of the *C. reinhardtii* PsaJ protein, and the predicted structure for each residue is shown in figure 5.4. From this prediction, PsaJ has no α-helical structure. The majority of the protein seems to be composed of β-sheet structures, separated by coils and one turn. The hydrophobic, membrane spanning region is also composed of beta sheets, with one coil section in the centre of the region. On either side, the hydrophobic region is flanked by coils. However, on the carboxyl side, a turn precedes the coil. The presence of β-sheet structures, and the lack of α-helices, in a thylakoid membrane protein is unusual. Most thylakoid membrane proteins are composed primarily of α-helices. It may be that this unusual aspect of the PsaJ structure is related to its function. Elucidation of the function may explain the reason for this atypical feature.
Figure 5.4. *C. reinhardtii* PsaJ protein sequence showing the predicted secondary structure of each amino acid residue. Residues predicted to form beta sheets are shown in black, residues predicted to form coils are shown in red, and those predicted to form turns are shown in blue.
5.5 Construction of the Null \textit{psaJ} plasmid

In \textit{C. reinhardtii}, the \textit{psaJ} gene is located upstream of the \textit{rps12} gene. Both genes are located on fragment 17 of an \textit{EcoRI} digest of the chloroplast genome. This fragment has been isolated and cloned into a pUC8 plasmid, creating plasmid p70-A (Lui et al., 1989). The \textit{psaJ} gene is flanked by two unique restriction sites, \textit{SpeI} and \textit{PacI}. Originally, it was intended that p70-A would be cut with these enzymes, and the \textit{aadA} cassette would be cloned in to replace \textit{psaJ}. However, difficulties were encountered when cutting with both enzymes, and so a two step plan was devised.

5.5.1 Cloning of \textit{aadA}.

p70-A was cut with \textit{SpeI}, and the plasmid ends blunt-ended with T4 DNA polymerase. The \textit{aadA} cassette was isolated from plasmid pUC-atpX-aadA, by digesting with \textit{SmaI} and \textit{EcoRV}. The 1.9 kbp \textit{aadA} fragment was purified by the GeneClean method. A ligation reaction suitable for blunt ends was performed with p70-A:SpeI and the \textit{aadA} cassette. \textit{E. coli} strain JM109 was transformed, and transformants were selected on 2YT or LB plates, containing appropriate amounts of spectinomycin. Transformants were selected, and grown for plasmid DNA extraction, as described in materials and methods.

5.5.2 Orientation of \textit{aadA}.

The ligation was blunt ended, and so the \textit{aadA} cassette will have been cloned into p70-A in two different orientations. Diagrams of both orientations are shown in figures 5.5A and 5.5B. A series of digestions were performed in order to orientate \textit{aadA}. The digestions performed, and the expected size of fragments is shown in table 5.1. A photograph of the restriction digests is shown in figure 5.6. One of the transformants with the \textit{aadA} cassette in the opposite orientation to \textit{psaJ}, called p70-A/aadA\leftrightarrow, was chosen to make the final construct.
Figure 5.5. The two possible orientations of the \textit{aadA} cassette in p70-N/\textit{aadA}--\textit{aadA} are shown. Figure 5.5A shows \textit{aadA} in the opposite orientation to \textit{psaJ}, while figure 5.5B shows \textit{aadA} in the same orientation as \textit{psaJ}. The restriction enzyme sites used to orientate \textit{aadA} are also shown.
<table>
<thead>
<tr>
<th>Enzyme(s)</th>
<th>p70-A</th>
<th>aadA in same orientation as <em>psaJ</em></th>
<th>aadA in opposite orientation as <em>psaJ</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>PstI</em></td>
<td>7.6 kbp</td>
<td>5.5kbp, 4kbp</td>
<td>6.5 kbp, 3kbp</td>
</tr>
<tr>
<td><em>XhoI</em></td>
<td>site not present</td>
<td>9.5 kbp</td>
<td>9.5kbp</td>
</tr>
<tr>
<td><em>PaeI</em></td>
<td>7.6 kbp</td>
<td>9.5 kbp</td>
<td>9.5kbp</td>
</tr>
<tr>
<td><em>PaeI</em> + <em>XhoI</em></td>
<td>7.6 kbp</td>
<td>1.52kbp, 7.97kbp</td>
<td>0.71kbp, 8.88kbp</td>
</tr>
<tr>
<td><em>PaeI</em> + <em>PstI</em></td>
<td>4.88 kbp, 2.72 kbp</td>
<td>0.62kbp, 4.88kbp, 4kbp</td>
<td>1.62kbp, 4.88kbp, 3kbp</td>
</tr>
<tr>
<td><em>PstI</em> + <em>XhoI</em></td>
<td>7.6kbp</td>
<td>5.5kbp, 0.91kbp, 3.09kbp</td>
<td>5.59kbp, 0.91kbp, 3kbp</td>
</tr>
</tbody>
</table>

**Table 5.1.** Table showing the restriction digests performed to orient the aadA cassette and the size of fragments expected for each orientation.
Figure 5.6. Restriction analysis of p70-A/aadA <-> transformants to orientate the aadA cassette in transformants selected for spectinomycin resistance. 1 µg plasmid DNA was digested with each enzyme and the fragments separated on a 1% agarose gel by electrophoresis. The gel was stained with ethidium bromide and visualised under UV light.

M- Ava I Bgl II markers (sizes are shown in kbp).
1-Transformant with aadA in opposite orientation to psaJ.
2-Transformant with aadA in the same orientation as psaJ.
Letters refer to enzyme(s): - a (Xho I), b (Pac I+ Pst I), c (Pac I+ Xho I), d (Pst I+ Xho I).
5.5.3 Creation of the Null \( psaJ \) plasmid.

The \( aadA \) cassette contains a \( XhoI \) site near the beginning of the fragment, and away from the coding region. After confirming that \( Nosi \) is an unique restriction site in \( p70-A/aadA \), the plasmid was cut with \( PacI \) and \( Nosi \). To prevent religation of any plasmid that was only cut once, and so still contains \( psaJ \), the restriction digest was purified using Gene Clean, and the larger fragment isolated (\( psaJ \) is contained on a 1.09kbp fragment). The 3' overhangs on the isolated fragment were blunted with T4 DNA polymerase. The plasmid was religated to itself, and used to transform \( E. coli \) strain JM109. Transformants were selected on 2YT or LB agar plates containing spectinomycin. Transformants were checked by \( PstI \) restriction digests, to confirm that \( psaJ \) had been removed. These are shown in figure 5.7.

5.5.4 Sequencing of the Null \( psaJ \) plasmid.

The null plasmid was sequenced to confirm that \( psaJ \) had been completely removed, and that \( aadA \) was in frame. The null plasmid, called \( pNullJ3 \), was purified by a Qiagen plasmid preparation, and sequenced with a 3'-primer designed to prime 120 base pairs downstream of the \( PacI \) site. The sequence obtained is shown in figure 5.8. The \( psaJ \) gene has been replaced with the \( aadA \) cassette, and the ATG start site of the cassette is marked.

5.6 Transformation of \( C. reinhardtii \) with the Null \( psaJ \) plasmid.

Attempts were made to transform wild type \( C. reinhardtii \) with \( pNullJ3 \), so that a mutant producing no PsaJ could be produced. However, this has not yet been successful.

5.7 Discussion.

Sequence comparison of PsaJ shows that this protein is conserved among species. Indeed,
Figure 5.7. *Pst* I restriction digest of possible null *psaI* mutants. The different sized fragments shows that the DNA in lane 2 is a null plasmid. Fragments were separated on a 1% agarose gel by electrophoresis. The gel was stained with ethidium bromide and fragments visualised under UV light.

M - *Ava I/BglII* markers (sizes are shown in kbp).
Figure 5.8. Sequencing gel of the *psaJ* null plasmid. The remains of the *PacI* site and the start codon of the *aadA* cassette (in green) are shown. The *rps12* sequence is shown in black and the *aadA* sequence is shown in red.

Sequencing was performed on plasmid DNA extracted by the Wizard miniprep procedure (see chapter 2), using the dideoxy chain termination method of Sanger et al. (1977), using a Sequenase II sequencing kit (USB), and the 3'-*psaJ* primer.
among closely related species the degree of conservation is startlingly high, being 95.2% similar between rice and maize. Clearly, if a protein is this highly conserved then it must have a valuable role to play.

Examination of the protein sequence shows the high degree of hydrophobicity of this spanning protein, which identifies it as a membrane protein. Although the “positive-inside rule” allows membrane to be orientated, the small size of the PsaJ protein makes this rule difficult apply. Nonetheless, tentative application of the rule orientates the protein with the amino end stromally exposed, and the carboxyl end lumenally exposed. The carboxyl end is also rather hydrophobic, and so probably interacts with another protein, rather than being free in the lumen. PsaJ has been shown to effect PsaF. In psaJ deletion mutants of Synechocystis sp. PCC6803, deletion of psaJ has also been shown to lead to a reduction in the steady state RNA levels of psaF. The amount of PsaF in the membranes of the mutant was also reduced (Xu et al., 1994). PsaF is a lumen protein, and it is possible that the hydrophobic carboxyl end of PsaJ, which is probably lumen exposed, may interact with this protein. the overall charge of this region is negative, and this may also serve to stabilise the PsaJ-PsaF interaction.
CHAPTER 6:- GENERAL DISCUSSION AND CONCLUSIONS.
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PSI is a large multi subunit complex, containing more than 12 polypeptides, encoded by genes located in both the chloroplast and nucleus. Little is known about how these various subunits are coordinated, and the overall interactions between the subunits is unclear. To obtain a clearer overall view of the photosystem, it would be beneficial if the functions of the unknown subunits could be identified.

The unicellular green alga, *C. reinhardtii*, is an ideal model organism for the study of PSI mutants. *C. reinhardtii* has many features (discussed in chapter 1) which makes it useful for this, but the primary reason is that, when supplied with a source of reduced carbon, it is able to dispense with photosynthesis. This property of *C. reinhardtii* allows photosynthetic mutants to survive. With the advent of the biolistics method for chloroplast transformation, the chloroplast genome of *Chlamydomonas* can be transformed relatively easily.

PsaA is one of the major subunits of PSI, and together with PsaB forms the backbone of the complex. PsaA contains a small region of amino acids that is among the most highly conserved in nature. This region contains the two cysteine residues that are believed to be involved in coordinating $F_X$. However, the roles of the other conserved amino acids are less clear. The role of certain of these amino acids was examined in Chapters 3 and 4. The role of subunits within a complex can be examined by creating mutants where that subunit is either deactivated or deleted. Chapter 5 describes attempts to create a null mutant of *psaJ*, a small chloroplast encoded protein. The exact role of PsaJ is unknown, and it was hoped that the creation of a null mutant would provide an insight into the possible role that it may play within the PSI complex.

In chapter 3 the role of the conserved residue aspartic acid 576 of PsaA was investigated. This residue is particularly important because, although cysteine 575 and cysteine 584
are generally believed to bind $F_X$, aspartic acid residues have been shown to serve as ligands to one or more iron atoms in some \([4\text{Fe}-4\text{S}]\) iron-sulphur centres (Canover et al., 1990). This aspartic acid residue may also help to stabilise the heterodimer by forming salt bridges with the neighbouring arginine residue. The possible roles that this aspartic acid residue plays were examined by substituting it with a leucine residue. This aspartic acid to leucine substitution resulted in a \(C.\ re\text{inhardtii}\) mutant that was unable to grow photoautotrophically. Examination of the quantity of PSI complexes in the D576L mutant, by green gel electrophoresis, revealed that the amount of PSI was vastly reduced, compared to that present in wild type cells. Western analysis also confirmed that the level of proteins PsaA/B, PsaC, and PsaF were reduced, compared to wild type \(C.\ re\text{inhardtii}\) cells.

Aspartic acid 576 is adjacent to cysteine 575, one of the cysteine residues believed to be involved in binding $F_X$. As such, it was expected that alteration of this residue would affect electron transfer through $F_X$. EPR analysis of the D576L mutant, however, showed that while electron transfer through $F_X$ was apparently not affected, the properties of $F_A/F_B$ were significantly altered. In the mutant, it appears that the electron is transferred to a single iron-sulphur centre, either $F_A$ or $F_B$, instead of both centres, as in wild type cells. The line width of the signals is also altered, and is narrower than that of wild type cells. This change in electron distribution and the narrowing of line width suggests changes may have occurred in the binding of PsaC to the heterodimer of PSI.

The mechanism of action of a particular mutation can be examined by creating second site suppressor mutants, which are capable of suppressing the original mutation. The original D576L mutation resulted in a loss of photosynthetic ability, so second site suppressors were selected for their ability to grow photosynthetically on minimal media. Green gel analysis of the suppressor mutants revealed that the amount of PSI complexes in the suppressors was increased, compared to the original D576L mutant. Western analysis also confirmed that the amount of PsaA/B, PsaC and PsaF in the suppressor mutants was
increased, compared to D576L, but reduced compared to wild type cells.

The altered EPR spectra of the D576L mutant suggested that changes had occurred in the binding of PsaC to the heterodimer of PSI. It seemed likely, therefore, that in the suppressor mutants the binding of PsaC to the heterodimer had been altered. Sequencing of the suppressor mutants chloroplast encoded genes, *psaA*, *psaB*, and *psaC*, which seemed to be the most likely candidates for a possible mutation, however, has shown that the suppressor mutation is not present on any of these genes. A small subunit of PSI, PsaJ, with no apparent function was also sequenced, but was found to be no different than that from wild type cells.

PSI contains both chloroplast and nuclear encoded subunits. As sequencing of the major chloroplast encoded PSI genes failed to identify the second site mutation, it became important that the mutation be isolated to the correct genome. Identifying nuclear or chloroplast mutations in *Chlamydomonas* sp. is relatively easy. *Chlamydomonas* sp. cells are either mt+ or mt-. All chloroplast DNA is inherited from mt+ cells, whereas all mitochondrial DNA is inherited from mt- cells. Nuclear DNA is inherited in a Mendelian fashion. Crossing of the two cell types results in four progeny cells. Each suppressor mutant (mt+) was crossed with a wild type strain (mt-). The backcross experiment showed that the suppressor mutation is, in fact, carried on a nuclear gene, rather than a chloroplast gene as was originally assumed. If the binding of PsaC had been altered, and a nuclear gene is responsible for it, the most likely candidates are PsaD or PsaE, both of which are closely associated with PsaC. All three proteins form a ‘cap’ on the stromal surface of the thylakoid membrane, and are in close association with each other.

However, if the binding of PsaC had been altered in the D576L suppressor mutants, one would then expect the EPR spectra of $F_A/F_B$ reduction to be different in both the original D576L mutant and the suppressor mutants. This is not the case. The spectra are essentially the same in both cases. Clearly, the binding of PsaC (and its subsequent effects on electron
transfer through $F_A/F_B$ has not been altered again in the suppressor mutants. This shows that the original idea that inhibition of photosynthesis was due to changes in electron transfer from $F_X$ to $F_A/F_B$ was incorrect.

Examination of the rate of forward electron transfer between $A_1$ and $F_X$ showed that the D576L mutant had a slower rate of electron transfer from $A_1$ to $F_X$ than wild type. In the suppressor mutants, the rate was found to recover. This suggests that the effect on electron transfer rates at this step is the factor responsible for lack of photosynthetic ability in the D576L mutant.

The EPR measurements suggest that although the binding of PsaC to the core heterodimer has been altered, this is not the main reason for the lack photosynthetic ability in the mutant. The D576L change still exists in the suppressors, which are effected in the same way as regards electron transport through $F_A/F_B$. The leucine substitution may have caused changes in the structure of PsaA, or that the uncharged leucine may have caused an alteration in the overall charge of that region, which may have an effect on $A_1$.

It would be valuable if the suppressor mutant mutation could be identified. Reconstitution experiments now need to be performed with PsaD and PsaE isolated from the suppressors. Reconstitution of PsaD and PsaC depleted D576L PSI complexes with each of these proteins would enable the altered component to be examined. If a mutation has occurred in either of these proteins, then a normal EPR spectrum for $F_A/F_B$ reduction would be seen, rather than the altered $F_A/F_B$ spectrum seen in the D576L mutant. Sequencing of the protein would then allow the change to be identified.

Chapter 4 reported on the successful creation of a series of random mutants in the conserved $F_X$ binding region of PsaA. This region contains a series of highly conserved amino acids. The high degree of conservation indicates that the amino acids have a vital role to play in the function of the complex. As it is impossible at present to predict with
any degree of accuracy the effect of substituting one amino acid for another, random mutagenesis is an effective tool for creating a large number of mutants within a defined region. Initially, it was assumed that it would be relatively straightforward procedure to create a fragment of DNA by PCR containing not only the random changes, but also a marker, that would allow for selection of the mutants. This would then circumvent the need for cloning, and would enable a library of mutants to be generated quickly. However, several different strategies failed to produce a transformable PCR product. It became apparent that the failure of the strategies used previously may have been due to the large size of the construct. A simpler approach, using a smaller PCR product containing only the random changes, which was then cloned into a plasmid containing a selection gene, was successfully implemented. Mutants were selected on the basis of their photosynthetic ability, as the aim was not to completely disrupt PSI, but to create subtle effects on the Fx binding region.

Three mutants were created, which had the following amino acid substitutions (wild type sequence is arginine, phenylalanine, proline, cysteine): N8 (tyrosine, lysine, glycine, cysteine), N9 (glutamine, isoleucine, threonine, cysteine), and N22 (serine, tyrosine, asparagine, cysteine). Green gel analysis showed that the mutants had slightly less PSI than wild type cells. EPR spectroscopy revealed that the rates of electron transfer through F_A/F_B were little different in the mutants, compared to wild type. However, this was expected, as the mutants are able to grow photoautotrophically.

All three amino acids chosen to be altered are very highly conserved among photosynthetic species. As such, it is expected that substituting these for other amino acids would effect the photosystem. Although minor, subtle changes were selected for, it is still expected that some differences between wild type cells and the mutants would be seen. However, this was not the case, despite substantial changes in charge and size. One of the most surprising things was that changing the proline residue did not appear to effect the the photosystem. Proline residues are usually found at turns, or ‘kinks’ in a protein, and
it was assumed that the high degree of conservation meant that altering this proline residue
would effect the photosystem. Webber et al. (1993) have also produced mutants of the
corresponding proline residue in \textit{psaB} in \textit{C. reinhardtii}. These mutants were also found to
assemble PSI correctly. These results confirm the results of Rodday \textit{et al.} (1995), who
changed the equivalent proline in PsaB to alanine and leucine. These \textit{C. reinhardtii}
mutants were found to be capable of PSI assembly and growth under photoautotrophic
conditions. However, the stability of the PSI reaction centre and the interaction with PsaC
in the mutants was decreased. Quinkal \textit{et al.} (1994) have also shown that when conserved
proline residues adjacent to the 2[4Fe-4S] ferredoxin of \textit{Clostridium pasteurianum} are
replaced, the reduction potential or enzyme function is not altered. The folding of the
protein is not altered either. However, when 2-D NMR studies were performed,
destabilisation of the structures most likely to be involved in hydrogen bonding within the
cluster domain was observed.

While each of the mutants N8, N9, and N22 have been shown to assemble PSI, and to
have electron transfer proteins similar to wild type cells, it would be interesting to
discover if the complex was as stable as that of wild type cells. If the changes to the
proline residue in these mutants causes the complex to behave in a similar fashion to the
mutant complexes of Quinkal and Rodday, then it is very likely that the PSI complexes
of N8, N9 and N22 will be less stable than wild type cells.

\textit{PsaJ} is a small subunit of PSI, approximately 4.5kDa in size. Its exact function is
unknown. In cyanobacteria, it has been shown that inactivation of \textit{psaJ} results in PsaE
being more vulnerable to chaotropic attack, and to the amount of PsaF being reduced.
Chapter 5 describes attempts to produce a null mutant of PsaJ in \textit{C. reinhardtii}, so that a
possible role could be assigned to PsaJ. Protein structure was also analysed so that possible
functions could be determined.

Sequence comparison of PsaJ shows that this protein is conserved among species, with the
degree of conservation being as high as 95.2% among closely related species, such as rice and maize. This high degree of conservation indicates that although the exact role of this protein is unknown, it must perform a valuable function to have been conserved so fully. Tentative application of the "positive-inside rule" orientates the protein with the amino end stromally exposed, and the carboxyl end lumenally exposed. The carboxyl end is also rather hydrophobic, and so probably interacts with another protein, rather than being free in the lumen. This may correspond to the results of Xu et al. (1994), where it was found that deletion of \textit{psaJ} in \textit{Synechocystis} sp. PCC6803 leads to a reduction in the steady state RNA levels of \textit{psaF}, and a reduced amount of PsaF in the membranes of the mutant. PsaF has been shown to be a luminal protein, and so the lumenally exposed hydrophobic carboxyl end of PsaJ may be able to interact with it, and help to stabilise it.

A null mutant of PsaJ has been created, with the \textit{psaJ} gene replaced with \textit{aadA}, conferring resistance to spectinomycin. The next stage is to transform wild type \textit{C. reinhardtii} with this recombinant plasmid, using the biolistics approach. Transformants would subsequently be selected for their resistance to spectinomycin. It would also be advisable to select transformants under dim light, in case the lack of PsaJ resulted in an impaired PSI, rendering the transformant incapable of photosynthesis. Once transformants are obtained, it is necessary to take them through several rounds of selection to single colonies, so that a homoplastic chloroplast genome is obtained. The mutants would then need to be sequenced, to confirm the absence of \textit{psaJ}. The photosynthetic ability of the mutants will also need to be established, so that the operational capability of the PSI complex can be determined. Western analysis of the mutants with PsaF antibodies should also be performed, to determine whether the situation that exists in \textit{Synechocystis} is also true for \textit{C. reinhardtii}. It would also be useful to perform Western analysis with antibodies raised against other subunits of PSI. Electron transfer through the PSI complex should also be examined by EPR. Although the PsaJ has been shown to be unnecessary for correct electron transfer in \textit{Synechocystis}, this may not be the case for \textit{C. reinhardtii}.
The results presented in this thesis have shown that aspartic acid 576 is a particularly important for PSI assembly and function. Alteration of aspartic acid 576 to leucine does not affect electron transfer through $F_X$. This seems to exclude the possibility of this residue acting as an alternative ligand for $F_X$. Instead, it has been shown to be important for the binding of PsaC, and for electron transfer through $F_A/F_B$. This D576L change also affects the $A_i$ reoxidation rate, which recovers in the second site suppressor mutants. The reason that the suppressor mutant regain their photosynthetic ability, while retaining the $F_A/F_B$ reduction spectra is unclear. However, leucine is an uncharged residue, compared to the negatively charged aspartic acid, and so it is possible that changes may have occurred in the overall charge of PsaA, which may have an effect on $A_i$. The second site suppressor mutation has been identified as being nuclear, and PS I contains several nuclear encoded subunits, who may be likely candidates. Identification of the suppressor mutation is now a priority, as this will provide more information about the interaction of aspartic acid 576 with the PSI complex.

A strategy has also been devised for random mutagenesis within a defined region. Using this strategy, three mutants in *C. reinhardtii* have been created. While these mutants are not dissimilar to wild type, further characterisation is required. A null *psaJ* construct has also been created. Transformation of *C. reinhardtii* with this construct should provide information about the function and interaction of PsaJ with PSI. The amino acid composition of PsaJ was also examined. It was found to be a relatively conserved, hydrophobic protein. Interestingly, the secondary structure of PsaJ reveals that it is more likely to be composed of $\beta$-sheets, rather than $\alpha$-helices, as most thylakoid membrane proteins are. This novel composition may have implications for the function of this protein.
CHAPTER 7: REFERENCES.
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