Proteins involved in the maintenance of the photosynthetic apparatus
in cyanobacteria and plants

A thesis submitted for the degree of Doctor of Philosophy
by
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ABSTRACT

Plant and algal chloroplasts evolved following symbiosis of a cyanobacterium-like photosynthetic prokaryote with the eukaryotic host. Although most genes have been transferred from the organelle genome to the nucleus the function of plastid proteins is often retained. It can be helpful therefore to complement the study of a plant gene by examining its role in a cyanobacterium or vice versa. Two genes were investigated in this way.

First was the predicted product of open-reading frame (ORF) sir0575 in Synechocystis sp. PCC 6803, a possible equivalent to the Arabidopsis thaliana protein Ape-1 (At5g38660). Similar genes are conserved in various photosynthetic organisms, suggestive of a continued function. *ape-1* mutant plants have deficient regulation of light harvesting and therefore the role of sir0575 deletion mutants was explored under different light conditions. Although an equivalent role was not identified in the cyanobacterium, there is evidence that the response to high light is perturbed in a *Synechocystis* insertional inactivation mutant of sir0575.

Second, a family of ATP-dependent metalloproteases encoded within the genomes of both *Synechocystis* and *A. thaliana* was investigated. The nucleic acid sequences for these are similar to the *Escherichia coli* FtsH but inactivation of homologous genes in the plant and cyanobacterium showed that they have important roles in photosynthesis. Using a chlorophyll biosynthesis double-mutant and light- or nutrient-deprivation experiments, the effect of inactivating *ftsH* genes on the turnover and assembly of the photosynthetic apparatus was monitored. This work showed that in *Synechocystis*, the product of the ORF sir0228 is needed in order to degrade photosystem II (PSII) not only after light-induced damage, as previously suggested, but under non-photoinhibitory conditions. Assays of dark-grown heterotrophic cells suggested that the PSII composition of cyanobacterial thylakoid membranes is regulated when photosynthesis is prevented, in the absence of light, and that the sir0228-encoded FtsH is vital for that PSII turnover process. Likewise, in *Arabidopsis*, the Var2 FtsH is necessary for turnover of PSII D1 protein damaged by high light, and it may also have a role in the turnover of the photosynthetic apparatus during senescence. Preliminary studies showed that Var2-2 mutant seedlings allowed to partially senesce were unable to remove PSII, much of their response to dark conditions being quite different to that of Col0 wild-type (WT) seedlings.

The effect of inactivating these FtsH proteins extends to photosystem I (PSI). The accumulation of PSI and pigments in sir0228* Synechocystis* after degradation of the photosynthetic apparatus was delayed, with a lag phase of 1–3 days compared with regreening WT. The function of sir0228 in PSI assembly may also involve trimer formation: cultures of sir0228* cells in trimer-favouring light conditions grow very poorly and lose PSI and chlorophyll. Unlike sir0228* Synechocystis, var2* A. thaliana* is not deficient in PSI but a delay in the return of pigment after etiolation requires more study.

The role of these proteins in the maintenance of the photosynthetic apparatus was established and appears to be conserved, in general, between the plant and cyanobacterium. Secondary effects of mutation upon carotenoid and fatty acid content in both *A. thaliana* and *Synechocystis* were also evident, suggesting that the numerous roles of proteases and chaperones in photosynthesis merit further investigation.
This thesis is dedicated to Ken Gordon, Elizabeth Bird and Gwen Callaway
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<td>$A_{750}$</td>
<td>Apparent absorption at 750 nm due to light scattering</td>
</tr>
<tr>
<td>AAA</td>
<td>ATPases associated with various cellular activities</td>
</tr>
<tr>
<td>ACP</td>
<td>Acyl carrier protein</td>
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<tr>
<td>$ape$</td>
<td>Acclimation of photosynthesis to the environment</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>c.f.u.</td>
<td>Colony-forming units</td>
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<td>Chl</td>
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<td>Columbia ecotype</td>
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<td>Filamentation temperature-sensitive</td>
</tr>
<tr>
<td>GL</td>
<td>Galactolipid</td>
</tr>
<tr>
<td>GLC</td>
<td>Gas–liquid chromatography</td>
</tr>
<tr>
<td>GT</td>
<td>Glucose-tolerant</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulphonic acid)</td>
</tr>
<tr>
<td>HL</td>
<td>High light</td>
</tr>
<tr>
<td>HLIP</td>
<td>High light-inducible protein</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>IM</td>
<td>Inner membrane</td>
</tr>
<tr>
<td>KanR</td>
<td>Kanamycin resistance cassette</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>LAHG</td>
<td>Light-activated heterotrophic growth</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LED</td>
<td>Light-emitting diode</td>
</tr>
<tr>
<td>LHC</td>
<td>Light-harvesting complex</td>
</tr>
<tr>
<td>lhca</td>
<td>Component of light-harvesting complex for photosystem I</td>
</tr>
<tr>
<td>lhcb</td>
<td>Component of light-harvesting complex for photosystem II</td>
</tr>
<tr>
<td>LL</td>
<td>Low light</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Mb</td>
<td>Megabases</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-Morpholino)ethanesulphonic acid</td>
</tr>
<tr>
<td>MGDG</td>
<td>Monogalactosyldiacylglycerol</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MV</td>
<td>Methyl viologen</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NASC</td>
<td>Nottingham <em>Arabidopsis</em> Stock Centre</td>
</tr>
<tr>
<td>ND</td>
<td>Not done</td>
</tr>
<tr>
<td>NPOQ</td>
<td>Nonphotochemical quenching</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>ORF</td>
<td>Open-reading frame</td>
</tr>
<tr>
<td>P700/P680</td>
<td>Primary electron donors of photosystem I/II</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phycobilisome</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PCC</td>
<td>Pasteur Culture Collection of Cyanobacteria</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>P&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Photosynthetic capacity; oxygen evolution in saturating light and carbon dioxide</td>
</tr>
<tr>
<td>PQ</td>
<td>Plastoquinone</td>
</tr>
<tr>
<td>psi</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>PSI/II</td>
<td>Photosystem I/II</td>
</tr>
<tr>
<td>q</td>
<td>Quenching</td>
</tr>
<tr>
<td>Q&lt;sub&gt;A/B&lt;/sub&gt;</td>
<td>Photosystem II plastoquinone electron acceptors A and B</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpa</td>
<td>Regulator of phycobilisome association</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>Rubisco</td>
<td>Ribulose-1,5-bisphosphate carboxylase–oxygenase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline-sodium citrate buffer</td>
</tr>
<tr>
<td>SP</td>
<td>Secretory pathway</td>
</tr>
<tr>
<td>SQDG</td>
<td>Sulphoquinovosyldiacylglycerol</td>
</tr>
<tr>
<td>SRP</td>
<td>Signal recognition particle</td>
</tr>
<tr>
<td>Tat</td>
<td>Twin-arginine translocation</td>
</tr>
<tr>
<td>T-DNA</td>
<td><em>Agrobacterium tumefaciens</em> DNA insert</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N', N', N', N'-Tetramethylethylene diamine</td>
</tr>
<tr>
<td>TES</td>
<td>Tris-ethylene diamine tetra-acetic acid-NaCl</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>t/m</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TMPD</td>
<td>Tetramethyl-&lt;i&gt;p&lt;/i&gt;-phenylene diamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>ycf</td>
<td>hypothetical chloroplast open-reading frame</td>
</tr>
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</table>
1. INTRODUCTION

1.1 Prologue

Many aspects of photosynthetic reactions and their regulation remain a mystery despite the understanding of photosynthesis as a whole. Although the electron transfer pathways are easily summarised, this apparent simplicity masks the complexity of the constant assembly, repair and adaptation of complicated molecular structures in response to small or large changes in the environment of the photosynthetic organism. Studying the role of proteins in both photosynthetic prokaryotes and eukaryotes is a route by which to explore these complicated networks of synthesis, turnover and regulation. The recent sequencing of a number of cyanobacterial and plant genomes allows us to confirm that many proteins are conserved between the two groups and enables us to examine how organisms, from the unicellular bacterium to the largest plants, photosynthesise effectively.

This thesis attempts to compare the role of two proteins in these regulatory and quality-control processes in *Synechocystis* and *Arabidopsis thaliana*. One, Ape-1, is involved in light harvesting and the other, FtsH, is apparently essential for turnover and assembly of components of the photosynthetic apparatus.

1.2 *Arabidopsis thaliana* as a model organism

In December 2000 the complete genome sequence of *A. thaliana* (Columbia ecotype; Col-0) was finally published [Arabidopsis Genome Initiative, 2000]. The plant has been used as an experimental model for many years – from the beginning of the last century. It has been a convenient experimental organism for so long because of its small size, profuse seed production and short generation time. In the 1980s, its relatively small genome (125 Mb) became another reason for its popularity with molecular biologists and geneticists. *A. thaliana* is now a fundamental part of plant biology, with huge volumes of information and well-tested protocols available. There are also numerous expressed-sequence-tag sequences, and many T-DNA and chemically-induced mutants are available, including the Var2-2 and Ape-1 mutants described here.

Despite the existence of so many mutants, only 9% of the proteins identified in the genome project were reported to have been characterised experimentally already. In fact,
only 69% of the predicted genes could be classified according to sequence similarity with proteins whose function was known. The authors of the *Arabidopsis* Genome Initiative (2000) report concluded that 30% of the proteins could not be assigned to a functional category. Although *A. thaliana* has been studied for such a long time, there clearly remains a great deal to be discovered.

The *Arabidopsis* Genome Initiative (2000) also predicted that up to 14% of proteins encoded by the nucleus are targeted to the chloroplast. These nuclear genes required by the organelle, in addition to its own, encode proteins ranging from structural components to *trans*-acting regulatory factors, all of which must be imported to the chloroplast from the host-cell cytoplasm. This makes a complex system. For example, it has been known for some time that a nuclear-encoded DNA-dependent RNA polymerase was necessary for the expression of housekeeping genes during early phases of chloroplast development [Leon & Arroyo, 1998], but the genome sequence of *A. thaliana* revealed that in total it encodes three nuclear DNA-dependent RNA polymerases plus four genes that encode single-subunit plastid-type RNA polymerases [*Arabidopsis* Genome Initiative, 2000].

A large part of the *A. thaliana* proteome is involved specifically in photosynthesis. Within the 154-Kb plastid genome of *Arabidopsis*, two-thirds of the 79 predicted genes encode photosynthetic membrane proteins. Meanwhile, 139 genes of the nuclear genome were predicted to function in photosynthesis [*Arabidopsis* Genome Initiative, 2000]. It is interesting to note, when comparing photosynthesis in prokaryotes and eukaryotes, that the components of metabolic pathways of *Synechocystis* and *Arabidopsis* are encoded by similar genes but the plant's genome sequence revealed multiple copies of many single-copy *Synechocystis* genes [*Arabidopsis* Genome Initiative, 2000]. It has been suggested that this may allow tissue-specific expression of proteins [Theologis et al, 2000], but some divergence of function could also have occurred.

In addition to the proteins that are needed for photosynthesis, vascular plants need to develop their different organs, e.g., for flowering, and various cell types. There are therefore unique proteins in plants that cannot be compared with equivalents in cyanobacteria. *Arabidopsis* contains hundreds of genes that are thought to operate in cell-wall synthesis, for example [*Arabidopsis* Genome Initiative, 2000]. Likewise, plants synthesise many secondary metabolites, and whereas *Synechocystis* encodes one cytochrome *p450* in its genome [Kaneko et al., 1996], for example, *A. thaliana* has a large family (approx.
of genes for these haem-containing enzymes that function in so many biosynthetic pathways \cite{Arabidopsis Genome Initiative, 2000}.

1.3 Cyanobacteria as model organisms

Cyanobacteria are key model organisms in the study of photosynthesis. The \textit{Synechocystis} genome sequence \cite{Kaneko et al., 1996} was the one of the first to be reported and, although it has over 20-fold the coding potential of many plastid chromosomes \cite{Sokolenko et al., 2002}, many components of the photosynthetic apparatus are comparable. The likely role of a cyanobacterium-like prokaryote as the original endosymbiont(s) that gave rise to the chloroplast makes comparison of photosynthesis in plants and cyanobacteria particularly pertinent and valid. Meanwhile, cyanobacteria have many advantages over plants for study, given their ease of culture. In particular, \textit{Synechocystis} \cite{Figure 1.1} has proved invaluable because of its ease of transformation and its early sequencing. The 3.57-Mb \textit{Synechocystis} genome \cite{Kaneko et al., 1996} contrasts with the 125 Mb of \textit{A. thaliana}, despite the latter being small in comparison with many plants.

\textbf{Figure 1.1.} \textit{Synechocystis} sp. PCC 6803 cells [reproduced from www.ibvf.cartuja.csic.es/].

\textit{Synechocystis} is spontaneously transformable because it is naturally competent for DNA uptake and has a high efficiency of homologous recombination \cite[for details, see Kufryk et al., 2002]{Kaneko et al., 1996}. Not only are mutants easily constructed therefore, but glucose-tolerant (GT) strains of \textit{Synechocystis} are also facultative photoautotrophs, so severe effects on photosynthesis do not always result in loss of viability if glucose is supplied as a carbon source \cite{Williams, 1988}. As exploited in experiments described below (Chapters 6, 7)
*Synechocystis* is particularly useful for the study of chlorophyll greening and biosynthesis of the photosystems because in cyanobacteria most chlorophyll is associated with reaction centre core complexes rather than with the peripheral antenna(e). Finally, the first crystal structures of components of the photosynthetic apparatus were from bacteria. The cyanobacterial data provide more detailed structural information than is currently available from plants, notably the recent high-resolution structures of photosystem I (PSI) and photosystem II (PSII) from *Synechococcus elongatus* [Jordan et al., 2001; Zouni et al., 2001].

### 1.4 Evolution of the chloroplast

The long-standing hypothesis that endosymbiosis is responsible for photosynthesis in eukaryotes is well-established. It is now generally accepted that the semi-autonomous organelles of eukaryotes such as the mitochondrion and the chloroplast resulted when one organism gained new functions from the symbiont [for review, see Douglas, 1998]. It was these new functions that enabled the precursor of green plants to become autotrophic, needing only light, water, air and some minerals to survive. Similarly, hypotheses about evolution of the mitochondrion attempt to explain the metabolic reasons why such mergers would take place. For example, symbiosis between a methanogen and a eubacterium able to excrete H$_2$ and CO$_2$ could become fixed as earth’s environment changed, the host’s dependence upon the molecular hydrogen produced by the symbiont selecting in favour of the merged ‘eukaryotic’ cell [Martin & Muller, 1998; Doolittle, 1998]. The publication of the sequence of the genome of *Rickettsia prowazekii* strongly supported the idea that one of its α-proteobacterial ancestors was the original mitochondrion [Andersson et al., 1998], but there has not been so convincing an identification of the exact organism that became the chloroplast. In particular, the difficulty tracking monophyletic or polyphyletic origins of the chloroplast has prevented a consensus being reached.

Chloroplasts in both plants and algae show varying amounts of reduction of their genomes, but in all cases most of the remaining genes encode photosystem subunits and components of the electron transport chain. In the *Arabidopsis* nuclear genome a high proportion of those proteins assigned to ‘metabolism’ and ‘energy’ categories were similar to known bacterial proteins, which supports the idea that many of the genes from a prokaryote-derived organelle have moved to the nucleus during ‘reductive’ evolution [Arabidopsis Genome Initiative, 2000]. It does seem likely that the original symbiont(s) was similar to a cyanobacterium. Some 806 predicted *Arabidopsis* proteins match 404 different *Synechocystis* proteins, for example, and a further 69 matched other partly-sequenced cyanobacterial...
genes [Arabidopsis Genome Initiative, 2000]. The most recent estimates suggest that a minimum of 18% of genes in Arabidopsis originated in a cyanobacterium [Martin, 2002]. Other pertinent features include the ribosomes of chloroplasts, which are 70S like prokaryotes, not 80S like cytoplasmic ribosomes [Snyder, 1985]. Chloroplast genes are also organised in the same way as prokaryotes: there are genes on both strands of the DNA, genes overlap in different reading frames, and there is co-transcription of functionally-related genes. There are similarities between chloroplast thylakoid lumenal targeting domains and cyanobacterial signal peptides [Smeekens et al., 1990] and, indeed, between the systems for thylakoid targeting and bacterial protein export [Schnell, 1998], suggesting that these pathways may be conserved from the original prokaryotic endosymbiont. The chloroplast is therefore very like cyanobacteria in character despite some contradictory features. Nearly one-fifth of the Arabidopsis chloroplast genes are thought to contain introns [Arabidopsis Genome Initiative, 2000], for example, a feature of eukaryotic cells.

It is unclear whether the uptake of the chloroplast-symbiont by the host occurred just once or independently in more than one organism because there are some striking differences between chloroplasts: this variable presence and absence of components of photosynthetic pathways in photosynthetic prokaryotes and organelles presents a complicated picture of the evolution of photosynthesis. As discussed below, whereas cyanobacteria possess phycobilisomes (PBS) for light-harvesting and green algae and plants contain membrane-intrinsic light-harvesting complexes (LHC), intermediate groups in terms of protein complexes and pigments occur. Red algae, for example Porphyridium cruentum, contain both PBS associated with PSII and a plant-like LHC associated with PSI, but have no appressed grana-type regions, chlorophyll b, nor protective xanthophyll cycle (see below) [Grabowski et al., 2000]. The diversity of molecular structures and pigments suggests no clear evolutionary pathway: a combination of lateral gene transfer and both gain and loss of structures or pigments through evolution seems the only plausible explanation for the complicated situation we see today, rather than the monophyletic origin favoured in earlier hypotheses [Doolittle, 2002].

1.5 Photosynthesis

The capture and conversion of light energy for biochemical energy is termed photosynthesis. This fundamental process has led from the development of primitive cells to the creation of an oxygenic photosynthetic form of life, profoundly changing the earth and resulting in the environment that exists today.
There are a number of different forms of photosynthesis, from that based on ion-pumping rhodopsins in the halobacteria to the anoxygenic reactions of green sulphur and non-sulphur bacteria, purple bacteria and heliobacteria [for review, see Blankenship, 2002]. The basic similarities in the chlorophyll-based photosystems suggest evolutionary links between them [e.g., Barber, 2002; Blankenship, 2002], but today the major proteins of photosynthesis may be simply divided by whether they ultimately reduce quinones (type II) or iron–sulphur clusters (type I). Whereas either a type I or a type II protein complex is found in most photosynthetic bacteria, plants, algae and cyanobacteria possess both, which work in series to oxidise water and produce reductant for chemical reactions in the cell. It is this classical chlorophyll-utilising form of light-driven electron transport found in the chloroplasts of plants and in cyanobacteria and their close relatives that is discussed here.

Oxygenic photosynthesis uses the energy from photons to move electrons across a membrane, a series of reactions transferring the electrons from water through to the strong reducing agent, reduced nicotinamide adenine dinucleotide phosphate (NADPH), a process coupled to the synthesis of adenosine triphosphate (ATP). The consumption of the ATP and NADPH in the formation of carbohydrates in the Calvin cycle then form the classical ‘dark’ reactions.

1.5.1 The Z-scheme

Much of the environment on earth today exists as a result of the conversion of carbon dioxide in the atmosphere to carbohydrates and the side-effect of oxygen production from water. The multiple steps of the process of oxygenic photosynthesis were only gradually elucidated, but the Z-scheme [Figure 1.2] proposed by Hill and Bendall (1960) summarised the knowledge of the time and is acknowledged as a historic achievement in the study of photosynthesis. The Z-scheme built on the understanding of the previous few decades, bringing together observations about the reaction centres and pigment antennae through to the enhancement effect. Although inevitably it has grown more complicated with definition, such as the allocation of roles to precise amino acids and discovery of additional components, the model stands today. Simply, it suggests the arrangement of two photosystems (PSII and PSI) in series, with intermediates in electron transport arranged according to midpoint redox potential [Figure 1.2]. Noncyclic electron flow in photosynthesis is shown, following light-induced charge separation in the reaction centre cores. Water is the electron donor for the process, the path progressing from water oxidation in the thylakoid lumen at the base of PSII, via the PSII core to the plastoquinone
pool and cytochrome $b/f$ complex, to plastocyanin/cytochrome $c_6$ and then to PSI which reduces ferredoxin/flavodoxin, finally providing electrons for the reduction of NADP$^+$ to NADPH.

Figure 1.2. The Z-scheme (top). Cartoon representing the complexes catalysing the reactions of oxygenic photosynthesis (below). The light-induced sequence transfers electrons from water at the manganese cluster, via tyrosine-161 of photosystem II (PSII) D1 protein, P680 chlorophyll(s), pheophytin, quinones $Q_A$ and $Q_B$, plastoquinone (PQ) pool, cytochrome $b/f$ complex then plastocyanin. From the P700 chlorophyll(s) in photosystem I (PSI), there is a second light-induced charge separation resulting in the flow of electrons via acceptor chlorophyll $A_o$, phylloquinone $A_1$, and iron–sulphur clusters $F_5$, $F_3$ and $F_0$ to ferredoxin (Fdx; or flavodoxin). LHC, Light-harvesting complex; NADP(H), nicotinamide adenine dinucleotide phosphate. [Reproduced from www.agron.iastate.edu/courses/agron317/; www.prenhall.com/horton/media/lib/media_portfolio/]
The current more detailed understanding of the electron transfer chain [for review, see Blankenship, 2001] is that charge separation occurs in PSI and PSII between a donor (P700 and P680 chlorophylls, respectively) and acceptor upon photons being harvested for excitation energy, the donor(s) in each case becoming a reductant upon excitation. In the case of PSII, it is known that a tyrosine (Tyr-161 or Yg) of the PSII ‘D1’ protein is the donor of the replacement electron re-reducing P680+, acting as a bridge in electron transfer between a water-splitting manganese cluster and the rest of PSII. It is the tyrosine rather than P680+ directly that causes the electron and proton to be taken from water in the water-oxidising complex. In PSI it is plastocyanin or cytochrome c, that becomes oxidised (see below for details).

1.6 Photosynthetic apparatus

All the pigment–protein complexes and the majority of the other components of the electron transport chain are located within the specialised membrane for photosynthetic reactions, the thylakoid membrane. A few are found on the surface of membranes. The arrangement of thylakoid membranes differs slightly between green plants/algae and cyanobacteria (discussed in more detail below, Section 1.6.4) but the components and operation are essentially the same in all oxygenic photosynthetic organisms. The ‘acceptor side’ of reaction centres is towards the stromal (or cytoplasmic in cyanobacteria) side of the thylakoid and the ‘donor side’ towards the thylakoid lumen. The electron transfer across the membrane is coupled with translocation of protons from stroma to lumen: it is this coupling of the electron flow in photosynthesis with the formation of a proton gradient across the thylakoid membrane that allows the operation of ATP synthase as mentioned above [for review, see Blankenship, 2002].

1.6.1 Subunits of photosystem II

PSII is the pigment–protein complex that catalyses the light-driven splitting of water into protons and oxygen. It is a ‘type II’ reaction centre, like that found and characterised early in photosynthetic research, in purple nonsulphur bacteria [Deisenhofer & Michel, 1989].

PSII occurs as a dimer and is localised in the thylakoid membrane along with the other major protein complexes of the photosynthetic apparatus. In higher-plant chloroplast thylakoids, the complex is localised largely in the grana, appressed, thylakoid membranes. This situation makes transport to the stroma-exposed regions necessary for assembly and...
repair (for more detail, see below); how functional and nonfunctional PSII is distributed has also been suggested to affect protection and energy dissipation mechanisms [for review, see Anderson, 2002].

**Figure 1.3.** Subunits of photosystem II (PSII). Left panel, Cartoon of plant PSII [by J. Nield; reproduced from www.ch.ic.ac.uk/klug/photosynthesis.html] showing reaction centre core-proteins D1 and D2 in red, chlorophyll a-binding CP43 and CP47 (in fact on opposite sides of the core) [see Barber, 2002] and the integral light-harvesting (Lhcb) proteins (green), and oxygen-evolving complex (at base of D1 and D2). Y, Tyrosine; Phe, pheophytin; Q, quinone; PQH$_2$, plastoquinol. Right panel [reproduced from Zouni et al., 2001], representation of a side view of cyanobacterial (Synechococcus elongatus) PSII from recent crystallographic work. D1, yellow; D2, orange; PsbH/I/K/L/X, blue; chlorophyll a head groups and haems, black line drawings; PsbO (33K protein), green β-sheet structure; cytochrome $c_{550}$, grey helical model.

High-resolution structural information has been available from various groups for some time [e.g., see Barber et al., 1999; Boekema et al., 1999; Rhee et al., 1998; Yakushevskaya et al., 2001; Zouni et al., 2001], and the crystal structure of cyanobacterial PSII has allowed some details about the subunits of the plant complex to be inferred. Within the reaction centre there is the heterodimer of D1 (encoded by *psbA*) and D2 (*psbD*), then the antenna proteins CP43 (*psbC*) and CP47 (*psbB*) proteins, and the oxygen-evolving complex at the lumen [the manganese cluster, (16/17, 23 and 33 kDa proteins; Figure 1.3]. The membrane-spanning chlorophyll a-binding CP43 and CP47 form the intrinsic antenna system of the PSII core, funnelling the excitation energy from the LHC to the reaction centre. CP47 also appears to be required for assembly of PSII centres, and it is thought that its large hydrophilic loop

26
may stabilise the water-oxidising complex. The functions of the components of PSII have been extensively studied and described although the precise composition of the primary donor remains enigmatic [for review, see Blankenship, 2002]. The roles of all the proteins are not fully elucidated, especially those of the small or peripheral subunits. The functions of PsbH, K, L and W (the latter being the only core reaction centre subunit of plants' PSII encoded by the nucleus) are not clear, for example, although they may have a role in the dimerisation of PSII. PsbH is also interesting because it is phosphorylated according to redox status [Allen, 1992]. The full subunit complement is not certain either: Kashino et al. (2002) recently identified a subunit, PsbQ, in a *Synechocystis* PSII preparation that was previously thought to exist only in the chloroplast photosystem. Otherwise, it is thought that PsbW, -P and -Y are present only in eukaryotes whereas PsbV (cytochrome *c*550) is only found in cyanobacteria and red algae [Blankenship, 2002].

Whereas the cyanobacterial PSII reaction centre only houses the extrinsic cytochrome *c*550, both systems contain the intrinsic cytochrome *b*559 (encoded by *psbE/F*) [Figure 1.3], the latter possibly acting in secondary electron transport via charge recombination rather than linear electron flow, to protect PSII against excess light, or perhaps serving as a docking site in the replacement of D1 protein [Barber & De Las Rivas, 1993; Jordan et al., 2001]. This highlights one of the features for which PSII is notorious: the key reaction centre protein, D1, is very susceptible to damage and has a uniquely high turnover rate [e.g., Kyle, 1985; De Las Rivas et al., 1992]. Part of the complexity of regulation of PSII is the co-ordinated removal and repair of the D1 protein (see Section 1.8.2).

**Light-harvesting**

Absorption of light by pigment molecules involves the conversion of photons into energy as excited electrons. The energy can then be transferred to other pigment molecules. In oxygenic photosynthetic organisms such as green plants and cyanobacteria, the pigment molecules of the antenna complexes capture actinic light and deliver excitation energy to PSI and PSII. The light-harvesting structures LHCI (for PSI) and LHCII (for PSII) of higher plant chloroplasts are formed by chlorophyll and carotenoid pigments noncovalently bound by the transmembrane proteins of the photosynthetic apparatus. Pigment–pigment interactions are under fine control so that energy loss is minimal: it has been stated that the organisation of light-harvesting in PSII is such that trapping can be virtually 100% efficient [Horton et al., 1996].
Several light-harvesting antenna complexes lie within PSII as a whole. LHCII binds approx. 70% of the chlorophyll in PSII, and is composed of the Lhcb proteins 1–3 [Figure 1.3]. Crystallisation showed the trimeric nature of LHCII, and that each monomer bound seven chlorophyll a, five chlorophyll b and two lutein molecules [for review, see Blankenship, 2002; Kuhlbrandt et al., 1994]. It absorbs light at around 650 nm [Keren & Ohad, 1998]. There are also three minor monomeric complexes in the peripheral antenna, CP24 (encoded by lhc6), CP26 (lhc5) and CP29 (lhc4), which together bind about 15% of the PSII chlorophyll [Ruban et al., 2003]. These appear to be involved in dissipation of energy to prevent damage to PSII. CP24, for example, is able to bind large amounts of violaxanthin, a pigment involved in nonphotochemical quenching (NPQ; see below) [Pagano et al., 1998 and references therein]. Interestingly, the minor component CP26 will form trimers to compensate for the loss of LHCII, even though it is normally monomeric [Ruban et al., 2003]. This shows the importance in photosynthesis of the correct structural organisation: ordered trimeric LHCs around PSII allows energy exchange between the PSII cores over the large chlorophyll antenna [Ruban et al., 2003]. The LHCII trimers have also been suggested to be stabilisers of the appressed grana membranes but other studies disagree [e.g., see Horton et al., 1996; Mustárdy & Garab, 2003; Yakushevsk et al., 2001].

1.6.2 Transfer from photosystem II to photosystem I

The electron carriers of the electron transfer chain are largely found in corresponding stoichiometry, but the importance of plastoquinone (PQ) [see Figure 1.3] is illustrated by the fact that it appears at a much higher ratio, measured variously at four- to eightfold the level of other individual components [for review, see Keren & Ohad, 1998]. The redox state of PQ is integral to PSII–PSI electron flow, and it appears to be a principal indicator (along with cytochrome b$_f$; see below) for many regulatory processes in photosynthesis, such as state transitions (see Section 1.7.2) [Pfannschmidt, 2003].

Cytochrome b$_f$ is the proton-translocating complex that catalyses electron transfer between the two photosystems, oxidising plastoquinol and reducing plastocyanin. It occurs as a dimer and is distributed relatively equally between the stromal lamellae and appressed grana lamellae of plant thylakoid membranes. In plants and cyanobacteria the major subunits are cytochrome f (encoded by petA) and cytochrome b (petB), the Rieske iron–sulphur protein (petC) and the subunit IV (petD) [for review, see Choquet & Vallon, 2000]. It also contains chlorophyll and carotenoid pigments, the roles of which are not known. As well as transferring the two electrons from plastoquinol to plastocyanin (or cytochrome c$_{552}$; see
below), the cytochrome $b_6f$ complex, like PQ, is integral to photosynthetic signal transduction pathways [Alfonso et al., 2000; Mao et al., 2002]. For example, a *Chlamydomonas* mutant lacking the complex cannot phosphorylate LHCII, a key regulatory mechanism [for reviews, see Keren & Ohad, 1998]. Only one of seven plant genes for cytochrome $b_6f$ subunits, petC, is not found in the *A. thaliana* chloroplast genome [Arabidopsis Genome Initiative, 2000], perhaps indicating the importance of the complex in these events. In cyanobacteria, where respiration is largely not located separately from photosynthesis, the cytochrome $b_6f$ complex also functions in the respiratory chain [Schmetterer, 1994].

The cytochrome $f$ subunit donates electrons to the small copper-containing protein, plastocyanin, which is then able to reduce the oxidised P700$^+$ of PSI. Versions of plastocyanin are very similar in all oxygenic photosynthetic organisms [for review, see Aitken, 1988]. An alternative intermediate, cytochrome $c_5$ ($c_{553-559}$), was known to be present in cyanobacteria and algae but was only recently identified in higher plants [Gupta et al., 2002; Wastl et al., 2002]. Both cytochrome $c_5$ and plastocyanin are localised in the thylakoid lumen where diffusion between grana and stromal complexes can occur as necessary.

### 1.6.3 Subunits of photosystem I

Like PSII, PSI is a thylakoid membrane pigment–protein complex. This photosystem is a ‘type I’ reaction centre, along with those found in green-sulphur bacteria and heliobacteria. Like PSII in higher plants, the PSI reaction centre is localised to a particular region of chloroplast thylakoids, in this case to the non-appressed stroma membranes and the grana margins [for reviews, see Anderson, 2002; Blankenship, 2002].

Much information about PSI is inferred from structural work using cyanobacteria. Here, PSI binds more pigment (96 chlorophyll $a$ molecules) than PSII (estimated at between 25 and 50 chlorophyll molecules) [e.g., Jordan et al., 2001; Myers et al., 1980; Rögner et al., 1990] and the recent crystal structure of the *Synechococcus elongatus* protein [Jordan et al., 2001] has confirmed its complexity. The cyanobacterial complex is composed of 12 different protein subunits: in *S. elongatus* the polypeptides of PSI are PsaA / B / C / D / E / F / I / J / K / L / M and X [Figure 1.4]. Plants contain an additional three or four subunits, G / H / N / O, but do not contain X, and angiosperms appear not to contain M [for review, see Scheller et al., 2001]. Along with the 96 chlorophyll molecules, there are also 31 other components: two phylloquinones, three iron–sulphur clusters, 22 carotenoids and four integral lipids.
Figure 1.4. Protein subunits and cofactors in a photosystem I monomer. Representations of side view (left) and stromal-view (right; crystallographic three-fold axis indicated by black triangle). Transmembrane \( \alpha \)-helices of membrane-intrinsic subunits represented as coloured cylinders; iron–sulphur clusters are orange/yellow [reproduced from Fromme et al., 2001].

PSI linear electron transport forms the second half of the Z-scheme, catalysing the transfer of electrons from plastocyanin or cytochrome \( \epsilon \), on the luminal side of the membrane, to ferredoxin or flavodoxin on the stromal side of the membrane. PSI also participates in the anaerobic process of cyclic electron transport, which contributes to the cross-membrane proton gradient and drives ATP synthesis but not NADPH formation. The central heterodimer of PsaA and PsaB is again intrinsic to the thylakoid membrane, and binds the primary electron donor(s) P700 and the acceptors \( \Lambda_i \), \( \Lambda_1 \), and \( \mathrm{F}_x \). Excitation energy from light brings about the singlet excited state of P700 that leads to charge separation and subsequent electron transfer, the electron transfer pathway then proceeding: chlorophyll \( \Lambda_i \) – phylloquinone \( \Lambda_1 \) – iron–sulphur cluster \( \mathrm{F}_x \) – iron–sulphur clusters \( \mathrm{F}_A/\mathrm{F}_B \). The cyanobacterial X-ray structure confirmed the widely-held view that P700 is a chlorophyll dimer, but the exact nature of the primary donor [for review, see Webber & Lubitz, 2001] and the precise route of electron transfer within the two possible pathways of the heterodimer remain the subject of some debate [e.g., Fromme, 2001; Muhiuddin et al., 2001; Scheller et al., 2001].

Many of the PSI subunits are transmembrane proteins, but PsaC, D and E are found on the stromal side of the thylakoid membrane, close to stromal loops of PsaA and PsaB.
[Figure 1.4]. The 8.9-kDa PsaC subunit provides ligands for the two terminal PSI electron acceptors, the iron–sulphur clusters $F_A$ and $F_B$. PsaD directly docks ferre(flavo)doxin and is essential for electron transfer to it, and may also stabilise PsaC. As with PSII, however, the role of many of the photosystem subunits has not been fully elucidated. For example, PsaE (8 kDa) is not essential for photoautotrophic growth, but it is thought to mediate the reduction of ferre(flavo)doxin and may be involved in cyclic electron transport around PSI in cyanobacteria [Yu et al., 1993]. Plants lacking the subunit were pale green and susceptible to photoinhibition [Varotto et al., 2000; for review, see Scheller et al., 2001]. As with PsaE, the roles of several subunits appear to differ in plants and cyanobacteria. Deletion of the 15.7-kDa PsaF, for example, prevents the binding of PSI and plastocyanin in Chlamydomonas, in which the plant/green algal subunit PsaN probably co-operates [Haldrup et al., 1998]. Arabidopsis plants lacking PsaF are barely viable, but an equivalent effect on plastocyanin has not been confirmed in cyanobacteria [see Scheller et al., 2001]. PsaF has also been suggested to affect the function of the phyloquinone(s) [Yang et al., 1998] and the binding of the PSI LHC [for review, see Scheller et al., 2001]. It is of interest in these studies because it, along with PsaD, was reduced in a Synechocystis FtsH mutant [Mann et al., 2000]. Finally, the PsaL subunit is also of note here because of its role in PSI trimerisation in cyanobacteria (see Chapter 6) but not in eukaryotes. It is generally argued that PSI in plants does not form trimers despite the presence of PsaL, but the 16.6 kDa subunit has long been thought responsible for trimerisation of cyanobacterial PSI [for review, see Chitnis, 2001]. The recent structures of S. elongatus PSI indeed showed PsaL near the axis of the PSI trimer, apparently forming most of the contacts between the adjacent monomers [Jordan et al., 2001].

Light harvesting

The PSI LHC delivers excitation energy from the antenna chlorophylls to the primary electron donor P700. The elucidated S. elongatus PSI structure [Jordan et al., 2001] contained its 96 chlorophylls along with 22 carotenoids: plant PSI, in contrast, contains an integral chlorophyll $a/b$-containing antenna, LHCI, as well as chlorophyll bound to the core proteins. LHCI is composed of proteins encoded by $lha1-4$, binding around 100 chlorophyll $a$ and $b$ molecules in addition to those found within the reaction centre core. Like LHClII, LHCI is an integral membrane complex based on a three-transmembrane helix structure. This aspect of light-harvesting proteins is of note since, despite the lack of intrinsic LHC in cyanobacteria, the three-helix structure is preserved within the cyanobacterial 'high-light inducible proteins' (HLIP) and chloroplast 'early light-inducible
proteins' (ELIP), suggesting an evolutionary link between apparently disparate antenna systems [for review, see Green & Dumford, 1996].

PsaG, one of the nucleus-encoded PSI subunits unique to eukaryotes, and PsaK, which is also present in cyanobacteria, have also been implicated in the binding of LHCl (and in state transitions) [Jensen et al., 2000] but this remains unclear since recent work showed spinach LHCl solely on the PsaE/F/J-side of PSI [Boekema et al., 2001b; for review, see Scheller et al., 2001]. The exact organisation of LHCl and the existence of heterogeneous subpopulations of PSI with different antenna compositions are also not clear. Grabowski et al. (2000) reported that P. cruentum LHCl trimers were found in sucrose density gradients, whereas Boekema et al. (2001b) observed eight monomers of LHCl in spinach PSI.

While considering the light-harvesting apparatus of PSI, it should also be remembered that state transitions (see below) involve the reversible association/dissociation of antenna complexes with the reaction centres. In the case of PSI, this has been suggested to involve its temporary association with Lhcb1 and -2, possibly via PsaH [Lunde et al., 2000], resulting in a PSI antenna composed of eight Lhca proteins plus some form of LHCII [for review, see Scheller et al., 2001].

1.6.4 Differences between plant and cyanobacterial photosynthetic apparatus

The work described here involves proteins found in both photosynthetic eukaryotes and prokaryotes, using A. thaliana and Synechocystis as model organisms. As implied above, the cyanobacterial photosynthetic apparatus contains protein complexes — PSI, PSII, cytochrome b6f and ATP synthase — that are functionally and structurally equivalent to the green-plant versions, except for the presence of the specialised light-harvesting apparatus, the PBS (see below) [Figure 1.5]. Many enzymes (e.g., ribulose-1,5-bisphosphate carboxylase-oxygenase; Rubisco) and factors involved in electron transport, (e.g., plastocyanin, ferredoxin) are very similar to those in plants. Indeed, the complexes as a whole when visualised by electron microscopy appear to be alike in plants and cyanobacteria [Boekema et al., 2001b]. As mentioned above, a few photosystem subunits present in plants are not present in cyanobacteria, and there are other small differences such as the three copies of the gene for the PSII D1 protein present in Synechocystis PCC 6803 [Williams, 1988], psbA1/2/3 (of which A2 and A3 are expressed, the latter more under high-intensity light) and two copies of the gene for D2 (psbD1/2). A significant difference is the arrangement of the thylakoid membranes in the chloroplast, which is physically distinct
from that in cyanobacteria. In plant chloroplasts, the thylakoid ultrastructure has evolved to comprise stacked (appressed, grana) and unstacked (stromal) regions, whereas in cyanobacteria the membranes can be simplistically described as forming concentric cylinders around the cell [Figure 1.6]. Despite their obvious importance, there remains debate over the precise arrangement of thylakoid membranes in both, how communication operates between different parts and where assembly and degradation occur for some proteins [for reviews, see Anderson, 2002; Mustardy & Garab, 2003]. In addition to the thylakoid ultrastructure, there is another important structural difference, as mentioned above: the PSI in *Synechocystis* and other cyanobacteria is present as monomers and trimers, but only monomers have been observed in plants.

Finally, contrary to the eukaryotic arrangement where respiration occurs within mitochondria, cyanobacterial respiration largely occurs in the same membranes as photosynthesis. The respiratory and photosynthetic electron transfer pathways both use the PQ pool and cytochrome b/f [Schmetterer, 1994], electron transport for respiration ultimately being diverted to cytochrome oxidase rather than PSI.

**Figure 1.5.** Photosystem II (PSII) in cyanobacteria [drawn by J. Nield, Imperial College]. Note the lack of intrinsic light-harvesting complex but the presence of the phycobilisome (PBS) antenna at the stromal surface of the thylakoid membrane. PBS and PSII not to scale.
Cyanobacterial light-harvesting

A critical difference when making comparisons of *Synechocystis* and *A. thaliana* is in the light-harvesting apparatus. In cyanobacteria the major pigments of the thylakoid membrane are chlorophyll *a*, *β*-carotene and zeaxanthin [e.g., Grabowski *et al.*, 2000] (see also Section 5.3). In contrast with plants and green algae, cyanobacteria (with the exception of prochlorophytes) contain no chlorophyll *b* and, instead of the LHC complexes, they (and some algae) contain PBS as the principal light-harvesting apparatus. The phycobiliproteins that make up the PBS are the major protein component of cyanobacterial cells and consist of the tetrapyrrole phycobilin chromophores linked with proteins [for review, see MacColl, 1998]. So-called linker polypeptides are not coloured but help in assembling the complex for most effective energy transfer [Glazer, 1988; Sidler, 1994].

The array of light-harvesting structures in photosynthetic organisms reflects the different conditions that they inhabit, maximising the absorption of light in different environments. In cyanobacteria, pigments suitable for varying light wavelengths in aquatic environments allow absorption of visible light of 495–650 nm [MacColl, 1998]. Some (although not *Synechocystis*) contain the red pigment–protein phycoerythrin, which absorbs between approx. 490 and 580 nm. In addition to phycoerythrin, and the principal pigment–protein in *Synechocystis* PBS, is phycocyanin (which absorbs at 615–640 nm) along with small quantities of allophycocyanin (absorbing at 650–655 nm). The PBS apparatus is arranged as a core of allophycocyanin around which is a fan of peripheral rods of phycocyanin [Figures 1.5, 1.7].
Allophycocyanin accepts excitation energy from phycocyanin and then transmits energy to the long-wavelength terminal emitters of the PBS, which finally transfer excitation to the reaction-centre core chlorophylls. It was previously thought that PBS dock only onto PSII but there is evidence that energy can also be transferred to PSI [Mullineaux 1992, 1994]. The transfer of energy between the PBS and reaction centres allows regulation of photosynthesis by directly altering docking to PSI or PSII [Figure 1.7] but how this occurs is not yet fully elucidated [e.g., Emlyn-Jones et al., 1999; McConnell et al., 2002].

Figure 1.7. Cartoon of phycobilisome structure showing allophycocyanin core (spheres) and phycocyanin (rods) diffusing over photosystems I and II [diagram by C. Mullineaux, University College London]. Photosystem I trimers not shown.

A further assembly for light-harvesting in cyanobacteria has also been described recently. In response to iron stress, where quantities of PBS and PSI may be reduced, *Synechocystis* 6803 was found to synthesise the CP43' (or IsiA) protein, which resembles the PSII core protein CP43. By forming an oligomeric ring-structure around PSI, the light-harvesting capacity of the cells appears to be greatly increased [Bibby et al., 2001; Boekema et al., 2001a]. This discovery, along with that of the ability of CP26 to compensate for loss of LHCII in plants [Ruban et al., 2003], suggests there may be more flexibility among photosynthetic light-harvesting than is normally described.

1.7 Acclimation to light

There is great interest in the adaptation of photosynthesis to the environment, especially since this is obviously an area where there is a practical application in crop science. One of
the best characterised responses to changes in the environment in photosynthetic organisms is that to the amount of incident light. Research over the last quarter-century has addressed the sites and mechanisms of light damage; the degradation and formation of proteins thus damaged; how damage is avoided; and how metabolic, adaptation and repair pathways are regulated in altered light levels.

Light not only provides the energy for the extraction of electrons from water and thus for the manufacture of ATP and reductant in photosynthetic organisms, but it also activates many photosynthetic regulatory pathways, protection mechanisms and protein synthesis. Light initiates and alters development: seedlings require light regulation for processes from chlorophyll production through to chloroplast biogenesis and the co-ordination of genes encoded within the nucleus and plastid. Although the redox state of the PQ pool is known to be a principal signal for acclimation in plants [Pfannschmidt, 2003; Pfannschmidt et al., 1999], sequencing the genome of *A. thaliana* resulted in about 100 genes being annotated as encoding proteins involved in light perception and signalling. Only about a third of these had roles already ascribed to them, such as the known photoreceptors, the red-/far red- and blue-/ultraviolet-A-light-absorbing phytochromes [*Arabidopsis* Genome Initiative, 2000].

Making the best use of absorbed light energy while avoiding oxidative damage caused by excessive excitation (i.e., photoinhibition) is clearly a major requirement for oxygenic photosynthetic organisms. Plants may change the angle of leaves or position of chloroplasts to optimise light absorption but, as essentially sessile organisms, they must respond to the local environment by altering growth or cell composition. This also applies to cyanobacteria, even to marine genera, since they must photosynthesise but are unable to modulate the intensity of the light they require other than by growing at different distances from illumination.

Changes made by photosynthetic organisms may be short-term or long-term. Short-term balancing of the energy distribution between the photosystems is possible, e.g., following reversible phosphorylation of the stroma-exposed N-terminal segments of LHCII polypeptides in plants, which regulates LHC coupling with the reaction centre cores. A conformational change in the complex as a result of the phosphorylation is thought to provoke dissociation of the mobile LHCII component in the state transition [Allen, 1992] (see below). Meanwhile, long-term changes require co-ordinated regulation of the
expression of many proteins, allowing plants to maximise their photosynthetic capacity in low-intensity light, whereas those exposed to high-intensity light need to increase the level of enzymes for $\text{CO}_2$ fixation and of electron transport components to utilise excess energy. Examples of such changes are the increase in the chlorophyll $a/b$-binding LHCs, particularly LHCII, in low light; in high light, LHCII is degraded whereas PSII, cytochrome $b_{59}$, ATP synthase and Calvin cycle enzymes increase. A common marker of acclimation in experiments is the chlorophyll $a:b$ ratio which rises in high light as a consequence of reduced LHCII, and the maximum rate of photosynthesis (photosynthetic capacity) which increases in parallel [for review, see Anderson, 1986].

Fluorescence yield is also a marker for acclimation because of redirected energy transfer from the LHC to PSII or between the two photosystems, fluorescence emission being observed mainly at PSII (although emission can be seen from PSI if cells are frozen at 77 K). Fluorescence is also a key method for observation of the dissipation of excess energy via quenching mechanisms. Where actinic light is excess to requirements for photochemistry, rapid energy transfer is obstructed and the energy of the excited electron is converted to another form and dissipated. 'Excess' light is a relative term: the light intensity, properties or availability of intermediate electron carriers and acceptors, or an imbalance between excitation of the photosystems because of the spectrum of actinic light can all determine the level of photochemical activity.

A constitutive safety mechanism to avoid the build-up of reactive species, such as long-lived singlet chlorophyll, triplet chlorophyll or singlet oxygen, is quenching via carotenoids [for review, see Niyogi, 2000]. Triplet chlorophyll is quenched via LHCII carotenoids and PSII $\beta$-carotenes which are closely associated with the photosystem chlorophylls. Carotenoids can quench singlet oxygen that forms as a result of triplet chlorophyll reacting with molecular oxygen. In addition, there are sustained quenching mechanisms that result in reduced quantum efficiency through acclimation (as mentioned above), termed $q$. The primary, regulated dissipation mechanism of NPQ ($q_{B}$) which can be rapidly-induced, however, is $q_{B}$ (named for membrane energisation) — the quenching of antenna fluorescence via harmless heat emission caused by a rise in membrane $\Delta p\text{H}$ [for review, see Horton et al., 1996]. This is distinct from the quenching described above that occurs as part of normal photochemistry, i.e., photochemical quenching ($q_{P}$), which reflects noncyclic electron transport. NPQ is a fast process that has been known for some time to be linked to carotenoid composition of the thylakoid membrane [Demmig-Adams & Adams, 1996].
with the PsbS protein (or CP22) now also shown to be essential for most NPQ [Li et al., 2000]. qE appears to function via the xanthophyll cycle, which is triggered via the increase in ΔpH because this activates the lumenal enzyme violaxanthin de-epoxidase. The mechanism for the consecutive, reversible, (de-)epoxidation of violaxanthin to antheraxanthin to zeaxanthin is known, but how quenching works is not fully elucidated. It is thought that formation of zeaxanthin may convert a light-harvesting pigment, violaxanthin, into a quenching (energy-sink) pigment, zeaxanthin, perhaps allowing the transfer of excitation energy from singlet chlorophyll to zeaxanthin [for review, see Niyogi, 2000]. It has also been proposed that the oligomerisation of LHC changes according to the level of zeaxanthin, aggregation of LHCII acting as a further (probably slower) mechanism of quenching [Horton et al., 1996; Horton, 2001]. However it occurs, the thermal dissipation of excess light at antenna level reduces the energy transfer to PSII, fluorescence emission and the formation of the harmful PSII triplet chlorophyll. There are further sinks for excess electrons, such as the reduction of thioredoxin, the water–water cycle around PSI and chlororespiration, but the relative contribution of these to dissipation mechanisms in vivo remains subject to debate [Niyogi, 2000].

The acclimation processes in cyanobacteria are similar in some respects but there are differences in NPQ in the apparent absence of any organised xanthophyll cycle. Cyanobacteria also respond differently to green plants and algae as a result of their different pigment composition. Under red or high-intensity light the amount of PSI/cell decreases in Synechocystis, with a resultant increase in the PSII:PSI ratio. In contrast, yellow light is strongly absorbed by the PBS so that the amount of PSI increases to compensate [for review, see Fujita 1994]. As implied by these reports, Fujita et al. (1994) suggested that it is the quantity of PSI that is altered in response to changing light conditions, principally by altering the level of synthesis.

The cyanobacteria that contain phycoerythrin are also able to adjust the ratio of phycocyanin to phycoerythrin in response to light wavelength, i.e., complementary chromatic adaptation. Green light provokes an increase in phycoerythrin content and red light an increase in phycocyanin [for review, see Tandeau de Marsac & Houmard, 1988]. Finally, state transitions, although apparently regulated by redox characteristics in a similar manner to plants [Mullineaux & Allen, 1990], operate differently as a consequence of the lack of intrinsic LHC. For more detail, see below.
1.7.1 Hypothetical proteins involved in regulation of light harvesting

Although the changes in the composition of the photosynthetic apparatus during acclimation have been well-characterised, the signals for this regulation and their transduction are not fully understood. With the aim of elucidating these mechanisms, the slr0575 open-reading frame (ORF) in *Synechocystis* was investigated. slr0575 was chosen because its predicted amino acid sequence was similar to that of a protein inactivated in an acclimation mutant. The mutant was identified among a set of T-DNA-disrupted *Arabidopsis* lines by monitoring photosynthetic efficiency in a chlorophyll-fluorescence imaging system (R. Walters and P. Horton, personal communication, 1999). The chlorophyll fluorescence parameter $\Phi_{PSII}$ (the quantum yield of PSII) is proportional to photosynthetic efficiency [for review, see Hall & Rao, 1999], a change in light conditions therefore revealing plants in which the $\Phi_{PSII}$ is different from WT, i.e., which cannot adapt properly. Amongst three mutants with aberrant $\Phi_{PSII}$ was the *ape*-mutant (acclimation of photosynthesis to the environment). The protein predicted to be encoded by the disrupted gene is 286 amino acids in length and estimated to be approx. 31.5 kDa in mass. Prediction programs such as ChloroP [www.cbs.dtu.dk/services/ChloroP] suggest that the Ape-1 protein is targeted to the thylakoid membranes: there is a 70–90-amino acid N-terminal extension (in contrast with the cyanobacterial version) probably forming the targeting peptide. Sucrose density gradients showed there was an increased tendency for aggregated LHC in the *ape*-mutant, resulting in the suggestion that Ape-1 may be involved in regulating LHCII as part of $q_t$ [Horton, 2001]. Western blots have not revealed the location of the protein, however, suggesting that it may be expressed only at low levels. Northern analysis shows constitutive expression of the gene in leaves, giving no clue to the role of the protein in changing light conditions, but the *ape*-1 mRNA was not present in roots and stems, implying a specific role in photosynthesis (R. Walters, personal communication, 2001).

As directed mutations in *Synechocystis* are relatively simple to make, the presence of a likely homologue of Ape-1 in this cyanobacterium made it an ideal organism in which to glean more information about the function of Ape-1.

1.7.2 State transitions

A second gene involved in the regulation of light harvesting was also of interest, namely *rpaC*, thought to mediate state transitions in cyanobacteria [Emlyn-Jones *et al.*, 1999]. As mentioned above, oxygenic photosynthetic organisms can balance the distribution of excitation energy between the two photosystems. The mobile pool of light-harvesting
apparatus moves from PSI to PSII or vice versa in a short-term (seconds to minutes), reversible acclimation mechanism known as the state transition. As described above, the transition is thought to be associated with the redox state of the PQ pool or cytochrome b$_6$f [e.g., Mao et al., 2002; Mullineaux & Allen, 1990] resulting, in green plants/algae, in the activation of a kinase that phosphorylates LHCII. In light for PSI (~710 nm), plastoquinol is oxidised. This is 'state 1', where LHCII is not phosphorylated and transfers energy to PSII. In contrast, LHCII–PSII-light (~650 nm; or for the PBS in cyanobacteria) causes the PQ pool to be reduced. Phosphorylated LHCII then dissociates from PSII, which may be monitored as a decrease in the fluorescence emitted: this is 'state 2'. As detailed above, the light-harvesting apparatus of cyanobacteria is the PBS rather than LHCII and the role of phosphorylation is also not clear, but in both plants and cyanobacteria there is evidence that the dissociated PSII light-harvesting apparatus is able to transfer a proportion of excitation energy to PSI [for reviews, see Allen & Forsberg, 2001; Fujita et al., 1994; van Thor et al., 1998]. In higher plants, this would involve migration between the appressed grana and the stromal domains of the thylakoid membranes. In Synechocystis, where the spatial arrangement is probably less complicated, work by Mullineaux et al. (1997) also supports the idea of the mobile, rather than spillover (where the PBS always remains associated with PSII), model of state transitions.

The state transition has also been postulated to be involved in the regulation of linear and cyclic electron flow, the latter favoured by state 2. It has been observed that phosphorylation of thylakoid membrane proteins causes some unstacking of appressed thylakoid membranes in spinach and Chlamydomonas, which in the latter was accompanied by redistribution of cytochrome b$_6$f. If these changes were to relate to activation of cyclic electron flow around PSI, state transitions may therefore be regulated by the need for ATP or, indeed, regulate ATP level [for review, see Keren & Ohad, 1998].

The regulatory mechanisms involved in state transitions are probably complex and are not as well characterised in cyanobacteria as in plants [see Allen, 1992; Keren & Ohad, 1998; Haldrup et al., 2001]. Of note, however, is the previously-constructed Synechocystis mutant with an inactivated sll1926 ORF, which causes a specific defect in state transitions [Emlyn-Jones et al., 1999]. The protein encoded by sll1926, termed RpaC, may therefore be a factor in the regulation of energy transfer from LHC to photosystems. Whether this is a protein involved only in PBS regulation was not known, and therefore the presence of RpaC homologues in green plants was also investigated.
1.8 Assembly and turnover of the photosynthetic apparatus

The principal area of investigation described here concerns the assembly and turnover of PSI and PSII. The regulation of photosynthesis is such a complicated process that much remains to be learned. The central reactions and subunits of photosynthesis are now relatively well understood and the mechanisms of light acclimatisation are becoming clearer, but many questions remain unanswered about the synthesis and assembly of the protein complexes of the thylakoid membranes. Although DNA transcription to mRNA and subsequent translation to protein are clearly steps where regulation can occur, there has been a gradually increasing appreciation of the fact that post-translational control of proteins is a key factor in the synthesis and maintenance of photosynthetic apparatus [for reviews, see Adam, 2000; Choquet & Vallon, 2000; Estelle, 2001; Wollman et al., 1999]. The marked increase in recent work exploring the proteases and chaperones that act in the thylakoid membrane indicates the growing acknowledgement that these apparently peripheral processes are in fact important regulatory functions, essential for optimal photosynthesis.

1.8.1 Assembly

Proteins that regulate the assembly of photosynthetic complexes may be important not only during chloroplast biogenesis but throughout the life of the organism. As alluded above, acclimation in changing environments involves the biogenesis and turnover of the thylakoid membrane complexes as well as regulating light-harvesting components and quenching mechanisms.

The growth in research in this area has provided numerous candidates for roles in assembly and turnover of the photosynthetic apparatus, but the synthesis of these membrane complexes is so complicated and highly regulated that the processes involved are far from defined. Biogenesis of the thylakoid complexes requires the synthesis of the subunit and cofactor constituents, their transport to the site of assembly and operation, protein folding and insertion into thylakoid membranes, cofactor binding such as iron–sulphur cluster assembly, and the formation of the oligomeric complex itself, stages which may or may not be temporally and spatially separated.

The transport processes that allow the targeting of polypeptides to organelles and the co-ordinated assembly of photosynthetic apparatus, via twin-arginine-translocation (Tat), secretory (Sec), signal-recognition particle (SRP) or spontaneous pathways, have been
extensively characterised and reviewed [Drew et al., 2003; Schnell, 1998; Smeekens et al., 1990]. Nevertheless, the precise site of assembly for photosystems in cyanobacteria, for example, remains subject to some debate: it has been reported that PSII subunits are present in the cyanobacterial plasma membrane, suggesting the possibility of assembly or repair of photosystems there [most recently discussed by Andersson and colleagues, e.g., Norling et al., 2001]. This remains an intriguing question as it is not known whether the thylakoid and plasma membranes are linked [for review, see Mustárdy & Garab, 2003].

Nucleus-encoded proteins normally also have cofactors incorporated after they have been imported into the chloroplast — these processes have evolved to allow correct formation of the photosystems not only in prokaryotes but, in plants and algae, to allow co-ordinated synthesis, transport and assembly of subunits encoded within the nucleus and the chloroplast genome. The pathways must not only be tightly regulated for efficient synthesis: where chlorophyll-binding proteins are involved there is the possibility of free chlorophyll, which could act as a hazardous photo-oxidant unless controlled. The latter stages of chlorophyll synthesis, and possibly also of carotenoids, have therefore been proposed to occur in the chloroplast envelope [see Hoober & Eggink, 1999]. Particular amino-acid residues of Lhcb bind two chlorophyll molecules before continuing transport within the chloroplast envelope inner membrane, whereas the PSII D1 protein is degraded if it does not bind chlorophyll $\alpha$ and $\beta$-carotene [Choquet & Vallon, 2000; Hoober & Eggink, 1999]. Chlorophyll has also been suggested to be necessary for cytochrome $b_{6}$f synthesis but etioplasts that lack the pigment have been found to contain low levels of the enzyme so this is not certain. This investigation of the subunit assembly of the cytochrome $b_{6}$f complex showed that subunit IV and cytochrome $b_{6}$ interact early, followed by cytochrome $f$ and then other small subunits, and last by the Rieske protein [for review, see Choquet & Vallon, 2000]. These reviewers suggest that cytochrome $b_{6}$f provides a model for photosystem assembly, the stabilisation of the apoproteins with their cofactors being a prerequisite for assembly and, if unassembled, subunits are rapidly degraded. It also appears that there is assembly-controlled synthesis of one subunit and the complex becomes increasingly stable as more subunits are added.

Some studies have been made of the hierarchy and interdependence of subunit assembly in the photosystems, although there is not necessarily the same effect of missing subunits in different genera of cyanobacteria nor between cyanobacteria and higher plants. For example, regarding PSI, Anabaena lacking PsaC was able to accumulate functional PSI whereas in Synechocystis 6803 (which contains two different copies of psaC genes only one of
which is transcribed) PsaC seems to determine assembly of the subunits PsaD and PsaE [Yu et al., 1995]. Accumulation of the PsaA–PsaB dimer is also necessary for the assembly of the latter two subunits. It has been shown that PsaC is not necessary for assembly of the core dimer plus PsaF in *Synechocystis*, nor for electron transport from P700 to F$_s$ [Yu et al., 1995], but PsaC is one of the proteins that continues to be encoded by the chloroplast genome in eukaryotes, suggesting that it is very important. Indeed, in contrast, in *C. reinhardtii*, inactivation of PsaC prevented the accumulation of the PSI reaction centre [for review, see Scheller et al., 2001]

PsaD has itself been found to be necessary for the binding of PsaC to the reaction centre or at least to stabilise it [Choquet & Vallon, 2000; Yu et al., 1995]. PsaD is nucleus-encoded in plants and algae, so is synthesised in the cytoplasm with a leader peptide targeting it to the chloroplast. In etiolated seedlings, it is the first nucleus-encoded subunit to accumulate in the thylakoid membrane. Its assembly is thought to be spontaneous: overexpressed recombinant PsaD apparently can be inserted into existing PSI complexes, replacing native PsaD after transient dimerisation of the two subunits [Minai et al., 2001]. This is of interest here, since an FtsH mutant was shown to contain reduced PsaD [Mann et al., 2000]. This subunit may also affect trimerisation, since structural studies have shown direct or indirect contact between it and PsaI and PsaL, which are located in the region where cyanobacterial PSI trimerisation occurs [for review, see Scheller et al., 2001]. Mutants lacking PsaL and trimers seem also to have destabilised PsaD, reflecting their close proximity within PSI [Jordan et al., 2001]. Notably, PsaF was present even when PsaC/D/E were missing or reduced, suggesting its incorporation is part of a different process [Yu et al., 1995]. It was PsaF as well as PsaD that was reduced in the FtsH mutant, however [Mann et al, 2000].

Since a *Chlamydomonas* mutant unable to make chlorophyll in the dark does not translate mRNAs for D1, D2, CP43 and CP47 until cells are returned to light conditions, it was assumed that chlorophyll synthesis is necessary for stabilising PSII assembly. Nevertheless, some subunits accumulated in etioplasts in the dark, such as PsbW, PsbH, cytochrome $b_{559}$, and the oxygen-evolving-complex polypeptides. The rapid rise of the synthesis of these subunits during subsequent regreening has also resulted in the suggestion that they serve as a nucleus for the chlorophyll-binding proteins [see Choquet & Vallon, 2000]. Another example of the hierarchy of assembly in PSII is the need for D2 in order for accumulation of the other core polypeptide, D1, whereas D1$^+$ mutants contain reduced levels of CP47. In contrast, not only has cytochrome $b_{559}$ been shown to accumulate without any chlorophyll-
binding subunits, in eukaryotes and prokaryotes, but it seems to be necessary for full PSII synthesis, particularly for the oxygen-evolving complex [for review, see Choquet & Vallon, 2000]. As in PSI, however, small subunits are often dispensable for assembly or function, e.g., in the case of Psbl [Ikeuchi et al., 1995]. Also as seen with PSI, mutations in structural genes seem to be tolerated better in Synechocystis than in Chlamydomonas [Scheller et al., 2001], although it is not known whether this reflects more active synthesis of the photosystem to compensate or a less efficient system for removal of incomplete complexes.

Besides the requirements for a hierarchy of subunit synthesis and for the proteins involved in membrane translocation/insertion [Drew et al., 2003; Schnell, 1998; Smeekens et al., 1990], others proteins are needed to act specifically in chaperoning assembly. Potential chaperones suggested to be involved in maintenance of the photosystems are the chloroplast-localised proteins Hcf136 and Hsp70, which apparently aid assembly and repair of PSII [Meurer et al., 1998]. Overexpression of Hsp70 protected PSII and underexpression (using antisense experiments) resulted in increased sensitivity of PSII to high light [for reviews, see Choquet & Vallon, 2000; Zhang & Aro, 2002]. The slr0399 gene product has likewise been implicated in PSII assembly, potentially as a chaperone of the quinone Qₐ into the reaction centre [Ermakova-Gerdes & Vermaas, 1999]. The proteins encoded by ycf3 and -4 and btpA have been implicated in PSI biogenesis, all three being located near/in the thylakoid membrane [Schwabe et al., 2001; Takahashi, 1998]. Chlamydomonas mutants in Ycf3, a hydrophilic protein, possess no PSI although PSI genes psaA, -B and -C are transcribed and PsaA and -B are translated, suggesting that Ycf3 must be an essential chaperone. Chlamydomonas mutants in the membrane-located Ycf4, which also transcribed psaA, -B and -C [Takahashi, 1998], and BtpA have only reduced levels of PSI rather than absent PSI [Schwabe et al., 2001], as seen in the Synechocystis slr0228 FtsH mutant [Mann et al., 2000] (see below). Interestingly, the degree of loss of PSI in these Chlamydomonas mutants was apparently dependent on the light or nutrient environment in which the cells were grown [Schwabe et al., 2001]. Meanwhile, Shen et al. (2002a,b) have shown that the cyanobacterial protein RubA is transiently associated with a heterogeneous population of PSI monomers, and that rubA\(^{−}\) Synechococcus 7002 mutants are unable to grow photoautotrophically and have no PSI electron transport activity. The authors suggest the RubA is needed specifically for the assembly of the F₉ iron–sulphur cluster.

Wilde et al. (2001) have suggested a role in PSI biogenesis for another hypothetical chloroplast protein homologue, Ycf37 (ORF slr0171), in Synechocystis 6803. ycf37 homologues are similar to ycf3 in domain structure and are found only in algae and not in plants. Mutants in ycf37 have a decreased PSI:PSII ratio and increased phycocyanin:chlorophyll ratio, with 77K
fluorescence emission spectra similar to those from the slr0228- mutant (see below) [Wilde et al., 2001]. In maize, mutants in the gene hcf47 were deficient in PSI and PSII, whereas a hcf44 mutant had reduced PSI only, possibly as a result of a defect in the synthesis or assembly of PsaC [for review, see Scheller et al., 2001]. There is an intriguing mutant of barley, viridis-viridis, that apparently contains only approx. 2% active PSI but in which transcript levels of PSI subunits are all normal. The reason for the loss of assembly is unknown [Scheller et al., 2001].

As described for cytochrome bf, the hierarchical accumulation of subunits of the complexes of the thylakoid membrane is governed by the translation of some subunits being limited by the presence of another polypeptide, even when the subunits' gene transcripts are present, i.e., assembly-controlled translation. How such feedback might operate has been reviewed by Choquet & Vallon (2000). What they term 'control by epistasy of synthesis' requires not only transcriptional/translational control but also the proteins that stabilise or chaperone assembly. The phenotypes of mutants in the numerous candidates proposed to be involved (described above) illustrate the complexity of the synthesis of the photosystems. The assembly processes are also tightly linked to degradation, and thus to protease activities in the chloroplast or thylakoid, where incomplete, misassembled or damaged complexes must be dealt with, especially in the case of PSII.

1.8.2 Turnover of photosystem II
As well as assembly of the photosynthetic apparatus in new cells, there is of course the need for some of the same processes in the maintenance and repair of already-formed complexes. This is an area where chaperone and protease activities are closely associated, but the proteins and processes involved in turnover, as with assembly, are often unknown or subject to much debate. There are examples of individual protein subunit turnover including subunits of ATP synthase in Chlamydomonas, which may undergo cycles of degradation and replacement [Choquet & Vallon, 2000] and, as mentioned above, one subunit of PSI can integrate into an existing complex [e.g., Minai et al., 2001]. The best-characterised process of assembly linked to turnover, however, is that of the PSII core subunit D1. As described above, the psbA-encoded D1 is one of the chlorophyll-binding proteins of the core of PSII. It is particularly vulnerable to the effects of high-intensity light and an efficient repair cycle exists to counter this [Figure 1.8].

Damage to the PSII reaction centre in fact appears to be inevitable, and not limited to high-intensity light. The capacity for photosynthesis and carbon fixation are dependent on
many environmental factors, from temperature to carbon dioxide and nutrient availability. Back electron flow, recombination of the primary charge-separated pair in PSII (P680–/pheophytin+) and formation of PSII triplet chlorophyll and singlet oxygen can therefore occur at all light intensities [for reviews, see Horton et al., 1996; Keren & Ohad, 1998]. D1 degradation does seem to occur under all conditions, but the rate of damage is higher under excess light although this is exacerbated by other factors such as drought and temperature stress.

To allow repair of the D1 polypeptide, there is disassembly, proteolysis and re-assembly of PSII, newly synthesised D1 being inserted into the partially disassembled but pre-existing PSII complexes. [Figure 1.8]. Despite the existence of such an organised process, its slow kinetics mean that the rate of damage can outweigh the rate of repair, resulting in photoinhibition, i.e., light-dependent inactivation of photosynthetic electron flow. While the rate of repair is insufficient, photosynthesis will decrease, and reactive species can accumulate and cause more widespread damage to the thylakoid membrane [see Horton et al., 1996], emphasising the importance of the proteins involved in governing PSII repair.

Where damage to PSII occurs has been the subject of some debate, whether it is on the PSII acceptor-side components (over-reduction of Q, leading to increased P680+/pheophytin− charge recombination; production of triplet chlorophyll 'P680, or
generation of singlet oxygen), or on the 'donor' side, where prevention of oxygen evolution may generate Tyr$_1^+$ and P680$^+$ which can then oxidise proteins or pigments, leading to damage and degradation [De Las Rivas et al., 1993; Jansen et al., 1993]. Electron acceptors operating at the $Q_A^-$ site (silicomolybdate) restore some PSII activity suggesting that the acceptor side is affected, but not the primary photochemistry of water oxidation [for review, see Keren & Ohad, 1998]. Charge recombination can occur when the light intensity causes more electrons to flow than the PQ pool can accept, or the activity of cytochrome $b_6f$/plastocyanin/PSI is impaired. The importance of singlet oxygen in causing photoinactivation is also shown by experiments with low-intensity light in anaerobic conditions, where PSII is not lost [for review, see Keren & Ohad, 1998].

However the damage occurs, conformational changes are thought to occur in photoinhibited PSII and D1 protein that mark the latter for proteolysis. Phosphorylation of the D1 protein seems to regulate its turnover in higher plants [Aro et al., 1993], although phosphorylation of D2 has been proposed to be involved in algae and mosses [see Keren & Ohad, 1998]. Monomerisation of the PSII dimer is then thought to occur, perhaps allowing access of proteases to the complex. It is thought that repair requires the dissociation of PSII antenna and core proteins [Figure 1.8]. The release of oxygen-evolving complex and manganese atoms into the thylakoid lumen, perhaps as a result of D1 degradation, conversion of cytochrome $b_{559}$ to its low-potential form and removal of CP43 from the complex have all been suggested to occur [for review, see Choquet & Vallon, 2000], followed by migration of the monomeric PSII from appressed to the nonappressed stromal lamellae, in the case of plants, again allowing access for repair.

The degradation may involve more than one cleavage step – also the subject of some controversy [e.g., Kanervo et al., 1998] – because in vitro and in vivo studies have showed transient intermediate degradation products. The initial primary cleavage site of D1 is proposed to be situated between the fourth and fifth loops in the protein, close to the $Q_b$ binding site and to the corresponding loop of the partner D2 protein [Mattoo et al., 1984]. Two degradation products have been seen, of 23 kDa and 10 kDa [for review, see Barber, 1995; Kanervo et al., 1998] followed by further complete degradation [Spetea et al., 1999]. Different studies, however, found three fragments of 29, 23.5 and 17 kDa were generated in vitro following radioactive labelling of the D1 protein and acceptor-side damage [Greenberg et al., 1987], or reported that under donor-side limitation of electron flow or ultraviolet-B
irradiation of thylakoid membranes, the initial cleavage appears to be at the C-terminus, close to the manganese cluster [De Las Rivas et al., 1992; for review, see Keren & Ohad, 1998].

Precursor pD1 protein is synthesised in situ by ribosomes at the (nonappressed) thylakoid membrane, followed by insertion into the membrane, processing to mature D1 which allows the light-dependent reactivation of the manganese cluster, migration back to the appressed lamellae (in plants) for reassociation with LHC, and dimerisation. The regulation of the synthesis of new D1 polypeptides is complicated but appears to be dependent upon degradation and removal of D1 from the complex [see Keren & Ohad, 1998], whereas degradation may also be related to synthesis of D1 in Synechocystis [Komenda et al., 2000] although some studies disagree [for review, see Keren & Ohad, 1998].

D1 is unique among the PSII subunits in having such a rapid turnover but it should be mentioned that the D2 protein has also been reported to be damaged and degraded under high light intensities, albeit later and to a lesser degree than D1. In particular, ultraviolet-B light that accompanies visible light may cause photoinactivation, damaging the manganese cluster and D2 protein more extensively than other PSII components [Keren & Ohad, 1998]. The repair of ultraviolet-B-damaged D1/D2 may be the same as that of high-light-damaged D1 [see Glatz et al., 1999]. CP43, CP47, cytochrome b559 and PsbW can also be degraded, especially in extreme high light [Choquet & Vallon, 2000; Kanervo et al., 1998]. In fact, Rubisco and NADPH:protochlorophyllide oxidoreductase are among a number of stromal enzymes that also exhibit increased turnover in the light [for review, see Adam, 2000].

PSI does not seem to be affected in the same way as PSII by high light, although electron flow via PSI to NADP⁺ may be reduced in photoinhibited thylakoid membranes in vitro, and under some circumstances PsA.B may be degraded [see Keren & Ohad, 1998]. An equivalent repair cycle to that for PSII is not known to exist, although it has been suggested that the lack of research into and identification of PSI photoinhibition is misguided [e.g., Sonoike, 1999, 2001].

1.9 Proteases and chaperones involved in photosynthesis

Until the sequencing of Synechocystis and Arabidopsis, most of the proteases and chaperones of the chloroplast and the photosynthetic complexes were a mystery. Comparison of predicted genes with those for characterised proteins, particularly prokaryotic proteases, now shows that the chloroplast is very well equipped for degradation, unfolding and
refolding of its proteins. Sokolenko and colleagues (2002) recently estimated that 2% and 1.2% of the predicted genes of *Synechocystis* and *A. thaliana*, respectively, encode proteases. That comprises approximately 60 in the cyanobacterium and around 300 in the plant. The *Arabidopsis* genome sequence showed a higher proportion of gene families of more than two members than seen in other eukaryotes [Arabidopsis Genome Initiative, 2000], and gene duplications seem to account for many of the extra proteases and chaperones in *A. thaliana*. Many of these in *Synechocystis* and *A. thaliana* are homologous, not surprising considering the evolutionary origin of the chloroplast, although both organisms contain a small complement that are unique to the prokaryote or plant [for review, see Sokolenko *et al.*, 2002].

Some of these proteins have been extensively investigated, such as various processing proteases that make a single endoproteolytic cut to reaction centre proteins, catalysing the removal of signal peptides and maturation of precursors to their final functional form. *Synechocystis* and *A. thaliana*, for example, both contain the carboxy-terminal peptidase CtpA, which processes precursor-D1 protein to mature D1 in most PSII-containing organisms, and numerous peptidases for modifying terminal amino acid residues [for review, see Sokolenko *et al.*, 2002]. The classical chaperone machines, DnaK and GroEL are also represented. The genome of *Synechocystis* 6803 contains two groEL-type genes (one of which occurs in an operon with its partner groES), analysis of which showed light- and temperature-upregulated transcription. Heat-stress also provokes upregulation of transcription of the *Synechocystis* heat-shock proteins Hsp70, -60, -17 and -14 [Glatz *et al.*, 1999]. These small chaperonins are not well-characterised, but Hsp17 was associated with thylakoid membranes coincident with increased resistance to light and heat stress and, in *Synechococcus* 7942 and *Synechocystis*, DnaK and GroEL chaperonins have also been found in association with thylakoid membranes [Glatz *et al.*, 1999; Kovács *et al.*, 2001]. An Hsp70 was also identified among several chaperone and assembly factors in the pea chloroplast [Peltier *et al.*, 2000] and has been found to be transiently associated with the Rieske iron-sulphur protein of cytochrome *b*$_{f}$ [for review, see Ostersetzer & Adam, 1997].

In addition to these, there are several other families of proteases and chaperones (the delineation of their activities often being somewhat blurred; see below) in common between *A. thaliana* and *Synechocystis* whose roles are not fully understood. Some previously-unknown proteins have been identified from mutant-screens where photosystem content was abnormal (see above, Section 1.8.1), or others where pigment content was aberrant (e.g., the ATP-dependent serine protease SppA) [Lensch *et al.*, 2001]. Meanwhile, some are
from known families of proteases and/or chaperones and seem to function not just in one pathway but in several. Examples of these are ATP-dependent proteases such as the Clp or FtsH proteins, which are processive proteases that can completely degrade their substrates [e.g., Kihara et al. 1999]. Another such group, Lon proteases, are present in Arabidopsis but are not dealt with here because there are few (perhaps two predicted to be chloroplast-localised) and there is little evidence for, nor even much information about, their function in photosynthesis [for review, see Adam & Clarke, 2002; Suzuki et al., 1997].

A number of the chaperones present in cyanobacteria have been suggested to be important in protection of the photosynthetic apparatus, an interesting suggestion being that interaction with some of them may enhance photoprotection of PSII. This is not unlikely, since their role as chaperones is in refolding and insertion of proteins into membranes. As mentioned above (Section 1.8.1), overexpression of the chloroplast-localised Hsp70 protected PSII in Chlamydomonas [for reviews, see Choquet & Vallon, 2000; Zhang & Aro, 2002]. Hsp17 and DnaK/DnaJ/GrpE chaperones can all aid protein refolding after stress [see Glatz et al., 1999]. The molecular chaperones and proteases control the folding of other proteins by recognising hydrophobic regions that become exposed because of mis- or unfolding [e.g., see Ellis, 2003]. Chaperones can assist by binding and thus preventing aggregation, or can actively aid refolding. Failure to refold can then result in proteolysis. Unlike the DnaK/GroE chaperones that act within larger protein-folding pathways, Clp and FtsH are able to both unfold substrate proteins and present them to their own proteolytic sites. The Clp (caseinolytic proteases) are ATP-dependent serine proteases (those with His-Asp-Ser catalytic sites) that typically form barrel-shaped complexes containing two types of subunit. The catalytically-active ClpP, which may also exist as a (probably) inactive form ClpR, forms a heptamer whereas the regulatory/chaperone component, ClpA/C/D/X , forms a hexameric ring. Synechocystis encodes four ClpP, plus partner, proteins. In A. thaliana about 10 ClpP-like proteins, plus numerous partner Clps, have been showed to be chloroplast-targeted [Sokolenko et al., 2002]. The proteins are not membrane-located and are found in the stroma.

The Clp-type proteases are known to function in chloroplasts removing unassembled or misfolded proteins [Peltier et al., 2001]. Cytochrome b$_6$f accumulates in ClpP-deficient Chlamydomonas during nitrogen starvation and ClpP was therefore proposed to be responsible for regulated degradation of the complex [Estelle, 2001; Majeran et al., 2001]. Clp mRNAs were also seen to accumulate in A. thaliana soon after dehydration, salinity
stress or during dark- etiolation [Simpson et al., 2003]. A ClpP and ClpB were associated with light acclimation in *Synechocystis*, and a ClpP with PBS degradation [Barker-Åstrom et al., 2001]. ClpB proteins in contrast are not found in the usual Clp high-molecular-weight complexes and are thought to act alone as chaperones under stress conditions [Sokolenko et al., 2002]. The roles of Clp proteins in photosynthesis still require exploration, however. There is certainly evidence that they are important. ClpP1 is the only protease encoded by the *A. thaliana* chloroplast genome [Arabidopsis Genome Initiative, 2000] and it is also maintained in algal chloroplast genomes [Estelle, 2001]. A ClpP1 mutant did not become homoplasmic in *A. thaliana* nor *Chlamydomonas*, furthermore, three of four ClpP *Synechocystis* knockout mutants would not segregate to homozygosity [Sokolenko et al., 2002].

Serine-type proteases are also represented by the Deg or Htr proteases in *Synechocystis* (where there are three) and *A. thaliana* (encoding 14). *E. coli* DegP (HtrA) is a heat-stress protein, the crystal structure of which has been determined recently [Krojer et al., 2002] revealing a homo-oligomer as seen with many of the proteases/chaperones, here of two staggered trimeric rings. Again, both chaperone and proteolytic activity has been proposed for Deg proteins [for review, see Adam, 2000]. The other family members are HhoA (DegQ) and -B (DegS), the latter being membrane-bound in *E. coli* and possibly also in *Synechocystis* [for review, see Sokolenko et al., 2002]. Of the multiple copies in *A. thaliana*, four to six were predicted to be chloroplast-targeted [Adam et al., 2001], and Peltier et al. (2000) confirmed the presence of a DegP in the *Arabidopsis* chloroplast proteome. DegP1, -5 and -8 were also recently found to be located in the thylakoid lumen [Schubert et al., 2002]. Each of the possible *Synechocystis* equivalents to DegP, DegQ and DegS was light-dependent for its expression [Funk & Adamska, 2002] but DegQ (HhoA) seems to be involved in heat-stress response [Sokolenko et al., 2002].

The intrinsic light-harvesting protein CP47 is degraded upon release of the protein from the PSII core and, although the effect was small and autocatalysis could not be ruled out, *in-vitro* experiments showed a small reduction in degradation when a serine-type protease-inhibitor was used [Psylinakis et al., 2002]. Serine-type proteases have also been implicated in the turnover of PSII by protease-inhibitor experiments [see Psylinakis et al., 2002]. Following further *in-vitro* work, the DegP2 protein was proposed to be involved in an initial guanosine triphosphate (GTP)-dependent cleavage step of D1-protein degradation in plants [Haußühl et al., 2001], although *in vivo* experiments contradict this, since intermediate-sized degradation products are not always seen (e.g., Section 8.4) [Bailey et al.,
Synechocystis mutants in DegP- and DegS-like proteins showed no drop in D1 degradation in light stress either, and a drop in D1 degradation in DegQ (HhoA)− cells was seen when 8 h high light was accompanied by heat shock [Funk & Adamska, 2002]. It should be noted, however, that the cyanobacterial proteins are rather divergent from the plant versions, e.g., A. thaliana DegP2 is 200 amino acids larger at the C-terminus than the Synechocystis version [Haußühl et al., 2001], so distinct roles would not be surprising.

### Table 1.1. AAA protein consensus motif.

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### 1.9.1 AAA proteins

Notably for this work, A. thaliana databases [see www.arabidopsis.org/] currently annotate about 33 predicted proteins as unknown AAA proteases (ATPases associated with various cellular activities) [Kunau et al., 1993] among the plant’s proteases, along with numerous other AAA proteins already identified as belonging to particular subgroups, such as the N-ethylmaleimide-sensitive vesicle-fusion factor (NSF) and p97 (or Cdc48) ATPases [Arabidopsis Genome Initiative, 2000; Figure 1.9]. Also amongst the AAA proteins encoded by A. thaliana are at least 11 FtsH ATPases (see Section 8.2).

AAA proteins are characterised by a minimal consensus motif, this region adjacent to the Walker motif of nucleotide triphosphatases being sufficient to identify a member of this protein family [Table 1.1; Patel & Latterich, 1998]. They do, in fact, contain very highly-conserved regions of amino-acid sequence but are nevertheless involved in diverse protein regulatory processes by virtue of aiding synthesis, degradation or transport. For example, members of the AAA family operate in membrane traffic, in secretion pathways (NSF) and peroxisome biogenesis (Pas1), whereas FtsH metalloproteases aid assembly of protein complexes in mitochondria. Many influence the cell division cycle: six of the 19S regulatory cap of the 26S proteosome are distinct but homologous AAA proteins – Sug1p, Sug2p, Yta1p, Yta2p, Yta3p, Yta5p – while some are microtubule-associated proteins (the AAA katanin disassembles tubulin whereas FtsZ, the prokaryotic tubulin, is a substrate for the E. coli FtsH) [Anilkumar et al., 2001; Arlt et al., 1996; Langer, 2000; Patel & Latterich, 1998; Swaffield & Purugganan, 1997].

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The genome sequence of *Synechocystis* has revealed novel AAA proteins that operate, like other proteases and chaperones, under stress conditions. For example, the novel protein encoded by ORF slr0374 has recently been shown to be upregulated under iron-stress and is part of a stress-regulated operon. The gene is thylakoid-associated, it is similar to *ycfA*6, and the operon in which it was identified appears to be conserved in other PBS-containing organisms [Singh & Sherman, 2002]. Stress-responsive operons containing FtsH genes in *E. coli* and *Helicobacter pylori* have also been identified [for review, see Schumann, 1999].

### 1.9.2 FtsH proteases

FtsH proteins are the only membrane-bound members of the AAA family. They are ubiquitous in prokaryotes and the organelles of eukaryotes, but the first FtsH was isolated and is characterised best in *E. coli*. Deletion of its single FtsH is lethal: it is the only essential protease in the bacterium [Tomoyasu *et al.*, 1993]. Although no simple knockout mutants are available, suppressor mutations or overexpression of the FtsH revealed various phenotypes, giving a clue to the many roles of FtsHs but also resulting in multiple synonyms for the gene. The first described was *hfbA*, high frequency of lysogeny, due to degradation of the bacteriophage λ CII transcriptional activator. There is also the filamentation-temperature-sensitive (*fisH*) effect (later found to be due to a secondary mutation caused by the chemical mutagenesis, in a penicillin-binding protein), tolerance against the colicin antibiotics (*toIZ*) and mRNA stability (*mrc*) [for review, see Schumann, 1999].

Both membrane and soluble proteins seem to be substrates of FtsH. Reports of its activity have been accumulating gradually, now including degradation of: the heat-shock transcription factor σ in *E. coli* and *Caulobacter crescentus*, the *α*-subunit of the *H*-ATPase, the membrane-bound translocation channel subunit SecY and the lipid synthesis *fpxC* deacetylase enzyme [Akiyama, 2002; Fischer *et al.*, 2002; Ogura *et al.*, 1999; Schumann, 1999]. Reports also describe FtsH mutants of *C. crescentus* being defective in stress response and cell-cycle control [Fischer *et al.*, 2002]. A defect in degradation or assembly of cytochrome *c* oxidase subunit 2 was reported in a yeast FtsH mutant (*Yme1*; also called the yeast Tat-binding analogue, *Yta11*) [Nakai *et al.*, 1995]. Meanwhile, a human-mitochondrial *Yme1* homologue, paraplegin, causes respiratory defects in mitochondria resulting in a hereditary and progressive form of paraplegia [see Langer, 2000]. The yeast FtsHs have even been implicated in removal of an N-terminal presequence for processing of a mitochondrial protein to its mature form [Esser *et al.*, 2002].
Figure 1.9. AAA families (top) [http://yeamob.pci.chemie.uni-tuebingen.de/ AAA]. FtsH hexamer (middle) [Brannigan et al., University of York; Karata et al., 2001] and domain structure (bottom). TM, transmembrane; SRH, ‘second region of homology’.
As alluded above, Yme1 is reported to act as a chaperone [see Adam, 2000]. Likewise, the chaperone activity of the yeast Yta10 and Yta12 FtsHs in mitochondria is required for assembly of the ATP synthase [Arlt et al., 1996]. Chaperone activity was also suggested by export defects in β-lactamase or the outer membrane protein OmpA in FtsH-depleted E. coli, and by the interaction of FtsH with some proteins without degrading them [Schumann, 1999]. In addition, overexpression of GroEL/ES or Hsp chaperones can alleviate defects in FtsH-depleted cells [see Suzuki et al., 1997].

The typical domain structure of FtsH proteins [Figure 1.9] comprises two transmembrane spans, two Walker-type ATP-binding domains, the 'second region of homology' (SRH; necessary for ATP hydrolysis), a zinc-binding domain (proposed to be the catalytic site) [Karata et al., 1999] and a carboxy-terminal coiled-coil structure also essential for activity but not ATP-binding [Shotland et al., 2000b]. This makes them distinct from the Clp proteases in containing all active sites in one molecule.

Single FtsHs are approx. 620–750 amino acids in length, generally about 70–80 kDa in size. The larger yeast FtsHs such as Yme1, or Yta10 and -12 together, form oligomers of about 850–1000 kDa [Langer, 2000; Suzuki et al., 1997], and the Synechocystis slr0228-encoded FtsH has also been shown to form a large complex [Silva et al., submitted]. The E. coli and Thermus thermophilus proteins have been crystallised as hexamers [Krzywda et al., 2002a,b; Niwa et al., 2002] [Figure 1.9] and these are the models for the structure of other family members.

The membrane location of FtsH metalloproteases gives them a special place amongst the chloroplast and cyanobacterial proteases. It is thought that FtsHs with two transmembrane domains are anchored so that a small amount of the N-terminus and most of the C-terminus protrudes from the membrane, with a loop of about 70 amino acids extending into the cytoplasm between the two membrane-spans. This was shown to be the case in digestion experiments using the thylakoid membrane of spinach and one of its FtsH proteins [Lindahl et al., 1996]. Although the majority of FtsH homologues contain two transmembrane spans, some are predicted to contain only one, e.g., Yme1 in Saccharomyces cerevisiae. The presence of some proteins predicted to have one and some two transmembrane helices among the FtsHs in photosynthetic organisms has led to the suggestion that FtsH complexes may face in opposite directions, one on each side of the membrane [Langer, 2000; Mann et al., 2000]. This is the situation in the inner membrane of...
yeast mitochondria, where a complex composed of FtsH proteins Yta10 and Yta12 faces
the matrix whereas the Yta11 (Yme1) FtsH complex faces the opposite way, into the
intermembrane space [for review, see Adam, 2000; Langer 2000]. The N-terminus/
membrane-spanning regions are also thought to allow oligomerisation [Makino et al., 1999;
Shotland et al., 2000b]. The periplasmic region may be needed for regulation of the *E. coli*
FtsH by the HflK and -C proteins (see Section 10.5) and particular sequence similarity in
this region seen only in photosynthetic organisms has also led to the suggestion that
regions near/within the transmembrane domains are important for substrate targeting (see
Chapter 8) [Bailey et al., 2002].

The ATP- and zinc-binding sites are exposed to the cytoplasm (or stroma of the
chloroplast). ATP hydrolysis is not thought to be essential for proteolysis of small peptides
(although it may be for proper substrate proteins in vivo) but is needed for the unfolding of
proteins or their extraction from the membrane [Niwa et al., 2002]. The *E. coli* FtsH
degradates substrates progressively as they are translocated across the membrane [Kihara et
al., 1999] and models of action for FtsHs propose that soluble or membrane-embedded
substrate proteins may be pulled through the large central pore [Langer, 2000; Niwa et al.,
2002]. It has been suggested that the protease catalytic site is accessible via a narrow
channel in the ring-shaped oligomer [Shotland et al., 2000b]. Indeed, structural analysis of
nucleotide-bound FtsH showed that the resultant and large conformational change induced
the ATP-binding region to shift towards the transmembrane region, facilitated by a flexible
domain between the two. Repetition of the movement is proposed to transport the
substrate protein from the central pore to protease domain [Niwa et al., 2002].

*Photosynthetic* FtsH proteins

One of the arguments for the importance of Clp proteases in photosynthesis is the fact that
a *clpP* gene remains in the *A. thaliana* chloroplast genome. The transfer of *ftsH* genes to the
nucleus is not complete, however. Although all *A. thaliana* FtsHs are nuclear-encoded, the
primitive red alga *Cyanidioschyzon merolae* encodes a FtsH metalloprotease in its chloroplast
genome that is most similar to cyanobacterial and other algal chloroplast-targeted FtsHs
[Itoh et al., 1999].
Figure 1.10. Multiple sequence alignment of *Synechocystis* PCC 6803 (sll/rxxxx) and *Thermosynechococcus elongatus* (tll/rxxxx) FtsH proteins, arranged according to similarity, with partial sequence of a cloned *Synechococcus* PCC 7002 (7002part) presumed slrl604 homologue. Note that the proteins occur in corresponding pairs, suggesting which is the likely equivalent FtsH in each species. Alignment carried out using ClustalW [www.ebi.ac.uk/clustalw].
The greater propensity of *Arabidopsis* than other eukaryotes to contain gene families of numerous members [*Arabidopsis* Genome Initiative, 2000] is also evident with AAA proteases. Before the *Arabidopsis* genome was complete it was known that two of its FtsH proteins, FtsH1 and Var2, and one immunologically-identified homologue in spinach, probably had roles in photosynthesis since they were reported to be targeted to the thylakoid membrane [Chen *et al.*, 2000; Lindahl *et al.*, 1996, Takechi *et al.*, 2000]. The two *Arabidopsis* FtsH proteins were also identified in an investigation of the plant's thylakoid-lumen proteome [Schubert *et al.*, 2002]. Soon after publication of the genome, Adam *et al.* (2001) then predicted nine potential genes to encode FtsH-like proteins. Subsequently, Sokolenko *et al.* (2002) identified 17, of which at least 11 seem to be chloroplast-targeted. Searching the *A. thaliana* genome with the Var2-FtsH amino-acid sequence now yields at least 32 similar proteins, of which at least 15 are predicted to be localised to the chloroplast, although these contain a mixture of structural features (see Section 8.2).

Sokolenko and colleagues (2002) propose that anomalous FtsH proteins such as those missing the zinc-binding site, for example, may form an inactive regulatory partner in the complexes, analogous to some Clp subunits. This is an attractive argument but the situation in Clp and FtsH proteins cannot be identical: the proteolytic FtsH metalloproteases investigated so far are quite unlike the active-partner ClpP proteins in that they already contain all the domains necessary for recognition, unfolding and proteolysis, needing no partner to supply a missing segment. Notably, these potentially inactive homologues are not seen in *Synechocystis*, suggesting their comparatively recent evolution in *Arabidopsis*.

There are four FtsH-type proteins in *Synechocystis*, encoded by ORFs slr0228, slr1604, slr1390 and slr1463, while recently published *S. elongatus* genome sequences [Nakamura *et al.*, *in press*] reveal a corresponding set. A very similar FtsH from *Synechococcus* 7002 was also cloned during the course of this work (Section 3.7) [Figure 1.10]. Of the four *Synechocystis* inactivation mutants, slr1604" and slr1390" would not segregate to homozygosity, and no phenotype was found for homozygous slr1463". The slr0228" cells, however, showed a photosynthetic phenotype and growth was reported to be reduced under even moderate light intensity [Mann *et al.*, 2000].

Not only does the number of FtsH homologues in *Synechocystis* and *A. thaliana* argue for their acting in photosynthetic pathways, but microarrays have often showed up- or downregulation of *ftsH* genes under conditions such as high-intensity light (*Synechocystis* slr0228 and slr1390, along with ClpB2) [Hihara *et al.*, 2001]. Although altered transcription
of FtsHs has also been seen under salinity stress [Simpson et al., 2003] and iron stress [Singh & Sherman, 2000], changes in transcription of an alfalfa FtsH were reported to be modulated specifically by high light or cold acclimation rather than as part of a generalised stress response [Ivashuta et al., 2002]. The slr0228- and slr1604-encoded *Synechocystis* FtsH proteins were also both found to be associated with a PSII preparation [Kashino et al., 2002]. Another study found that an FtsH can degrade unassembled Rieske iron–sulphur protein *in vitro*, although under these circumstances degradation by this immunologically-identified FtsH was apparently dependent on zinc but was not stimulated by ATP [for review, see Choquet & Vallon, 2000; Ostersetzer & Adam, 1997]. The *Capsicum annuum* PftF (plastid fusion and/or translocation factor) [Summer & Cline, 1999b] seems to be involved in biogenesis of chromoplasts [Hugueney et al., 1995], lending weight to arguments that FtsHs may be involved in biogenesis of chloroplasts [Chen et al., 2000]. In the following work, therefore, the role in photosynthesis was investigated for the slr0228 FtsH in *Synechocystis* and for Var2 in *A. thaliana*. 

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2. METHODS

2.1 ORGANISMS AND GROWTH CONDITIONS

2.1.1 Cyanobacteria and algae

The Pasteur Culture Collection of Cyanobacteria (PCC) Synechocystis species 6803 was the principle cyanobacterium used in experiments. More than one ‘wild type’ (WT) is currently in use in laboratories: behaviour of both a GT and a motile strain was investigated (see Table 2.1). Synechocystis deletion mutants used were deficient in PSII (PsbD\textsuperscript{CD-}), the dark chlorophyll biosynthesis pathway (ChIL-) [Wu & Vermaas, 1995], desaturases (DesA/D\textsuperscript{+}) [Tasaka et al., 1996], or the ORFs slr0575 and slr0228 (see Chapters 3, 4). A second slr0228\textsuperscript{-} Synechocystis previously constructed in the motile strain was also utilised [Mann et al., 2000]. All strains used are listed in Table 2.1.

DNA from Synechococcus spp PCC 7002 and 7942, Calothrix sp. PCC 7601, Dactylococcopsis salina and Anabaena cylindrica, the green alga Chlamydomonas reinhardii and from the unicellular red alga Cyanidium caldarium was used in RpaC experiments. Sources are listed in Table 2.1.

Table 2.1. Strains of photosynthetic micro-organisms used in this work.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synechocystis</td>
<td></td>
</tr>
<tr>
<td>Synechocystis sp. PCC 6803 (GT)</td>
<td>W. Vermaas, Arizona State University</td>
</tr>
<tr>
<td>Synechocystis sp. PCC 6803 (motile)</td>
<td>N. Mann, University of Warwick (from PCC)</td>
</tr>
<tr>
<td>PsbD\textsuperscript{CD-} Synechocystis 6803 (GT)</td>
<td>W. Vermaas, Arizona State University</td>
</tr>
<tr>
<td>ChIL- Synechocystis 6803 (GT)</td>
<td>W. Vermaas, Arizona State University</td>
</tr>
<tr>
<td>DesA/D\textsuperscript{-} Synechocystis 6803 (GT)</td>
<td>N. Murata, National Institute of Basic Biology, Okazaki</td>
</tr>
<tr>
<td>PsaL- Synechocystis 6803 (GT)</td>
<td>C. Aspinwall, University College London</td>
</tr>
<tr>
<td>slr0575- Synechocystis (GT)</td>
<td>This work</td>
</tr>
<tr>
<td>slr0228- Synechocystis (GT, PsbD\textsuperscript{CD-} and ChIL-)</td>
<td>This work</td>
</tr>
<tr>
<td>slr0228- Synechocystis (motile)</td>
<td>S. Bailey, University of Warwick</td>
</tr>
<tr>
<td>Other:</td>
<td></td>
</tr>
<tr>
<td>Synechococcus sp. PCC 7002</td>
<td>D. Bryant, Pennsylvania State University</td>
</tr>
<tr>
<td>Synechococcus sp. PCC 7942</td>
<td>PCC</td>
</tr>
<tr>
<td>Calothrix sp. PCC 7601</td>
<td>M. Ashby, University of the West Indies</td>
</tr>
<tr>
<td>Dactylococcopsis salina</td>
<td>T. Walsby, University of Bristol</td>
</tr>
<tr>
<td>Anabaena cylindrica</td>
<td>D. Adams, University of Leeds</td>
</tr>
<tr>
<td>Chlamydomonas reinhardii</td>
<td>S. Purton, University College London</td>
</tr>
<tr>
<td>Cyanidium caldarium</td>
<td>D. Vernon/S. Brown, University of Leeds</td>
</tr>
</tbody>
</table>

GT, Glucose-tolerant; PCC, Pasteur Culture Collection of Cyanobacteria.
2.1.2 Arabidopsis thaliana

The strain of *A. thaliana* used as WT was a line derived from the original Columbia ecotype, the Col-0 bulk ecotype [Nottingham Arabidopsis Stock Centre (NASC) stock no. N1092]. Var2-2 seeds were also supplied by the NASC (Var2-2, stock no. N272). Initial experiments for the cloning of potential *rpAC* homologues in *A. thaliana* were conducted using DNA extracted from an Institute for Arable and Crop Research (Rothamsted) strain kindly donated by J. Pearson (University College London).

2.1.3 Cyanobacteria growth conditions

*Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942 and *A. cylindrica* were grown in BG11 medium [Castenholz, 1988] supplemented with 10 mM sodium bicarbonate, sodium thiosulphate [BDH, Poole, Dorset, UK] and 2-[(2-hydroxy-1,1-bis[hydroxymethyl]ethyl)amino]ethanesulphonic acid [Sigma, St Louis, Missouri, USA]. For solid media, BG11 was supplemented with 1.5% bacteriological agar [Difco, Sparks, Maryland, USA]. *Synechococcus PCC 7002* was grown in A+ medium [Schluchter *et al.*, 1996], with 1.5% agar for plate culture as above. Where required, glucose was added at a concentration of 5 mM. Antibiotics were added to media as detailed in Table 2.2.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin [Sigma]</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Chloramphenicol [Sigma]</td>
<td>25 µg/ml</td>
</tr>
<tr>
<td>Erythromycin [Sigma]</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Kanamycin [Merck, Darmstadt, Germany]</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Spectinomycin [Sigma]</td>
<td>50 µg/ml</td>
</tr>
</tbody>
</table>

Plates and liquid media were incubated at 30°C. Light intensity for liquid cultures was approx. 10 µmol/m²/s unless stated otherwise, and the orbital incubator was set to shake at 100 rpm. Cultures were not aerated by bubbling unless specified. Agar plates were illuminated at 5–10 µmol/m²/s.

2.1.4 Escherichia coli growth conditions

DH5α *Escherichia coli* [details in Brown, 1991] or ‘Epicurian Coli’ XL2-Blue Ultracompetent *E. coli* [Stratagene, La Jolla, California, USA] was grown at 37°C in Luria Bertani (LB) medium supplemented with ampicillin or kanamycin as required [Sambrook *et al.*, 1989].
2.1.5 Arabidopsis thaliana growth conditions

*Arabidopsis* was grown according to the guidelines developed by the NASC [nasc.life.nott.ac.uk/protocols/]. Seeds were sowed on the surface of multipurpose compost and overnight stratification (breaking seed dormancy) was carried out at 4°C to make growth synchronous. Plants were grown at 20±4°C, 16 h light. When large plants and delayed flowering was required for biochemical evaluation (since *A. thaliana* is very small and this can be problematic when producing sufficient material for experimental purposes), ‘short days’ of 12 h light were used to repress flowering. Illumination was low because of the sensitivity of Var2-2 to high light: plants were positioned in the growth chamber [Procema, Twickenham, Middlesex, UK] to receive 35 μmol/m²/s light.

Plants grown on agar media were sterilised and sowed by pipette in sterile 0.7% agarose [Sigma] onto half-strength (2.2 g/l) Murashige and Skoog medium [Sigma] in 1% plant cell culture-tested agar [Sigma]. The method for seed sterilisation was adapted from Bauwens *et al.* (1997). Seed was placed in a sterile Eppendorf tube and 1 ml 70% ethanol added. After gentle shaking for 2 min the seeds were allowed to settle. The ethanol was removed and 1 ml 5% v/v sodium hypochlorite [BDH, Poole, Dorset, UK] plus 0.1% Tween 20 [Sigma] was added. The tube was inverted every few minutes for 15 min to mix, and then seeds allowed to settle before removing the sterilising solution. Seeds were rinsed four times in the same manner with water before there were added to molten agarose for sowing.

**Bulking up seeds**

After ordering seed lines from NASC, several Col-0 and Var2-2 plants were grown through to flowering, and self-fertilisation of strains ensured by containing each inflorescence in an individual tube [Beta Tech, Ghent, Belgium]. When siliques began to ripen, Col0 and Var2-2 seeds were harvested and separated from other plant material using separate sieves. The seed was stored in the dark at room temperature.

2.1.6 Synechocystis growth experiments

Parallel cultures for growth experiments were adjusted to the same cell density according to cell scattering at 750 nm (λ<sub>750</sub>) at the subculture before and at the start of the growth experiment. Cultures of identical volumes (usually 50 ml) were grown in 100 or 200 ml flasks, in duplicate or triplicate as specified below.

Growth was measured by cell counting using a haemocytometer or by measuring cell density according to λ<sub>750</sub> [Unicam UV2 spectrophotometer, Cambridge, UK] and
calibration of the values to cell counts ($A_{750}$ of 1.0 is equivalent to $1.52 \times 10^8$ c.f.u./ml in the Unicam spectrophotometer). Doubling times during the exponential growth phase were calculated using linear regression on log plots [SigmaPlot; Statistical Packages for the Social Sciences, Chicago, Illinois, USA].

**Competition assay**

A competition assay was carried out as described by Ivleva et al. (2000) to follow the growth of WT and slr0575" mutant (see Chapter 3) *Synechocystis* in mixed culture.

To first observe growth rates in switching light intensities, parallel subcultures of WT and slr0575" *Synechocystis* were adjusted to equal cell density according to $A_{750}$. Growth curves were carried out in light that was altered from approx. 10 to 75 $\mu$mol/m$^2$/s every 24 h, for replicate slr0575" cultures with and without kanamycin to eliminate the possibility of any effect of the antibiotic on response. For the competition assay, an equal number of cells from WT and slr0575" cultures (grown in under standard conditions) were subcultured into duplicate 100-ml mixed cultures. (The slr0575" strain was first gently pelleted and resuspended in BG11 without kanamycin to avoid contaminating the mixed culture.) Cultures were subjected to the low light–high light switches approximately every 24 h. PCR was carried out on extracted genomic DNA (see Section 2.2.1) from the mixed cultures with primers for slr0575 and the kanamycin resistance cassette [Table 2.3] from $t=0$. Densitometric analysis using Optimas software [Media Cybernetics, Silver Spring, Maryland, USA] allowed the proportion of PCR product from either WT or insertionally-inactivated slr0575 to be monitored in the mixed cultures, thus revealing the proportion of each strain present over time in the changing light environment. For further details, see Section 3.4.2).

### 2.1.7 Light quality

**High light and low light conditions**

High-light conditions for photoinhibition or acclimation experiments were provided using custom-made light rigs supplying high-intensity white light (1300 $\mu$mol/m$^2$/s), one loaned by P. Nixon (Imperial College) and one constructed in-house. Both were water-cooled and contained magnetic plates to stir cultures, to ensure that a constant temperature was maintained. Where low light was required, neutral density (grade 0.6) filters [Lee Filters, Andover, Hampshire, UK] were used to give 2 $\mu$mol/m$^2$/s. Light intensity was measured using a LI-250 light meter [LI-COR, Lincoln, Nebraska, USA].
For high-light experiments, *Synechocystis* cultures were diluted to $A_{750}$ of approx. 0.1 to ensure that shading of cells within the culture could not be significant.

High light experiments with *A. thaliana* were conducted using the same high-light apparatus, but plants grown on agar were maintained at 22°C by positioning them in a tray of temperature-controlled water.

*Altered-spectrum light*

For light of particular wavelength, for exciting chlorophyll or PBS specifically, red [Medium Red; Rosco, London, UK] or yellow [Lee Green in combination with Deep Straw; Lee Filters] filters to give 600–700 or 450–660 nm light, respectively, were used to mask lights in the incubator. Red filters allowed light of intensity of 17 $\mu$mol/m$^2$/s and yellow filters 11 $\mu$mol/m$^2$/s.

Blue filters [Just Blue 079; Lee Filters] were used with cultures of slr0228$^-$ and DesA$^-$/Des$^-$ *Synechocystis*, giving a light intensity of 4 $\mu$mol/m$^2$/s to follow growth rates and production of monomers and trimers (Chapter 6). Neutral density filters (light intensity 5 $\mu$mol/m$^2$/s) were used on a further set of flasks to control for the effect of low light on growth.

**2.1.8 Chlorosis assays**

*Synechocystis*

Cyanobacteria can be provoked to degrade their photosynthetic apparatus by transferring them to phosphate- or nitrogen-deficient media (N. Mann, personal communication, 2000). Parallel subcultures of exponential-growth-phase WT and mutant were gently pelleted (3800 rpm for 8 min using a MSE Mistral 1000 benchtop centrifuge) [Global Medical Instrumentation, Albertville, Minnesota, USA] and resuspended in BG11−N (NaNO$_3$ omitted) or −P (without K$_2$HPO$_4$). These were supplemented with 5 mM glucose where GT *Synechocystis* strains were used. Cultures were incubated under standard conditions and samples taken during chlorosis. When cultures had yellowed (a few days for −N and up to 4 weeks for −P) the cells were pelleted once again and subcultured into whole BG11 for regreening. Time-course samples were again taken for measurement of growth rates, pigments and photosystem content and activity.

A second technique for removal of the photosynthetic apparatus without the disadvantages of widespread effects of nutrient deficiency was the use of the ChIL$^-$ mutant (for details,
see Chapter 7). The strain lacks the dark chlorophyll biosynthesis pathway and, therefore, incubation of cultures in the dark under heterotrophic conditions allows the gradual chlorosis of cells. Regreening occurs upon replacement of the cultures in the light.

To grow Chl^- cultures, 250 ml exponentially-growing cells were subcultured 1:1 in new BG11 supplemented with 5 mM glucose. After 24 h growth with glucose in the light, cultures were transferred to light-activated heterotrophic growth (LAHG) conditions (see Chapter 7). A light period of 15 min each day (white light; approx. 10 µmol/m^2/s) was supplied during dark treatment, and the cultures were sparged with filtered air. Time-course samples were made for quantification of growth rates and pigments, and photosystem content and activity. Glucose and BG11 were renewed in dark-treated cultures after 3 days, and supplementary BG11 was added when the cultures were returned to light.

*Arabidopsis thaliana*

Weaver & Amasino (2001) showed that primary leaves of *Arabidopsis* seedlings that have developed cotyledons and their first two true leaves are able to regreen upon return to normal illumination after beginning to senesce during a period of dark treatment. By treating Col0 and Var2-2 seedlings in this way, the loss and return of photosynthetic pigments and apparatus could be followed.

Seedlings were germinated on agar plates as above. When the synchronously growing plants had developed two primary leaves they were transferred to a dark growth chamber for 1 week. At t=0 and at 8 days (upon return to light), samples of leaves were taken to test oxygen evolution and prepare thylakoid membranes for quantification of PSI. Samples were then taken at 3-day intervals. Control seedlings of Col0 and Var2-2 grown under standard 16 h light–8 h dark cycles were tested in parallel.

### 2.2 MOLECULAR BIOLOGY TECHNIQUES

Routine DNA manipulation was carried out according to Sambrook *et al.* (1989). DNA was stored at −20°C in 10 mM Tris–HCl [Sigma] pH8 (for genomic DNA) or TE buffer [10 mM Tris–HCl pH8, 1 mM ethylene diaminetetra-acetic acid (EDTA); BDH].

DNA was separated on gels of concentration 0.6–1.2% agarose according to Sambrook *et al.*, 1989. DNA was visualised using ultraviolet light on the Gel-Doc system [UVP, Upland, California, USA]. Gibco BRL 1 Kb Ladder [Invitrogen, Paisley, Scotland, UK] or λ_HindIII
[Invitrogen] were used as molecular markers on DNA gels. Markers for protein electrophoresis were Rainbow Recombinant Protein Molecular Weight Markers of 10–250 kDa [Amersham Life Science, Little Chalfont, Buckinghamshire, UK]. Unless specified otherwise, centrifugation for molecular biology techniques was in the Heraeus Biofuge Pico microcentrifuge [Kendro Laboratory Products, Bishop’s Stortford, Hertfordshire, UK].

Restriction digests were conducted using enzymes supplied by New England Biolabs [Beverly, Massachusetts, USA] in the manufacturer's recommended buffer.

2.2.1 Genomic DNA

*Synechocystis* 6803, *Synechococcus* 7942 and 7002, *A. cylindrica* and *C. caldarium* genomic DNA was extracted from cells harvested from dense culture by centrifugation. The cell pellet was resuspended in 400 µl TES buffer [5 mM Tris–HCl pH8.5, 5 mM EDTA, 50 mM NaCl] and incubated at 37°C for 15 min with 100 µl of 50 mg/ml lysozyme [Sigma]. The manufacturer's protocol was then followed for the DNeasy Plant DNA kit [Qiagen, Crawley, West Sussex, UK] from the addition of RNase A onwards.

Total *Arabidopsis* DNA was prepared using the same kit, after breaking up plant tissues in liquid nitrogen using a pestle and mortar.

Concentration of DNA was quantified where necessary using the λ_HindIII marker of known concentration for comparison or, where more accuracy was required, the absorbance at 260 nm ×50 gives the concentration of double-stranded DNA (µg/ml) [Sambrook *et al.*, 1989].

2.2.2 Plasmid DNA

Plasmids used were pBluescript SK+/- [Stratagene] and pUC4K [Amersham Pharmacia Biotech, Piscataway, New Jersey, USA]. Plasmid DNA was prepared using Qiagen mini- and midi-kits according to the manufacturer’s instructions.

2.2.3 DNA purification

DNA was purified using the gel extraction technique from Tris–acetate–EDTA gels [Sambrook *et al.*, 1989] and the Qiagen Gel Extraction kit, or by using the Qiagen PCR Purification kit where gel extraction was not necessary. DNA was concentrated using ethanol precipitation [Sambrook *et al.*, 1989] as required.
2.2.4 Sequence data

The *Synechocystis* strain 6803 genome was searched and analysed using the WWW database CyanoBase [www.kazusa.or.jp/cyano/; Kaneko et al., 1996]. The *A. thaliana* genome was searched and analysed, when it became available, using the facilities at the Arabidopsis Genome Initiative [www.arabidopsis.org/] and TIGR [www.tigr.org/tdb/at/at.html].

The program DNASIS [Hitachi, San Francisco, California, USA] was used for analysis of DNA and protein sequence data. BLAST searches were carried out at the WWW site of the National Centre for Biotechnology Information [www.ncbi.nlm.nih.gov/]. Alignments were made using ClustalW [www.ebi.ac.uk/clustalw/] and transit peptides predicted using ChloroP [www.cbs.dtu.dk/services/ChloroP/].

2.2.5 Mutagenesis strategies

Several *Synechocystis* mutants (details in Chapters 3, 4) were made by the same strategy, essentially according to Williams (1988). Briefly, a forward primer was designed according to the sequence upstream of the ORF and a reverse primer downstream of termination codon, with a convenient restriction enzyme site near the middle of the fragment. The PCR-amplified fragment was cloned into pBluescript and the ORF mutated by insertion of the kanamycin resistance gene (Kan^R^) from pUC4K. The appropriate *Synechocystis* strain was transformed with the construct and selection made for kanamycin resistance. Transformed colonies were streaked out to allow full segregation and the genotype confirmed by PCR and/or Southern blot. Details of methods follow.

2.2.6 PCR

Primers [Table 2.3] were synthesised by Perkin-Elmer Applied Biosystems [Boston, Massachusetts, USA] or by MWG Biotech [Ebersberg, Germany]. PCR was carried out using the Expand High Fidelity PCR reagents [Roche, Lewes, East Sussex, UK] with *Taq* and *Pwo* (proofreading) DNA polymerases, according to the manufacturer's protocol. The GeneAmp PCR system 2400 [Perkin Elmer] thermocycler was used and cycles devised according to Newton (1995).
Table 2.3. Sequence of primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’ )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degenerate primers for sU1926</td>
<td></td>
</tr>
<tr>
<td>rpaC forward</td>
<td>CGG GTA CCN GCN AAY TGY MGN TTY GAY CAY TGG</td>
</tr>
<tr>
<td>rpaC reverse</td>
<td>CGG AGC TCR TCN SWY TCN CAN GGN ACR AAR TCN A</td>
</tr>
<tr>
<td>sU1926-2 forward*</td>
<td>GAY ATH TAY GGN CAR ATH ATH GTN TGG</td>
</tr>
<tr>
<td>sU1926-2 reverse*</td>
<td>RTG RTT NAR NAR NGT NGT NGT NAC RAA NS</td>
</tr>
<tr>
<td>rpaC primers</td>
<td></td>
</tr>
<tr>
<td>sU1926-1 forward</td>
<td>CGG GTA CCT TTG TIT GGG GCA GGG AC</td>
</tr>
<tr>
<td>sU1926-1 reverse</td>
<td>CGA GCT CTT AAT CGG ACT CCA CCG GGA C</td>
</tr>
<tr>
<td>slr0575 primers</td>
<td></td>
</tr>
<tr>
<td>slr0575-2f</td>
<td>GGG GTA CCC TAT TGC CGT GGG ACA ATT TGC</td>
</tr>
<tr>
<td>slr0575-2r</td>
<td>CGA GCT CAA GAT TTG GTC AAG CAC GTC</td>
</tr>
<tr>
<td>Competition assay primers</td>
<td></td>
</tr>
<tr>
<td>(for kanamycin cassette)</td>
<td></td>
</tr>
<tr>
<td>pk3</td>
<td>GAT TTT GAG ACA CAA CGT GGC</td>
</tr>
<tr>
<td>pk7</td>
<td>GAC TTG ACG GGA CGG GGC C</td>
</tr>
<tr>
<td>FtsH primers</td>
<td></td>
</tr>
<tr>
<td>ftsH-1 forward</td>
<td>CGC TGT GAA GAA AGG GAA AGT CGA G</td>
</tr>
<tr>
<td>ftsH-1 reverse</td>
<td>CTG AAT CGG GGA CAC ATA TGA AC</td>
</tr>
<tr>
<td>ftsH-2 forward</td>
<td>TCG GTA CCC TCA TCT CCC ACC CCT AAA ACC</td>
</tr>
<tr>
<td>ftsH-2 reverse</td>
<td>CGA GCT CCT CTC GCA CCT CAG CAT CTA C</td>
</tr>
<tr>
<td>slr0228-1 forward</td>
<td>GGT ACC CAA ACC CTC AAC CAG CTA CTA ACC G</td>
</tr>
<tr>
<td>slr0228-1 reverse</td>
<td>GAG CTC GAT CCA CCA AGC GAT CAA CCA C</td>
</tr>
<tr>
<td>Primers for sequencing</td>
<td></td>
</tr>
<tr>
<td>pBluescript constructs</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>AAT TAA CCC TCA CTA AAG GG</td>
</tr>
<tr>
<td>T7</td>
<td>GTA ATA CGA CTC ACT ATA GGG C</td>
</tr>
</tbody>
</table>

*Designed with reference to *Synechocystis* sU1926 and *Anabarna* putative homologue (see Chapter 3).

Code for redundancies in degenerate primers: M (A/C); R (A/G); W (A/T); S (G/C); Y (C/T); K (G/T); V (A/C/G); H (A/C/T); D (A/G/T); B (C/G/T); N (A/G/C/T).

2.2.7 Cloning

The fragment to be cloned was digested to prepare the ends of the DNA for ligation as required. Digestion of vector was checked by agarose-gel electrophoresis and then enzyme activity destroyed by heating the reaction mix at 65°C for 10 min. For blunt-end cloning, the linearised vector was treated with calf intestinal alkaline phosphatase [New England Biolabs] (1 μl added and incubated at 37°C for 1 h) to prevent recircularisation. Following gel extraction to purify the vector and fragment DNA, the ligation mixture was set up to contain fourfold the number of copies of insert as vector (which was diluted to approx. 20–60 ng/μl). Either 1/20 or 1/10 volume of ligase [Stratagene] was added to the reaction mix for sticky or blunt cloning, respectively, with buffer and ATP according to the supplier’s protocol [Stratagene]. The mixture was incubated overnight at 16°C for sticky-end ligations or at room temperature for blunt-end ligations.
2.2.8 Transformation of *Escherichia coli*

If using DH5α *E. coli* cells, an overnight culture was grown up in 10 ml LB broth to stationary growth phase. An aliquot of 750 μl was used to inoculate a second 10 ml LB culture and this was incubated for 3 h at 37°C, shaking at 200 rpm. The cells were pelleted at 4000 rpm/4°C for 5 min [Eppendorf 5403 centrifuge; Cambridge, UK] and resuspended in 10 ml chilled, sterile 100 mM MgCl₂. After incubation on ice for 5 min, the cells were pelleted as before and resuspended in 1 ml chilled, sterile 100 mM CaCl₂. The competent cells were left on ice or refrigerated overnight.

Competent cells were transformed by adding approx. 1 μg plasmid construct in 5 μl TE buffer to 100 μl cells and incubating on ice for 30 min, at 42°C for heat shock for exactly 1 min, then a further 10 min on ice. After adding 1 ml prewarmed LB broth the transformed cells were incubated with shaking for 1 h at 37°C and then spread on selective LB agar plates.

If using the Stratagene XL2 strain (see above), the manufacturer’s protocol was followed for preparation of cells and transformation.

For blue–white selection to detect insertion of a DNA fragment into the pBluescript cloning site and successful transformation of bacteria, 100 μl of 10 mM isopropyl-1-thio-β-D-galactoside [Sigma] and 100 μl of 2 mg/ml (in dimethyl formamide) 5-bromo-4-chloro-3-indolyl-β-D-galactoside [Sigma] were added to each LB agar plate. Antibiotic selection was used to confirm transformation of *E. coli* with vector containing the kanamycin resistance cassette.

2.2.9 Transformation of *Synechocystis*

*Synechocystis* strains were transformed according to Williams (1988). Briefly, an overnight liquid culture in exponential growth phase was adjusted to approx. 4×10⁸ cells/ml. DNA (1–5 μg genomic; 2–10 ng cloned gene in plasmid construct) in TE was added to 100 μl cells and incubated for 4 h at 30°C in the light. Transformed cells were then plated on BG11 agar (plus glucose in the case of PSII⁺ strains) and after 3 days overlaid with 0.6% agar containing sufficient selection antibiotic (kanamycin) to give a final concentration of 50 μg/ml in each plate. *Synechocystis* contains multiple copies of its genome and therefore can maintain mutated and intact copies of a gene in selective conditions [Williams, 1988]. It is important that transformants be passaged through several generations on selective plates.
to ensure homozygous mutants. Colonies selected were therefore subcultured through at least four plates in order to ensure full segregation of the mutation to all copies of the genome.

### 2.2.10 Hybridisation techniques

Electrophoresis of digested genomic DNA was carried out according to Sambrook et al. (1989). Depurination to fragment the DNA in the agarose gel was carried out in 0.25 M HCl (10 min) and the DNA was then denatured (1.5 M NaCl, 0.5 M NaOH; two 15 min washes) and the gel rinsed in neutralising solution (1.5 M NaCl, 0.5 M Tris–HCl pH 7.2, 0.001 M EDTA; two 15 min washes). The manufacturer’s protocol was followed for overnight capillary blotting of DNA to a Hybond-N+ membrane [Amersham Life Science].

The Stratagene Prime-It random primer kit was used for preparing radiolabelled probe and hybridisation of probe was carried out using the method of Church & Gilbert (1984). Using reagents supplied in the Stratagene kit, approx. 1 µl DNA for probe template and 5 µl random primer mixture in 9.5 µl distilled water were denatured by heating for 5 min at 100°C and were then cooled on ice. The denatured DNA was mixed with 5 µl 2'-deoxyadenosine-5'-triphosphate/2'-deoxyguanosine-5'-triphosphate/thymidine-5'-triphosphate mix in reaction buffer, 0.5 µl Klenow enzyme and 2.5 µl [α-32P]-2'-deoxycytidine-5'-triphosphate (111 TBq/mmol) [Amersham Life Sciences] and incubated for 10 min at 37°C. The reaction was stopped by addition of 200 µl of Church solution [7% (w/v) sodium dodecyl sulphate (SDS) [BDH], 0.01 M EDTA, 0.05 M sodium phosphate buffer pH 7.2] and boiling for 5 min.

The blot was moistened with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) before it and 20 ml prewarmed Church solution were placed in a hybridisation bottle [Hybaid, Teddington, Middlesex, UK] for prehybridisation at 65°C for at least 3 h. The prehybridisation buffer was replaced with 20 ml fresh warmed Church solution. The labelled probe was added to the bottle for incubation at 65°C overnight. The standard washing procedure for homologous probe was a rinse with 1% SDS followed by two 15-min washes at 65°C with buffer containing 10% (w/v) SDS and 0.04 M sodium phosphate buffer pH 7.2. Stringency of hybridisation and blot washing was altered [Bryant & Tandeau de Marsac, 1988], to allow sequence of lower similarity to bind probe where desirable, by adjusting the temperature of incubation and proportion of SDS in buffer.
After hybridisation, membranes were exposed to Kodak Biomax MS X-ray film [New Haven, Connecticut, USA] in intensifying cassettes at –80°C until satisfactory images could be developed using the X-OMAT automatic developer [Kodak].

2.2.11 Sequencing

DNA sequences were checked using automated cycle sequencing [ABI Prism 377 DNA sequencer] operated by A. Casal/L. Wood.

2.3 BIOCHEMICAL TECHNIQUES

2.3.1 Preparation of *Synechocystis* thylakoid membranes

*Synechocystis* thylakoid membranes were prepared by a method adapted from Rögnert et al. (1990). Briefly, several litres of dense cultures were harvested by centrifugation (15 000 g, 4°C, 10 min) using a GSA/SLA-1500 rotor in the Sorvall RC-5B centrifuge [Du Pont, Wilmington, Delaware, USA]. Cells were resuspended in 25% glycerol, 10 mM MgCl₂, 50 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (HEPES) [Sigma] pH7.6. The cells were broken by French Press (15 000 psi, two or three times) and centrifuged at 12 000 g, 4°C, for 15 min in a SS34 rotor in the RC-5B centrifuge. Thylakoid membranes were washed and harvested from the supernatant by ultracentrifugation (two spins of 16 000 g, 4°C, 45 min) using a T647.5 rotor in the OTD65B centrifuge [Du Pont]. Membranes were resuspended in *Synechocystis* thylakoid buffer (20% glycerol, 10 mM CaCl₂, 10 mM MgCl₂, 20 mM 2-(N-morpholino)-ethanesulphonic acid pH6.5, 0.5 M mannitol).

2.3.2 Preparation of *Arabidopsis* thylakoid membranes

*Arabidopsis* leaf tissue was weighed and then ground using a chilled pestle and mortar with chilled grinding buffer (0.33 M sorbitol [Aldrich, Milwaukee, Wisconsin, USA], 5 mM MgCl₂, 5 mM EDTA, 10 mM HEPES pH7.6). The homogenate was strained twice through four layers of muslin and centrifuged at 4000 rpm at 4°C for 10 min [Eppendorf centrifuge 5403]. The pellet was resuspended in a few millilitres of chilled wash buffer (0.33 M sorbitol, 1 mM MgCl₂, 1 mM EDTA, 50 mM HEPES pH7.6) and centrifuged as before. The pellet was osmotically shocked by resuspension in chilled 5 mM MgCl₂ and incubation for approx. 1 min before addition of an equal volume of chilled 0.66 M sorbitol. The thylakoid membranes were finally pelleted as above and resuspended in *Arabidopsis*.
HEPES pH7.6) before freezing in liquid nitrogen for storage at −80°C if not for immediate use.

2.3.3 Fractionation of Synechocystis thylakoid membranes

After measuring chlorophyll content, thylakoid membranes were diluted with Synechocystis thylakoid buffer to 1 mg/ml chlorophyll where possible, or diluted so that strains to be compared were of identical concentration. β-dodecyl maltoside [Sigma] was added (to 2%) and membranes stirred in darkness at 4°C for 30 min. After ultracentrifugation (330 000 g, 4°C 1 h in a TLA100.3 rotor in the DTL100 ultracentrifuge) [Beckman, High Wycombe, Buckinghamshire, UK] to remove insoluble material, the chlorophyll content of the supernatant was checked again. Equal preparations were loaded onto 22 ml sucrose density gradients (10–50% sucrose in 20 mM Tricine pH8.0, 10 mM CaCl₂, 10 mM MgCl₂, 0.5 M mannitol, 0.04% β-dodecyl maltoside) and centrifuged for approx. 16 h at 160 000 g, 4°C in 70Ti rotor in the L7 ultracentrifuge [Beckman].

2.3.4 Pigment content

For estimation of pigment content of Synechocystis cultures, cells were pelleted (13 000 rpm 10 min) and resuspended in 100% methanol, or for thylakoid membranes the solubilised extract was diluted in 100% methanol. Both were spun to remove debris and absorption spectra of the methanol extract was recorded (equation 1) [Porra et al., 1989] in the Unicam spectrophotometer. The ratio of phycocyanin to chlorophyll was calculated from absorption spectra of whole cell samples measured using a SLM Aminco DW2000 spectrophotometer [SLM Instruments, Urbana, Illinois, USA] (see below) using the formulae of Myers et al. (1980) (equations 2, 3).

\[
\text{Chl in methanol (µg/ml): } (A_{665} - A_{750}) \times 12.6 \quad [1]
\]

\[
\text{Chlorophyll in whole cells (µM): } ((1.0162 \times A_{678}) - (0.063 \times A_{625})) \times 1000/68 \quad [2]
\]

\[
\text{Phycocyanin in whole cells (µM): } ((1.0162 \times A_{625}) - (0.2612 \times A_{678})) \times 1000/111 \quad [3]
\]

Estimation of chlorophyll content in A. thaliana was carried out according to Hipkins & Baker (1986) and Porra et al. (1989). Leaves were macerated in liquid nitrogen and then pigment was extracted with Tris–Cl (pH8.0)-buffered 80% acetone. Samples were briefly centrifuged (3000 rpm, 2 min) [MSE Mistral 1000] to remove cell debris. The concentration of chlorophyll \(a\) (equation 4), chlorophyll \(b\) (equation 5) or total chlorophyll

73
(equation 6) was calculated from spectra recorded using the Unicam spectrophotometer according to Porra et al. (1989).

1. Chlorophyll $a$ in buffered 80% acetone (µg/ml): \(12.25 \times A_{663.6} - 2.55 \times A_{646.6}\) [4]
2. Chlorophyll $b$ in buffered 80% acetone (µg/ml): \(20.31 \times A_{646.6} - 4.91 \times A_{663.6}\) [5]
3. Total chlorophyll in buffered 80% acetone (µg/ml): \(17.76 \times A_{646.6} + 7.34 \times A_{663.6}\) [6]

The carotenoid content of the FtsH-mutant Synechocystis and A. thaliana was also investigated by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC; see below).

2.3.5 Lipid analysis

Six litres each of WT and slr0228− GT Synechocystis were cultured for preparation of thylakoid membranes (see above). Likewise, leaves of mature compost-grown Col0 and Var2-2 A. thaliana plants were harvested and thylakoid membranes prepared as above. Synechocystis and A. thaliana WT and mutant samples were adjusted to equal chlorophyll concentrations and frozen at -80°C.

Lipid extraction was carried out essentially according to Kruse et al. (2000). In all evaporation steps, samples were gassed with CO$_2$ to prevent oxidation. Methanol (30 ml) was added to each sample and the solutions were then filtered through glass filters. The process was repeated with chloroform and then with acetone, leaving proteins and starch on the filter. The filter was rinsed with methanol and each sample was then evaporated under vacuum. The dried samples were washed with 1:5 methanol:chloroform and evaporated as before, and finally resolubilised in 6 ml 1:1 methanol:chloroform.

Thin-layer chromatography

The purified lipids from the thylakoid membranes were resolved into their constituent components by TLC for qualitative analysis. Spots of each sample were pipetted onto duplicate silica gel TLC plates [F234; Merck] and lipids resolved over 1.5 h using 75 ml chloroform, 13 ml methanol, 9 ml acetic acid and 3 ml water. A phosphatidylcholine (PC) control was also run on the phospholipid plate. Separated galactolipids and phospholipids were stained with anthron or molybdenum oxide, respectively [Kruse et al., 2000]. Bands were identified with reference to Sato & Murata (1988).
TLC plates were set up as above for carotenoid separation, but run for 2 h in a mobile phase of 100 ml benzene, 12 ml isopropanol and 0.25 ml water.

2.3.6 Saponification of lipids for fatty acid analysis

To prepare thylakoid-membrane fatty acids, the lipid samples (above) were evaporated and then boiled in 95% methanol with 0.5 M sodium hydroxide in distillation flasks. To separate polar from nonpolar lipids, approx. 60 ml petroleum-ether was added, the samples shaken vigorously and then allowed to settle. This was repeated so that the water-soluble (green) phase and the carotenoid-containing phase could be collected using separating funnels.

The yellow phase was used for carotenoid analysis by HPLC using an RP-18-column [Merck] and a Kontron HPLC system [Optimize Technologies, Oregon City, Oregon, USA], according to Hirschberg & Chamovitz (1994) and Li et al. (2002). The green solution from the alkaline hydrolysis was further treated with HCl. After acidification to liberate fatty acids, separation of these samples with petroleum-ether was again carried out, and the petrol-ether fraction retained. After washing with water three times in the separating funnels to remove all acid, the purified fatty acids were evaporated. A further wash and evaporation with 1:1 methanol:chloroform and then hexane was followed by solubilisation in 100 µl hexane, yielding concentrated samples for GLC [Li et al., 2002]. Final preparation was carried out according to Kruse et al. (2000) and samples were resolved using a Hewlett Packard 5890 gas chromatograph [General Medical Instrumentation].

2.3.7 Protein gel electrophoresis

SDS–polyacrylamide gels were prepared and run essentially according to Laemmli (1970) using Mini-Protean equipment [Bio-Rad, Hercules, California, USA]. The resolving gel contained 12% (w/v) acrylamide [Sigma], 0.35 M Tris–HCl pH8.8, 11.5% (v/v) glycerol [BDH], 0.1% (w/v) SDS, 0.05% (v/v) N', N', N', N'-tetramethylethylenediamine (TEMED) [Sigma] and 0.005% (w/v) ammonium persulphate [BDH]. A few drops of water-saturated butanol were applied to the top of the gel which was then allowed to polymerise for 30 min. After rinsing the top of the gel with water, the stacking gel [6.5% (w/v) acrylamide, 62 mM Tris–HCl pH6.7, 0.1% (w/v) SDS, 0.25% (v/v) TEMED, plus 0.06% (w/v) ammonium persulphate] and sample comb were applied and the gel allowed to polymerise.
Protein samples were loaded after dilution with an equal volume of Laemmli sample buffer [Sigma; 20% (v/v) glycerol, 0.004% (w/v) bromophenol blue, 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 0.12 M Tris—HCl pH 6.8], heating to 100°C for 2 min and then centrifugation (5000 g, 2 min) to remove insoluble material. The running buffer contained 1.8% (w/v) Tris—HCl, 8.64% glycine [Sigma] and 0.6% (w/v) SDS.

Gels were stained for 20 min in a solution of 0.1% (w/v) Coomassie Blue [Sigma] in 45% (v/v) methanol and 9% (v/v) glacial acetic acid. Destaining solution of 7% (v/v) methanol with 7% (v/v) glacial acetic acid was left on the gel overnight, or until bands were clearly visible, with gentle shaking.

2.4 SPECTROSCOPY

2.4.1 Fluorescence spectroscopy: Synechocystis
For 77K fluorescence emission spectra, samples of Synechocystis cells were adjusted to 10 μg/ml chlorophyll with growth medium. Cells were then injected into 4 mm-diameter silica tubes and dark-adapted for 10 min to ensure photosystems adapted to state 2 [see Mullineaux & Allen, 1990] before freezing in liquid nitrogen. Fluorescence emission was measured across wavelengths 620—750 nm on a Perkin Elmer LS50 luminescence spectrometer. The excitation wavelength was at 435 nm for chlorophyll or 600 nm for PBS. Slit widths were 5 nm for excitation and emission.

Time-course measurements
State transitions can be monitored in Synechocystis using room-temperature time-course fluorescence spectroscopy. Samples of low-light-grown cultures were adjusted to 3 μg/ml chlorophyll and measurements made in a 3 ml cuvette on the LS50 luminescence spectrophotometer using an Olympus [Southall, Middlesex, UK] light source and fibre optics, according to Emlyn-Jones et al. (1999). Slit widths were set at 5 nm for excitation light and 15 nm for emission. Cells were first adapted to state 2 in 620 nm light. Fluorescence emission at 680 nm was then monitored as cells thus adapted to state 2 were induced to switch to state 1 and back using a chlorophyll-exciting red light [RG665 filter; Schott, Stafford, UK].

2.4.2 Fluorescence spectroscopy: Arabidopsis
Arabidopsis samples were prepared in the dark or using a dark-room green light only.
Arabidopsis leaf tissue was weighed and then ground in liquid nitrogen using a pestle and
mortar. After homogenising with a known quantity of chilled grinding buffer (0.33 M sorbitol, 5 mM MgCl₂, 5 mM EDTA, 10 mM HEPES pH7.6) the samples were injected into a glass capillary tube and refrozen immediately in liquid nitrogen. Alternatively, extracted thylakoid membranes (see Section 2.3.1 above) were frozen in tubes as above, or fluorescence was measured directly from frozen leaves using a fibre optic light source and receiver [Perkin Elmer].

The excitation wavelengths used for *A. thaliana* samples were 435 nm (chlorophyll *a*) and 480 nm (chlorophyll *b*). Slit widths were set at 5 nm. In the case of whole-leaf fluorescence, leaves were mounted on black cardboard and floated, frozen, on a bath of liquid nitrogen (leaves were covered in a shallow layer of the liquid). The fibre-optic light source and receiver were positioned to give maximal fluorescence readings. The excitation slit width was 15 nm and emission slit width was 10 nm. A 540 nm short pass filter [Ealing, Rocklin, California, USA] was used on the excitation light source for all *A. thaliana* measurements to remove the fluorescence artefact coinciding with the PSI peak in chlorophyll *b* (480 nm) spectra.

### 2.4.3 Absorption spectra

Cyanobacterial whole cell samples were adjusted to 5 μg/ml chlorophyll where possible. Absorption spectra were measured using the SLM Aminco DW2000 spectrophotometer across 400–750 nm.

### 2.4.4 Data manipulation

Where direct comparison was required, fluorescence emission spectra and absorbance spectra from WT and mutant cells were manipulated to allow plots to be overlaid. Using SigmaPlot, background fluorescence or light-scattering was removed by subtracting the minimum value from spectra. In the case of 77K fluorescence emission spectra, only relative fluorescence may be analysed because the absolute fluorescence values are unreliable. Therefore, fluorescence emission spectra were normalised to the peak arising from PSI or PSII pigments as appropriate.

### 2.4.5 Flash spectroscopy

PSI and PSII content were estimated spectrosopically from the flash-induced oxidation and re-reduction of P700 in whole cell samples (*Synechocystis*) or thylakoid membranes (*A. thaliana*), in apparatus custom-built by P. Rich (University College London). Note that
this method results only in concentrations of functional reaction centres and may not agree with assays that detect the absolute number of functional plus nonfunctional photosystems.

For flash spectroscopy, wide-spectrum light was supplied from the Xenon flash lamp [Perkin Elmer Optoelectronics, Fremont, California, USA] using BG39 filters on the two light pipes from the flash unit. P700 activity was measured at 703 nm using a 695 nm cut-on filter and a 695–707 nm narrow-band interference filter on the photomultiplier tube. The sample was activated by eight flashes (each of 6 μs half-peak-width), and 10 or 20 transients (according to the chlorophyll concentration of the sample: more are necessary for weaker samples) were averaged to produce the final result.

The magnitude of P700 signal size in samples frozen with 5% DMSO was confirmed to match that of unfrozen samples in flash spectroscopy and, therefore, time-course samples with 5% DMSO were accordingly frozen in liquid nitrogen and stored for later analysis where timing dictated in time-course assays.

The contribution of PSI to each trace was evaluated by the addition of 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to a sample to remove PSII activity; i.e., any rereduction of P700 because of concurrent PSII activity is prevented. The difference between the +/− DCMU spectra therefore gives an estimate of PSII activity whereas the +DCMU spectrum is the result of only PSI activity. A millimolar extinction coefficient of 64 /mM/cm [Hiyama & Ke, 1972] was used to calculate the concentration of P700.

2.4.6 Electron paramagnetic resonance

Whole-cell samples of *Synechocystis* were prepared according to Cammack (1988). Cells were gently centrifuged (3800 rpm, 9 min) [MSE Mistral 1000] and pellets were resuspended in BG11 plus 5 mM EDTA (to chelate free manganese) to be as concentrated as possible, approx. 200 μg/ml chlorophyll. Where WT and mutant samples were to be compared, matching chlorophyll concentrations or cell densities were used. Samples of 0.3 ml were injected into quartz electron paramagnetic resonance (EPR) tubes of 3 mm internal diameter, dark-adapted for 20 min and frozen in the dark in liquid nitrogen. EPR studies were performed using a Jeol RE1X spectrometer [Kyoto, Japan] fitted with a helium cryostat [Oxford Instruments, Abingdon, Oxfordshire, UK]. Samples were subjected to alternating light (illuminated with 150 W light source) and dark measurements to examine whether the light-induced P700 signal was reversible.
2.5 BIOPHYSICAL TECHNIQUES

2.5.1 Oxygen electrode

Two Clark-type oxygen electrodes were used during the course of this work. The first electrode and control box [Rank Brothers, Bottisham, Cambridgeshire, UK] were assembled and operated according to Hipkins & Baker (1986), connected to a chart recorder. The alternative Hansatech electrode [model DW2 for liquid or LD1/2 for gas phase; Kings Lynn, Norfolk, UK] was operated through a personal computer according to the manufacturer's instructions. One millilitre or 2 ml of whole cells of *Synechocystis* were used in the electrodes, respectively, at a chlorophyll concentration of 3 μg/ml, unless stated otherwise. Cyanobacteria were gently pelleted (3800 rpm 8 min) [MSE Mistral 1000] and resuspended in fresh BG11 for all measurements. Hamilton syringes were used to inject chemical mediators into the reaction chamber without disturbing the reactions. The electrode was maintained at 30°C for cyanobacteria and 22°C for *A. thaliana* leaves using a water-jacket connected to a circulating waterbath.

The light supply was filtered through a red filter [Ealing] or, in the Hansatech equipment, red light was supplied by LEDs (wavelength, 650 nm). The reference value for oxygen concentration of air-saturated water for calculations was supplied by the software or was taken from references within Allen & Holmes (1986).

The leaf disk chamber was set up following the manufacturer's instructions [Hansatech] with moistened capillary matting below the leaves and operated according to Walker (1987). As carried out by Eastman & Camm (1995), leaves were first subjected to low light of 10 μmol/m²/s for 3 min, dark for 10 min to measure dark respiration rates and for dark adaptation, and then to light for recording oxygen evolution.

*Whole-chain electron transport*

For samples of cyanobacteria, saturating light was checked by ensuring that the oxygen evolution gradient was not reduced when a 63% neutral density filter [Balzers, Milton Keynes, Bedfordshire, UK] was positioned in front of the light source. Two light intensities were used in all experiments to ensure that light was saturating. Serial measurements were always made for recordings for leaves in actinic light of 370 and 460 μmol/m²/s to ensure that oxygen evolution or uptake was not light-intensity-dependent, and 500 and 980 μmol/m²/s for cyanobacteria. To ensure that oxygen evolution was not CO₂-limited,
10 mM sodium bicarbonate was supplied to liquid culture, and 200 μl of 1 M sodium bicarbonate (pH9) was soaked onto capillary matting in the leaf disk chamber.

**PSI activity**

Light-dependent oxygen uptake (the Mehler reaction) was used as measure of PSI activity. Methyl viologen (MV; 50 μM), to catalyse the transfer of electrons from PSI to oxygen, was added to cells incubated in the dark at 30°C for 15 min before testing [Mullineaux & Allen, 1990]. PSI activity alone was monitored by inactivating PSII using 10 μM DCMU, preventing electron flow from Q$_{a}^{-}$ to Q$_{b}$. Ascorbate (5 mM) was added as electron donor for P700, with tetramethyl-β-phenylene diamine (TMPD; 0.1 mM) to mediate electron transfer from outside the cells (see Table 2.4) [Allen & Holmes, 1986].

**Table 2.4. Additions made to oxygen electrode reactions.**

<table>
<thead>
<tr>
<th>Mediators</th>
<th>Stock concentration</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-(3,4-dichlorophenyl)-1,1-dimethylurea [Sigma]</td>
<td>10 mM</td>
<td>10 μM</td>
</tr>
<tr>
<td>Ascorbic acid [Sigma]</td>
<td>0.5 M</td>
<td>5 mM</td>
</tr>
<tr>
<td>Methyl viologen [Sigma]</td>
<td>50 mM</td>
<td>50 μM</td>
</tr>
<tr>
<td>Sodium bicarbonate, pH9.0 [Sigma]</td>
<td>1 M</td>
<td>10 mM</td>
</tr>
<tr>
<td>Tetramethyl-β-phenylene diamine [Aldrich]</td>
<td>10 mM</td>
<td>0.1 mM</td>
</tr>
</tbody>
</table>

2.5.2 **Fluorescence microscopy**

Leaves from Col0 and Var2-2 *A. thaliana* were gently torn to give unicellular layers of tissue suitable for fluorescence microscopy. Chlorophyll fluorescence from chloroplasts was photographed under using an Axiophot [Zeiss, Welwyn Garden City, Hertfordshire, UK] confocal microscope equipped with a mercury lamp [Zeiss]. Images were acquired with a digital camera [Hamamatsu, Welwyn Garden City, Hertfordshire, UK] and Openlab 2.5 software [Improvision, Coventry, Warwickshire, UK].
3. HYPOTHETICAL PROTEINS INVOLVED IN LIGHT-HARVESTING

3.1 Introduction
The necessity to adapt to a constantly altering light environment has resulted in numerous regulatory pathways in photosynthetic organisms that balance energy supply and utilisation. The possibility of over-reduced electron transport components and therefore potential oxidative damage is avoided by altering antenna size, reaction centre content and carbon dioxide fixation, and by changing the amount of energy dissipation, for example as heat [for reviews, see Anderson, 1986; Horton et al., 1996; Keren & Ohad, 1998]. Short-term adaptation occurs within minutes via such mechanisms as the state transition or quenching; long-term changes take hours or days as cells regulate the composition and function of the photosynthetic apparatus. Although the general mechanisms of high-light acclimation have been investigated there remains much to be discovered. Microarrays show that the level of transcription of hundreds of genes changes during adaptation [for example, Hihara et al., 2001] and the role of the many proteins and signal transduction pathways involved are not well understood.

Two proteins thought to be involved in light adaptation have been identified recently, one, RpaC, acting in short-term regulation in *Synechocystis* and the other, Ape-1, regulating the behaviour of light-harvesting apparatus in *A. thaliana*. The work presented below was intended to answer the question of whether these proteins acted in the same manner in eukaryotic and prokaryotic photosynthetic organisms, attempting to elaborate the results obtained in one system with those from another.

3.2 The role of the *Synechocystis* homologue of the *Arabidopsis* Ape-1 protein
Three mutants defective in distinct aspects of acclimation were identified using a fluorescence imaging system to examine seedlings from a T-DNA-disrupted *A. thaliana* library (R. Walters, P. Horton, personal communication, 1999). The gene disrupted in the first of the mutants, *ape-1* (acclimation of photosynthesis to the environment) was sequenced and found to be similar to a predicted ORF in *Synechocystis* 6803.
It seems very likely that the Ape-1 protein has a role in photosynthesis. In *A. thaliana*, although there was no discernable difference between the *ape-1* mutant and WT under low-light conditions, upon transfer of plants from 100 to 400 μmol/m²/s light [Figure 3.1], the mutant responded less to the change than WT in its chlorophyll *a:b* ratio, *F₅/F₅* fluorescence ratio (a measure of PSII efficiency) and *Φₚ₅*. The mutant also had a 60% higher *F₅* value 1 day after transfer, and there was a slightly increased *P₅* (the maximum rate of oxygen evolution per chlorophyll in CO₂-saturated conditions) in high-light-grown *ape-1* plants compared with WT (R. Walters, personal communication, 1999) [also Walters *et al.*, 2003]. Since there was normally no difference in the quantity of PSII (although there was slightly less in mutant than WT after any change in illumination intensity), and the mutant was still capable of rapidly reducing its *P₅* and chlorophyll *a:b* upon moving plants from high to low light, Walters and Horton postulate that the principal defect is not in acclimation itself but in LHCII regulation, perhaps its degradation. This hypothesis is supported by the presence on sucrose density gradients of stably aggregated LHCII. Aggregated LHCII is associated with NPQ; if there was sustained NPQ in the *ape-1* plants the drop in the photochemical efficiency and quantum yield of PSII could indeed occur in the mutant.

**Figure 3.1.** Response over time of oxygen evolution (*P₅*; μmol O₂/mol chlorophyll/s) and chlorophyll *a:b* ratio in *ape-1* and wild-type (WT) *Arabidopsis thaliana*. Low-light (LL)-grown plants were transferred to high light (HL) (left) and HL-grown plants transferred to LL (right). (○) WT *P₅*; (●) *ape-1* *P₅*; (□) WT chlorophyll *a:b*; (■) *ape-1* chlorophyll *a:b*. Data are mean ± standard error. (Figure supplied by R. Walters, University of Oxford.)
The ORF predicted to encode a protein similar to Ape-1 in the *Synechocystis* genome is slr0575. An insertional inactivation mutant was therefore made by disrupting slr0575 with a kanamycin resistance gene. The slr0575" cells were then examined for any photosynthetic phenotype, particularly with reference to effects seen in the *ape-1* plants, using spectroscopy and biochemical methods. Sequence databases were also searched and a reduced-stringency Southern blot carried out to find other homologues of the gene.

### 3.2.1 slr0575 predicted structure

The protein predicted to be encoded by slr0575 is 184 amino acids in length. Hydropathy analyses by PSIPRED and TMPRED [ca.expasy.org/tools/] suggest that the protein would contain one transmembrane helix [Figure 3.2] from residue 5–28 in-to-out, and possibly another from residue 30–51 out-to-in. It should be noted that the latter prediction cannot be absolutely correct, since two amino acids are insufficient to form the external loop between transmembrane domains.

### 3.2.2 slr0575 sequence analysis

Figure 3.2 shows the predicted amino acid sequence of the protein encoded by slr0575. There are no recognisable functional domains nor motifs in the predicted amino-acid sequence from the *Synechocystis* ORF or from the *A. thaliana* *ape-1* gene. The Ape-1 sequence, however, is predicted to contain a chloroplast targeting sequence by TargetP or SignalP [ca.expasy.org].

The putative protein appears to be widespread and, notably, all similar proteins appear to be found in photosynthetic organisms [Figure 3.2]. Complete-genome databases of plants, green algae and photosynthetic bacteria reveal several proteins with >30% amino-acid identity [Figure 3.2], the other cyanobacterial proteins often approx. 70% identical. No similar protein was found in existing red algal sequences, however, although these are currently limited in availability.

Interestingly, the ORF is immediately downstream of the only cytochrome p450 gene predicted within the *Synechocystis* genome (ORF slr0574) but the slr0575 gene does not appear to be part of an operon.
Figure 3.2. (a) Predicted secondary structure of predicted protein encoded by the Synechocystis 6803 open-reading frame (ORF) slr0575. (b) Sequence alignment of representative closest matches to slr0575. Matches were all in photosynthetic organisms as above and also in Nostoc punctiforme, Synechococcus sp. WH 8102; Prochlorococcus marinus subsp. pastoris str. CCMP1378, Lotus japonicus and Chlamydomonas reinhardtii. Abbreviations: Arabidopsis thaliana clone MBB18, Q9FFV6 on chromosome 5; Anabaena PCC 7120 ORF alr3596; Thermosynechococcus elongatus ORF ttl0792; Prochlorococcus marinus str. MIT 9313; Trichodesmium erythraeum.
Figure 3.3. Southern blot showing PCR-amplified open-reading frame slr0575 hybridisation only with two lanes of wild-type *Synechocystis* DNA (~5 Kb band). The membrane also contained total DNA from *Arabidopsis thaliana* and *Synechococcus* strains PCC 7002 and 7942 (all *Not*I-digested).

3.2.3 Southern blot for slr0575 homologues

The similarity of genes for components of the photosynthetic apparatus, even between prokaryotes and eukaryotes, has previously allowed chloroplast genes to be used as hybridisation probes in cyanobacteria [see Williams, 1988]. Therefore, Southern blots were carried out using the *Synechocystis* slr0575 fragment as a probe to look for homologous sequence in genomic DNA from the alga *C. caldarium* and the cyanobacteria *Synechococcus* 7942 and 7002. Only *Synechocystis* 6803 produced a band in these blots; neither of the other organisms’ DNA produced bands, nor did *A. thaliana*, in which homology at the nucleotide level was not sufficient to bind probe, at moderate-stringency washes at least [Figure 3.3].

3.3 Creation of a slr0575-null strain of *Synechocystis*

An slr0575 deletion mutant was constructed by taking advantage of the ability of *Synechocystis* to take up external DNA from its environment and undergo homologous recombination [for details, see Kufryk et al., 2002]. First, the ORF slr0575 was amplified using PCR, and the ends of the 0.8 Kb DNA fragment digested with *Asp*718 and *Sac*I to make them compatible with *Asp*718/*Sac*I-cut pBluescript vector. After ligation and
transformation in \textit{E. coli}, the resultant plasmid was cut in the centre of slr0575 using the restriction enzyme \textit{MseI}. The kanamycin-resistance cassette was cut from pUC4K using \textit{HincII}. It was then blunt-end ligated into the linearised vector in order to disrupt the slr0575 coding region [Figure 3.4]. The successful disruption of the gene was checked by sequencing the pBluescript-slr0575-Kan construct. Transformation of \textit{Synechocystis} with the plasmid resulted in kanamycin-resistant cells, suggesting that the kanamycin-resistant copy of slr0575 had successfully replaced the genomic version. Colonies were serially subcultured until segregation was complete. Because there are multiple copies of the \textit{Synechocystis} genome it is important to check that homoplasmicity has been achieved, as detailed by Williams (1988); this was done using PCR [Figure 3.5] and Southern blot. The correct insertion of the disrupted fragment in the kanamycin-resistant mutant (transformant 8-1:4) was again confirmed by sequencing [Figure 3.6].

The same construct was used for transformation of the PSII\textsuperscript{−} strain [see Table 1] of \textit{Synechocystis} [Figure 3.7]. The resultant mutant is not described here but it segregated successfully, suggesting that the effect of inactivating slr0575 was not sufficient to cause further growth defects in this sensitive strain.

3.4 \textbf{Photosynthesis in slr0575\textsuperscript{−} \textit{Synechocystis}}

The growth rate and presence of functional photosynthesis were established in the mutant slr0575\textsuperscript{−} cells. The normal composition of photosystems and carotenoids in the mutant was confirmed in comparison with WT using sucrose density gradients of solubilised thylakoid membranes [Figure 3.8]. Since the phenotype of the \textit{A. thaliana ape-1} mutant manifests itself in LL–HL transitions, the acclimation of the slr0575\textsuperscript{−} \textit{Synechocystis} strain was also monitored under various light conditions.

3.4.1 \textbf{Growth}

Growth curves were constructed using duplicate cultures, one set supplemented with glucose to highlight any change in growth rate that might result from a photosynthetic defect. There was no difference in either medium, however, between the growth or doubling times of WT and slr0575\textsuperscript{−} cells under standard growth light of approx. 10 \(\mu\text{mol/m}^2/\text{s}\) light intensity [Table 3.1; Figure 3.9].
Figure 3.4. Insertional inactivation of open-reading frame (ORF) slr0575 showing position of kanamycin resistance cassette (KanR) within gene. The PCR-amplified slr0575 fragment was 842 bases including added KpnI (before primer 1, **bold** in sequence) and SacI (after primer 2, **grey** in sequence) sites. KanR was ligated into the central MscI site (**orange** in sequence). Primers were external to ORF, the start site of which is highlighted in green in sequence above and termination site in red. Sequence from Cyanobase [www.kazusa.or.jp/cyano/cyano.html]. For primer sequences see Table 2.3.
Figure 3.5. Segregation of mutant gene copies. PCR of wild type (WT) and insertional inactivation mutants, (a) slr0575<sup>−</sup>: 0.8 Kb WT and 2.0 Kb disrupted; (b) slr0228<sup>−</sup>: 0.9 Kb WT and 2.1 Kb disrupted; (c) slr0228<sup>−</sup>/Chll<sup>−</sup>: 0.9 Kb WT and 2.1 Kb disrupted. T, Transformant.
Figure 3.6. Sequence from *Synechocystis* transformant 8-1-4 (tr8-1-4). Glucose-tolerant wild-type cells transformed with slr0575 kanR-pBluescript DNA. Sequence (using primer slr0575-2f) [Table 2.3] aligned with open-reading frame slr0575 and kanamycin resistance cassette sequence (kanR) using ClustalW [www.ebi.ac.uk/clustalw/index.html]. Bases in orange show *MscI* cut site for insertion of kanR. *, Identical base.

...Sequence of transformant (Tr8-1-4) failed at this point.

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Figure 3.7. PsbCD$_2$D$_2^-$ (left) and slr0575/PsbCD$_2$D$_2^-$ (right) Synechocystis showing unimpaired growth with the additional mutation of slr0575. Cultures were grown on BG11 agar.

Figure 3.8. Sucrose density gradients of solubilised thylakoid membranes from wild-type (WT) and slr0575~ Synechocystis grown under standard incubation conditions. Note orange carotenoids, top photosystem II (PSII) with photosystem I (PSI) monomer band, then lowest band of PSI trimers. Identity of extracted bands confirmed using 77K fluorescence emission spectra.
Table 3.1. Growth rate during exponential growth phase of wild-type (WT) and slr0575" *Synechocystis* in BG11 with or without glucose.

<table>
<thead>
<tr>
<th></th>
<th>Doubling time (h±SEM)</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>No glucose</td>
<td>14.0±1.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.5±1.7</td>
</tr>
</tbody>
</table>

SEM, Standard error of the mean; WT, wild-type.

Growth was also measured during exponential growth phase in high light-conditions since it was at higher light intensities that *ape-1*" plants were adversely affected. In the cyanobacterial mutant, however, growth was again comparable with WT [Figure 3.9] when cultures were transferred to light of approx. 800 μmol/m²/s for 12 h.

### 3.4.2 Competition assay

It is known that the transfer of cultures from low- to moderate-light-intensity incubation can result in the up- or downregulation of dozens of *Synechocystis* genes [Hihara *et al.*, 2001]. To investigate further whether slr0575 is one of the genes involved in acclimation to changes in light intensity, the effect of a light-switching regime on the growth of the mutant was investigated. Although exponential-phase growth had not revealed a difference in low-intensity light and very high-intensity light, a subtle effect of the slr0575 mutation might become discernable over a longer timescale during more variable light conditions, as shown in a competition assay developed by Ivleva *et al.* (2000).

This long-term mixed culture assay involved growing a culture containing both WT and mutant under an incubation light switched between 11 and 75 μmol/m²/s every 24 h. The effect of the mutation on viability was measured by following the proportion of WT and mutant slr0575 ORF that could be PCR-amplified over the course of the experiment.

WT and slr0575" *Synechocystis* were subcultured in parallel at least twice and matched for cell scattering (A_750) at each subculture. To begin the competition assay, the cultures were allowed to reach exponential growth and matching cell density (monitored by A_750 measurements). Duplicate 50-ml mixed cultures were then prepared from 25 ml of each strain, after gentle pelleting and resuspension of cells in 25 ml fresh BG11 (to remove kanamycin from the mutant culture). Two further matched, mixed cultures were used to provide samples for DNA preparation at t=0.
Figure 3.9. Growth curves for wild-type (WT) and slr0575 \textsuperscript{-} Synechocystis. Cell scattering measurements (as c.f.u./ml where calibrated with plate counts) for cultures with/without glucose or (bottom panel) in high-intensity light (800 \(\mu\)mol/m\(^2\)/s) and return to low light (10 \(\mu\)mol/m\(^2\)/s; shaded).
Light conditions in the incubator were then switched between 11 and 75 \, \mu\text{mol/m}^2/\text{s} every 24 h (72 h over weekends) for 25 days. At approx. 72 h intervals, 500 \, \mu\text{l} of each culture was removed to inoculate a new 50 ml culture, and the remainder of the mixed culture used to prepare DNA (for Methods, see also Section 2.1.6). PCR was then carried out on the DNA from each timepoint using primers [see Table 2.3] for each end of slr0575 (slr0575-2f/2r for amplifying the WT ORF) and from one end of slr0575 plus a primer for the kanamycin resistance cassette (slr0575-2f/pK7 for the mutant ORF). This partial mutant fragment was needed because previous optimisation of the mixed PCR had showed that the entire disrupted mutant ORF could not be amplified reliably because of the common phenomenon in PCR of preferential amplification of small products, so that WT was always over-represented (data not shown).

To evaluate the proportion of WT to mutant DNA, and therefore WT to mutant cells in the mixed culture, densitometric analysis [Optimas] was carried out on WT (830 bp) and mutant (548 bp) bands in the photographs of PCR-product gels. The amount of DNA in each band was also calculated with reference to a known quantity of \( \lambda_{\text{HindIII}} \) DNA run as a marker as a secondary check on the values. The ratio of WT:mutant was plotted over time [Figure 3.10].

### 3.4.3 Effect of alternating low-high light

Mutating the slr0575 ORF does not greatly affect growth rates when the light intensity is switched from low to high every day. The doubling times were calculated from the slightly different periods of exponential growth for each strain (there was a small extension of the mutant’s lag phase) [Figure 3.10] for WT (9.6±0.1 h) and the slr0575\(^{−}\) mutant (14.3±3.9 h). Although these times were slightly different, when repeated, growth curves showed WT doubling at 14.5±4.2 h and the mutant at 14.9±3.9 h.

Ivleva et al. (2000) also found that short-term growth curves did not result in different growth rates in the \textit{Synechocystis} mutants. They did report, in contrast, that long-term mixed culture growth analysis demonstrated some reduction in fitness of mutants (which had altered PSII D1 proteins) when they could observe no difference by other methods. Comparing WT and slr0575\(^{−}\) \textit{Synechocystis} in such a competition assay, however, suggested no reduction in the proportion of mutant or WT cells present over 25 days of alternating light conditions. The quantity of WT or mutant product amplified by PCR of DNA from the mixed culture showed an approximately constant ratio over the period of the assay. This was the case when PCR products were quantified by comparison with bacteriophage \( \lambda \).
DNA or by densitometric analysis. The best correlation by regression analysis ($r^2 0.06$) showed no relationship between variation in the WT:mutant ratio and the duration of the assay [Figure 3.10].

These results confirm previous data from the low- and extreme-high-light cultures: the slr0575 mutant does not grow more slowly under conditions where it must acclimate to altered light intensity.

### 3.4.4 Pigment content

A mutation affecting light harvesting might affect the pigment composition in *Synechocystis*, indeed, as described previously, small changes in the chlorophyll *acb* ratio were seen in response to changes in light conditions in the *A. thaliana ape-1* mutant. To monitor pigment content in the WT and slr0575- cyanobacteria, absorption spectroscopy was used. Spectra of intact cells of WT *Synechocystis* show three major peaks, those at 435 nm and 680 nm corresponding to chlorophyll and that at 625 nm is due to phycocyanin in the PBS. Carotenoids contribute to the region 450–500 nm [Figure 3.11].

The amount of chlorophyll and phycocyanin per cell in the slr0575 mutant was compared with WT, applying the formulae of Myers *et al.* (1980) to absorbance spectra from whole *Synechocystis* cells. Low-light-grown cells (10 μmol/m²/s) proved to have identical pigment contents [Figure 3.11].

Once again, the response of the mutant to high light was investigated, this time using the pigment content as an indication of changes to the photosynthetic apparatus. Many tetrapyrrole synthesis genes are downregulated within 15 min of transfer to high-intensity light. It has been previously documented [Hihara *et al.*, 2001] that the chlorophyll and phycocyanin content of *Synechocystis* decreases to two-thirds within a few hours of transfer to high light, with gradual recovery only after approx. 24 h. This pattern of decrease and then increase was seen in both WT and the slr0575- mutant [Figure 3.12], suggesting that the latter was able to respond adequately to the change to more intense light.
Figure 3.10. (a) Growth of wild-type (●) versus slr0575^- (○) Synechocystis in alternating 11 and 75 μmol/m²/s light (upper panel). One assay shown but experiment repeated twice with duplicate cultures (see Section 3.4.3). (b) Ratio of wild-type to slr0575^- DNA amplified in PCR of total DNA from a mixed culture grown in alternating 11 and 75 μmol/m²/s light (lower panel). Linear regression analysis (orange) r² 0.06.
Figure 3.11. (a) Absorbance spectrum of wild-type (WT) and slr0575⁻ Synechocystis. Background cell scattering subtracted. (b) Pigment composition of WT versus slr0575⁻ in low (10 μmol/m²/s light) estimated according to Myers et al. (1980) and divided by $A_{750}$ to compensate for cell density of each sample. Chl, Chlorophyll; PC, phycocyanin.
Figure 3.12. Response of wild-type (WT) and slr0575 \textit{Synechocystis} to light of 100 μmol/m²/s. Growth before t=0 at approx. 10 μmol/m²/s. Chlorophyll (top) and phycocyanin (bottom) were calculated according to Myers \textit{et al.} (1980).
3.4.5 Fluorescence spectroscopy

In tandem with pigment measurements by absorbance spectroscopy, the activity of the photosystems was monitored directly via fluorescence. Fluorescence spectroscopy is a useful non-invasive indicator of whole-chain electron flow \textit{in vivo}. Cyanobacteria have a short generation time and the photosystem stoichiometry can be altered reasonably quickly. The changed PSI:PSII ratio can then be seen in fluorescence spectroscopy when \textit{Synechocystis} responds to a change in light quality [for review, see Chitnis, 2001]. The technique is also used for the investigation of the function of photosystems, e.g., a reduction in rate or a blockage to electron flow past PSII induces increased PSII fluorescence.

At room temperature, the fluorescence from PSI is very weak (as energy is trapped very fast by this reaction centre), but at liquid-nitrogen temperature fluorescence from both photosystems is increased [Rijgersberg & Amesz, 1980] since the PSI pathway becomes less efficient at low temperatures and fluorescence from a proportion of the chlorophylls can be seen [Butler, 1961]. In 77K fluorescence emission spectra, emission maxima of approx. 685, 695 (CP43 and CP47 respectively) and 725 nm (PSI) are generated in light of 435 nm [for example, see Figure 3.13], corresponding to the Soret band of chlorophyll \(a\) absorption [see Murakami, 1997, and references within]. In contrast, excitation at around 600 nm (the edge of the phycocyanin absorption band, peaking at 620 nm) results in three peaks: the first, broad, peak at approx. 650 nm is fluorescence from phycocyanin (645 nm) and allophycocyanin (665 nm), the second is from the PBS terminal emitters plus PSII (680 nm) and the third is from PSI (725 nm). A shoulder at 690 nm emanates from the PSII core.

3.4.6 77K Fluorescence emission spectra

Figure 3.14 shows the ratio of fluorescence from PSI and PSII in 77K fluorescence emission spectra from dark-adapted (ruling out changes because of state transitions) WT and slr0575\(^{-}\) cells incubated first in low light (10 \(\mu\)mol/m\(^2\)/s) then transferred to high light (100 \(\mu\)mol/m\(^2\)/s) for 48 h and then returned to low light again until 72 h. In cyanobacteria, the PSI:PSII ratio usually decreases when cells are transferred to high light [Fujita \textit{et al.}, 1994], when \textit{psa} genes are greatly downregulated [Hihara \textit{et al.}, 2001], and it is known that fluorescence parallels closely any changes in the photosystem stoichiometry [Murakami, 1997]. The drop followed by a rise in the PSI:PSII fluorescence seen here is therefore exactly as expected [Figure 3.14], confirming the absorbance data that showed acclimation to high light in the slr0575\(^{-}\) mutant.
Figure 3.13. Fluorescence from wild-type (WT; black line) and slr0575 (dashed orange line) *Synechocystis* after incubation in 100 µmol/m²/s light in 77K fluorescence emission spectra in 435 nm excitation light. Peak 685–695, photosystem II; peak 725, photosystem I.

Figure 3.14. Ratio of photosystem I (PSI):photosystem II (PSII) fluorescence from wild-type (WT) and slr0575 *Synechocystis* after incubation in 10–100–10 µmol/m²/s light. Peak height at approx. 720 nm (PSI) versus 690 nm (PSII) recorded from 77K fluorescence emission spectra in 435 nm excitation light. Mean (± standard error) ratios from a minimum of two cultures are shown.
Figure 3.15. Ratio of fluorescence from photosystem II (PSII):photosystem I (PSI) in wild-type (WT; ■) and slr0575^-^Synechocystis (○) after incubation in red or yellow light to monitor the ability of the photosynthetic apparatus to adjust to changes in light quality. Cultures grown under otherwise standard incubation conditions. Samples taken at 0, 1, 2, 3, 24, 48 h. PSII fluorescence was measured at about 690 nm and that from PSI at about 720 nm in 77K fluorescence emission spectra. Excitation light, 435 nm.

Chlorophyll or phycobilisome light

To examine the ability of WT and slr0575^-^ to acclimate to different wavelengths of light, fluorescence emission spectra were recorded from cells transferred to red (for chlorophyll, state 1) or yellow (for PBS; state 2) [Mullineaux & Allen, 1990] light for 48 h. Figure 3.15 shows that both WT and the strain with inactivated slr0575 adjusted the PSII:PSI ratio up under red light and down under yellow light, i.e., there was increased PSII to compensate in chlorophyll light and increased PSI to compensate when the PBS pigments were favoured.

State transitions

Along with other photosynthetic organisms, cyanobacteria are able to adapt rapidly to changes in their light environment by a mechanism known as the state transition [Fork & Satoh, 1983]. This is the regulation of the distribution of light energy between the two reaction centres by changes to the LHC (see Chapter 1). Since aspects of light harvesting
are impeded in the *A. thaliana* ape-1 mutant, are state transitions altered in the *Synechocystis* slr0575− strain? To find out, 77K fluorescence emission spectra were used to monitor changes in the distribution of energy between the reaction centres in light conditions chosen to induce state transitions.

State 1 is characterised by illumination preferentially absorbed by PSI and efficient energy transfer to PSII: in state 2, light best absorbed by PSII results in increased efficiency of energy transfer to PSI [for review, see Van Thor *et al.*, 1998]. Figure 3.16 shows that WT cells (*a, b*) that had been subjected to red light (state 1, PSI excitation) demonstrate increased amplitude of PSII fluorescence emission compared with cells in state 2 (black lines), which reflects more energy transfer from the PBS to PSII. In slr0575− cells (*c, d*) it can be seen that the state transition operates in the same way. This can be seen in both 600 nm (*d*) and (a less dramatic change) 435 nm (*c*) spectra. The insertional inactivation of the slr0575 ORF, therefore, appears not to impede this aspect of the regulation of light-harvesting.

3.4.7 Oxygen evolution

To assess the photosynthetic ability of the slr0575 mutant, whole-chain electron transport was assayed in the form of oxygen evolution measurements in saturating carbon dioxide. The mean (± standard error; SEM) light-saturated oxygen evolution was the same in WT (143±8 μmol O₂/mg chlorophyll/h) as in slr0575− (147±19 μmol O₂/mg chlorophyll/h) when the cells had been grown in low light intensities of 2–9 μmol/m²/s [Figure 3.17]. There is no indication that, under ideal growth conditions, the mutant is impaired in photosynthetic electron transport.

In *ape-1* *A. thaliana*, oxygen evolution was slightly higher at high irradiances than in WT (R. Walters, personal communication, 1999). After incubation in extreme high light, there was also a difference in oxygen evolution between WT and slr0575−.

Plotting the changes in oxygen evolution over time [Figure 3.18], the oxygen evolution rate per chlorophyll in the slr0575− mutant appears to adjust more slowly than WT to increased illumination, at least for the first few hours. Then, when the prolonged damage after 48 h growth in extreme high light (>850 μmol/m²/s) had resulted in reduced oxygen evolution in WT, the slr0575− cells evolved significantly more oxygen (*t* test *P=0.01, 4 degrees of freedom; d.f.).
This difference was not consistently reflected in 77K fluorescence emission spectra (described above) recorded at the same sampling times as those for oxygen evolution measurements. Although small differences were sometimes seen in fluorescence spectra of cells subjected to high light [e.g., Figure 3.13], the mean ratio of fluorescence from PSII was comparable in WT and slr0575 mutant after 48 h growth at photoinhibitory light intensity and again during the recovery in low light at 72 h (data not shown). During recovery in LL, however, the oxygen evolution remained significantly different (t-test $P=0.01$, 5 d.f.) between WT and slr0575, with the slr0575 yield being somewhat lower.

**Figure 3.16.** 77K fluorescence emission spectra from wild-type (WT) (a, b) and slr0575 (c, d) *Synechocystis* adapted to state 2 (dark) then state 1 (red light). Excitation light was 435 nm (a, c) or 600 nm (b, d). Relative fluorescence was normalised to the photosystem I peak (~720 nm) in the 435 nm spectrum or to the phycocyanin/allophycocyanin peak (~660 nm) in 600 nm spectrum, in both cases after background fluorescence was subtracted.
Figure 3.17. Oxygen evolution from wild-type (WT) and slr0575 Synechocystis. Measurements made from five (WT) and two (slr0575) cultures grown under standard incubation conditions and tested under saturating light in the oxygen electrode.

Figure 3.18. Oxygen evolution for wild-type (WT) and slr0575 Synechocystis cultures grown in low light (L.L.) then extreme high light, before recovery in L.L. Mean (± standard error) calculated from minimum of four measurements in a minimum of two experiments, except for 2 h timepoint (only one datapoint available) Measurements made under saturating light with 10 mM sodium bicarbonate.
3.5 Discussion (slr0575)

The *Synechocystis* ORF predicted to encode the protein most similar to *A. thaliana* Ape-1 was inactivated by insertion of an antibiotic resistance cassette. With reference to the phenotype of the Ape-1 mutant, i.e., defective regulation of light harvesting, the composition and behaviour of the *slr0575* *Synechocystis* was investigated in low-, high- and altered spectrum-light conditions. The results generally suggested that the protein was not expressed or the function was too subtle to produce an obvious phenotype, but oxygen evolution measurements of plants subjected to high light do indicate that further investigation might be fruitful.

Photosynthesis in the mutant was comparable to WT under standard incubation conditions, as measured by oxygen evolution and fluorescence emission. The mutant was able to function under photoinhibition and could regulate light harvesting by state transitions. It is often the case that studies of knockout mutants yield no clues to the function of the mutated gene [for review, see Mullineaux, 2001]. Secondary pathways, up/downregulation of other pathway components or redundancy among genes and proteins can compensate for the loss of just one protein, so that phenotypes become impractical to detect.

It was altered light conditions that revealed a probable role for the Ape-1 protein in regulation of light-harvesting in *A. thaliana*, but low–high light treatment of the *Synechocystis* mutant produced more ambiguous results about the function of the cyanobacterial protein, if there is one. Growth was not affected adversely when light was alternated daily from low to high intensity. After transfers to high light and in low-light recovery, all measurements of antenna function (e.g., pigment composition and energy transfer measured by fluorescence from PSI and PSII) suggested that there was little effect of inactivating the slr0575 protein in cyanobacteria. In contrast, monitoring the whole-chain electron transport did reveal some differences. The measurements should be repeated with additional timepoints to provide more detail about the response of the mutant, and further studies of functional electron transport would perhaps be useful. For example, flash spectroscopy and EPR might allow the further dissection of any effect of the mutation on individual components of photosynthetic electron transport after changing light conditions. It may be that subtle effects of removing a protein acting within signal transduction networks for environmental sensing would be manifested differently in plants and cyanobacteria, with a light-harvesting phenotype in *Arabidopsis* and a more direct electron-transport phenotype in *Synechocystis*. 
There are many similarities between plants and cyanobacteria in terms of regulation and light-harvesting. Despite the presence of chlorophyll-containing LHC or PBS, groups of other light-harvesting-associated proteins, e.g., the cyanobacterial HLIPs and some LHC polypeptides and the ELIPs of chloroplasts, seem to be similar [Green & Dumford, 1996; Montanè & Kloppstech, 2000]. Nevertheless, the fundamental difference in light-harvesting and other variations between acclimation and protection mechanisms could mean that there is a difference in the use or utility of slr0575/ape-1 gene products between *Synechocystis* and *A. thaliana*. Cyanobacteria appear not to have a xanthophyll cycle and NPQ is different to that in plants (there is no PsbS), for example; if the Ape-1 protein was involved in this aspect of acclimation then its role must be somewhat divergent from its likely genetic precursor.

Microarray data revealing high-light-regulated genes in *Synechocystis* did not identify slr0575 mRNA among those transcripts whose presence rose or fell in 300 μmol/m^2/s light. It is not possible to rule out the regulation of slr0575 by changing light, however, since this microarray had a high level of background fluorescence, allowing only relatively highly-transcribed genes to be identified [Hihara et al., 2001]. Signal transduction components and transcription factors were indeed feared to be omitted by the authors of this study. The microarray data are not encouraging when studying *Synechocystis* slr0575, but the ape-1 mRNA appeared to be transcribed constitutively despite its apparent part in the control of light-harvesting. The transcription of slr0575 also might not be light-regulated.

The fact that similar predicted proteins are encoded by the genomes of other photosynthetic organisms that have been fully sequenced suggests that the gene is well-conserved and would therefore, presumably, retain a function and be expressed. ORFs have been seen to be degraded when no longer in use, e.g., the *Prochlorococcus* phycoerythrin gene *cpeP* [Ting et al., 2002], so the existence of numerous full-length homologues of slr0575 again suggests that it remains functional. If this work were to be continued, molecular biological studies of transcription and translation would be essential to confirm the expression of slr0575 in *Synechocystis*. The detection of the slr0575 mRNA would confirm that further studies were worthwhile, and an antibody to the Ape-1 protein is also available which could be used, or a new antibody produced, for *Synechocystis*.

A competition assay with greater variation in light level could be considered, since the trend was for a slight (not significant) decrease in the proportion of mutant DNA in the mixed culture [Figure 3.10]. Quantification of PSI and PSII would help identify the reason
for altered electron transport, while the response of the slr0575 mutant to other environmental stimuli (carbon dioxide and temperature, for example) may also be helpful to investigate.

3.6 Is the regulator of light-harvesting RpaC found in other photosynthetic organisms?

The RpaC (regulator of PBS association) protein required for state transitions was identified previously [Emlyn-Jones et al., 1999] by disruption of the sll1926 ORF in Synechocystis 6803. Subsequently, the sequencing of the genome of the cyanobacterium Anabaena was completed, revealing a likely RpaC homologue. There is currently no experimental evidence that explains how the protein might regulate light-harvesting, but further good matches can also now be identified in Thermosynechococcus elongatus and in Trichodesmium erythraeum [Figure 3.19]. Two other possible homologues are present in Chlorobium and Prochlorococcus marinus. Further evidence that the small (~10 kDa) protein acts in light harvesting comes from recent microarray data. A DNA microarray assay that examined high-light-regulated gene expression in Synechocystis [Hihara et al., 2001] confirmed that transcription of rpcflL was downregulated throughout a period of exposure to 300 µmol/m²/s light, in common with a set of genes involved in light absorption and photochemistry.

The most similar proteins to RpaC appear only in cyanobacterial genomes (although the presence of similar sequence in a Chlorobium gene perhaps indicates at least a common functional domain elsewhere) according to current sequence data. This distribution suggests potential involvement with PBS light-harvesting apparatus. If, however, similar proteins are present in prokaryotes or eukaryotes with principally chlorophyll light-harvesting, RpaC-type proteins might have a general role in pathways regulating antennae in photosynthesis. Southern blots of low stringency and PCR were therefore carried out on various prokaryotes and eukaryotes in order to identify potential homologues, along with sequence-database searches as genome data became available during the course of this work.
3.7 Identification of \textit{rpaC} homologues

3.7.1 PCR
It was intended to investigate the function of eukaryotic versions of RpaC in plants, if the protein appeared to be present. Neither standard nor degenerate PCR, however, amplified any products from plant DNA (\textit{A. thaliana}) or green algal DNA (\textit{C. reinhardtii}), however. Very few sequence data were available for other photosynthetic organisms at the start of this project. To explore further the distribution of RpaC, PCR was carried out on DNA from \textit{Synechococcus} PCC 7002 and 7942, \textit{Calothrix} PCC 7601, \textit{D. salina}, \textit{A. cylindrica}, \textit{C. reinhardtii} and \textit{C. caldarium}. PCR using primers for the sU1926 ORF (primers sU1926-1f and -1r) [Table 2.3] amplified the 0.5 Kb \textit{rpaC} in \textit{Synechocystis} 6803 but in \textit{Synechococcus} 7942, a 1.7 Kb product and in \textit{Synechococcus} 7002, 0.4 and 1.0 Kb products were amplified. Neither showed significant homology to \textit{rpaC}: the sequenced 1 Kb product from \textit{Synechoococcus} 7002 was most similar to a number of O-methyl transferase genes and the 0.4 Kb product was clearly part of a previously-unidentified \textit{Synechococcus} 7002 \textit{fbH} gene (see Introduction; Figure 1.10). Degenerate PCR (primers sU1926-2) [Table 2.3] on DNA from \textit{Synechococcus} 7942, \textit{Calothrix} 7601 and \textit{C. caldarium} resulted in products of 0.3–0.5 Kb. Neither the 1.7 Kb \textit{Synechococcus} 7942 product nor the products from degenerate-PCR primers were sequenced successfully.

3.7.2 Hybridisation
Southern blots were carried out on digested genomic DNA from \textit{Synechococcus} 7002 and 7942, \textit{C. reinhardtii}, \textit{A. thaliana}, using PCR-amplified sU1926 as probe. No bands were produced in low-stringency Southerns, suggesting that any equivalent genes to RpaC were either not present or too dissimilar to the probe for hybridisation to occur.

3.7.3 Database homology searches
The sequencing of the \textit{A. thaliana} genome was completed during the course of this work. BLAST searches on the sequence data confirmed the negative results from PCR and Southern blots. Only one rather poor match is identified in similarity searches of the \textit{A. thaliana} genome with the RpaC predicted amino-acid sequence [Figure 3.19]. The region (At1g73180) is broken by a second methionine in part of the sequence that is well-conserved in the cyanobacterial versions, and only a proportion of the highly conserved residues are present. It is unlikely that this is a plant equivalent of \textit{rpaC}.

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**Figure 3.19.** Alignment of predicted amino-acid sequence of the open-reading frame sll1926 from *Synechocystis* 6803 (a) with putative homologues in *Anabaena* (All2971), *Synechococcus* (*Thermosynechococcus elongatus*) and *Trichodesmium erythraeum* (b). Other good matches (not shown) were found in *Chlorobium* and *Prochlorococcus marinus*. (c) sll1926 versus the region containing the most similarity in the *Arabidopsis thaliana* genome (Atlg73180). Identical residues (*) and in orange), conserved (;) and semi-conserved (.) substitution are indicated according to standard ClustalW format [www.ebi.ac.uk/clustalw/index.html].
3.8 Discussion (RpaC)

PCR products of appropriate size were amplified from cyanobacterial and red algal DNA suggesting, like the sequence data, that RpaC is present only in PBS-containing organisms. There is no evidence for similar proteins in green plants or algae and no recognisable motifs are identified by sequence-analysis programs [ca.expasy.org/tools/]. The distribution of the most similar RpaC-like proteins in organisms that contain PBS for light-harvesting suggest that it does indeed have a precise role regulating that apparatus: RpaC was therefore not amenable to parallel investigation in plants and cyanobacteria and was not studied further.
4. CREATION OF slr0228-NULL MUTANTS

4.1 Introduction
Since the mechanisms of electron transfer are largely conserved between prokaryotic and eukaryotic photosynthetic organisms, the regulatory and support systems for photosynthesis might also be expected to be similar in the chloroplast and cyanobacteria. The completion of the *A. thaliana* genome sequence confirms this: although there is often gene duplication so that the plant contains multiple copies of *Synechocystis* homologues, probable equivalent protease and chaperone sequences are easily recognised [for review, see Sokolenko et al., 2002 and references within]. The work presented in the following chapters attempts to analyse the function of apparently equivalent FtsH-type proteases in *Synechocystis* and *A. thaliana*, with the aim of using data from one to support study of the other. First, the FtsH proteins in *Synechocystis* are considered.

Four proteins encoded by the *Synechocystis* genome were predicted to be FtsH-like proteases [Kaneko et al., 1996]. Insertional inactivation mutants of the *ftsH* genes were investigated recently by Mann et al. (2000). The slr1604" and slr1390" cells did not segregate to homozygosity, suggesting that these are essential for viability of *Synechocystis*. Of the two mutants that did segregate, the slr0228" strain of motile *Synechocystis* 6803 was the only one that demonstrated a clear photosynthetic phenotype. Initial characterisation revealed that loss of the slr0228-encoded protein results in an altered photosystem and pigment composition but the cause of this was not known.

Work here was continued using the strain constructed previously [Mann et al., 2000] but, in order to investigate further the nature of changes caused by the mutation, a slr0228" mutant was also created in a GT strain of *Synechocystis*. This strain has the advantage over motile *Synechocystis* in its ability to grow heterotrophically, of great benefit in assays where components of the photosynthetic apparatus are inactivated. A second mutation was also made in a *Synechocystis* strain that cannot synthesise chlorophyll in the dark (ChL") [Wu & Vermaas, 1995]. This mutation allows rapid chlorosis and regreening of the cyanobacterium in dark-incubation (with a daily 10-min photoperiod). The effect of insertional inactivation of the slr0228 ORF on degradation and synthesis of the photosystems could therefore be investigated without resort to nutrient deprivation methods (see Chapter 6).
>Synecocystis sp. strain PCC6803 ORF slr0228 (2529009-2530892)

Figure 4.1. Insertional inactivation of the open-reading frame (ORF) slr0228 showing position of insertion of kanamycin resistance cassette (KanR) within gene. The fragment of slr0228 amplified by PCR was 922 bases including the added KpnI (Asp718, before primer 1, red in sequence) and SacI (after primer 2, grey in sequence) sites. Primers designed to avoid a KpnI site (underlined in sequence) within the gene. KanR was ligated into the centre of the cloned fragment in an MspI site (orange in sequence). Sequence from Cyanobase [www.kazusa.or.jp/cyano/cyano.html]. For primer sequences see Table 2.3.
Figure 4.2. Sequence from *Synechocystis* transformant 6-4:2 after transformation of glucose-tolerant wild-type cells with slr0228-kan-pBluescript DNA. Transformant sequence ('forward'; using primer slr0228-lf) [Table 2.3] aligned with open-reading frame slr0228 using ClustalW [www.ebi.ac.uk/clustalw/index.html]. Sequence begins at approx. 900 nt at the position of the sh0228-lf primer and continues until the gene is disrupted by insertion of kanamycin resistance cassette sequence at Mscl restriction site (in orange). Sequence from the reverse primer slr0228-lr (not shown) confirmed correction insertion, running (3'→5') from approx. 1770, at the C terminus of sLr0228 to 1149 at the end of the kanamycin cassette [see Figure 4.1]. Identical bases (*) are indicated.
4.2 Insertional inactivation of slr0228

An slr0228 deletion mutant was constructed taking advantage of the ability of *Synechocystis* to take up external DNA from its environment and undergo homologous recombination [for details, see Kufryk et al., 2002]. The construction of the mutant is summarised in Figure 4.1. The ORF slr0228 was amplified using PCR with primers slr0228-1f and -1r [Table 2.3]. The ends of the 0.9-Kb DNA fragment were digested with *Asp*718 (isoschizomer of *KpnI*) and *SacI* to make them compatible with *Asp*718/*SacI*-cut pBluescript vector. After ligation and then transformation into DH5α *E. coli*, the resultant plasmid was cut in the centre of the slr0228 sequence using the restriction enzyme *MscI*. The linearised construct was treated with phosphatase to prevent recircularisation.

The kanamycin-resistance cassette was prepared by digestion from pUC4K using *HincII*. It was then blunt-end-ligated into the cut slr0228–pBluescript construct in order to disrupt the slr0228 coding region. Once again the plasmid was transformed into DH5α *E. coli*. Transformation of the GT *Synechocystis* strain with the slr0228kan–pBluescript construct then resulted in kanamycin-resistant cells, suggesting that the kanamycin-resistant copy of slr0228 had successfully replaced the genomic version.

Colonies were serially subcultured until segregation was complete: because there are multiple copies of the *Synechocystis* genome [Williams, 1988], it is important to check that homozygosity has been achieved; this was done using PCR [Figure 3.5]. The correct insertion of the disrupted fragment in the kanamycin-resistant mutant (transformant 6-4:2) was again confirmed by sequencing [Figure 4.2].

4.3 Double mutants

The slr0228 construct disrupted with the kanamycin-resistance cassette was also used to transform motile (not described further here), PSII− and ChlL− *Synechocystis* exactly as above. Segregation was successful in the ChlL− cells [Figure 3.5], but multiple attempted transformations of the PSII− strain resulted in colonies that did not survive continued culture on kanamycin. It is possible that this mutation is too severe in PSII− cells, which were not able to sustain the additional changes resulting from the missing protease.
4.4 Confirmation of phenotype

It is already known that disruption of slr0228 in *Synechocystis* results in an altered ratio of fluorescence emission from PSI and PSII in 77K fluorescence emission spectra [Mann et al., 2000]. To confirm that the new (GT and ChL" mutants) demonstrated a comparable phenotype, absorbance [Figure 4.3] and 77K fluorescence emission spectra [Figure 4.4] were recorded.

Excitation at 435 nm in 77K fluorescence emission spectroscopy resulted in lower fluorescence emission from the PSI peak of the slr0228" or slr0228"/ChL" cells than from the PSI peak of WT or ChL" cells, relative to the PSII fluorescence. Absorbance spectra demonstrated the same effect, with an decreased chlorophyll:phycoeyanin absorbance ratio [Figure 4.3]. Differences in the quantity of photosystems and pigments per cell were also similar to those previously noted (see Chapter 5 for detailed characterisation).

4.5 Discussion

Mutants with insertional inactivation of the slr0228 ORF were constructed successfully in GT and ChL" *Synechocystis*, but not in the PSII" strain. Initial characterisation showed the expected altered pigment and photosystem ratio in fluorescence and absorbance spectra. The effect of loss of the slr0228-encoded FtsH on photosynthesis, particularly the synthesis of PSI and turnover of PSII, was investigated in detail (see Chapters 5–7).
Figure 4.3. Absorbance of whole-cell samples from new slr0228<sup>−</sup> mutants in glucose-tolerant wild-type and ChIL<sup>−</sup> *Synechocystis*. Note the changes to the chlorophyll:phycocyanin absorbance ratio. Spectra normalised by subtraction of background cell scattering.
Figure 4.4. 77K fluorescence emission spectra to confirm the phenotype of the new slr0228− glucose-tolerant (GT) (a) and slr0228/ChlL− (b) mutants. Note altered photosystem I (PSI) to photosystem II fluorescence and the small shift in the PSI peak (see Section 6.3.2). Spectra normalised to PSI peak (approx. 720 nm) after background fluorescence subtracted.
5.1 Introduction

Preliminary work using a motile *Synechocystis* strain [Mann *et al.*, 2000] has already suggested a role for the slr0228-encoded FtsH in photosynthesis, since the slr0228" mutant showed reduced levels of PSI and some changes to pigment levels. The pigment content and fluorescence characteristics of the GT slr0228" mutant were analysed to confirm that it was comparable to the strain used previously (Chapter 4), and both the GT and motile mutant were studied further to clarify the effect of the loss of the slr0228-encoded FtsH on the photosynthetic apparatus.

Growth and pigment levels were monitored with particular attention to the carotenoid composition of the mutant since it often appeared aberrant during biochemical assays. To determine the effect of the altered PSII:PSI and pigment ratios in slr0228" *Synechocystis*, its electron transport was examined using the oxygen electrode and the function of light-harvesting was investigated using time-course and 77K fluorescence emission spectroscopy. Finally, the mutant and WT were subjected to damaging light intensities to compare the photoinhibition of PSII and PSI, using measurements of oxygen evolution and uptake. The function of PSI before and after high light treatment was followed using EPR.

5.2 Growth of slr0228" *Synechocystis*

The growth of WT and slr0228" *Synechocystis* is comparable under standard incubation conditions [Figure 5.1; Table 5.1] although there is a small difference in very low light, presumably because lower chlorophyll levels in the slr0228" cells (see below) put the strain at a disadvantage in these conditions.

<table>
<thead>
<tr>
<th>Strain/light level</th>
<th>Doubling time (h) ±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μmol/m2/s</td>
<td></td>
</tr>
<tr>
<td>WT (motile)</td>
<td>37.1±3.5</td>
</tr>
<tr>
<td>slr0228&quot; (motile)</td>
<td>38.0±1.7</td>
</tr>
<tr>
<td>2 μmol/m2/s</td>
<td></td>
</tr>
<tr>
<td>WT (GT)</td>
<td>65.7±11.5</td>
</tr>
<tr>
<td>slr0228&quot; (GT)</td>
<td>89.4±10.3</td>
</tr>
</tbody>
</table>

Table 5.1. Growth rates of slr0228" mutants in standard and low-light incubation.

Doubling rate and standard error of the mean (SEM) calculated from linear regression in exponential phase of growth using SigmaPlot. WT, Wild-type; GT, glucose-tolerant.
Figure 5.1. Growth of wild-type (WT) and slr0228^ motile strains of Synechocystis in 10 µmol/m²/s light. Doubling times (dt) calculated for exponential growth (data between 24 and 144 h).

Figure 5.2. Mean (± standard error) content of photosystem I (PSI) in wild-type (WT) and slr0228^ Synechocystis calculated using flash spectroscopy of P700 (see Methods for detail). Coefficient for moles of PSI from Hiyama & Ke (1972).
5.3 Pigment and photosystem content

It was already reported that chlorophyll content of slr0228 motile Synechocystis is approx. 80% that of the motile WT strain, whereas PBS content is correspondingly increased [Mann et al., 2000]. This slr0228 GT strain of Synechocystis has similar pigment characteristics to those of the motile strain [Table 5.2]: in GT slr0228 cells, chlorophyll is at 86% the level of WT, whereas phycocyanin in the WT is correspondingly only 87% the level seen in the mutant. The same effect is seen in Chl− versus slr0228/Chl− cells, although it is slightly less marked [Table 5.2; Figure 4.3].

All the major polypeptide components of the photosynthetic apparatus were comparable between WT and slr0228−, as seen in SDS–PAGE of thylakoid membranes (not shown). More precise quantification of PSI was carried out (see below) using flash spectroscopy.

### Table 5.2. Mean pigment and photosystem 1 (PSI) composition of wild-type (WT) versus slr0228− and Chl− versus slr0228/Chl− Synechocystis cells.

<table>
<thead>
<tr>
<th>Pigment/photosystem</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Chlorophyll (molecules/cell)</td>
<td></td>
</tr>
<tr>
<td>WT:slr0228−</td>
<td>1.21×10^8±2×10^6</td>
</tr>
<tr>
<td>Chl−:slr0228/Chl−</td>
<td>1.2</td>
</tr>
<tr>
<td>Phycocyanin (molecules/cell)</td>
<td></td>
</tr>
<tr>
<td>WT:slr0228−</td>
<td>5.3×10^7±2×10^6</td>
</tr>
<tr>
<td>Chl−:slr0228/Chl−</td>
<td>0.9</td>
</tr>
<tr>
<td>PSI (n/cell)*</td>
<td>124 000±8000</td>
</tr>
<tr>
<td>Chlorophyll (molecules/PSI)*</td>
<td>133±4</td>
</tr>
</tbody>
</table>

Mean ± standard error of mean (SEM) calculated from a minimum of two sets of cultures. Pigment content measured using the SLM Aminco DW2000 spectrophotometer and deconvoluted using the formulae of Myers et al. (1980). *Photosystem content measured using flash spectroscopy and the extinction coefficient of PSI from Hiyama & Ke (1972), and chlorophyll content measured from methanol extracts [Porra et al., 1989].

5.3.1 Photosystem content

When the PSI content per cell was measured using flash spectroscopy [Figure 5.2; Table 5.2] it confirmed what was previously calculated using absorption difference spectra with the motile slr0228− mutant [Mann et al., 2000]. The altered PSI:PSII ratio appears to be due to a large reduction in PSI content: the GT slr0228− strain contained only half the PSI/cell of WT. Correspondingly, as well as in 77K fluorescence emission spectra in 435 nm
excitation, light of 600 nm produces higher emission from the PBS/PSII terminal emitter peak (approx. 680 nm) than in WT, relative to the chlorophyll peak at 720 nm, reflecting the reduced amount of PSI and increased PBS/cell [Figure 5.3]. Although chlorophyll/cell is reduced, the number of chlorophyll molecules per PSI calculated from the flash spectroscopy data is increased (133±4/PSI in WT versus 231±56/PSI in slr0228'), again reflecting the reduced content of that reaction centre in slr0228− cells [Figure 5.2].

The ChIL− strain was reported [Wu & Vermaas, 1995] to be unaffected by the loss of its light-independent chlorophyll biosynthesis pathway under standard incubation conditions. The measurement of PSI in ChIL− here, however, showed a reduction in PSI/cell compared with WT, suggesting that it is affected to some degree, at least under these rather low-light incubation conditions. Although the amount of PSI/cell in the ChIL− strains in these measurements did not show the dramatic loss of PSI associated with the slr0228 mutation, the chlorophyll levels do if measured using methanol extracts [Porra et al., 1989] (see also Section 7.4 for discussion of pigment quantification). The slr0228/ChIL− double mutant had more chlorophyll/PSI compared with ChIL−, like the slr0228− mutant compared with WT. The altered fluorescence spectra [Figures 4.4, 5.3] and pigment ratios [Table 5.2] also confirmed that the slr0228/ChIL− double mutant is comparable to the GT slr0228− strain, despite some differences between WT and ChIL− cells.

5.3.2 Carotenoids

During the course of this work, biochemical assays of both *Synechocystis* and *A. thaliana* FtsH mutants often showed a striking difference in the colour of cell extracts suggestive of altered carotenoid content. Sucrose density gradients, for example, showed a difference in colour and quantity of carotenoids in the upper band in samples of slr0228− and WT solubilised thylakoid membranes (loaded on the basis of equal quantity of chlorophyll) [Figure 5.4]. TLC of thylakoid-membrane carotenoids confirmed this difference [Figure 5.5]. When investigated using HPLC, both *Arabidopsis* (see Chapter 8) and *Synechocystis* mutants were found to contain less β-carotene: slr0228− *Synechocystis* also contains abnormally high myxoxanthin levels [Table 5.3; Figure 5.6].
Figure 5.3. 77K fluorescence emission spectra showing the altered photosystem I to photosystem II emission ratio in the glucose-tolerant (GT) slr0228" (a) and slr0228/ChlL" (b) mutants. Spectra normalised to the phycocyanin/allophycocyanin peak (approx. 650 nm) after background fluorescence subtracted.
Figure 5.4. Sucrose density gradients of solubilised thylakoid membranes from wild-type (WT) and slr0228– Synechocystis. (a) Cells grown under standard incubation conditions and loaded on the basis of equal chlorophyll. Note orange carotenoids (particularly dark in slr0228– gradient) above top band of photosystem II and photosystem I (PSI) monomers. The lowest band is PSI trimers. (b) WT, slr0228– and PsaL– Synechocystis grown under blue light. WT and PsaL– loaded with equal chlorophyll. No PSI trimers were visible in the small amount of thylakoid membranes it was possible to extract from blue-light-grown slr0228– (magnified region of gradient shown, far right). Extracted phycobilisome pigments also shown on left to demonstrate altered composition in slr0228–. Identity of extracted bands confirmed using 77K fluorescence emission spectra.
Figure 5.5. (a) Thin-layer chromatogram (TLC) of total lipids stained with anthron (for galactolipids; GL) and (b) carotenoid TLC, of thylakoid membranes from Col0 and Var2-2 *Arabidopsis thaliana* and wild-type (WT) and slr0228 *Synechocystis*. GL have poor contrast because plate faded before scanning but all lanes contained comparable lipids in mutant and respective WT. Monogalactosyl diacylglycerol (MGDG) contributes approx. 50% total GL, the composition of the others (DGDG, digalactosyl diacylglycerol; SQDG, sulfoquinovosyl diacylglycerol) depending upon growth conditions [Glatz et al., 1999]. See Table 5.3 (Chapter 9 for quantification of fatty acids).
Figure 5.6. High-performance liquid chromatography (HPLC) of thylakoid membrane carotenoids from *Arabidopsis thaliana* wild type (WT; Col0) and Var2-2, and *Synechocystis* (glucose-tolerant; GT) WT and slr0228. Assignment of pigments by O. Kruse (personal communication, 2001). NB. After the separation of carotenoids from chlorophylls some of the latter may remain as impurities in HPLC, producing peaks. Changes in chlorophyll molecules following harsh extraction procedures can also result in artificial isomers, as seen in the *Synechocystis* results [Braumann & Grimme, 1981].
Table 5.3. Carotenoid composition of WT (GT) and slr0228− *Synechocystis.*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Myxoxanthophyll</th>
<th>Echinenone</th>
<th>Zeaxanthin</th>
<th>β-carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>9 (8.0±1.0 %)</td>
<td>&lt;1 (1.0±0.5 %)</td>
<td>39 (34.0±1.5 %)</td>
<td>67 (57.0±2.0 %)</td>
</tr>
<tr>
<td>slr0228−</td>
<td>20 (18.0±1.0 %)</td>
<td>&lt;1 (1.0±0.5 %)</td>
<td>45 (40.0±1.5 %)</td>
<td>47 (41.0±2.0 %)</td>
</tr>
</tbody>
</table>

*Values calculated using extinction coefficients in acetone [see Hirschberg & Chamovitz, 1994].

NB. Differences between percentage ratios and total values are due to the fact that the loss of one particular pigment does not mean that one of the other pigments is automatically increased.

WT, Wild type; GT, glucose-tolerant; SEM, standard error of the mean.

Figure 5.7. Carotenoid–xanthophyll biosynthesis pathway. Xanthophyll-cycle carotenoids (in plants) are show in boxes. Grey text, reduced in Var2-2 (Chapter 8) and slr0228− strains; bold text, increased in slr0228− cells. *In *Synechocystis.* [After Davison et al., 2002 and Lagarde & Vermaas, 1999]

Increased carotenoid biosynthesis is part of the general response to stress in photosynthetic organisms [e.g., Davison et al., 2002; for review, see Hirschberg & Chamovitz, 1994]. It is possible therefore that differences seen in carotenoids in the slr0228− *Synechocystis* are caused by increased photo-oxidative damage because of the mutant’s reduced PSII function (see Section 5.6). It is also possible, however, that the slr0228− (and indeed Var2; Chapter 8) FtsH has a regulatory role in the biosynthesis of membrane components such as carotenoids: it is known that a function of the single *E. coli* FtsH is regulation of lipid biosynthetic pathways [Ogura et al., 1999] (see Chapter 9). It is interesting that the amount of β-carotene, a carotenoid early in the biosynthetic pathway, is decreased whereas myxoxanthophyll, synthesised after β-carotene [Figure 5.7], is increased. There is another parallel here with *E. coli*, since its FtsH degrades lipid biosynthesis enzymes to achieve downregulation [Ogura et al., 1999]. The loss of FtsH in *Synechocystis* might therefore result
in increased production of intermediates such as carotenoids made late on in biosynthetic pathways. Of note also, myxoxanthophyll is a polar molecule that must span the bilayer and may rigidify membranes [Várkonyi et al., 2002]: instead of or as well as reflecting photodamage, these changes in themselves may affect function of photosynthetic membranes.

5.4 Light harvesting

There are clearly a number of differences in the pigmentation of cells lacking the slr0228-encoded FtsH, but how functional is the mutant's light-harvesting antenna? The different quantities of PBS/cell has already been noted between the motile slr0228" and WT Synechocystis strains [Mann et al., 2000], a feature also found in the GT mutant which also has high phycocyanin levels. 77K fluorescence emission spectra suggest that the antenna is correctly assembled, but the high fluorescence from the 680 nm peak (from the PBS terminal emitters and PSII) in 600-nm phycocyanin excitation light may indicate some level of dissociation of antennae from reaction centres [Figure 5.3].

5.4.1 State transitions

Initial characterisation of the motile slr0228" Synechocystis mutant [Mann et al., 2000] led to the suggestion that it may be defective in a further aspect of light harvesting, namely state transitions (P. Nixon, personal communication, 2001). As described above, fluorescence changes are a useful way to monitor the activity of each photosystem and therefore follow the increases and decreases in emission that occur during state transitions. These were therefore investigated in the slr0228" GT strain using liquid-nitrogen-temperature spectroscopy and time-course spectra at room temperature.

5.4.2 State transitions in 77K fluorescence emission spectra

The higher fluorescence normally seen in slr0228" PSII relative to WT PSII does not mask the effect of the state transition. The adaptation from state 2 to 1 is seen in slr0228" cells as in WT: there is a rise in fluorescence from the PSII peak [(a) left-hand peak or (b) middle peak in WT, and corresponding peaks (c, d) for slr0228"; Figure 5.8] as the efficient transfer of energy to PSII is favoured because of the change to red light absorbed by PSI.
Figure 5.8. 77K fluorescence emission spectra from wild-type (WT) \((a, b)\) and slr0228\(^{-}\) \((c, d)\) *Synechocystis* adapted to state 2 (dark) then state 1 (red light). Excitation light was 435 nm \((a, c)\) or 600 nm \((b, d)\). Relative fluorescence normalised to photosystem II peak \((~720\text{ nm})\) in 435 nm spectra or to phycocyanin/allophycocyanin peak \((~660\text{ nm})\) in 600 nm light, in both cases after background fluorescence subtracted.
Figure 5.9. State transition of wild-type (WT) and slr0228^- glucose-tolerant Synechocystis. Cells of equal chlorophyll concentration (3 µg/ml) adapted from state 2 (in 620 nm light) to state 1 (light 1; red RG665 filter) [Schott] and back in room-temperature time-course assay of fluorescence emission at 680 nm.
5.4.3 Time-course spectra

Whereas the 77K fluorescence emission spectra suggested normal state transitions, room-temperature time-course measurements of slr0228− fluorescence were not exactly the same as those of WT.

Fluorescence emission at 680 nm was monitored from cells grown in low light (1—2 μmol/m²/s) and exposed to 620 nm light for phycocyanin absorption. Cells thus adapted to state 2 for 60 s were then induced to switch to state 1 by changing to chlorophyll-exciting red light ("light 1"). When this light provokes the state 1 transition, rising emission can be seen in the WT trace as the absorption by PSII gradually increases. Upon switching off the light, there is an increase in fluorescence as PSII reaction centres are closed (as the PQ pool is reduced after a decrease in excitation of PSI); this is followed by a gradual decay in fluorescence when absorption by PSII decreases during the transition back to state 2 [Figure 5.9].

There are a number of variations in the room-temperature spectra between WT and slr0228− cells. First, the slr0228− mutant fluorescence-baseline is higher than that of WT. This is probably because slr0228− cells contain more phycocyanin, phycobilins being responsible for most background fluorescence [Mullineaux & Allen, 1988]. The higher PSII:PSI ratio in slr0228− could also result in slightly altered electron-transport function, causing different levels of background fluorescence.

When light 1 is switched on, there is a gradual increase in fluorescence from the slr0228− and WT cells, indicating the state 1 transition. Before that, however, there is an immediate and rapid rise in fluorescence from the mutant cells immediately upon light 1 illumination [Figure 5.9]. In the WT there is only the slower rise. Such immediate responses in these spectra result from PSII trap closure or opening: it is the slower changes that result from state transitions [Fork & Satoh, 1983]. It would appear, therefore, that in the absence of the slr0228 FtsH, there is PSII trap closure when the red light is switched on (immediate fluorescence from PSII).

What is the reason for this? Since the slr0228− cells' PSII:PSI ratio is higher than in WT, it is more likely that the PSII quinone acceptors and PQ pool can be overloaded with electrons: there are fewer PSI centres to receive electrons. "Light 1 on" may therefore cause PSII trap closure and the immediate rise in fluorescence seen here.
Figure 5.10. Oxygen uptake (via the Mehler reaction) using methyl viologen, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, ascorbate and tetramethyl-p-phenylene diamine and oxygen evolution from wild-type (WT) and slr0228\textsuperscript{+} \textit{Synechocystis} under saturating light conditions. Mean (±standard error) measurements taken from one culture for uptake and three cultures for evolution.

Figure 5.11. Dark respiration levels (oxygen uptake in the dark) of wild-type (WT) versus slr0228\textsuperscript{+} \textit{Synechocystis} in the oxygen electrode. Mean (±standard error of the mean) of eight measurements presented.
Figure 5.12. Mean oxygen evolution (a) and uptake (b; via the Mehler reaction) during incubation of motile wild-type (WT) and slr0228− Synechocystis in 1300 μmol/m²/s light. Oxygen evolution measured with sodium bicarbonate; photosystem I activity isolated in the oxygen electrode using methyl viologen, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, tetramethyl-p-phenylene diamine and ascorbate.
A third difference in the room-temperature spectra is that the later peak of increased fluorescence from PSII (at "light 1 off") seen in WT is missing in slr0228" cells [Figure 5.9]. This is the closure of PSII in WT, followed by the gradual fall in fluorescence of the state 2 transition. When light 1 is switched off, it seems plausible that in slr0228" cells the initial fluorescence peak does not occur because most PSII have been overloaded and are closed already.

5.5 Oxygen evolution

Although some characteristics of light-harvesting and composition of the thylakoid membranes is altered, the slr0228" mutant is able to photosynthesise adequately under standard incubation conditions. Oxygen evolution measurements correspond with the quantification of photosystems by flash spectroscopy (Section 5.3.1) in both GT versus slr0228" and ChlL" versus slr0228/ChlL" strains (data not shown for latter).

Under conditions of saturating light and carbon dioxide, there was a slight difference in whole-chain electron transport measured by oxygen evolution rate/chlorophyll, which might be expected, given the decrease in chlorophyll per cell and increase in PSII levels relative to PSI in the slr0228— strain. The mean (±SEM) values were not significantly different (t test; not shown), however, between in WT of 146 (±16) and of 249 (±65) μmol O₂/mg chlorophyll/h in slr0228" cells [Figure 5.11].

Mean PSI activity measured as the level of light-dependent oxygen uptake using MV/DCMU/ ascorbate/ DCMU ascorbate/ TMPD (see Section 2.5.1) in slr0228" was only half (48 μmol O₂/mg chlorophyll/h) the level seen in WT (123 μmol O₂/mg chlorophyll/h) cells [Figure 5.11], as observed in flash spectroscopy (Section 5.3.1).

The levels of PSII certainly allowed comparable oxygen evolution in the two strains whereas PSI measured by the Mehler reaction corresponded with flash spectroscopy measurements. The level of dark respiration was also different, however, proving to be higher and more variable in slr0228" than WT cultures, however [Figure 5.11]. The mean (±SEM) oxygen uptake by WT cultures under standard conditions in the oxygen electrode when not illuminated is 38 ± 10 μmol O₂/mg chlorophyll/h whereas the mean in slr0228 cells is 86 ± 24 μmol O₂/mg chlorophyll/h, but the variability of the mutant produces a difference significant only at P=0.1 (t test, 14 d.f.). A difference in dark respiration could be explained by the imbalance of PSI and PSII biosynthesis and turnover (Chapters 6, 7). This could alter respiration rates by causing perturbed redox control and/or ATP or NADPH availability,
and the balance of linear to cyclic electron flow could be affected in these circumstances. Further investigation would be needed to clarify this, especially given the altered chlorophyll levels upon the basis of which the respiration measurements were compared.

5.6 Photoinhibition

It has recently been shown that the slr0228 FtsH is involved in the turnover of the PSII D1 protein after high light damage. The photoinhibition of PSII in slr0228~*Synechocystis* is covered in detail elsewhere [Silva et al., submitted]. Measurements were made in this work as part of the investigation of PSI photoinhibition and effect of membrane lipid composition on phenotype (see Chapter 9). The photoinhibition of PSII in *A. thaliana* is described in Chapter 8.

5.6.1 Photoinhibition of PSI

High-light damage of PSI is not believed to be the routine event that is photoinhibition of PSII. PSI function has been shown to decrease, however, especially in conjunction with cold temperatures [for review, see Scheller et al., 2001]; in addition, PSI photoinhibition may be underestimated when high concentrations of MV are used in measurements [Sonoike, 1999]. It has been suggested that PSI is also more vulnerable to high-light damage, like PSII, in the absence of FtsH (S. Bailey, personal communication, 2002). Western blots have showed [Mann et al., 2000] that the PSI subunits PsaD and PsaF were reduced in the motile slr0228~ strain compared with WT, perhaps making its PSI more vulnerable to damage. This was investigated by measuring oxygen uptake via the Mehler reaction (see Methods) in the oxygen electrode and by EPR, to monitor PSI activity in WT and slr0228~* cells subject to intense light of >1000 μmol/m²/s.

5.6.2 Oxygen uptake in photoinhibition

The motile WT and slr0228~*Synechocystis* strains were used for these assays since it was in these strains the previous observations had been made. Samples of parallel cultures were adjusted to a concentration of 10 μg/ml chlorophyll and tested before and after exposure to 1300 μmol/m²/s light intensity. PSI activity was isolated in the oxygen electrode using MV, DCMU, TMPD and ascorbate (see Section 2.5.1). Whole-chain electron transport was measured by oxygen evolution with the only addition to the electrode chamber being sodium bicarbonate. Both measurements were conducted in saturating light.

After 1 h of 1300 μmol/m²/s light, the level of oxygen evolution had dropped in WT and oxygen evolution had declined to zero in slr0228~ (motile) *Synechocystis*. At this point, light-
dependent oxygen uptake had increased in both, although slr0228− levels remained about half those seen in WT [Figure 5.12]. After over 2 h incubation in high light, PSI measured in this way remained functional since oxygen uptake continued at normal levels, although decreased since the 1 h timepoint. There is no indication, therefore, that PSI is more vulnerable to inactivation in slr0228− cells than in WT, even in light sufficient to damage PSII beyond the cells' capability for repair.

To be sure that there is no effect on PSI photoinhibition, the assay could be repeated with even higher light intensity to further magnify any difference between WT and FtsH mutant on PSI behaviour. As mentioned above, Sonoike (1999) also reported that PSI photoinhibition is underestimated if too much MV is used in assays: adjusting the concentration of MV might also therefore improve the chance of investigating this phenomenon.

5.6.3 Electron paramagnetic resonance

EPR is particularly useful for monitoring the activity of metalloproteins and free radicals, and is therefore often employed in the study of photosynthesis. For example, the oxidation state of iron–sulphur proteins can be monitored in low-temperature EPR spectroscopy. For these experiments, whole-cell samples of cyanobacteria were used to investigate the activity of P700.

The intensity of the EPR signal is an indication of the number of intact chains of electron transfer [Cammack, 1988]. Photoinhibited samples were therefore investigated by this method, to determine the level of functional PSI in slr0228− compared with WT cells. Since MV is not used in the EPR measurements to isolate PSI activity, the masking of PSI photoinhibition possible in oxygen uptake measurements [Sonoike, 1999] would not apply in EPR spectra.

After 2 h incubation in 1130 μmol/m²/s light there was a large difference in the magnitude of the P700 signal in difference spectra from WT and slr0228− cells [Figure 5.13]. Per cell, the slr0228− signal was two thirds that of WT; per chlorophyll, the signal would be approx. 85% that of WT (each slr0228− cell has much less chlorophyll than WT). The amount of functional PSI is therefore lower in slr0228− cells, but the reduction is no greater than that indicated in flash spectroscopy nor that in oxygen electrode assays. In both WT and mutant, the light-induced P700 signal was reversible, suggesting that the reaction centre core is fully
functional. As indicated by the oxygen electrode data, the inactivation of slr0228 FtsH does not appear to greatly alter the capacity of *Synechocystis* PSI to function in high light.

Figure 5.13. Electron paramagnetic resonance spectra of P700 in wild-type (WT) and slr0228 motile *Synechocystis* after >2 h incubation in 1130 μmol/m²/s light. Samples were adjusted for equal cell concentration: the slr0228 signal is two-thirds that of WT. If the chlorophyll concentration is factored in, the slr0228 signal would be 85% that of WT. P700 activity was measured at 14.8 K, with modulation amplitude 0.2 mT, microwave power 10^3 mW and a microwave frequency of 9.094 GHz).

5.7 Discussion

The role in photosynthesis of FtsH-like proteins in *Synechocystis* was suggested by N. Mann (University of Warwick), who observed that a subgroup of the proteases was found only in oxygenic photosynthetic organisms (see Introduction). The finding that levels of PSI were reduced [Mann et al., 2000] added weight to this.

To further characterise the effect of inactivation of the slr0228 FtsH, and therefore try to provide further information about the function of other photosynthetic FtsHs, the photosynthetic apparatus and function was studied in slr0228 *Synechocystis*. Flash spectroscopy was used to quantify PSI (the PSII component in flash spectroscopy was too
small to measure accurately), and TLC and HPLC were used to examine carotenoid composition which appeared to be aberrant. The photoinhibition of PSI was examined because there had been previous indications of some effect. EPR studies of the mutant were of interest not only in photoinhibition studies but also because there have been suggestions of a role for a FtsH protein in removing unassembled Rieske iron–sulphur centres [Ostersetzer & Adam, 1997]: EPR could be used to pinpoint more closely where iron–sulphur centre function was impeded.

This work showed that not only is there a phenotype in the slr0228− GT and ChlL− strains consistent with that seen in motile Synechocystis, but the loss of the protein’s function in the assembly and/or degradation of the photosynthetic apparatus appears to have widespread effects. Not only is the PSI content reduced, but levels of chlorophyll, phycocyanin, β-carotene and myxoxanthophyll are altered, dark respiration levels are higher, and the regulation of light-harvesting is affected, probably because of the changes to the photosystems. Finally, the recovery of PSII from photoinhibition is reduced [Silva et al., submitted, Chapter 9].

Despite the loss of function, growth rates were unaffected under standard incubation conditions and light- and CO₂-saturated oxygen evolution was comparable. This is consistent with the ycf7 mutant in Synechocystis which also had a reduced PSI:PSII ratio but showed normal oxygen evolution [Wilde et al., 2001]. EPR spectra also suggested that PSI and component iron–sulphur centres (data not shown) were functioning correctly in slr0228−, although reduced in number.

It is perhaps unexpected that there is such a dramatic effect of the deletion of the slr0228 FtsH in Synechocystis 6803, given that there are four FtsH-type proteins and a multitude of other proteases in the organism that might act as functional substitutes. The widespread effects on photosynthesis of the insertional inactivation of slr0228 suggest that the encoded protein may have multiple roles within the thylakoid membrane. Previously, authors have suggested that FtsHs may act in various ways in quality-control of proteins [Schumann, 1999] raising the question, is the slr0228 FtsH a molecular chaperone as well as a protease?

To discover more about the roles of the slr0228 FtsH, the behaviour of the mutant Synechocystis was followed during the degradation and reassembly of the photosynthetic apparatus. It was hoped that this would show more clearly whether photosynthetic FtsHs act as chaperones and proteases, or both, for proteins of the thylakoid membranes.
6. IS FTSH A CHAPERONE FOR PSI ASSEMBLY?

6.1 Introduction
As mentioned above (and see Chapter 9), research concurrent to this showed that the motile slr0228\textsuperscript{−} \textit{Synechocystis} strain is compromised in its ability to repair high-light damage [Silva et al., submitted]. This, together with the work presented here in non-photoinhibited \textit{Synechocystis} (Chapter 7) and in the \textit{Arabidopsis} Var2-2 FtsH mutant (Chapter 8) [Bailey et al., 2002], suggests that a subset of FtsH proteins are involved in the turnover of PSII. This is not surprising; the PSII D1 protein is continuously degraded and replaced in high light [e.g., Aro et al., 1993; De Las Rivas et al., 1992; Kyle, 1985] and FtsH proteins are recognised as proteases that remove damaged proteins [e.g., Asahara et al., 2000; Herman et al., 1998]. In addition to effects on PSII, double mutants with an inactivated slr0228 ORF in a PSII\textsuperscript{−} background could not be created, suggesting an additional deleterious effect on transformants. Since some FtsH proteins can also act as molecular chaperones [for review, see Schumann, 1999], two other features of the slr0228\textsuperscript{−} phenotype were of interest. First, there is a marked reduction in the level of PSI per cell in the slr0228\textsuperscript{−} \textit{Synechocystis} which posed the question of whether the slr0228 FtsH has an additional role in the assembly of the reaction centre. Second, during the course of experiments, similarities were noted between slr0228\textsuperscript{−} and a PsaL\textsuperscript{−} strain of \textit{Synechocystis} grown in the same laboratory. PsaL has a role in the trimerisation of PSI in cyanobacteria [Chitnis & Chitnis, 1993; Schluchter et al., 1996] and therefore the effect of the loss of the slr0228 FtsH on PSI trimers was also investigated.

6.2 Nutrient deficiency assays
How can the synthesis of PSI be monitored? The GT and ChlL\textsuperscript{−} strains of \textit{Synechocystis} are useful, as mentioned above (Chapter 4), in that they can tolerate chlorosis to some degree while remaining viable. Depriving the WT and FtsH mutant of light (see Chapter 7, 8) or nutrients as described by Rippka (1988), might allow comparison of the loss and reassembly of the photosynthetic apparatus. Here it was found that subculturing GT \textit{Synechocystis} into either phosphate- or nitrogen-deficient BG11 resulted in reversible chlorosis of cultures. This allowed the pigment and photosystem levels to be measured during their decline, and then their recovery followed upon return of the culture to complete medium. To monitor the effect of inactivating the FtsH, doubling times, fluorescence, flash and absorbance spectra and oxygen evolution were compared in WT and slr0228\textsuperscript{−} cells.
6.2.1 Phosphate deprivation: motile strains

The first observation of reduced PSI content was in motile Synechocystis so the first assays were carried out in the motile strains. Although not ideal because of their obligately autotrophic nature, they did undergo slow chlorosis followed by regreening.

Parallel 90-ml cultures of motile WT and slr0228\textsuperscript{-} were matched for cell density (according to A\textsubscript{750}). Cells were gently pelleted and resuspended in BG11 that contained no source of phosphate (BG11-P). The cultures were incubated until they appeared to have lost most of their chlorophyll, approx. 2 weeks. Cultures remained relatively dense (WT and slr0228\textsuperscript{-} A\textsubscript{750} approx. 0.4–0.6) as they lost their green colouration, becoming yellow during this process.

![Fluorescence emission spectra](image)

**Figure 6.1.** 77K fluorescence emission spectra (excitation 435 nm) from duplicate cultures of chlorotic wild-type (WT) and slr0228\textsuperscript{-} motile strains of *Synechocystis* after growth in phosphate-deficient medium. Spectra normalised to photosystem I after background fluorescence subtracted.

Time-course samples were taken for absorbance and 77K fluorescence emission spectra. The latter show that, although much chlorophyll was lost, the cells retain at least some
functional photosynthetic reaction centres since the standard fluorescence peaks remain, although overall fluorescence emission was low [Figure 6.1]. Presumably the motile *Synechocystis*, not able to grow heterotrophically, would rely greatly on photosynthesis to remain viable, and would degrade other proteins before losing all photosystems. Nevertheless, chlorosis was sufficient to follow regreening.

To regreen cells, 50 ml of chlorotic cultures was gently pelleted as above and resuspended in 200 ml complete BG11. Time-course samples were taken as before to follow the return of normal pigmentation to the cultures.

**Regreening rates**

In all assays, when pigment concentration was corrected for cell density and plotted over time, the WT cells demonstrated a linear pattern of regreening. In the slr0228^- cultures, however, there was a clear lag period of 1–2 days before a substantial increase in the pigments' concentrations [Figure 6.2]. Rates of regreening could be calculated conveniently from linear regression plots to compare the effect statistically [Table 6.1], although it should be noted that this is not an ideal representation of the mutant (nonlinear) data. Despite this, these regression plots were reproducible, always showing that the motile *Synechocystis* WT accumulated pigments after chlorosis at almost twice the rate of slr0228^- cells. Interestingly, this applied to the return of both chlorophyll and phycocyanin.

**Table 6.1. Rates of regreening in motile WT and slr0228^- *Synechocystis* after phosphate chlorosis.**

<table>
<thead>
<tr>
<th></th>
<th>Rate of regreening (µM/A750/h)^*</th>
<th>Ratio of regreening rates (WT:slr0228^-)^t</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chlorophyll</td>
<td>Phycocyanin</td>
</tr>
<tr>
<td></td>
<td>WT      slr0228^-        WT      slr0228^-        Chlorophyll Phycocyanin</td>
<td></td>
</tr>
<tr>
<td>Motile strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assay 1</td>
<td>0.13    0.08</td>
<td>0.09          0.04</td>
</tr>
<tr>
<td>Assay 2</td>
<td>0.09    0.04</td>
<td>0.07          0.03</td>
</tr>
<tr>
<td>Assay 3</td>
<td>0.08    0.05</td>
<td>0.06          0.04</td>
</tr>
<tr>
<td>Mean (±SEM)</td>
<td>1.9 (±0.3)</td>
<td>1.9 (±0.2)</td>
</tr>
<tr>
<td>GT strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assay 1</td>
<td>0.04    0.02</td>
<td>ND            ND</td>
</tr>
<tr>
<td>Assay 2</td>
<td>0.08    0.07</td>
<td>0.051         0.038</td>
</tr>
<tr>
<td>Assay 3</td>
<td>0.05    0.04</td>
<td>0.033         0.039</td>
</tr>
<tr>
<td>Mean (±SEM)</td>
<td>1.4 (±0.2)</td>
<td>1.1 (±0.2)</td>
</tr>
</tbody>
</table>

^*Return of pigments monitored using absorbance spectra; pigment contents according to Myers *et al.* (1980) corrected for cell density (A750) of samples and time (rates/h). Rates estimated from linear regression analysis [SigmaPlot].

^t Test for motile strains, chlorophyll *P*=0.1; phycocyanin *P*=0.02 (4 degrees of freedom; d.f.). t Test for GT and motile strains combined, chlorophyll *P*=0.1; phycocyanin *P*=0.05 (10 and 8 d.f., respectively).

WT, Wild type; GT, glucose-tolerant; ND, not done; SEM, standard error of the mean.
Figure 6.2. Regreening of motile *Synechocystis* after chlorosis in phosphate-deficient medium. Chlorophyll (a) and phycocyanin (PC; b) accumulate more slowly in the slr0228<sup>−</sup> cells (○) than in WT(▲). Pigment concentration was corrected for cell density ($A_{750}$). See Assay 1 in Table 6.1 for rate of regreening.
In motile *Synechocystis*, the estimated rates of regreening of the slr0228" cells were slower than those of WT. This supported a role for the slr0228 FtsH in the assembly of pigment-containing complexes. It seemed possible, however, that there would be side-effects of phosphate deprivation on widespread metabolic pathways which might affect the results, especially in the motile strain of *Synechocystis* which is unable to dispense with photosynthesis in the presence of glucose. Its requirement to maintain autotrophic growth probably allowed only slow degreening. Chlorosis in the GT strain, in contrast, might be quicker, less damaging and cause fewer complicating side-effects since the medium could be supplemented with glucose.

### 6.2.2 Phosphate deprivation: glucose-tolerant strains

The slr0228" GT mutant (constructed as described in Chapter 4) was grown in phosphate-deficient medium as above, but with 5 mM glucose added. Chlorosis occurred within 2 weeks. For regreening, cells were returned to complete medium, again supplemented with glucose. Time-course samples for absorbance, flash and 77K fluorescence emission spectra were collected.

In the first assay the GT *Synechocystis* cells were enumerated during chlorosis using haemocytometer (total) cell counts and plate counts. These were $1.3 \times 10^6$ cells/ml and $2.5 \times 10^6$ c.f.u./ml in GT WT, respectively, and $5.9 \times 10^5$ cells/ml and $1.2 \times 10^5$ c.f.u./ml in GT slr0228" cells, respectively, showing that the cultures remained dense and viable.

**Regreening rates**

Regreening was slower in the GT slr0228" *Synechocystis*, as seen before in the motile mutant. Chlorophyll accumulated in the WT at 1.4-fold the rate of slr0228" [Table 6.1]. The faster rate of return of phycocyanin was not so marked in the GT WT, in contrast with motile strains where phycocyanin return was always slower in slr0228".

### 6.2.3 Photosystem I content

Most of the chlorophyll in *Synechocystis* is localised to PSI [Jordan *et al.*, 2001]. It might be inferred, therefore, that a slower return of chlorophyll to chlorotic cells indicates that PSI is also synthesised more slowly. To investigate this, samples were taken for the quantification of PSI during the regreening phase of one assay, after growing the GT strains in BG11–P until yellowed. Using flash spectroscopy to quantify functional PSI in the samples, the return of the reaction centre could be monitored over the duration of regreening [Figure 6.3].
Figure 6.3. Return of photosystem I (PSI) after subculture of chlorotic wild-type (WT) and slr0228 \(^{-}\) glucose-tolerant *Synechocystis* into BG11+phosphate \((t=0)\). PSI content calculated using flash spectroscopy of P700 (see Methods). Coefficient for moles of PSI from Hiyama & Ke (1972). Mean ± standard error given where sufficient material for duplicate samples. Trendlines calculated using polynomial regression in Microsoft Excel; correlation coefficients shown.

In this assay, the rate of return of chlorophyll in the samples was confirmed to be slower in the FtsH mutant. Using linear regression on flash spectroscopy data, as above, molecules of chlorophyll/c.f.u. in WT increased at 1.4-fold the rate of slr0228 \(^{-}\) [WT, 7.9\(\times\)10\(^4\)/c.f.u./h \((r^2 0.825)\) versus slr0228 \(^{-}\), 5.7\(\times\)10\(^4\) molecules/c.f.u./h, respectively \((r^2 0.743)\)].

As with the return of chlorophyll, the quantity of PSI in the slr0228 \(^{-}\) mutant (GT) remained static/decreased for longer than in WT after subculture to phosphate-containing medium. The lag phase was then followed by an increase in PSI, as seen with pigment re-accumulation. Rather than using polynomial line-fitting as shown here, if linear regression is used on the most linear period 72–144 h, WT PSI was accrued at 1.8-fold the rate of slr0228 \(^{-}\) PSI [WT, 1173 PSI /colony-forming unit (c.f.u.)/h \((r^2 0.944)\) versus slr0228 \(^{-}\), 657 PSI /c.f.u./h \((r^2 0.828)\)]. These are preliminary data only, and the difficulty in achieving
sufficient chlorophyll for flash spectroscopy measurements (large quantities of chlorotic cells needed to be centrifuged even to provide 6 µg chlorophyll) resulted in insufficient sampling timepoints to follow PSI closely. This experiment should be repeated to produce more datapoints and replicate samples.

6.2.4 Summary: phosphate deprivation
Using phosphate-deficient medium, it was possible to follow the loss and return of photosynthetic apparatus in these cyanobacteria. Regreening in complete medium suggested that the slr0228\(^{-}\) is impaired in its ability to return to functional photosynthesis after the degradation of the photosynthetic apparatus.

The slower return of chlorophyll and the preliminary results quantifying PSI suggested that the inactivation of this FtsH might affect the assembly of PSI, explaining the reduced content of that photosystem in slr0228\(^{-}\) cells. The return of phycocyanin to the cultures was more variable, but in the motile strains it was always slower than in WT. Why this should be is not clear: slr0228\(^{-}\) cells contain more, not less, phycocyanin per cell than WT which contradicts the likelihood of any inherent difficulty in PBS assembly. It may be that the PBS are affected by widespread consequences of reduced photosystem content or the nutrient deficiency: starved non-heterocyst-forming cyanobacteria certainly break down PBS quickly because they are a major nitrogen store [Baier \textit{et al.}, 2001]. It seems possible that there are more severe side-effects of phosphate starvation in the absence of the slr0228-encoded protein, and this affected the phycocyanin content of regreening cells.

6.2.5 Nitrogen deprivation of slr0228
It was hoped that a quicker system for provoking the breakdown of the photosynthetic apparatus might produce a ‘cleaner’ method of degreening and regreening cyanobacterial cells. Nitrogen deficiency produces chlorotic cells much faster than phosphate deficiency (N. Mann, personal communication, 2000) and was therefore used as an alternative method for regreening assays with the GT \textit{Synechocystis} strains. Using glucose-supplemented BG11 once again, it might be possible to specifically degrade the photosynthetic apparatus rather than universally damaging the cells.

As before, cells were gently centrifuged to remove them from complete medium before resuspension in BG11 (plus 5 mM glucose) from which all nitrogen-containing components had been omitted. As hoped, chlorosis resulted very quickly in WT. After only a few days, cells were sufficiently chlorotic to be returned to complete medium for
regreening. In contrast with WT, however, chlorosis took longer to develop in the slr0228⁻ culture. This is very marked in the appearance of the cultures [Figure 6.4] at 2 or 3 days N-starvation. Interestingly, the phosphate-deficient cultures had not showed the dramatically slower loss of chlorophyll that occurred in slr0228⁻ cells in BG11–N, although a slightly slower rate of degreening was sometimes observed (data not shown). Since the PBS are known to be degraded in response to nitrogen starvation [Baier et al., 2001], the marked pigmentation of the slr0228⁻ mutant might reflect a defect in PBS turnover or its regulation.

When the BG11–N cultures were examined in more detail during development of chlorosis [Figure 6.5a, d], absorbance spectra from the FtsH mutant showed a rather stable level of pigment/cell for the first few days, particularly of phycocyanin before its eventual decline, whereas in the WT both chlorophyll and phycocyanin dropped gradually over 0–4 days [Figure 6.5]. During regreening there was again a delay (here 3 days) in slr0228⁻ compared with WT before chlorophyll and phycocyanin were accumulated to any degree.

**Figure 6.4.** Flasks of slr0228⁻ (left) and wild-type (right) glucose-tolerant *Synechocystis* under (a) standard incubation conditions and (b) after 3 days nitrogen-deficient BG11.
Figure 6.5. Pigment concentration (corrected for cell density; $\Delta_{750}$) of wild-type (WT) and slr0228~ glucose-tolerant *Synechocystis* during nitrogen-deficient chlorosis and regreening in complete BG11. Duplicate cultures’ chlorophyll \((a, b)\) and phycocyanin \((c, d)\) content during chlorosis \((a, c)\) and regreening \((b, d)\) respectively. Note that the slr0228~ mutation results in less chlorophyll and more phycocyanin per cell even at $t=0$.

6.2.6 Photosynthetic function during regreening

Since it was difficult to produce samples of chlorotic cells with sufficient chlorophyll for flash spectroscopy, oxygen evolution was used to further investigate the function of photosynthesis during regreening, upon return of the cells to complete medium after nitrogen-deficiency.

Confirming the previous results in the phosphate deprivation experiment, oxygen evolution ceased quickly during chlorosis and then returned upon subculture to complete BG11 (plus glucose) [Figure 6.6]. Once again, the GT slr0228~ mutant was unable to photosynthesise effectively until 2–3 days after the WT; a distinct lag period is visible on the graph of oxygen evolution over time, as in the previous measurements of pigment and PSI.
Figure 6.6. Oxygen evolution of glucose-tolerant wild-type (WT) and slr0228\textsuperscript{−} \textit{Synechocystis} during nitrogen chlorosis (−nitrogen) and regreening (+nitrogen). During chlorosis, there was oxygen uptake (respiration) but no oxygen evolution, represented as negative values. All measurements (mean ± standard error; SEM) are light–dark rates from duplicate cultures made in saturating light in the presence of 10 mM sodium bicarbonate; samples were equalised to 5 μg/ml chlorophyll. Note the lag in recovery of slr0228\textsuperscript{−} cells, in parallel with slow return of pigments [Figure 6.5].

6.2.7 Summary: nitrogen deprivation

As in phosphate-deprivation experiments, the mutation of the slr0228 FtsH resulted in altered behaviour of the photosynthetic apparatus during nitrogen-deprivation. The aim of the nutrient deprivation studies was to examine assembly of PSI in the slr0228 insertional inactivation mutant but chlorosis as well as regreening proved to be defective. The cells’ control of pigment levels was affected so that, even under starvation conditions, a store of nitrogen was not utilised properly. Mutants with impaired PBS degradation have been identified in studies by using nitrogen-deficiency for screening [e.g., Baier \textit{et al.}, 2001] and it would be very interesting to explore further this aspect of slr0228 functions. Phycocyanin is a marker for PBS content, the degradation of phycocyanin paralleling the loss of phycobiliproteins as a whole. The NblR protein of \textit{Synechococcus} 7942 controls the number
of PBS present, and the nblA1 and nblA2 genes of Synechocystis are transcribed within 3 h of nitrogen starvation. A NblR−Synechococcus mutant had less rapidly-degrading PBS, and died quickly under nitrogen starvation [Baier et al., 2001], showing that the impediment to pigment regulation in the slr0228 mutant could have serious consequences. As discussed later (Chapter 8), the effect on pigment levels may be more complicated if related to degradation of PSII in slr0228− Synechocystis.

With regard to assembly of PSI, the delayed accumulation of pigments and of functional photosynthesis upon the return of chlorotic slr0228− cells to BG11+N support the previous data from the BG11±P assays. There was a reproducible and rather severe effect upon the synthesis of the photosynthetic apparatus in heterotrophically growing slr0228− cells: the quantification of chlorophyll and PSI did suggest that there may be a deficiency in PSI assembly.

6.3 Photosystem I assembly: blue light

There is reduced abundance of PsaD and PsaF in slr0228− (motile) cells [Mann et al., 2000] so it seems plausible that this FtsH is a molecular chaperone for subunit assembly in PSI. A further effect of the slr0228− mutation on PSI was also observed, however. In sucrose density gradients of solubilised thylakoid membranes, there always appeared to be a slightly more dense PSI monomer/PSII band in the slr0228− sample than in that from WT [Figure 5.4] even from cells grown under standard incubation conditions. This prompted the question of whether inactivation of the slr0228-encoded FtsH affects PSI timerisation or whether it reflected the increased PSII:PSI ratio. Although the necessity for the PsaL subunit for PSI trimers is well-known [Chitnis & Chitnis, 1993], regulatory protein(s) that might be responsible for the assembly of trimers, or protease(s) for their turnover or division, have not been identified. An effect upon trimerisation could perhaps account for some pigment and PSI defects in slr0228− Synechocystis.

6.3.1 Growth under blue light

Cyanobacteria moved to low-blue light from moderate-intensity light tend to increase the amount of PSI trimers relative to monomers (P. Fromme, personal communication, 2001). Blue filters were therefore used on liquid cultures of GT WT and slr0288− Synechocystis to magnify any effect of the inactivation of the slr0228 FtsH. Exponentially-growing duplicate or triplicate parallel cultures were first grown under moderate light intensity (approx. 20 μmol/m²/s) and then blue filters were added, resulting in blue light of intensity 4 μmol/m²/s.
When grown in blue light, it was clear that slr0228<sup>−</sup> and WT adapted differently to the altered light conditions. The growth of the mutant was severely impaired, never producing the dense, dark green cultures typical of <i>Synechocystis</i>. The mean doubling time of triplicate WT cultures in blue light was 43±1 h whereas that of the slr0228<sup>−</sup> cells was 78±8 h [Figure 6.7].

The effect of low light intensity was investigated to be sure that the slow growth was not resulting from the reduced light levels under blue filters. When neutral density filters that reduced the light to approx. 5 μmol/m<sup>2</sup>/s were used, however, similar growth rates were seen in WT and slr0228<sup>−</sup> [Figure 6.7].
Figure 6.8. 77K fluorescence emission spectra from wild-type (WT) and slr0228 glucose-tolerant *Synechocystis* grown in low light (LL, top panel) and blue light for 5 days (lower panel). Note the small right-shift of WT photosystem I (PSI) peak in blue light that is not matched in the slr0228 culture, and large photosystem II peak in slr0228'. Excitation light 435 nm; spectra normalised to PSI peak after background fluorescence subtracted.
6.3.2 77K fluorescence emission spectra

Examining blue-light cultures in more detail, 77K fluorescence emission spectra revealed that the slr0228 mutant had an extremely large PSII:PSI fluorescence ratio compared with WT [Figure 6.8]. (The increased PSII:PSI ratio seen was even larger than that normally seen [Figure 6.8].) This meant that the slr0228 cells’ PSII was either nonfunctional, there was more PSII than in WT, or there was less PSI than in WT.

The WT spectrum remained much the same during growth in blue light, which was surprising. The amount of PSII/cell in Synechocystis might be expected to increase to compensate for the chlorophyll-exciting blue light best-absorbed by PSI, even if more PSI trimers were formed concurrently as suggested (P. Fromme, personal communication, 2001). There was one change to confirm that WT had responded to blue light, however. The 77K fluorescence emission spectrum in 435 nm excitation light from PSI trimers (extracted from sucrose density gradients; see below) shows a PSI peak at a slightly longer wavelength than that from the extracted PSI monomer/PSII band [see, for example, Figure 6.9]. This shift was seen in the whole-cell spectra from blue-light-incubated cultures: before blue light treatment the PSI peak was at approx. 724 nm; after blue light incubation for 5 days, the PSI peak was at approx. 726 nm, suggesting an increased proportion of trimers. In contrast, the slr0228 cells showed no movement of the peak from its initial position [Figure 6.8], indicating no increase in the trimer:monomer ratio. Interestingly, this is also the case in the trimer band from fractionated slr0228 thylakoids [Figure 6.9]. What causes this difference remains unclear.

![Figure 6.9](image-url)

Figure 6.9. 77K fluorescence emission spectra from sucrose density gradient bands from solubilised thylakoid membranes. Upper band is composed of photosystem II (peak at 680–690 nm) with photosystem I (PSI) monomers (peak at approx. 721 nm) whereas the lower band is composed of PSI trimers (peak at ≥723 nm). Note the shift in PSI peak in wild-type (WT) between monomers and trimers. Excitation light, 435 nm. Spectra normalised to PSI peak after background fluorescence subtracted.
Figure 6.10. 77K fluorescence emission spectra from wild-type (WT) and PsaL\textsuperscript{−} *Synechocystis* grown in blue light for 11 days. Excitation light, 435 nm. Spectra normalised to PSI peak after background fluorescence subtracted.

PsaL\textsuperscript{−} *Synechocystis* thylakoid membranes were included in the blue-light assays as a comparison strain unable to form trimers (see below); like the slr0228\textsuperscript{−} FtsH mutant, the PsaL\textsuperscript{−} cells grew more slowly under blue light and produced a static PSI peak in the 435 nm 77K fluorescence emission spectrum, lacking the shift to longer wavelength after incubation that would indicate the presence of trimers [Figure 6.10].

6.3.3 Sucrose density gradients in blue light

To observe the physical presence of the trimers and monomers rather than just their fluorescence, sucrose density gradients were carried out after growth in blue light. Although a change was seen in fluorescence spectra of samples made very soon after the transfer (≤4 days), sufficient material for preparing thylakoid membranes was available only after several weeks’ growth and production of several litres of cells. In the case of slr0228\textsuperscript{−} cells, growth was so impeded that it was never possible to obtain a sample with sufficient photosynthetic apparatus for a reasonable density gradient, in particular to allow loading on an equal chlorophyll basis [Figure 5.4].
PsaL− thylakoid membranes showed only one band, the PSI monomers with PSII, as expected [Figures 5.4, 6.10]. The poor slr0228 gradient appeared to be most similar to PsaL−: there was no evidence of a PSI trimer band at all. The only band evident below the carotenoids was the PSI monomer plus PSII band. If the slr0228− mutant was able to respond like WT and produce trimers, a quite different gradient would be expected: even if another reason prevented thylakoid membrane assembly in general, a response comparable to WT would have produced at least a lower band albeit a faint one.

The WT solubilised thylakoid membranes could be seen to contain more trimers relative to monomers after growth under blue light than under standard incubation conditions [Figure 5.4]. Since the 77K fluorescence emission spectra from whole cells (see above) suggested that there was not a large change in the relative amounts of PSI and PSII in WT it seems that, under blue light, either some of the extant population of PSI monomers is assembled into trimers or it is degraded and new PSI synthesised and assembled as trimers. Either way, there is no great difference in the ratio of PSI to PSII in WT which would be seen in the relative sizes of the fluorescence spectra peaks.

6.3.4 Quantification of photosystem I and pigments in blue light

Flash spectroscopy was used to quantify functional PSI in cultures grown in blue light [Figure 6.11]. This showed that there was a drop and then recovery in both WT and the slr0228− mutant. The already-reduced PSI content in slr0228− followed by a further decrease, however, leaves little (functional) PSI/cell remaining. The effect of blue light on the mutant is so severe that between 5 and 8 days no samples could be made for flash spectroscopy because there was insufficient chlorophyll present in the cultures (the bulk of the cultures would have been needed to concentrate the chlorophyll sufficiently for measurements). Following prolonged incubation in blue light, the mutant recovered PSI/cell like WT but, as in nutrient deficiency experiments, the recovery was later than in WT. (NB. Growth was slower in the FtsH mutant compared with WT, resulting in the lack of photosystems in total on density gradients.)

Chlorophyll levels in each strain reflected changes in PSI [Figure 6.12]. Chlorophyll dropped slightly and recovered in WT but decreased greatly in the mutant and did not recover. This explains why the cultures are extremely pale after prolonged incubation in blue light. In contrast, phycocyanin levels changed little in slr0228− and WT. The small decrease and recovery of phycocyanin seen in WT might explain why 77K fluorescence spectra do not show any difference in fluorescence emission ratios from PSI/PSII in WT:
if PSII and the PBS were to decrease somewhat as PSI/chlorophyll decreases, no significant change would be detected in relative fluorescence emission.

The retention of phycocyanin in slr0228 corresponds with the increased PSII peak in 77K fluorescence emission spectra. The relatively stable phycocyanin levels in this FtsH mutant are also in line with the findings of Yu et al. (1999), who previously reported that a chlorophyll-synthesis mutant, when grown in the dark to provoke chlorosis, retained its phycocyanin even when chlorophyll was depleted. Here, however, the mutant did seem to have a further difference: altered PBS pigment composition. When sucrose density gradients were prepared, the PBS washed off during preparation from slr0228 cells grown under blue light were a different colour to those from WT and PsaL cells [see Figure 5.4]. Altered levels of PSII and PSI would alter regulation and composition of the light-harvesting antennae, perhaps causing this effect, but it would be interesting to examine this further.

**Figure 6.11.** Quantification of functional photosystem I (PSI) by flash spectroscopy in wild-type (WT) and slr0228 glucose-tolerant *Synechocystis* cultures grown in blue light. Mean ± standard error of duplicate measurements made in duplicate cultures. The content of PSI was calculated using flash spectroscopy of P700 (see Methods), using the coefficient for moles of PSI from Hiyama & Ke (1972).
Figure 6.12. Pigment content of wild-type (WT) and slr0228 Synechocystis during growth in blue light. Chlorophyll and phycocyanin levels estimated according to Myers et al. (1980) from DW2000 absorbance spectra and divided by $A_{750}$ to compensate for cell density of each sample.
6.4 Discussion

The first facet observed of the slr0228-null phenotype was a reduction in the level of PSI, with slightly lower levels of the PSI subunits PsaD and PsaF [Mann et al., 2000]. Why there was less PSI was not clear. Later work has showed a severe effect on PSII of mutation of photosynthetic FtsHs [Silva et al., submitted; Bailey et al., 2002] (Chapters 7, 8) raised the question of whether altered PSII behaviour causes side-effects amongst which is a reduction in PSI/cell. Nevertheless, AAA proteins are known for their multiple roles in cells facilitating the degradation and assembly of protein complexes (see Introduction) [for reviews, see Langer, 2000; Schumann, 1999]. In addition, a PSII/slr0228- mutant could not be constructed, suggesting a function of the slr0228 protein beyond PSII. Could the slr0228 FtsH act as a molecular chaperone, therefore, in addition to its role in the turnover of PSII?

Growing WT and slr0228- in altered light and nutrient environments allowed the synthesis of PSI to be studied. All the tests carried out support the hypothesis that the slr0228-encoded FtsH is involved in the assembly of functional photosynthetic apparatus. Growth of the slr0228- mutant is not slower than WT under standard incubation conditions; in contrast, the lag phase in the recovery of each of the features tested here suggests that there is a specific loss of function when reassembly of the reaction centres is required (which is then compensated for between 1 and 3 days after the lag phase).

Both phosphate- and nitrogen-deficiency experiments proved useful in provoking chlorosis of Synechocystis cultures and showed a marked delay in the return of pigmentation (chlorophyll, and phycocyanin to some degree), function (oxygen evolution) and PSI itself during regreening.

By studying the behaviour of a chlorophyll-depleted culture of a ChlL/PSI- strain of Synechocystis, Wu & Vermaas (1995) observed that at the very beginning of regreening the rate of chlorophyll synthesis is independent of the presence of PSI (the complex with the largest chlorophyll requirement). CP43 is reported to be relatively stable in the membrane even upon deletion of some PSII components [Wu & Vermaas, 1995 and references therein], and it has been suggested therefore that initially chlorophyll accumulates and binds to chlorophyll-binding proteins not associated with either photosystem core. Wu and Vermaas (1995) found, however, that the phase of regreening from 6 h to 5 days is lower in amplitude in the absence of PSI, and confirmed that in WT the same period of 6 h to 5 days was when functional PSII and PSI are formed. These findings correlate with the
lower chlorophyll-regreening seen here in the slr0228- cells compared with WT; with slower PSI assembly, chlorophyll accumulation would be lower.

The slower return of PSII as well as PSI could not be ruled out by the nutrient deficiency experiments. However, although PSII turnover is affected by loss of the slr0228 FtsH, the synthesis and function of PSII appear unchanged under non-photoinhibitory conditions (flash spectroscopy data not shown, and see regreening data in Chapter 7). Monitoring the oxygen uptake in the Mehler reaction using the oxygen electrode would reveal a specific effect on PSI, and quantifying PSII using labelled atrazine could rule out an effect on synthesis of PSII.

In the BG11–N assays the loss of the PBS (as indicated by phycocyanin levels) was also aberrant. As mentioned above, this may be related to the slow turnover of PSII, or the slr0228 FtsH may be a protease involved in the degradation of the PBS. Other candidates for PBS turnover in cyanobacteria have been identified by other authors [SppA by Lensch et al., 2001; ClpP1 by Barker-Åstrom et al., 2001], but this does not rule out the involvement of a FtsH protease, singly or perhaps as part of a complex with other proteases.

In each of these experiments the effect of the slr0228- mutation, e.g., a lag phase in mutant regreening or in the breakdown of PBS, was eventually followed by recovery. This suggests that another protein(s) can partially substitute for the function of the slr0228 FtsH. Since the *Synechocystis* genome encodes four FtsH-type proteins, one or more of the other three may be able to replace the slr0228-encoded subunits in the FtsH oligomer, or an alternative pathway may eventually compensate for the loss of the FtsH. Detailed molecular biological investigation of the temporal and spatial expression of the alternative FtsHs and the interactions between them would help elucidate their functions.

The lower levels of PsaD and PsaF in the motile slr0228- *Synechocystis* strain [Mann et al., 2000] do support the idea that the slr0228 FtsH may have a function as a chaperone in PSI synthesis at the level of assembly of subunits into the complex. The effect of blue light on the mutant, however, suggests an additional or alternative role for the protein in PSI trimerisation. The presence of trimers of PSI in cyanobacteria makes them distinct from PSI in plants. Why cyanobacteria have trimers and plants do not is often explained from the point of view that the plant integral LHC prevents trimerisation [for discussion, see Boekema et al., 2001b], but the regulation and reason for PSI trimers remaining or having developed in cyanobacteria is uncertain. Trimer-deficient *S. elongatus* does grow more slowly
than WT [Fromme et al., 2001]. One hypothesis for their function is that, because protein complexes are densely packed in the thylakoid membranes of cyanobacteria, trimerisation of PSI may be a way of preventing uncontrolled excitation energy transfer from PSII [for review, see Mullineaux, 1999].

How the cyanobacterial PSI trimers are assembled is also not known, beyond the involvement of PsaL, which may be necessary for optimal light-harvesting in both plants and cyanobacteria [Fromme et al., 2001]. Whether the level of PsaL is changed in the slr0228" mutant is not known.

Using blue light does seem to provoke greater trimerisation, although the lack of detail available about the formation of monomers or trimers of PSI makes the interpretation of the blue-light data complicated. Although blue-light-grown WT fluorescence emission spectra do not show a dramatically altered PSI:PSII fluorescence [Figure 6.8], there was the typical trimer-associated shift in the PSI peak and the sucrose density gradients also showed more trimers than monomers. It is not known if a population of PSI monomers is directly converted to trimers, or whether monomers are removed while new PSI is formed as trimers. The flash spectroscopy data did show the loss of some functional PSI but the fluorescence spectra suggest either no net loss of PSI/cell or, alternatively, the amount of PSII per cell may decrease at the same time as PSI turnover occurs. Oxygen evolution during the blue-light assays did decrease (data not shown) and the phycocyanin level dropped slightly, so a change in PSII/cell is possible.

Both the blue-light-grown WT and slr0228" mutant showed a reduction in PSI in flash spectroscopy but the WT recovered quickly. The slr0228" strain normally has much-reduced levels of PSI under standard conditions and, therefore, the loss of any proportion of the photosystem may severely affect the cells. The mutant certainly grew very poorly in blue light. The lack of efficient PSII turnover (see Chapter 7, 9) in slr0228 concomitant with a decrease in PSI/cell presumably accounts for the dramatic increase in its PSI:PSII fluorescence ratio in the mutant in blue light, in contrast with WT [Figure 6.8].

Interestingly, the loss of PSI/cell in blue light seems to be faster (in both WT and mutant) than would be possible via mere dilution of PSI during the growth of cells that have stopped synthesising the reaction centre, suggesting an active process of degradation of PSI in *Synechocystis*. Although the cycle by which PSII is assembled and repaired is well-studied because of the interest in how the D1 protein is damaged, it is not known if there is such a
mechanism for turnover for PSI. Sonoike (1999, 2001) has postulated that PSI photoinhibition is more common than was previously thought, so a parallel repair cycle may be necessary.

The slr0228 FtsH does not seem to be necessary for removal of PSI, unlike PSII. Without the slr0228 FtsH, however, there are fewer trimers of PSI under conditions where the WT has increased its trimer:monomer ratio; the content of PSI in the slr0228^− mutant is much-reduced; and the assembly of PSI and return of oxygen evolution are both impeded after chlorosis. This marked and reproducible effect upon the return of chlorophyll, PSI and functional photosynthesis to regreening slr0228^− *Synechocystis* and the behaviour of the strain in blue light strongly implicate this photosynthetic FtsH in the assembly of PSI as well as repair of PSII. Not only do photosynthetic FtsHs act as proteases but they facilitate the assembly of the photosynthetic apparatus as well.
7 INVESTIGATING THE ROLE OF slr0228 FTSH USING CHL\textsuperscript{L\textsuperscript{-}} \textit{SYNECHOCYSTIS}

7.1 Introduction
Photosynthetic bacteria are among the organisms that are able to synthesise chlorophyll by both light-dependent and -independent pathways. A useful strain of \textit{Synechocystis} that is unable to synthesise chlorophyll in the dark has been made by mutating the \textit{chl} gene that encodes one of three polypeptides of the light-independent protochlorophyllide oxidoreductase [Wu & Vermaas, 1995]. Growth of the strain in LAHG (see below) causes the loss of most pigment and photosystems but the cells continue to be viable [Wu & Vermaas, 1995].

The Chl\textsuperscript{L\textsuperscript{-}} strain of \textit{Synechocystis} appeared to be an ideal mutant with which to investigate the regreening of chlorotic cyanobacteria and further elucidate the role of photosynthetic FtsHs as molecular chaperones. Although the nutrient deprivation assays (Chapter 6) supported a role for the slr0228 FtsH in PSI assembly, chlorosis assays that avoided the starvation side-effects of phosphate and nitrogen deficiency might aid a more clear understanding of the process. A double mutant, slr0228/Chl\textsuperscript{L\textsuperscript{-}}, was therefore constructed as detailed (Chapter 4) and compared with Chl\textsuperscript{L\textsuperscript{-}} during regreening following chlorosis. When these assays were carried out, the role of the slr0228 FtsH in the regulation of PSII content also became a subject of study.

7.2 LAHG in slr0228/Chl\textsuperscript{L\textsuperscript{-}} \textit{Synechocystis}
Duplicate Chl\textsuperscript{L\textsuperscript{-}} and Chl\textsuperscript{L}/slr0228\textsuperscript{-} cultures were subcultured in parallel, adjusted to give the same cell density and supplemented with 5 mM glucose (and antibiotics as appropriate). Experience with these strains suggested that, to ensure viability, it was necessary to allow upregulation of genes for heterotrophic growth, so cultures were grown for 24 h growth in the medium plus glucose before they were incubated in the dark with a 10-min light period each day (LAHG).

Before studying the regreening behaviour in the slr0228/Chl\textsuperscript{L\textsuperscript{-}} mutant, it became apparent that its ability to remove the photosynthetic apparatus was greatly affected. The presence of pigments, photosystems and photosynthetic activity during LAHG was monitored, therefore.
Some chlorophyll always remained after LAHG in Chll- cells, presumably a proportion from before LAHG conditions began and some synthesised in the short light period each day. After approx. 5 days, however, substantial loss of pigment was observed in Chll-, in great contrast with the Chll-/slr0228' cultures which appeared dense and pigmented [Figure 7.1]. Chll- strains cannot make chlorophyll in the dark so the difference must be due to a change in the breakdown of photosynthetic apparatus in the absence of the slr0228 FtsH.

![Image of cyanobacteria cultures](image_url)

**Figure 7.1.** After growth with glucose for 24 h (a), light-activated heterotrophic growth (LAHG) conditions for 4 days result in chlorosis in Chll- (b), but not in slr0228/Chll- (c), cultures. The cyanobacteria must be grown during LAHG in flasks bubbled with filtered air and supplemented frequently with fresh medium to maintain viable cultures.

7.2.1 77K fluorescence emission spectra

What photosynthetic apparatus remains in the double mutant after LAHG incubation, causing the continued pigmentation of the cultures? Wu & Vermaas (1995) documented the loss of almost all pigment and photosystems until the usual characteristics of photosystem fluorescence were no longer discernable in 77K fluorescence emission.
spectra, but chlorosis could not be carried out to this extreme here: it became apparent that very long periods of LAHG incubation were required to cause chlorosis in the slr0228/ChlL⁻ double mutant.

77K fluorescence emission spectra showed that the amplitude of fluorescence diminished over time in LAHG conditions, although PSI and PSII peaks remained even after growth in the dark for 1 week. Spectra from 435 nm excitation light showed that fluorescence emission in the slr0228/ChlL⁻ double mutant (although reduced overall after 6 days LAHG treatment) was greatly increased from PSII compared with PSI, in contrast with ChlL⁻ [Figure 7.2].

Under 600 nm excitation, the fluorescence was also markedly increased from the 680 nm peak from PSII and the PBS terminal emitters in the slr0228/ChlL⁻ double mutant compared with ChlL⁻ [Figure 7.3] after LAHG treatment. Such high fluorescence from this peak in 77K spectra is thought to indicate PBS decoupled from reaction centres [Mullineaux, 1994].

These spectra reinforce the observations from nitrogen deficiency studies and work described here [Chapter 8; Bailey et al., 2002] and elsewhere [Silva et al., submitted] (Chapter 9): without the slr0228 FtsH the photosynthetic apparatus of Synechocystis is not removed properly. Specifically, PSII turnover seems to be defective.
Figure 7.2. 77K fluorescence emission spectra from (a) duplicate ChlL⁺ (A/B) and slr0228/ChlL⁻ (C/D) cultures, and (b) after 6 days' growth in the dark plus glucose. Spectra normalised to photosystem I peaks after background fluorescence subtracted; excitation light 435 nm.
Figure 7.3. 77K fluorescence emission spectra in 600 nm excitation light from (a) duplicate ChlL⁻ (A/B) and slr0228/ChlL⁻ (C/D) cultures; and (b) after 6 days’ growth in the dark plus glucose. Spectra normalised to the phycocyanin/allophycocyanin peak after background fluorescence subtracted.
7.2.2 Pigment content during LAHG

Measurement of pigment levels was difficult during the chlorosis caused by LAHG in the ChlL⁻ strains. First, pigment content, along with other photosynthetic parameters, often decreased upon the addition of glucose to cultures. Second, there was a discrepancy between the chlorophyll levels calculated using methanol extracts and whole-cell samples.

Methanol extracts made at the time of sampling were measured using the Unicam spectrophotometer and these showed that the chlorophyll content [according to Porra et al., 1989] dropped in ChlL⁻ but not in slr0228/ChlL⁻, an increasingly dramatic difference over time corresponding with the appearance of the cultures [Figure 7.4]. When absorbance measurements were made of whole-cell samples using the DW2000 spectrophotometer and the pigment content calculated using the coefficients from Myers et al. (1980), however, the quantity of chlorophyll and phycocyanin thus determined was incorrect. In contrast with the appearance of the cultures, the per-cell (calculated) pigment levels did not decrease greatly [Figure 7.5]. This suggests that the use of $A_{750}$ for equalising the results of the Myers' formulae on cell density will not produce accurate pigment/cell estimates when used for cells with unusual light-scattering properties. This might result from altered thylakoid membrane content, for example: indeed, thylakoid membranes do appear to be reduced in chlorotic ChlL⁻ seen in electron microscopy (S. Møller, C. Mullineaux, personal communication, 2003).

An interesting point to note from the whole-cell calculated pigment levels, however, is the difference between the phycocyanin content of ChlL⁻ and slr0228/ChlL⁻. Assuming that the comparison of whole-cell spectra from ChlL⁻ and slr0228/ChlL⁻ is valid, despite the specific problems with quantification, it appears that after LAHG conditions the phycocyanin content of the strains diverged greatly. As before in nitrogen deficiency assays, the amount of phycocyanin per cell in the FtsH mutant, slr0228/ChlL⁻ Synechocystis, remained high compared with ChlL⁺, even increasing by 5 days' LAHG. In contrast, in ChlL⁻ Synechocystis the phycocyanin level dropped during LAHG as might be expected.
Figure 7.4. Ratio of mean chlorophyll/ml in duplicate cultures of ChlL− and slr0228/ChlL− *Synechocystis* during light-activated heterotrophic growth. Chlorophyll dropped in ChlL− cultures but remained stable in slr0228/ChlL−. Chlorophyll content estimated from the absorbance of methanol extracts measured in the Unicam spectrophotometer [Porra et al., 1989], i.e., not corrected for cell density. N.B. Diluting the cultures to match the cell densities on *A*250 at t=0 resulted in a large difference in initial chlorophyll concentration producing the high ratio at the start of this assay.
Figure 7.5. Mean pigment content (± standard error) of ChlL− (▲) and slr0228/ChlL− (○) Synechocystis before and during light-activated heterotrophic growth (LAHG; shaded area). Glucose (5 mM) added 24 h before LAHG. Chlorophyll (a) and phycocyanin (b) levels estimated according to Myers et al. (1980) from DW2000 absorbance spectra and divided by A750 to compensate for cell density of each sample.
Figure 7.6. Oxygen evolution (a) and light-dependent oxygen uptake (b) in the Mehler reaction in ChlL− (grey) and slr0228/ChlL− (orange) Synechocystis during light-activated heterotrophic growth (LAHG). No t=0 measurement of uptake was made. Glucose (5 mM) added 24 h before LAHG. Measurements made under saturating light with sodium bicarbonate for oxygen evolution; 3-(3,4-dichlorophenyl)-1,1-dimethylurea, methyl viologen, ascorbate and tetramethyl-p-phenylene diamine for uptake. All data are mean (± standard error) light–dark measurements.
7.2.3 Oxygen evolution during LAHG

Oxygen evolution under saturating light in the oxygen electrode was used as a measure of PSII activity during LAHG. As with pigment levels, this measure proved difficult to use under LAHG conditions with the Chl\textsuperscript{L} strains. The addition of glucose to cultures often resulted in lower oxygen evolution in Chl\textsuperscript{L} and slr0228/Chl\textsuperscript{L} \textit{Synechocystis} than during photoautotrophic growth, resulting in rather variable measurements and large standard errors. Further experiments are needed to include more replicates and sampling points which would improve the reliability of the data.

Broadly, during LAHG, oxygen evolution stopped in the Chl\textsuperscript{L} strain at or before 5 days, as would be expected from its chlorotic appearance. In contrast, whole-chain electron transport remained stable or increased in the double mutant, even after 9 days of incubation in the dark [Figure 7.6]. This corresponds with the continued pigmentation and the increased PSII peak seen in the 77K fluorescence emission spectra, supporting the hypothesis that PSII degradation was not carried out in the absence of the slr0228 FtsH in \textit{Synechocystis}.

7.2.4 Photosystem I content during LAHG

PSI activity was evaluated by measuring light-dependent oxygen uptake in the Mehler reaction in the oxygen electrode and by flash spectroscopy. Oxygen uptake remained relatively stable in both Chl\textsuperscript{L} and slr0228/Chl\textsuperscript{L} after 5 days of LAHG [Figure 7.6], suggesting that the turnover of PSI was slower than that of PSII. Once again, however, variability between samples made it difficult to produce small errors of mean values.

Although Wu & Vermaas (1995) were able to use LAHG to remove almost all the photosynthetic apparatus in their studies, a compromise was necessary here to maintain viability of both Chl\textsuperscript{L} and slr0228/Chl\textsuperscript{L} cultures. Although GT \textit{Synechocystis} should be able to grow heterotrophically, it was found that viability under LAHG conditions was difficult to maintain if chlorosis was carried through to complete loss of chlorophyll in Chl\textsuperscript{L} cultures. Therefore, LAHG conditions were used only until oxygen evolution stopped or decreased greatly. This ensured that regreening could be reliably carried out (see below) and that any changes seen should not be the result of any cells dying during the experiments. In addition, so that quantification of PSI during regreening (using flash spectroscopy) could be carried out in parallel in Chl\textsuperscript{L} and slr0228/Chl\textsuperscript{L}, treatment of the cultures was offset: the double mutant was incubated under LAHG for 10 days, whereas the dark-incubation of the Chl\textsuperscript{L} culture began only at day 6.
suggested would allow a significant degree of chlorosis to occur in each. (Both were supplemented with glucose 24 h before LAHG and again upon return to low light after LAHG.)

Confirming oxygen uptake, flash spectroscopy measurements showed that over 4 days’ LAHG, the number of PSI centres per ChlL− cell remained fairly stable at around $1 \times 10^5$/cell (as mentioned above, cells were not left to deplete all their pigment and photosystems). The level of PSI did eventually decline somewhat in slr0228/ChlL− cells, suggesting no deficiency in the ability to remove that photosystem, but most of the decrease was seen after 4 d. The difference between it and the ChlL− cells is probably due to the increased period of LAHG rather than any difference in the ability to degrade PSI [Figure 7.7].

Flash spectroscopy also confirmed that the slr0228/ChlL− Synechocystis maintains chlorophyll at around 200 molecules per PSI, even after more than 1 week of LAHG (data not shown). In these LAHG assays, the mean chlorophyll in ChlL− cells decreased from 200–300 to approx. 100 molecules per PSI over 4 days of LAHG (data not shown).

Figure 7.7. Quantification of functional photosystem I (PSI) by flash spectroscopy in ChlL− and slr0228/ChlL− Synechocystis cultures grown under light-activated heterotrophic growth (LAHG). Mean (± standard error) content of PSI was calculated using flash spectroscopy of P700 (see Methods) using the coefficient for moles of PSI from Hiyama & Ke (1972). Glucose (5 mM) added at 24 h before LAHG conditions started.
Figure 7.8. Oxygen evolution (a) and uptake in the Mehler reaction (b) in ChlL⁻ (grey) and slr0228/ChlL⁻ (orange) *Synechocystis* during recovery in low light after light-activated heterotrophic growth (LAHG). Cultures supplemented with glucose (5 mM). Measurements made under saturating light with sodium bicarbonate for oxygen evolution; 3-(3,4-dichlorophenyl)-1,1-dimethylurea, methyl viologen, ascorbate and tetramethyl-p-phenylene diamine for uptake. All data are mean (± standard error) light–dark measurements; no measurements of oxygen evolution at 48 h in ChlL⁻.
7.3 Regreening after LAHG

After the prolonged activity of PSII in the slr0228/ChlL− double mutant during LAHG, the oxygen evolution measurements remained stable during recovery in low light [Figure 7.8]. The ChlL− cultures, in contrast, had degraded PSII and the recovery of oxygen evolution could be seen over time in measurements in the oxygen electrode [Figure 7.8].

The reverse was the case in PSI measurements using the Mehler reaction. Although there was little change in the mean oxygen uptake/cell during 4 or 5 days’ LAHG, the slr0228/ChlL− mutant was subjected to further dark treatment so that chlorosis could eventually occur and then regreening be monitored. At this point PSI activity had declined. Return of the FtsH double mutant to low light was then followed by the usual lag phase, compared with ChlL−, this time in the return of oxygen uptake levels [Figure 7.8]. In fact, in slr0228/ChlL−, oxygen uptake in the Mehler reaction continued to decrease for 3 days after return to low-light conditions with recovery in PSI activity only after 4–5 days. This was also seen in the flash spectroscopy measurements [Figure 7.7].

As before, flash spectroscopy also allowed the chlorophyll per reaction centre to be estimated. These figures suggested that when the ChlL− cultures were returned to low light, the chlorophyll/PSI doubled to approx. 200. In contrast, at 10 days LAHG the quantity of PSI had begun to decrease in slr0228/ChlL− cells, resulting in twice the amount of chlorophyll/PSI seen in ChlL after LAHG. Since the PSI recovery was then slower than in ChlL−, the chlorophyll/PSI was consequently unusually high for the first 3 days of low-light recovery (data not shown). As before, this indicated a lag in the recovery of the FtsH mutant after chlorosis, specifically in PSI reaccumulation rather than chlorophyll.

In the ChlL− strain, the Mehler reaction produced stable oxygen uptake after return to low-light incubation in ChlL−. This is as expected since PSI was apparently not greatly reduced during LAHG according to either oxygen uptake or flash spectroscopy. In contrast with oxygen electrode data, however, flash spectroscopy measurements indicated a further decline in functional PSI for several days after the return to low-light conditions [Figure 7.7]. More experiments would be needed to determine whether variability within samples was the cause of the discrepancy between the two methods.
7.4 Discussion

The purpose of using chlorosis in the Chl$^{-}$ and slr0228/Chl$^{-}$ strains was to answer the question of whether regreening was affected by insertional inactivation of the slr0228 FtsH. The effect of the LAHG treatment on the cultures was so striking, however, that the loss of pigment and photosynthetic activity was also followed during development of chlorosis. Interestingly, not only did double-mutant cells remain green after dark incubation, the surface of the aerated cultures became rather foamy, presumably from starch production, suggesting further side-effects of insertional inactivation of the slr0228-encoded FtsH on its metabolism in the dark.

As could be seen from the appearance of the cultures [Figure 7.1], the slr0228/Chl$^{-}$ double mutant was not able to reduce its pigment and photosystem content in parallel with the Chl$^{-}$ strain. LAHG conditions were necessary for approx. 11 days before any great loss of photosystem activity was seen in the oxygen electrode and using flash spectroscopy. Pigment analysis was difficult but it appears that both phycocyanin and chlorophyll were slow to respond to dark incubation in slr0228/Chl$^{-}$. Although phycocyanin continued to be present and the fluorescence emission from PSII in 77K spectra (under 435 and 600 nm excitation) suggested that the PSII reaction centre was properly assembled, the abnormally high emission at 680 nm in 600 nm excitation light showed that at least some of the PBS was probably decoupled from the reaction centres in slr0228/Chl$^{-}$. The reason for this is unclear: it would be interesting to investigate further if the PSII centres still present were targeted for removal in the double mutant but could not be degraded in the absence of the FtsH, resulting in high fluorescence emission from decoupled PBS.

As reported by Wu & Vermaas (1995), the Chl$^{-}$ mutant did not evolve oxygen after LAHG treatment. Oxygen evolution returned to normal levels after 4 days in these experiments, whereas in the slr0228/Chl$^{-}$ cells, oxygen was produced at relatively stable levels throughout LAHG (under saturating light in the oxygen electrode).

As noted previously, it is known that there is a severe effect on slr0228$^{-}$ mutants in photoinhibitory conditions because the repair of the D1 protein is impeded. The marked increase in the slr0228/Chl$^{-}$ PSII:PSI fluorescence emission ratio during LAHG and the continued oxygen evolution by the cells both support the idea that this FtsH is essential for PSII to be removed from cells. These results, however, broaden the hypothesis from a protease necessary for repair after high-light damage to a protease that is specific for PSII, vital for the correct regulation of PSII content under any circumstances. There was clearly
no photo-oxidative damage occurring under dark incubation conditions, yet the PSII activity of ChlL− declined during heterotrophic growth: the double mutant with the inactivated FtsH seemed unable to remove the photosystem. It seems that the slr0228-encoded FtsH is essential for correct maintenance of photosystem stoichiometry because of its action on PSII. A further fascinating implication of this work is that, since oxygen evolution continued in the slr0228/ChlL− cells, (at least some of) the PSII that should be degraded remains functional.

In addition to this, the synthesis of PSI — originally the intended object of examination in these experiments — was found to be delayed after chlorosis, as in nutrient deficiency assays. Yu et al. (1999) found that some fluorescence from PSI could be detected with 6 h of the synthesis of chlorophyll in LAHG cells that had been returned to normal light conditions. PSII components were evident after 12 h. Here, the return of photosynthetic activity monitored using the oxygen electrode suggested a delay in the return of PSI in the absence of the slr0228-encoded FtsH that corresponded with the delay seen in the P and N deficiency assays. These experiments therefore further supported the role of the slr0228 FtsH in PSI assembly.

Despite the interesting effects on PSII turnover and PSI assembly, which correspond with other assays of photosynthesis in slr0228− mutants, the ChlL− strain proved difficult to work with. In particular, to ensure viable cultures for regreening assays it was necessary to curtail chlorosis. As well as compromising on the period of LAHG and/or viability of the two strains, the addition of glucose altered various parameters used to measure photosynthetic activity. To improve the data from these assays, more replicates are required and the viable cell count should be monitored during the course of experiments.

There is a further complication in such chlorosis assays: the effect of pigment availability on photosystem turnover and assembly should be considered. If the slr0228 FtsH were to prevent the removal of chlorophyll from the thylakoid membranes or the reaccumulation of the pigment during regreening, then there would be an effect on photosystem degradation and biosynthesis. Chlorophyll was lost from slr0228− cells during growth in blue light (Chapter 6) suggesting that the failure to remove pigments in LAHG is not a specific defect associated with the inactivated FtsH gene. In the case of PSII, also, the need for chlorophyll for D1 synthesis, for example, is disputed [e.g., He & Vermaas, 1998; Kim et al., 1994]. In addition, since only a proportion of chlorophyll is lost in slr0228− mutants, it seems unlikely that chlorophyll regulation could cause the very marked, reproducible effect
on PSII during nutrient- or dark-provoked chlorosis, or during photo-oxidative damage. D1 degradation was not impaired in lincomycin-treated WT (motile) cells [Silva et al., submitted], suggesting that synthesis of the protein is not the limiting factor in its turnover.

In the case of regreening, the accumulation of both chlorophyll and phycocyanin is perturbed. Rather than a specific effect of chlorophyll, for example, causing a delay in photosystem accumulation, the fact that both pigments are affected might suggest a generalised response resulting from perturbed regulation of photosynthesis in the absence of the slr0228 FtsH. Since the activity and quantity of PSI seems to be specifically delayed during regreening, the evidence from these assays most strongly supports a role for the FtsH in photosystem assembly rather than pigment regulation.

An approach to quantify the effect of pigment availability would be to study the recovery of PSI (quantified by flash spectroscopy) and PSII (quantified by labelled atrazine-binding) in chlorotic slr0228/ChlL^- cells subjected to light for a range of time intervals and then returned to LAHG conditions. This might allow the dependence of photosystem assembly on varying amounts of re-accumulated pigment to be determined.
8 FTSH IN ARABIDOPSIS THALIANA

8.1 Introduction

Before publication of the *A. thaliana* genome sequence, Lindahl *et al.* (1996) had already showed hybridisation between a protein in spinach thylakoid membranes and antibodies to the *E. coli* FtsH, resulting in the identification of an *Arabidopsis* ftsH cDNA. The protein predicted to be encoded (‘FtsH1’) appeared very similar to the bacterial version. Since then, analysis of the completed *A. thaliana* genome sequence has become possible, revealing that, in fact, it encodes numerous proteins similar to the *E. coli* FtsH protease [Table 8.1].

As previously mentioned, a subset of FtsH proteases has been proposed to exist that are found only in oxygenic photosynthetic organisms [Bailey *et al.*, 2002; Mann *et al.*, 2000], amongst which is not only the slr0228-encoded FtsH in *Synechocystis* but a few of those that occur in *A. thaliana*. In particular, an 81-amino acid segment of an *A. thaliana* FtsH gene named var2 is similar to part of slr0228. The conserved residues [Figure 8.1] are not found in the *Arabidopsis* FtsH1 [Lindahl *et al.*, 1996], *E. coli* FtsH or the other three *Synechocystis* FtsH homologues. As well as investigating the function of the cyanobacterial slr0228-encoded protein, therefore, the *Arabidopsis* Var2 FtsH protein was chosen for study. Some of the work on Var2 is reported in Bailey *et al.* (2002).

8.2 FtsH proteins in Arabidopsis thaliana

Fifteen FtsH-like proteins that are predicted to be chloroplast-targeted were identified in BLAST searches of the *A. thaliana* genome with the Var2 amino-acid sequence. All are nucleus-encoded. Searching with a protein containing ATP-binding motifs [Table 8.1] results in many matches with other ATP-binding proteins, but the best matches all appear to be AAA proteins, most of which are organelle-targeted and many of which have zinc-binding motifs like that of the ‘prototype’ FtsH in *E. coli*. In Table 8.1, the search results with best BLAST similarity scores are listed by predicted site of action and the presence of expected FtsH domains. Those predicted to be targeted to the chloroplast that have two transmembrane spans and a clear zinc-binding site are presented first, followed by those that are still predicted to be membrane-bound, the unique feature of FtsH proteases.

This search is in agreement with work by various authors recently, all of whom suggest that there are several chloroplast FtsH proteases, from nine [Adam *et al.*, 2001] to 10 [Sakamoto *et al.*, 2002] or, as in Table 8.1, 11 or more particularly strong candidates [see also Sokolenko...
et al., 2002). This search also showed that amongst the putative FtsHs and AAA proteases in *Arabidopsis* there are subsets predicted to have only one transmembrane region (as seen in some yeast-mitochondrial FtsHs) and those that contain two predicted membrane spans [for review, see Langer 2000]. It is tempting to speculate that this allows orientation of FtsH proteins with catalytic sites on each face of the membrane. Since it is known that the hexamers of FtsH-like proteins can contain two distinct but very similar subunits (e.g., Yta10p with Yta12p in *S. cerevisiae* mitochondrial AAA protease oligomers) [for review, see Langer, 2000], it would also be very interesting to know whether the diversity of FtsH homologues in *A. thaliana* allows similar flexibility in the assembly of its (probable) hexamers.

Figure 8.1. Alignment of predicted amino-acid sequence from between the transmembrane domains of a subset of FtsH proteins found only in oxygenic photosynthetic organisms. FtsH-like protein encoded by gene on Cl, chromosome 1 (At1g06430) or C5, chromosome 5 (At5g15250), of *Arabidopsis thaliana*. 

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Table 8.1. FtsH-type proteins identified in *Arabidopsis thaliana* from the *Arabidopsis Genome* Initiative sequence database.

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</tbody>
</table>

FtsH-like proteins in *A. thaliana* annotated in the TAIR database, plus 'FtsHX' notation where suggested by Adam et al. (2001). All include ATP-binding sites but presence of zinc-binding sites and predicted t/m regions varies.

*One t/m region in TAIR database; two by manual TMPRED.
†Zn-binding identified in database but not visible in sequence.
C, Chloroplast, M, mitochondrion; SP, secretory pathway; G, Golgi; PftF, *Capsicum annuum* FtsH; t/m, transmembrane domain (predicted by TAIR database: very tentative); Zn, zinc-binding motif (my search); CAD, conserved ATPase domain [Swaffield & Purugganan, 1997].

Serendipitously, *var2* was one of three *A. thaliana* FtsH genes whose sequence was available at the start of this work, before the plant's genome was completed, and also in which a mutant (Var2-2) plant had been identified (see below). The 695 amino-acid, 74-kDa *Var2* FtsH is encoded by a 2-kb gene (3.4 kb including introns as well as five exons), and is predicted to be targeted to the thylakoid by the Tat pathway and a chloroplast transit peptide. It has the AAA module composed of Walker A and B motifs at
261–276 and 322–329 amino acids, the so-called second region of homology (366–384 amino acids) and zinc-binding domain further towards the N-terminus [Figure 8.2].

Var2 is particularly similar to PftF from *Capsicum annuum*, a protein suggested to be involved in vesicle fusion and/or protein translocation during chromoplast differentiation [Hugueney *et al.*, 1995], leading Chen *et al.* (2000) to conclude that Var2 functions in thylakoid membrane biogenesis as a factor that mediates vesicle fusion events. The Var2 amino acid sequence, particularly the C-terminal regions, is distinct from the Lindahl *et al.* (1996) FtsH1 identified in the spinach chloroplast, but recent work confirmed that the Var2-FtsH is also targeted to the thylakoid membrane [Chen *et al.*, 2000]. During the course of this work, two groups investigating variegated *Arabidopsis* mutants identified that the mutated var2 gene and cause of variegation was an FtsH homologue [Chen *et al.*, 2000; Takechi *et al.*, 2000]. It was cloned by chromosome walking, and numerous var2 alleles have now been sequenced [Chen *et al.*, 2000; Sakamoto *et al.*, 2002; Takechi *et al.*, 2000]. var2-2 was one of the most debilitating alleles resulting in only 1% of WT levels of the var2-encoded FtsH being visible in Western blots [Chen *et al.*, 2000]. This is perhaps surprising since Var2-2 has one conservative amino-acid substitution (R191K) at the end of the second transmembrane domain. Chloroplast import experiments using *in-vitro*-translated Var2 protein indicated that the protease was localised on the thylakoid membrane with its C-terminus facing the stroma, and Northern and Western blots showed Var2 expressed only in photosynthetic tissues [Chen *et al.*, 2000] or in all tissues [Takechi *et al.*, 2000]. As there is often cross-reaction between FtsH antibodies, it is moot which protein was in fact bound by the *E. coli* antibodies in the work by Lindahl *et al.* (1996).

### 8.3 Characteristics of Var2-2

The ethyl-methanesulphonate-mutagenised Var2-2 strain of *A. thaliana* (with Col0 the parent line) is available [NASC]. As mentioned above it is severely variegated, particularly in early pairs of true leaves and in high-intensity light, suggestive of a photosynthetic phenotype [Figure 8.3]. The characteristics of plants with the 'Var2-2' mis-sense mutation were compared with WT (Col0), therefore, to investigate the function of the Var2 FtsH. The pigment composition, photosynthetic function and the dark turnover of the photosystems were examined as in *slr0228* *Synechocystis*, using absorbance, fluorescence and flash spectroscopy, oxygen evolution and a senescence assay. The effect of the mutation on the resistance of *A. thaliana* to photoinhibition was also carried out, in collaboration with S. Bailey (University of Warwick) [Bailey *et al.*, 2002].
Figure 8.2. Alignment of Arabidopsis thaliana FtsH proteins Var2 and FtsHl with the Synechocystis slr0228-encoded protein (in order of similarity to Var2). FtsHl is 43% identical to Var2. Identical residues (*), conserved (;) and semi-conserved (.) substitutions are indicated according to standard ClustalW format [www.ebi.ac.uk/clustalw/index.html]. The Tat pathway recognition site, ATP-binding Walker A and B boxes, the second region of homology and the HExGH zinc-binding site are underlined (in that order). L/M F V G between the A and B boxes appears to be the conserved motif of the unfolding/translocation mechanism in Clp, Hsl or FtsH proteases [Niwa et al., 2002].
8.3.1 Growth of Var2-2

Under the growth conditions used here, germination rates for Col0 and Var2-2 were similar, but after production of the first pair of true leaves, growth was markedly slower in the mutant. Within 4 weeks, the Col0 plants have three–four pairs of true leaves and the rosette is about 3.5 cm in diameter, whereas that of Var2-2 plants is approx. 1.5 cm and there are only half the number of true leaves.

Unusually for variegated mutants of *Arabidopsis,* the cotyledons of Var2-2 seedlings are fully green. The reason for this remains a complete mystery. The first few sets of true leaves of Var2-2, in contrast, are almost entirely white (including petioles) with a small cluster of green cells. Later leaves have a gradually increasing proportion of green until, at flowering, leaves on the plants are almost entirely green. Another unexplained feature of the mutant is that under the growth-conditions used here, coincident with flowering, the rosette of leaves becomes much larger and variegation dramatically less severe. These mature plants’ leaves remain mottled and speckled, however, with white tips. Although Takechi *et al.* (2000) found that the var1-*A* allele they were studying did cause variegation of siliques, in Var2-2 plants the rest of the plants’ organs were not visibly affected by the mutation: flowering appeared normal and the siliques were not variegated but were slightly smaller than those in WT.

Whereas the Col0 life cycle is completed within 16 or 17 weeks under our growth conditions, Var2-2 reaches maturity and produces seeds by about 21 weeks. The mutant
plant's rosette remains smaller at maturity, reaching only approx. 6 cm in diameter [Figure 8.3].

The basis for the variegation in this mutant is unknown. Variegated plants may have both WT and mutant genotypes in different plant tissues or, alternatively, defective plastids can form in only a proportion of a plant's cells despite mutations in nuclear genes. If variegation is due to the action of a nuclear recessive gene, although a plant has a uniform genotype throughout its cells, it has been reported that the phenotype can be expressed only in some areas. Var2-2 apparently has such a nuclear-gene mutation [e.g., see references within Chen et al., 2000]. Corresponding with this, plastids appear to be normal in the green sectors of leaves but in the white sectors there is only a small amount of chlorophyll fluorescence [Figure 8.4].

8.3.2 Pigment content

Chlorophyll

The variegation of the Var2-2 mutant will clearly affect the total chlorophyll per leaf. The quantity of pigment from mature leaves ground in liquid nitrogen, calculated according to Porra et al. (1989), was indeed much greater in WT. In Col0, the mean chlorophyll \((a+b)\) was 1062 ± 108 µg/g leaf tissue, versus 679 ± 85 µg/g leaf tissue in Var2-2. This is significantly different at \(P=0.0355\) [Table 8.2].

The ratio of chlorophyll \(a\) to \(b\) is often used to characterise the photosynthetic apparatus. For example, low-light-grown plants have fewer PSII but very large light-harvesting antennae, and therefore tend to have lower chlorophyll \(a:b\) ratios [for review, see Porra 2002]. The same chlorophyll \(a\) to \(b\) ratio in Col0 and Var2-2 would suggest the antenna of PSII and PSI:PSI content was comparable, but the mean ratio is 2.8 in Col0 and 3.3 in Var2-2. The difference is not significant between Col0 and Var2-2 [Table 8.2], however, so the PSII antenna or ratio of photosystems is probably not greatly different in the two strains under the growth conditions used here.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chlorophyll (µg/g ± SEM)</th>
<th>Chlorophyll (a:b) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col0</td>
<td>1062±108</td>
<td>2.8±0.2</td>
</tr>
<tr>
<td>Var2-2</td>
<td>679±85</td>
<td>3.3±0.2</td>
</tr>
<tr>
<td>(P)</td>
<td>0.0355 (8 d.f)*</td>
<td>0.1646 (10 d.f)†</td>
</tr>
</tbody>
</table>

*Significantly different (\(t\) test).
†Not significantly different (\(t\) test).

SEM, Standard error of the mean; d.f., degrees of freedom.
Figure 8.4. Fluorescence microscopy of Var2-2 *Arabidopsis thaliana*. Chlorophyll fluorescence from chloroplasts was photographed using a confocal microscope. Top panel, low-magnification surface of leaf showing normal ultrastructure. Bottom panel, chloroplast fluorescence showing that normal plastids are formed in Var2-2 (also see Inset). The dark area is a variegated sector of the leaf where there are fewer developed chloroplasts and less fluorescence.
Carotenoids

As mentioned in Section 5.3.2 it was frequently observed that the FtsH mutants produced brown- or orange-pigmented cell extracts during biochemical assays. TLC of thylakoid-membrane carotenoids confirmed that this difference was related to the composition of photosynthetic pigments [Figure 5.5] in both the Synechocystis str0228 and A. thaliana Var2-2 strains. When describing the variegated plants they found to be var2+, Chen et al. (2000) reported that there was no accumulation of carotenoid precursors in the mutant leaves, suggesting intact carotenoid biosynthesis pathways. Here, however, HPLC of thylakoid membranes did reveal a difference in carotenoid profile, showing both Arabidopsis and Synechocystis (see Section 5.3.2) mutants to contain less β-carotene than their respective WT [Table 8.3; Figure 5.6]. The amount of lutein per chlorophyll in Var2-2 A. thaliana was also slightly lower than that in Col0, although not affected as a proportion of total carotenoids.

Table 8.3. Carotenoid composition of Col0 and Var2-2 Arabidopsis thaliana.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Neoxanthin (ng/µg chlorophyll (%))</th>
<th>Violaxanthin (ng/µg chlorophyll (%))</th>
<th>Lutein (ng/µg chlorophyll (%))</th>
<th>Zeaxanthin (ng/µg chlorophyll (%))</th>
<th>β-carotene (ng/µg chlorophyll (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col0</td>
<td>6 (4.0±0.5 %)</td>
<td>14 (9.0±1.0 %)</td>
<td>69 (45.0±2.0 %)</td>
<td>16 (11.0±1.0 %)</td>
<td>46 (32.0±1.5 %)</td>
</tr>
<tr>
<td>Var2</td>
<td>7 (6.0±0.5 %)</td>
<td>15 (12.0±1.0 %)</td>
<td>60 (48.0±2.0 %)</td>
<td>15 (12.0±1.0 %)</td>
<td>26 (22.0±1.5 %)</td>
</tr>
</tbody>
</table>

*Values calculated using extinction coefficients in acetone [Hirschberg & Chamowitz, 1994].

NB. Differences between percentage ratios and total values are due to the fact that the loss of one particular pigment does not mean that one of the other pigments is automatically increased.

Table 8.3, Standard error of the mean.

As mentioned with reference to Synechocystis pigment composition, carotenoids are part of general stress responses in photosynthetic organisms [for review, see Hirschberg & Chamovitz, 1994] and therefore changes in the levels of these pigments could be a consequence of the FtsH phenotype as much as a cause of it in Var2-2 plants. In terms of specific photosynthetic roles that might be impaired, β-carotene quenches triplet chlorophyll in PSII so Var2-2 A. thaliana may be impaired in its ability to reduce overexcitation. In addition, carotenoids are a key route of energy dissipation through the xanthophyll cycle. Xanthophylls participate in the quenching of chlorophyll triplet states, and antheraxanthin and zeaxanthin aid the thermal dissipation of the excited singlet state of antenna chlorophylls [for review, see Horton et al., 1996]. Parallel work by S. Bailey (using plants from this laboratory) showed that, as with overall carotenoid composition, the xanthophyll cycle in Var2-2 was slightly different from Col0. The parameter qB, which is calculated from fluorescence yields after periods of dark and illumination [for review, see Horton et al., 1996] in Var2-2 was half that measured in Col0 at moderate and high irradiance: Col0 qB at 300 µmol/m²/s was 0.95 ± 0.04 versus 0.52 ± 0.07 in Var2; at
1800 μmol/m²/s ColO qₑ was 2.15 ± 0.11 compared with 1.25 ± 0.18 (S. Bailey, personal communication, 2001). This is interesting because lutein is reduced in Var2-2 and lutein deficiency has been shown to result in partial loss of qₑ [Pogson et al., 1998]. Reduction in qₑ in the FtsH mutant could affect the susceptibility of plants to PSII photoinhibition: the thermal dissipation of excess light at antenna level lessens energy transfer to PSII, fluorescence emission and formation of the harmful PSII triplet chlorophyll that can then inactivate the reaction centre [e.g., Niyogi, 2000; Santabarbara et al., 2002].

8.3.3 Thylakoid membrane protein composition
SDS–PAGE was carried out on thylakoid membranes prepared from mature leaves of ColO and Var2-2 plants. Coomassie-Blue staining revealed that the protein composition of the photosynthetic membranes was comparable, with all bands seen in ColO present in Var2-2 and suggestive of approximately the same quantities of protein components [Figure 8.5].

8.3.4 Photosystem content
In slr0228~ Synecocystis there is a much-reduced content of PSI. Interestingly, this appears not to be the case in the Arabidopsis mutant. Functional photosystems were enumerated (per μg chlorophyll) using flash spectroscopy of thylakoid membranes prepared from ColO and Var2-2 seedlings of age 3 weeks [Figure 8.6]. The samples were stored before quantification. It should therefore be noted that, although the concentration of chlorophyll in each sample was verified after flash spectroscopy measurements, pigments and thylakoid membranes could have been degraded during frozen storage and thawing of the samples or during spectroscopy and the delay thereafter before acetone extraction of chlorophyll. As with other assays where samples were stored during assays, the correction of photosystem content to pigments only quantified later may result in some error. Figures given here should be considered most reliable for comparison between WT and mutant.

The (functional) photosystem content proved to be approximately the same in ColO and Var2-2. There was a small decrease in the PSII:PSI ratio in Var2-2 compared with ColO [Table 8.4] in the leaves tested, but this was not significant although it corresponds with the slightly higher mean chlorophyll ab ratio, also not significant, seen in the variegated mutant. More samples would be necessary to show if this was always the case, particularly in adult leaves since these measurements were made in seedlings.
Figure 8.5. Coomassie Blue-stained thylakoid membrane proteins of Col0 and Var2-2. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of solubilised thylakoids loaded at 0.2 μg/ml chlorophyll.
Figure 8.6. (a) Photosystem (PS) content/μg chlorophyll in Col0 and Var2-2 Arabidopsis seedlings (mean ± standard error). Difference only significant for PSI ($P=0.065$). (b) Oxygen evolution from Col0 and Var2-2 Arabidopsis under saturating light (mean ± standard error). The difference between the mean values is not significantly different ($P=0.332$; t test, 12 degrees of freedom).
Table 8.4. Functional photosystem I and photosystem II content in thylakoid membranes of Col0 and Var2-2 Arabidopsis thaliana seedlings, measured by flash spectroscopy.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean ± SEM photosystem content/μg chlorophyll</th>
<th>PSII:PSI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean photosystem content/μg chlorophyll</td>
<td>PSI*</td>
</tr>
<tr>
<td>Col0</td>
<td>4.4 ± 0.4 \times 10^{11}</td>
<td>1.8 ± 0.3 \times 10^{11}</td>
</tr>
<tr>
<td>Var2-2</td>
<td>4.9 ± 0.7 \times 10^{11}</td>
<td>4.3 ± 1.2 \times 10^{11}</td>
</tr>
<tr>
<td></td>
<td>0.5655</td>
<td>0.0650</td>
</tr>
</tbody>
</table>

1 μg chlorophyll equates to ~7 \times 10^{14} molecules. For each PSI plus PSII (and light-harvesting complexes), flash spectroscopy data suggested there were a total of 2.8±0.6\times10^3 chlorophyll molecules in Col0 versus 1.6±0.4\times10^3 in Var2-2 (mean ±SEM; P=0.1147; not significantly different because of variation in samples).

*Only PSI significantly different (7 degrees of freedom in t test).

PS, Photosystem; SEM, standard error of the mean.

Flash spectroscopy data also allow pigment content to be compared; the mean chlorophyll content (total for both photosystems and LHC together) in Var2-2 was again almost half that of Col0 per PSI plus PSII, but the variation between measurements resulted in low statistical significance [Table 8.4].

8.3.5 Oxygen evolution

Photosynthesis was evaluated in Col0 and Var2-2 A. thaliana using the leaf-disk oxygen electrode chamber to monitor oxygen evolution [Figure 8.6]. It was found that, using whole leaves, the oxygen uptake was higher than oxygen evolution, giving negative slopes in both saturating light and the dark. Light–dark calculations were therefore necessary to estimate the net rate of oxygen evolution per chlorophyll.

The mean oxygen evolution thus calculated from detached Var2-2 leaves (mean, 142 μmol O₂/mg chl/h) was slightly higher than that from Col0 leaves (mean, 113 μmol O₂/mg chl/h) but the difference was not significant [Table 8.5]. It may be accounted for by the lower chlorophyll content of Var2-2 but similar photosystem content, producing an artificially elevated per-chlorophyll figure in the variegated mutant.

Table 8.5. Mean oxygen evolution from leaves of Col0 and Var2-2 Arabidopsis thaliana.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean oxygen evolution ± SEM</th>
<th>t (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μmol O₂/mg Chl/h)</td>
<td></td>
</tr>
<tr>
<td>Col0</td>
<td>113 ± 18</td>
<td>-1.0121 \ (P=0.3315)*</td>
</tr>
<tr>
<td>Var2-2</td>
<td>142 ± 22</td>
<td></td>
</tr>
</tbody>
</table>

*Not significantly different (12 degrees of freedom in t test).

Chl, Chlorophyll; SEM, standard error of the mean.
8.3.6 *Fluorescence emission characteristics*

The use of low-temperature fluorescence spectroscopy for analysis of leaf tissue is not as straightforward as that with microbial cells because of the need to homogenise material for injection into capillary tubes. A method was developed therefore by which 77K fluorescence emission spectra could be measured in whole leaves using a fibre-optic light source and receiver to monitor fluorescence from leaves frozen in liquid nitrogen. Excitation at 435 nm for chlorophyll \( a \) or 480 nm for chlorophyll \( b \) then results in two peaks of fluorescence emission, at approx. 680 nm for PSII and approx. 730 nm for PSI. These spectra demonstrate the absence of photosynthetic activity in variegated areas of the mutant's leaves [Figure 8.7]. When the spectra were normalised, however, there remained an abnormally large difference in the PSII:PSI fluorescence ratio between Var2-2 and Col0, probably due to reabsorption of PSII emitted fluorescence magnifying the ultimate PSI emission in Col0 leaves, which contain more chlorophyll [Figure 8.7].

Using ground leaf tissue, it was possible to optimise the concentration of pigments in samples to prevent fluorescence emission from PSII being absorbed as excitation light by PSI, and thus altering the magnitude of the fluorescence peaks. As greater variability was found in spectra from duplicate samples of both ground leaves and from thylakoid membranes of *Arabidopsis* than in whole-cell *Synechocystis* samples, a range of concentrations of samples were analysed to discover the most effective leaf-preparation method for the technique. It was found that <25 mg of Col0 leaf tissue ground in 5 ml grinding buffer or thylakoid membranes prepared from 15 mg leaf tissue were most reliable, and twice the amount of leaf tissue was used from Var2-2 to compensate for its diminished chlorophyll levels [Figure 8.8]. In addition, to ensure that PSI and PSII fluorescence measurements were reproducible, multiple samples were prepared for each assay to give mean fluorescence ratios, and all were prepared under a very dim green light to ensure that all samples were in state 1 (see State transitions, Section 1.7.2).

PSI and PSII are usually present in plant chloroplasts in similar quantities, although here (see above) the Var2-2 mutant's PSI content was slightly higher than that of Col0. When the ratio of fluorescence from PSII and PSI (from multiple thylakoid preparations) were compared, there was also small increase in the mean PSI:PSII fluorescence ratio (significant at \( P<0.01 \)) in Var2-2 plants [see Table 8.6]. There was no dramatic variation, however, between individual fluorescence emission spectra, suggesting that in general the WT and Var2-2 photosystem function and light-harvesting antennae were comparable when plants were grown in relatively low-light (30 \( \mu \text{mol/m}^2/\text{s} \)) [Figure 8.9].
Figure 8.7. 77K fluorescence emission spectra from whole leaves of Col0 and Var2-2 Arabidopsis thaliana. Excitation at 480 nm for chlorophyll $b$. Dotted spectrum, Var2-2 photosystem II (PSII) fluorescence normalised (norm.) to that of Col0 after background fluorescence subtracted: note different PSII:PSI fluorescence ratio caused by reabsorption of PSII fluorescence.

Figure 8.8. Varying quantities of leaf material to investigate reabsorption of emitted fluorescence, in 77K fluorescence emission spectra from Var2-2 leaves ground in liquid nitrogen. Excitation 435 nm; spectra normalised to PSII after background fluorescence subtracted.
Table 8.6. Mean fluorescence emission from photosystem I versus photosystem II in thylakoid membranes of Col0 and Var2-2 *Arabidopsis thaliana*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean PSI:PSII fluorescence ratio (± SEM)</th>
<th>t (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col0</td>
<td>0.83 (± 0.04)</td>
<td>-2.933 (P=0.0089)*</td>
</tr>
<tr>
<td>Var2-2</td>
<td>1.06 (± 0.05)</td>
<td></td>
</tr>
</tbody>
</table>

*Significantly different (18 degrees of freedom in t test).
PS, Photosystem; SEM, standard error of the mean.

Figure 8.9. 77K fluorescence emission spectra from Col0 (black line) and Var2-2 (orange dashed line) *Arabidopsis thaliana* thylakoid membranes. Excitation was at 435 nm for chlorophyll *a*. Peaks were normalised to photosystem II after subtraction of background fluorescence.

There are clearly some differences in photosynthetic function between the *A. thaliana* Var2-2 mutant and Col0. As with the *Synechocystis* slr0228<sup>+</sup> strains, the mutant plant is able to photosynthesise normally to a large degree but there are a number of variations between it and WT. In this case, besides the obvious variegation and drop in total chlorophyll content, the Var2-2 carotenoid composition was altered (and parallel work by S. Bailey showed an impaired xanthophyll cycle), and there was a small decrease in fluorescence emission from PSII relative to PSI. Although neither difference was significant, the higher mean chlorophyll *a:b* ratio and the lower mean PSII:PSI ratio do match significant changes
in the fluorescence emission and the PSI content. The number of functional PSI centres/chlorophyll was significantly higher although, as with the slightly increased mean oxygen evolution, this may be (all or partly) caused by the lower chlorophyll levels in Var2-2. Whether these changes are a direct effect of the missing FtsH or are a side-effect of it, there is some indication that photosynthetic function is altered in Var2-2 mutants. This is consistent with the changes seen in slr0228**Synechocystis** although, in contrast with the **Synechocystis** mutant, *A. thaliana* Var2-2 does not seem to be impaired in PSI synthesis.

### 8.4 Response of Var2-2 to an altered light environment

Light levels affected the degree of variegation of Var2-2 strains. Whereas Col-0 plants would normally be grown at 130 μmol/m²/s in our growth chambers, when optimising conditions for growth of the Var2-2 plants it became clear that they required light levels of approx. 30 μmol/m²/s for best growth. Takechi et al. (2000) later reported that they found it necessary to grow four strains of Var2 (Var2-1, Var2-6, Var2-7 and Var2-8) at 70 μmol/m²/s. Interestingly, it has also been noted that high temperatures and low light conditions retard the growth of var2-2 mutants and result in plants that are nearly all-green [Chen et al., 2000] so there is clearly some light-related effect of the mutation of this FtsH.

To investigate the ability of the Var2-2 *A. thaliana* to respond to an altered light environment, Col0 and Var2-2 plants were subjected to moderate and extreme light intensity and their response monitored using 77K fluorescence emission spectroscopy. Work in parallel by S. Bailey measured the ratio of maximum to variable fluorescence (*Fₘ/Fᵥ;* a measure of PSII efficiency) to monitor photoinhibition [for review of method, see Hall & Rao, 1999], also using Western blots to follow the repair of the PSII D1 protein.

#### 8.4.1 Fluorescence spectra in high-intensity light

Plants typically contain equimolar amounts of active photosystems, but those grown under high light do increase the amount of PSII (along with the chlorophyll *a/b* ratio) while the amount of LHCII drops. This adaptation in the form of a changing photosystem stoichiometry is more often seen in young than old plants [for review, see Chitnis, 2001], so young plants were used for assays of response to high-intensity light here. For high-light treatment, 25-day-old seedlings grown in half-strength Murashige and Skoog agar were subjected to approx. 1600 μmol/m²/s light in a water-cooled apparatus for approx. 2 h. 77K fluorescence emission spectra were then recorded as described above, using thylakoid membranes prepared from Col0 and Var2-2 leaves.
Figure 8.10. Mean PSII:PSI fluorescence ratio of plate-grown *Arabidopsis thaliana* Col0 and Var2-2 mutant during low light (LL), high light (2 h at 1600 μmol/m²/s) and LL recovery for approx. 4 h. Fluorescence emission measured from 77K fluorescence emission spectra with 435 nm excitation light. Differences between mean fluorescence ratio in these plants not significant by *t* test.

In both strains, the mean fluorescence ratio from PSII:PSI was reduced after high-light treatment for approx. 2 h, although the mean ratio was slightly lower in Var2-2 seedlings than Col0 seedlings [Figure 8.10]. The difference was not significant, however [Table 8.7]. Neither strain increased the activity of PSII relative to PSI during the following 4 or 5 h recovery under standard (low-intensity light) growth conditions, the mean PSII:PSI fluorescence ratio again remaining rather lower in Var2-2 than in Col0.

**Table 8.7.** Fluorescence emission from Col0 and Var2-2 *Arabidopsis thaliana* after 2 h high-intensity light and 4 h low-light recovery (excitation light 435 nm).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean PSII:PSI fluorescence ratio (± SEM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LL</td>
</tr>
<tr>
<td>Col0</td>
<td>0.52±0.04</td>
</tr>
<tr>
<td>Var2-2</td>
<td>0.50±0.04</td>
</tr>
</tbody>
</table>

*None significantly different (12 degrees of freedom in *t* test).

PS, Photosystem; SEM, standard error of the mean; LL, low light (30 μmol/m²/s); HL, high light (approx. 1600 μmol/m²/s).
The peaks at 685 and 695 nm in 77K fluorescence emission spectra correspond to emission from CP43 and CP47, which funnel excitation energy from the light-harvesting antenna to PSII. The reduction in fluorescence in both Col0 and Var2-2 from PSII within 2 h of very high light intensity may correspond with a drop in LHCII content, reducing the energy directed to CP43 and CP47, thus reducing the PSII:PSI fluorescence ratio. PSII damage would also occur under this high-intensity light (1600 \(\mu\)mol/m\(^2\)/s), contributing to the decrease in PSII activity.

The 77K fluorescence emission spectra in high light suggested that the Var2-2 mutant could sense high-light conditions and regulate light-harvesting and photosynthetic function in the absence of this FtsH. The fluorescence kinetics \((F_o/F_m)\) of Var2-2 and Col0 plants were also comparable after growth under standard conditions (data not shown). When detached leaves were subjected to high light intensities, however, \(F_o/F_m\) measurements in the FtsH mutant revealed a severe photoinhibition phenotype. Photoinhibition was monitored using a fluorimeter (work by S. Bailey) to measure room-temperature fluorescence and calculate \(F_o/F_m\). These data showed that Var2-2 leaves suffered a greater magnitude of photoinhibition at moderate (300 \(\mu\)mol/m\(^2\)/s) and at very high (1800 \(\mu\)mol/m\(^2\)/s) light, and recovery from photoinhibition was slower in Var2-2 than in Col0 [Figure 8.11] (S. Bailey, personal communication, 2001). In Col0 the drop in \(F_o/F_m\) levelled out after approx. 2 h, whereas in Var2-2 there was a drop in \(F_o/F_m\) but no recovery. Using a protein synthesis inhibitor, lincomycin, to prevent new D1 protein being made, and thus repair PSII damaged by high light, the \(F_o/F_m\) response of Col0 and Var2-2 became comparable, showing a drop without any subsequent recovery. This suggests that the D1 repair cycle is always faulty in Var2-2: even without lincomycin, the mutant seemed unable to repair damaged PSII. Western blots by S. Bailey [Figure 8.11] using plants from this laboratory confirmed that the D1 protein was correctly degraded in Col0 in high light. In Var2, in contrast, the D1 protein was normally present but after photoinhibitory treatment it was not removed. Interestingly, there was some effect on the D2 protein also. It has been suggested that the degradation of D2 and D1 are indeed coupled [Jansen et al., 1999].
Figure 8.11. (a, b) Maximum photochemical efficiency of photosystem II (PSII) in Col0 and Var2-2 *Arabidopsis thaliana* (mean±standard error). [Work by S. Bailey, University of Warwick.] (a) Col0 $F_v/F_m$ is stable at moderate light (300 $\mu$mol/m$^2$/s; △), but decreases in high light (HL; 1800 $\mu$mol/m$^2$/s; ▲) before recovering. Var2-2 is more susceptible than Col0 to photoinhibition of PSII at both moderate light (○) and HL (●). -60 min, overnight dark adaptation; 0 min, end of 1-h light treatment. (b) $F_v/F_m$ of PSII in Col0 (△) and Var2-2 (○) leaves after HL with or without lincomycin. (c) Western blot. With lincomycin, Col0 leaves have decreased D1 protein relative to untreated leaves. After HL and lincomycin, D1 is almost absent. With no lincomycin, D1 is replaced in Col0 even in HL (see lower panel). In Var2-2, levels of D1 remain stable. Even in HL with lincomycin, D1 is not lost. Turnover of the D2 protein mirrors that of D1, but the minor subunit PsbS remained constant showing that the reaction centre as a whole was not faulty.
8.5 PSII turnover in Var2-2

8.5.1 Senescence assay

Not only do FtsHs found on the thylakoid membranes appear to function directly in photosynthesis but several studies and microarrays to date [e.g., Hihara et al., 2001; Kanesaki et al., 2002; Simpson et al., 2003; Singh & Sherman, 2000] have shown changes in the level of transcription of the same proteins during stress conditions in general. These might be investigated by triggering senescence, a highly ordered process of degradation in cells that culminates in the translocation of useful cellular components to other areas of the plant [for review, see Yoshida, 2003].

After senescence begins, photosynthesis is downregulated, followed by upregulation of salvage pathways. Leaf proteins decline and an increase in protease activity has been documented [Zavaleta-Mancera et al., 1999a]. During dark-induced etiolation and senescence, therefore, the removal of components of the photosynthetic apparatus and the initiation of stress-response functions might be expected to involve the Var2 FtsH, and differences may be seen between mutant and WT *A. thaliana*. Unfortunately, although senescent ageing leaves of *Nicotiana* can recover if the apical set of leaves is removed from the plant [Zavaleta-Mancera et al., 1999a,b], most accounts suggest that *A. thaliana* is more difficult to control in this manner. In the absence of an FtsH mutant in *Nicotiana*, however, a previously reported method of inducing senescence in *Arabidopsis* was attempted. Weaver & Amasino (2001) have carefully detailed which leaves of *A. thaliana* can be induced to degrade the photosynthetic apparatus and which cannot. They showed that senescence is not a reversible process in *A. thaliana* except in seedlings that have only one pair of primary leaves — plants that have developed cotyledons and their first two true leaves can regreen upon return to normal illumination after beginning to senesce during a period of dark treatment.

Col0 and Var2-2 seedlings were tested to confirm whether they could be treated in this way. Seedlings were grown in the dark for 7 days after development of primary leaves, and could recover upon return to normal growth conditions. Interestingly, the appearance of the leaves suggested that the loss of pigment was slower in Var2-2 than Col0 seedlings during this initial experiment. The loss and return of photosynthetic pigments and apparatus were therefore followed using the oxygen electrode and flash spectroscopy in a further time-course assay.
8.5.2 Pigment content

It should be noted that the analysis of pigment levels may be problematic in senescence assays with chlorotic seedlings. Standard methods of pigment analysis can be difficult where the content is outside the ideal range of absorbance (0.4—1.0) [Kouřil et al., 1999 and references therein]. In this assay where senescence causes a drastic loss of chlorophyll, pigment values should therefore be considered to be approximate.

Chlorophyll content was quantified in seedlings at $t=0$, after 8 days' growth in the dark and at 3 and 6 days thereafter, using buffered acetone extraction [Porra et al., 1989]. The chlorotic appearance of dark-treated Col0 plants was again more marked than in Var2-2 [Figure 8.12]. Despite the etiolated appearance of the seedlings, the mean chlorophyll in acetone extracts from Col0 samples after 8 days growth in the dark was only slightly less than at $t=0$ showing that the pigment was not quickly removed in the dark. In Var2-2, in contrast, the mean pigment was higher after 8 days with no light, although measurements were variable [Figure 8.13]. Zavaleta-Mancera et al. (1999b) previously showed that yellowed (adult) senescent leaves contained approx 70 μg chlorophyll/g leaf tissue (reduced from 650 μg/g in presenescent tissue), very similar to the 50 μg/g seen here in Col0. Even though there always remains some chlorophyll in early senescence therefore, the mean total chlorophyll in Var2-2 was higher than before: after 8 days' dark treatment chlorophyll/g leaf was increased to over twice the amount at $t=0$.

By 3 days' light-recovery, the Col0 dark-treated seedlings were able to increase the mean chlorophyll level to almost double that at $t=0$, but Var2-2 seedlings were slower to recover from dark treatment, showing decreasing pigment/g at 3 and 6 days after return to low-light incubation.

The chlorophyll $a:b$ ratio in the seedlings at $t=0$ reflected the mean values obtained with mature plants: even at seedling stage where the primary pair of true leaves has developed, the Var2-2 chlorophyll $a:b$ ratio was increased compared with Col0 [Figure 8.14]. In fact, the difference was rather more severe at this stage (mean ± SEM: Col0, 2.5 ± 0.2; Var2, 3.7 ± 0.2). After 8 days in the dark, the chlorophyll $a:b$ ratio of senescent Col0 and Var2-2 the ratio had dropped, as reported previously: Zavaleta-Mancera et al. (1999a) reported a ratio of 1.7 in senescent Nicotiana leaves. This suggests that both strains were reacting to dark conditions and etiolation—senescence responses had begun.
After dark-treatment, the Var2-2 chlorophyll \( a:b \) ratio dropped again, representing either a rise in chlorophyll \( b \) or a drop in chlorophyll \( a \), whereas the \( a:b \) ratio of Col0 rose during light recovery. As mentioned above, the total chlorophyll was also decreased in Var2-2 at this point [Figure 8.13] but the reduced chlorophyll \( a:b \) ratio appeared to be a result of particularly reduced chlorophyll \( a \). Relative to \( t=0 \), the Var2-2 plants contained less chlorophyll \( a \); even though they initially retained high pigment levels after dark-conditions there was a drop through dark growth and light recovery in the chlorophyll \( a:b \) ratio.

### 8.5.3 Photosystem content

Flash spectroscopy was carried out as before using cyanobacteria, but to produce liquid samples for ease of measurement, thylakoid membranes were prepared from \( A.\ thaliana \) leaves. As described (Section 2.4.6) the oxidation and rereduction of P700 was used to quantify PSI, and DCMU was used to isolate PSI activity alone. PSII content was also quantified as described (Section 2.4.6). This showed an unchanging level of photosystem content in the light-grown control seedlings [Figure 8.15], even during the development of the very young plants. In contrast, in chlorotic seedlings, there was no PSII activity at all in the Col0 seedlings after 8 days' growth in the dark, nor PSI activity at this point [Figure 8.16]. After 3 days' recovery in the light, Col0 PSII had returned to normal.
Figure 8.13. Effect of dark incubation on Col0 and Var2-2 Arabidopsis. (a) Mean (± standard error) chlorophyll in light (t=0) and after 8 days in the dark before return to light. (b) Oxygen evolution in light-grown controls and (c) in seedlings dark-treated as before. Where large error bars would distort appearance of graph only (+/-) bar is shown.
Figure 8.14. Mean (± standard error) chlorophyll $a:b$ ratio in Col0 and Var2-2 *Arabidopsis thaliana* seedlings, calculated using coefficients from Porra *et al.* (1989). At $t=0$ seedlings had developed the primary pair of true leaves. Top, Light-grown control plants; bottom, seedlings grown in the dark from 0–8 days.
Figure 8.15. Light-grown controls: photosystem I (PSI) and photosystem II (PSII) content of Col0 and Var2-2 seedlings of Arabidopsis thaliana quantified by flash spectroscopy of P700 (see Methods for details). At t=0, seedlings had developed only the primary true leaves.
Figure 8.16. Senescence treatment: photosystem I (PSI) and photosystem II (PSII) content of Col0 and Var2-2 seedlings of Arabidopsis thaliana grown in the dark to induce senescence and then returned to standard conditions, quantified by flash spectroscopy of P700 (see Methods for details). At t=0, seedlings had developed primary true leaves only.
In Var2-2 the effect of chlorosis was different: after dark senescence the PSII level remained the same and did not diminish during light recovery, remaining high [Figure 8.16]. The level of detection of PSI, however, was not sensitive enough to quantify any functional PSI in Var2-2 at 3 days recovery in light. This corresponds with the drop in chlorophyll $a:b$ ratio at this time which was due to loss of chlorophyll $a$.

### 8.5.4 Oxygen evolution

It has been shown that photosynthetic activity normally parallels the loss of pigment in senescent leaves [e.g., Zavaleta-Mancera et al., 1999a]. The leaf-disk chamber was used, therefore, to monitor changing levels of whole-chain electron transport as measured by oxygen evolution. Since the seedlings were very small, numerous leaves were laid out on the chamber's matting and then chlorophyll extracted after electrode measurements were complete, to produce a chlorophyll content to aid later comparisons of data. As before, oxygen uptake was higher than oxygen evolution in the seedlings so light–dark measurements were used to give net oxygen evolution. Where senescence occurred and oxygen evolution stopped, therefore, negative values result, representing only dark respiration [see Figure 8.13].

The calculation of oxygen evolution at $t=0$ in the very young seedlings also proved rather problematic: the level of oxygen evolution per $\mu g$ chlorophyll was abnormally high [Figure 8.13]. The demand of young leaves for sugars declines as they mature and develop their photosynthetic apparatus [Zavaleta-Mancera et al., 1999a] causing the respiration rate to change, and meanwhile oxidation of plastoquinol by PSI is reduced [Niyogi, 2000 and references therein]. It became apparent that the distinct metabolic characteristics of young seedlings and older plants made comparative measurements difficult between $t=0$ and later points, although comparisons between Col0 and Var2-2 should still be valid.

In light-grown control Col0 seedlings the oxygen evolution remained very high at 8 days, but at 11 days, WT and Var2-2 plants were comparable. Var2-2 oxygen evolution then decreased in seedlings at 2 weeks after development of primary leaves. In the plants grown in the dark for 8 days, the mean oxygen evolution was reduced, as might be expected. The mean level was in fact higher in dark-grown Var2-2 than in Col0. Variability between the seedlings resulted in some positive and some negative measurements, represented by a rather wide standard error [Figure 8.13]. Both strains recovered comparably to evolve oxygen at 3 days after seedlings were returned to standard growth conditions.
The results indicated that Var2-2 plants were more likely to maintain some functional whole-chain electron transport than Col0 plants but there was variability between measurements. The experiments would need to be repeated to determine the most effective period of dark treatment and more samples should be included to improve the power of statistical analysis – the variation seen in measurements here may result from plants being detrimentally affected by too long a dark period. In addition, more samples might allow a higher rate of oxygen evolution without the need for light–dark calculations: subtracting the dark respiration assumes that a similar level of oxygen uptake occurs during the light and dark which would not be the case, although clearly some considerable oxygen uptake does occur in young seedlings in the light.

8.6 FtsH1

The 2.1-Kb (3.5 Kb with introns) gene for FtsH1 [Table 8.1, Figure 8.1] encodes a 712-amino acid, 76 kDa protein. After the A. thaliana ftsH1 cDNA was described [Lindahl et al., 1996] the same authors suggested that this FtsH1 was responsible for the PSII degradation they saw in in-vitro studies of D1 turnover [Lindahl et al., 2000]. Work here was carried out on the mutant of the var2 FtsH because it was readily available from the NASC but also because it was noted to be so similar to the shr0228-encoded Synechocystis protein already identified to be important in photosynthesis [Mann et al., 2000]. Although the in-vivo experiments described above suggested that the Var2 FtsH was important for photosynthesis it was ftsH1, not var2, that was identified in the DNA array carried out by Kurth et al. (2002) in their investigation of A. thaliana genes whose transcription level was altered in light compared with dark conditions. Takechi et al. (2000) also noted that var2 transcription levels were rather stable, with only a small degree of light-regulation. During the course of the work on Var2, attempts were made to isolate a T-DNA-mutagenised FtsH1 in A. thaliana mutant libraries. The comparison of the two strains would be helpful in characterising the specificity of the photosynthetic FtsHs and perhaps elucidating the degree of redundancy in their roles.

8.6.1 Identification of FtsH1 mutants

Thousands of T-DNA-generated transformants have been produced in Arabidopsis, with insertions randomly distributed in the genes and genome. Currently, neither the Salk (signal.salk.edu/cgi-bin/tdnaexpress) nor the GABI-Kat (www.mpiz-koeln.mpg.de/~GABI-Kat/GABI-Kat_homepage.html) public databases of T-DNA mutants list an FtsH1 line, although several are available with an insertion several hundred bases 5′, which may disrupt the transcription of the gene.
Since none were available publicly, a screening programme at the University of Wisconsin was used to identify pools of mutants with insertions in the FtsH1 gene. Sets of transformed lines are available at the facility which are mixed in subpools then made available for PCR analysis. DNA from 17 *A. thaliana* FtsH1 PCR products (using T-DNA and FtsH1 primers) from pools of T-DNA mutagenised *A thaliana* were supplied by the University of Wisconsin T-DNA library for Southern blotting to confirm identity.

Southern blots were carried out on the pooled DNA using a PCR-amplified probe specific to FtsH1 (2.3 Kb of the gene, including introns, amplified with FtsH-2f and -2r; Table 2.3) Two lanes contained positive controls (lanes 16 and 17) but four pools contained very strong hybridisation bands (lanes 1, 2, 3, 6) [Figure 8.17], suggesting that T-DNA mutants of the gene do exist in the library. The pools of lines now need to be reduced: this work was ongoing at the time of writing. If work were to be continued it would also be worthwhile confirming whether the position of the insertion in the Salk and GABI-Kat lines is useful and examining the phenotype of potential mutants.

![Figure 8.17. Southern blot of PCR products supplied by the University of Wisconsin from pools of T-DNA-mutagenised *Arabidopsis thaliana*. The probe was PCR-amplified genomic *ftsH1*. +, Positive control pool; P, probe used as positive for control for hybridisation; 1K, marker lane.](image)

### 8.7 Discussion

In *Arabidopsis*, only four loci have been identified to give rise to a variegation phenotype, *im* (immutans), *cm* (chloroplast mutator), *var1* [Sakamoto et al., 2002], and *var2*. The plastid autonomy and lack of maternal inheritance of the plastid defect in Var2 mutants are reported to make *var2* distinct among other cases of nuclear gene-induced variegation,
forming a novel class of variegation mutant [Takechi et al., 2000]. This work shows that
Var2 is an important protein in photosynthesis, even among the dozen or more possible
FtsH homologues in *A. thaliana*. As the work on D1 turnover was being carried out [Bailey
et al., 2002], Takechi et al. (2000) speculated that, because of the functions of prokaryotic
FtsHs, *Arabidopsis* FtsH proteases might degrade photo-oxidatively damaged proteins of
the thylakoid membranes. Meanwhile, Chen et al. (2000) concurrently proposed that the
Var2 FtsH would be involved in the biogenesis of chloroplasts. From the *in-vivo* studies
carried out here in the Var2-2 mis-sense mutant, a role in turnover of damaged
photosynthetic proteins can be confirmed. Unlike the *Synechocystis* equivalent, however, a
specific function in assembly of thylakoid membrane complexes does not seem so likely,
although this should be investigated further with closer sampling timepoints since
chlorophyll reaccumulation was delayed in Var2-2 compared with Col0 after return to light-
growth conditions.

As expected from the variegated leaves, the chlorophyll content was greatly reduced in
mature Var2-2 plants under standard growth conditions. Although accumulation of
chlorophyll has been reported to be delayed in the mutant, the chlorophyll biosynthesis
pathway apparently remains intact. Chen et al. (2000) suggest that the variable pattern of
variegation in the mutant is due to different factors regulating var2 gene expression and
mediating rates of cell and plastid division. They hypothesise that plastids with sufficient
functional Var2 protein are green, whereas plastids that contain less than a threshold
amount of Var2 activity are white. It has also been proposed that the loss of the Var2
protein reduces the overall level of FtsH proteins in *A. thaliana* and this therefore causes a
variegated phenotype [Takechi et al., 2000].

Here, fluorescence microscopy of Var2-2 showed faint chlorophyll fluorescence even from
the limited plastids in white areas [Figure 8.3]. Electron microscopy confirmed that these
sectors in Var2-2 plants are heteroplastidic, containing highly vacuolated plastids without
proper membrane structure but with rudimentary lamellar structures, as well as some
apparently fully developed chloroplasts [see Chen et al., 2000; Takechi et al., 2000]. In the
green sectors of the Var2-6 strain, Takechi et al. (2000) also noted that chloroplast granal
stacks were less dense than in WT, and that plastid size was more variable in general.

Despite the effects of variegation and large reduction in chlorophyll level, 77K
fluorescence emission spectra showed that the light-harvesting antenna size and function in
Var2-2 is generally comparable with WT plants. Nevertheless, there are other small changes
in the pigment composition, in carotenoids and chlorophyll \( a:b \) ratio, and there are slightly different fluorescence emission characteristics. Parallel work by S. Bailey confirmed a specific effect of the mutation on PSII function, showing that Var2 is essential for \( A. thaliana \) to degrade the reaction centre D1 protein following high-light damage. In Var2-2 plants, the PSII D1 (and D2 to some extent) protein was not degraded, the impaired D1 repair cycle increasing susceptibility to photoinhibition of PSII, and there was also slower recovery from photoinhibition [Bailey et al., 2002]. There was an effect on xanthophyll-cycle function as well as the small alterations in carotenoid content, but as the rates of PSII photoinactivation in Col0 and Var2-2 were the same in the presence of lincomycin and in 77K fluorescence emission spectra, the changes in quenching probably did not cause the photoinhibition phenotype. The data regarding the D1 protein are supported by work [Silva et al., submitted] (and see Section 9.3.1) showing the same effect in slr0228\(^-\) \textit{Synechocystis}.

It is interesting, in the light of the PSII-repair and -photoinhibition phenotype, that Var2 expression has not been shown to be particularly responsive to light. Northern blot analysis of \textit{var2} by Takechi and colleagues (2000) did show slightly lower levels of transcription in Col0 grown under dim light than under 70 \( \mu \text{mol/m}^2/\text{s} \) light, but this study, in contrast with that by Chen \textit{et al.} (2000), found that \textit{var2} mRNA accumulated in all tissues including roots. The need for Var2 beyond a role in processes provoked or regulated directly by light is indicated by the results of the senescence assay. Despite the difficulties using \textit{Arabidopsis} for these assays compared with \textit{Nicotiana}, it was possible to trigger and reverse senescence, presumably by altering the sink–source balance in the seedlings and/or the cytokinin distribution in these young seedlings. As seen in the slr0228/Chl\(^+\) \textit{Synechocystis} double mutant, PSII was not removed in the dark in the absence of the FtsH in Var2-2 seedlings. Meanwhile, functional PSI was affected by the dark treatment: although Var2-2 did retain PSI activity at 8 days’ dark incubation, later, a DCMU-resistant component of the P700 oxidation/rereduction spectra could not be detected during light recovery, perhaps suggesting a delayed adverse response causing PSI defects in seedlings poorly regulated to adjust properly to their changing environment. In Col0, in contrast, PSI and PSII function were absent after dark treatment as might be expected, both returning within 3 days of transfer to standard growth conditions.

In the absence of light it has been assumed that levels of the D1 protein of PSII are stable, but during senescence there obviously is a controlled process of removal of the complexes of the photosynthetic apparatus. In addition, a large proportion of D1 is removed under
low, non-photoinhibitory light intensity [Jansen et al., 1999]. Using pulse-chase radiolabelling of a WT grass (Festuca pratensis), Hilditch et al. (1986) found that there was light and dark degradation of the D1 protein, but that in a ‘stay-green’ mutant, light degradation occurred but dark degradation did not. There remained some PSII activity, in parallel. The authors of this study therefore not only proposed the existence of a process for dark turnover of D1, but that there are independent pathways for light and dark degradation. This may partially be the case, but the evidence from this study (see also Ch1L in Section 7.2) suggests that the Var2 FtsH is involved in both processes in A. thaliana.

The composition of the thylakoid membrane can also be indicated by the chlorophyll $a:b$ ratio, which did not change greatly in the WT dark-treated seedlings. The small drop seen during the dark-period corresponds with that seen in senescent [Koufíl et al., 1999] and in shade plants, which would typically have a chlorophyll $a:b$ ratio of 2–3 [Anderson, 1986; Porra, 2002]. This is because plants grown in insufficient light reduce the PSII:PSI ratio, but increase the amount of PSII LHCII (which has a relatively low chlorophyll $a:b$ ratio) [Anderson, 1986]. The small rise seen in the Col0 chlorophyll $a:b$ ratio during light recovery corresponds with the reverse process.

Var2-2 seedlings, in contrast, showed a declining ratio through dark treatment then light recovery. This corresponds with the retention of PSII components but does not match the return of PSI activity 6 days’ after the return of seedlings to light-incubation. While the chlorophyll $a:b$ ratio does once again indicate a different response in Var2-2 to that seen in WT (perhaps the persistence of pigment-stabilised LHCII even after return to the light; see below), it should be noted the chlorophyll $a:b$ ratio can change inconsistently during senescence, perhaps because of chlorophyll $a$ and $b$ interconversion, as suggested by Zavaleta-Mancera et al. (1999a). The effect of the persistence of chlorophyll in Var2-2 is also unknown but presumably this could result in stabilisation of reaction centre components. Even when photosynthetic activity is lost, proteins can remain in mutants unable to remove chlorophyll: the N terminus alone of LHCII has been shown to be partially degraded in such a case, the rest of the membrane-embedded protein being stabilised by the continued presence of chlorophylls between the transmembrane helices [Zavaleta-Mancera et al., 1999b].

Notably one of the unknown steps in the removal of chlorophyll in plants during senescence involves an ATP-dependent membrane protein. It would be interesting to know if this is another AAA family member. Chlorophyll degradation begins with the
formation of chlorophyllide and then loss/removal of the central magnesium. The opening of the macrocycle to form a linear tetrapyrrole, the so-called red chlorophyll catabolite (RCC) is achieved by pheophorbide \( \alpha \) oxidase, and the unknown ATP-dependent protein is thought to be involved in removal of RCC across the chloroplast envelope [Matile \textit{et al.}, 1987, 1999; Schellenberg \textit{et al.}, 1990; Thomas, 2002]. There is a notable distinction between higher plants and unicellular photosynthetic organisms in this chlorophyll degradation process: the latter are able to remove toxic pigment byproducts into the surrounding medium, whereas plants must use detoxification processes, e.g., in the vacuole [Matile \textit{et al.}, 1988]. This may affect the severity of the consequences of pigment retention in Var2-2 and \textit{slr0228}: plants unable to produce the RCC become highly photosensitised, showing the importance of controlled removal of photosynthetic pigments [Mach \textit{et al.}, 2001]. The recovery of Var2-2 mutants was slower than Col0 in terms of PSI behaviour, return to normal chlorophyll composition and oxygen evolution, suggesting long-term effects of its altered senescence responses.

The effect of the 8 days' dark incubation on whole-chain electron transport in Var2-2 was in contrast with other documented chlorophyll-retaining mutants. The amount of chlorophyll retained during senescence is not always associated with maintenance of photosynthetic activity; in 'stay-green' \textit{Glycine} mutants \( P_{\text{max}} \) was reduced even though chlorophyll and LHCII remained [Guiamet \textit{et al.}, 2002]. The fact, then, that here both pigment and at least some photosynthetic activity were retained suggests that the photosynthetic phenotypes of FtsHs are not directly caused by side-effects of chlorophyll (or PBS) retention but that FtsH proteases have a direct effect on the photosystems. The reduced (or variable) oxygen evolution in the Var2-2 seedlings during senescence but continued function of PSI and PSII was also in agreement with the findings of McRae \textit{et al.} (1985). During senescence of the primary leaves of beans, they noted a reduction in linear electron transport that was more marked than the reduction in the activity of either PSI or PSII independently. These authors suggested that this is a result of limited inter-photosystem electron transport. Since thylakoid membranes have been observed to become less stacked (but remain identifiably distinct membrane structures) during senescence, they also confirmed that this was not a side-effect of changes in thylakoid membrane fluidity or diffusion of mobile electron carriers (see also Chapter 9).

To fully elucidate the changes occurring during etiolation and regreening in the absence of Var2, this preliminary assay requires repetition with more replicates and sampling timepoints. Using sucrose-supplemented medium allows chlorophyll to fall more quickly
[Simpson et al., 2003], which may help clarify the specific role of pigments during senescence. Also, since senescence in *Nicotiana* species is more easily controlled it would be worthwhile attempting to procure a potentially equivalent FtsH mutant: the larger plants would also aid biochemical and biophysical assays.

A role in senescence processes is worth investigating further because Var2 is notable among the *A. thaliana* FtsH proteins in its particular similarity to the *C. annuum* Pftf FtsH. Pftf is a thylakoid protein that aids the differentiation of plastids in the pepper, promoting vesicle formation in the transitions of chloroplasts into chromoplasts, a process involving pathways also activated in senescence [Hugueney et al., 1995; Summer & Cline, 1999a,b]. As observed in work in *ChlL*-Synechocystis, it is difficult to separate experimentally the processes involved in protein turnover and protein synthesis for cell differentiation. It may be that the terminology for these biological processes confuses the issue as well. For example, senescence is seen to be cell-death process but in fact early stages in etiolation, recovery from etiolation and biogenesis of thylakoid membrane complexes could all involve the same proteolytic (or unfolding) or chaperone (or unfolding or refolding) functions. This is in line with the observation that both Var2 and Pftf are constitutively expressed.

A role for Var2 in mitochondrial development should be investigated as part of further work. This is because FtsH-type proteins not only appear to be important in mitochondria as in chloroplasts [see Table 8.1], but because the chloroplast mutator (*chm*) mutant has a deficiency in mitochondrial gene expression which results in variegation, i.e., functional mitochondria may be required for proper chloroplast development [Sakamoto et al., 1996]. Mitochondrial malfunction is unlikely to be the cause of the Var2-2 phenotype since Takechi et al. (2000) found that mitochondria in the Var2-6 mutant appeared normal from electron microscopic examination, but any effect should be ruled out.

Further investigation of the other *A. thaliana* FtsHs may explain a recurrent conundrum in all the FtsH research carried out so far. This is the fact that most phenotypic effects seem to be ameliorated or corrected eventually: as with the slr0228-* mutant, the effect of the missing Var2 FtsH was severe and reproducible, but there appeared to be a degree of redundancy allowing eventual recovery, for example, from high-light damage. There may be proteins that can substitute for Var2 function, or alternatively, the processes catalysed by the FtsH(s) may occur slowly even in its/their absence. This has been noted in other degradation processes, e.g., even in the absence of the pheophorbide *a* oxygenase, some
pheophorbide chlorophyll degradation product is accumulated [references within Thomas, 2002]. It would be useful to identify which FtsH proteins are localised and where on the thylakoid membranes, or associated with the Arabidopsis photosystems and their components, although care would be required in the choice of antibody or probe. The cyanobacterial and plant FtsH proteins have highly conserved regions which have previously proved problematic in hybridisation methods. Takechi et al. (2000) found that a full-length cDNA produced cross-hybridisation with mRNA from other genes, presumably encoding other FtsH proteins, only achieving more specific hybridisation by using the 5' end only of var2 as a probe. Silva et al. (submitted) and others (S. Bailey, personal communication, 2001) found the same problem in Western blots, and this cannot be ruled out in the Western blots of Lindahl et al. (1996). Finally, the interaction and potential for formation of hetero-oligomers between Var2, Var1 and FtsH1, the FtsH proteins known to be active on the thylakoid membranes, and the identity of protein partners that regulate their proteolytic and/or chaperone functions, are obvious further questions to be answered.
9. **FTSH AND LIPIDS**

9.1 **Introduction**

Unlike *Synechocystis* or *A. thaliana*, *E. coli* possesses only one FtsH protein, which has multiple roles in the bacterium. One of these is the regulation of lipid biosynthesis. It appears that the *E. coli* FtsH degrades the product of *envA* (also called *lpxC*), the deacetylase enzyme at the committed step of lipid A synthesis [Ogura *et al.*, 1999]. The regulation of this enzyme is at an important point in membrane biosynthetic pathways as it determines whether phospholipids or lipopolysaccharide are synthesised.

As well as making up the membranes essential for compartmentalising organelles, lipids in plants form storage compounds, signalling and defence molecules and leaves' protective layers. They are of interest in the study of the *var2*- and *slr0228*-encoded FtsHs in *A. thaliana* and *Synechocystis* because lipids serve numerous other functions in photosynthesis [for review, see Dörmann & Benning, 2002]. Lipids act as structural components of the thylakoid membrane, where the proteins of photosynthesis are inserted, translocated and carry out electron transfer. The recent crystal structure of cyanobacterial PSI also revealed integral lipid molecules, one monogalactosyldiacylglycerol (MGDG) and four phosphatidylglycerol molecules in each PSI monomer [Jordan *et al.*, 2001]. The question therefore arises, do the *slr0228* or *Var2* FtsH proteins act upon a lipid biosynthesis enzyme and is photosynthetic function perturbed because of that rather than a specific role for FtsH in photosynthesis?

Since the *Synechocystis* genome encodes four FtsH proteins and the *A. thaliana* genome even more (see Chapter 8), it seems likely that all the functions of the *E. coli* FtsH need not be maintained by each cyanobacterial or plant version. Nevertheless, to rule out an effect of altered lipid composition on the photosynthetic phenotype of the FtsH mutants, the fatty acid composition of the thylakoid membranes of the *slr0228* strain and of *Var2-2* *A. thaliana* was investigated. A *Synechocystis* desaturase mutant was also analysed in parallel with *slr0228* in photoinhibition and trimerisation experiments. Some of this work is described in Silva *et al. (submitted).
9.1.1 Fatty acids of photosynthetic membranes

The cyanobacterial cell contains two types of membrane, the thylakoid and plasma membranes. The two are different from each other in composition. Thylakoid membranes are composed primarily of galactolipids, primarily MGDG followed by digalactosyldiacylglycerol (DGDG) and sulphoquinovosyldiacylglycerol (SQDG) [Glatz et al., 1999]. The fatty acids of cyanobacteria are predominantly unbranched chains of 14, 16 or 18 carbons with 0–3 double bonds, with 16:0 (palmitic acid) and 16:1 (palmitoleic acid) amongst the most common [for review, see Sato & Murata, 1988].

The lipids of plant membranes typically contain 16 or 18 carbons, but plants also have some unusual unsaturated fatty acids that are found only in storage triacylglycerols in seeds, and additional fatty acids of eight to 32 carbons in length that are accumulated for storage lipids or epicuticular wax. Plant thylakoid membranes contain high concentrations of polyunsaturated fatty acids, a large proportion of which are the polyunsaturated linoleic (18:2\(^{\Delta 9,12}\)) and α-linoleic (18:3\(^{\Delta 6,9,12}\)) acids [Sommerville et al., 2000].

9.2 Lipid analysis

Thylakoid membranes were prepared from *Synechocystis* cells and *A. thaliana* (see Section 2.3.1–2). Lipid and fatty acid extraction was carried out as in Kruse et al. (2000). The extracted lipid to chlorophyll ratio again confirmed that these FtsH mutants contain less chlorophyll than WT (data not shown). The galactolipid and phospholipid content of extracts was qualitatively investigated using TLC, staining with anthron or molybdenum oxide, respectively. Fatty acid composition was analysed using GLC (for details, see Section 2.3.6).

Interestingly, spots of extracts for lipid TLC plates were clearly different between the FtsH mutants and their respective WT: pigments retained in the mutants’ extracts were much more yellow/brown than the WT extracts, which were green, as would be expected. This was especially marked in slr0228\(^{\circ} \) material (data not shown). The altered pigmentation of extracts suggested that carotenoid composition might vary considerably in the mutants, so TLC plates were run to separate photosynthetic pigments (see Sections 5.3.2, 8.3.2).

The TLC plates stained for phospholipids or galactolipids showed that each mutant contained comparable lipids to WT. In *A. thaliana* samples, phosphatidylcholine and phosphatidylglycerol were present in each; the *Synechocystis* membranes contain phosphatidylglycerol only (data not shown; plate deteriorated before scanned).
Galactolipids visible in all four samples were MGDG and DGDG; SQDG was rather faint. Less Var2-2 sample was loaded than Col0 on this plate on a per-chlorophyll basis but the strength of galactolipid bands was equal to Col0 when stained, consistent with the much lower chlorophyll:lipid ratio [Figure 5.5].

9.2.1 Fatty acid analysis

Further analysis of fatty acids by GLC [Figure 9.1], however, showed that the slr0228" and Var2-2 thylakoid membranes contained a greater proportion of saturated fatty acids than the WT membranes [Figure 9.2]. Decreases in the level of unsaturated 18-carbon fatty acids were matched by an increase in the proportion of 16:0 molecules. In the case of Var2-2, there was also a correspondingly greater proportion of less-unsaturated 18-carbon fatty acids.

The mutants lacking an FtsH protein could therefore be affected in thylakoid membrane function because of altered membrane characteristics [described in cyanobacteria by Sippola et al., 1998; Tasaka et al., 1996; in Arabidopsis by Vijayan & Browse, 2002].

9.3 Does altered membrane composition affect photosynthesis in the FtsH mutants?

Since the composition of the thylakoid membrane could affect behaviour of proteins involved in photosynthesis, it was important to determine the effect of the changes in fatty acids in slr0228" Synechocystis. To do this, a mutant with severely saturated membranes was tested in parallel with slr0228" and WT Synechocystis in photoinhibition and blue-light assays. Any comparable effect on either PSII or PSI could then be identified.

The mutant used was the DesA/D" strain (kindly donated by N. Murata). DesA/D" Synechocystis is missing the acyl-lipid desaturases that introduce double bonds into the Δ12 and Δ6 positions of C18 fatty acids; it is therefore missing all fatty acids with double bonds at those positions. The DesA/D" cells have a much more severe fatty-acid phenotype than that of the slr0228" cells [Figure 9.2], having no polyunsaturated fatty acids at all, only the mono-unsaturated 18:1ω9 fatty acid. As in the slr0228" strain, there is no effect on lipid class, with a galactolipid content comparable to WT [Tasaka et al., 1996].

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Figure 9.1. Gas chromatogram of fatty acids in thylakoid membranes from *Arabidopsis thaliana* (Col0 and Var2-2; left panels) and *Synechocystis* [wild type (WT) and slr0228*; right panels]. See Figure 9.2 for quantitative analysis.
Figure 9.2. Fatty acid composition of wild-type (WT) and FtsH mutants in *Synechocystis* (a) and *Arabidopsis thaliana* (b), analysed by gas–liquid chromatography. 16:0, palmitic acid; 16:1, palmitoleic acid; 16:3, hexadecatrienoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2(Δ9,12), linoleic acid; 18:3(Δ6,9,12), γ-linoleic acid; 18:3(Δ9,12,15), α-linoleic acid; 18:4(Δ6,9,12,15), octadecatetraenoic acid.
### 9.3.1 Photosystem II turnover

Under high-intensity light, as long as the rate of repair of PSII exceeds the rate of damage, the photoinactivation and degradation of the D1 protein should not result in a significant change in oxygen evolution or carbon fixation. If there were similar reductions in oxygen evolution in high light in DesA/D^− and slr0228^- cells, the altered fatty acid composition could be responsible for the D1-turnover phenotype of FtsH mutants.

Parallel subcultures of WT, slr0228^- and DesA/D^- *Synechocystis* were therefore prepared and adjusted to the same cell density according to *A*^\*^595. When in exponential growth phase, duplicate 20 ml cultures of 4 μg/ml chlorophyll were incubated (at 30°C) in photoinhibitory light of approx. 1400 μmol/m^2^/s for 4.5 h and then allowed to recover in light of 10 μmol/m^2^/s overnight (19 h). Light-saturated oxygen evolution of whole cells (plus 10 mM NaHCO_3) was measured at intervals.

After 2 h in 1400 μmol/m^2^/s illumination, oxygen evolution had declined to almost zero in the slr0228^- cells, whereas WT and DesA/D^- continued to photosynthesise [Figure 9.3a]. This experiment was repeated to confirm the effects, with the same results. By 3 h the slr0228^- mutant was always unable to evolve oxygen, although respiration continued, whereas WT and DesA/D^- cells maintained a fairly even evolution of around 200 μmol O_2/mg chlorophyll/h.

Studies of the recovery of *Chlamydomonas* from photoinhibition showed that after high light (magnitude approx. 1000 μmol/m^2^/s) for some hours, replacement of D1 cannot be maintained and recovery is only possible after incubation in low light for 1–3 h [Keren & Ohad, 1998]. Even after overnight recovery in low light, the FtsH mutant remained severely affected (yellowed cultures), in contrast with the WT and desaturase mutant (remained green) [Figure 9.3b].

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Figure 9.3. Wild-type (WT), slr0228− and DesA/D− (left–right) glucose-tolerant *Synechocystis* cells incubated in low light (10 μmol/m²/s), then 2 h light of approx. 1400 μmol/m²/s and recovery in low light (19 h at 10 μmol/m²/s). (a) Oxygen evolution. Results from duplicate cultures are shown. (b) Appearance of cultures after overnight recovery.

### 9.3.2 PSI assembly

The lack of photoinhibition phenotype in the DesA/D− cells suggested that PSII repair was not greatly affected by increased saturation of the thylakoid membranes, at least under the conditions tested in these experiments. What about PSI? Work had already suggested that PSI assembly is affected if the slr0228 FtsH is inactivated (Chapters 6, 7), putting the mutant at a particularly severe growth disadvantage when incubated in blue light. The blue-light experiments were therefore repeated including DesA/D− cells to investigate whether altered fatty acid composition would also cause retarded growth.
Figure 9.4. Growth of wild-type (WT), slr0228 and DesA/D glucose-tolerant Synechocystis under blue (a) and neutral density (ND; b) filters.
9.3.3 Growth

Figure 9.4 shows the growth of WT, slr0228- and DesA/D- cultures when grown in blue light. Rapidly-growing cultures, which had been subcultured twice to ensure that cells were growing exponentially upon transfer to blue light, should have produced linear growth almost immediately. This was only the case with WT cells, followed by DesA/D-, but slr0228- cell scattering (A750) dropped at first before recovering. The exponential growth phase of triplicate cultures upon transfer to blue light, however, gave similar mean doubling times (± SEM) for all cultures, of 108 ± 13 h for WT, 103 ± 9 h for slr0228- and 98 ± 18 h for DesA/D-.

The major difference appeared not to be growth rate itself (note, however, that this is only during the first few days after transfer to blue light) but that slr0228- cells never achieved any significant cell density despite prolonged incubation under blue filters, in contrast with DesA/D- which reached an A750 comparable to WT [Figure 9.4]. The poor growth of slr0228- was very noticeable upon observation of its pale cultures, whereas WT and DesA/D- appeared normal despite the relatively long doubling times, presumably an effect of rather low-intensity light resulting from the use of filters. Low light was not itself responsible for the effect on slr0228- since the cultures all reached identical cell densities by 9–13 days of incubation at approx. 5 μmol/m²/s under neutral density filters [Figure 9.4].

9.3.4 77K fluorescence emission spectra

Cultures grown in blue light were then examined using 77K fluorescence emission spectra since this would show any dramatic effect upon PSI under blue light. Under standard incubation conditions, the DesA/D- cultures produced spectra with a lower PSI:PSII ratio, like slr0228-, but under blue-light incubation there was once again little difference between DesA/D- and WT [Figure 9.5]. slr0228- cells, as before, demonstrated a greatly-increased PSII peak relative to PSI after blue-light incubation and failed to produce the characteristic right-shift of the PSI peak usually seen in populations of PSI trimers. The large difference in PSI:PSII ratio in the slr0228- mutant is discussed in more detail in Chapters 5 and 6.
Figure 9.5. 77K fluorescence emission spectra from wild-type (WT), slr0228\textsuperscript{–} and DesA/D\textsuperscript{–} glucose-tolerant *Synechocystis* grown in low light (LL, top panel) and blue light for 5 days (lower panel). Note the small right-shift of WT photosystem I peak in blue light that is not matched in the slr0228 culture, and large photosystem II peak in slr0228\textsuperscript{–}.
9.4 Discussion

These results suggest that even a mutant with more severely saturated membranes than slr0228− is able to maintain its D1 protein repair-cycle after hours of intense light. In fact, it is known that at temperatures of < 20°C (below the 30°C for optimal growth of Synechocystis) the DesA/D− mutant is affected in its ability to counteract photoinhibition, specifically to repair PSII efficiently [Tasaka et al., 1996]: even with these multiple mutations, an additional temperature stress is required to impede membrane function when there is altered lipid composition. In these experiments, oxygen evolution of the DesA/D− strain at 30°C in extreme high light was comparable with that of WT cells, despite the increased level of fatty acid saturation. The slr0228− mutant strain, in contrast, has less severely affected thylakoid membrane fatty acids but was unable to maintain full-chain electron transport in photoinhibitory light.

With regard to PSI, although the DesA/D− strain did not produce identical growth curves to WT, its growth was certainly not impaired like that of the slr0228− cultures (for discussion of the change in slr0228−, see Chapter 6). It would be interesting to investigate further the cause of the initially high PSI:PSII ratio seen in DesA/D− 77K fluorescence emission spectra, but the response of DesA/D− to blue light does not suggest that the more saturated membranes of slr0228− are the cause of a marked PSI phenotype.

All the tests comparing DesA/D− with slr0228− and WT cells have showed that DesA/D− Synechocystis is able to photosynthesise like WT under standard incubation conditions and it can adapt to its environment more effectively than the slr0228 mutant. This evidence suggests that, despite small changes in the fatty acid composition of slr0228− cells, it is not the lipid phenotype that is responsible for severe changes in photosynthesis in the mutant.
10. DISCUSSION

10.1 Summary of findings

Three proteins hypothesised to be involved in the maintenance of the photosynthetic apparatus in both prokaryotes and eukaryotes were studied in this work. Since a homologue of the *Synechocystis* RpaC protein appeared not to be present in *A. thaliana*, it was not investigated further. The slr0575 ORF of *Synechocystis*, however, was insertionally inactivated in an attempt to augment information gained about a likely plant equivalent, the *A. thaliana* Ape-1 protein. Unlike the observations made with the *Arabidopsis ape-1* mutant, there was no clear indication of a role in the regulation of light-harvesting for the cyanobacterial protein. Finally, the investigation of two FtsH proteins, the product of slr0228 in *Synechocystis* and *var2* in *A. thaliana*, showed that they do have some equivalent roles in the bacterium and the chloroplast. In *Arabidopsis* and *Synechocystis*, their respective FtsH protein is necessary for PSII turnover. Notably, PSII is not degraded in both dark and photoinhibitory conditions in FtsH mutants, in contrast with WT. As might be expected, however, the presence of numerous FtsH proteins in the plant appears to have allowed divergence of function so that a role in PSI assembly in *Synechocystis* is probably not conserved in *A. thaliana*. The proteins encoded by slr0228 and *var2* are necessary for correct carotenoid and fatty acid composition in both plant and cyanobacterium, however, implying the involvement of such 'photosynthetic' FtsH proteins in many pathways, as seen with the best-studied FtsH, that in *E. coli*.

10.2 slr0575 in *Synechocystis*

Free-living organisms, such as *Synechocystis* and *Chlamydomonas*, are favoured experimental models for photosynthesis but their different ability to respond to their environment, for example via chemotaxis or phototaxis, inevitably results in differences in the regulation of photosynthesis between such single-celled organisms and multicellular, sessile plants. The comparison of *Synechocystis* and *A. thaliana* proved to be a useful approach in the investigation of FtsH proteins, but the difference in functions of the Ape-1 protein in *Arabidopsis* and *Synechocystis* confirmed that, despite the inheritance of so many proteins from the cyanobacterium-like ancestor of the chloroplast, there is often diversification of function. Nevertheless, the protein predicted to be encoded by *Synechocystis* slr0575 has a number of likely homologues in *Prochlorococcus, Thermosynechococcus, Anabaena* and *Trichodesmium*, suggesting that it is still, or was very recently, fulfilling a role in the organisms.
Photosynthesis seemed to be fully functional in the slr0575\(^{-}\) mutant, which was able to adapt to low, high, red and yellow light, conditions that require the regulation of light-harvesting and photosystem content and activity. The mutant was also able to perform state transitions, a short-term adaptation mechanism, suggesting functional signal transduction pathways. There is perhaps the indication of some role in photosynthesis apart from the conservation of similar genes in photosynthetic organisms. There were small but reproducible and significant changes in oxygen evolution early in adaptation to very high intensities of light and during recovery immediately afterwards in low-light conditions. More detailed examination of the response of each of the components in the electron transfer chain would be needed to elucidate the nature of the changes that resulted in a delayed increase in photosynthetic capacity during adaptation to high-intensity light, and why the increased oxygen evolution continued for longer upon return to low light than in WT cells. It is possible that, as seen in the ape-1 mutant, there were small changes in PSII content, although corresponding differences in PSII:PSI seen in 77K fluorescence emission spectra were not consistent. Alternatively, regulation of the pigment antennae may be perturbed in slr0575\(^{-}\). If other proteins and pathways were able to compensate in the adaptation to light of different spectral quality, only a small effect on photosynthesis in some circumstances might be noted, as here. The regulation of light harvesting is not yet elucidated in terms of regulatory proteins, promoters or important sequence domains. Indeed, no recognisable motifs were identified in or around the slr0575 sequence, even though a number of similar genes already exist in the currently limited supply of whole-genome databases. None of a series of microarray studies published recently have revealed a change in transcription levels of the slr0575 ORF, suggesting that it is either constitutively expressed at a low level, expressed at levels too low to detect, or is not expressed at all under any of the conditions so far tested. Any further investigation of this gene should use Northern analysis or reverse-transcription–PCR to demonstrate that slr0575 is active, particularly under photoinhibitory light conditions since this is the best indicator so far of a function. In addition, the up- and down-regulation of photosystem subunits in Northern and/or Western blots might show why oxygen evolution rates are different from WT under changing light environments.

10.3 FtsH proteins in *Synechocystis* and *A. thaliana*

Insertional inactivation mutants of the ORF slr0228 were made in GT and ChlL\(^{-}\).* Synechocystis* to allow the turnover and reassembly of the photosynthetic apparatus to be studied in the cyanobacterium. The effect of an apparently equivalent mutation, that of *nap2* in *A. thaliana*, was also studied for comparison.
The effect on the photosystems of inactivating this one protein — of the four putative FtsH proteins in *Synechocystis* — was very specific and reproducible, but the deletion also affected numerous facets of photosynthesis. The phenotype was severe, particularly in terms of the ability of the mutant to degrade PSII and to adapt to growth under conditions apparently requiring a larger population of PSI trimers. Nevertheless, despite the reduced PSI content, reaction centres and PBS appeared to be assembled and fully functional under ideal growth conditions. Signal transduction pathways allowed state transitions and response to high light. The Var2-2 *A. thaliana* mutant was also able to photosynthesise adequately under standard growth conditions, but its ability to remove PSII was impeded, as in the slr0228" cyanobacterial mutant.

### 10.3.1 Pigment and photosystems

The observations from the previous insertional inactivation of slr0228 in a motile *Synechocystis* strain [Mann et al., 2000] were confirmed. The GT slr0228" mutant produced the same distinctive absorbance and 77K fluorescence emission spectra resulting from reduced chlorophyll (86% of WT), increased phycocyanin (115%) and reduced PSI (46%) content. In addition, the mutant's growth in low light was impaired, and there was higher and more variable dark respiration. There was decreased oxygen uptake via the Mehler reaction and P700 activity in EPR, which corresponded with reduced PSI content. An increased mean oxygen evolution was not significantly different from WT, but the increased number of chlorophyll molecules per PSI complicates the interpretation of several of these measurements of photosynthetic function that are normalised on chlorophyll content.

Var2-2 plants were also found to contain reduced (to about 60%) chlorophyll content per weight of leaf tissue compared with Col0, the parental strain of *A. thaliana*. Microscopy confirmed that normally-formed chloroplasts were present in green sectors of leaves but chlorophyll fluorescence was reduced, although not completely absent, from white sectors. The reason for the variegation in the Var2-2 mutant, i.e., why assembly of chloroplasts is functional in some areas and not in others, remains unclear.

The thylakoid membrane composition was comparable in Var2-2 and Col0 by examination of SDS–PAGE gels of solubilised thylakoid membranes, but Var2-2 PSI/µg chlorophyll was slightly increased as judged by P700 activity in flash spectroscopy. This is the converse finding to that in slr0228" *Synechocystis*, but it was confirmed by a higher PSI:PSII
fluorescence ratio in 77K fluorescence emission spectra. A corresponding increase in the chlorophyll $ab$ ratio was not significantly different, nor was the increased mean oxygen evolution/chlorophyll.

In both the cyanobacterial and the plant FtsH mutant, the carotenoid content was altered. There proved to be a reduced level of $\beta$-carotene but increased myxoxanthophyll and zeaxanthin in slr0228" *Synechocystis* thylakoid membranes. In Var2-2 thylakoid membranes, $\beta$-carotene again was the most dramatically altered, with only about half (per chlorophyll) to two-thirds (as a proportion of a reduced total quantity of carotenoids) the quantity found in Col0. Lutein was also reduced per chlorophyll, but the content remained approximately the same as in WT as a proportion of total carotenoids. Significantly, two molecules of lutein are found within the LHCII complex [Horton et al., 1996] and lutein is needed for $q_E$ [Pogson et al., 1998], possibly explaining the reduced $q_E$ seen in Var2-2 [Bailey et al., 2002]. Carotenoid depletion has also been reported to inhibit recovery of PSII (see below): in *Chlamydomonas*, inhibitors of phytoene desaturase that prevented synthesis of carotenoids stopped reaccumulation of the D1 protein during photoinhibition. The authors also suggested a role for $\beta$-carotene in destabilising PSII and triggering D1 degradation after high-light damage [Trebst & Depka, 1997].

The altered carotenoid composition in these two FtsH mutants might be the cause or the result of other changes in the thylakoid membrane. If these FtsHs were to operate directly in the regulation of carotenoid synthesis pathways, or in regulation of the xanthophyll cycle in *A. thaliana*, their mutation might cause or exacerbate the PSI and PSII phenotypes. Studies of other carotenoid mutants, however, suggest that such severe phenotypes are seen only in strains that have lost multiple pigments or which suffer complete loss of one pigment since, for example, $\alpha$-carotene-derived xanthophylls can substitute for those synthesised via $\beta$-carotene [Niyogi et al., 1998 and references therein]. Therefore, the minor changes due to these mutated FtsHs might not be expected to cause the extreme, specific phenotypes in PSI and PSII seen here (see below).

It is possible that the altered carotenoids are a symptom of other changes. The slr0228" and Var2-2 mutants may be subject to sustained high-light damage because of their inability to repair PSII properly, and light stress is known to cause changes in carotenoids. For example, the xanthophyll-rich ELIP and algal homologue Cbr are induced in pea plants and *Dunaliella*, respectively, during light stress when they probably act in protection of PSII [Green & Durnford, 1996; Jin et al., 2001]. It would be of interest to identify the cause of
the change in carotenoids, whether it be induction of ELIP-type proteins and their equivalents or changes in the carotenoid biosynthetic pathways.

Although the change to carotenoid content was minor in Var2-2 and slr0228\(^-\), it occurs in addition to a decrease in chlorophyll content in both mutants, and as well as an increase in phycocyanin content in slr0228\(^-\). Amongst other such subtle changes there was an abnormally high PSII/PBS terminal emitter peak from slr0228\(^-\) cells under 600 nm excitation light in 77K fluorescence emission spectra. Also suggestive of slightly perturbed electron transport was the aberrant fluorescence seen in time-course studies of state transitions in the cyanobacterial mutant. Although slr0228\(^-\) regulated the energy channelled via PSII and PSI in state 1 and 2, fluorescence characteristics in the early moments of the state transition suggested that it is easier to overload the PQ pool or the quinone acceptors of PSII with electrons in slr0228\(^-\) because there is less PSI to take them away. These indications of subtly different electron transport function were also noted in the higher respiration rate and higher background fluorescence in slr0228\(^-\).

### 10.3.2 Photosystem II turnover

One of the most severe effects of the mutation of slr0228 or var2 was that on PSII turnover. Work here and concurrent to this showed that neither slr0228\(^-\) [Silva et al., submitted] nor Var2-2 [Bailey et al., 2002] could maintain functional photosynthesis in prolonged high light. As previously discussed (Section 1.8.2), the PSII protein PsbA, or D1, has a high rate of turnover because of photoinactivation. To prevent photoinhibition, damaged D1 needs to be removed from the reaction centre complex and be replaced with a newly synthesised D1 protein. The Var2-2 mutant was unable to degrade the D1 protein in photoinactivated PSII, but there was not a significant difference in the rate of damage itself, as seen in the similar response of Var2-2 and Col0 in 77K fluorescence emission spectra (or in lincomycin-treated leaves in work by S. Bailey). Not only did the inability to repair PSII affect the mutants in high-intensity light, however; the effect extended to turnover of the photosynthetic apparatus under non-photoinhibitory conditions.

The effect of FtsH inactivation on the removal of pigments and PSII was particularly marked in chlorosis of slr0228\(^-\) during nitrogen deprivation and of the slr0228/ChlL\(^-\) double mutant. The appearance of slr0228/ChlL\(^-\) after LAHG was a dramatic indication of its inability to degrade the photosynthetic apparatus in the absence of the slr0228-encoded FtsH. The mutation of the slr0228 ORF does not necessarily result in an inability to reduce chlorophyll levels, as seen in blue-light growth of slr0228\(^-\) when the amount of
chlorophyll/cell dropped rapidly and permanently. The effect of inactivated slr0228 seems to be the loss of turnover of PSII, with the side-effect of some retained pigments in addition. Oxygen evolution continued in these green double-mutant cultures during LAHG, suggesting that the PSII removed in ChlL− would have been functional, with interesting implications for the systems of targeting and removal of PSII.

The ChlL− mutant seemed an ideal system for the study of chlorosis and regreening. The mutant is not identical to WT in the level of PSI, however (under these incubation conditions at least), and both the ChlL− and slr0228/ChlL− double mutant were difficult to work with. Rather variable data resulted from the difficulty in maintaining full viability of both cultures through extensive chlorosis, so that the dramatic effects seen in most cultures were masked by large errors when all the samples were analysed together. In addition, changes to the thylakoid membranes and cell scattering characteristics seem to be responsible for the difficulty compensating for Δ750 when using the equations from Myers et al. (1980) to deconvolute whole-cell pigment contents in these assays.

The results of preliminary senescence assays in A. thaliana, however, did correspond with those from the slr0228/ChlL− chlorosis assays. Chlorophyll in Var2-2 seedlings did not decrease in parallel with that in Col0 after 8 days growth in the dark and, interestingly, the chlorophyll ab ratio continued to drop in the variegated mutant, as seen in other ‘stay-green’ plants which retained LHCII in particular [Zavaleta-Mancera et al., 1999a,b]. Which components of the photosynthetic apparatus were retained by the Var2-2 plants still needs to be fully examined, but measurement of photosystem activity here once again suggested that the inactivated FtsH prevented removal of PSII. Neither PSI nor PSII activity (quantified by flash spectroscopy) was reduced in Var2-2 at 8 days’ dark treatment but P700 activity was reduced upon return to normal light incubation. In Col0 the activity of both photosystems was reduced below the detection limit for flash spectroscopy at 8 days’ dark incubation.

In normal senescence in plants, after flowering, the degradation of cytochrome b6f is thought to occur first, followed by a decrease in PSI and PSII and then ATP synthase, although the mechanisms of this thylakoid protein catabolism are not yet understood [Guimé et al., 2002]. As with slr0228/ChlL−, more Var2-2 than Col0 seedlings evolved oxygen after dark treatment (although there was some variability), corresponding also with increased chlorophyll levels. If cytochrome b6f is degraded first, and is subject to a different proteolytic pathway from PSII (suggested to be via a ClpP protease) [Majeran et al., 2001],
the variability in oxygen evolution measurements might be explained by some decrease in other components of the electron transport chain. As with the ChlL− chlorosis assays, however, it was difficult to maintain full viability during such harsh treatment. The experiments need to be carried out on a larger scale to allow more sampling points, and minimisation of variability and error bars during analysis of the data.

The work here on FtsH in both cyanobacteria and plants, however, supports the idea that the same pathway operates for breakdown of the D1 protein in most/all circumstances and this depends upon an FtsH protease. Whether the high light-induced inactivation and degradation of PSII and its turnover in low light are related has been subject to some debate [e.g., Keren & Ohad, 1998]. The process by which D1 (and indeed other proteins of the thylakoid membranes) is targeted for removal, has not been conclusively explained. As mentioned previously, even the site of damage of the D1 protein in high light is not agreed. Amongst the views published are the theories that a conformational change in D1 is caused by active oxygen species or other radicals which triggers degradation of the protein, perhaps by revealing a cleavage site for a protease. A site of endoproteolytic cleavage has been proposed to exist close to the Qb pocket or cleavage of the D1 protein may be governed by Qb, e.g., cleavage only occurs when PQ occupies the Qb site since mutants were unable degrade D1 if they could not oxidise plastoquinol owing to inactivation of cytochrome b6f, plastocyanin or PSI [see Kanervo et al., 1998; Keren & Ohad, 1998; Komenda & Barber, 1995; Mattoo et al., 1984].

There is some evidence that phosphorylated D1 is more stable (see below), and a chemical process without involvement of light damage/active oxygen species has been proposed to exist to explain the D1 degradation seen in darkness in Synechocystis [Kanervo et al., 1998], and in experiments with Chlamydomonas [see Keren & Ohad, 1998]. In this work in vivo in Synechocystis, the loss of photosystems and pigments in ChlL− also suggested that PSII turnover is not restricted to light conditions. The high PSII:PSI fluorescence in slr0228/ChlL− suggests a constitutive pathway for removal of PSII in ChlL−, where the PSII:PSI fluorescence ratio was not increased. Growth conditions for this strain did include a 10-min photoperiod every day, however, and it could be argued that its mutated chlorophyll synthesis pathway could also have an effect on the stability of PSII.

The D1 protein is certainly turned over in light of wavelengths broader than those used by PSII – from ultraviolet-B through to far-red – and in very low light conditions. Some degradation of the D1 and D2 proteins is correlated with photon flux, since >25% total
degradation in the plant *Spirodela* occurs in 5 μmol/m²/s light, rising to 57% in <100 μmol/m²/s, to >90% of the degradation occurring at <750 μmol/m²/s. The turnover of D1 and D2 is also multiphasic, however, suggesting that it is controlled by more than one parameter. Whether the existence of phases of degradation is related to heterogeneous populations of PSII (e.g., phosphorylated D1 differing in its susceptibility to photoinhibition) is not known [Aro et al., 1992; Jansen et al., 1999]. In cyanobacteria, there are also differences in the susceptibility of D1 depending upon which version of the polypeptide is expressed [e.g., see Komenda et al., 2000; Sane et al., 2002]. Given the lack of photoinhibition in dark incubation, the assumption would be that the *Synechocystis* PSII in LAHG contained the less resistant form of D1, and its turnover in Chl− implies that it can be recognised by the slr0228-encoded FtsH without a photodamage-induced targeting mechanism. Perhaps a conformational change does occur and provokes rapid replacement of D1 when there is excess excitation, whereas there is a secondary slower pathway whereby cells have a mechanism for altering PSII:PSI when synthesis of photosystems is downregulated. Since sampling times here were convenient for these long-term assays, it would be interesting to repeat some of the measurements at reduced time-intervals in both plants and cyanobacteria, in dark and light incubation conditions, to establish the relative rates of removal of PSII due to photoinhibition or merely (potentially) constitutive turnover. This might confirm whether the two processes exist or indeed occur by the same mechanism.

The reduced D2 as well as D1 degradation seen here in Western blots (by S. Bailey) support the fact that turnover of the two core proteins is coupled, although quite how is not known [Jansen et al., 1999]. Notably, Jansen et al. (1999) suggested that since D2 is degraded in addition to D1 in one of every three degradation events, the process of turnover must occur by at least two different routes, especially if, as is postulated, the presence of D2 in the PSII core is necessary for binding of new D1. The possibility of secondary routes for the repair cycle might explain why the slr0228− and Var2-2 plants can eventually recover from photoinhibition, and why they can photosynthesise adequately under low–moderate light intensities.

The selective nature of the proteolysis of PSII proteins is not contested; there are numerous studies demonstrating that particular protein complexes and their subunits are regulated by specific and distinct pathways. For example, the D1 protein does not become a target for proteolysis until partially assembled with D2 and CP47 [for review, see Choquet & Vallon, 2000]. Further, whereas some PSII core proteins were degraded in barley with a
half-life of 10–15 h, the half-life of the D1 protein can be as short as 2–3 h [for review, see Hoober & Eggink, 1999]. In contrast, the proteins of the oxygen-evolving complex, despite release into the thylakoid lumen upon D1 turnover, may not be degraded for up to 24 h [Keren & Ohad, 1998]. Meanwhile, a ‘stay-green’ mutation, gtf, in Glycine max partially inhibits the breakdown during senescence of LHCII but not the degradation of the cytochrome b6f complex, PSI nor ATP synthase [Guiamét et al., 2002], implying that these proteins’ turnover is also by different pathways.

The proteases involved have not been well-defined as yet, and there are clearly numerous proteases available. Mistargeted oxygen-evolving complex 33-kDa protein can be degraded by an enzyme in the stroma whereas the Rieske iron–sulphur centre and other proteins have been shown to be controlled by thylakoid-localised proteases [Ostersetzer & Adam, 1997]. Despite the proposal for an FtsH degrading PSII in 1996 [Lindahl et al., 1996], various groups have suggested other candidates for the particular protease responsible for PSII turnover. Much of the work has been in vitro and the evidence reported has been contradictory, however, often focusing on the presence of D1 degradation fragments. Although a primary cleavage event is reported to occur between helices IV and V of D1 [De Las Rivas et al., 1993], resulting in a 23.5 KDa fragment (N-terminal) and a 16 KDa (C-terminal) breakdown product, in immunological analysis and pulse-chase experiments [Greenberg et al., 1987], additional degradation products of 14, 13 and 10 KDa have been reported [Salter et al., 1992]. The 22/23 and 10 KDa fragments have often been identified [see Kanervo et al., 1998]. As part of this work, however, Western blots (by S. Bailey) of D1 turnover in Col0 and Var2-2 did not reveal any sizeable partial breakdown fragments even in the Var2-2 strain. He & Vermaas (1998) also did not see partial D1 fragments when the precursor protein was degraded in experiments with a PSI/ChlL- strain. The number of specific proteolytic steps therefore remains subject to debate. It is known that the D1 repair process is temperature-dependent [Aro et al., 1990], however, and that it is mediated by a factor(s) sensitive to protease inhibitors [De Las Rivas et al., 1993; Kanervo et al., 1998]. Some work suggested that ATP was not necessary for an initial cleavage step, which would exclude AAA+ proteins including FtsH and Clp ATPases [for review, see Keren & Ohad, 1998], but others maintain that a first cleavage step is GTP-dependent and a second is ATP-dependent [Lindahl et al., 2000; Spetea et al., 1999]. A relatively well-accepted theory now is that FtsH recognises the new carboxyl terminus formed because of the initial GTP-dependent cleavage of the D1 protein into 23 and 10 kDa fragments, and the FtsH is thought to be necessary for extracting the damaged D1 as well as degrading it [Estelle, 2001; Haubühl et al. 2001, Lindahl et al., 2000]. Although the evidence from studies of
slr0228 and var2 confirm the role of FtsH in vivo, the lack of partial breakdown products does not help resolve this part of the debate.

Membrane-bound proteases have long been suggested to be involved, since experiments with serine proteases and sonication with high- and low-salt washes suggested a protease integral to the thylakoid membrane [Kanervo et al., 1998]. As discussed previously, FtsH proteases are unique in their membrane location. Salter et al. (1992) also suggested that the protease would be an integral part of PSII, and indeed D1 degradation has been reported in isolated PSII particles [for review, see Keren & Ohad, 1998]. Notably and convincingly, in Synechocystis, the slr0228 and slr1604 proteins are the only proteases so far found bound to PSII in preparations of the photosystem [Kashino et al., 2002]. This evidence contradicts the suggestions of Salter et al. (1992) that a serine protease may be responsible, with a binding site on the CP43 protein, and the (in-vitro) work of Haußühl et al. (2001), who proposed that in Arabidopsis the DegP2 serine protease carried out the primary cleavage step in D1 degradation. In addition, the closest Synechocystis homologue of DegP2 appears to be the HtrA protein, but Funk & Adamska (2002) did not find a convincing effect on D1 repair in an htrA− mutant of the cyanobacterium. Some evidence for a role of these proteases in stress or light-damage comes from work by Silva et al. (2002) where a triple Htr mutant of Synechocystis exposed to high-intensity light showed reduced oxygen evolution and reduced D1 removal in immunoblotting experiments.

Despite the debate about the proteases involved in the regulation of PSII, it is difficult to argue with the impaired recovery from photoinhibition seen in the slr0228− and Var2-2 FtsH mutants. Although the Synechocystis mutant has much-reduced PSI level, with concomitant small changes that affect photosynthetic function, the fact that a var2 mutant showed the same phenotype makes a case for FtsH proteins being involved in turnover of D1 in photoinhibited PSII. Likewise, despite difficulties in assays with slr0228/ChIL− and senescent Var2-2, both sets of experiments showed PSII was retained when the respective WT strains degraded their photosynthetic apparatus. Although the conclusive determination of site and mechanism of action of the slr0228 or Var2 proteins would be ideal, the isolation of slr0228 and slr1604 bound to eluted PSII complexes certainly suggests a direct interaction and therefore role in the maintenance of the reaction centre.

Other questions remain, however. During chlorosis in BG11−N, slr0228− failed to degrade its PBS for 2 days. Whether this is related to the lack of PSII turnover or a separate function in maintenance of the antenna as well as photosystem remains to be seen.
10.3.3 Photosystem I assembly

Initial work in motile slr0228" showed an effect of inactivating the FtsH on PSI [Mann et al., 2000]. This was supported by the failure here to make an additional mutation in slr0228 in Synechocystis suggesting a role beyond PSII. The suggestion that PSI photoinactivation was increased in slr0228" Synechocystis (S. Bailey, personal communication, 2002) was not substantiated, however, since oxygen uptake via the Mehler reaction and P700 activity in EPR were not greatly affected by >2h incubation in extremely high-intensity light.

Nutrient deficiency assays in slr0228" and senescence assays in Var2-2 allowed the regreening as well as chlorosis of the photosynthetic apparatus to be monitored. The preliminary work in Var2-2 showed a return to functional photosynthesis on the same timescale as Col0, but there was a marked effect upon PSI synthesis in slr0228". Nutrient deficiency assays using BG11-P and -N both showed delayed return of chlorophyll, phyocyanin, whole-chain electron transport as judged by oxygen evolution, and PSI. Linear regression analysis was used to analyse how consistent the slower regreening was, revealing that chlorophyll and PSI reaccumulated in WT at 1.4–1.9-fold the rate of slr0228". Although useful for analysis, however, the profile of all regreening studies was not linear but showed a lag phase of 24–76 h before the start of recovery in the mutant. This suggested a distinct failure in reassembly before a recovery, rather than a non-specific effect or slower growth. (Indeed, growth rates were the same in WT and slr0228" under all but the most severe conditions.) It would be useful to study in more detail when subunits and complexes returned to the thylakoid membranes and at smaller time-intervals, as carried out by Wu & Vermaas (1995) in their ChlL- regreening studies. The time-scale of recovery of pigments and photosystems here matched their studies, however, and supported the hypothesis that inactivated slr0228 prevented reassembly of PSI, resulting in the lower levels of PSI seen in slr0228" cultures.

Regreening of ChlL- and slr0228/ChlL- after LAHG also supported the nutrient deficiency assays. After chlorosis, slr0228/ChlL- oxygen uptake via the Mehler reaction decreased, only increasing and stabilising at 4 days after return to normal light-incubation conditions. Chlorophyll levels corresponded with this; after LAHG there were abnormally high numbers of chlorophyll/PSI, only reducing at 3 days as PSI was reaccumulated. The effect in the slr0228/ChlL- double mutant contrasted with the ChlL- strain, which maintained constant oxygen uptake (via the Mehler reaction) throughout LAHG and afterwards,
suggesting that PSI was not removed very quickly during dark-provoked chlorosis, despite the dramatic loss of chlorophyll.

In Var2-2, recovery from senescence did not seem to be affected in the same way, suggesting that the additional FtsH proteins present in the plant allow for greater specificity of function. It should be noted, however, that there was a delayed effect on PSI activity in both Var2-2 and slr0228/ChlL. Flash spectroscopy of samples after return to low light showed P700 activity decreased beyond detection in Var2-2, and a continued decrease in slr0228/ChlL, before recovery. Whether this reflects continued consequences of incorrectly regulated photosynthetic apparatus is not known and needs further study.

Although the only evidence for this here is a reduction in chlorophyll recovery after reversed senescence, the Var2 protein has been suggested to be necessary for chloroplast biogenesis [Chen et al., 2000]. How the plants with the severe var2-1 allele form normal chloroplasts in green sectors when this severe mutation results in no detectable mutant protein at all, and why this would happen in some areas of the leaf and not others, remains unexplained [Chen et al., 2000].

When grown under standard incubation conditions, the Var2-2 mutant, apparently equivalent to the slr0228 mutant in Synechocystis in terms of PSII, in fact has more PSI than WT, not less. A difference between Synechocystis and A. thaliana in assembly of PSI would not be surprising although the difference is the reverse of that normally seen, where the prokaryotes often seem to sustain photosystems even with deleterious mutations whereas eukaryotes are more severely affected. There are numerous examples: where the ycf4 mutant in Synechocystis has reduced PSI content, ycf4 C. reinhardtii mutants lose all functional PSI. This has also been seen for PSII: for example, psbK mutants in cyanobacteria have functional PSII whereas PSII is degraded in psbK C. reinhardtii. Anabaena and Synechocystis lacking PsaC accumulated functional PSI whereas the lack of PsaC in Chlamydomonas prevented accumulation of any PSI [Schelle et al., 2001; Takahashi, 1998; Wollman et al., 1999; Yu et al., 1995], but this is an interesting example in that the Synechocystis PSI was missing PsaD and PsaE as well as PsaC [Yu et al., 1995]. The level of PsaD (and PsaF) was also reduced in the absence of the slr0228-encoded protease [Mann et al., 2000]. Although the assembly of PSI was subject to a lag phase in slr0228, the identification of specific PSI subunits to find which, if any, were reduced still requires investigation. The assembly of trimers of PSI was examined, however, and in this the observation of reduced PsaD is again of interest because structural studies have shown direct or indirect contact between it
and PsaI and PsaL, all located in the region where cyanobacterial PSI trimerisation occurs [for review, see Scheller et al., 2001].

It was suggested that blue light be used to increase the level of PSI trimerisation in *Synechocystis* (P. Fromme, personal communication, 2001). 77K fluorescence emission spectra and sucrose density gradients in WT showed that the population of trimers did indeed increase. In contrast, slr0228⁻ *Synechocystis* was unable to respond to blue-light conditions. The population of trimers did not seem to increase in the mutant, an effect on PSII turnover again appeared to occur, and growth was severely impeded, suggesting multiple effects of slr0228 inactivation under these conditions.

Comparison of 77K fluorescence emission spectra from WT grown in moderate-intensity full-spectrum light and from after they were moved to low-intensity blue light suggested little change in the PSI:PSII ratio. Fractionation of thylakoid membranes on density gradients did show an increase in the population of trimers compared with monomers, however. The spectra from WT therefore suggest that the monomers initially present were either degraded to be replaced by trimers or converted to trimers, so that overall PSI levels relative to PSII do not change substantially. Since flash spectroscopy showed a drop in PSI/cell in WT, PSII content must decrease somewhat too (the level of PSII was too low for accurate measurement using flash spectroscopy), thus maintaining the fluorescence emission ratio. In line with this, WT chlorophyll and phycocyanin measured in absorbance spectra both decreased slightly before recovery. PSI/cell in WT also recovered after only a few days in blue light.

PSI content was also reduced in slr0228⁻ moved to low-intensity blue light but this may be a more deleterious effect in the mutant since PSI levels are already less than in WT. The recovery in PSI/cell was difficult to follow because of the need for large cultures to generate sufficient sampling volumes, but measurements corresponded with the occurrence of another lag phase in PSI recovery in blue light. The mutant was so severely affected that cell numbers never increased as they would in full-spectrum light. This was the most dramatic phenotype seen in the slr0228 mutant apart from the effect on PSII photoinhibition. In slr0228⁻, the chlorophyll content also dropped dramatically, with no recovery, whereas phycocyanin levels were more stable.

The greatly decreased PSI:PSII ratio seen in slr0228⁻ 77K fluorescence emission spectra results from a decrease in PSI as revealed by flash spectroscopy, but a failure to degrade
PSII could also contribute — if PSII in slr0228^- could not be reduced as it was inferred to in WT, an element of the defective PSII turnover would also increase the large peak of PSII fluorescence emission observed in 435 nm spectra. This spectrum is very like that seen in Chl^- cultures during chlorosis, where PSII degradation was severely impeded. The altered phycocyanin/PBS content visible in extracts from thylakoid membranes of the blue-light-grown slr0228^- mutant also suggested a difference in PBS composition, although this was not investigated further. Why trimers of PSI are needed in cyanobacteria is not known: the consequence of no trimers in PsaL^- _Synechocystis_ is not severe under normal growth conditions although growth of the mutant in blue light was reduced compared with WT (C. Aspinwall, personal communication, 2002), as with slr0228^- . Nevertheless, it is clear that some aspect of this blue-light growth coinciding with increased PSI trimerisation in WT greatly reduced the viability of slr0228^- . A role for the slr0228-encoded FtsH in trimerisation would explain the lack of an effect on PSI in Var2-2 _A. thaliana_, but more work is necessary to dissect the changes occurring in PSI and under blue light.

Further interesting implications of the work on PSI are the need for a process for active removal of PSI. In PSI photoinhibition, disassembly and resynthesis of entire PSI is thought necessary for repair [see Scheller et al., 2001], distinct from the subunit replacement that can occur for PSII. The 1–3 day period of recovery seen in WT is in line with PSI recovery from photoinhibition which is thought to take several days [Scheller et al., 2001].

### 10.4 Compound effects of a missing FtsH

The effect on the level of fatty-acid saturation and carotenoid composition in both the slr0228^- and Var2-2 FtsH mutants illustrates the difficulty of elucidating the precise roles of FtsH proteins. Although all phenotypic effects were reproducible and, in the case of PSII turnover and blue-light growth, very severe, the numerous other effects suggest many functions for the slr0228^- and _var2_ -encoded FtsHs and/or various side-effects. The many roles of the single FtsH protein in _E. coli_ [Figure 10.1] are also an indication that these proteases/chaperones can be regulatory factors in pathways as diverse as membrane synthesis, transcriptional regulation, stress response networks and membrane transport systems. The possibility of crosstalk between signal transduction networks in bacteria leading to complex regulatory networks has been proposed [for review, see Hellingwerf et al., 1998]. It must be considered that the complicated effects of mutation of slr0228, and indeed _var2_, although marked and reproducible, impact upon diverse regulatory networks in _Synechocystis_ or _A. thaliana_.

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The changes to fatty acids cannot be easily dismissed at a time when lipid bilayers have been accepted as being more than merely the inert solvent for more interesting membrane proteins. As well as the identification of lipid components of the photosystems, lipids have been suggested to aid in the folding of membrane proteins, perhaps acting as molecular chaperones for membrane proteins [Drew et al., 2003 and references therein]. Experiments using the DesA/D" mutant, however, show that the severe effects of slr0228" on PSII photoinhibition and blue-light growth are not due to increased fatty acid saturation. What does cause the altered saturation levels of fatty acids was not investigated: it may be an indirect effect of FtsH inactivation or the indication of a direct role of FtsH proteins in membrane synthesis. Since the amount of phospholipids appeared to be comparable in WT and mutant in Synechocystis and A. thaliana, it seems unlikely that either the slr0228- or var2-encoded FtsH proteins is the regulator of lipid biosynthesis exactly as in E. coli, where FtsH controls the branch point between lipopolysaccharide and phospholipid biosynthetic pathways [Ogura et al., 1999].
The many roles of FtsH in *E. coli* [Figure 10.1] suggests other avenues for investigation in clarifying the roles of the slr0228 and Var2 FtsH proteins. The *E. coli* FtsH may regulate the protein translocation ability of the bacterium, since overexpressed SecY is pulled from the membrane and degraded by the FtsH [Akiyama, 1998a,b; Drew *et al.*, 2003; Kihara *et al.*, 1999]. A role in translocation would not be surprising; the bacterial GroEL complex has also been shown to interact with the Sec pathway via translocase SecA, suggesting a role in post-translational membrane protein targeting [see Drew *et al.*, 2003] as well as its role in chaperoning protein reassembly [Iametti *et al.*, 2001; Kessel *et al.*, 1995].

The role of the *E. coli* FtsH in the degradation of mRNA should also be remembered when considering the function of similar proteins in plants and cyanobacteria. Mutation of the *E. coli* FtsH resulted in an increase in the decay rate of bulk mRNA [for review, see Schumann, 1999]. Meanwhile, other AAA proteins, such as the 26S proteasome subunit, Sugl, have DNA helicase activity. The ATPase of Sugl is also stimulated by some RNAs. Notably, the well-conserved residues of the SRH region of FtsH are in the same position in the structure as the region for DNA-binding on such helicases [Karata *et al.*, 1999].

The functions of other FtsH and AAA proteins do not contradict the importance of the slr0228- and nar2-encoded proteins in photosynthesis. Indeed, they add weight to the idea that FtsHs may be important regulators of thylakoid membrane composition. Proteases are well known members of eukaryotic regulatory networks, degrading cyclins and inhibitors of CDKs for cell-cycle control, via the ubiquitin pathway [for reviews of plant networks, see Hellman & Estelle, 2002; Vierstra, 2003]. There is also increasing evidence that proteins like FtsH have chaperone abilities in addition to their proteolytic activities. The complicated regulation of the formation of thylakoid membrane protein complexes requires co-ordinated expression and assembly but also degradation of unassembled or damaged components. The possibility that FtsH proteins are necessary for chaperoning synthesis and aiding removal of proteins might account for the numerous effects and severe phenotypes of mutants.

10.5 A model for action?

Although AAA ATPases act in diverse pathways, a common theme is the folding/unfolding and disassembly of protein complexes. Notable examples are the 26S proteasome, the metalloproteases including FtsH that unfold substrates in an ATP-dependent manner, or the AAA katanin which disrupts tubulin–tubulin interactions to degrade microtubules [see Karata *et al.*, 2001]. Members of the wider ‘AAA+’ family
include further ring-shaped complexes, such as the *E. coli* equivalent of the eukaryotic 26S proteasome, HslVU. Energy-dependent degradation always seems to be carried out by such multi-subunit complexes, which provide interesting models of action for the FtsH proteins studied here. As seen with the slr0228- and *nar2*-encoded FtsHs, these complexes are needed for normal growth and development, for housekeeping functions, and also for stress responses. The structures of AAA+ proteins are now well-understood and are so similar that it is possible to infer which regions and domains correspond in other members of the group [for review, see Ogura & Wilkinson, 2001] — crystal structures are available for the HslU proteasome equivalent from *E. coli* and *Haemophilus influenzae* [Sousa et al., 2000], RuvB and FtsH from *Thermus thermophilus* [Niwa et al., 2002], Hsp100/ClpB [Li & Sha et al., 2002] amongst others, as well as the *E. coli* FtsH itself [Krzywda et al., 2002a,b].

Eukaryotic cells target proteins for degradation by attaching ubiquitin, which results in their being digested in the 26S proteasome. This, like Clp proteases, contains proteolytic (20S) components and regulatory (19S) proteins that include ATPases. Whereas the ClpAP complex contains ClpP proteolytic subunits and ClpA ATP-hydrolysing chaperone subunits, the FtsH complex contains all active sites on single subunits. Increasing numbers of these ring-shaped complexes have had chaperone as well as proteolytic activities described. Such assemblies with dual chaperone and protease function may be capable of sensing subunit availability for protein complexes then regulating and catalysing either assembly or degradation as appropriate. Braun et al. (1999) showed *in vitro* that even the proteasome can refold denatured substrates in an ATP-dependent manner, and that this chaperone activity was not as strict in its requirement for ubiquitination as the proteolytic activity of the enzyme. The 19S regulatory particles, containing the AAA ATPases, appear to be the site of the chaperone activity [for review, see also Zwicki & Baumeister, 1999]. Translocation through the pore is thought to use energy from hydrolysis of ATP in both FtsH and ClpA and ClpX [see Karata et al., 2001], but short peptides may be translocated through the hexamer without nucleotide hydrolysis [see Karata et al., 2001].

Dougan et al. (2002) recently summarised the observations of various groups that the wide AAA+ family (including Clp/ClpP family as well as AAA proteins) of proteins are modulated in their activities by partner proteins. It is the adaptor proteins that regulate the range of substrate proteins and activity, e.g., for *E. coli* ClpXP to degrade σ5 starvation factor, the adaptor protein RssB is required for binding and degradation. RssB is part of a two-component response, requiring phosphorylation for activation. The domains bound by activator proteins appear to vary, from N-terminal domain to AAA module for example,
within the \textit{E. coli} AAA+ proteins. It is possible therefore, that the particular similarity seen in a subgroup of FtsH proteins only found in photosynthetic organisms is an indicator of a recognition domain for particular substrates or modulators. It is known that the activities of FtsH proteins can be similarly modulated by others. FtsH in \textit{E. coli} forms a complex with HflK and HflC (probably via their periplasmic domains) [Akiyama \textit{et al.}, 1998b], with which a further protein YccA (of unknown function) can transiently associate [Kihara \textit{et al.}, 1999]. HflK-C is thought to dissociate upon binding of an appropriate substrate, thus acting as a regulator of FtsH activity [for reviews, see Akiyama, 2002; Schumann, 1999]. These bacterial Hfl proteins are similar to the eukaryotic prohibitins [Bacher \textit{et al.}, 2002], which are integral mitochondrial membrane proteins that assemble with the yeast mitochondrial FtsHs [Steglich \textit{et al.}, 1999]. Likewise, in the presence of SpoVM in \textit{Bacillus subtilis}, FtsH does not degrade the sigma factor $\sigma^{22}$ [Schumann, 1999].

The presence of numerous FtsH proteins in photosynthetic organisms and the 'leaky' effect of \textit{var2} mutation producing heterogeneous areas of variegation also supports the hypothesis that the ring-shaped proteases can sometimes compensate for one another. This would explain some of the contradictory findings of studies of proteases in photosynthesis. Why does mutation of ClpP, the slr0228 FtsH and SppA each result in reduced degradation of the PBS? Why is there so much confusion over the degradation of the PSII D1 protein? There certainly appears to be overlap between the substrate specificity of Clp and FtsH proteases in \textit{E. coli}: derivatives of the bacteriophage \textit{$\lambda$} repressor protein CI with short nonpolar C-termini were degraded by purified \textit{E. coli} FtsH or by ClpAP or ClpXP [Hermann \textit{et al.}, 1998]. In fact, FtsH, Clp and Lon proteases co-operate in the pathway that commits the bacteriophage to lysis or lysogeny, degrading \textit{$\lambda$} regulator-proteins CII and CIII, O, and P proteins, respectively [Adam, 2000; Schumann, 1999; Shotland \textit{et al.}, 2000a]. FtsH is apparently the major protease responsible for degradation of the $\sigma^{22}$ factor, but nevertheless, Clp and Hsl proteases are able to degrade it [see Akiyama, 2002]. Likewise in the mitochondrion, Lon and FtsH proteases have both been found able to chaperone the same processes, namely assembly of cytochrome $c$ oxidase and the F$_{1}$F$_{0}$-ATPase. Interestingly, bacterial Lon co-operates with the DnaK chaperone whereas mitochondrial Lon co-operates with Hsp70 or a DnaJ homologue to degrade abnormal proteins [Adam & Clarke, 2002; Nakai \textit{et al.}, 1995; Rep \textit{et al.}, 1996]. The Deg serine proteases contain multiple PDZ domains, involved in protein–protein interactions [Krojer \textit{et al.}, 2002] but such interactions for the Clp or FtsH proteins are not known [Suzuki \textit{et al.}, 1997].
Therefore, whereas some proteins may be quite specifically degraded by one or another FtsH, some may be less affected by mutation of FtsH because of overlapping activity of proteases and chaperones. It is interesting to note that the chaperone GroEL, which is highly expressed in endosymbionts, has been proposed to buffer against the mutations that can accumulate from symbioses, which result from small population sizes, lack of recombination and transmission through population bottlenecks (e.g., maternal transmission) [Fares et al., 2002]. Perhaps this is part of the explanation for the vast number of proteases and chaperones present in chloroplasts as opposed to freeliving photosynthetic bacteria. Nevertheless, equivalent enzymes in Synechocystis and A. thaliana that probably arose by gene duplication now seem to have acquired distinct functions. It remains an intriguing possibility, and an obvious target for future research, that the increased number of FtsH proteins in cyanobacteria and Arabidopsis compared with E. coli allows the proteases to be positioned on either side of membranes to co-operate in degrading/unfolding/refolding protein complexes (as seen in mitochondria), or for subunit composition to vary according to activity.

Evidence from these studies suggests that FtsH proteins have numerous roles in the thylakoid membrane, and these probably include chaperone as well as protease functions. It would be interesting to discover more about the proteins that interact with the slr0228- and var2-encoded FtsHs, and indeed the other FtsHs of the thylakoid membranes. Double mutants of other ftsH genes in GT Synechocystis and A. thaliana might aid this. The subunit composition of FtsH hexamers as well as further substrates might be examined. The work of Ostergartner & Adam (1997) suggested a role in Rieske iron–sulphur centre degradation, for example. Potential adaptor proteins could also be investigated. To further elucidate the role of Var2 in senescence, a magnesium chelatase mutant of Arabidopsis is available (J. Brusslan, personal communication, 2002) which, crossed with Var2-2, would allow easier production of chlorosis in the plant. Finding FtsH mutants in Nicotiana might also allow improved senescence assays, since this plant appears more amenable to cycles of chlorosis and regreening. Probing the cellular location of the plant FtsH proteins (including FtsH1) has begun in other groups [Sakamoto et al., 2002] and this will aid the delineation of those with chloroplast functions. Cyanobacterial and plant FtsH proteins are implicated in stress responses, in addition to their precise photosynthetic functions, by the altered carotenoids seen here and in various microarrays: this might be examined relatively easily using Northern blots and further mutant studies. The evidence from this and concurrent work confirms that FtsH proteins are vital for maintenance of the photosynthetic apparatus, but these proteins present many fascinating avenues for more study.
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