The Maintenance and Expression of Foreign Genes in the Chloroplast of *Chlamydomonas*

A thesis submitted for the degree of
Doctor of Philosophy
by

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"When you have eliminated all which is impossible, then whatever remains, however improbable, must be the truth"

Sir Arthur Conan Doyle - The Adventures of Sherlock Holmes
Thesis Abstract

The chloroplast of the unicellular green alga Chlamydomonas reinhardtii is readily amenable to molecular genetic analysis. Using particle-gun-bombardment, exogenous DNA can be introduced into the chloroplast where it will recombine into the organellar genome. Coupled with the high levels of homologous recombination this allows precisely targeted insertion of introduced DNA anywhere into this 196 kbp genome. Several different chloroplast expression vectors have been constructed in order to introduce and ectopically express foreign genes within the Chlamydomonas chloroplast. One of these genes, aphA-6, a eubacterial aminoglycoside antibiotic resistance gene, confers resistance to kanamycin and amikacin in transformed cells and so can be used as a dominant selectable marker for chloroplast transformation. Experiments to demonstrate the utility of this new marker to chloroplast molecular genetics were also carried out.

The same expression vector was used in an attempt to express the protochlorophyllide oxidoreductase (POR) gene from Synechocystis sp. PCC 6803 in a chlorophyll-less Chlamydomonas double mutant (pc-ly-7) lacking active POR. In addition, the tufA gene encoding the protein elongation factor EF-Tu, from the plastid of the Apicomplexan Plasmodium falciparum was introduced into the chloroplast. This was carried out in order to attempt a functional replacement of the endogenous chloroplast EF-Tu. The results of the introduction and expression of these two foreign genes are discussed.

Finally, an experiment is described which attempted to isolate cells in which chloroplast DNA had transferred to the nuclear genome. It was hoped that this would serve as a model for the process occurring throughout chloroplast evolution by which the majority of plastid genes have become nuclear encoded. To achieve this the 'ble' selectable marker for nuclear transformation in Chlamydomonas, conferring zeomycin resistance, was introduced into the chloroplast genome where it was not functional. Cells in which this marker had moved to the nuclear genome were then selected by their ability to survive on zeomycin-containing medium. The results of this screen are discussed.
To Harriet, who will appreciate the end far more than the beginning, with love.
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For donating antibodies and plasmids I’d like to thank W. Trevor Griffiths for the *Synechocystis por* gene and antibodies, R. J. M. (Iain) Wilson for the *Plasmodium tufA* gene and antibodies (and also for his support), and Patrice Courvalin for providing the *aphA-6* gene.

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<td>μE</td>
<td>microEinstein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>bAA</td>
<td>psbA aphA-6 transformants</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs (kbp: kilobase pairs)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumen</td>
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<tr>
<td>Chlide</td>
<td>chlorophyllide</td>
</tr>
<tr>
<td>cpDNA</td>
<td>chloroplast DNA</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons (kDa: KiloDaltons)</td>
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<tr>
<td>DAPI</td>
<td>4‘,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dATP</td>
<td>2’ deoxyadenosine 5’-triphosphate</td>
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<tr>
<td>DCMU</td>
<td>3-(3,4,-Dichlorophenyl)-1,1-diethylurea</td>
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<tr>
<td>dCTP</td>
<td>2’ deoxycytidine 5’-triphosphate</td>
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<tr>
<td>ddATP</td>
<td>2’,3’ dideoxyadenosine 5’-triphosphate</td>
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<tr>
<td>ddCTP</td>
<td>2’3’ dideoxyctytidine 5’-triphosphate</td>
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<td>ddGTP</td>
<td>2’3’ dideoxyguanosine 5’-triphosphate</td>
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<tr>
<td>ddTTP</td>
<td>2’3’ dideoxythymidine 5’-triphosphate</td>
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<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>dGTP</td>
<td>2’ deoxyguanosine 5’-triphosphate</td>
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<tr>
<td>dH₂O</td>
<td>distilled water</td>
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<td>DNA</td>
<td>deoxyribonucleic acid (cDNA: copy DNA; rDNA: ribosomal DNA)</td>
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<td>dNTP</td>
<td>2’ deoxynucleoside 5’-triphosphate</td>
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<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
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<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
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<tr>
<td>dTTP</td>
<td>2’ deoxythymidine 5’-triphosphate</td>
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<tr>
<td>EDTA.Na₂</td>
<td>ethylenediamine tetraacetic acid. disodium salt</td>
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<td>EPR</td>
<td>electron paramagnetic resonance</td>
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<td>EtBr</td>
<td>ethidium bromide</td>
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<td>GM</td>
<td>genetically modified</td>
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<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
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<td>IPTG</td>
<td>isopropylthio-β-galactosidase</td>
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<tr>
<td>IR</td>
<td>inverted repeat</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani medium</td>
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<tr>
<td>MBN</td>
<td>mung bean nuclease</td>
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MOPS 3-[N-Morpholino] propane-sulfonic acid
mt mating-type
NADP nicotinamide adenine dinucleotide phosphate
NADPH reduced nicotinamide adenine dinucleotide phosphate
ORF open reading frame
P680 primary electron donor in PSII
P700 primary electron donor in PSI
PAGE polyacrylamide gel electrophoresis
Pchlide protochlorophyllide
PCR polymerase chain reaction
PEG polyethylene glycol
PSI photosystem one
psi pounds per square inch
PSII photosystem two
RNA ribonucleic acid (tRNA: transfer RNA; rRNA: ribosomal RNA)
rpm revolutions per minute
RuBisCo ribulose bisphosphate carboxylase oxygenase
SD Shine-Dalgarno
SDS sodium dodecyl sulphate
SSC saline sodium citrate
ssDNA single stranded DNA
TAP tris-acetate phosphate
TBE tris borate EDTA
TBS tris buffered saline
TE tris-EDTA
TEMED tetramethylethylenediamine
TMV tobacco mosaic virus
Tris tris(hydroxymethyl)aminoethane
U unit
UTR untranscribed region
UV ultraviolet
v/v volume for volume
w/v weight for volume
WT wild-type
X-gal 5-bromo-4-chloro-3-indolyl-β-D-galactosidase
ycf hypothetical chloroplast open reading frame
Y_D a tyrosine electron donor in PSII
Chapter 1

Introduction
CHAPTER 1 INTRODUCTION

1.1 The chloroplast and its genome

In this first section of the introduction an overview will be given of chloroplasts as organelles and their importance within the host organism. In order to give an idea of the various functions of the chloroplast, its gene content and expression will be described. Finally, the evidence supporting the hypothesis that the chloroplast is of endosymbiotic origin will be summarised.

1.1.1 Structure and function of the chloroplast

Chloroplasts are cell organelles, found within plants and eukaryotic algae, which contain the complete set of components necessary for photosynthetic function. The number of chloroplasts per cell varies greatly, from a single large (5-10 μm diameter) chloroplast in *Chlamydomonas* sp. to around 200 small (1-3 μm diameter) chloroplasts in plant leaf mesophyll cells. Although the primary function of the chloroplast is as a photosynthetic carbon fixation factory it is also the site of amino acid, nucleotide, starch, fatty acid, pigment and lipid biosynthesis. The chloroplast is bounded by between two and four membranes, depending on the species of the host. For example, chlorophyte algae and land plant chloroplasts are bounded by two membranes whereas euglenophytes have three and other algae such as the cryptophytes have four (reviewed in Gray, 1992). The chloroplast also contains another, internal membrane known as the thylakoid membrane. The thylakoid membrane is formed as highly folded stacks known as grana which contain all the energy generating systems of the chloroplast. The chemiosmotic mechanisms of the photosynthetic apparatus are unique to chloroplasts and photosynthetic bacteria. This makes them the sole providers of the organic material which fosters life on earth.

Chloroplasts are just one of a supergroup of organelles known as plastids; proplastids being the developmental precursors to chloroplasts. In the dark, leaf plastids can differentiate to form etioplasts, which are deficient in thylakoid membranes but contain arrays of prolamellar bodies arranged in quasicrystalline lattices. Upon transfer to light, there is a large increase in the complement of thylakoid membranes and photosynthetic proteins as the etioplasts mature to become fully developed chloroplasts (Fig. 1.1). As well as chloroplasts, plastids can develop to become a variety of other organelles. These include starch-rich amyloplasts found in roots, chromoplasts whose carotenoids are
responsible for the colouration of many flowers and fruits, and oil-containing elaioplasts.

The chloroplast envelope has been discovered to have three main functions (reviewed by Joyard et al., 1991). Firstly, the inner envelope is responsible for metabolite transport between the cytoplasm and the chloroplast stroma. The second, highly complex function of the envelope is to transport into the chloroplast the large numbers of plastid-localised proteins that are encoded by the nuclear genome (reviewed by Soll and Alefsen, 1993; Robinson and Klosen, 1994; Haucke and Gottfried, 1997). Thirdly, the chloroplast envelope is the site of biosynthesis of several vital components of the plastid. For example, glycerolipids such as phosphatidylglycerol and monogalactosyldiacylglycerol (which accounts for over half the glycerolipid content of thylakoids and the inner envelope) synthesis occurs via enzymes that are associated with the chloroplast envelope. Moreover it appears that plastids are the major site of fatty acid synthesis within the cell.

In combination, the stromal and thylakoid localised components of the photosynthetic machinery use light to convert carbon dioxide and water to carbohydrates and oxygen (Hall and Rao, 1994). The thylakoid membrane, consisting of flattened vesicles stacked into regular aggregates called grana, is the site of photosynthetic electron transport (the 'Z-scheme') and photophosphorylation (Fig. 1.2). These reactions are carried out by four major multisubunit protein complexes termed photosystem I (PSI), photosystem II (PSII), the cytochrome b₆/f complex, and the ATP synthase complex (Nugent, 1996). PSII, the cytochrome b₆/f complex and PSI are the three complexes that couple photochemical excitation of electrons to electron transport from H₂O to NADP⁺. PSII uses photochemical energy to produce plastoquinol and O₂ from plastoquinone and H₂O. PSI mediates the light-driven production of oxidised plastocyanin and NADPH, from reduced plastocyanin and NADP⁺. The cytochrome b₆/f complex couples the reduction of plastocyanin by plastoquinol to H⁺ transport. A key component of the thylakoid membrane and photosystems I and II is the pigment chlorophyll. Chlorophyll, which is synthesised in the chloroplast (Fig. 4.1), is highly abundant in the thylakoid making it is the most abundant pigment on earth. The chloroplast stroma contains the soluble enzymes involved in the Calvin-Benson cycle of C₃ photosynthesis. The principal component of the stroma and the enzyme responsible for carbon dioxide fixation is ribulose bisphosphate carboxylase oxygenase (RuBisCO). In many photosynthetic organisms RuBisCO has a molecular weight of ca. 560 kDa and is composed of eight large subunits (ca. 52 kDa, invariably chloroplast encoded) and eight small subunits (ca. 14 kDa, usually nuclear encoded).
Morphologically, chloroplasts appear to divide by binary fission (Pyke, 1997). Using electron microscopy an electron dense isthmus between dividing chloroplasts, termed the 'plastid-dividing (PD) ring', has been identified (Kuriowa et al., 1981). Until recently molecular data concerning the nature of the the PD ring was lacking. However, the recent discovery, and downregulation using antisense expression, of homologs to the bacterial $ftsZ$ gene in *Arabidopsis* has provided insight into this problem (Osteryoung and Vierling, 1995; Osteryoung et al., 1998). In this plant FtsZ proteins are targeted both to the plastid and the cytosolic compartments (Osteryoung et al., 1998). This suggests that the PD ring is composed of elements on the interior and exterior of the plastid.

![Figure 1.1 Developmental pathways of chloroplasts in higher plants (Gillham, 1994). Proplastid, etioplast and chloroplast stages are described in the text. Proplastids or etioplasts exposed to short pulses of light (2 minutes) separated by long periods (98 minutes) form protoplasts that contain few thylakoids, no chlorophyll $b$, and no grana. Once exposed to continuous light protoplasts rapidly mature into chloroplasts.](image)
Figure 1.2 Detailed model of the Z-scheme including structural information on the organisation of the protein complexes involved in the electron and proton transport within the thylakoid membrane of green plants (F. Morais, Ph.D thesis).

**Abbreviations:**

**PSII:** Capital letters indicate the products of psb (PSII) genes; P680 = primary donor; Q_A, Q_B = secondary plastoquinone acceptors; PQ = plastoquinone

**Cyt b\_f:** FeS = Reiske centre; \( h_p \) = high potential; \( l_p \) = low potential; Q = quinone molecule.

**PSI:** Capital letters indicate the products of psa (PSI) genes; P700 = primary donor; PC = plastocyanin; \( A_0 \) (Chl), \( A_1 \) (phyloquinone) = primary and intermediate electron acceptors of PSI; \( F_A, F_B \) and \( F_X \) = bound FeS centres; Fd = ferredoxin.
1.1.2 Chloroplast genome structure

Chloroplasts, together with mitochondria, are distinct from all other intracellular organelles in that they contain their own genetic apparatus. It is generally accepted that the reason for this anomaly is that these organelles have a distinct evolutionary history. Both chloroplasts and mitochondria are believed to be the result of an endosymbiotic event between a progenitor eukaryotic cell and a bacterium. This hypothesis and evidence supporting it will be reviewed in section 1.1.4. In this century, the genetic systems of both these organelles have been shown to be inherited in a non-Mendelian fashion. However, it was as a result of a chloroplast associated trait that, in 1909, Baur and Correns separately published the first reports of non-Mendelian inheritance based on studies of variegation in higher plants (Baur, 1909; Correns, 1909; for discussion see Gillham, 1994; Birky Jr, 1995). Landmark studies by Sager demonstrating extranuclear inheritance in *Chlamydomonas* will be discussed in section 1.2.2.

A chloroplast DNA species was initially demonstrated by CsCl gradient centrifugation in the early 1960's (Chun *et al.*, 1963; Sager and Ishada, 1963). The first visualisation of circular DNA molecules from isolated chloroplasts was achieved in 1971 using chloroplasts from the green alga *Euglena gracilis* (Manning *et al.*, 1971). As well as its circular conformation, studies such as this demonstrated the polyploid nature of the chloroplast genome. The question which then arose was were these multiple copies identical? Restriction map evidence from several algal and higher plant species (for example Bedbrook and Bogorad, 1976) confirmed that the bulk of circular chloroplast DNA molecules are identical in sequence. Obviously, the next goal in understanding the nature of chloroplast DNA was to sequence a complete genome. This milestone was first achieved in 1986 with the complete sequences of the chloroplast genomes from tobacco (Shinozaki *et al.*, 1986) and the liverwort *Marchantia polymorpha* (Ohyama *et al.*, 1986). Table 1.1 lists the fully sequenced plastid genomes to date.

The most notable result of these sequencing projects overall is the plasticity of the plastid genome. Sequencing of chloroplast DNA from representative organisms has produced an invaluable guide to the evolution of algae and plants. For example, the alga *Cyanophora paradoxa* is often described as a "living fossil" because it contains cyanobacterium-type plastids termed cyanelles. The cyanelle genome of *C. paradoxa* mirrors this morphological observation in that it contains several cyanobacterial features, including a much more complete set of genes than in higher plants (30% more...
<table>
<thead>
<tr>
<th>Organism</th>
<th>Genome Size (bp)</th>
<th>Number of protein coding genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nicotiniana tabacum</em> (Tobacco)</td>
<td>155,844</td>
<td>76</td>
<td>Shinozaki <em>et al.</em>, 1986</td>
</tr>
<tr>
<td><em>Marchantia polymorpha</em> (Liverwort)</td>
<td>121,024</td>
<td>84</td>
<td>Ohyama <em>et al.</em>, 1986</td>
</tr>
<tr>
<td><em>Oryza sativa</em> (Rice)</td>
<td>134,525</td>
<td>76</td>
<td>Hiratsuka <em>et al.</em>, 1989</td>
</tr>
<tr>
<td><em>Epifagus virginiana</em> (nonphotosynthetic parasitic flowering plant)</td>
<td>70,028</td>
<td>21</td>
<td>Wolfe <em>et al.</em>, 1992a</td>
</tr>
<tr>
<td><em>Euglena gracilis</em> (green alga)</td>
<td>143,170</td>
<td>58</td>
<td>Hallick <em>et al.</em>, 1993</td>
</tr>
<tr>
<td><em>Pinus thunbergii</em> (Black pine)</td>
<td>119,707</td>
<td>69</td>
<td>Wakasugi <em>et al.</em>, 1994</td>
</tr>
<tr>
<td><em>Zea mays</em> (Maize)</td>
<td>140,387</td>
<td>76</td>
<td>Maier <em>et al.</em>, 1995</td>
</tr>
<tr>
<td><em>Cyanophora paradoxa</em> (non-green alga)</td>
<td>135,599</td>
<td>136</td>
<td>Stirewalt <em>et al.</em>, 1995</td>
</tr>
<tr>
<td><em>Porphyra purpurea</em> (red alga)</td>
<td>191,028</td>
<td>200</td>
<td>Reith and Mullholland, 1995</td>
</tr>
<tr>
<td><em>Odontella sinensis</em> (diatom/brown alga)</td>
<td>119,704</td>
<td>124</td>
<td>Kowallik <em>et al.</em>, 1995</td>
</tr>
<tr>
<td><em>Plasmodium falciparum</em> (Malarial parasite)</td>
<td>34,682</td>
<td>23</td>
<td>Wilson <em>et al.</em>, 1996</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em> (green alga)</td>
<td>150,613</td>
<td>78</td>
<td>Wakasugi <em>et al.</em>, 1997</td>
</tr>
<tr>
<td><em>Guillardia theta</em> (Cryptophyte alga)</td>
<td>124,524</td>
<td>141</td>
<td>Douglas and Penny, 1999</td>
</tr>
</tbody>
</table>

Table 1.1 Fully sequenced plastid genomes. Adapted from (Martin and Herrmann, 1998).
genes than tobacco); seven genes encoding phycobiliproteins; one gene with homology to \textit{ftsW}, possibly involved in cyanelle division or cell wall biosynthesis; genes coding for both the large and small subunits of RuBisCo (Stirewalt \textit{et al.}, 1995). Characterisation of genes unique to this unusual alga may help elucidate details of the endosymbiotic event that produced the progenate plastid.

Of the two other nongreen algae whose chloroplast genomes are fully sequenced the red alga, \textit{Porphyra purpurea}, contains the largest chloroplast genome sequenced to date (191,028 bp). Its genome codes for 251 genes and ORFs, approximately double the number typically found in higher plants (Reith and Mullholland, 1995). The additional genes mainly encode biosynthetic functions such as synthesis of amino acids, fatty acids, pigments and thiamine. These genes have presumably been lost, replaced, or transferred to the nucleus in other species (section 1.1.4). Neither in \textit{Porphyra purpurea}, nor the diatom \textit{Odontella sinensis}, have any introns or RNA editing been detected in chloroplast genes (Kowallik \textit{et al.}, 1995; Reith and Mullholland, 1995). In contrast 38\% of the chloroplast genome of the green alga \textit{Euglena gracilis} is represented by introns, including unusual introns known as "twintrons", which are introns within introns (Hallick \textit{et al.}, 1993). It has been suggested that the introns in \textit{Euglena} are the descendants of mobile genetic elements that have invaded the chloroplast genome (Hallick \textit{et al.}, 1993). This hypothesis is supported by the fact that these introns are found in unique locations not found in other chloroplast DNAs, in intercistronic spacers and within other introns. An alternative hypothesis for the origin of introns is that they have facilitated the assembly of new genes from the functional domains of more ancient genes and so become stuck at their intragenic location (Darnell and Doolittle, 1986; Gilbert \textit{et al.}, 1986; Dorit \textit{et al.}, 1990). This is often referred to as exon shuffling. It can be postulated that the former, invasion hypothesis, rather than exon shuffling is more correct for intronless chloroplast genomes such as \textit{Porphyra purpurea} and \textit{Odontella sinensis} whose ancestor(s) might not have been exposed to the invading element.

The other green alga for which a complete chloroplast sequence is available is \textit{Chlorella vulgaris} (Wakasugi \textit{et al.}, 1997). Although this species lacks \textit{ndh} genes, it contains several \textit{ycf} genes which occur in higher plants but not in non-green algae, suggesting \textit{Chlorella} is related more closely to higher plants than its non-green counterparts. Interestingly, the \textit{Chlorella} chloroplast genome contains two genes related to the \textit{E. coli} genes \textit{minD} and \textit{minE}. In \textit{E. coli} expression of the \textit{minC/D/E} locus is required for the placement of a division septum at the midpoint of the rod shaped cell. Overexpression of \textit{Chlorella minD} in \textit{E. coli} causes aberrant cell division (Wakasugi \textit{et al.}, 1997), therefore analysis of \textit{minD} and \textit{minE} in \textit{Chlorella} may further understanding of the mechanism of chloroplast division at cytokinesis.
Unlike mitochondria, it appears that, with the possible exception of *Euglena* (Hallick *et al.*, 1993), persistence of the chloroplast genome is essential to the maintenance of the organelle. Plants treated with inhibitors of chloroplast gene expression, while producing nonphotosynthetic bleached tissue, maintain DNA containing etioplasts. While mutagenised copies of essential chloroplast genes in *Chlamydomonas* remain in a heteroplasmic state and are lost as soon as selective pressure is removed (e.g. Boudreau *et al.*, 1997a). Therefore, the plastid genome must contain some essential function which has not yet be discovered. *Epifagus virginiana*, commonly known as beechdrops, is a flowering plant that is parasitic on the roots of beech trees and is completely nonphotosynthetic. *Epifagus* may help illuminate the answer to this problem as its highly reduced plastid genome is functional and contains only 42 genes, at least 38 of which code for components of the gene expression apparatus of the plastid (Wolfe *et al.*, 1992a). The *E. virginiana* plastid genome does not contain any genes coding for components of photosynthetic or chlororespiratory systems. The reason for the persistence of the *Epifagus* plastid genome must be the expression of at least one of the four genes not involved in maintenance and expression of the genome. Only two of the four ORFs have a clear homology to any known genes. The first is *accD* encoding the plastid homolog of the β subunit of the carboxyltransferase component of *E. coli* acetyl-CoA carboxylase, which catalyses the first committed step in fatty acid synthesis. The second is *clpP*, encoding the plastid homologue of the proteolytic subunit of the ATP-dependant Clp protease of *E. coli* and may function in processing, turnover or even import of plastid proteins. Either of these genes may be the focus of selective pressure that maintains the whole genome (Wolfe *et al.*, 1992a).

Another phylum containing a highly reduced plastid genome is the *Apicomplexa*. The discovery that the malarial parasite *Plasmodium falciparum* and other members of the *Apicomplexa* contained a second species of cytoplasmic DNA was unexpected (Palmer, 1992; McFadden and Waller, 1997). Although the lineage is thought to have shared a common ancestor with the dinoflagellates (Palmer, 1992), it was only upon sequencing of the *P. falciparum* 35 kb DNA that its plastidic origin was confirmed (Wilson *et al.*, 1996; Kohler *et al.*, 1997b). The *P. falciparum* plastid genome is similarly reduced in size but bears little relation to its *Epifagus* counterpart apart from the saturation of genes involved in gene expression. Apart from these genes, also present is a degenerate *clpC* gene and up to eight ORFs. Several of these ORFs are small and may not be protein coding, whereas ORF 470 is conserved among red algae and the diatom *Odontella sinensis*, although its function is unknown (Williamson *et al.*, 1994). As with
Epifagus, the reason for the maintenance of the genome is unknown and the answer presumably lies in the identity of its ORFs.

The chloroplast genome of higher plants appears to be less divergent than that of the algae. Most have an inverted repeat segregating a large single copy (LSC) and a small single copy (SSC) region, although exceptions include pea, broad bean and alfalfa (Sugiura, 1992). The \textit{ndh} genes, which have been shown to express components of the chlororespiratory chain (Burrows \textit{et al.}, 1998; Kofer \textit{et al.}, 1998b), are common to higher plant chloroplast genomes (apart from \textit{Pinus thunbergii}, Wakasugi \textit{et al.}, 1994) but absent among other plastid containing species. This would seem counter to conventional thinking that the algae are "less evolved" than flowering plants. Assuming that \textit{ndh} genes were present in a common ancestor and have not been acquired during the evolution of higher plants, then they must either have been lost or transferred to the nucleus in lower species (Martin \textit{et al.}, 1998). Complete loss of the chlororespiratory complex would seem strange considering it has been retained in so many species and is presumably advantageous to the cell (section 1.2.2.2). Screening for the presence of \textit{ndh} genes in the nuclear genome of algae or \textit{Pinus thunbergii} may solve this enigma.

1.1.3 Chloroplast gene content and expression

Chloroplast genomes range in size from 120-200 kbp and code for between 120-240 genes. Until recently it was possible to divide the genes contained on chloroplast genomes into two classes: genetic system genes and photosynthetic genes. The sequencing of a greater number of chloroplast, and especially algal chloroplast genomes, has identified novel ORFs with homology to genes with biosynthetic and cell division functions. As yet, most of these ORFs have only been characterised by sequence comparison and so ideas of their true function are speculative. Therefore, instead of discussing these novel genes individually I will include them in a third section entitled, ORFs of putative or unknown function.

1.1.3.1 Genetic system genes

In all chloroplast genomes studied many components of the gene expression machinery have been discovered. Does this observation mean that the chloroplast is a self sufficient organelle, able to express genes on its own without importing any components from the cytosol? One of the aims of analysing genetic system genes have
been to answer this question. All 61 possible codons are used in chloroplast genes and the number of tRNA genes ranges from 27 in *Euglena gracilis* (Hallick *et al*., 1993) to 36 in *Cyanophora paradoxa* (Stirewalt *et al*., 1995). Theoretically, the minimum number of tRNA species needed for translation of all 61 codons (64 minus 3 stop codons) is 32, if normal wobble base-pairing occurs in codon-anticodon recognition (Alberts *et al*., 1989). It is possible that chloroplasts with less than this number use expanded codon-anticodon pairing (Sugiura, 1992; Hallick *et al*., 1993) or that they import cytosolic tRNAs (Morden *et al*., 1991; Wolfe *et al*., 1992c). tRNA import must occur in the nonphotosynthetic plastid of *Epifagus virginiana* as its genome contains only 17 tRNA species (Wolfe *et al*., 1992a).

The complete set of rRNAs necessary to compose the 70S ribosome are usually encoded by the chloroplast genome. In general, these are arranged in an operon in the same order as that found in *E. coli* (16S-23S-5S) (Sugiura, 1992). In contrast to rRNAs, only one third of the sixty ribosomal proteins are encoded by the chloroplast genome (Tanaka *et al*., 1986). The ten ribosomal proteins of the *rpl23* operon are clustered in the same order to homologous genes in the *E. coli* S10, *spc* and α operons, even though several of the chloroplast genes contain introns (Tanaka *et al*., 1986). This suggests that genes for ribosomal proteins in chloroplasts and *E. coli* evolved from a common ancestral set (Sugiura, 1992).

Several translational factors are also expressed by the chloroplast genome. *tufA*, coding for Elongation Factor-Tu, is found in the chloroplast genome of algae but not in the chloroplast DNA of any higher plant sequenced to date (Baldauf and Palmer, 1990b). A *tufA* chloroplast homologue has been found in the nuclear genome of tobacco, and this is presented as evidence for its evolutionary transfer to the nucleus (Baldauf *et al*., 1990a; Baldauf and Palmer, 1990b). Two other translational factors present in the chloroplast of several species are *infA*, encoding Initiation Factor-1, and *clpP*, encoding the proteolytic subunit of an ATP-dependant protease responsible for degrading incomplete polypeptides and unassembled proteins.

The final group of genetic system genes coded for by the chloroplast genome are the RNA polymerase genes. The chloroplast genome encodes an *E. coli*-type RNA polymerase with genes coding for α (*rpoA*), β (*rpoB*), β′ (*rpoCl*) and β″ (*rpoC2*) polypeptides. As far back as 1970 it was suggested that in maize two different types of RNA polymerases exist (Bogorad and Woodcock, 1970). More recently, work using barley mutants with plastids lacking ribosomes suggested the presence of a functioning non-chloroplast-encoded RNA polymerase (Hess *et al*., 1993). Subsequently, evidence
produced using transgenic tobacco plants containing an insertionally inactivated \textit{rpoB} gene has definitively proven the existence of a second nuclear encoded plastid transcription system (Allison \textit{et al.}, 1996). Interestingly, this nuclear encoded transcription system preferentially transcribes genetic system, rather than photosynthetic genes (Hajdukiewicz \textit{et al.}, 1997). A similar experiment which attempted to delete a chloroplast RNA polymerase gene in \textit{Chlamydomonas} gave a contrasting result: the disrupted gene could not be completely deleted in all 80 copies of the chloroplast genome (Fischer \textit{et al.}, 1996). This result is discussed further in chapter 6.

In answer to the initial question it must be concluded that the genes encoded in the chloroplast are not sufficient to produce a complete genetic system apparatus. As well as factors discussed above such as RNA polymerases and ribosomal proteins, other genes for essential components of the genetic apparatus are missing from the chloroplast genome. These include initiation factors, tRNA synthetases, ribonucleases and DNA polymerases. Examples of several of these classes of genes have been found in the chloroplast genome of the red alga \textit{Porphyra purpurea} (Reith and Mullholland, 1995). This suggests that these absentees have transferred to the nucleus, or been replaced by nuclear gene products in the common ancestor of most plastids.

\textbf{1.1.3.2 Photosynthetic genes}

Photosynthetic gene products are the most intensely studied of all plastid proteins. Many of the proteins composing the photosynthetic apparatus were isolated and identified long before their genes were cloned and sequenced.

By far the most abundant stromal protein in chloroplasts (constituting approximately half of leaf proteins) is RuBisCo, making it probably the most abundant protein on earth. Due to its function as the key enzyme of carbon fixation much research has been aimed at elucidating the structure and function of this molecule. In chloroplasts, RuBisCo is composed of eight identical chloroplast encoded large subunits (50-55 kDa) and eight identical nuclear encoded small subunits (12-15 kDa) (Hall and Rao, 1994). Exceptionally, \textit{rbcS}, encoding the small subunit of RuBisCo, has been found in the chloroplast genome of brown and red algae (e.g. Stirewalt \textit{et al.}, 1995). In these cases it is located downstream, and forms an operon with \textit{rbcL}, as occurs in Cyanobacteria (Kaneko \textit{et al.}, 1996).
The so called "light reactions" of photosynthesis all occur within four complexes located in the thylakoid membrane within the chloroplast. The four complexes are photosystems I and II (PSI, PSII), the cytochrome $b/f$ complex and ATP synthase. At least half of the genes coding for components of these complexes in extant eukaryotes are nuclear. It appears though that the core proteins in each of these complexes remain chloroplast encoded. Hypothetical reasons for this biased gene transfer will be discussed in section 1.1.4.

Finally, the somewhat enigmatic $ndh$ genes mentioned in section 1.1.2 could almost form their own group of chloroplast genes. The predicted amino acid sequence of the $ndh$ genes resembles that of the components of the respiratory chain NADH dehydrogenase from mitochondria (Ohyama et al., 1986; Shinozaki et al., 1986; Hiratsuka et al., 1989). In tobacco $ndhA-F$ have been shown to be actively expressed, and two of these genes ($ndhA$ and $ndhB$) contain introns (Matsubayashi et al., 1987). Therefore, it is believed that $ndh$ genes express the components of a chlororespiratory pathway, as demonstrated biochemically in Chlamydomonas (Bennoun, 1982). This hypothesis has recently been proven correct using reverse genetics techniques in tobacco. Two separate groups used insertional mutagenesis to knock-out $ndh$ genes in the chloroplast via biolistic transformation (Burrows et al., 1998; Kofer et al., 1998b). Both groups concluded that the plastid $ndh$ gene products have a respiratory function and act to reduce the plastoquinone pool using stromal reductant. Whether these genes have been transferred to the nucleus in algae such as Chlamydomonas, which lack $ndh$ genes in the chloroplast, remains to be seen.

1.1.3.3 ORFs of putative or unknown function

All plastid genomes contain ORFs whose predicted amino acid sequence does not match that of any previously identified chloroplast proteins. Some of these ORFs can be assigned a putative function based on homology with genes of known function from bacterial, nuclear, or mitochondrial genomes. Others only have homology with unidentified reading frames in the plastid genomes of other organisms, and these are known as $ycf$ genes (hypothetical chloroplast reading frames). In higher plants, the fact that many $ycf$ genes are conserved may evidence their essential function (Maier et al., 1995). Although several of the $ycf$ genes probably code for unidentified components of either the genetic or photosynthetic apparatus, others almost certainly have different functions. Epifagus virginiana is a non-green dicotyledonous flowering parasitic plant that is completely non-photosynthetic and lacks any photosynthetic or chlororespiratory
genes in its plastid genome (dePamphilis and Palmer, 1990; Wolfe et al., 1992a). As discussed in section 1.1.2, only four of its plastid genes are not involved in gene expression (Wolfe et al., 1992a; Wolfe et al., 1992b), and so at least one of these must be the reason for the maintenance of gene expression apparatus in the plastid. All four of these ORFs are conserved in several higher plant plastid genomes (Maier et al., 1995) suggesting that one or all may have an unknown non-bioenergetic function in plastids (Wolfe et al., 1992a).

The chloroplast genome of several algal species contain ORFs which are indicative of their bacterial origin. As discussed in section 1.1.2, the chloroplast genome of the green alga *Chlorella vulgaris* contains two genes related to the *E. coli* genes minD and minE involved in placement of the division septum (Wakasugi et al., 1997). As well as several *ycf* genes the *C. vulgaris* chloroplast genome contains ORF1720, which has partial homology with the *ftsH* product from *E. coli* and the *ycf2* product from higher plants (Wakasugi et al., 1997). *E. coli* *fts* mutants (filamentation temperature sensitive) fail to septate when grown at high temperatures (de Boer et al., 1990) implying that chloroplast division may occur by a similar mechanism to cell division in bacteria (section 1.1.1). Similarly, the chloroplast genome of the nongreen alga *Cyanophora paradoxa* contains a homologue of *ftsW* (Stirewalt et al., 1995).

The technique of choice for studying the function of unknown genes is to produce "knock-out" mutants using reverse genetics. The easiest plastid containing organism in which to carry out such studies is *Chlamydomonas reinhardtii* (section 1.2.1). Analysis of ORFs specific to this alga is discussed in section 1.2.3.

In conclusion, a proportion of chloroplast ORFs are undoubtedly previously undetected components of the photosynthetic or gene expression machinery. But, evidence from *Epifagus* and several algal species (see also section 1.2.1), suggests that some plastid ORFs remain as "black boxes". Determination of the function of the products of these putative genes is one of the great challenges in plastid molecular biology.

1.1.3.4 Chloroplast gene expression

Co-ordination of the different classes of chloroplast genes described in the previous section must involve tight control of gene expression. For example, correct construction of the photosynthetic complexes must be preceded by coordinate expression of the genes encoding the many proteins of each complex. As well as co-ordination with each
other, chloroplast gene expression must be synchronised with that of nuclear genes whose products are destined for the plastid. Expression of genes, or groups of genes, must also be regulated according to the developmental stage of the organism. In this section some of the general mechanisms currently understood to regulate plastid gene expression will be described. A more in depth discussion specifically relating to *Chlamydomonas* chloroplast gene expression is given in section 1.2.4.

In contrast to nuclear genes, transcriptional regulation appears to exert a general effect on chloroplast gene expression, rather than a specific effect on individual genes (Mayfield *et al*., 1995; Rochaix *et al*., 1998). Plastid gene promoters often contain consensus bacterial like -35 (TTGACA) and -10 (TATAAT) sequences (Mayfield *et al*., 1995), although identification of many of these sequences may reflect an initial bias towards studying photosynthetic genes (see below). Mutational studies of higher plant (Gruissem and Zurawski, 1985a; Gruissem and Zurawski, 1985b) and algal (Klein *et al*., 1992) chloroplast gene promoters have determined that the -35 element may not always be necessary for efficient transcription. As described in section 1.1.3.1 there is now molecular evidence in support of the idea of two types of RNA polymerase, a chloroplast encoded bacterial type polymerase and a second nuclear encoded polymerase (Hess *et al*., 1993; Allison *et al*., 1996). Hajdukiewicz *et al*., (1997) divided chloroplast promoters into three classes depending on whether they are transcribed by both the plastid and nuclear encoded RNA polymerase (class II), the plastid encoded polymerase alone (class I), or the nuclear encoded polymerase alone (class III) (Hajdukiewicz *et al*., 1997). Further studies are needed to determine whether these classes can be applied to all plastid genes in all species.

The type of message transcribed from chloroplast genes appears to be largely different between algal and plant species. In general, chloroplast genes of higher plants are organised in clusters and co-transcribed as polycistronic pre-mRNAs which are then processed, whereas algal genes are frequently transcribed under their own promoter (Westhoff and Herrman, 1988; Sugita and Sugiura, 1996; Rochaix *et al*., 1998). How these poly or monocistronic messages are subsequently processed is a major method of control of chloroplast gene expression. This control being exerted by the products of nuclear genes (see below).

Although of bacterial origin, plastid genes frequently contain introns, which can be divided into three groups depending on the mechanism of splicing. Occasionally, as in the case of *Euglena gracilis* chloroplast genes, introns contain other introns, termed "twintrons" (Hallick *et al*., 1993). Several chloroplast introns contain ORFs that encode endonucleases or RNA-splicing maturases (reviewed in Saldanha *et al*., 1993). Introns...
splicing, though prevalent in chloroplast genes, has not been shown to significantly affect levels of gene expression (Mayfield et al., 1995). Chloroplast transcripts occasionally undergo RNA editing. RNA editing is defined as the process that changes the primary nucleotide sequence of an RNA molecule from that encoded by the corresponding gene (for reviews see Pring et al., 1993; Gray and Covello, 1993b). Chloroplast gene RNA editing usually involves the conversion of a C-to-U base to produce the correct translatable message. Examples include the rpl2 gene in maize and psbl in tobacco (Hoch et al., 1991; Kudla et al., 1992) which both edit an ACG codon to AUG, producing an initiation codon.

Chloroplast transcription units mostly contain short inverted repeats at their 3' ends, which can potentially fold into stem-loop structures, and it is the processing of these 3' ends that may be one of the keys to understanding the control of chloroplast gene expression (reviewed in Mayfield et al., 1995; Sugita and Sugiura, 1996). Initially, it was thought that these 3' inverted repeats acted as transcriptional terminators, but they are now known to act as RNA-processing signals (Sugiura, 1992). Polyadenylation of mRNA 3' ends also plays a role in accelerating the degradation of chloroplast mRNA (e.g. Kudla et al., 1996).

The final level at which chloroplast gene expression can be controlled is that of translation. Light can severely affect the rate of translation of chloroplast mRNAs, for example, increasing protein accumulation from 100 to 10 000 fold during light-induced greening of plastids (Rochaix, 1992; Mayfield et al., 1995). As with transcription, translation in the chloroplast has both prokaryotic and eukaryotic-like traits. Prokaryotic features include 70S ribosomes, homology between bacterial and chloroplast rRNAs, and Shine-Dalgarno sequences in many chloroplast mRNAs. However other features, such as translation not being linked to transcription and stable mRNAs with long half lives existing as ribonucleic protein complexes hint at a more eukaryotic-type translation system. Nuclear mutants affecting chloroplast gene translation suggest that control of chloroplast gene translation is exerted by nuclear encoded factors. Much of this work has been carried out using C. reinhardtii and so this area will be covered in section 1.2.4.
1.1.4 The origin of the chloroplast genome

In the previous section, some of the bacterial-like features of plastid gene expression were discussed. In this section, further evidence that suggests plastids to be of bacterial origin will be described. It was these features that led Margulis to propose the endosymbiont hypothesis to account for the origin of chloroplasts and mitochondria (Margulis, 1970). This hypothesis proposes that chloroplasts and mitochondria originated from bacterial progenitors that entered into an association with, and were ultimately incorporated into, a primitive eukaryotic cell. At the time this hypothesis was based solely on morphological data as DNA sequencing had not yet developed (Sanger et al., 1977), and so no genetic data was available. Genetic data provided by the sequencing of organelle genes has enabled much greater insight into the origin of organelle genomes, and therefore organelles themselves (extensively reviewed by Gray and Doolittle, 1982; Gray, 1989; Gray, 1992; Gray, 1993a; Leblanc et al., 1997).

Molecular data confirms, beyond doubt, that plastids originated as bacterial endosymbionts. One of the questions that remains is whether plastids arose from a single, or multiple, primary endosymbiotic events. Although all plastids contain chlorophyll a, they are extremely diverse when comparing accessory pigments (chlorophylls b, c, etc.) and numbers and type of surrounding membranes (two, three or four, except C. paradoxa - see section 1.1.2), (Gray, 1992). Pigment diversity has been the basis for arguments of a multiple primary origin of plastids, where different plastid types arose from the symbiosis of different types of bacteria containing the corresponding pigments (Gray, 1993a; Reith, 1996). These hypotheses have now been shown to be incorrect, based on comparisons of plastid 16S RNA and protein sequences, which support a single origin regardless of pigmentation (Martin et al., 1992; Morden et al., 1992), plastids sharing a common ancestor with the blue-green algae (Gray, 1992).

Current data suggests a single primary plastid origin, but still inconclusive is the question of secondary plastid origins. A secondary plastid origin is the consequence of a non-photosynthetic protist taking up a photosynthetic one, which loses all of its cellular structure save its plastid, which is subverted to use by the host cell. Secondary endosymbiosis is assumed to account for the origin of plastids surrounded by more than two membranes, such as the chromophytes which have four, and Euglena spp., which has three (Reith, 1996). These extra membranes are postulated to be the vestiges of one or both of the plasma membrane of the endosymbiont and the phagosome membrane of the host. rRNA and protein data have determined the plastids of several
species including *Euglena* sp. (Henze *et al.*, 1995) and *Cryptomonas* φ (Douglas *et al.*, 1991) to be the result of secondary endosymbiosis.

Although organelle gene structure is similar to that of bacteria (section 1.1.3), there is an obvious disparity between the size and gene content of the respective genomes. For example, the closest bacterial relative to plastids Cyanobacteria, contain around 3000 genes (Kaneko *et al.*, 1996) whereas chloroplast genomes contain between 60-200 genes (Table 1.1). The loss of so much genetic information is accounted for by redundancy and subsequent loss of organellar genes due to replacement by nuclear counterparts (Brennicke *et al.*, 1993; Thorsness and Webber, 1996). Suprisingly, a mass of data now suggests that these nuclear counterparts are the result of transfer of organelle genes to the nucleus (reviewed by Thorsness and Webber, 1996). That massive organelle to nuclear gene transfer has occurred is conclusive, what is less certain is how and why it occurred and if this mysterious process is still active.

Answers to whether organelle to nuclear gene transfer is still occurring, and possibly how it is facilitated, may come from the characterisation of genes that have been transferred. For example, isolation of fragments of plastid DNA in the nuclear genome of tomato suggested a possible mechanism of integration involving direct repeats (Pichersky *et al.*, 1991). Also, evidence for transfer via an RNA intermediate has been observed based on the presence of edited versions of mitochondrial genes in the nucleus of soybean (Covello and Gray, 1992) and *Petunia* (Blanchard and Schmidt, 1995). Rather than being an ancient event plastid-nuclear gene transfer, even if not still occurring, has happened relatively recently: *rpl22*, encoding the chloroplast ribosomal protein CL22, is present in the chloroplast genome of all land plants examined except legumes. Moreover, in pea a functional copy of *rpl22* is known to be located in the nuclear genome (Gantt *et al.*, 1991). Similarly, the *tufA* gene which is located in the chloroplast genome in algae and encoding elongation factor Tu (EF-Tu), is absent from the chloroplast genome of higher plants. It has however been identified in the nuclear genome of the higher plant, *Arabidopsis* (Baldauf *et al.*, 1990a; Baldauf and Palmer, 1990b). Both these studies demonstrate transposition of genetic information from the plastid to the nucleus relatively recently in the evolution of land plants.

Obviously, nuclear localised organelle genes such as those described above are the result of transpositions which evolution has honed over millions of years. The transposition event though, is a multistep process (discussed in Brennicke *et al.*, 1993; Kadowaki *et al.*, 1996; Thorsness and Webber, 1996; Martin and Herrmann, 1998) of which transfer and integration of genetic material from the organelle to the nucleus are
the initial events. The transferred gene must then be adapted to expression in the
nucleus and also targeted to the organelle in question. These hypothetical events and
experimental data relevant to them are discussed in chapters 5 and 6.

The final question as to why plastid genes have transferred to the nucleus can only be
speculated about. Benefits of plastid genes being expressed in the nucleus may include
more efficient and faithful replication, increased regulatory control, and co-ordination
with other cellular metabolic activities (Thorsness and Webber, 1996). Also, the effect
of Muller’s ratchet - the rapid accumulation of deleterious mutations in asexual
populations - may be a motivating factor to transposition of genetic information (Martin
et al., 1998). The rapid accumulation of deleterious mutations has been observed in
metazoan mitochondrial genomes (Lynch, 1996) and endosymbiotic bacteria (Moran,
1996). Although intergenic recombination between plastid genomes may alleviate the
deleterious effect of Muller’s ratchet it may still be beneficial for plastid genes to be
released from their asexual environment. Perhaps the increase in ploidy of the
protosymbiont genome to the hundreds of copies present in land plant plastids was
motivated by the benefits of intergenic recombination. The increase in copy number
may reduce the need to transfer genes to the nuclear genome. Whatever the case, a more
puzzling question may be not why plastid genes have been transferred to the nucleus
but why any genes remain in the plastid at all? Some possible reasons may be
(Goldschmidt-Clermont, 1998a):

1. Certain polypeptides cannot be transported across the plastid envelope and therefore
must be expressed within the organelle. The rbcL gene encoding the large subunit of
RuBisCo (see section 1.1.3.2) would be a good candidate for this explanation as it is
encoded in the plastid in all photosynthetic species. To test this hypothesis an rbcL
mutant was engineered in tobacco, which resulted in a pale, nonphotosynthetic
phenotype (Kanevski et al., 1999). This mutant was then transformed in the nucleus
with an expression construct containing rbcL linked to a plastid transit peptide sequence
(allotropic expression, Nagley and Devenish, 1989). The transformed lines were green
and accumulated RuBisCo, albeit at reduced levels, and some were capable of reduced
photosynthetic growth. Therefore, it would seem that there is no absolute bar to
expression of rbcL in the nucleus. Why then rbcL is retained in the plastid, especially
when rbcS, encoding the small subunit of RuBisCo, is nuclear encoded is a mystery.

2. Polypeptides that contain too many (more than three) hydrophobic transmembrane
helices cannot be imported into the plastid (Popot et al., 1994) and so cannot become
nuclear encoded. Using a similar method to that described above the psbA gene (which
is always encoded in the plastid genome) encoding the D1 polypeptide of PSII, which
has five transmembrane spans, was allotropically expressed in the nucleus of tobacco. The \textit{psbA} gene used was a mutant copy that conferred atrazine resistance, and transgenic plants displayed a transient atrazine resistance phenotype (Cheung \textit{et al.}, 1988). Also, low levels of the modified gene product were detected immunologically in thylakoid membranes. These low levels may have been due to competition with the endogenous D1, as the plastid encoded \textit{psbA} gene was still present. Alternatively, supporting the above hypothesis, the hydrophobicity of the polypeptide may have hindered its import into the plastid resulting in reduced levels of transgenic D1 in thylakoids.

3. \textit{Genes may remain located in the plastid for regulatory reasons.} It has been postulated that genes for the core subunits of the photosynthetic apparatus are retained in the chloroplast so as to be easily regulated by a putative redox sensing mechanism (Allen, 1993). Although, this would not account for the maintenance of a plastid DNA in nonphotosynthetic parasites such as \textit{Epifagus virginiana} (Wolfe \textit{et al.}, 1992a).

4. \textit{The plastid genome is still in a state of flux and in future more or possibly all of its genes will be lost or transferred to the nucleus.} For obvious reasons this hypothesis is not easily testable.

In summary, the origin of the chloroplast genome, and as a corollary the chloroplast, is now much less mysterious than it has ever been. There is now general agreement that plastids arose from a single primary endosymbiotic event between a cyanobacteria and a progenitor unicellular eukaryote. Even the origin of the secondary endosymbiosis of several algal genera is becoming discernible with the sequencing of more plastid genomes (e.g. Martin \textit{et al.}, 1998). Unfortunately, the subject of plastid - nuclear gene transfer remains largely unresolved, but more sequence data from a wider range of species combined with ultrastructural and biochemical information may in future help solve these problems.
1.2 The chloroplast of *Chlamydomonas*

1.2.1 *Chlamydomonas reinhardtii* as a model organism

The genus *Chlamydomonas* comprises unicellular, biflagellate green algae and accounts for the largest of the 33 genera in the family Chlamydomonadaceae (Harris, 1989). Species of the genus *Chlamydomonas* are distinguished from other unicellular Volvocales by the presence of a cell wall, a pair of apical flagella, and a basal chloroplast surrounding one or more pyrenoids (Harris, 1998). *Chlamydomonas* species are to be found in a multitude of environments but the standard laboratory strains of *C. reinhardtii* all derive from a zygospore isolated from a soil sample collected in Massachusetts in 1945 (Harris, 1998). The reason for so much interest in this unassuming alga is its exceptional utility as a model organism, earning it the nickname the "green yeast" (Rochaix, 1995).

*Chlamydomonas reinhardtii* is the most commonly studied species of *Chlamydomonas* due to its ability to grow non-photosynthetically on a reduced carbon source. This property makes it possible to isolate viable non-photosynthetic mutants, a property that is lacking in higher plant models such as *Arabidopsis*. The main ultrastructural features of *C. reinhardtii* include a single nucleus, a pair of apical flagella (typically 1.5 to 2x the length of the cell body), multiple mitochondria, a single large cup-shaped chloroplast also containing a pyrenoid, and an eyespot (Fig. 1.2). To a greater or lesser degree, the structure of all these components has been investigated in *C. reinhardtii* by the use of mutants in which their morphology is altered. Compared to higher plants, photosynthetic research using *Chlamydomonas* also benefits from the ability of *Chlamydomonas* to synthesise chlorophyll in the dark. Chlorophyll is such a major component of the photosynthetic complexes that without it these complexes cannot properly assemble. Using *Chlamydomonas* it is possible to grow photosynthetic mutants in complete darkness that assemble mature chloroplasts, the initial effect of light on the mutated complex(es) can then be quantitatively examined.
Figure 1.3 Diagram of electron micrograph of a *C. reinhardtii* cell. C: chloroplast; E: eyespot; ER: endoplasmic reticulum; F: flagella; G: golgi; M: mitochondrion; N: nucleus; P: pyrenoid; V: vacuole. From a drawing by Keith Roberts.
In common with most model organisms, *C. reinhardtii* has a relatively short life cycle (Fig. 1.4). Vegetative cells are haploid, negating the need to backcross mutants to produce homozygous strains. With a doubling time of about eight hours, large numbers of cells can be grown quickly, stationary phase being reached at approximately 1-2 x 10^7 cell/ml (Harris, 1989). If provided with a light source, *C. reinhardtii* cells can be cultured in simple media containing only mineral salts with carbon dioxide as the sole carbon source (phototrophic growth). With the addition to the medium of a reduced carbon source such as acetate, cells can be grown in the dark (heterotrophic growth) or in the light (mixotrophic growth). Cell division can be synchronised by growing cells in a 12:12 hour light-dark cycle. Cell division mutants have been isolated (Warr, 1968).

*C. reinhardtii* are a heterothallic species comprising of which there are two mating types (*mt* or *mf*). Gametogenesis can be induced in the laboratory by nitrogen deprivation and blue light. Mating begins with flagella pairing which initiates a cascade of events including lysis of gametic cell walls, contact of mating-type structures at cell apices, and fusion of gametic cytoplasms. Subsequent resorption of flagella, nuclear and chloroplast fusion, are followed by secretion of a hard impermeable wall creating a durable zygospore within 24 hours of mating. If incubated in the correct conditions the zygospores will germinate (at which point meiosis takes place) within a week to produce four haploid progeny - the 'tetrad' (reviewed in Harris, 1998). Nuclear genes are inherited in a Mendelian fashion and segregate 2:2 among tetrad products. Chloroplast genes are inherited uniparentally from the *mt* parent in most zygotes, while mitochondrial genes are inherited uniparentally from the *mf* parent. Uniparental inheritance in *Chlamydomonas* will be discussed in section 1.2.2.

![Figure 1.4 The life cycle of *C. reinhardtii*. (Harris, 1989)](image-url)
Genetic analysis of Chlamydomonas

*Chlamydomonas* was identified as an alga with potential for carrying out genetic analysis as early as 1918, when Pascher reported segregation of genetic differences in crosses of two *Chlamydomonas* strains (discussed in Harris, 1989). Unfortunately, these studies were not followed up, and it was not until the 1940's that sexually competent *Chlamydomonas* strains were properly isolated and analysed in the laboratory (Smith, 1946). Using these isolates the first isolation and genetic analysis of *Chlamydomonas* mutants was achieved by Lewin (Lewin, 1949; Lewin, 1951). Lewin's experiments were carried out using *C. moewusii*, which is unable to grow non-photosynthetically. Contemporaries of Lewin favoured *C. reinhardtii* for its ability to grow in the dark on an organic carbon source, and for this reason it continues to be the *Chlamydomonas* species of choice (section 1.2.2).

Classical genetic analysis of *Chlamydomonas* is well defined and routine, but the power of the organism lies in its use for both classical and modern (reverse) genetics. Reverse genetics is based on the ability to transform an organism with exogenous genetic information. *C. reinhardtii* has the unique boast of being the only organism to date to have been transformed in all three genomes - nuclear, chloroplast and mitochondrial.

The nuclear genome of *C. reinhardtii* has been estimated to have a size of 7-10 x 10^8 bp. Seventeen linkage groups have been mapped in this alga (Harris, 1989; Harris, 1998; Silflow, 1998). Unfortunately, chromosome cytology is poor and chromosomes can at best be resolved as four co-migrating bands on pulsed-field gels (reviewed in Harris, 1998). Transformation of the nuclear genome is possible using a multitude of techniques including biolistics, electroporation, vortexing with silicon carbide whiskers or vortexing with glass beads (recently reviewed in Stevens and Purton, 1997; Kindle, 1998; Lumbreras and Purton, 1998b). The later method (Kindle, 1990), is most commonly practised due to its ease of use. Also, a variety of auxotrophic or drug resistance selectable markers have been developed to expedite selection of nuclear transformants (Kindle, 1998). In common with *Arabidopsis* (Bouchez and Herman, 1998), homologous recombination of transformed DNA occurs at a very low rate in the nuclear genome of *C. reinhardtii* (Gumpel *et al*., 1994). Even though the enzymatic mechanism necessary for homologous recombination has been demonstrated as active in vegetative cells (Sodiende and Kindle, 1993). This low level of homologous recombination makes gene replacement difficult as transformed DNA inserts predominantly at random sites in the genome (Debuchy *et al*., 1989; Kindle *et al*., 1989). However, this property can be advantageous in the enabling tagging of
insertionally mutagenised genes and has led to isolation of genes encoding a wide variety of functions (reviewed in Kindle, 1998). Finally, after initial problems possibly as a result of codon bias or gene silencing, expression of foreign genes in the nucleus is becoming a practical possibility (Stevens et al., 1996; Cerutti et al., 1997a; Cerutti et al., 1997b; Lumbreras et al., 1998a).

Genetic analysis and manipulation of the chloroplast genome is one of the main themes of this introduction and will be discussed fully in subsequent sections.

The mitochondrial genome of *C. reinhardtii* consists of a 15.8 kbp linear molecule with inverted repeats at its ends and is present in multiple copies per cell. The genome has been completely sequenced and contains thirteen genes (Vahrenholz et al., 1993). These genes code for components of the respiratory enzyme complexes, tRNAs, rRNAs and a reverse transcriptase possibly involved in DNA replication (reviewed by Remacle and Matagne, 1998). Several mutants of mitochondrial genes have been isolated and are conditionally lethal when grown in the dark. The mitochondrial genome has been transformed with partially purified mitochondrial DNA using particle gun bombardment to rescue the *duml* mutant to respiratory competence (Randolph-Anderson et al., 1993).

Historically *Chlamydomonas* has been used as a model system for studying two areas, photosynthesis and flagella function, and not without good reason. Although distantly related, the photosynthetic machinery of *C. reinhardtii* (reviewed by Olive and Wollman, 1998), and higher plants are extremely similar. The flagellum of *C. reinhardtii* (reviewed by Johnson and Rosenbaum, 1993; Dutcher, 1995; Johnson, 1995) is also virtually indistinguishable at the ultrastructural level from that of higher organisms, including human sperm. But, with the increasing number of tools available, the nearly complete sequencing of the chloroplast genome and the possible sequencing of the nuclear genome in the near future, *C. reinhardtii* will soon be the most multifaceted model in cell biology.
1.2.2 Uniparental inheritance of the chloroplast genome

The story of organellar inheritance begins in 1909, nine years after the rediscovery of Mendel's laws. In this year Correns and Baur independently demonstrated that variegation in plant leaves could be inherited in a non-Mendelian fashion (Baur, 1909; Correns, 1909; discussed by Gillham, 1994; Birky Jr, 1995). Although they could not have known it at the time the source of the novel inheritance patterns was mutations in plastid genes. These studies were thoroughly followed up during the 1920-30s but the science of organelle genetics did not begin in earnest until the 1950s (reviewed by Gillham, 1994). In 1949 Ephrussi and collaborators discovered respiration deficient 'petite' mutations in bakers yeast, which commonly exhibited non-Mendelian inheritance (summarised in Ephrussi, 1953). Unfortunately, these strains proved to be pleiotropic and similar in their effects on respiratory phenotype, hindering genetic analysis of the mitochondrial genome. Also, in 1952 non-Mendelian inheritance was described in the filamentous fungi *Neurospora*. This slow growing mutant, named *poky*, exhibited uniparental-maternal inheritance patterns and had a respiratory-deficient phenotype (Mitchell and Mitchell, 1952). However, the most elegant and what would subsequently prove to be the most tractable demonstration of "cytoplasmic" inheritance was in *C. reinhardtii*. In the early 1950s Sager isolated two different antibiotic resistant mutants of *C. reinhardtii*, one which was resistant to low levels of streptomycin (*sr-1*), and a second which was resistant to high levels of streptomycin (*sr-2*). Genetic analyses of these two strains showed that the inheritance pattern of *sr-1* followed Mendelian rules while *sr-2* was nearly always inherited uniparentally from the *mt* strain (Sager, 1954). This was a clear demonstration of a stable mutation that was inherited "in an extrachromosomal manner" (Sager, 1954). In retrospect, it seems strange that Sager made no predictions that the carrier of these mutations should be DNA, but it must be remembered that this was at a time when only a year before Watson and Crick had published their model of the structure of DNA (Watson and Crick, 1953).

Significantly, this work defined an experimental system in which the question of non-Mendelian uniparental inheritance could be easily addressed (Fig. 1.5). This led to the isolation of more mutations which were inherited in a uniparental fashion (reviewed in Gillham, 1994). It is now known that the *sr-2* mutation is localised within the *rps12* gene of the chloroplast genome (Liu *et al.*, 1989).
By the beginning of the 1960s *Chlamydomonas* was again leading the way in organelle genetics with the first microscopic evidence of areas of the chloroplast binding to DNA specific stains (Ris and Plaut, 1962). In the next year two groups described the isolation of chloroplast DNA by CsCl density gradient centrifugation (Chun *et al.*, 1963; Sager and Ishada, 1963). Uniparental inheritance from the *mt* strain was now well documented in *Chlamydomonas*, but the mechanism of this inheritance was still completely unknown. Work throughout the 1970-80s illustrated that the solution lay in the fate of the *mt* chloroplast genome during mating. The results of DNA labelling, molecular biological and fluorescence microscopy studies (reviewed in Armbrust, 1998), concluded that shortly after the fusion of gametes (within 40 minutes of zygote formation, before nuclear or chloroplast fusion), chloroplast DNA from the *mt* cell is actively degraded.

The next stage in the quest to comprehend the mechanisms of uniparental inheritance is currently being investigated and is far from fully understood. Viz., how is the chloroplast DNA from the *mt* gamete selectively degraded and what are the molecular mechanisms underlying this process. The most parsimonious hypothesis to explain this phenomenon is that at some point during gametogenesis the chloroplast genome in *mt* cells is somehow "tagged". During zygote formation this tag protects the *mt* chloroplast DNA from degradation (by a putative DNase), while the unprotected *mt* chloroplast DNA is destroyed (reviewed by Gillham, 1994). This process is believed to be controlled by the mating-type locus. The mating-type locus is found on the left arm
of linkage group VI and spans nearly 850 kbp (Ferris and Goodenough, 1994). It is a highly complex region of suppressed recombination in which twelve genes, five required for life cycle events and seven housekeeping genes, have so far been identified (Armbrust et al., 1993; Ferris and Goodenough, 1994; Armbrust et al., 1995; Ferris and Goodenough, 1997). As well as organelle inheritance, gamete differentiation and zygote maturation are also controlled by the mating-type locus (Goodenough et al., 1995). The region has been studied intensively by Ferris and Goodenough who found that it is identical in $m^+$ and $m^-$ cells except for a central domain of about 190 kbp, designated the R domain. The R domain contains chromosomal rearrangements resulting in segments unique to one or other mating type (Ferris and Goodenough, 1994). These rearrangements are presumably the cause of the suppressed recombination in this region, allowing permanent differentiation of R domain genes in each mating type (Ferris and Goodenough, 1994). A complete discussion of the functions of the mating-type locus is beyond the scope of this introduction (reviewed in Goodenough et al., 1995; Armbrust, 1998), but one gene located on it is of particular relevance. $ezyl$ (early zygote) is one of several genes expressed specifically at the time of zygote formation. The $ezyl$ gene is located in multiple tandemly repeated copies at the mating-type locus in both mating-type plus and minus cells (Armbrust et al., 1993). One of the methods developed by Sager to disrupt the normal pattern of uniparental inheritance was treatment with ultraviolet light (UV) just prior to mating (discussed in Gillham, 1994). This treatment prevents the selective degradation of $m^-$ chloroplast DNA. Importantly, UV irradiation of $m^+$ but not $m^-$ gametes selectively disrupts accumulation of $ezyl$ mRNA, whereas expression of a zygote-specific cell wall gene did not show the same mating-type linked specificity (Woessner and Goodenough, 1989). Therefore, $ezyl$ expression correlates with the absence of $m^-$ chloroplast DNA destruction in UV treated zygotes, suggesting it to be involved in degradation of chloroplast DNA during zygote formation. Adding weight to this hypothesis is the observation that Ezyl protein is localised specifically to the chloroplast nucleoids in the early zygote, as identified by immunofluorescence of DAPI-stained cells (Armbrust et al., 1993). Whether Ezyl itself is a nuclease or acts as an activator for a putative nuclease, and how $m^+$ chloroplast DNA is specifically protected from its action is still unknown.

Although biparental inheritance does occur (e.g. Baur, 1909), uniparental inheritance of organelle genes is seen in the majority of species (Kuroiwa, 1991; Birky Jr, 1995). As discussed above, uniparental inheritance of chloroplast genes in Chlamydomonas is achieved by selective degradation of the chloroplast genome from one of the gametes. Other green alga such as Ulva mutabilis achieve the same end by destroying the whole chloroplast organelle from one gamete in the zygote (Braten, 1971; Braten, 1973). In isogamous species such as Chlamydomonas the mechanism of uniparental inheritance is
non-obvious. In contrast, in anisogamous species such as humans the female gamete is far larger than its male counterpart (oogamy), and the latter does not contribute any cytoplasmic organelles to the zygote (Birky Jr, 1995). Obviously, there are many variations on the mechanism of uniparental inheritance of cytoplasmic genes, but why has the mechanism evolved in the first place? Surely it would be easier and energetically favourable for the zygote to inherit cytoplasmic genes from both gametes, rather than actively destroying half of this genetic information. Several hypotheses have been presented to account for the evolution of uniparental inheritance of organelle genes (reviewed by Birky Jr, 1995). One particularly attractive idea, is that inheritance of two sets of organelles produces conflict between the opposing sets of cytoplasmic genes (Anderson, 1992; Hurst, 1992). Therefore, it is less costly to inherit organelle genes from one gamete in order to avoid the fallout of potential organelle "warfare". This hypothesis has also been extended to account for the occurrence of only two sexes in most species genes (Hurst, 1992; Hurst and Hamilton, 1992).

1.2.3 The *Chlamydomonas reinhardtii* chloroplast genome

1.2.3.1 Genome structure

The *C. reinhardtii* chloroplast genome is 196 kbp in size and contains approximately 120 genes (Fig. 1.6). Each cell contains a single chloroplast harbouring around 80 copies of its genome (Chiang and Sueoka, 1967; Gillham, 1978). When visualised with DNA specific stains such as DAPI (4',6-diamidino-2-phenylindole), the polyploid chloroplast genome is seen to be sequestered into between 8-10 DNA-protein complexes known as nucleoids (e.g. Kuriowa et al., 1981). A characteristic feature of chloroplast DNA, the *C. reinhardtii* genome also contains a large (21 kbp) inverted repeat (IR) containing a cluster of rRNA genes as well as *psbA*, encoding the D1 polypeptide of PSII. Unlike higher plants which typically have large and small single copy regions, the *C. reinhardtii* IR separates two single copy regions of approximately equal size. The IR also enables the *C. reinhardtii* chloroplast genome to form two isomeric conformations, which have been shown to be present in an approximately 50:50 ratio in vivo (Harris, 1989). Also, the high frequency of symmetrical mutations and lack of sequence divergence between the repeats argues that the chloroplast DNA can form a dumbbell configuration allowing a copy correction mechanism to maintain the mutual fidelity of the repeats (reviewed in Harris, 1989). Mutations in the chloroplast genome were initially mapped using biparental zygotes from crosses. Biparental zygotes, where progeny contain chloroplast DNA from both gametes, was
first identified by Sager (1954) and are the rare exception to uniparentally inherited chloroplast genomes. The first chloroplast genes in *C. reinhardtii* were identified in 1978 (Malnoe and Rochaix, 1978), and since then genetic mapping has been superseded by physical mapping. The overall structure of the *C. reinhardtii* chloroplast genome is very different from that of higher plants and even from other *Chlamydomonas* species (Harris, 1989; Boudreau et al., 1994). The mechanism and control of chloroplast DNA replication has been studied by several groups (reviewed in Sears, 1998). Two potential replication origins, as evidenced by electron microscopic visualisation of replication bubbles or displacement loops (D-loops), have been observed in *C. reinhardtii* chloroplast DNA (Waddell et al., 1984). One of these (oriA), was also shown to be active as an initiator of DNA synthesis *in vitro* (Wu et al., 1986). The presence of recombination hotspots (e.g. Newman et al., 1992) and mobile elements (e.g. Fan et al., 1995) have also been demonstrated in the *C. reinhardtii* chloroplast genome.
Figure 1.6 The chloroplast genome of *C. reinhardtii*. Adapted from Boudreau *et al.*, (1994).
1.2.3.2 Gene content

Due to its utility as a model organism, sequencing of the *C. reinhardtii* chloroplast genome has been carried out piecemeal since the early 1980s, and only a small fraction now remains unsequenced. The genes so far identified on the *C. reinhardtii* chloroplast genome are summarised below according to the categories discussed in section 1.1.3 (i.e. genetic system genes; photosynthetic genes; ORFs of putative or unknown function). Unless otherwise stated the data discussed is taken from Boudreau et al., (1994), in which the chloroplast genomes of *C. reinhardtii* and *C. moewusii* were mapped and compared.

The prokaryotic-type chloroplast ribosome consists of a protein-RNA complex. The *C. reinhardtii* chloroplast genome encodes all the rRNA species (16S, 7S, 3S, 23S, and 5S in this order) necessary for the ribosome. 7S and 3S are unique to *Chlamydomonas*. The rDNA genes are found in a highly conserved cluster on the inverted repeat. In contrast, the *C. reinhardtii* chloroplast genome only encodes about one third of the protein subunits necessary to construct the ribosome. Of the ribosomal protein genes present, the genes in the *rpl23* operon have the same conserved order as the *rpl23* operon of bacteria and higher plants (Tanaka et al., 1986). Also, though they have not all been identified, the *C. reinhardtii* chloroplast DNA in parallel with other algae, is thought to contain all the tRNA species required for translation in the organelle (see section 1.1.3.1 for discussion). Genes for the β, β', and β" subunits of the *E. coli*-type RNA polymerase have been identified in the *C. reinhardtii* chloroplast genome (*rpoB1*, *rpoC1*, and *rpoC2* respectively). Whether or not *rpoA* is present in the chloroplast genome, or has become nuclear encoded, awaits completion of the chloroplast genome sequence and/or its isolation from nuclear DNA. The possibility of a second nuclear encoded chloroplast RNA polymerase in *Chlamydomonas*, homologous to that found in higher plants, is considered in chapter 6.

The photosynthetic gene content of the *C. reinhardtii* chloroplast genome is similar to that of higher plants, with several notable exceptions. Genes are present coding for many of the core subunits of PSI and PSII, the cytochrome *b/f* complex and ATP synthase. Notably, *psaA* is split into three exons which are spread throughout the genome (Boudreau et al., 1994). The three exons are spliced in *trans* which involves several unidentified nuclear and one chloroplast factor, *tscA*, a RNA species which is proposed to act as a linker to *psaA* exons 1 and 2 (section 1.2.4.3, Goldschmidt-Clermont et al., 1990; Goldschmidt-Clermont et al., 1991b). In addition, *C. reinhardtii* also contains three chloroplast encoded genes involved in light-independent reduction of
protochlorophyllide to chlorophyllide. This mechanism is absent in angiosperms which rely solely on the light dependent enzyme protochlorophyllide oxidoreductase (Smith and Griffiths, 1993; Reinbothe and Reinbothe, 1996). The products of the genes *chlL*, *chlB*, and *chlN*, in conjunction with as yet unidentified nuclear encoded factors, catalyse this reaction (Choquet *et al*., 1992; Suzuki and Bauer, 1992; Liu *et al*., 1993). Another unusual feature of *C. reinhardtii* chloroplast genes is the presence of putative protein introns. *rps3*, *rpoC2a*, *cemA* and *clpP* are all much larger than homologous genes in other species and contain long, in frame insertions juxtaposed with conserved coding sequence (Huang *et al*., 1994; Rochaix, 1996). Transcripts of these genes are not processed at the RNA level. Therefore it is postulated that the insertions are excised from the nascent polypeptide, which is spliced together to produce the mature form of the protein (Rochaix, 1995).

The *C. reinhardtii* chloroplast genome contains around eight *ycf* genes as well as several ORFs unique to this alga (Boudreau *et al*., 1994). *ycf3* and *ycf4* form part of a polycistronic transcript and have both been inactivated using insertional mutagenesis (Boudreau *et al*., 1997b). Both genes were found to be essential for photosynthetic growth and required for accumulation of PSI, although not stably associated with it. Similar deletion of *ycf8* determined this gene to be a novel PSII subunit but only to be necessary for optimal PSII activity under adverse growth conditions (Monod *et al*., 1994). Therefore, it appears that several *ycf* gene products may play a role as accessory photosynthetic proteins rather than core components of the photosynthetic complexes. This may be why their products have not been previously isolated by biochemical methods. In contrast to *ycf3* and *ycf4*, mutants of *C. reinhardtii* transformed with an insertionally inactivated ORF1995 maintained copies of the wild-type chloroplast gene (Boudreau *et al*., 1997a). The predicted polypeptide coded by ORF1995, is highly basic and is suggested to be involved in binding of the chloroplast DNA to the chloroplast envelope or thylakoid membrane. Also ORF1995 may be homologous to *ycf1*, one of the four ORFs maintained in the plastid genome of *Epifagus Virginiana* (Wolfe *et al*., 1992a; Wolfe *et al*., 1992b). Its possible conservation in the plastid genome of *Epifagus Virginiana* suggests that ORF1995 may have a novel non-bioenergetic or gene expression function. Again, using insertional inactivation ORF2971 was found to be similarly essential as transformants remained heteroplasmic (Watson and Purton, unpublished). Further experiments suggest that this protein may also be involved in binding of chloroplast DNA.
1.2.4 C. reinhardtii chloroplast gene expression

1.2.4.1 Transcriptional regulation

Transcriptional regulation is the first stage in the cascade of checkpoints that regulate gene expression. Levels of chloroplast transcripts are known to fluctuate coordinately with endogenous controls such as the cell cycle and circadian rhythm, and also with external stimuli such as light and growth medium. Measuring the transcript level of a particular gene by northern analysis does not give a direct measure of the rate of transcription as the rate of mRNA degradation also affects its steady state levels. Transcription rates are measured directly either by in vitro run-on transcription assays or by in vivo pulse labelling (Nickelson, 1998). Although work in C. reinhardtii has mainly focused on post-transcriptional regulation of gene expression, studies have been undertaken in order to understand how factors such as light, the cell cycle and circadian rhythms affect the rate of transcription of chloroplast genes (reviewed in Stern and Drager, 1998). Often, the greatest obstacle in such studies is delineating the effect each of these factors has on transcription rate. Hwang et al. (1996) synchronised cell division using 12 hour-light/12 hour-dark cycles, then placed cultures under continuous light or dark to assess the effect of any endogenous rhythm on chloroplast gene transcription. They found that the levels of tufA mRNA showed a dramatic oscillation with a period of 24 hours for at least two or three cycles after transfer to continuous light or dark respectively (Hwang et al., 1996). This circadian rhythm of mRNA accumulation was mainly due to oscillation in the level of transcription of tufA (Hwang et al., 1996). How circadian rhythm regulates the transcription of chloroplast genes is unknown. Two early studies used synchronously dividing cells, entrained by a 12 hour-light/12 hour-dark regime, to analyse the rates of transcription of chloroplast genes throughout the cell cycle (Herrin et al., 1986; Leu et al., 1990). Both studies concluded that the rate of transcription of the chloroplast genes changed throughout the cell cycle, and therefore that chloroplast gene expression is coupled, via transcription, to the cell cycle. Incisively, Salvador et al. (1993a) were critical of these studies describing how it is not possible to distinguish between light/dark, endogenous, and cell cycle-control of gene expression in light/dark synchronised cultures. To circumvent this problem Salvador and colleagues grew high density, asynchronous cultures in a light/dark cycle, thereby obliterating the observance of cell cycle effects. In this study the transcription rates of seven chloroplast genes of various functions were compared at different times in the light and dark (Salvador et al., 1993a). The conclusion reached was that light/dark cycles regulate the transcription of chloroplast genes collectively: maximum and minimum rates of transcription of all the genes analysed occurred at the
beginning and toward the end of the light period respectively. Whether these effects are a direct response to light/dark stimuli or are the result of circadian or other endogenous rhythms (apart from cell cycle), which are entrained by the light/dark period, is unknown.

Another observation from the study by Salvador et al. (1993a) was that although light and dark have general effects on transcription rates, gene specific effects were also observed to be superimposed on this overall pattern (Salvador et al., 1993a). One might suppose that these gene specific effects on transcription are activated by nuclear factors, targeted to the chloroplast whose own gene expression is light/dark controlled. Many analogous trans-acting factors have been isolated which interact with and control transcript stabilisation and translation (see below). Anomalously, of the many C. reinhardtii mutants effected in chloroplast gene expression only one has been isolated to date that has a reduced rate of transcription (Hong and Spreitzer, 1994). This mutant is affected in expression of rbcL, although the accuracy of these results has been questioned (Rochaix, 1996; Nickelson, 1998). Assuming that such transcriptional regulatory factors exist, the dearth of mutants affected in them may be because the target gene or genes are essential for cell viability. This problem could be overcome by the isolation of temperature-sensitive mutants effecting chloroplast gene transcription. Alternatively, differential transcription of photosynthetic genes, such as psaB, and genetic system genes, such as tufA (Salvador et al., 1993a), may be the result of their transcription by different types of RNA polymerase (section 1.1.3.1).

Cis-acting elements regulating transcription of chloroplast genes are usually found upstream of the start of transcription. These chloroplast gene promoters often consist of at least one of the E. coli-type -35 (TTGACA) and -10 (TATAAT) promoter elements. Genes such as rbcL and rnl6 contain slightly degenerate versions of both consensus sequences, while others such as atpB and psbA contain only the -10 element (Klein et al., 1992). The -10 element is most common and differs slightly from the E. coli consensus in the form of a palindromic "TATAATAT" sequence (Klein et al., 1992). Klein et al. (1992), used a chimeric construct with various sections of the atpB promoter fused to the E. coli uidA (β-glucuronidase, GUS) reporter gene. They observed levels of transcription equal to the endogenous atpB gene using a fragment 22 bp upstream and 60 bp downstream of the atpB transcription start site (Klein et al., 1992). Obviously, at least for atpB, there is no cryptic -35 promoter element, as a reduced promoter is able not only to promote normal levels of transcription but also to initiate transcription at the usual site (Blowers et al., 1990). A more unusual promoter element was discovered to effect transcription of rbcL: Experiments using the uidA gene
fused to the rbcL promoter severely retarded transcription of the chimeric construct unless an element between 126 bp and 170 bp after the start of transcription of rbcL, (well within the rbcL coding sequence) was included (Blowers et al., 1990; Klein et al., 1994).

The ability to transform the chloroplast with the type of chimeric reporter genes described above makes Chlamydomonas a powerful model system for studying chloroplast gene expression in vivo. Whether the type of transcriptional control by remote elements seen for rbcL (Klein et al., 1994) is a phenomenon general to chloroplast gene expression remains to be seen, though trnEl was determined in vitro to have an internal promoter (Jahn, 1992). Chlamydomonas chloroplast promoters not only resemble their E. coli counterparts but can also drive their own, and chimeric gene expression when transformed into E. coli (Hanley-Bowdoin and Chua, 1987; Thompson and Mosig, 1987; Goldschmidt-Clermont, 1991a).

Chloroplast genes in most species, including C. reinhardtii, are flanked at the 3' end by an inverted repeat sequence that can potentially form a stem loop structure. As is the case in bacteria, these repeats were initially thought to act as transcriptional terminators (Blowers et al., 1993). Using chimeric reporter genes containing 3' ends from different chloroplast genes, in vivo run-on transcription experiments subsequently showed this not to be the case (Rott et al., 1996; reviewed in Stern and Drager, 1998). 3' ends of mature mRNAs in Chlamydomonas are probably formed by 3' end processing of longer precursors, a process that is affected by the presence of stem loop structures (see below).

Finally, DNA topology may effect the rate of transcription of chloroplast genes. Thompson and Mosig (1990) showed that light can induce changes in the structure of chloroplast chromosomes (Thompson and Mosig, 1990). They used a DNA-intercalating dye which differentially binds DNA according to its structure (more dye is bound to underwound DNA than is bound to supercoiled DNA). On exposure to UV light, this dye induces crosslinking of chloroplast DNA as a function of the extent of its intercalation. If denatured and quickly renatured, crosslinked strands are far more likely to reanneal with their complementary partner, providing an assay for chromosomal structural changes.
1.2.4.2 RNA processing and stability

RNA processing

The interaction of chloroplast transcripts with regulatory factors prior to translation plays a major part in controlling gene expression in *Chlamydomonas*. How transcript stability is regulated will be described below. Firstly though, the steps leading to production of the mature transcript will be briefly discussed. Unlike their bacterial progenitors, chloroplast genes commonly contain introns. *Chlamydomonas* spp. contain both group I and group II introns, grouped according to their differing structures and mechanisms of splicing (reviewed by Herrin *et al.*, 1998). Group I and group II introns are defined as those introns found mainly in organelle genes, group III introns being located mainly in eukaryotic nuclear genes. Certain group I and II introns have been shown to self-splice *in vitro*, the pre-RNA molecule self-catalysing its own excision from the transcribed gene (Herrin *et al.*, 1998). However, *in vivo* splicing of most chloroplast introns is probably supported by ancillary proteins (Saldanha *et al.*, 1993). Among the *Chlamydomonas* genus, group I introns have been found only in ribosomal RNA and photosynthetic genes (Herrin *et al.*, 1998). Also, several chloroplast gene introns contain ORFs encoded within the intron but in frame with the exon coding sequence. For example, two of the four introns in the *C. reinhardtii psbA* gene and the single intron in *rnl* contain ORFs which encode DNA endonucleases involved in intron mobility (Erickson *et al.*, 1984; Rochaix *et al.*, 1985).

The only group II introns described so far in *Chlamydomonas* spp. are the two introns of the PSI gene *psaA*, encoding one of the two related polypeptides found at the centre of PSI. Unlike other organisms where *psaA* exists as a single unit, in *C. reinhardtii* *psaA* consists of three exons which are scattered throughout the chloroplast genome. Exon 1 is located on the complementary strand to exons 2 and 3 and all three exons are flanked by group II intronic sequences, whose splicing relies on multiple *trans*-acting accessory factors (Kuck *et al.*, 1987). Many PSI mutants previously isolated were found to be deficient in *psaA* expression and specifically in splicing of the three disparate exons (Choquet *et al.*, 1988). These mutants could be grouped into three classes depending on whether they were defective in the conjoining of exons 1 and 2, exons 2 and 3, or both these events (Goldschmidt-Clermont *et al.*, 1990). These and other data suggested that the three *psaA* exons are transcribed separately and spliced together *in trans* to form the mature transcript. The mechanism of assembly of the mature transcript is thought to involve ligation, *in trans*, of intron sequences flanking the three exons, which are then spliced out to produce a continuous coding sequence.
At least 13 unlinked nuclear and one chloroplast loci have been found to be involved in the trans-splicing of *psaA* in *C. reinhardtii*, suggesting many individual proteins are essential to co-ordinate the splicing mechanism (Goldschmidt-Clermont *et al.*, 1990). The identities of the 13 nuclear loci remains unknown, while the chloroplast locus (*tscA*) was found to encode a small RNA. It is proposed that this RNA molecule bridges the two halves of intron 1, found at the 3' terminus of the exon 1 transcript and the 5' terminus of the transcript of exon 2 (Fig. 1.7) (Goldschmidt-Clermont *et al.*, 1991b). Dissection of the mechanism of splicing of *psaA*, apparently unique to *C. reinhardtii*, poses the intriguing question of whether the split intron structure reflects an intermediate stage in evolution between group II introns (in which splicing is catalysed in cis by the different intron domains) and eukaryotic introns (in which splicing is catalysed by trans-acting RNA-protein complexes, the snRNPs) (Mets and Rochaix, 1998).

**Figure 1.7** The mechanism of *psaA* mRNA trans-splicing in *C. reinhardtii* (Herrin *et al.*, 1998). For description of the mechanism see text. A, B, and C are the three classes of nuclear encoded factors needed for splicing, A: factors specific for splicing the second intron, B: factors required for splicing of both introns, C: factors specific for splicing of the first intron. The same or similar factors may also be involved in earlier steps of transcript stabilisation and processing.
Aside from splicing, another mechanism active in the production of mature chloroplast gene transcripts is 3'-end processing. In higher plants chloroplast genes are mainly co-transcribed under a single promoter to form polycistronic messages which are subsequently processed to produce mature, single gene transcripts (e.g. Westhoff and Herrman, 1988). This is in contrast to Chlamydomonas, where chloroplast genes are usually flanked by their own individual promoters and so transcribed individually (reviewed in Stern and Drager, 1998). Although, co-transcribed Chlamydomonas chloroplast genes have been identified (e.g. Choquet et al., 1988; Monod et al., 1992; Levy et al., 1997). As mentioned in section 1.2.4.1, stem loop structures found at the 3'-end of chloroplast genes are ineffective transcriptional terminators. However, these inverted repeats have been shown to serve as barriers against exonucleolytic degradation of upstream sequences and to mediate correct 3'-end formation (Stern and Drager, 1998). In summary, although the 3'-end of chloroplast gene transcripts interact with stabilising factors, the stem loop structure may not be involved in this interaction per se.

**RNA stability**

The mechanism of regulation of transcript stability of chloroplast genes in Chlamydomonas differs somewhat from the equivalent mechanism in higher plants. In plants, a single nuclear mutation can often cause a general decrease in chloroplast transcript levels due to loss of a factor that stabilises these RNAs (Meurer et al., 1996). The situation in Chlamydomonas differs in that a nuclear mutation can affect the transcript stability of a single chloroplast gene. Some chloroplast transcripts are even stabilised by more than one nuclear encoded factor. For example, C. reinhardtii nuclear mutants F54 and ncc1 both affect the transcript stability of the atpA gene (Drapier et al., 1992). Chlamydomonas photosynthetic mutants that lack the transcript of a specific chloroplast gene, have been frequently isolated. Whereas analogous higher plant mutants are affected in the mRNA stability of multiple unlinked genes. The ability of C. reinhardtii to grow non-photosynthetically has enabled the isolation of many different nuclear mutants affected in the stability of photosynthetic gene transcripts (Table 1.2), (Kuchka et al., 1989; Sieburth et al., 1991; Drapier et al., 1992; Monod et al., 1992; Gumpel et al., 1995). This type of mutant is characterised by normal expression of all chloroplast genes bar one gene or operon, which has low or undetectable levels of mRNA, while exhibiting wild-type rates of transcription (reviewed in Rochaix, 1996; Nickelson, 1998). The cis-acting factors interacting with these putative nuclear encoded transcript stabilising proteins do not appear to conform to any consensus and can lie

55
within any area of the mRNA molecule (e.g. Lee et al., 1996). The isolation in
*Chlamydomonas*, of the *trans*-acting factors that interact with these *cis*-elements to
stabilise chloroplast transcripts, lags behind similar studies in higher plants (Sugita and
Sugiura, 1996). Even so, several nuclear encoded proteins have been identified that
bind specific regions in the 5′ leader of *C. reinhardtii* chloroplast gene transcripts and
stabilise their expression *in vivo* (Danon and Mayfield, 1991; Zerges and Rochaix,
1994; Hauser et al., 1996). Genetic analysis and cloning of the genes encoding these
transcript stabilisation factors should produce significant insights into the mechanisms
of chloroplast gene expression.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Chloroplast gene affected</th>
<th>Photosynthetic complex affected</th>
<th>RNA level in % wild-type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GE2.10</td>
<td><em>psbB</em></td>
<td>PSII</td>
<td>0</td>
<td>Sieburth et al., 1991</td>
</tr>
<tr>
<td>222E</td>
<td><em>psbB</em></td>
<td>PSII</td>
<td>0</td>
<td>Monod et al., 1992</td>
</tr>
<tr>
<td>6.2z5</td>
<td><em>psbC</em></td>
<td>PSII</td>
<td>0</td>
<td>Sieburth et al., 1991</td>
</tr>
<tr>
<td>nac2-26</td>
<td><em>psbD</em></td>
<td>PSII</td>
<td>0</td>
<td>Kuchka et al., 1989</td>
</tr>
<tr>
<td>Mφ14</td>
<td><em>psbD</em></td>
<td>PSII</td>
<td>0</td>
<td>Purton et al., unpublished</td>
</tr>
<tr>
<td>ncc1</td>
<td><em>atpA</em></td>
<td>ATP synthase</td>
<td>10</td>
<td>Drapier et al., 1992</td>
</tr>
<tr>
<td>thm24</td>
<td><em>atpB</em></td>
<td>ATP synthase</td>
<td>0</td>
<td>Drapier et al., 1992</td>
</tr>
<tr>
<td>Mφ11</td>
<td><em>petA</em></td>
<td>cytochrome b6/f</td>
<td>0</td>
<td>Gumpel et al., 1995</td>
</tr>
<tr>
<td>Mφ37</td>
<td><em>petB</em></td>
<td>cytochrome b6/f</td>
<td>5</td>
<td>Gumpel et al., 1995</td>
</tr>
<tr>
<td>F16</td>
<td><em>petD</em></td>
<td>cytochrome b6/f</td>
<td>0</td>
<td>Drager et al., 1998</td>
</tr>
</tbody>
</table>

*Table 1.2* Nuclear mutants of *C. reinhardtii* affected in chloroplast mRNA

Finally, one under-studied process that may be involved in the control of mRNA
stability in *Chlamydomonas* is polyadenylation. Recent evidence has shown that in
spinach polyadenylation of *petD* mRNA promotes degradation of the message, and so
may act as a mechanism for regulation of chloroplast transcript degradation (Kudla et
al., 1996). Whether mRNA polyadenylation, which is normally associated with nuclear
gene expression, occurs in the *Chlamydomonas* chloroplast awaits investigation.
1.2.4.3 Translational regulation

Unlike bacterial gene expression, transcription and translation of chloroplast genes do not occur concomitantly. As described above, chloroplast gene transcripts are stabilised and processed by trans-acting factors that are largely unidentified. Targeting of these factors to specific transcripts is signalled by cis-elements, frequently found in the 5' and 3' UTRs. Once stable and correctly processed, the mature transcript is translated into the nascent polypeptide via prokaryotic-type translation apparatus. The structure of the chloroplast ribosome is very similar to its prokaryotic counterpart. However the mechanism of translational initiation may differ. For example, the *C. reinhardtii* 16S rRNA is highly conserved and contains anti-Shine-Dalgarno (SD) sequences, whereas several chloroplast genes are missing complementary SD sequences and when present are hyper-variable in position and nucleotide composition (Gillham, 1994). Translation is a key step in control of chloroplast gene expression and several mutants of *C. reinhardtii* affected in chloroplast translation have been isolated and characterised (reviewed in Hauser *et al.*, 1996; Rochaix, 1996). A model has been proposed for the thylakoid localised translation of chloroplast membrane proteins based on *in vitro* and mutant analysis of transcript binding/stabilising factors (Rochaix, 1996). This model is summarised below (detailed discussion in Nickelson and Rochaix, 1994).

The two core proteins of PSII, involved in primary charge separation and electron transport, are D1 and D2, encoded by the chloroplast genes *psbA* and *psbD* respectively. The 74 base 5' UTR of the *psbD* transcript was found to interact *in vitro* with a 47 kDa protein. Whereas, once the 5' UTR is processed to 47 bases the transcript did not interact, indicating the binding motif for the 47 kDa protein is in the region cleaved by processing (Nickelson and Rochaix, 1994). mRNA binding proteins in the range of 47 kDa have also been reported to interact with several other chloroplast gene transcripts (Danon and Mayfield, 1991; Zerges and Rochaix, 1994; Hauser *et al.*, 1998). Stability of the *psbD* mRNA is specifically affected by mutations at the nuclear locus *NAC2*, probably because the *psbD* 5' leader interacts with the product of the *NAC2* gene (Kuchka *et al.*, 1989). The *Nac2* cDNA has recently been cloned and the main portion of its predicted polypeptide contain motifs that are known to mediate protein-protein interactions. The C-terminus of the Nac2 protein contains a hydrophobic stretch capable of forming a single putative membrane spanning helix. Nac2 has therefore been tentatively characterised as a peripheral membrane spanning protein (Nickelson and Rochaix unpublished, in Nickelson, 1998). Combining these data, it was proposed that the 47 kDa protein described above is bound to the 5' end of the *psbD* mRNA, and also interacts with the Nac2 factor. Nac2 is thought to be membrane...
associated, causing the whole complex to be anchored to either the thylakoid or chloroplast envelope by the hydrophobic tail of Nac2 (Fig 1.8). A putative endonuclease then thought to process the 5' leader of the $psbD$ mRNA, excising it from the Nac2/47 kDa protein complex. The mature $psbD$ transcript is thereby freed from the Nac2/47 kDa complex allowing it access to the 30S ribosomal subunit, whose binding might otherwise be blocked by Nac2. 5' processing also allows the Nac2 complex to be recycled and bind another $psbD$ mRNA molecule. Due to the recent association of the $psbD$ mRNA with the Nac2/47 kDa membrane-bound complex, the nascent D2 polypeptide is able to insert directly into the thylakoid membrane. Similar models have been proposed to account for the generalised translation of all chloroplast membrane and stromal proteins (Hauser et al., 1998).

Figure 1.8 Model for the function of the $psbD$-translational stabilation factor, $Nac2$ (Nickelson and Rochaix, 1994). Details described in text. endo: putative endonuclease that processes the $psbD$ mRNA 5' end at position -47 (arrow).
1.3 Manipulating chloroplast genes

1.3.1 Chloroplast transformation

In this section I will describe the evolution of techniques for plant transformation with specific reference to transformation of the chloroplast genome. Most of the plethora of techniques which have found success in transformation of bacterial and animal cells have, at one time or another, been attempted in plants. One of the limiting factors specific to plant transformation is the presence of a cell wall. One way to remove this barrier is to enzymatically degrade the cell wall to produce a protoplast. Protoplasts have been used extensively as a host for the introduction of exogenous DNA, and once transformed can be used to regenerate mature, transgenic plants (Vasil, 1983).

Techniques used to transform protoplasts include temperature shock (Potrykus et al., 1985), use of surface binding agents such as PEG or calcium phosphate (Lorz et al., 1985), electroporation (Fromm et al., 1985; Ou-Lee et al., 1986), phagocytosis of transforming DNA via liposomes, and microinjection (reviewed in Steinbiss and Broughton, 1983). The problem with protoplast transformation is that it is limited to those species which are amenable to the isolation and culture of protoplasts. Initially workers were unable to produce protoplasts from several commercially important groups such as cereals and legumes (Steinbiss and Broughton, 1983). These difficulties have now been overcome and protoplasts from species such as maize, rice and wheat have been transformed using electroporation (Fromm et al., 1985; Ou-Lee et al., 1986) and heat shock (Potrykus et al., 1985). Nuclear transformation of dicotyledonous species can also be achieved using the parasitic bacterium *Agrobacterium tumefaciens*. *A. tumefaciens*, the causative agent of crown gall disease, naturally infects and subsequently transforms the nuclear genome of several species of dicotyledonous plants as part of its life cycle. Using recombinant techniques the genes normally transferred to the host can be replaced with a foreign gene(s), such that the bacterium is fooled into transferring this gene(s) instead (reviewed by Zupan and Zambryski, 1995; Tinland, 1996; Azpiroz-Leehan and Feldmann, 1997) *A. tumefaciens* mediated transformation has been used extensively, but due to host specificity cannot be used for transformation of most monocotyledonous species.

The chloroplast genome has proven more recalcitrant to transformation than its nuclear counterpart. To enter the plastid compartment DNA must gain access past the cell wall and three membranes before eventually reaching its target. Early attempts to circumvent this problem included production of "cybrid" plants. Cybrids are the outcome of specialised protoplast fusion resulting in a plant containing the nuclear genome from
one protoplast but chloroplasts (or mitochondria) from the fusion partner (reviewed by Galun and Aviv, 1986). To achieve this feat protoplasts from a donor strain have their nuclei inactivated by irradiation or removed by micromanipulation. These donor protoplasts are then fused with an untreated recipient resulting in a cybrid containing one functional nuclear genome (from the recipient), and a mixture of organelles from donor and recipient cells. Plant regeneration inevitably leads to segregation of the donor and recipient organelles. The resultant mature plants will contain one or other, or occasionally both types of chloroplast. Cybrid experiments have been used to transfer specific chloroplast encoded traits such as drug resistance or pigmentation deficiency to a new strain, or to determine whether a particular phenotype is conferred by the plastid or the nuclear genome (reviewed by Galun and Aviv, 1986; e.g. Koop et al., 1992).

Other early attempts at chloroplast transformation focused on introduction of DNA into chloroplasts of isolated protoplasts (Daniell and McFadden, 1987). Transfer of the 'transplastome' to a different strain was then achieved by protoplast fusion to an albino recipient, a technique termed 'organelle cloning' (Koop et al., 1992).

Definitive chloroplast transformation, (i.e. uptake of exogenous genetic material, which is expressed to confer a novel phenotype, thereby "transforming" the organism) has been most successfully achieved using particle-gun bombardment (see below). Several other methods have been employed to achieve the same goal, with varying degrees of success (Table 1.3). Chloramphenicol resistance (CmR) and chloramphenicol acetyltransferase activity were demonstrated in the chloroplasts of tobacco plants transformed with a CmR gene using A. tumefaciens (De Block et al., 1985; Venkateswarlu and Nazar, 1991). Weber et al., (1989) described the use of a UV-laser microbeam to temporarily pierce, and allow subsequent uptake, of DNA into isolated chloroplasts of Brassica napus (L.). A. tumefaciens and laser beam mediated plastid transformation have not gained popularity, whereas a more reliable method would appear to be polyethylene glycol (PEG) mediated plastid transformation. PEG mediated protoplast transformation has proven a successful method for transforming the nuclear genome in plants (Lorz et al., 1985). The same technique has since been used to transform the plastid genome in Nicotiana spp. with a rDNA conferring antibiotic resistance (O'Neill et al., 1993), and a bacterial antibiotic resistance gene (Koop et al., 1996; reviewed by Kofer et al., 1998a). In C. reinhardtii glass bead transformation, a method highly efficient for obtaining nuclear transformants, was successfully used to obtain chloroplast transformants (Kindle et al., 1991). Although genuine chloroplast transformants were obtained, the rate of transformation was between 1-2 orders of magnitude lower than the particle-bombardment method, and also required enzymatic removal of the cell wall (Kindle et al., 1991).
<table>
<thead>
<tr>
<th>Method used for DNA (or RNA) introduction into the chloroplast</th>
<th>Host organism(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. tumefaciens</em> mediated</td>
<td>Tobacco</td>
<td>De Block et al., 1985; Venkateswarlu and Nazar, 1991</td>
</tr>
<tr>
<td>UV-laser microbeam mediated</td>
<td>Isolated chloroplasts of <em>Brassica napus</em> (L.).</td>
<td>Weber et al., 1989</td>
</tr>
<tr>
<td>Polyethylene glycol (PEG) mediated</td>
<td>Tobacco</td>
<td>O’Neill et al., 1993; Koop et al., 1996</td>
</tr>
<tr>
<td>Glass bead agitation</td>
<td><em>C. reinhardtii</em></td>
<td>Kindle et al., 1991</td>
</tr>
<tr>
<td>Galistan expansion microsyringe (GEF)</td>
<td>Tobacco</td>
<td>J. C. Gray, unpublished</td>
</tr>
<tr>
<td>Particle-gun-bombardent</td>
<td>Algae and higher plants</td>
<td>See text</td>
</tr>
</tbody>
</table>

**Table 1.3 Techniques used to transform the chloroplast**

The methods described above for transferring exogenous DNA into chloroplasts have met with limited success. This is mainly due to complexity of technique, and host specificity. Methods such as PEG and laser-beam mediated transformation rely on protoplasts, whose preparation is involved and only possible for a minority of species. The use of *Agrobacterium* vectors suffers from the limited host range of the bacterium, which excludes many commercially valuable monocotyledonous plants. Because it is not limited by such constraints, particle-gun-bombardment was envisaged as a possible novel method for delivering exogenous genetic material into the chloroplast (reviewed by Sanford, 1988). Particle-gun-bombardment (or biological-ballistics, "biolistics") was first demonstrated by Sanford and colleagues (Klein et al., 1987; Sanford et al., 1987). In these studies a gun powder discharge was used to fire a macroprojectile, tipped with tungsten microprojectiles, down a gun barrel aimed at onion (*Allium cepa*) epidermal cells. Once the macroprojectile reached the end of the barrel it hit a stopping plate containing a small aperture which allowed the tungsten microprojectiles to pass through and penetrate the onion tissue. The tungsten microprojectiles were coated with tobacco mosaic virus (TMV) RNA, which upon delivery into the cell was thought to disperse in the cytoplasm. Three days after bombardment, crystalline inclusions typical of TMV could be seen by microscopic examination in 30-40% of cells that contained macroprojectiles. Therefore, these cells had been transformed with, and were
transiently expressing the TMV RNA. The authors proposed that this method of
delivery of substances into living cells was superior to existing methods in terms of
"scope of application, efficiency, and practicality" (Sanford et al., 1987), and arguably
have been proven correct.

A year after this first demonstration of transient transformation of plant tissue using
biolistics, the technique was used to stably transform the chloroplast in C. reinhardtii
(Fig. 1.9) (Boynton et al., 1988), and mitochondria in Saccharomyces cerevisiae
(Johnston et al., 1988; for review see Butow and Fox, 1990). Stable chloroplast
transformation was achieved by bombarding a non-photosynthetic C. reinhardtii mutant
containing a deletion in the atpB gene with wild-type copies of atpB DNA (Boynton et
al., 1988). Transformed cells were seen to grow photoautotrophically on minimal
medium and contain wild-type levels of α and β subunits of the ATP synthase complex
(Boynton et al., 1988). The rate of transformation was relatively low (2-4 x 10⁶), but
did not pose a problem as it was possible to bombard large numbers of cells (6 x 10⁶).
It was postulated that the introduced wild-type copy of atpB replaced the deletion allele
in the mutant by a double crossover or gene conversion event (Boynton et al., 1988).

This and similar studies (Blowers et al., 1989; Boynton et al., 1990), paved the way
for a new era in chloroplast biology, that of chloroplast reverse genetics. Chloroplast
morphology in C. reinhardtii (one single large cup-shaped chloroplast per cell) and
higher plants (approximately 100 small chloroplasts per cell) suggested biolistic
transformation of the higher plants may be more involved than the alga. However,
demonstration by separate groups of investigators that biolistics could be used to stably
transform the chloroplast in tobacco (Svab et al., 1990; Ye et al., 1990; Staub and
Maliga, 1992), bore out the principle of the utility of biolistic transformation (Sanford et
al., 1987). Moreover, Debuchy et al. (1989) showed that particle-gun-bombardment
could also be used for transformation of the C. reinhardtii nuclear genome.

Since the "taming of the plastid genome" (Chasan, 1992) by biolistic transformation,
delivery of genetic material into the genomes of a multitude of previously impregnable
organisms has been achieved. These include Drosophila embryos (Baldarelli and
Lengyel, 1990), fertilised fish eggs (Zelenin et al., 1991), mouse and chicken
epidermal cells (Fynan et al., 1993), amphibian limb epidermal tissue (Maden, 1994),
and Xenopus embryos and ovary tissue (Cheng and Joho, 1994; Koster et al., 1996).
Several of the reports are only preliminary studies, but it is obvious that the biolistic
device has universal application.
Figure 1.9 Chloroplast transformation of *Chlamydomonas* using particle-gun-bombardment.
1.3.2 Transformation strategies

As described in the previous section, the advent of biolistics has made the chloroplast genome routinely accessible to genetic engineering. Particle-gun-bombardment allows genetic material to be delivered into a cell or tissue without pre-treatment, and does not suffer from problems of host specificity. Once inside, the exogenous genetic material must be expressed in order to confer a novel phenotype. A cell expressing the introduced material is then referred to as a "transformant". In this section I will consider the variety of genes and genetic techniques used to produce chloroplast transformants.

Once genetic material has been delivered into the chloroplast it can be either expressed transiently or incorporated into the organelle genome and stably expressed. In the former case the introduced material is temporarily expressed but is not incorporated into, or recognised by the chloroplast genome replication machinery and so is rapidly lost on cell division. In the latter case the introduced DNA must be incorporated, either homologously or heterologously, into the chloroplast genome. Homologous recombination of introduced DNA is an unpredictable process, occurring readily in some species such as the nuclear genome in budding yeast, but rarely in others, such as the nuclear genome in *C. reinhardtii* and *Arabidopsis* (Bouchez and Herman, 1998; Davies and Grossman, 1998). The reasons for such disparities are not well understood. Moreover, lack of homologous recombination of transforming DNA in an organism can severely hamper "gene knockout" experiments which are routine in other models (discussed in Kindle, 1998). Homologous recombination does occur readily in the chloroplast genome of *C. reinhardtii* enabling "chloroplast DNA surgery" (Rochaix, 1995), meaning introduced DNA can be targeted to a precise location anywhere within the genome. Fragments of homologous chloroplast DNA delivered into the cell will recombine with their genomic partner. Alternatively, heterologous DNA can be inserted into the chloroplast genome by flanking it with adjacent stretches of chloroplast DNA. If, as readily occurs, a double cross-over event takes place between the flanking DNA and its genomic partner the heterologous DNA will be incorporated at the same time.

Stable transformation of extrachromosomal DNA in the *C. reinhardtii* chloroplast has only been reported once (see below), and is probably atypical in this organism (Kindle et al., 1994).

A prerequisite for the isolation of chloroplast transformants is that the introduced DNA should, when expressed, confer a selectable phenotype normally absent in the host strain. In *Chlamydomonas* several different classes of gene have been used as a "marker" of chloroplast transformation. Many studies, have used reinstatement of the
ability to grow photosynthetically in a non-photosynthetic mutant as a marker for transformation (e.g. Boynton et al., 1988). This type of transformation strategy uses a host strain with a single mutation in a chloroplast-encoded photosynthetic gene. A functional copy of the corresponding gene is introduced into this strain which restores photosynthetic capability. This type of strategy can even be refined by varying the light intensity under which transformants are selected, to introduce photosynthetic genes with specific mutations. For example, Redding et al. (1998), used moderate light levels to differentiate between transformants containing point mutations in one or other, or both psaA and psaB. The host strain used contained deletions in psaA and psaB and was unable to grow under any light levels. Upon co-transformation with copies of psaA and psaB both containing point mutations, only strains containing both the subtly altered genes were able to grow under moderate light (Redding et al., 1998). Strains containing only one of the introduced genes remained sensitive to moderate light levels. An advantage in using photosynthetic growth as a marker for transformation, where the host strain contains a deletion in a photosynthetic gene, is that false positives (i.e. photosynthetic revertants) are rare.

An alternate type of marker used for chloroplast transformation is drug resistance. Point mutations in chloroplast rRNAs frequently confer resistance to aminoglycoside antibiotics such as streptomycin, spectinomycin and erythromycin. Transformation of wild-type cells using mutant rDNAs can be used to isolate chloroplast transformants by selection for their antibiotic resistant phenotype (Newman et al., 1990; Kindle et al., 1991; Newman et al., 1991; Roffey et al., 1991). Mutations in the PSII gene psbA, that confer resistance to herbicides such as metribuzin or DCMU have also been used as selectable markers (Newman et al., 1991; Przibilla et al., 1991). Marker genes conferring drug resistance are superior to photosynthetic markers (see above), in that they can be used in any strain rather than one specific mutant. However, both types of markers are of limited use in that transforming DNA is always restricted to insertion in one specific region of the genome, adjacent to the marker. Also, psbA and 16S rRNA mutations conferring herbicide and antibiotic resistance respectively, have both been shown to affect photosynthetic activity (Heifetz et al., 1997). Such indirect effects need to be taken into account, especially when using these markers to study photosynthetic function.

One limitation of both the photosynthetic and drug resistance markers described above is the need to isolate an appropriate photosynthetic mutant host or a mutated gene conferring drug resistance. These prerequisites severely restrict the number and type of genes open to manipulation. One strategy which circumvents this problem is to introduce novel mutations by co-transformation (Kindle et al., 1991; Newman et al.,
Kindle et al. (1991) simultaneously transformed two plasmids, one containing the wild-type \textit{atpB} gene and a second containing a mutant 16S rDNA conferring spectinomycin resistance, into an \textit{atpB} deletion mutant host strain. Transformants were initially selected on spectinomycin-containing medium, and then transferred to minimal medium to assess their ability to grow photosynthetically. Although initially not selected for this phenotype, approximately three-quarters of the transformants were capable of photosynthetic growth, having incorporated both the mutated 16S rDNA and wild-type \textit{atpB} genes (Kindle et al., 1991).

For \textit{Chlamydomonas} chloroplast transformation, both photosynthetic and point mutation gene markers have now been superseded by a bacterial antibiotic resistance marker gene. The \textit{aadA} gene, encoding resistance to the aminoglycoside antibiotics spectinomycin and streptomycin, is sandwiched between a \textit{C. reinhardtii} chloroplast promoter and 3' UTR (Goldschmidt-Clermont, 1991a). When introduced into the chloroplast genome this chimeric cassette readily expresses the aminoglycoside 3'-adenyl transferase (AAD) protein. Although in a foreign environment, AAD is able to inactivate spectinomycin/streptomycin allowing the cell to survive in medium containing these antibiotics. The \textit{aadA} cassette has several obvious advantages over the types of marker described previously (reviewed in Goldscmidt-Clermont, 1998b). Primarily, it is portable. i.e. by flanking the cassette with the appropriate DNA, it can be targeted to any site in the genome. This means that any chloroplast gene can be deleted or investigated by site-directed mutagenesis by inserting \textit{aadA} into the appropriate construct. \textit{aadA} can also be used as a reporter for studying chloroplast gene expression (section 1.3.4 Goldschmidt-Clermont, 1991a; Klein et al., 1992).

Due to the absence of a second portable marker, strategies have been developed to remove \textit{aadA} from the chloroplast genome. "recycling" of the \textit{aadA} cassette enables secondary mutations to be introduced into the chloroplast genome of the same strain. \textit{aadA} can be introduced and subsequently removed from the chloroplast genome in one of two ways: Firstly, the \textit{aadA} cassette can be juxtaposed with short direct repeats. In a chloroplast transformant containing this modified \textit{aadA} cassette, removal of selective pressure permits excision of the cassette by recombination of the repeats (Fischer et al., 1996). This phenomenon occurs readily, leading to loss of the cassette after a few rounds of subcloning (Fischer et al., 1996). The same result can also be achieved by disrupting the coding sequence of an essential ORF by inserting the \textit{aadA} cassette (\textit{aadA}::\textit{eORF}). For the cell to remain viable, all 80 copies of an essential chloroplast gene cannot be inactivated. The \textit{aadA}::\textit{eORF} vector is co-transformed with a second plasmid containing the gene of interest (\textit{\textit{gene} x'}), such as a mutagenised photosynthetic
gene. Selective pressure forces gene x to reach homoplasy, whereas aadA::eORF remains heteroplasmic because all copies of the essential ORF cannot be inactivated for the cell to remain viable. On removal of selective pressure the homoplasmic gene x remains stably incorporated in the chloroplast genome while the heteroplasmic aadA cassette is rapidly lost (Fischer et al., 1996). Although these methods complicate experimental design they do provide a system for introducing serial mutations into the same strain.

Another difficulty in the manipulation of some chloroplast genes is their large size due to the presence of introns (section 1.2.4.2). In C. reinhardtii, psbA is located on the inverted repeat and contains four introns, complicating its study by site-directed mutagenesis. Synthetic, intronless versions of psbA, when introduced into the chloroplast have been shown to complement deletions in the endogenous copy (Johanningmeier and Heiss, 1993; Mayfield et al., 1994; Minagawa and Crofts, 1994). These experiments also demonstrate the feasibility of using cDNAs to study intron containing chloroplast genes.

Extrachromosomal genetic elements such as plasmids and artificial chromosomes are commonly used as vectors for transformation. Isolation of plasmids or other extrachromosomal elements from the chloroplast of wild-type Chlamydomonas spp. has so far not been reported (Harris, 1989). Autonomous replicating plasmids are potentially useful for studying chloroplast biology as they allow stable transformation without modification of the genome. The only report of a stable extrachromosomal element in the chloroplast of C. reinhardtii is of a high copy number (approximately 200 copies per cell), episomal tandem repeat sequence (Kindle et al., 1994). This episomal DNA was apparently the result of transformation of an atpB deletion strain with a truncated copy of atpB, lacking a 3' inverted repeat thought to affect RNA stability. Strains containing the episomal tandem repeat had a more photosynthetically robust phenotype than the deletion mutant, presumably as a result of the additional copies of (a truncated) atpB. This phenomenon may be unique to the atpB locus as similar reports using other chloroplast genes have not been forthcoming. However, the high copy number of the episomal DNA might potentially be adapted for overexpressing genes in the Chlamydomonas chloroplast. A more conventional extrachromosomal element was isolated during transformation experiments in tobacco. The NICE1 (Nicotiana plastid extrachromosomal) element is a circular 868 bp DNA molecule derived from the rDNA region and contains two imperfect direct repeats present in the plastid genome (Staub and Maliga, 1994b). A vector derived from the NICE1 element was introduced into the tobacco chloroplast and appeared to be present both extra- and intrachromosomally in
transformed lines. Whether this vector is strictly extrachromosomal or is being continually excised from transformed genomes is unclear (Staub and Maliga, 1994b).

1.3.3 Chloroplast reverse genetics

The ability to deliver exogenous DNA into the chloroplast and efficiently select transformants has made it possible to manipulate chloroplast genes in a variety of ways. The transformation of organisms with genes which have been manipulated in vitro is known broadly as "reverse genetics". The most common application of chloroplast reverse genetics has been gene inactivation. This enables one to observe the phenotypic effects of removal of a specific chloroplast gene product, and so gain insight into its function e.g., the effect that deletion of one of the core subunits of PSII has on assembly of the photosystem. In the last decade a large proportion of Chlamydomonas chloroplast genes, and some tobacco chloroplast genes, have been inactivated in this way (Webber et al., 1995; Erickson, 1996; Rochaix, 1997). Gene inactivation can be achieved either by disrupting or deleting a section of the gene of interest with a selectable marker (Fig. 1.10). To date, most targeted chloroplast gene inactivations in Chlamydomonas using this reverse genetic approach have utilised the aadA selectable marker (section 1.3.2). The C. reinhardtii chloroplast genome is present in approximately 80 copies per cell (Harris, 1989), while initial transformants probably only contain a few copies of the introduced construct. Therefore, when introducing recessive mutations such as gene disruptions, transformants must be subcloned on selective medium. This subcloning favours survival of progeny with increasing numbers of copies of the selectable marker, resulting in one of two outcomes. If the gene is not essential, such as a photosynthetic gene when cells are supplied with a reduced carbon source, all 80 copies can be inactivated (e.g. O'Connor et al., 1998). Alternatively, if the gene is essential, wild-type and mutant copies will never completely segregate but will remain heteroplasmic, always maintaining active copies of the gene. In this case, removal of selective pressure leads to rapid loss of the mutant allele (e.g. Fischer et al., 1996).
**Figure 1.10** Various methods for disruption of chloroplast genes using the *aadA* cassette, a dominant selectable marker encoding spectinomycin resistance. The marker can be used to knock-out the gene of interest either by replacement (panel b) or disruption (panel c) by selection for spectinomycin resistance. More subtle changes can be introduced by placing the marker adjacent to a site-directed mutant copy of the gene (panel a). SpcS: cells sensitive to spectinomycin; SpcR: cells resistant to spectinomycin; gene x-m: site-directed mutant copy of the gene x.

*C. reinhardtii* has been the main conduit for the disruption or alteration of chloroplast genes encoding components of the photosynthetic apparatus (reviewed in Webber *et al.*, 1995; Erickson, 1996; Rochaix, 1997). *psbK* encodes the small 4 kDa K polypeptide of PSII (Ruffle and Sayre, 1998), and its inactivation is a typical example of the power of *Chlamydomonas* reverse genetics for studying the photosynthetic reaction centres. Biochemical data previously suggested that the K polypeptide was not part of the core complex of PSII but may be peripherally associated (Erickson, 1998). Disruption of *psbK* using the *aadA* cassette resulted in transformants that were unable to grow...
photosynthetically and accumulated PSII to only 10% of wild-type levels (Takahashi et al., 1994). When taken together, the genetic and biochemical data suggest the K polypeptide is essential for the stable accumulation and/or assembly of PSII (Takahashi et al., 1994).

Subtle interactions within and between proteins, and between proteins and accessory pigments of the photosynthetic complexes can be studied by site-directed mutagenesis. In C. reinhardtii this has been achieved by linking the aadA cassette to a site-directed mutant copy of a photosynthetic gene, then transforming this construct by selection on spectinomycin-containing medium. In a proportion of the transformants recombination will occur with the endogenous gene outside the mutagenised region, thereby incorporating the mutations along with the aadA cassette (Fig. 1.10). This strategy was used to introduce site-directed mutations into the Fe-S\textsubscript{x} iron-sulphur centre of PSI (Hallahan et al., 1995). Fe-S\textsubscript{x} is a crucial membrane bound component of the PSI electron transfer chain. The PSI core complex centres around a heterodimer encoded by the genes psaA and psaB, and contains a highly conserved region within which Fe-S\textsubscript{x} is thought to be bound by specific cysteine residues (Evans and Nugent, 1993). Particle-gun bombardment was used to introduce mutated copies of psaA such that the proposed Fe-S\textsubscript{x} binding cysteine was changed to aspartate or histidine. The effect these alterations had on the structure and function of PSI was then probed using electron paramagnetic resonance spectroscopy (Evans and Nugent, 1993). Studies such as this involve screening of initial transformants to confirm presence of the mutation and then subcloning to obtain homoplasmic lines. A more efficient strategy is to use a strain containing a deletion in the gene of interest as a host for the introduction of site-directed mutations. Any phenotype resulting from the introduced changes is then visible immediately as it will be present in a null background, negating screening and subcloning of the transformant (Stevens and Purton, unpublished; Minagawa and Crofts, 1994).

Aside from gene inactivation, another application of chloroplast transformation has been the use of reporter genes to study the elements controlling chloroplast gene expression. Commonly foreign, bacterial genes are used as reporters and so discussion of these in Chlamydomonas will be included in the next section (1.3.4).

Before the advent of biolistics many Chlamydomonas chloroplast mutants had been isolated after standard UV-light or chemical mutagenesis (Harris, 1989). Particle-gun bombardment enables mapping of these randomly generated mutants by successive introduction of contiguous fragments of wild-type chloroplast DNA, i.e. complementation. Using this approach the loci responsible for the phenotype of several
of these 'traditional' mutants have been mapped and the gene affected identified (e.g. Goldschmidt-Clermont et al., 1991b; Ketchner et al., 1995). One such study mapped the mutation responsible for the defective accumulation of the ATP synthase complex in the mutant FUD16. This mutant was known to accumulate barely significant levels of (C)F1, the extrinsic soluble portion of ATP synthase responsible for nucleotide binding and ATP catalysis (Strotmann et al., 1998). Using biolistic transformation, the (responsible) mutation was mapped and found to be a pair of missense codons in the gene atpA, encoding the α subunit of ATP synthase (Ketchner et al., 1995). An interesting secondary phenotype in this mutant was that rather than being degraded the defective α subunit overaccumulated and formed, in association with the β subunit, inclusion bodies within the chloroplast (Ketchner et al., 1995). These inclusion bodies were detectable immunologically and ultrastructurally, and were the first demonstration of such structures in chloroplasts or mitochondria. However, the reason that this specific mutation produces inclusion bodies is not understood, especially when considering that the expression of chloroplast genes is usually tightly controlled at the post-transcriptional level (section 1.2.4 and Rochaix, 1996).

Manipulation of chloroplast genes via deletion/disruption and reporter constructs has provided valuable insight into the molecular machinery of the chloroplast. Such studies will surely continue, ideally resulting in a bank of C. reinhardtii strains in which every non-essential chloroplast gene has been inactivated. Other, more isolated studies have demonstrated how molecular genetic techniques developed in other systems easily translate to the Chlamydomonas chloroplast genome. Chen et al. (1993) used particle-gun bombardment to introduce copies of the petD gene with mutations in the initiation codon. Instead of ATG the mutated copies contained AUU or AUC in the same position. petD encodes subunit IV of the cytochrome b6/f complex and is required for photosynthetic electron transport, therefore initiation codon mutations in petD might affect transformants' ability to grow photosynthetically. Although only a small decrease was seen at room temperature, at 35 °C transformants accumulated <5 % as much subunit IV as wild-type cells grown at the same temperature and were unable to grow on minimal medium (Chen et al., 1993). Introduction of initiation codon mutations to chloroplast genes, and identification of phenotypic suppressors, should provide insight into the mechanisms controlling chloroplast translation initiation.

Finally, aside from alteration or removal, chloroplast transformation can also be used to add amino acids to chloroplast encoded proteins. Recently, Sugiura et al. (1998) added a His-tag encoding oligonucleotide to the 3' terminus of psbD. The six histidine residues at the carboxy-terminus of the D2 protein of PSII did not the affect
photosynthetic ability of the transformants but enabled the core complex to be rapidly isolated on a Ni²⁺ affinity column (Sugiura et al., 1998). The isolated complex retained a high oxygen-evolving activity (Sugiura et al., 1998).

1.3.4 Foreign gene expression in the chloroplast

The expression of foreign genes in the chloroplast is possible only because plastids contain an independent genetic system. Of course, most of the proteins within the chloroplast are synthesised in the cytosol and post-translationally targeted to the stroma. Foreign genes expressed in the nucleus can mimic this process and be targeted to the chloroplast by the addition of a chloroplast transit peptide (Verwoert et al., 1994; Shikanai et al., 1998). Verwoert et al. (1994) introduced the E. coli fabD gene, encoding malonyl coenzyme A-acyl carrier transacylase (MCAT), into the nuclear genome of transgenic rape and tobacco seeds using A. tumefaciens-mediated transformation. MCAT is part of the fatty acid synthetase system that enables de novo fatty acid biosynthesis in bacteria and higher plants (chloroplasts). It is hoped that transgenic alteration of fatty acid synthesis in plants will permit manipulation of the oil content of seeds in plants such as rape. By combining a developmentally regulated chimeric promoter and transit peptide sequence MCAT was targeted to the plastid in a seed-specific and developmentally specific manner (Verwoert et al., 1994). Gene expression is generally down-regulated in proplastids and foreign genes expressed within the chloroplast may be repressed at particular stages of development, such as in the seed. Expression levels of foreign genes introduced into the nuclear genome and targeted to the plastid are controlled according to the chimeric promoter used. Therefore, such genes can be specifically expressed at high levels in proplastids or etioplasts at times when the expression of transgenes located in the plastid would be suppressed. However, this section will from now on be concerned with foreign genes introduced and expressed within the plastid organelle. The foreign genes expressed in the chloroplast to-date are listed in Table 14.
<table>
<thead>
<tr>
<th>Gene (protein)</th>
<th>Source organism</th>
<th>Host organism</th>
<th>Transient / stable expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>aadA</em> (aminoglycoside 3&quot; adenytransferase, AADA)</td>
<td>Enterobacteriaceae</td>
<td><em>C. reinhardtii</em></td>
<td>Stable</td>
<td>Goldschmidt-Clermont, 1991a</td>
</tr>
<tr>
<td><em>uidA</em> (β-glucuronidase, GUS)</td>
<td><em>E. coli</em></td>
<td><em>C. reinhardtii</em></td>
<td>Stable</td>
<td>Sakamoto <em>et al.</em>, 1993</td>
</tr>
<tr>
<td><em>recA</em> (RecA)</td>
<td><em>E. coli</em></td>
<td><em>C. reinhardtii</em></td>
<td>Stable</td>
<td>Cerutti <em>et al.</em>, 1995</td>
</tr>
<tr>
<td>Luciferase</td>
<td><em>Renilla</em></td>
<td><em>C. reinhardtii</em></td>
<td>Stable</td>
<td>D. Herrin, unpublished</td>
</tr>
<tr>
<td><em>aphA-6</em> (3'-aminoglycoside phosphotransferase, APH(3')VI)</td>
<td><em>Acinetobacter baumannii</em></td>
<td><em>C. reinhardtii</em></td>
<td>Stable</td>
<td>This study</td>
</tr>
<tr>
<td><em>cat</em> (chloramphenicol acetyltransferase, CAT)</td>
<td><em>E. coli</em></td>
<td>cucumber (etioplasts); <em>Nicotiana tabacum</em> (cultured NT1 cells)</td>
<td>Transient</td>
<td>Daniell and McFadden, 1987; Daniell <em>et al.</em>, 1990</td>
</tr>
<tr>
<td><em>rbcL/rbcS</em></td>
<td><em>Anacystis nidulans</em></td>
<td>cucumber (etioplasts)</td>
<td>Transient</td>
<td>Daniell and McFadden, 1987</td>
</tr>
<tr>
<td><em>aadA</em> (aminoglycoside adenyltransferase, AADA)</td>
<td>Enterobacteriaceae</td>
<td><em>Nicotiana tabacum</em></td>
<td>Stable</td>
<td>Svab and Malaga, 1993</td>
</tr>
<tr>
<td><em>kan</em> (neomycin phosphotransferase, NPTII)</td>
<td><em>E. coli</em></td>
<td><em>Nicotiana tabacum</em></td>
<td>Stable</td>
<td>Carrer <em>et al.</em>, 1993</td>
</tr>
<tr>
<td><em>uidA</em> (β-glucuronidase, GUS)</td>
<td><em>E. coli</em></td>
<td>wheat leaves and calli; <em>Nicotinaina plumbaginifolia</em> (isolated chloroplasts); <em>Nicotiana tabacum</em></td>
<td>Transient; transient; stable;</td>
<td>Daniell <em>et al.</em>, 1991; Koop <em>et al.</em>, 1992; Staub and Maliga, 1993; McBride <em>et al.</em>, 1994</td>
</tr>
<tr>
<td><em>crylA(c)</em> (crystal toxin)</td>
<td><em>Bacillus thuringiensis</em></td>
<td><em>Nicotiana tabacum</em></td>
<td>Stable</td>
<td>McBride <em>et al.</em>, 1995</td>
</tr>
<tr>
<td><em>rbcL</em> (Rubisco large subunit)</td>
<td><em>Helianthus annuus</em> (sunflower)</td>
<td><em>Nicotiana tabacum</em></td>
<td>Stable</td>
<td>Kanevski <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>EPSPS (5-enolpyruvyl shikimate-3-phosphate synthase)</td>
<td>petunia</td>
<td><em>Nicotiana tabacum</em></td>
<td>Stable</td>
<td>Daniell <em>et al.</em>, 1998</td>
</tr>
<tr>
<td><em>gfp</em> (Green fluorescent protein, GFP)</td>
<td><em>Aequorea victoria</em></td>
<td><em>Nicotiana tabacum</em></td>
<td>Transient</td>
<td>Hibberd <em>et al.</em>, 1998; Knoblauch <em>et al.</em>, 1999</td>
</tr>
</tbody>
</table>

Table 1.4 Foreign genes expressed in the chloroplast
In order to recombine with, and be stably expressed in the chloroplast genome a foreign gene must be situated within a 'transformation cassette'. The transformation cassette varies according to the desired level of expression and location of the gene within the chloroplast genome. However, in general, all transformation cassettes will include the following elements (Fig. 1.11): 1. The promoter, untranslated leader and translation initiation ATG codon from a constitutively expressed (usually chloroplast) gene. 2. Restriction enzyme sites on or around the ATG where the coding sequence from the foreign gene can be inserted. 3. The 3' UTR from a chloroplast gene which will cause the chimeric mRNA to be recognised as an endogenous gene, thereby stabilising expression of the foreign gene transcript. 4. A selectable marker adjacent to the chimeric gene allowing phenotypic selection of transformants. 5. Chloroplast DNA, homologous to the region in which the foreign gene is to be inserted, flanking the chimeric gene which will permit homologous recombination of the transformation cassette. Once constructed, the transformation cassette can be delivered into the chloroplast (using particle-gun-bombardment), where it will inserted into the chloroplast genome via homologous recombination (Fig 1.10). Selection pressure is then used to isolate homoplasmic strains where every copy of the chloroplast genome contains the transformation cassette.
Figure 1.11 Generalised transformation cassette for the introduction of a foreign gene into the chloroplast genome. mcs: multiple cloning site; UTR: untranslated region.
1.3.4.1 Novel methods for elucidating chloroplast gene function and control

Molecular genetic analysis of chloroplast gene function using foreign genes has mainly been restricted to a few select organisms. The vast majority of studies have been performed in either the green alga *Chlamydomonas reinhardtii* or the dicotyledonous plant, tobacco (*Nicotiana tabacum*). *C. reinhardtii* is used for its properties as a model plant (section 1.2.1), and because it was the first organism in which the chloroplast was routinely transformable (1.3.1), and able to incorporate foreign DNA (Blowers *et al.*, 1989). However, the *C. reinhardtii* chloroplast does not pass through different developmental stages and does not become etiolated in the absence of light. Therefore, although production and analysis of transformants is more time consuming, plants such as tobacco or *Arabidopsis thaliana* are commonly used for studies of plastid development or tissue-specific gene expression.

**Selectable markers**

Foreign gene expression in the chloroplast has most frequently been applied to the production of selectable markers. Heterologous antibiotic resistance genes are usually used as selectable markers as they have the advantages of being dominant and portable, i.e. they can be targeted to any position in the chloroplast genome. The first of these genes to be adapted for use was the eubacterial gene *aadA* in the chloroplast of *C. reinhardtii* (section 1.3.2). The *aadA* gene has also been used in the same way to transform the chloroplast in tobacco leaves (Svab and Malaga, 1993; Zoubenko *et al.*, 1994). Transformed leaf sections are transferred to regeneration medium containing spectinomycin and initially become bleached, but resistant green shoots and calli expressing *aadA* appear after three to eight weeks. A second selectable marker for chloroplast transformation in tobacco has also been developed using the bacterial *kan* gene, conferring kanamycin resistance (Carrer *et al.*, 1993).

**Reporter genes**

Reporter genes are foreign coding sequences whose expression, at the level of transcription or translation, can be easily monitored. When housed within the controlling elements of an endogenous gene, expression of a reporter gene can provide insight into the function of these *cis*-acting elements. Selectable markers make useful
reporter genes: In *C. reinhardtii* the *aadA* gene has been used as a reporter gene to assess the level of expression conferred by different chloroplast promoters (Goldschmidt-Clermont, 1991a). The relative degree of antibiotic resistance of strains containing antibiotic resistance genes can be used as a crude measure of the level of gene expression. Also, unlike GUS, assaying antibiotic resistance is non-destructive. Enzymatic activity of another bacterial antibiotic resistance gene, chloramphenicol acetyltransferase (*cat*), has been used as a chloroplast reporter gene in cultured tobacco cells (Daniell *et al.*, 1990).

The *E. coli uidA* (β-glucuronidase, GUS) gene has been used extensively as a reporter of gene chloroplast gene expression. It has been used mainly to analyse transcriptional rates and mRNA stability conferred by 5' and 3' elements of various chloroplast genes (Blowers *et al.*, 1990; Blowes *et al.*, 1993; klein *et al.*, 1994). The expression of chimeric *uidA* reporter genes has also been used to investigate the regulation of chloroplast gene expression by light (Staub and Maliga, 1993; Salvador *et al.*, 1993b; Staub and Maliga, 1994a). There have also been isolated reports in which *uidA* has been used as a reporter of post-transcriptional chloroplast gene expression by assaying GUS enzymatic activity (Sakamoto *et al.*, 1993; Staub and Maliga, 1994a).

Ideally, reporter genes should allow real-time monitoring of gene expression in living cells. Luciferase and green fluorescent protein (GFP) fulfil this requirement and recently have both been expressed in the chloroplast. Hibberd *et al.* introduced a version of *gfp* modified for nuclear gene expression, into the tobacco chloroplast by particle-gun-bombardment. Using conventional fluorescence or confocal microscopy transient expression of GFP was detected in the chloroplast 2-4 days after bombardment (Hibberd *et al.*, 1998). The demonstration of *gfp* expression in the plastid opens the way to its use as a reporter of gene expression; a transformation marker; and as a fusion protein, a real-time monitor of protein targeting. Although there are as yet no reports of *gfp* expression in the chloroplast in *C. reinhardtii*, luciferase from the coral *Renilla* has been expressed (D. Herrin, unpublished). Upon addition of its substrate, coelenterazine, luciferase luminescence could be detected and quantified in liquid culture or in colonies. In future both *gfp* and luciferase should enable more immediate and accurate analysis of chloroplast gene expression.

Foreign gene expression has also been used to investigate RNA processing by observing whether heterologous gene transcripts are correctly processed. The results of these studies have been contrasting. For example, when exon II of the *Petunia hybrida* mitochondrial *coxII* gene was expressed in tobacco chloroplasts RNA editing of the
transcript at the normal sites was not detected (Sutton et al., 1995). However, when chimeric RNAs containing a mitochondrial intron from *Scenedesmus obliquus* or the *atpF* gene of spinach were expressed in the *C. reinhardtii* chloroplast, the *Scenedesmus obliquus* intron was correctly spliced but the spinach intron was not (Herdenberger et al., 1994; Deshpande et al., 1995).

In a novel set of experiments Cerutti et al. (1995) expressed the *recA* gene from *E. coli* in the chloroplast of *C. reinhardtii*. *E. coli* RecA expression dramatically increased the frequency of plastid DNA recombination, but had no effect on DNA repair or cell survival (Cerutti et al., 1995). Therefore, the chloroplast probably uses a bacterial-type mechanism for recombination of its genome. Presumably the genes for components of the *C. reinhardtii* chloroplast recombination machinery have been transferred to the nuclear genome and are post-translationally targeted to the chloroplast. Because of the size of the nuclear genome many of the genes expressing proteins that are targeted to the chloroplast have not been identified. This study demonstrates the potential for identifying these products by expressing a homologue in the chloroplast.

1.3.4.2 Overexpression of heterologous proteins

The ability to introduce and express foreign genes in the chloroplast has great potential for the over-expression of heterologous proteins. The chloroplast genome has a natural aptitude for high level gene expression because in plants it is often present at up to 10,000 copies per cell. Expression of chloroplast genes is not simply dependent on copy number but involves a complex interaction of *cis* and *trans*-acting elements affecting almost every stage of expression (sections 1.1.3.4 and 1.2.4). Therefore, to release the potential levels of expression of the huge ploidy of chloroplast transgenes these factors must also be taken into account.

The most direct application of over-expression of foreign genes in the chloroplast is industrial. Not only is there the potential for high levels of expression, but the recombinant protein also comes pre-packaged in the plastid. Chloroplast-bound gene products are less likely to be subject to secondary modifications, such as glycosylation, which might be added during the processing of cytosolic proteins. With respect to industrial applications, the most suitable organism for the large scale production of chloroplast recombinant protein is an alga such as *Chlamydomonas*. *Chlamydomonas* can be grown rapidly in large quantities and, when grown photoautotrophically, needs little more than a mixture of minerals to be added to the growth medium. Cell wall-less
*Chlamydomonas* strains are also available which would simplify purification of the recombinant protein. For the over-expression of hydrophobic proteins a possible approach using *Chlamydomonas* might be to add a thylakoid targeting sequence to the foreign gene. The recombinant protein would saturate the thylakoid membrane causing severe retardation in the assembly of the photosynthetic complexes. However, *C. reinhardtii* can grow heterotrophically when acetate is added to the medium. The deleterious affect of the recombinant protein on photosynthesis could thereby be circumvented. Isolation of thylakoid membranes, rich in the recombinant protein, could then be used as a simple initial stage of purification.

Although all the factors affecting expression genes are not understood, several heterologous proteins have already been overexpressed in plastids. Under the control of the rRNA operon promoter, *Prrm*, the *crylA(c)* gene, encoding *Bacillus thuringiensis* (*Bt*) crystal toxin, was expressed in transgenic tobacco plants which accumulated 3-5% of their total soluble protein as CrylA(c) protoxin (McBride et al., 1995). *Bt* toxins are powerful insecticides and *Bt* genes have been used, via transformation into the nuclear genome, to produce transgenic strains of commercially valuable plants. However, the level of accumulation of CrylA(c) in the plastid transformants was remarkably high. As a result an insect species (*Spodoptera exigua*, beet armyworm), normally resistant to CrylA(C) protoxin suffered a high rate of mortality when fed on the plastid transgene containing strains (McBride et al., 1995). High levels of GUS (2.5% of total protein) and neomycin phosphotransferase (NPTII) (1% of total protein) expression were also detected in chloroplast transformants containing *uidA* and *kan* genes respectively (Carrer et al., 1993; Staub and Maliga, 1993; McBride et al., 1994).

Aside from the commercial value in overexpressing foreign genes in the chloroplast, the same technology could be adapted to improving the nutritional value of plants. The quasi-bacterial nature of chloroplast gene expression may permit the introduction of whole operons encoding heterologous metabolic pathways. Heterologous expression of two different foreign genes consecutively from the same chimeric promoter has already been achieved in tobacco chloroplasts (Daniell et al., 1998). Transgenic plastids (transplastomes) could be used to house a variety of novel biosynthetic pathways. Perhaps such technology could lead to the production of 'fortified crops' producing vitamins or nutritional valuable cofactors within the plastid of crop plants.
1.3.4.3 Manipulation of metabolic pathways

**Improving enzymatic efficiency**

Another potential application of chloroplast foreign gene expression is the manipulation of enzyme efficiency. Increasing the efficiency of photosynthesis in plants using molecular genetics is a long term aim of much of the research carried out in this field. Moreover, the plastid is not only the site of CO₂ fixation, but also houses the synthesis of amino acids, nucleotides, fatty acids, pigments, lipids and starch (section 1.1.1). The production of each of these requires distinct biosynthetic pathways involving many enzymes, which also have the potential for transgenic manipulation. One method for increasing enzyme efficiency is to perform site-directed-mutagenesis of putative key residues. However, this strategy commonly has unpredictable, and usually deleterious affects on the efficiency of the enzyme. In this case the use of a foreign gene can be beneficial as its product has already been optimised, although in a different organism, over millions of years of evolution. A good example of this is the enzyme ribulose 1,5-bisphosphate carboxylase (RuBisCo). Large variation exists between RuBisCo from different species making it an obvious candidate for transgenic manipulation. Recently, Kanevski *et al.* introduced chimeric *rbcL* genes from the cyanobacterium *Synechococcus* PCC6301 and sunflower (*Helianthus annuus*) into the plastid of tobacco. The transplastomic lines containing cyanobacterial *rbcL* failed to express the gene beyond the level of mRNA. In contrast, the sunflower gene was fully expressed and the synthesised protein associated with the endogenous tobacco small subunit to form an active holoenzyme (Kanevski *et al.*, 1999). Substrate affinity and turnover of the hybrid RuBisCo were reduced compared to the wild-type enzyme. Even so, the ability of a heterologously expressed large subunit to form hybrid RuBisCo demonstrates that this important enzyme is open to manipulation.

**Introduction of novel pathways**

One of the great hopes of plastid transformation is the benefits of the introduction of truly 'foreign' genes, conferring completely novel properties to the host. Research into the expression of foreign transgenes in the nuclear genome of plants has rapidly become commercialised. Similar development in the field of plastid foreign gene expression is in its infancy but holds as much, if not more potential as nuclear transgenics. The paradigm in this area is the production of crops containing transplastomes conferring herbicide or pesticide resistance. The efficacy of this approach was demonstrated last
year by Daniell et al. (1998), with the production of transplastomic tobacco plants conferring herbicide resistance. In this study a mutant version of the petunia EPSPS gene (5-enol-pyruvyl shikimate-3-phosphate synthase) conferring resistance to the herbicide glyphosate was introduced into the tobacco plastid genome (Daniell et al., 1998). When sprayed with varying concentrations of glyphosate the transplastomic plants were found to be resistant to extremely high concentrations (5 mM) of the herbicide.

Expression of herbicide/pesticide resistance genes in the plastid has several advantages over their expression in the nucleus:

• Plastid localised heterologous genes have the potential to be highly expressed due to the huge copy number of the plastid genome (5000 - 10,000 copies/cell). This has already been utilised to overexpress Bt protoxin in tobacco (see above), and is suggested to be the basis of the exaggerated resistance of petunia EPSPS chloroplast transformants to glyphosate (Daniell et al., 1998).

• Many of the target proteins or enzymes of herbicides are compartmentalised within the plastid.

• Insecticide-resistance genes such as Bt toxin are bacterial, and so they are naturally more adapted to expression in the plastid than the nucleus.

• DNA introduced into the nuclear genome integrates at random necessitating screening of large numbers of transformants that do not have suppressed expression due to positional effects. Screening is also needed to remove transformants where the introduced gene has mutagenised an endogenous gene. Although certain deleterious phenotypes will be obvious or lethal, others may be more subtle and consequently missed. Also, large scale screening to isolate appropriate transformants is also labour intensive and costly. In contrast, chloroplast transgenes can be targeted to a (intergenic) region of the genome known to be benign.

• The chloroplast genome is maternally inherited in many plant species, including many crops. Therefore, foreign genes incorporated in the plastid genome cannot be transferred via pollen in these species.

This final point has been the cause of some debate (Bilang and Potrykus, 1998; Stewart and Prakash, 1998; Daniell and Varma, 1998). One of the concerns about GM plants is the possible leakage of transgenes into the environment. The introduction of herbicide or pesticide resistance genes, by cross pollination, into pernicious weed species has the potential to create an environmental catastrophe. Expression in the plastid rather than the nucleus has long been suggested as a way of containing transgenes (Maliga, 1993;
Maliga et al., 1993; McBride et al., 1994). This is because in many species of flowering plants plastid inheritance is exclusively through the maternal line. Practically, this means that plastids are not passed through the pollen, and so plastid transgenes cannot be dispersed by cross-pollination with other varieties or species. It is this point, reiterated by Bilang and Potrykus (1998) in a commentary on the work by Daniell and colleagues (1998) that sparked controversy. The commentary was criticised for apparently suggesting that chloroplast transformation provided a panacea to stopping transgene escape in all species (Stewart and Prakash, 1998). The authors retorted by confirming that they suggested "maternal inheritance of foreign genes in MOST crops, when they are engineered via the chloroplast genome" (Daniell and Varma, 1998). As with many controversial technologies the actual potential of transplastomic crops to contain transgenes will only be known after large scale trials. However foreign gene expression in the chloroplast may become the method of choice for producing future GM crops.

As mentioned above, the quasi-bacterial nature of chloroplast gene expression could be utilised to express whole operons encoding novel metabolic pathways (Bilang and Potrykus, 1998). One tentative but attractive application of this property is the introduction of bacterial genes catalysing nitrogen fixation into the chloroplast (Maliga, 1993). The so called 'nitroplasts' would be able to carry out photosynthesis during the day and, using the resulting carbohydrates, fix nitrogen at night. Only certain bacteria are able to fix nitrogen although some species, such as \textit{Rhizobium}, form symbiotic interactions with leguminous plants. GM crops containing nitroplasts would not need nitrogenous fertilisers to be added to the soil, producing a significant saving as well as benefiting the environment.

1.3.4.4 Foreign gene expression in the chloroplast - future prospects

One of the axioms of modern science is that progress is limited by the availability of technology. This is exemplified by the effect of biolistic technology on the understanding of chloroplast molecular genetics. Biolistic technology has also provided a means of introducing foreign DNA into the plastid compartment. The ability to stably transform and express heterologous genes within the plastid has brought, and will continue to bring, many rewards. However, this optimism should be tempered with a realisation that there are still several elements of plastid foreign gene expression that are not understood. Attempts at expression of foreign genes in the chloroplast are not always successful, although the reasons for the lack of expression are usually difficult
to discern (e.g. Kanevski et al., 1999). In an ideal situation expression of a foreign gene or genes in the chloroplast would be routine and even controllable. Although many of the factors affecting plastid foreign gene expression, particularly in model organisms are understood, it cannot be assumed a priori that heterologous DNA will be expressed. Understanding of the limiting factors will most likely come from studying the control of expression of endogenous chloroplast genes. Due to the benefits above most of this fundamental knowledge has been, and will continue to be revealed using the green alga *Chlamydomonas* as a model for other organisms. In contrast, novel technological developments benefiting plastid foreign gene expression are likely to be achieved, because of their applicability to commercial crops, in higher plants such as tobacco. This multidisciplinary approach will doubtless bring many advances in the understanding and application of plastid foreign gene expression. Some of the potential avenues for this progress are as follows:

1. One useful tool for plastid foreign gene expression would be the development of plasmids or other extrachromosomal elements that replicate autonomously within the organelle. This would allow stable transformation of the chloroplast without disruption of the genome. Also, the introduced foreign DNA could be intentionally lost by removal of selective pressure for the extrachromosomal element. There have been a few reports of extrachromosomal DNA in plastids, although no stable replicating vectors have yet been described (Boynton et al., 1988; Staub and Maliga, 1994b; Suzuki et al., 1997).

2. A very recent technological development which has been applied to plastid foreign gene expression is the galistant expansion microsyringe (GEF). The GEF uses heat induced expansion of galistant (a liquid metal alloy of gallium, indium and tin) within a glass syringe to expel sample through a tip diameter of about 0.1 μm (J. C. Gray, unpublished). This minute syringe was used to inject *gfp* constructs into tobacco chloroplasts, resulting in transient expression of GFP within 24 hours. Interestingly, GFP fluorescence spread to adjacent chloroplasts from the one injected over 72 hours after injection. These data provide support for the idea of transient connections between chloroplasts allowing transfer of macromolecules between chloroplasts in the same cell (Kohler et al., 1997a). The same *gfp* chloroplast expression vector has also been used to obtain transient expression in other plastid types such as amyloplasts of potato tubers and chromoplasts of marigold petals, carrot roots and pepper fruits via particle-gun-bombardment (Hibberd et al., 1998). This was an important achievement as, until recently, the ease with which chloroplast transgenic technology will transfer to other plastid types has not been known.
3. With regard to transgenic crops, foreign gene expression in the chloroplast has several advantages over expression in the nucleus, some which have been described. As discussed previously, this will undoubtedly be an area of accelerated progress. One of the drawbacks of transplastomic plants is that plastid genes are always expressed to some extent throughout the life cycle of the organism. Whereas, nuclear genes are often tightly regulated by tissue-specific and/or developmental controls. One way to exert tissue-specific developmental control of plastid transgenes was devised by McBride et al (1994). In this study the bacterial *uidA* gene was introduced into the tobacco chloroplast under the control of the phage T7 gene 10 promoter. Previously a chimeric T7 RNA polymerase containing a chloroplast transit sequence had been introduced into the nuclear genome of the host strain. GUS expression in the chloroplast was found to be dependent on the presence of the nuclear encoded chimeric T7 polymerase (McBride et al., 1994). This system could be adapted by placing the chimeric T7 polymerase under the control of a tissue-specific or developmentally regulated promoter. This would allow, for example, expression of a plastid transgene in the leaf which would be lethal if expressed during embryonic stages of development.
1.4 Aims of this research

This thesis presents the results of novel investigations into the introduction and expression of foreign genes in the chloroplast of *C. reinhardtii*.

i. The bacterial gene, *aphA-6*, was introduced into the chloroplast genome in order to effect its heterologous expression. Results are presented regarding the ability of the foreign enzyme encoded by *aphA-6* to be functionally expressed in the chloroplast. Subsequent results are described in which *aphA-6* is developed as a novel dominant selectable marker for chloroplast transformation.

ii. With the aim of instigating a system for its analysis using site-directed mutagenesis, the NADPH-protochlorophyllide oxidoreductase encoding gene (*por*) from *Synechocystis* sp. PCC6803 was introduced into the chloroplast. The results of analysis of transformants, with regards to transgenic expression of *por*, are presented. Separately, the *tufA* gene encoding Elongation factor Tu from the plastid of *Plasmodium falciparum*, was introduced into the chloroplast genome. Results are presented as to the ability of the foreign gene to functionally replace its *C. reinhardtii* chloroplast counterpart.

iii. The *ble* cassette, a dominant selectable marker for nuclear transformation, was introduced into the chloroplast genome. The results of screens designed to isolate strains in which *ble* had escaped from the chloroplast to the nuclear genome are presented.
Chapter 2

Materials and Methods
2 MATERIALS AND METHODS

2.1 Media and reagents

2.1.1 Growth media

Details of the medium used for bacterial culture are summarised in Table 2.1. Media used to culture *C. reinhardtii* are detailed in Table 2.3. Where necessary, growth medium was solidified with 2% bactoagar (Difco). Solutions and media were sterilised by autoclaving at 15 psi, 121 °C for 20 minutes, or in the case of heat sensitive reagents, passed through a 0.1 µm pore-diameter filter and stored in aliquots at -20 °C.

<table>
<thead>
<tr>
<th>Table 2.1 E. coli growth medium</th>
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<tr>
<td><strong>For 1 litre</strong></td>
</tr>
<tr>
<td>Bactotryptone</td>
</tr>
<tr>
<td>Yeast extract</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
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<td>dH₂O</td>
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<th>Table 2.2 E. coli strains</th>
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</thead>
<tbody>
<tr>
<td><strong>Strain</strong></td>
</tr>
<tr>
<td>DH5α</td>
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<tr>
<td>JM109</td>
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Table 2.3 *C. reinhardtii* growth media

<table>
<thead>
<tr>
<th></th>
<th>TAP medium</th>
<th>Tris minimal medium</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td>975 ml</td>
<td>975 ml</td>
</tr>
<tr>
<td>Tris</td>
<td>2.42g</td>
<td>2.42g</td>
</tr>
<tr>
<td>4x Beijerink Salts¹</td>
<td>25 ml</td>
<td>25 ml</td>
</tr>
<tr>
<td>1M (K)PO₄ ph 7.0²</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Trace Elements³</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>~1 ml to pH 7.0</td>
<td>-</td>
</tr>
<tr>
<td>Concentrated HCl</td>
<td>-</td>
<td>~1 ml to pH 7.0</td>
</tr>
<tr>
<td>References</td>
<td>Gorman and Levine, 1965</td>
<td>Surzycki, 1971</td>
</tr>
</tbody>
</table>

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¹ 4x Beijerink Salts:

16g NH₄Cl (1.6g NH₄Cl for TAP 1/10N medium)
2g CaCl₂
4g MgSO₄

dissolve in 1 litre distilled H₂O

² 1M (K)PO₄:

250 ml 1M K₃HPO₄
170 ml 1M KH₂PO₄ (titrate to pH 7.0)

³ Trace Elements:

i. Dissolve in 550 ml distilled water in the order indicated below, then heat to 100 °C

11.4 g H₂BO₄
22 g ZnSO₄·7H₂O
5.06 g MnCl₂·4H₂O
4.99 g FeSO₄·7H₂O
1.61 g CoCl₂·6H₂O
1.57 g CuSO₄·4H₂O
1.1g (NH₄)₆ MoO₄·4H₂O

ii. Dissolve 50 g EDTA·Na₂ in 250 ml H₂O by heating and add to the above solution. Reheat to 100 °C. Cool to 80-90 °C and adjust to pH 6.5-6.8 with 20% KOH.

iii. Adjust to 1 litre. Incubate at room temperature for two weeks and allow a rust coloured precipitate to form. The solution will change from green to purple.

iv. Filter through three layers of Whatman No.1 paper under suction until the solution is clear. Store at 4°C.
2.1.2 Reagents and enzymes

The radiolabelled 2'deoxyribo nucleoside 5'triphosphates (dNTPs); \((\alpha_{-}^{32}P)\) 2'deoxyctydine 5'triphosphate (dCTP) and \((\alpha^{35}S)\) 2'deoxyadenosine 5' triphosphate (dATP) were purchased from Amersham International. Oligonucleotides were custom synthesised by PE-Applied Biosystems, UK (Cheshire). Restriction endonucleases and DNA modifying enzymes and polymerases were purchased from; New England Biolabs (Hitchin), Stratagene (Cambridge), Promega (Southampton) and Boehringer Mannheim (Lewis). The various antibiotics used (kanamycin monosulphate; amikacin; gentamycin sulphate; chloramphenicol; spectinomycin; ampicillin) were all purchased from Sigma Chemical Co. (Dorset).

All common laboratory reagents were of the highest grade available, and where no supplier is mentioned, were purchased from Sigma Chemical Co. (Dorset).

2.2 Chlamydomonas descriptions and maintenance

2.2.1 C. reinhardtii strains

The wild-type (WT) C. reinhardtii strains CC-1021 mating type+ (mt+), the cell wall deficient strain cw15(mt-) (Davies and Planskitt, 1971), the wild-type strain CC-12 (mt-), and the NADPH-protochlorophyllide oxidoreductase mutants pc-1 (mt+) and pc-ly-7 (mt-) were obtained from Dr E. Harris at the Chlamydomonas Genetics Center at Duke University, North Carolina, USA. The photosynthetic mutant ΔpsbH (Bst-opp) and the control strain MluI (O'Connor et al., 1998), were produced and are maintained in this laboratory.

2.2.2 Growth and maintenance of C. reinhardtii strains

C. reinhardtii strains were maintained on Tris-acetate phosphate (TAP) medium (Table 2.3) solidified with 2% (w/v) bacto agar under a photon flux of 20 μE/m²/s at 18 °C and were streaked to fresh plates every 6-8 weeks. Working stocks were maintained at 45 μE/m²/s at 25 °C and these were restreaked weekly. Liquid cultures were grown in the appropriate medium in Erlenmeyer flasks in a Gallenkamp illuminated orbital
incubator in the light (80 μE/m²/s) at 25 °C and aerated by shaking at 150 rpm. Starter cultures of 10 ml volume of the appropriate medium were inoculated from stock cultures into 25 ml Sterilin tubes (Bibby Sterilin Ltd, Glamorgan) and grown to stationary phase (1-2 x 10⁷ cells/ml), and an appropriate amount of this culture was subsequently used to inoculate a larger volume of medium. Appropriate aseptic techniques were employed throughout.

2.2.3 Measurement of cell density

The cell density of *C. reinhardtii* in liquid culture was determined by removing a 1 ml sample from a well-mixed culture and adding to it 10 μl of tincture of iodine (0.25 g iodine in 95% ethanol). High density cultures were diluted 1/10 before counting. Duplicate 10 μl aliquots of cells killed in this way were then counted using a haemocytometer (Webber Scientific International Ltd) on a Wild Heerbrugg microscope at x400 magnification and multiplied by 10⁴ (or 10⁵ if diluted) to give cell density/ml.

2.2.4 Mating *C. reinhardtii ble* transformants

*C. reinhardtii ble* (117) transformants (mating-type plus) and WT CC-12 (mt-) were crossed according to the methods developed by Levine and Ebersold (1960) and detailed in Harris (1989), although resulting zygotes were not dissected (Levine and Ebersold, 1960). Cells were streaked at 1-2 day intervals on TAP plates three times to ensure exponential growth. To obtain gametes a large loop-full of cells of both mating types was streaked onto TAP 1/10N plates (Table 2.3). These plates were incubated in the light at 25 °C for 3-4 days. Cells were then resuspended in phosphate buffer (10 mM sodium phosphate, pH 7.0) to approximately 2 x 10⁷ cells/ml and incubated in the light for approximately 30 minutes to allow flagella regeneration. Equal volumes of *ble* 117 (mt+) and WT CC-12 (mt-) (~2 ml each) were then mixed in a sterile 50 ml flask. At 1, 2, and 3 hour intervals 100 μl of the mating mixture was spotted onto TAP 3% plates and left in the light overnight, then incubated in the dark for one week at 25 °C to allow zygotes to mature. After this time the majority of unmated gametes were removed from the plate by scraping the surface of the agar with a sterile razor blade. The remaining unmated gametes were then killed by holding the plate over chloroform for 30 s. Zeomycin resistant daughter cells were then selected for by overlaying the plate with 0.7 % TAP containing 20 μg/ml zeomycin and incubating in the light at 25 °C.
2.2.5 Screening *C. reinhardtii* for spontaneous resistant or revertant strains

Screening for *C. reinhardtii* spontaneously antibiotic resistant strains or revertants of chlorophyll biosynthesis mutants was carried out as follows. 100 ml of the appropriate strain was grown to a concentration of ~1 x 10^7 cells/ml. The precise concentration was then calculated by measuring cell density (section 2.2.3). 2 x 50 ml of cells were then centrifuged in Falcon tubes in an Eppendorf 5403 centrifuge at 4000 rpm for 5 minutes. The cell pellets were then resuspended in ~5 ml of medium. To this cell concentrate was added soft TAP agar (0.7%, cooled to ~40 °C), where appropriate supplemented with antibiotic, to a total volume of 50 ml (x2) and mixed by inversion. Both tubes of cells were then poured onto the surface of a large (20 cm x 20 cm) petri dish containing the appropriate medium. After the cell-agar mixture had set the plate was inverted and incubated under the appropriate conditions.

2.3 Bacterial descriptions and maintenance

2.3.1. *Escherichia coli* strains

The *E. coli* strains used and their genotypes are listed in Table 2.2. Both strains are available from Stratagene (Cambridge).

2.3.2 Growth and maintenance of *Escherichia coli*

*E. coli* strains were maintained in the short term by growing overnight at 37 °C on LB medium (Table 2.1) solidified with 2% (w/v) bacto-agar (supplemented with the appropriate antibiotics where applicable). In the short term *E. coli* cultures were stored at 4 °C. Long term storage of *E. coli* cultures was in the form of frozen glycerol stocks: 1.2 ml of an overnight culture was mixed with 0.8 ml of sterile glycerol in a 2 ml screw cap Eppendorf tube, and stored at -70 °C.
2.4 Recombinant nucleic acid techniques

2.4.1 Restriction endonuclease digestion

Restriction digestion of plasmid DNA (0.5–2 μg) using 5–10 units of restriction endonuclease was performed in buffer supplied by the manufacturer. Incubation times and temperatures were as recommended by the enzyme supplier. Restriction digestion of *C. reinhardtii* DNA was carried out using 20 units of restriction endonuclease for 2 hours, or overnight at the recommended temperature. In the former case, an additional 10 units of enzyme were added and the digestion allowed to proceed for a further hour to ensure complete digestion.

2.4.2 Agarose gel electrophoresis of DNA

DNA fragments were separated by electrophoresis through agarose gels (1% w/v) cast in 0.5 x TBE buffer (45 mM tris-HCl, 45 mM boric acid, 1.25 mM EDTA.Na₂). Ethidium bromide was included in gels at 0.5 μg/ml. Samples were mixed at the appropriate ratio with 6x loading buffer which contained 40% (w/v) glycerol, 0.1% (w/v) SDS, 0.1mM EDTA.Na₂, 0.01% (w/v) bromophenol blue, 0.01% (w/v) xylene cyanol. Gels were run submerged in TBE at 100 V constant voltage for 1-2 hours in a Hoefer 10 cm cooled minigel apparatus or, overnight in a Hybaid 30 cm maxigel apparatus at 50 V (usually for Southern analysis). Either 0.5 μg of bacteriophage λ DNA digested with *HindIII* or 1 kb or 100 bp DNA ladders (MBI Fermentas) were used as size markers. Fragments were visualised under UV light at 302 nm and photographed using a UVP Gel Documentation System (CA, USA), DNA fragments of unknown length were sized by comparison with the standard DNA ladders described.

2.4.3 Agarose gel electrophoresis of RNA

A denaturing 1.2% agarose gel was prepared by melting 4.8 g agarose in 300 ml DEPC-treated water (0.1% DEPC-ddH₂O, incubated at 37 °C overnight then autoclaved to remove DEPC). Once the mix had cooled to ~50 °C, 40 mls of 10x MOPS buffer (0.2 M MOPS, 80 mM NaOAc, 15 mM EDTA, pH 7.0, using 10 M NaOH) and 65 ml of formaldehyde (40%) were added and the gel was poured into the casting tray. Samples were mixed at the appropriate ratio with 2x loading buffer (made by mixing 12
μl 10 mg/ml EtBr, 300 μl 10x MOPS buffer, 80 μl formaldehyde (40%) and 300 μl formamide, stored at -20 °C) and heated to 65 °C for 5 minutes before loading. Gels were run submerged in 1x MOPS at 50 V constant voltage overnight in a Hybaid 30 cm maxigel apparatus while recirculating the buffer (with 10 minutes running prior to loading samples). RNA was visualised under UV light (302 nm) and sized by comparison with 0.2-10 kb RNA ladder (Sigma).

2.4.4 Ligation of DNA

For blunt ended ligations, reaction mixtures (10 μl) included up to 4 μg insert DNA, 0.5 μg of vector DNA and 1 unit of T4 DNA ligase in buffer supplied by the enzyme manufacturer. Ligations were carried out at 37 °C for one hour. For ligation of DNA involving cohesive termini, more insert DNA was included with 2 units of T4 DNA ligase, and the reactions carried out at 18 °C overnight.

2.4.5 Dephosphorylation of DNA, ssDNA end removal, and ssDNA end filling

Dephosphorylation of DNA 5'-ends was achieved using calf intestinal alkaline phosphatase (Boehringer-Manheim): Approximately 2 μg of linearised purified plasmid DNA was resuspended in 90 μl ddH2O. 10 μl of 10x reaction buffer and 1 μl alkaline phosphatase were added and the reaction incubated at 55 °C for 1 hour then at 75 °C for 5 minutes to inactivate the phosphatase.

Mung Bean Nuclease (MBN), a single stranded DNA/RNA endonuclease, was used to remove 3' and 5' overhangs from the cohesive termini of DNA fragments or linearised plasmids. The DNA to be treated was resuspended in 15.5 μl ddH2O. To this, 2 μl of 10x MBN buffer and 2.5 μl MBN (diluted to 1.5 U/μl in MBN dilution buffer) were added and the mixture incubated at 37 °C for 30 minutes.

To convert 5'-overhangs of DNA fragments or linearised plasmids to blunt end termini either the Klenow fragment of DNA polymerase I or T4 DNA polymerase were used. A typical reaction mixture using Klenow would be: 5 μg DNA + 5 U Klenow + 5 μl Klenow buffer + dATP, dCTP, dGTP, dTTP all at 33 μM + ddH2O to a final volume.
of 50 μl. The reaction mixture would then be incubated at 25 °C for 15 minutes. Using T4 DNA polymerase, 0.2-5 μg of restriction digested DNA was resuspended in T4 polymerase 1x buffer (Promega) containing 100 μM of each of dATP, dCTP, dGTP, dTTP and 0.1 mg/ml BSA in a 20 μl reaction volume. 5 U of T4 DNA polymerase per microgram of DNA were then added and the mixture incubated at 37 °C for 5 minutes. The reaction was terminated by incubation at 75 °C for 10 minutes.

2.4.6 Recovery of DNA fragments from agarose gels

DNA fragments were recovered from agarose gels using a QIAquick Gel Extraction Kit (Qiagen Ltd, UK) according to the manufacturers protocol and resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, Na₂ pH 8.0) or sterile ddH₂O.

2.4.7 Transformation of Escherichia coli

A method derived from that of Cohen et al. (1972) was used to introduce plasmids into competent E. coli strains (DH5α or JM109 - see Table 2.2) (Cohen et al., 1972). 100 ml of a stationary phase overnight culture of the appropriate strain was used to inoculate 10 ml of LB medium (supplemented with antibiotics where appropriate) and this was grown at 37 °C in an orbital shaker at 150 rpm for 3 hours. Cells were harvested by centrifugation in an Eppendorf 5403 centrifuge at 4,000 rpm for 5 minutes, resuspended in 10 ml of cold (4 °C) 100 mM MgCl₂ and incubated on ice for 5 minutes. Cells were pelleted as above and then resuspended in 0.1 volume of cold (4°C) 50 mM CaCl₂. Following incubation on ice for a further 30 minutes, 100 μl aliquots of cells were transferred to 1.5 ml Eppendorf tubes and to these were added the transforming plasmid (typically 5 μl of a ligation reaction or 0.1 μg of a parental plasmid). The competent cell/plasmid mixture was incubated on ice for 30 minutes and then heat shocked at 42 °C for 90 seconds. After cooling briefly on ice, 900 μl of LB medium was added to the cells which were then incubated at 37 °C for 30-45 minutes to allow expression of plasmid-encoded antibiotic resistance markers. The transformed cell suspension was then plated to LB-2% agar plates supplemented with appropriate antibiotic(s). For pUC based vectors (Vieira and Messing, 1982), agar plates were also pre-treated with 4 ml of a 200 mg/ml stock solution of the non-substrate lac inducer
IPTG, and 40 µl of a 20 mg/ml in dimethylformamide stock solution of the chromogenic substrate X-gal as described in Sambrook et al. (1989) to allow blue/white selection. The plates were incubated at 37 °C overnight and resultant recombinants were picked to 10 ml of LB medium supplemented with the appropriate antibiotic(s).

Controls were also included as part of each transformation experiment. These were either a 'cells only' control when transforming parental plasmid DNA, or when transforming a ligation reaction, control reactions lacking host vector, insert, or DNA ligase were used.

2.5 Preparation of nucleic acids

2.5.1 Isolation of plasmid DNA

Small scale preparations of plasmid DNA ('minipreps') were isolated from transformed E. coli using the alkaline SDS method (Birnboim and Doly, 1979). 10 ml of LB containing appropriate antibiotic(s) was inoculated with the bacteria containing the plasmid by picking a colony from a plate. The culture was incubated at 37 °C overnight and the bacteria were pelleted by centrifugation. The cells were resuspended in 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 50 mM EDTA.Na₂), incubated at room temperature for 5 minutes and transferred to an Eppendorf tube. 200 µl of freshly made solution II (1% SDS, 0.2 M NaOH) was added in order to lyse the cells. After a 5 minute incubation at room temperature, 150 µl of solution III (2 M acetic acid, 3 M potassium acetate) was added to precipitate chromosomal DNA and cell debris, and the samples were then left on ice for 30 minutes. The debris was pelleted by centrifugation for 10 minutes and the supernatant removed to a fresh tube. The centrifugation was then repeated for 5 minutes if the supernatant was not to be phenol extracted. The resulting supernatant was then extracted with an equal volume of phenol/chloroform (this stage was omitted when using DH5α): one volume of TE (10 mM Tris-HCl, 1 mM EDTA.Na₂, pH 8.0) saturated phenol and one volume of chloroform:isoamyl alcohol (24:1; v/v) was added and the phases mixed by vortexing. Following centrifugation for 5 minutes in a microcentrifuge the upper, aqueous phase was transferred to a fresh Eppendorf. An equal volume of isopropanol (propan-2-ol) was then added to the supernatant and mixed by inversion. Plasmid DNA was pelleted by centrifugation for 15 minutes in a microcentrifuge. The supernatant was discarded and the pellets were washed with 70% (v/v) ethanol and then dried in a Rotovac
vacuum drier. DNA pellets were finally resuspended in 50 µl TE pH 8.0 or ddH$_2$O, 1 mg/ml RNaseA.

Larger scale isolation of plasmid DNA was performed using a Qiagen maxiprep kit according to manufacturer’s instructions (Qiagen Ltd, UK); finally resuspending the DNA in 200 µl of TE pH 8.0. The concentration of the DNA was determined by diluting 2 µl of the concentrated stock into 1 ml of ddH$_2$O and measuring the absorbance at 260 nm. DNA at a concentration of 1 mg/ml gives a reading of 20 absorbance units at 260 nm, therefore the concentration could be calculated and the sample diluted to a working concentration of 1 mg/ml.

2.5.2 Preparation of *C. reinhardtii* DNA

Three different methods were employed to prepare *C. reinhardtii* DNA according to the level of purity needed. The first method produces a crude cell lysate that can be used as a template for PCR reactions: A colony or small loop of cells was picked from a plate and resuspended into 50 µl ddH$_2$O plus 3.5 µl proteinase K (20 mg/ml). The mixture was then incubated at 60 °C for one hour then 90 °C for 10 minutes in a thermal cycler (Hybaid) to inactivate the proteinase K. 5 µl of this lysate was then used in PCR reactions.

A second method used for preparation of *C. reinhardtii* DNA to use as a template for PCR is described in Newman *et al.* (1991). A mid-size loop of cells was scraped from a plate and resuspended into 0.5 ml TEN buffer (10 mM Tris-HCl, 10 mM EDTA,Na$_2$, 150 mM NaCl, pH 8.0) in an Eppendorf tube (Newman *et al.*, 1990). The cells were then resuspended completely by vortexing and pelleted by spinning for 10 seconds in a microcentrifuge. The supernatant was subsequently aspirated and the cells resuspended in 150 µl ddH$_2$O and 300 µl of SDS-EB buffer (2% SDS, 400 mM NaCl, 40 mM EDTA,Na$_2$, 100mM Tris-HCl, pH 8.0) added. This mixture was then vortexed briefly and incubated on ice for 5 minutes. This was proceeded by a single extraction using 350 µl of phenol/chloroform (section 2.5.1) followed by a single extraction with chloroform:isoamyl alcohol (24:1; v/v). The DNA in the recovered aqueous phase was subsequently precipitated by adding an equal volume of isopropanol (propan-2-ol). The DNA was recovered by centrifuging the tubes for 10 minutes in a microcentrifuge to pellet the DNA. The supernatant was discarded and the pellets were washed with 70%
(v/v) ethanol and then dried in a Rotovac vacuum drier. DNA pellets were finally resuspended in 50ml TE pH 8.0 or ddH₂O, 1 mg/ml RNaseA. 1 µl of this preparation was used a template for PCR.

To prepare less contaminated *C. reinhardtii* DNA for Southern analysis a rapid 'miniprep' method adapted from Rochaix *et al.* (1988) was used (Rochaix *et al.*, 1988). Cells from 10 ml of a culture at a density of 1 x 10⁷ cells/ml were harvested by centrifugation in a 25ml Sterilin sample tube (Bibby Sterilin Ltd, Glamorgan) at 4000 rpm for 5 minutes in an Eppendorf 5403 centrifuge. The supernatant was discarded and the cell pellet resuspended in 1ml of TAP medium and transferred to a 1.5ml Eppendorf tube then repelleted by centrifugation in a microcentrifuge. Again the supernatant was discarded and the cell pellet was resuspended in 0.35 ml of TEN buffer (50 mM EDTA.Na₂ pH 8.0, 20 mM Tris-HCl pH 8.0, 0.1 M NaCl) (Rochaix *et al.*, 1988). 50 µl of pronase at 10 mg/ml and 25 µl of 20% sodium dodecyl sulphate (SDS) were added and the cells were incubated at 55°C for 2 hours. 2 µl of diethylpyrocarbonate (DEPC) was added and incubation continued for a further 15 minutes at 70°C in a fume hood. The tube was briefly cooled on ice and 50 µl of 5 M potassium acetate added. The contents of the tube were mixed by shaking thoroughly and the tubes were incubated on ice for a further 30 minutes. The precipitate which formed was removed by centrifuging the tubes for 15 minutes in a microcentrifuge and transferring the supernatant to a fresh Eppendorf tube. Contaminating proteins were removed by performing between 2-4 phenol/chloroform extractions as detailed in section 2.5.1. DNA was precipitated and resuspended as described in the previous method. 5-15 µl were used for Southern blot analysis.

**2.5.3 Preparation of *C. reinhardtii* RNA**

Total RNA from *C. reinhardtii* was prepared according to a method adapted from Goldschmidt-Clermont *et al.* (1990). 10 ml of cells were grown to a density of 2-4 x 10⁶ cells/ml. The cells were then pelleted by centrifugation in an Eppendorf 5403 centrifuge at 13,000 rpm for 5 minutes. The supernatant was poured off and the cell pellet resuspended in 0.6 ml TEN.SDS (0.2 M Tris-HCl pH 8.0, 0.5 M NaCl, 0.01 M EDTA, 0.2% SDS) (Goldschmidt-Clermont *et al.*, 1990). This suspension was then 'freeze-thawed' three times in liquid nitrogen and a 37°C water bath respectively. Cell debris was then removed by extraction with 0.6 ml phenol/chloroform (section 2.5.1).
Finally RNA is precipitated by the addition of 1.4 ml ethanol and centrifuging in a microcentrifuge for 10 minutes. The resultant pellet was washed with 70% ethanol, dried in a Rotovac vacuum drier and resuspended in 50 µl DEPC treated ddH₂O in a RNase free Eppendorf. The relative concentration of each sample was estimated by running 1 µl on a 1% agarose gel (section 2.4.2). Approximately 10 µl was used for northern analysis.

2.6 Filter hybridisation of nucleic acids

2.6.1 Transfer of nucleic acids to nylon membranes

DNA fragments that had been separated by agarose gel electrophoresis were transferred to Hybond-N nylon membranes (Amersham International) in a method derived from that of Southern (1975). Following the production of a photographic image of the DNA as described in section 2.4.2, the gel was soaked in 1.5 M NaCl, 0.5 M NaOH for 30 minutes to denature the DNA duplex to single strands. Gels were then soaked 2 x 30 minutes in a neutralising buffer (1 M Tris-HCl pH 8.0, 1.5 M NaCl). The gels were then assembled on a blotting stack using 20 x SSC (3 M NaCl, 0.3 M sodium citrate) as the transfer buffer. Two sheets of Whatman 3MM paper (pre-soaked in 20 x SSC) were used as wicks to draw the SSC from a reservoir to a supported glass plate. The gel was placed onto the 3MM and any remaining wick exposed at the edge of the gel was masked with Nescofilm (Nipon Shoji Kaisha Ltd - Japan) to prevent SSC transfer buffer bypassing the gel. A sheet of Hybond-N cut to the exact size of the gel was placed on top of it and on this were placed three sheets of 3MM (again cut to the exact size). Finally, a stack of paper towels approximately 5 cm in height was placed on the stack and the whole assembly was weighed down with a glass plate and a 0.5 kg weight. Transfer was allowed to take place for 15 hours following which the filter was baked in an envelope of 3MM at 80 °C for 1 hour to immobilize the DNA on the filter. Membranes were subsequently stored in the 3MM envelope at room temperature.

RNA fragments that had been separated by agarose gel electrophoresis were also transferred to Hybond-N nylon membranes (northern blot) in a manner very similar to that described for Southern blots. However, RNA is single stranded and so the gels did not need to be incubated in denaturing or neutralising solution. Instead, RNA-containing agarose gels were photographed, then washed for 30 minutes in DEPC treated ddH₂O before transfer to the nylon membrane.
2.6.2 Radiolabelling DNA probes

DNA fragments to be used as probes were labelled using the Prime-It II Random Primer Labelling Kit (Stratagene) using random 9-mer primers and $\textit{exo}^-$ Klenow DNA polymerase, essentially as per the manufacturer's instructions.

2.6.3 Hybridisation of DNA probes to membrane bound nucleic acids

Nylon filters from Southern or northern blots were blocked by incubation in 40 ml of pre-hybridisation solution (6x SSC, 0.5% SDS, 5x Denhardt's reagent, 100 µg/ml denatured sheared salmon sperm DNA) in Hybaid hybridisation bottles in a Hybaid dual hybridisation oven at 65 °C for 4 hours. The pre-hybridisation solution was discarded and 15 ml of hybridisation buffer (6x SSC, 0.5% SDS, 5x Denhardt's reagent, 0.01 M EDTA, 100 µg/ml denatured sheared salmon sperm DNA) containing the radiolabelled DNA probe (section 2.6.2), was added to the bottle. DNA and RNA blots were hybridised overnight in Hybaid hybridisation bottles in a Hybaid dual hybridisation oven at 65 °C. Blots were sandwiched between plastic gauzes to equalise hybridisation over the whole filter. Following hybridisation, filters were washed sequentially in excess volumes of 2 x SSC, 0.1% (w/v) SDS for one hour initially at room temperature, then increasing to 65 °C, then in 0.1 x SSC, 0.1% (w/v) SDS for one hour continuously at 65 °C. Filters were sealed in Saran wrap (Dow chemical Co.) and hybridised probe was detected by autoradiography at -70 °C using Kodak (Herts) X-ray film in intensifying cassettes (Genetic Research Instruments, Dunmow).

2.7 Protein analysis

2.7.1 Preparation of protein from \textit{C. reinhardtii}

To prepare a crude whole cell protein extract 10 ml cultures of \textit{C. reinhardtii} were grown to a cell density of 4-6 x 10^6 cells/ml and pelleted by centrifugation at 4,000 rpm for 5 minutes in an Eppendorf 5403 centrifuge. After removal of the supernatant the cell pellet was resuspended in approximately 0.5 ml of medium. The chlorophyll

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4 50x Denhardt's reagent (1% w/v Ficoll - type 400, 1% w/v polyvinylpyrrolidone, 1% w/v BSA -fraction V)
concentration of each sample was then calculated (section 2.9) and the equivalent of 20 μg of sample added to an equal volume of 5x loading buffer (0.3125 M Tris-HCl pH 6.8, 50% w/v glycerol, 10% w/v SDS, 0.025 w/v bromophenol blue, 25% w/v 2-mercaptoethanol) and the mixture heated at 65 °C for 10 minutes to lyse cells. Insoluble material was removed by brief centrifugation (30 seconds) in a microcentrifuge prior to loading on the gel.

To separate cytosolic and membrane bound proteins a similar volume of cells was used and resuspended in 1 ml of 5 mM HEPES pH 7.5. 50 μl of Complete Protease Inhibitor (Boehringer Mannheim) was added to each sample. The chlorophyll concentration of each sample was then calculated (section 2.9). Samples were then sonicated for 30 seconds and cell lysis checked under a Wild Heerbrugg microscope at x400 magnification. Samples were then centrifuged for 10 minutes and the supernatant removed to a new tube. Cytosolic protein concentration was then determined using the BCA protein assay (Biorad). Approximately 20 μg of cytosolic and 20 μg chlorophyll equivalent of membrane protein were added in a 1:5 ratio to 5x loading buffer (see above) and the mixture incubated at 65 °C for 5 minutes before loading.

2.7.2 SDS-polyacrylamide gel electrophoresis (PAGE) of proteins

SDS-PAGE was carried out according to the method of Laemmli (1970) (Laemmli, 1970). The percentage of polyacrylamide used in the resolving gel was altered according to the molecular weight of the protein of interest. The stacking gel was 3.75 polyacrylamide (acrylamide:bis-acrylamide ratio of 29:1), 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, and the resolving gel was varying concentrations of polyacrylamide (acrylamide:bis-acrylamide ratio of 29:1), 0.3 M Tris-HCl (pH 8.8), 0.1% SDS. Gels were polymersied by the addition of TEMED (tetramethylethylenediamine, 15 μl) and 10% ammonium persulphate (200 μl). The reservoir buffer was 0.025 M Tris-HCl (pH 8.3), 0.192% glycine, 0.1% SDS. Gels were run using a Biorad 20 x 20 cm slab gel apparatus. Samples were electrophoresed at a constant voltage of 100 V overnight. Amersham full range Rainbow recombinant protein molecular weight markers and ECL biotinylated protein molecular weight markers (Amersham) were used as molecular weight standards.
2.7.3 Transfer of proteins to nitrocellulose membrane and immunodetection

Proteins separated by SDS-PAGE were blotted onto Hybond nitrocellulose membrane using a semi-dry trans blotter (Biorad) by the method of Towbin (Towbin et al., 1979). The transfer buffer was 20% (v/v) methanol, 25 mM Tris, 192 mM glycine, and transfer was performed at 20 V for 1 hour.

Filters were blocked with 5% low fat skimmed milk powder in TTBS (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% Tween-20) and probed with the appropriate antibody. Primary antibodies were detected using ECL Western blotting detection reagents (Amersham International), essentially via the manufacturer’s protocol.

2.8 *Chlamydomonas* transformation

2.8.1 Nuclear transformation with the *ble* marker

Transformation of the *C. reinhardtii* nuclear genome using the *ble* marker was achieved using the glass bead method (Kindle, 1990). Wild-type cells were grown to no more than 2 x 10⁶ cells/ml (section 2.2.3) in 400 ml of TAP medium (section 2.1.1) in a 1 litre Erlenmeyer flask. Cells were then transferred to sterile 200 ml nalgene centrifuge bottles and centrifuged in a Sorval GSA rotor at 4,000 rpm for 5 minutes. The supernatant was then removed and the cells resuspended to a concentration of 2 x 10⁸ cells/ml. 0.3 g of sterile glass beads (0.4 mm, BDH), 300 µl cells (at 2 x 10⁸ cells/ml), and 2 µg of plasmid DNA or ~1 µg PCR product DNA were then added to a Sterilin tube and vortexed in a Vortex genie-2 (Scientific Industries) vortex for 15 seconds at top speed. The transformation mix was then diluted to 20 ml with TAP and incubated for 18 hours in a Gallenkamp illuminated orbital incubator in the light (80 µE/m²/s) at 25 °C, with shaking to aerate the cells. The cells were then pelleted by centrifugation and resuspended in ~0.5 ml TAP. Finally, the cells were spread onto a TAP 2% agar plate in 4 ml of melted soft TAP agar (0.6%, cooled to ~40 °C) both of which contained zeomycin at 20 µg/ml. The plates containing the putative transformants were then incubated at 25 °C in the light (45 µE/m²/s). Transformant colonies usually appeared after 7-9 days.
2.8.2 Chloroplast transformation by particle-gun bombardment

Chloroplast transformation was carried out as described (Boynton et al., 1988), using the PDS-1000 helium-driven particle gun (Biorad). DNA coated microprojectiles were prepared by adding 0.5 ml of 70% ethanol to 6 mg of 1.0 μm gold particles (Biorad) or tungsten (tungsten M10 powder; mean particle size 1 μm with a range of 0.2-5 μm, Sylvania Chemicals and Metals, Towanda, PA, USA) and vortexing for 3 minutes. The suspension was then incubated for 15 minutes at room temperature. The microprojectiles were next pelleted by pulsing in a microcentrifuge. The supernatant was then aspirated and the pellet resuspended in ddH₂O, vortexed for 1 minute, allowed to settle for 1 minute and then re-pelleted by pulsing in a microcentrifuge. This wash was then repeated twice more. On the third wash the pellet was resuspended in 100 μl of sterile 50% glycerol (gold particles final concentration 60 mg/ml). This suspension was used fresh or was stored at 4 °C until needed. Before coating with DNA, the suspension was vortexed for 3 minutes to disrupt agglomerated particles. 50 μl of microparticles were then transferred to a new tube (enough for 8 transformations).

While vortexing between additions the following were added: 3.5 μl plasmid DNA (1 μg/μl), 50 μl CaCl₂ (2.5 M), 20 μl spermidine (0.1 M). The mixture was then vortexed for a further 2 minutes and allowed to settle for 1 minute before pelleting by pulsing in a microcentrifuge. The supernatant was then discarded and the DNA-coated microparticles washed once in 70% ethanol and twice in absolute ethanol before finally resuspending (by gentle vortexing and tapping the tube) in 48 μl of absolute ethanol. For each transformation, 6 μl of DNA-coated microparticles were spread onto a macrocarrier disc (Biorad) and allowed to dry for 5 minutes in a laminar flow hood. Every transformation experiment included at least two control plates which had been bombarded with microparticles prepared in the same way except that ddH₂O was used in place of DNA.

Transformations using chimeric aphA-6 cassettes were carried out as follows: Cells were grown up to 1 x 10⁶ cells/ml, then 10 mls of cells were pelleted by centrifugation, resuspended in ~0.5 ml TAP and spread on to sterile nylon filters (Hybond) on TAP plates. These plates were then bombarded with microparticles coated with the appropriate plasmid using an 1100 psi rupture-disc and approximately 27 inches Hg vacuum using a JAVAC DS 150 high vacuum pump (Aust.). After transformation the plates were incubated at 21 °C out of direct light for 24 hrs. The cell covered filters
were then aseptically transferred to fresh TAP plates, containing either kanamycin at 65 μg/ml or amikacin at 150 μg/ml and incubated at 25 °C in bright light (45 μE/m²/s) for 1-2 weeks. Transformation and selection for ΔpsbH photoautotrophic mutants using the *aphA-6* marker was performed in the same way except transformation plates were covered with Whatman 3MM paper. Putative transformant colonies were then picked onto TAP + kanamycin at 100 μg/ml. Homoplasmic transformants were obtained by restreaking primary transformants 3-4 times on TAP + kanamycin at 100 μg/ml and TAP + amikacin at 150 μg/ml plates. In liquid medium *aphA-6* transformants were grown in TAP + 2 μg/ml kanamycin.

Transformation involving rescue of photoautotrophic mutants was achieved by preparing cells in the same way as for *aphA-6* transformations, but using TAP only plates from which, after 24 hours the filters were transferred to Tris-min plates to select for photosynthetically competent colonies.

Chloroplast transformation using the *aadA* cassette (Goldschmidt-Clermont, 1991a) was carried out in the same way as the *aphA-6* marker with the following alterations. Pelleted cells were resuspended in ~0.5 ml TAP then 0.5 ml molten soft TAP agar (0.4% bactoagar, cooled to ~40 °C) was added and the suspension poured onto a TAP containing spectinomycin (100 μg/ml), spread over the agar surface by swirling and allowed to set. After particle-gun-bombardment the plates were incubated immediately at 25 °C in bright light (45 μE/m²/s). Transformant colonies were picked onto fresh TAP + 100 μg/ml spectinomycin plates and selected for homoplasmy by restreaking primary transformants 3-4 times on TAP + spectinomycin at 100 μg/ml.

2.9 Chlorophyll assays

Assays of chlorophyll concentration were carried out by adding 20 μl of chloroplast suspension to 10 ml of 80% acetone. This suspension was then mixed and filtered through Whatman 3MM paper. The absorbance of duplicate samples was then determined at 645, 647, 652, 653 and 664 nm and an average absorbance calculated for each wavelength. The equations of Arnon, (1949) and Porra *et al.*, (1989) were then used to calculate chlorophyll concentrations.
2.10 Electron paramagnetic resonance (EPR) spectroscopy

Highly concentrated suspensions of whole cells were used for EPR analysis of photosystem II reaction centres. 200 ml of cells were grown to 2-4 \times 10^6 cells/ml. A chlorophyll concentration assay was then performed (section 2.9). The cells were then pelleted by centrifugation in a Sorval GSA rotor at 3000g for 10 minutes and the cells resuspended in 20 mM Tris-HCl, 5 mM EDTA.Na\_2, pH 8.0 buffer (approximately 30 ml). This step was then repeated twice and then the cells were finally respuspended to a concentration of 2-3 mg/ml chlorophyll. Approximately 0.3 ml of sample was then injected into an EPR tube, dark adapted for at least one hour on ice, and then frozen in liquid nitrogen. EPR analysis was performed using a Jeol REIX spectrometer with an Oxford Instrument liquid helium cryostat.

2.11 The polymerase chain reaction

Amplification of DNA fragments by PCR (Mullis and Faloona, 1987) was performed in a Hybaid Thermal Cycler using Vent DNA polymerase (New England Biolabs). All reactions were in a total volume of 50 µl. Reactions typically contained: 1 µg genomic DNA or 10 ng plasmid DNA template, 50 µM of each dATP, dCTP, dGTP and dTTP, 1 µM of each oligonucleotide primer, 1U Vent DNA polymerase, 1x Vent reaction buffer (Promega) and made up to volume with ddH\_2O. Reactions were overlaid with 50 µl of mineral oil to prevent evaporation. Conditions of the reaction cycles were determined by the length and composition of the oligonucleotide primers and by the predicted length of the product.

2.12 DNA sequencing and sequence analysis

DNA and protein sequence analysis was carried out using DNASTAR LaserGene Navigator software (DNASTAR Inc.). Protein alignments were carried out using the Lipman-Pearson algorithm (Lipman and Pearson, 1985).

The majority of DNA sequencing was carried out on an automated Perkin-Elmer ABI Genetic Analyser 310. However, manual sequencing was also performed via a dideoxy chain termination derived method (Sanger et al., 1977), using the Sequenase II
sequencing kit (USB) and (α-35S)dATP (Amersham). Denatured plasmid DNA was used as a template for the sequencing reaction (Chen and Seeburg, 1985). 10 μg of plasmid DNA, made up to a volume of 18 μl with ddH₂O was used as template and prepared by adding 2 μl of 2 M NaOH and heating to 68 °C for 20 minutes to denature the DNA strands. The DNA was precipitated by adding 10 μl of 3 M sodium acetate pH 6.0, 100 μl of absolute ethanol and incubating at -20 °C for 30 minutes. The DNA was pelleted in a microfuge, washed with 70 % ethanol, dried under vacuum and resuspended in 7 μl ddH₂O. For the sequencing reactions, 1μl of oligonucleotide primer at 40 ng/μl and 2 μl of Sequenase reaction buffer were added to the denatured plasmid DNA. The mix was heated to >65 °C for 2 minutes and allowed to cool slowly to <35°C to allow annealing of the primer to the plasmid template. Sequencing was then performed according to the manufacturer’s protocol. The sequencing reactions were heated to 80 °C for 2 minutes and half of each sample loaded onto a 6 % polyacrylamide (acrylamide:bis-acrylamide, 19:1), 7 M urea, 1x TBE gel (Scotlab-Strathclyde) which had been polymerised chemically using 0.025 % TEMED and 0.025 % ammonium persulphate. The gel was run under 35 V constant voltage using a 30 cm vertical gel apparatus (Cambridge Electrophoresis). Gels were fixed in 10 % methanol, 10 % acetic acid and dried onto Whatman 3 MM paper (Bio-Rad Gel Drier, 80 °C), and autoradiographed overnight at room temperature.
Chapter 3

The Development of a Novel Selectable Marker for Chloroplast Transformation
3 THE DEVELOPMENT OF A NOVEL SELECTABLE MARKER FOR CHLOROPLAST TRANSFORMATION

3.1 Introduction

As in other model organisms molecular genetic analysis of the *Chlamydomonas* chloroplast genome has been greatly enhanced by the development of dominant selectable markers. In contrast, the first example of chloroplast transformation in *Chlamydomonas* used a photoautotrophic mutant host strain. This strain was bombarded with a wild-type copy of the defective *atpB* gene and transformants were selected for by their ability to grow photoautotrophically (Boynton *et al.*, 1988). Rescuing photosynthetic mutants with the wild-type copy of the affected gene has since been used in several studies as a means of transformant selection (reviewed in Boynton and Gillham, 1993; Erickson, 1996). Subsequent strategies used variants of rRNA genes (Newman *et al.*, 1990; Kindle *et al.*, 1991; Newman *et al.*, 1991; Roffey *et al.*, 1991) or the PSII gene *psbA* (Przibilla *et al.*, 1991; Newman *et al.*, 1992), conferring antibiotic resistance and herbicide resistance respectively, as markers of transformation. The first dominant selectable marker to be developed for *Chlamydomonas* chloroplast transformation used the bacterial *aadA* gene (Goldschmidt-Clermont, 1991a). The 'aadA cassette', conferring resistance to spectinomycin and streptomycin, is now ubiquitous among studies of chloroplast gene function in *Chlamydomonas* (Rochaix, 1997). The *aadA* gene has also been adapted for use as a selectable marker for chloroplast transformation in higher plants (Svab and Malaga, 1993) and in the nuclear genome of *Chlamydomonas* (Cerutti *et al.*, 1997a).

Unfortunately, in contrast to other model systems, new dominant selectable markers for *Chlamydomonas* chloroplast transformation have not been forthcoming. The current lack of dominant markers (apart from the *aadA* cassette) is becoming increasingly problematic: strains containing *aadA* cannot be studied further by subsequent transformation as they are already spectinomycin/streptomycin resistant. This problem can be circumvented to some degree by methods which re-use the *aadA* cassette (Fischer *et al.*, 1996). Although useful, such methods require a more involved transformation protocol and are useless for re-transformation of ordinary *aadA*-containing mutants produced before the publication of this method.

This chapter describes the development of a novel selectable marker for chloroplast transformation in *Chlamydomonas* based on the 3'-aminoglycoside phosphotransferase encoding *aphA-6* gene, originally isolated from *Acinetobacter baumannii* (Martin *et al.*, 1997a).
Like the *aadA* cassette, the new marker uses a bacterial gene (*aphA-6*), flanked by chloroplast controlling elements. However, in contrast to *aadA*, it confers resistance to the aminoglycoside antibiotics kanamycin and amikacin. *aphA-6* was selected from a literature review which aimed to identify candidate antibiotic resistance genes which might be developed as novel markers for chloroplast transformation. It was thought that to act effectively as a marker for chloroplast transformation in *C. reinhardtii*, such a gene would ideally possess the following attributes: (1) Confer resistance to substrates affecting prokaryotic, rather than eukaryotic cells. Therefore, such substrates would specifically effect the chloroplast (but not the mitochondrial). (2) Have a similar codon bias to *C. reinhardtii* chloroplast genes, i.e. favouring AT over GC nucleotides, in order to limit potential down-regulation of expression of the foreign gene due to rare codon usage. (3) Confer resistance to several different substrates. A selectable marker conferring resistance to more than one substrate enables elimination of spontaneous resistant strains. (4) Have been expressed previously in a heterologous system.

The *aphA-6* gene appeared to fulfil these criteria: It confers resistance to aminoglycoside antibiotics specifically affecting 70S ribosomes (Lambert *et al.*., 1988); its AT:GC ratio is 67:33 (Martin *et al.*., 1988); it was isolated from a nosocomial strain of *A. baumannii* resistant to kanamycin and structurally related aminoglycoside antibiotics including amikacin (Lambert *et al.*., 1988); it has been shown to have activity when expressed in *E. coli* (Martin *et al.*., 1988). Also, the coding sequence of *aphA-6* is relatively small (777 bp, Martin *et al.*., 1988), making cloning straightforward. Finally the product of *aphA-6*, APH(3')-VI, has phosphorylating activity against gentamycin A/B, kanamycin A/B/C, amikacin, tobramycin, neomycin A/B/C, paromycin, butirosin and ribostamycin (Lambert *et al.*., 1988), several of which are commonly available and inexpensive.

### 3.2 Results

#### 3.2.1 Construction and sequencing of the *aphA-6* cassette

In order to analyse the effect of different controlling elements on the expression of foreign genes, three different chloroplast expression cassettes were constructed. The cassettes were located within the vector p72B-HS, at an intergenic region downstream of *psbH* so as to limit possible interference with expression of endogenous genes. Each expression cassette contains upstream sequence the 5' untranslated leader and ATG from a different, highly expressed chloroplast gene. This is followed by a series of restriction enzyme sites and a 437 bp fragment containing the 3'-end of the *rbcL* gene.
The *aphA-6* coding sequence was inserted into all three of these cassettes. Detailed descriptions of the cloning of each of the *aphA-6* chloroplast expression vectors is included in appendix II. Figure 3.1 shows schematically the structure of each of the vectors described in the text.

Throughout construction, each plasmid was partially sequenced to confirm its structure. Also, the *psbA5*:::*aphA6*:::*rbcL3* cassette was amplified from *pskKmR* (section 3.2.7), using primers MLU5/MLU3 (appendix I.i) and the resulting product completely sequenced. Figure 3.2 shows the DNA sequence of the cassette and the predicted amino acid sequence of APH(3')-VI. The APH(3')-VI sequence perfectly matches the previously published sequence (Martin *et al.*, 1988), apart from a two amino acid addition at the N terminus, which is a vestige of the cloning strategy used.
Figure 3.1 *C. reinhardtii* chloroplast expression vectors and structures of plasmids used for transformation of *aphA-6*. Thin full line represents linearised section of chloroplast DNA in pUC8, bars represent segments of DNA inserted into plasmid vectors. Black bars: position of *psbN* and *psbH* in p72B-HS; hatched bar: 5' part of *rbcL*, *atpB* and *psbA* genes respectively; stipled bar: 3' part of *rbcL* gene; chequered bar: *aphA6* coding sequence. Panel a: *aphA6* under various controlling elements. Open bar: 5' part of *rbcL*, *atpB* or *psbA* genes. Panel b: Inactivation of *psbH*. Dotted line: pBluescript SK(+).
**Figure 3.2** DNA sequence of psbA5':::aphA6::rbcL3' cassette ('aphA-6 marker' cassette). Primers used to amplify cassette from p72-psbA-aphA6 shown in bold; *psbA* 5' promoter region shown in blue; *aphA-6* coding sequence shown in red; *rbcL3' sequence shown in green; amino acid sequence of APHA(3')-VI protein shown in capitals; selected restriction enzyme sites underlined.
3.2.2 Levels of spontaneous resistance to aminoglycoside antibiotics in *C. reinhardtii*

In order to develop *aphA-6* as a selectable marker it was first essential to assay the rates of spontaneous resistance in *C. reinhardtii* to substrates of the aminoglycoside phosphotransferase APH(3')-VI, the product of *aphA-6*. To do this $1 \times 10^9$ stationary phase wild-type CC-1021(mt+) cells were plated onto large (20 cm x 20 cm) TAP plates containing a particular aminoglycoside antibiotic (all at 100 μg/ml). The antibiotics were selected from those shown to be modified by APH(3')-VI, *in vitro* (Lambert *et al.*, 1988). The results of this assay are shown in Table 3.1. Spontaneous resistance to kanamycin and amikacin was relatively high (one resistant colony per $1 \times 10^4$ - $1 \times 10^5$ cells), whereas resistance to gentamycin was extremely low (less then one resistant colony per $1 \times 10^8$ cells) and resistance to chloramphenicol was not detected. Although phosphorylated by APH(3')-VI (Lambert *et al.*, 1988), neomycin was not tested due to its low solubility making it impractical for routine usage.

<table>
<thead>
<tr>
<th>Antibiotic (all at 100 μg/ml)</th>
<th>Chloramphenicol</th>
<th>Gentamycin sulphate</th>
<th>Kanamycin monosulphate</th>
<th>Amikacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of resistant colonies (out of $1 \times 10^9$ cells)</td>
<td>0</td>
<td>5</td>
<td>$1.7 \times 10^4$</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>Rate of resistance per cell per generation (number of resistant colonies/total number of cells)</td>
<td>0</td>
<td>$5 \times 10^9$</td>
<td>$1.7 \times 10^5$</td>
<td>$1 \times 10^4$</td>
</tr>
</tbody>
</table>

Table 3.1 Rate of spontaneous resistance to aminoglycoside antibiotics in *C. reinhardtii* wild-type CC-1021(mt+) cells. All antibiotics at 100 μg/ml, $1 \times 10^9$ cells assayed for each antibiotic.

3.2.3 Chloroplast expression constructs containing *aphA-6* confer kanamycin resistance in *E. coli*

Several studies have demonstrated that chloroplast gene 5' UTRs are able to promote gene expression in *E. coli* (Hanley-Bowdoin and Chua, 1987; Thompson and Mosig,
Figure 3.3 Kanamycin resistance of *E. coli* (DH5α) transformed with *aphA*-6 coding sequence under various controlling elements. UT: Untransformed DH5α; SK: DH5α transformed with pSKaphA-6; rbcL: DH5α transformed with p72-rbcLII-aphA6; atpB: DH5α transformed with p72-atpB-aphA6; psbA: DH5α transformed with p72-psbA-aphA6; 235: DH5α transformed with pAT235.
1987; Goldschmidt-Clermont, 1991a). Therefore, it was decided to test whether *E. coli*, transformed with the different chloroplast expression constructs containing *aphA-6* (Fig. 3.1), displayed the appropriate antibiotic resistance phenotype. Initially, *E. coli* (DH5α) which had been transformed with the various plasmid vectors, using ampicillin as the selective antibiotic, were re-streaked onto LB agar plates containing increasing concentrations of gentamycin sulphate between 0.05 μg/ml and 100 μg/ml. These plates were incubated overnight at 37 °C then assessed for presence or absence of new colonies. Using gentamycin sulphate no difference was observed between DH5α transformed with the chimeric *aphA-6* constructs, and a control plasmid lacking the *aphA-6* coding region. All were able to grow up to, but not above, 1 μg/ml gentamycin. Therefore, this assay was repeated using plates containing kanamycin (sulphate) in the range of 2 μg/ml to 40 μg/ml. As expected, untransformed cells and cells containing the control plasmid lacking *aphA-6* were unable to grow even at the lowest kanamycin concentration used (2 μg/ml) (Fig. 3.3). Whereas, DH5α transformed with the plasmid pAT235 (Martin *et al.*, 1988), containing *aphA-6* under its native promoter, were viable up to the highest concentration (40 μg/ml) (Fig. 3.3). DH5α transformed with the three chimeric *aphA-6* constructs conferred differing levels of kanamycin resistance as follows: p72-rbcXII-aphA6 up to 2 μg/ml; p72-atpB-aphA6 up to 10 μg/ml; p72-psbA-aphA6 up to 20 μg/ml (Fig. 3.3). Unexpectedly, DH5α transformed with pSKaphA6, which is the *aphA-6* coding sequence ligated into the multiple-cloning-site in pBluescript SK(+), conferred viability up to 10 μg/ml kanamycin. This was assumed to be due to residual expression of *aphA-6* from the *lac* promoter, sited upstream from the multiple-cloning-site. This could have occurred even without IPTG (a gratuitous inducer of the *lac* promoter) included in the medium as in the *E. coli* strain DH5α the copy number of pSK plasmids is greater than the number of molecules of the *lac* repressor.

3.2.4 High levels of aminoglycoside antibiotic resistance in indirectly selected *aphA-6* transformants

In order to assess the expression of *aphA-6* under various controlling elements, p72-rbcXII/atpB/psbA-aphA6 were all transformed into the PSII− host strain ΔpsbH (Bst-
Table 3.2 Levels of kanamycin (panel a) and amikacin (panel b) resistance in *ΔpsbH* strain transformed with *aphA-6* coding sequence under different controlling elements. 


### a

<table>
<thead>
<tr>
<th>Kanamycin concentration (µg/ml)</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>150</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>rbcL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>atpB</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>psbA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

### b

<table>
<thead>
<tr>
<th>Amikacin concentration (µg/ml)</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>150</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>rbcL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>atpB</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>psbA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
opp), (O’Connor et al., 1998). In ΔpsbH (Bst-opp), psbH has been inactivated by the insertion of the aadA cassette into the coding sequence. Therefore, transformants were selected by their ability to grow photoautotrophically on Tris-min medium (lacking a reduced carbon source). Aminoglycoside antibiotic resistance in transformants containing aphA-6 under each of the three controlling elements was then assessed by ability of the transformant to grow as single colonies on TAP medium containing increasing concentrations of kanamycin or amikacin (Tables 3.2). p72-rbcXII-aphA6 and p72-psbA-aphA6 transformants were able to grow on at least 200 μg/ml kanamycin, whereas p72-atpB-aphA6 transformant were only viable on less than 100 μg/ml kanamycin. In contrast, growth of wild-type cells was completely inhibited at and above 50 μg/ml kanamycin (Table 3.2a). The results for amikacin were less clear in that all three types of transformant grew on at least 200 μg/ml amikacin, while wild-type were viable to some degree between 100-200 μg/ml amikacin (Table 3.2b). From these results it was decided to use p72-psbA-aphA6 in attempts to obtain aminoglycoside antibiotic resistant transformants, i.e. to develop the p72-psbA-aphA6 cassette as a dominant selectable marker for chloroplast transformation.

3.2.5 aphA-6 is present in the chloroplast genome of directly selected transformants

In transformations using p72-psbA-aphA6 plasmid DNA and WT-CC1021(mt+) as a host strain it was possible to isolate antibiotic resistant colonies by selection on TAP medium containing either kanamycin at 65 μg/ml or amikacin at 150 μg/ml. To initially detect the presence of aphA-6, four of these putative transformants isolated using kanamycin were selected and PCR analysis carried out on a crude cell lysate using the primers APHA5/APHA3. As seen in figure 3.4, a band of the expected size, and the same as that produced from the plasmid control, is amplified from all four putative aphA-6 transformants. Whereas, none is produced from wild-type cells. To confirm this result total DNA from the same four transformants was digested with EcoRI, separated by agarose gel electrophoresis and transferred to nylon membrane by Southern blot. This blot was then probed with the aphA-6 gene (Fig.3.5). Two bands of around 1.2 and 4.5 kbp hybridise in all four transformants. This is good evidence that not only is the aphA-6 cassette present within these transformants but also that it has incorporated into the chloroplast genome. psbH is located on EcoRI fragment 19.
(4.2 kbp), with the addition of the *aphA-6* cassette (containing an *EcoRI* site in the *aphA-6* gene), the two bands demonstrate that the cassette has recombined into the expected location in the chloroplast genome.

**Figure 3.4** PCR amplification of *aphA-6* from wild-type cells transformed with p72-psbA-aphA6. M: 1 kb ladder (MBI Fermentas); 1-4: Four p72-psbA-aphA6 transformants, in WT CC1021(mt+) host, genomic DNA used as template for PCR; Pl: p72-psbA-aphA6 plasmid DNA used as template for PCR; WT: WT CC-1021(mt+) total genomic DNA used as template for PCR.
Figure 3.5 Southern analysis of p72-psbA-aphA6 transformants in a wild-type host. Total genomic DNA was digested with EcoRI, Southern blot was probed with aphA-6 coding sequence. 1-4: Four p72-psbA-aphA6 transformants in WT CC-1021(mt+) host; PI: p72-psbA-aphA6 plasmid DNA; WT: WT CC-1021(mt+) total genomic DNA digested with EcoRI.
Figure 3.6 Kanamycin and amikacin resistance in *aphA*·6 transformants. 1-4: Four p72-psbA-aphA6 transformants in WT CC-1021(mt+) host; WT: wild-type CC-1021(mt+); TAP+Ak150: TAP medium supplemented with amikacin at 150 μg/ml; TAP+Km100: TAP medium supplemented with kanamycin at 100 μg/ml.
To confirm the observation from indirectly selected \textit{aphA-6} transformants (section 3.2.4), that the heterologously expressed APH(3')-VI inactivates amikacin as well as kanamycin, these four \textit{aphA-6} transformants were grown on amikacin containing plates. In figure 3.6 it is seen that all four transformants grow on TAP + amikacin (150 \text{\textmu}g/ml) and TAP + kanamycin (100 \text{\textmu}g/ml), whereas wild-type growth is inhibited on both these antibiotics.

3.2.6 Expression of \textit{aphA-6}

To confirm that \textit{aphA-6} transformants were transcribing the foreign gene, total RNA from the \textit{aphA-6} containing mutants bAA 1/3/6 (see below) was isolated, separated by agarose gel electrophoresis and hybridised to a nylon membrane by northern blot. This blot was then probed with \textit{aphA-6} DNA (Fig. 3.7). Approximately equal quantities of RNA was loaded in each well. As expected, the probe hybridises to RNA from all three transformants, but not at all to RNA isolated from wild-type cells. A band at around 2.8 kb is seen in all three transformants, but is much feinter in bAA 1 (Fig. 3.7). Conversely, RNA from bAA 1 hybridises to the probe at approximately 1.1 kbp, but an equivalent band is not seen in the other two transformants (Fig. 3.7). These data are discussed in section 3.3.

\textbf{Figure 3.7} Expression of \textit{aphA-6} in p72KmR (bAA) transformants. Total RNA from p72KmR transformants bAA 1,3,6 probed with \textit{aphA-6} DNA. WT: total RNA from WT CC1021(mt+).
3.2.7 Insertional mutagenesis using *aphA-6*

To confirm that the *aphA-6* cassette is a truly portable marker, i.e. that its expression is not due to positional effects of nearby chloroplast DNA sequence, the 
psbA5'::aphA6::rbcL3' cassette was isolated by PCR and inserted into the *EcoRV* site in pBluescript SK(+) (details of cloning in Appendix II). The primers used contained engineered restriction sites so that when the cassette was cloned into pBluescript SK(+) the new plasmid (pskKmR) would contain convenient sites flanking the cassette to enable its easy excision (Fig. 3.1). The sequence of the *aphA-6* cassette within pskKmR was then determined (Fig. 3.2). In order to confirm its utility the *aphA-6* cassette was excised from pskKmR as a *Smal* fragment and inserted into a unique *BstXI* site in *psbH* in the opposite orientation, to produce p72KmR (Fig. 3.1). Wild-type CC-1021 (mt+) cells were then bombarded with p72KmR plasmid DNA and kanamycin resistant colonies selected. Kanamycin resistant strains were checked for the presence of the *aphA-6* gene by PCR and, once its presence had been confirmed, were taken through four rounds of streaking to single colonies on selective medium. The photoautotrophic competence of six of these transformants, denoted 'bAA 1-6' (*psbA* *aphA-6* transformants), was then examined by comparing their growth in the light on solid medium containing acetate as a reduced carbon source (TAP medium) and on minimal medium (Tris-min medium). As seen in figure 3.8, bAA 1/3/6 are unable to grow on minimal medium in common with ApsbH (Bst-opp), a similar mutant generated using the *aadA* cassette (O'Connor *et al.*, 1998). Whereas, wild-type cells are able to grow on minimal medium. As expected, all the strains are viable on TAP medium and only bAA 1-6 are viable on TAP + kanamycin. As bAA 2/4/5 remained photosynthetically competent it may be possible that these strains were spontaneously kanamycin resistant, having arisen while streaking to homoplasmy on selective media. To test this hypothesis, PCR analysis was carried out on a crude cell lysate from bAA 1-6 and wild-type using the primers APHA5/APHA3. This analysis confirms the presence of *aphA-6* in all the bAA strains (Fig. 3.9). Therefore bAA 2/4/5 must be heteroplasmic for the insertionally inactivated copy of *psbH*.

In order to confirm the expected absence of photosystem II in these transformants electron paramagnetic resonance (EPR) spectroscopy was carried out on mutants bAA 3, ΔpsbH (Bst-opp), and on wild-type CC-1021 (mt+) cells. All samples were dark adapted for 1 hour prior to freezing in liquid nitrogen. YD is a tyrosine residue of PSII that can donate an electron to P680+. Therefore, an absent YD+ signal indicates lack of functional PSII in the samples. In EPR traces from bAA 3 and ΔpsbH (Bst-opp), the YD+ signal was absent. Upon illumination at 4.7 K the PSI reaction centre chlorophyll
special pair P700* signal was observed in all three strains. This indicates that the two PSII" strains contain functional PSI (Hallahan et al., 1995), in similar amounts to wild-type (Fig. 3.9).

Figure 3.8 Phenotypic analysis of bAA putative photosynthetic mutants. Spot tests of bAA 1-6 grown on TAP; minimal medium (Tris Min); TAP supplemented with kanamycin at 100 µg/ml (TAP + Km100). 1-6: bAA transformants 1-6; ΔpsbH: ΔpsbH (Bst-opp) photosynthetic mutant; WT: WT CC-1021(mt+). All plates were incubated at 25 °C in bright light (45 µE/m²/s).
Figure 3.9 Genotypic analysis of putative bAA mutants. PCR amplification of the \textit{aphA-6} gene. M: marker DNA ladder; 1-6: bAA transformants 1-6 DNA used as template for PCR; P: p72-\textit{psbA-aphA6} plasmid DNA used as template for PCR; WT: WT CC-1021(mt+) used as template for PCR.
Figure 3.10 EPR analysis of bAA 3. Panel a: EPR trace from bAA 3 dark adapted cells showing absence of the $Y_D^+$ signal (blue line) and, upon illumination at 4.7 K, presence of the P700$^+$ signal (pink line) indicating absence of PSII and presence of PSI respectively. Panel b: ΔpsbH (Bst-opp) EPR trace, $Y_D^+$ signal absent and P700$^+$ signal present. Panel c: WT CC-1021(mt+) EPR trace, $Y_D^+$ signal and P700$^+$ signal both present.
3.3 Discussion

The above results have shown that under the correct controlling elements the foreign gene, \textit{aphA-6}, can be functionally expressed in the \textit{Chlamydomonas} chloroplast. The high AT content (67\%) and previous demonstration of its heterologous expression in \textit{E. coli} (Lambert \textit{et al}, 1988) suggested that \textit{aphA-6} may be suitable to such a role.

A common problem with the use of antibiotic resistance as a marker of transformation is the prevalence of spontaneous resistance to the substrate used. Even if this rate is relatively low it can still mask the identification of transformants due to the inefficiency of many methods of transformation. Most aminoglycoside antibiotics interact with the ribosome causing inhibition of protein synthesis or misreading during translation of the mRNA transcript (Franklin and Snow, 1975; Garrod \textit{et al}, 1981). Due to the precise nature of the interaction between a ribosomal subunit and an antibiotic molecule, in a single base-pair mutation a ribosomal gene can confer resistance against the drug. For example, streptomycin resistance in \textit{C. reinhardtii} can be conferred by a single point mutation in the chloroplast encoded S12 ribosomal protein (Liu \textit{et al}, 1989). For this reason, the extent to which \textit{C. reinhardtii} becomes spontaneously resistant to aminoglycoside antibiotics inactivated by APH(3')-VI was investigated. The high rates of spontaneous resistance to kanamycin and amikacin (Table 3.1) indicate that resistance to these drugs is probably conferred by a single point mutation in a ribosomal gene. The extremely low rate of spontaneous resistance to gentamycin might imply that multiple mutations in the chloroplast ribosome are needed to confer resistance. An alternative explanation might be that this drug inhibits protein synthesis on both chloroplast and mitochondrial ribosomes. The likelihood of resistance conferring mutations being present in the ribosomes of both organelles in the same cell being relatively low. Both organelles perform prokaryotic-type protein synthesis and so should be similarly affected by aminoglycoside antibiotics. Why particular antibiotics affect only the chloroplast and not the mitochondrial protein synthetic machinery in \textit{C. reinhardtii} is not understood. The absence of any colonies spontaneously resistant to chloramphenicol may imply a similarly stringent action to gentamycin. Alternatively, the fact that chloramphenicol causes arrest of cell growth (by inhibiting translation, Franklin and Snow, 1975), rather than inducing cell death, may impede isolation of strains spontaneously resistant to this drug.

Initially the chimeric \textit{aphA-6} gene was incorporated into the chloroplast genome via an indirect method, linking it to \textit{psbH} and obtaining transformed colonies via complementation of a \textit{psbH} mutant. Even though selected for their ability to grow

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photoautotrophically, these strains showed high levels of resistance to kanamycin and amikacin, two of the aminoglycosides which \( \text{aphA-6} \) is reported to inactivate (Martin et al., 1988). Also, strains containing the atpB5':\( \text{aphA-6}\):rbcL3' cassette showed lower levels of kanamycin resistance than those containing rbcL or \( \text{psbA} \) controlling elements. Of these latter cassettes, one may confer a higher level of resistance than the other, as both strains were viable at the highest antibiotic concentrations tested (200 \( \mu \text{g/ml} \)). As such, the expression of \( \text{aphA-6} \) has been used as a reporter to determine the levels of expression imparted by each controlling element. The differing levels of resistance conferred by the three elements concur to some degree with relative rates of transcription of the genes from which they are derived (Blowers et al., 1990). All three constructs also promoted expression of \( \text{aphA-6} \) when transformed in \( \text{E. coli} \) resulting in kanamycin resistant colonies (Fig. 3.3). Previous studies have also demonstrated that chloroplast promoters have activity in \( \text{E. coli} \) (Hanley-Bowdoin and Chua, 1987; Thompson and Mosig, 1987; Goldschmidt-Clermont, 1991a). This result should be practically useful when manipulating the \( \text{aphA-6} \) marker \textit{in vitro}, i.e. enabling \( \text{aphA-6} \)-containing plasmids to be shuttled between \textit{Chlamydomonas} and \( \text{E. coli} \) using kanamycin as the selective substrate. The varying levels of kanamycin resistance conferred by each chloroplast promoter in \( \text{E. coli} \) reflects the similarity between bacterial and chloroplast gene expression systems.

Subsequently it was shown that the psbA5':\( \text{aphA6}\):rbcL3' cassette can be used as a directly selectable marker for chloroplast transformation using either kanamycin or amikacin as the selective antibiotic. Initial PCR (Fig. 3.4), and then Southern analysis (Fig. 3.5) confirmed the presence of \( \text{aphA-6} \) in strains transformed in this way. In four randomly picked transformants resistance to kanamycin was directly linked to presence of a single copy of \( \text{aphA-6} \) in the chloroplast genome (Fig. 3.5/6). Kanamycin resistant \textit{Chlamydomonas} strains have been previously isolated with mutations affecting the small subunit of the chloroplast ribosome (Harris, 1989). Initially, it was thought the prevalence of such strains may impede selection for \( \text{aphA-6} \) transformants on kanamycin due to high levels of spontaneous resistance (Table 3.1). In actual experiments however, the background appeared to be much lower than expected, usually around 10% of putative transformant colonies being spontaneously resistant. Unexpectedly, the spontaneous resistant colonies that appear when selecting on TAP + kanamycin at 65 \( \mu \text{g/ml} \) were consistently dose-dependant, such that none of these colonies when transferred to TAP + kanamycin at 100 \( \mu \text{g/ml} \) was ever found to be viable.
Finally, it has been shown previously that *Chlamydomonas* strains containing an insertionally mutagenised copy of *psbH*, using the *aadA* cassette, do not assemble photosystem II (O'Connor *et al.*, 1998). The *psbA5':aphA6::rbcL3'* cassette was inserted at the same position within the coding sequence of *psbH* as was the *aadA* cassette by O'Connor *et al.* (1998) (Fig. 3.1). If *aphA-6* was acting correctly as a selectable marker then transformation of wild-type cells with this construct, and subsequent subcloning on selective medium, should produce the same PSII' phenotype. Kanamycin resistant strains transformed with p72KmR were subcloned on kanamycin and amikacin containing medium. Three out of the six transformants (bAA 1-6) were unable to grow photoautotrophically (Fig. 3.8). EPR analysis, a technique which directly monitors unpaired electrons, was then used to detect whether functional PSII was present in one of these putative PSII mutants. bAA 3 lacked the *Y_D^+* signal (Fig. 3.10), indicating absence of PSII and confirming that insertional mutagenesis of *psbH*, using the *aphA-6* cassette, results in the expected PSII' phenotype. Northern analysis of these mutants showed RNA species from the three non-photosynthetic mutants (bAA 1,3,6) hybridised to the *aphA-6* probe (Fig. 3.7). The pattern of this hybridisation though is somewhat perplexing. In *C. reinhardtii*, *psbH* is known to be transcribed on three different mRNAs of 900, 600 and 500 residues each (Johnson and Schmidt, 1993). In the bAA transformants one would expect to see the *aphA-6* monocistronic message, beginning from the chimeric *psbA* promoter, and this is likely to be the 1.1 kb band in bAA 1. The 2.8 kbp bands seen in bAA 3 and bAA 6 were initially thought to be a redundant *psbH* transcript containing the *aphA-6* insertion. However, the predicted size of such a transcript is at most approximately 2.4 kb (the 900 base *psbH* transcript with a 1.5 kbp *aphA-6* cassette insertion). Reasons for the identical (non-photosynthetic) phenotypes, but differing *aphA-6* transcript patterns in bAA 1, and bAA 2/3 can only be speculated upon. As discussed in section 1.2.4.2, many factors, both chloroplast and nuclear encoded, affect the stability and processing of chloroplast mRNAs in *C. reinhardtii*. Perhaps bAA 1 contains some secondary mutation which affects transcript stability of *aphA-6*. Also, northern analysis using a double stranded DNA probe detects both sense and antisense messages, complicating the resulting hybridisation pattern. Ideally, further analysis such as RNase protection experiments would determine the exact size of the *aphA-6* sense transcript(s), but unfortunately time was limited.
Chapter 4

Introduction of Two Novel Foreign Genes into the Chloroplast of *C. reinhardtii*
4 INTRODUCTION OF TWO NOVEL FOREIGN GENES INTO THE CHLOROPLAST OF C. REINHARDTII

4.1 Introduction

In this section the aim was to use the ease of transformation of the *Chlamydomonas* chloroplast to express the genes for two foreign proteins. This would be done by transforming the foreign genes into the chloroplast and assessing the functionality of the heterologous proteins.

This approach would first be used in order to develop a system for the heterologous expression of the *por* gene, encoding the enzyme NADPH-protochlorophyllide oxidoreductase (POR), from *Synechocystis* sp. PCC 6803. POR or 'light-dependent protochlorophyllide reductase' catalyses the reduction of protochlorophyllide (Pchlide) to chlorophyllide (chlide). This reaction is the penultimate step in the biosynthesis of chlorophyll (Fig. 4.1). The dependence on light for greening in angiosperms is a direct result of light being an essential cofactor for POR. In addition, non-flowering land plants, algae and photosynthetic bacteria all contain a light-independent protochlorophyllide reductase that does not rely on light as a cofactor (Schulz and Seng, 1993). Figure 4.2 shows an alignment of the amino acid sequences of POR from *C. reinhardtii* and *Synechocystis* sp. PCC 6803. The two polypeptides are 52% similar (excluding the transit peptide sequence from *C. reinhardtii* POR) and are identical at key residues such as tyrosine 189 and lysine 193 (in *Synechocystis* POR), within the proposed active site (Wilks and Timko, 1995). Therefore it is possible that *Synechocystis* POR synthesised in the *C. reinhardtii* chloroplast may retain some of its enzymatic activity.
Figure 4.1 Overview of chlorophyll biosynthesis. Adapted from Smith and Griffiths, (1993)/Reinbothe and Reinbothe, (1996). Panel a: Overview of the chlorophyll biosynthetic pathway. ALA: 5-aminolaevulinic acid. Panel b: Light and NADPH-dependent reduction of Pchlide to Chlide by POR.
In order to study POR in vivo via site-directed mutagenesis, a chloroplast expression vector containing the *Synechocystis* por gene was introduced into the yellow-in-the-light/yellow-in-the-dark mutant *pc-ly-7*. The *Chlamydomonas* mutant *pc-ly-7* lacks both the light-dependent and light-independent POR (Harris, 1989). A routine system for the heterologous expression of *por* in the *Chlamydomonas* chloroplast would allow extensive characterisation of the enzyme using a molecular genetic approach. Using this system, transformants in which the heterologous POR is functional could be selected by their ability to synthesise chlorophyll, and hence carry out photosynthesis. The results of this screen are presented.

**Figure 4.2** Amino acid sequence alignment of POR from *Synechocystis* sp. PCC 6803 (Suzuki and Bauer, 1995) and *C. reinhardtii* (Li and Timko, 1996). The first 83 residues of the *C. reinhardtii* sequence (putative transit peptide sequence) are not shown. Conserved residues are shown in blue. Dotted lines represent gaps in sequence. 6803: *Synechocystis* sp. PCC 6803 sequence; Cr: *C. reinhardtii* sequence. Sequence similarity = 52%.
The second gene discussed is the *tufA* gene, encoding the highly conserved protein elongation factor EF-Tu, from the plastid of the Apicomplexan *Plasmodium falciparum*. EF-Tu specifically binds aminoacyl-tRNA, components of the ribosome, guanine nucleotides, a magnesium ion, and another protein elongation factor, elongation factor Ts (EF-Ts) (described in Alberts *et al.*, 1989). EF-Tu is regulated by the binding of GTP and GDP (Berchtold *et al*., 1993). Binding of GTP activates EF-Tu causing it to form a ternary complex with aminoacyl-tRNA and transports the complex to the A site of the messenger RNA-programmed ribosome. Subsequent GTP hydrolysis leads to the release of EF-Tu-GDP from the protein synthesis machinery. Protein synthesis cannot continue until EF-Tu has dissociated from the ribosome. This delay increases the fidelity of polypeptide synthesis because incorrectly bound tRNAs with the wrong codon-anticodon base-pairing lack the stability to remain bound during the delay. EF-Tu thereby increases the ratio of correct to incorrect amino acids incorporated into the nascent polypeptide (Fig. 4.3).

![Figure 4.3](image_url)

**Figure 4.3** The role of elongation factor Tu in the initial stages of protein synthesis (Alberts *et al*., 1989).
Recently it has been demonstrated that many members of the parasitic Apicomplexan genus have a third DNA-containing organelle (reviewed by Lang-Unnasch et al., 1998). The origin of this organelle is somewhat enigmatic although insight has been gained by phylogenetic analysis of its genome, suggesting it is monophyletic (Denny et al., 1998) and of plastidic origin (Wilson et al., 1994). Initial analysis suggested the genome of this organelle, or 'apicoplast', to be of rhodophyte ancestry (Williamson et al., 1994). However, a more recent study comparing the nucleotide and protein sequence of the tufA gene from the apicoplast of three Apicomplexans to that of forty other species, including red and green algae, cyanobacteria and eubacteria, determined it to be of green algal origin (Kohler et al., 1997). However, further evidence concerning the question of the ancestry of this organelle is much needed.

In order to test the hypothesis that the plastid genome of Apicomplexans and green algae are phylogenetically closely related, a gene replacement experiment was carried out. This involved replacing the Chlamydomonas chloroplast tufA gene with its homologue from the apicoplast of the malarial parasite Plasmodium falciparum. The primary amino acid sequence of the two proteins share 50% identity overall (Fig. 4.4) and are completely identical in residues G19HVDHGK25; D81CPG84; N136KED139 and S174AL176 involved in binding the phosphoryl, Mg²⁺ and guanine residues of GTP respectively (Kjeldgaard and Nyborg, 1992). The residues defining the GDP binding pocket are also conserved (G24, N136, K137, D139, S174, L176). In a less well conserved region the two proteins share an insertion (residues 181-190) specific only to plastid versions of EF-Tu (Wilson et al., 1996). Therefore, it is not inconceivable that the P. falciparum plastid EF-Tu could complement its C. reinhardtii homologue, albeit at a reduced efficiency. It was assumed that the ability of the apicoplast tufA gene to functionally replace its green algal counterpart would depend on the degree of divergence of the two plastids. Also, the apicoplast, which appears to be essential for the viability of its host, has great potential as a target for chemotherapeutic agents (Fichera and Roos, 1997). Difficulties in handling erythrocytic P. falciparum cultures could make a transgenic Chlamydomonas strain carrying an apicoplast gene a useful heterologous system for testing such drugs.
Figure 4.4 Protein sequence alignment of EF-Tu from *C. reinhardtii* (Baldauf and Palmer, 1990b) and *P. falciparum* (Wilson et al., 1996). Common residues shown in blue. CR: *C. reinhardtii* sequence; PF: *P. falciparum* sequence. Similarity = 50%. Gaps (-), introduced for maximum alignment.
4.2 Results

4.2.1 Construction of *por* transformation cassettes

Construction of plasmid p72B and chloroplast expression vectors p72-rbcX and p72-psbA are described in appendix II. To produce p72-rbcX-por the *por* gene coding sequence was amplified from p6803 (obtained from Dr W. T. Griffiths, University of Bristol) by PCR using primers POR51 and POR31 (appendix I) incorporating *Bsp*HI and *Sal*I sites respectively. This PCR product was digested with *Bsp*HI/*Sal*I and ligated into p72-rbcX, which had been previously digested with NcoI/SalI, to produce p72-rbcX-por (Fig. 4.5). p72-psbA-por was produced in the same way but the PCR product was ligated into p72-psbA rather than p72-rbcX (Fig. 4.5).

![Diagram of plasmid structures](image)

**Figure 4.5** Structure of plasmids used for transformation of *Synechocystis por*. Thin full line represents linearised section of chloroplast DNA in pUC8, bars represent segments of DNA inserted into plasmid vectors. Black bars: position of *psbN* and *psbH* in p72B-HS; hatched bar: 5' part of *rbcL* and *psbA* genes; stipled bar: 3' part of *rbcL* gene; chequered bar: *por* coding sequence. Open bar: 5' part of *rbcL* or *psbA* genes.
4.2.2 Transformation of \textit{pc-ly-7} with \textit{p72-rbcX-por}

In an attempt to rescue the yellow-in-the-light/yellow-in-the-dark double mutant \textit{pc-ly-7}, \textit{p72-rbcX-por} plasmid DNA was coated onto tungsten microparticles and transformed into \textit{pc-ly-7} using particle-gun-bombardment. Cells were transformed on TAP medium and allowed to recover for 24 hrs in dim light (20 µE/m²/s) at 25 °C. They were then transferred to Tris-min plates and incubated in bright light (45 µE/m²/s) at 25 °C until green colonies appeared.

Several green (chlorophyll synthesising) putative transformants were obtained and were initially tested to check the phenotype was not a result of a reversion of the \textit{y-7} (yellow-in-the-dark) mutation. To do this, transformants 8, 9, 10 and 11 were spotted onto TAP plates and grown in the dark. As expected the putative transformants became yellow in colour appearing similar to the original \textit{pc-ly-7} mutant, whereas wild-type cells remained green (Fig. 4.6).

In order to detect the presence of the introduced cassette DNA was isolated from the same four putative transformants, Southern blotted and probed with the \textit{Synechocystis} sp. PCC 6803 \textit{por} gene. DNA from the putative transformants did not hybridise to the probe. Therefore, it was assumed the ability of these cells to synthesise chlorophyll must come from a reversion of the \textit{pc-1} mutation.

4.2.3 Characterisation of \textit{lpcr} in putative \textit{p72-rbcX-por} transformants

The \textit{pc-1} mutation affecting the light-dependant NADPH:protochlorophyllide oxidoreductase in \textit{C. reinhardtii} has previously been characterised (Li and Timko, 1996). It was found to be the result of a two base-pair deletion in the nuclear encoded \textit{lpcr} gene (Fig. 4.7a). This causes a shift in the reading frame of the POR precursor leading to premature termination of translation. To assess the presence or absence of this mutation a 344 bp region of the \textit{lpcr} gene was amplified from the putative transformants 10 and 11, wild-type and \textit{pc-1} mutant cells by PCR using primers LPCR5 and LPCR3 (Fig. 4.7b). The resulting products were sequenced and aligned to the published sequence of \textit{C. reinhardtii lpcr} (Fig. 4.8) The part of the \textit{lpcr} gene sequenced in both putative transformants is identical to wild-type and the published sequence. Importantly, both putative transformants contain the CA base-pairs absent from the \textit{lpcr} gene in the \textit{pc-1} mutant. Presumably this is the cause of the light-
Figure 4.6 Putative p72-rbcX-por transformants in pc-1y-7 host cannot synthesise chlorophyll in the dark. Cells spotted onto TAP medium and grown in dark for one week at 25 °C, and then transferred to the light. 1, 2, 3 and 4: putative p72-rbcX-por transformants 8, 9, 10, and 11 respectively. wt: wild-type CC-1021(mt+).
dependent chlorophyll synthesis in the putative transformants rather than the presence of a heterologous chloroplast-encoded POR.

Figure 4.7 PCR amplification of the nuclear l-per gene from putative p72-rbcX-por transformants. Panel a: DNA sequence in region of l-per gene amplified. Underlined sequence: regions against which primers were designed; CA: base-pairs deleted in pc-l mutant; *: termination codon in pc-l mutant due to frameshift. Panel b: Agarose gel of PCR reactions to amplify the above region. M: DNA size marker; 8, 10, 11: putative p72-rbcX-por transformant DNA used for PCR; WT: WT CC-1021(mt+) genomic DNA used for PCR; PC: pc-l genomic DNA used for PCR.
It is possible that the putative transformants were actually all clones of a single rare reversion event of the \textit{pc-1} mutation. In an effort to assay the rate of reversion of the \textit{pc-1} mutation in the \textit{pc-ly-7} strain approximately $5 \times 10^8$ \textit{pc-ly-7} cells were plated separately onto TAP and Tris-min plates and incubated at 22 °C in bright light (45 μE/m$^2$/s). No green colonies appeared on either plate. Also, the above transformation was repeated several times using p72-rbcX-por and p72-psbA-por plasmid coated onto gold microparticles. Selection for transformants was attempted under dim light (20 μE/m$^2$/s) and bright light (45 μE/m$^2$/s), but no putative transformants or chlorophyll synthesising revertants were isolated.

4.2.4 Transformation of p72-psbA-por into the photosynthetic mutant \textit{ΔpsbH}

It was not possible to rescue the \textit{pc-ly-7} mutant using any of the vectors containing the \textit{Synechocystis} \textit{por} (see above). Therefore, it was decided to test whether introduction of the gene was possible when rescue of the host strain was not reliant on its expression. As such, the vector p72-psbA-por (Fig. 4.5) was introduced into the strain \textit{ΔpsbH} (Bst-opp) (O'Connor \textit{et al.}, 1998) by particle-gun bombardment. \textit{ΔpsbH} (Bst-opp) contains an insertionally mutagenised copy of \textit{psbH},. Transformants therefore were selected for photosynthetic growth on Tris-min medium where p72-psbA-por, containing a wild-type copy of \textit{psbH}, had been incorporated. Several putative transformants were selected and streaked-out to single colonies three times on Tris-min medium in order to isolate homoplasmic strains. DNA was then isolated from four of these strains, digested with \textit{EcoRI/PstI} and analysed by Southern blot probing with the \textit{Synechocystis} sp. PCC 6803 \textit{por} gene (Fig. 4.9). A band of just over 2 kbp is seen in transformants 3, 5 and 6 confirming the presence of the \textit{por} gene in these strains. These strains were also found to be homoplasmic for the introduced construct.

4.2.5 Western analysis of p72-psbA-por transformants

Southern analysis of the p72-psbA-por transformants demonstrated that the \textit{por} gene had been incorporated into the \textit{C. reinhardtii} chloroplast genome. To determine whether the heterologous gene was being expressed western analysis was performed on three of the transformants with wild-type and \textit{pc-1} as controls. The protein preparations for each sample were separated into membrane and cytosolic fractions and run separately, only
L-PCR: cctgccctccccaccccttgccacacacag atg gcc ctc acc atg tcc
M A L T M S
GCC AAG TCC GTG AGC GCC CGC GCC CAG GTG TCC AGC AAG GCC CAG
A K S V S A R A Q V S S K A Q

WT: cctgccctccccaccccttgccacacacag atg gcc ctc acc atg tcc
M A L T M S
GCC AAG TCC GTG AGC GCC CGC GCC CAG GTG TCC AGC AAG GCC CAG
A K S V S A R A Q V S S K A Q

PC-1: cctgccctccccaccccttgccacacacag atg gcc ctc act gtc cgc
M A L T V R
CA GTC CGT GAG CGC CGC CGC CCA GTT GTC CAG CAA GGC CCA GGC
Q V R E R P P G V Q Q G P G

10: cctgccctccccaccccttgccacacacag atg gcc ctc acc atg tcc
M A L T M S
GCC AAG TCC GTG AGC GCC CGC GCC CAG GTG TCC AGC AAG GCC CAG
A K S V S A R A Q V S S K A Q

11: cctgccctccccaccccttgccacacacag atg gcc ctc acc atg tcc
M A L T M S
GCC AAG TCC GTG AGC GCC CGC GCC CAG GTG TCC AGC AAG GCC CAG
A K S V S A R A Q V S S K A Q

Figure 4.8 The pc-1 mutation in putative p72-rbcX-por transformants has reverted to the wild-type allele. Only part of the 342 bp PCR product shown, both transformants l-pcr genes contain the ‘CA’ base-pairs missing in the pc-1 mutant. l-pcr: Genbank sequence of l-pcr (U36752, Li and Timko, 1996); wt: Nucleotide sequence of PCR product amplified from WT CC-1021(mt+) genomic DNA; pc-1: Nucleotide sequence of PCR product amplified from pc-1 mutant genomic DNA; 10/11: Nucleotide sequence of PCR product amplified from putative p72-rbcX-por transformants 10/11 genomic DNA; *: position of ‘CA’ deletion; →: translation continues to end of gene; ——> : translation terminates early.
Figure 4.9 Southern analysis of genomic DNA from p72-psbA-por transformants demonstrates the presence of the *Synechocystis* *por* gene in transformants 3, 5 and 6. Total genomic DNA from four transformants and wild-type was digested with *EcoRI* and *PstI* and probed with the *Synechocystis* *por* coding sequence. 2, 3, 5 and 6: p72-psbA-por transformants in ΔpsbH (Bst-opp) host strain; +ve: *Synechocystis* sp. PCC 6803 *por* coding sequence (positive control); WT: wild-type CC-1021(mt+) (negative control).
cytosolic fractions are shown (Fig. 4.10). The antibody used was polyclonal and raised against *Synechocystis* sp. PCC 6803 POR (obtained from Dr W. T. Griffiths, University of Bristol). A single band is seen at approximately 36 kDa in the wild-type and the three p72-psbA-por transformants, but is absent in the *pc-l* strain (Fig. 5.10). This band is assumed to be the ca. 36 kDa *C. reinhardtii* POR protein cross-reacting with the *Synechocystis* antibody. A second band of the size of the *Synechocystis* POR (ca. 35 kDa) was not seen in the cytosolic or membrane fractions of any of the transformants in this or in repetitions of this analysis.

**Figure 4.10** Western analysis of *Synechocystis* por transformants. Cytosolic cell fractions were run on a 10% (w/v) SDS-polyacrylamide gel and transferred to nitrocellulose membrane as described in the methods. M: ECL biotinylated molecular weight markers (Amersham); 3, 5, 6: p72-psbA-por transformants; PC: *pc-l*; WT: wild-type CC-1021(mt+).
4.2.6 Construction of tufA transformation cassettes

Details describing the construction of the vectors shown in figure 4.11 are contained in appendix II. Essentially, two 700-900 bp fragments of chloroplast DNA flanking the C. reinhardtii tufA gene were amplified and cloned into pBluescript SK(+). The tufA gene from the apicoplast of *P. falciparum* was then inserted between the two fragments. Finally, the *aadA* cassette was cloned downstream of the *P. falciparum* gene to produce pPftfA. This construct enabled the *P. falciparum* tufA gene to be targeted to, and to replace the C. *reinhardtii* chloroplast tufA gene by homologous recombination. Also, a similar vector, PΔtfa, was produced in the same way as pPftfA but without insertion of the *P. falciparum* tufA gene. Transformation of pΔtfa was used to delete the *C. reinhardtii* tufA gene by selection for the *aadA* cassette.
Figure 4.11 Structure of plasmids used to replace and delete the *C. reinhardtii* *tufA* gene. Thick black line: *C. reinhardtii* chloroplast DNA; black boxes: chloroplast encoded genes; dotted lines: regions of chloroplast DNA amplified by PCR; thin black lines: pBluescript SK(+) sequence; hatched box: *P. falciparum* *tufA* gene; stippled box: *aadA* cassette.
4.2.7 Transformation of the *P. falciparum* *tufA* gene and *C. reinhardtii* *tufA* deletion constructs

Both pPfDtA and pAtfA were transformed into wild-type CC-1021(mt+) cells on TAP + spectinomycin containing plates by particle-gun bombardment. Putative transformants were initially checked for the presence of the introduced cassette by Southern analysis and then taken through four rounds of streaking to single colonies on TAP + spectinomycin (100 μg/ml).

4.2.8 Confirmation of presence of *P. falciparum* *tufA* gene in putative transformants

To confirm the presence of the *P. falciparum* gene total DNA from five putative pPfDtA transformants was isolated, digested with *NdeI/PstI* and Southern blotted using the *P. falciparum* *tufA* gene coding sequence as a probe (Fig. 4.12). Putative transformants 2, 3, 4 and 5 all contain a single band of the expected size (1650 bp) that hybridises to the probe. Therefore, transformants 2, 3, 4 and 5 have incorporated the introduced *P. falciparum* *tufA* gene at the expected locus within the chloroplast genome.

4.2.9 The chloroplast encoded *tufA* gene is essential for cell viability

It is assumed that the *C. reinhardtii* *tufA* gene is essential for chloroplast, and hence cell viability, although there has been no experimental evidence to confirm this. Therefore, pAtfA transformant A (after being taken through four rounds of selective streaking on TAP + spectinomycin) was re-streaked on TAP and TAP + spectinomycin. Total DNA from these cells, grown on selective and non-selective medium, was then isolated. Insertionally mutagenised copies of essential chloroplast genes are known to be rapidly lost once the selective pressure is removed (Goldschmidt-Clermont, 1991a; Huang et al., 1994; Fischer et al., 1996; Boudreau et al., 1997a). Therefore, if the *C. reinhardtii* *tufA* gene is essential for cell viability the introduced deletion construct should not only remain heteroplasmic under continued selective pressure, but should be rapidly lost if selection is removed. Southern blot analysis was performed on genomic DNA isolated from these cells which had been digested with *BamHI/EcoRI*. A PCR product was then produced as a template for a probe which would hybridise to both pAtfA and wild-type *tufA* genes. This was done by amplifying a 109 base-pair region of non-coding DNA
Figure 4.12 The *P. falciparum* *tufA* gene is present within the chloroplast genome of four putative pPftfA transformants. Total genomic DNA from transformants and wild-type was digested with *NdeI/PstI* and probed with the *P. falciparum tufA* coding sequence. 1-5: genomic DNA from pPftfA transformants in WT CC-1021(mt+) host; +ve: *P. falciparum tufA* coding sequence (positive control); A/B: genomic DNA from pAtfA transformants in wild-type CC-1021(mt+) host; WT: wild-type CC-1021(mt+) DNA.
just downstream of \textit{tufA} gene coding sequence using primers CRTF35 and CRTFKPN (appendix I). When hybridised with this probe, a 1.3 kbp band is seen in all the transformants and the wild-type, corresponding to copies of the genome containing the uninterrupted \textit{tufA} gene (Fig. 4.13). In p\Delta t\textit{fA} transformants A and B a second band also hybridises which is lost when transformant A is grown on non-selective medium, and corresponds to the heteroplasmic deletion construct (Fig. 4.13). p\Delta t\textit{fA} transformant C is assumed to be a strain that has acquired resistance to spectinomycin through random mutation. These results demonstrate that \textit{tufA} is essential because p\Delta t\textit{fA} transformants remain heteroplasmic and the introduced construct is rapidly lost on removal of selective pressure.

\textbf{4.2.10 The \textit{P. falciparum} tufA gene is unable to functionally replace its \textit{C. reinhardtii} counterpart}

To assess whether the \textit{P. falciparum} tufA gene had replaced its endogenous chloroplast counterpart, pPftA transformant 4 was taken through two further rounds of streaking to single colonies on selective and non-selective medium in the same way as described in the previous section. DNA was then isolated from these cells. This DNA and that from transformants 2, 3, 4, and 5 after four rounds of streaking, was digested with \textit{Ndel/PstI}, filter hybridised by Southern blot and probed simultaneously with the \textit{P. falciparum} and \textit{C. reinhardtii} tuf\textit{A} gene coding sequences (Fig. 4.14a). The introduced \textit{P. falciparum} tuf\textit{A} gene can be seen, as previously demonstrated in section 4.2.8, at around 1650 bp in transformants 2, 3, 4, and 5. However, it is lost after a single round of sub-cloning on non-selective medium. It is likely therefore that the introduced cassette is not completely replacing the endogenous tuf\textit{A} gene. This is confirmed by the fact that the \textit{C. reinhardtii} tuf\textit{A} gene (higher molecular weight band at around 7 kbp) is maintained even after six rounds of selection on spectinomycin containing medium.

The loss of the introduced cassette in pPftA transformant 4 grown on non-selective medium was confirmed by stripping this blot and reprobing it with the \textit{aadA} coding sequence (Fig. 4.14b). This probe hybridised with a band of around 3 kbp. The same pattern of maintenance with selection and loss on removal of selective pressure can be seen with this fragment, confirming that the introduced cassette cannot replace the endogenous tuf\textit{A} gene.
Figure 4.13 The pAtfA cassette remains persistently heteroplasmic proving the *tufA* gene is essential to cell viability. Southern analysis of pAtfA transformants in a WT CC-1021(mt+) host, genomic DNA digested with *BamHI/EcoRI* and probed with 109 bp probe complementary to non-coding DNA downstream of the *tufA* gene coding sequence. A-C: pAtfA transformant DNA after four rounds on TAP + spectinomycin containing medium; (A)-5R/6R: pAtfA transformant number 6 after 5/6 rounds on TAP (T) or TAP + spectinomycin containing medium (S). WT: Wild-type CC-1021(mt+) genomic DNA; ad+: *aadA* coding sequence.
Figure 4.14 The *P. falciparum* *tufA* gene cannot completely replace the endogenous copy and is rapidly lost without continuous selection. Southern analysis of genomic DNA from transformants digested with *NdeI/PstI* and probed with *P. falciparum* *tufA* coding sequence/*C. reinhardtii* *tufA* coding sequence (panel a), and *aadA* coding sequence (panel b). Pf+/ad+: *P. falciparum* *tufA* /*aadA* coding sequence respectively; 2-5: pPftfA transformants DNA after four rounds of selection on TAP + spectinomycin containing medium; (4)-5R/6R: pPftfA transformant number 4 after five/six rounds on non-selective TAP (T) or selective TAP + spectinomycin (S) containing medium; WT: wild-type CC-1021(mt+) genomic DNA.
4.2.11 Western analysis of pPftfA transformants

A polyclonal antibody (αMap1) has recently been raised against *P. falciparum* EF-Tu by R. J. M (Iain) Wilson and colleagues (N.I.M.R., Mill Hill). It was decided to use this antibody to detect heterologous expression of the *P. falciparum* tufA gene in the pPftfA transformants. Whole cell extracts from light and dark grown pPftfA transformants 2, 3 and wild-type were used. The concentration of each sample was estimated by measuring chlorophyll concentration (pPftfA 1: 0.99 mg/ml, pPftfA 2: 0.83 mg/ml, wild-type: 0.97 mg/ml). Only western analysis of dark grown cells is shown (Fig. 4.15), as no bands were seen in either the transformants or wild-type cells grown in the light. From the dark grown *P. falciparum* tufA transformants a band just below 50 kDa is seen. A much feinter band of the same size is seen in the dark-grown wild-type even though all the samples were of approximately equal concentration. Unfortunately, part of the wild-type band was cut from the filter during the analysis but at least half of it can still be seen.

![Western blot image](image)

**Figure 4.15** Transformants containing the *P. falciparum* tufA gene synthesise the heterologous protein. Immunoblot analysis of dark-grown whole cell extracts from pPftfA transformants 2 and 3 and wild-type CC-1021(mt+). Cell extracts were run on a 10% (w/v) SDS-polyacrylamide gel and transferred to nitrocellulose membrane as described in the methods. The western blot was probed with the αMap1 antibody, raised against *P. falciparum* EF-Tu.
4.3 Discussion

The intent in this chapter was to introduce \textit{por} and \textit{tufA} into the chloroplast genome in order to effect their heterologous expression. However, the purpose of expressing the two genes was not the same. The \textit{por} gene encoding NADH-protochlorophyllide oxidoreductase (POR) from \textit{Synechocystis} sp. PCC 6803 was introduced to instigate a system for studying the enzyme by site-directed-mutagenesis. In contrast, the \textit{tufA} gene, encoding the elongation factor-Tu (EF-Tu), from the plastid of \textit{Plasmodium falciparum} was introduced to determine whether it could functionally replace its chloroplast counterpart.

One technical difficulty with the heterologous expression of \textit{por} in the chloroplast of wild-type \textit{C. reinhardtii} is that its activity would be masked by that of the endogenous POR. Several \textit{C. reinhardtii} mutants are available which are affected in the synthesis of POR. The 'y' series mutants are affected at one of the several loci coding for components of the light-independent POR and have a 'yellow-in-the-dark' (chlorophyll-less) phenotype (Choquet \textit{et al.}, 1992; Suzuki and Bauer, 1992; Liu \textit{et al.}, 1993). The mutant \textit{pc-1} is affected in the single gene, \textit{lpcr}, coding for the light-dependant POR (Li and Timko, 1996). The \textit{pc-1} strain is still able to synthesise chlorophyll, but only around half the amount in the dark and around one third the amount in the light of that in wild-type cells (Ford \textit{et al.}, 1981). The double mutant \textit{pc-1y-7} is a result of crossing two of these mutants and is completely unable to synthesise chlorophyll. Therefore, if the cyanobacterial enzyme is functional in the \textit{C. reinhardtii} chloroplast its expression should rescue the \textit{pc-1y-7} mutant, such that it can synthesise chlorophyll in the light.

The \textit{Synechocystis} sp. PCC 6803 \textit{por} gene was placed downstream of promoters from the two highly expressed chloroplast genes \textit{rbcL} and \textit{psbA} (Fig. 4.5). Both of the expression constructs containing these promoters were known to be effective in expressing another foreign gene, \textit{aphA-6} (Chapter 3). Rescue of the \textit{pc-1y-7} mutant to a green-in-the-light phenotype was attempted by transformation with both these vectors under a variety of conditions (section 4.2.3). Green colonies were isolated from one of these transformations, but were later found to be revertants at the \textit{pc-1} locus. Unexpectedly, it was not possible to isolate any more such revertants, even when large numbers of \textit{pc-1y-7} cells were analysed. The only explanation for this anomaly is that the revertants were isolated as a result of particle-gun-bombardment using tungsten microparticles, whereas later transformations used gold. Tungsten is known to be toxic and mutagenic (Russell \textit{et al.}, 1992), and so it was assumed that its mutagenic properties induced reversion of the CA base-pair deletion (Fig. 4.8).
It appeared that neither of the *por* expression constructs were able to rescue the *pc-ly-7* mutant to be able to synthesise chlorophyll. Perhaps topological differences between the *Chlamydomonas* and cyanobacterial *POR* inhibited the heterologous enzyme from catalysing the conversion of protochlorophyllide to chlorophyllide (Fig. 4.1).

Alternatively, the *Synechocystis* *POR* enzyme may not have been forming the correct tertiary structure in the foreign environment of the chloroplast stroma or might not have been targeted correctly to its site of action (associated with the thylakoid membrane, Dahlin *et al.*, 1995). Unlike these explanations, one possibility that could be easily tested was that the foreign protein was not being stably expressed. To determine whether *Synechocystis por* could be expressed in the chloroplast it was transformed, under the control of the *psbA* promoter, into a different mutant (Δ*psbH*) where rescue did not rely on heterologous expression of functional *POR* (section 4.2.4). Preliminary western analysis of these transformants did not detect the heterologous protein (Fig. 4.10). However, further analysis of these transformants is needed. This is discussed in section 6.3.2. Also, a second band corresponding to the 34 kDa protein seen using antibodies raised against the *C. reinhardtii* *POR* (Li and Timko, 1996) was not seen in any of the transformant or wild-type strains. This is probably due to the differing sensitivity of the antibodies used in this study, raised against the *Synechocystis* *POR*, to that of those raised against the wheat *POR* (Li and Timko, 1996).

As mentioned above, the aim in this chapter was to achieve heterologous expression of both *por* and *tufA*. Both these proteins serve vital functions in completely different cellular processes. Whereas *POR* functions in a light dependant manner to catalyse one of the reactions in chlorophyll biosynthesis, EF-Tu plays a crucial role in polypeptide synthesis (Fig. 4.3). A vector was designed to enable replacement of the *C. reinhardtii* *tufA* gene with that from the *P. falciparum* plastid (Fig. 4.11). Upon recombination into the chloroplast genome, expression of the Apicomplexan gene would be controlled by the same elements as the endogenous *tufA*. This should preclude problems in timing and level of expression that may occur had the gene been under the control of a chimeric promoter. A deletion construct (pΔ*tfA*) was also produced to test whether the *C. reinhardtii* EF-Tu was essential for cell viability (Fig. 4.11). Transformation and selection for this deletion construct resulted in heteroplasmic transformants that retained wild-type copies of *tufA* as well as deleted copies. Upon removal of selection the deleted copies of *tufA* were rapidly lost from the transformants (section 4.2.9). This phenomenon is well known in *C. reinhardtii* and is indicative of essential chloroplast genes such as ORF 472 (Goldschmidt-Clermont, 1991a; Fischer *et al.*, 1996), *clpP* (Huang *et al.*, 1994), and ORF 1995 (Boudreau *et al.*, 1997a). Therefore, it had been
confirmed that the chloroplast encoded protein EF-Tu is essential for cell viability. Interestingly, the wild-type and deleted copies were present in approximately equal amounts, as estimated by the intensity of the respective bands on the Southern blot (Fig. 4.13). Presumably cells must retain a minimum of half the usual number of wild-type copies of the tufA gene to remain viable. Whether this is because there is redundancy in the level of wild-type expression of EF-Tu, or that transformants are somehow able to upregulate the level of expression of the remaining copies is unknown.

Once pPftfA transformants, containing the Apicomplexan tufA gene, had been obtained they were taken through several rounds of sub-cloning on selective medium. These strains were then analysed to confirm the presence of the foreign gene and to determine whether it had replaced its C. reinhardtii counterpart (section 4.2.10). All the pPftfA transformants analysed retained copies of the C. reinhardtii tufA gene (Fig. 4.14a). Also, once selective pressure was removed the foreign gene was rapidly lost (Fig. 4.14a/b), in a similar manner to the deletion construct (see above). To determine whether the P. falciparum gene was being expressed western analysis was performed on two of the pPftfA transformants (section 4.2.11). Although only preliminary, this analysis does suggest that there was expression of the P.falciparum EF-Tu (Fig. 4.15). The predicted molecular weights of the P. falciparum and C. reinhardtii EF-Tu's are Mr 46,642 and Mr 45,747 respectively. Therefore, it is unlikely that the two proteins would be visibly distinct on an SDS-PAGE gel. The αMap1 antibody is known to cross-react with E. coli EF-Tu (R. J. M. Iain Wilson, personal communication), it is therefore likely that it also cross-reacts with the C. reinhardtii EF-Tu. In figure 4.15 a feint band can be seen in the wild-type at the same position as the much more intense bands of the two transformants. As the loading of each sample was similar (section 4.2.11) it is concluded that heterologous P. falciparum EF-Tu was being expressed in the two transformants. The feint band produced in the wild-type was produced by the αMap1 antibody cross-reacting with the endogenous C. reinhardtii EF-Tu. C. reinhardtii EF-Tu must still be present in the transformants as they remained heteroplasmic (section 4.2.10), but this band would indistinguishable from the P. falciparum EF-Tu as the molecular weights of the two proteins are very similar. Since the foreign protein was being expressed it must be concluded that it was not able to functionally replace the endogenous EF-Tu. The meaning of these results is discussed further in section 6.3.2.
Chapter 5

Transposition of DNA from the Chloroplast to the Nucleus in *Chlamydomonas*
5.1 Introduction

An endosymbiotic basis for the origin of mitochondria and chloroplasts was suggested nearly thirty years ago by Margulis (Margulis, 1970). It proposed that these organelles were originally free living bacteria which were engulfed by early eukaryotic cells lacking such structures. A large body of experimental evidence now supports this hypothesis and has led to its general acceptance as the explanation for the origin of chloroplasts and mitochondria (reviewed by Gray and Doolittle, 1982; Gray, 1992). An unexpected consequence of this endosymbiosis appears to have been the reduction of the proto-organelle genome to a fraction of its original postulated size. The majority of the genes encoded by the original symbiont having either been transferred to the nucleus, or lost entirely and functionally replaced by pre-existing nuclear genes. At present, any explanations of the motivation behind this massive migration of genetic information have been purely speculative (section 1.1.4). For example, perhaps organellar genes transplanted to the nucleus permit their tighter control and concerted expression with the rest of the cellular machinery. Aside from such theoretical discussions, gene transfer between organelle and nuclear genomes was not demonstrated at the DNA sequence level until the early 1980's. The first clear example of this was the isolation of contiguous rearranged portions from mitochondrial genes (the small ribosomal protein gene var1, the cytochrome b gene cox/box, and ori/rep sequences involved in mitochondrial DNA replication) in the nuclear genome of Saccharomyces cerevisiae (Farrelly and Butow, 1983). Subsequently, many examples of organellar to nuclear gene transfer has been documented involving both mitochondrial (e.g. Van den boogaart et al., 1982; Gellissen et al., 1983; Blanchard and Schmidt, 1995) and chloroplast DNA (e.g. Timmis and Steele Scot, 1983; Pichersky et al., 1991; Ayliffe and Timmis, 1992; Blanchard and Schmidt, 1995). DNA discovered to have been transferred relatively recently in evolutionary history suggests that this process is ongoing (section 1.1.4 Baldauf and Palmer, 1990b; Gantt et al., 1991; Blanchard and Schmidt, 1995).

How the process of organellar to nuclear gene transfer occurs is unknown. It is generally assumed that genetic material translocated to the nucleus at some stage adapts to expression in the new environment and acquires the appropriate organelle transit sequence. Once it has been effectively expressed and targeted to the organelle, the now redundant organellar gene becomes inactivated by random mutation and is eventually
lost (Brennicke et al., 1993, Gray, 1992 #111). Obviously, there are many unsolved questions as to the mechanisms by which each of these steps might occur. Not least among these is how genetic material exits the organelle and is taken up and incorporated into the nuclear genome. Is the process active or is genetic material simply spilt from the organelle and enters the nucleus by diffusion? Is the translocation vector DNA or RNA? [The presence of edited versions of mitochondrial genes in the nuclear genome (Covello and Gray, 1992; Blanchard and Schmidt, 1995), has led to the suggestion that the transfer may, in some cases, involve an RNA intermediate]. The only experimental system to enable closer analysis of this enigmatic process demonstrated escape of DNA from the mitochondria to the nucleus in the budding yeast Saccharomyces cerevisiae (Thorsness and Fox, 1990). This involved transforming a ρ⁰ yeast strain with a plasmid containing the nuclear URA3 gene, the mitochondrial COX2 gene and the origin of replication of the yeast 2μ plasmid. A synthetic ρ strain was isolated which contained this plasmid only within the mitochondria, and so was auxotrophic for uracil. By selection on uracil free medium this strain could be shown to produce Ura+ prototrophs, where the plasmid containing the URA3 gene had moved to the nucleus. The rate of this escape was relatively high (~2 x 10⁵ per cell per generation), whereas escape of COX2 in the opposite direction (nucleus to mitochondria) was at least 100,000 times less. Subsequently nuclear mutations were isolated which increased the rate of escape of DNA from mitochondria to the nucleus. One of these mutations was shown to be in a gene designated YME1, which encodes a protein homologous to E. coli FtsH, suggesting Yme1p may play a role in mitochondrial fusion and/or division.

The following experiments were designed to set-up an analogous system for studying escape of DNA from the chloroplast to the nucleus in C. reinhardtii (Fig. 5.1). This would be done by introducing the 'ble' cassette, a dominant selectable marker for nuclear transformation which confers zeomycin resistance in C. reinhardtii (Stevens et al., 1996), into the chloroplast. The lack of a bacterial-type chloroplast expression sequence would inhibit the expression of ble in the chloroplast. Once homoplasmic chloroplast transformants were obtained, the aim was to screen large numbers of cells for resistance to zeomycin, conferred by ble sequence that had escaped from the chloroplast and transferred to the nucleus. If this 'transposition' could be observed at a high enough frequency it would then be possible to screen for mutants in which the rate of escape is altered.
Figure 5.1 Experimental design to isolate cells where DNA has escaped from the chloroplast to the nucleus genome. Zm\textsuperscript{S}: cells zeomycin sensitive; Zm\textsuperscript{R}: cells zeomycin resistant. chloroplast: chloroplast; n: nucleus

5.2 Results

5.2.1 Construction of ble chloroplast transformation cassettes

In order to introduce ble into the chloroplast of C. reinhardtii it first needed to be flanked by adjacent sections of the chloroplast genome, so that the introduced cassette would be incorporated by homologous recombination. To achieve this the RBCS2\textsuperscript{5\prime}::ble::RBCS2\textsuperscript{3\prime} EcoRI cassette from pSP108 (Stevens et al., 1996), containing the ble gene coding sequence flanked by 5\' and 3\' UTRs from the nuclear gene RBCS2, was ligated into the unique EcoRI site in pBa3-AX downstream of psaA exon 3 (psaA3) to produce pBa3-AX-ble (Fig. 5.2). pBa3 was assembled from the 6.9 kbp BamHI fragment from the C. reinhardtii chloroplast genome (Bam13), cloned into
pBluescript SK(-) which had previously had the 18 bp Smal/EcoRV fragment excised from its multiple cloning site. pBa3-AX was then made by cutting pBa3 with AvrII/XbaI and religating the plasmid. pBa3-AX-117 (see section 5.2.6) was produced similarly, by inserting the EcoRI fragment from pSP117 (in which the ble gene has had two copies of RBCS2 intron 1 inserted into it Lumbreras et al., 1998a) into the same unique EcoRI site in pBa3-AX (Fig. 5.2).

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**Figure 5.2** Structure of vectors used for introduction of the ble cassette into the chloroplast genome. Thin line represents linearised BamHI fragment 13 of chloroplast DNA in pBluescript SK(-). Black bar: position of exon 3 of psaA. RBCS25':ble::RBCS23' and ble (117) cassettes excised from pSP108 and pSP117 respectively as EcoRI fragments and inserted into unique EcoRI site of pBa3-AX. Hatched bars: 5' and 3' elements from RBCS2; stipled bar: ble coding sequence; chequered bars: RBCS2 intron 1 (I).
5.2.2 Production and confirmation of homoplasmic pBa3-AX-ble chloroplast transformants

To enable selection of pBa3-AX-ble chloroplast transformants the host strain C575D was used (Hallahan et al., 1995). This strain contains site-directed mutations in *psaA* exon 3, causing a C→D change in the amino acid sequence of the core photosystem I protein PSI-A. C575D cells are unable to grow photosynthetically due to the site-directed-mutations in *psaA-3*. Therefore, replacement by the wild-type copy contained on pBa3-AX-ble would allow transformed cells to be selected for by their ability to photosynthesise. If the recombination event occurs outside the ble cassette then this will also be incorporated into the chloroplast genome of transformed cells, and the aadA cassette will be replaced (causing the cells to become sensitive to spectinomycin). pBa3-AX-ble plasmid DNA was coated onto tungsten microparticles and transformed by particle-gun-bombardment onto a lawn of C575D cells grown on TAP medium. After incubation for 24 hours at 25 °C in dim light (20 µE/m²/s) bombarded cells were transferred to Tris-Min medium and then incubated at 25 °C in bright light (45 µE/m²/s). Several photoautotrophic putative transformant colonies were isolated and these were taken through three rounds of streaking to single colonies in order to obtain homoplasmic transformants. pBa3-AX-ble transformant strains should be homoplasmic in order to give the maximum number of copies of ble in the chloroplast and to prevent possible loss of the ble cassette by copy correction.

Initial confirmation that these transformants contained the ble cassette within the chloroplast genome was carried out by PCR analysis (Fig. 5.3). One primer homologous to chloroplast DNA downstream of *psaA-3* (PSAA3) and one homologous to the *RBCS2* 3' region of the ble cassette (BLE3) were used in the reaction (appendix I). A product of the expected size (1.4 kbp) was amplified from all nine putative transformants (Fig. 5.3).

To confirm the PCR analysis and determine whether the transformants were homoplasmic, total DNA was isolated from all nine transformants, digested with *BamHI*, separated by gel electrophoresis and membrane-hybridised by Southern blot. Initially the blot was probed with the ble cassette in order to detect the presence of ble in the chloroplast. As expected, this probe hybridised to a 1.7 kbp and a 6.8 kbp band in all nine transformants (Fig. 5.4a), due to the presence of a *BamHI* site at the terminus of the ble coding sequence (Fig. 5.2). Once the presence of the ble cassette had been confirmed the membrane was stripped and re-probed with the 1.5 kbp *BamHI/EcoRI*
Figure 5.3 PCR analysis of genomic DNA from putative ble chloroplast transformants. Using a primer homologous to chloroplast DNA downstream of psaA-3 and one to the 3' region of RBCS2, a 1.4 kbp PCR product was amplified from all nine pBa3-AX-ble transformants (1-9). M: DNA size marker; Pl: pBa3-AX-ble plasmid DNA used as template for PCR (positive control); WT: WT CC-1021(mt+) genomic DNA used as template for PCR (negative control). Black bar: exon 3 of psaA; hatched bars: 5' and 3' elements from RBCS2; stipled bar: ble coding sequence.
Figure 5.4 The ble cassette is present and homoplasmic in nine pBa3-AX-ble transformants. Total genomic DNA from pBa3-AX-ble transformants 1-9 and wild-type CC-1021(mt+) was digested with BamHI and probed with the 1.3 kbp EcoRI fragment from pBa3-AX-ble (panel a); the 1.5 kbp EcoRI/BamHI fragment from pBa3-AX-ble (panel b). +ve: 1.3 kbp EcoRI fragment from pBa3-AX-ble; WT: WT CC-1021(mt+) genomic DNA.
fragment from pBa3-AX-ble (Fig. 5.2), consisting of chloroplast DNA downstream of the introduced ble cassette. In wild-type DNA this probe should hybridise to the 6.9 kbp Bam13 fragment of the chloroplast genome, whereas in C575D (the host strain for the pBa3-AX-ble transformation) the Bam13 fragment is increased to 8.8 kb due to the presence of the aadA cassette, the marker used to introduce the site-directed mutations (Hallahan et al., 1995). Therefore, if any of the pBa3-AX-ble transformants were heteroplasmic the probe should hybridise to the 1.7 kbp fragment seen previously (Fig. 5.4a), and the 8.8 kbp fragment of the C575D host. In all nine transformants the probe only hybridised with 1.7 kbp fragments (Fig. 5.4b), whereas in the wild-type control only the 6.9 kbp fragment hybridised, as expected. Therefore, the presence and homoplasmic state of the ble cassette in the chloroplast genome has been confirmed in all nine transformants.

5.2.3 pBa3-AX-ble chloroplast transformants are unable to grow on zeomycin containing medium

An essential element of the assay for escape of chloroplast DNA to the nucleus is the inability of the RBCS25':ble::RBCS23' cassette to confer zeomycin resistance when maintained in the chloroplast. Theoretically this is unlikely as the ble gene is flanked by nuclear controlling elements which have little or no activity in the chloroplast. Also, any Ble protein present in the chloroplast would have to be transferred to the nucleus, in order to inhibit the nuclease activity of zeomycin on nuclear DNA (Calmels et al., 1993). To confirm this assumption pBa3-AX-ble transformants 1, 2 and 3 were spotted onto TAP and TAP + zeomycin (10 μg/ml) plates together with two bona fide ble nuclear transformants (Fig. 5.5). All three ble chloroplast transformants were completely inhibited from growing on zeomycin containing medium, confirming that the presence of the RBCS25':ble::RBCS23' does not confer resistance to zeomycin. As expected the two ble nuclear transformants grew well on TAP + zeomycin (10 μg/ml) (Fig. 5.5).

5.2.4 Nuclear transformation of the ble cassette amplified from chloroplast transformants confers zeomycin resistance

Before assaying for escape of DNA to the nucleus in ble chloroplast transformants, it was decided to confirm that the chloroplast localised ble cassette was still viable as a
nuclear marker. To do this PCR amplification of the ble cassette was carried out from pBa3-AX-ble transformants 1 and 4, in the same way as described in section 5.2.2. These two PCR products as well as pBa3-AX-ble plasmid DNA, and a no-DNA control, were then used to transform the nuclear genome of the cell wall-less strain cw15(mt-) using the glass bead method (Kindle, 1990). ble transformants were selected for on TAP + zeomycin (10 μg/ml). Figure 5.6 shows the results of this transformation, which demonstrate that the ble cassette amplified from pBa3-AX-ble chloroplast transformants is still able to act as a marker for nuclear transformation. The pBa3-AX-ble plasmid DNA also produced zeomycin resistant transformants, whereas no transformants were produced if ble DNA is absent from the transformation protocol.

![TAP vs TAP + Zeo experiment](image)

**Figure 5.5** ble cassette chloroplast transformants are unable to grow on zeomycin containing medium. C1-C3: pBa3-AX-ble transformants 1-3; N1/N2: Two RBCS25′:ble::RBCS23′ nuclear transformants; TAP: TAP medium; TAP + Zeo: TAP medium containing zeomycin at 10 μg/ml.
Figure 5.6 The ble cassette, amplified from pBa3-AX-ble chloroplast transformants, is still active as a nuclear transformation marker. CP tf 1/4: cw15(mt-) transformed with the RBCS25'::ble::RBCS23' cassette amplified from pBa3-AX-ble transformants 1/4; No DNA: cw15(mt-) transformed without DNA as a negative control; pBa3-AX-ble: cw15(mt-) transformed with pBa3-AX-ble plasmid DNA as a positive control.
5.2.5 Attempts at the isolation of strains where ble has transferred from the chloroplast to the nuclear genome

Although, as mentioned previously, the rate of escape of DNA from the mitochondria to the nucleus in yeast is relatively high (~2 x 10^5 per cell per generation), (Thorsness and Fox, 1990), it was not known what the equivalent rate of transfer from the chloroplast to the nucleus might be. Therefore, any assay for transfer of ble from the chloroplast to the nucleus would have to be extremely sensitive in case the rate of transfer was very low. With this in mind, all the assays described in Table 5.1 (apart from mated cells), were carried out by growing 100 mls of pBa3-AX-ble transformant 4 to late log phase (0.5-1 x 10^7 cells/ml), then plating these cells on large (20 cm x 20 cm) TAP plates in soft (0.7 %) TAP agar. Unfortunately, under the various conditions described in Table 5.1, zeomycin resistant colonies were never isolated. The only exception being when cells were grown at 33 °C. However, in this case zeomycin resistant colonies were also isolated from the wild-type control to a similar order, so this result was assumed to be due to the mutagenic affect of the extreme incubation temperature. Similar assays carried out using ble nuclear transformants yielded lawns of confluentely growing cells.

All the assays described in Table 5.1 should be sensitive enough to detect zeomycin resistant colonies at a level 10,000 times greater than the rate of escape of DNA from the mitochondria to the nucleus seen in yeast (Thorsness and Fox, 1990). One reason why zeomycin resistant colonies might not have been isolated was the relative efficiency of the ble marker. Introduction of two copies of RBCS2 intron 1 into the coding sequence of the ble gene in pSP108 has been shown to increase nuclear transformation efficiencies by up to twenty times (Lumbreras et al., 1998a). Therefore, it was decided to re-introduce this enhanced version of the ble marker into the chloroplast, in the hope that it would be more easily detected if it escaped to the nuclear genome.

5.2.6 Production and confirmation of homoplasmic pBa3-AX-117 chloroplast transformants

The plasmid pBa3-AX-117 (Fig. 5.2), containing the enhanced pSP117 version of ble (Lumbreras et al., 1998a), was transformed into the host strain C575D using particle-gun-bombardment and photoautotrophic transformants selected in the same way as pBa3-AX-ble transformants (section 5.2.2). After streaking-out through three rounds on Tris-min medium to produce putative homoplasmic transformants the presence of the ble (117) cassette was detected by PCR using primers RBCS25 and RBCS23 (Fig.
<table>
<thead>
<tr>
<th>Method of selection</th>
<th>Results</th>
<th>pBa3-AX-ble transformants</th>
<th>pBa3-AX-117 transformants</th>
<th>WT CC-1021 (mt+) control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubated on TAP +20 µg/ml zeomycin plate at 25°C in bright light.</td>
<td>No colonies</td>
<td>No colonies</td>
<td>No colonies</td>
<td></td>
</tr>
<tr>
<td>Incubated on TAP +5 µg/ml zeomycin plate at 25°C in bright light.</td>
<td>Several zeomycin resistant 'palmelloid' colonies recovered.</td>
<td>ND</td>
<td>No colonies</td>
<td></td>
</tr>
<tr>
<td>Incubated on TAP +7.5/12.5/15 µg/ml zeomycin at 25°C plates in bright light.</td>
<td>No colonies</td>
<td>ND</td>
<td>No colonies (TAP +10 µg/ml zeomycin only)</td>
<td></td>
</tr>
<tr>
<td>Cells grown in liquid culture at 33°C then incubated on TAP +10 µg/ml zeomycin plate at 25°C in bright light.</td>
<td>4.9 x 10⁵ cells plated-out, 49 colonies recovered.</td>
<td>ND</td>
<td>5.8 x 10⁶ cells plated-out, 121 colonies recovered.</td>
<td></td>
</tr>
<tr>
<td>0.5 ml cells incubated at 42°C for 120 s, then inoculated into 100 mls TAP, grown to stationary phase (1x10⁷ cells/ml) at 25°C. Culture then incubated on TAP +10 µg/ml zeomycin plate at 25°C in bright light.</td>
<td>No colonies</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Mated with WT CC-12 (mt-) cells. Plate containing zygotes overlain with 0.7% TAP agar +20 µg/ml zeomycin and incubated at 25°C in bright light.</td>
<td>ND</td>
<td>Areas of un-mated cells able to grow once zeomycin had been depleted.</td>
<td>Areas of un-mated cells able to grow once zeomycin had been depleted.</td>
<td></td>
</tr>
<tr>
<td>Cells grown in liquid culture at 30°C then incubated on TAP +20 µg/ml zeomycin plate at 25°C in bright light.</td>
<td>ND</td>
<td>No colonies</td>
<td>No colonies</td>
<td></td>
</tr>
<tr>
<td>Cells grown in continuous liquid TAP for eight weeks, then incubated on a TAP +15 µg/ml zeomycin plate at 25°C in bright light.</td>
<td>ND</td>
<td>No colonies</td>
<td>No colonies</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.1** Results of attempts to isolate pBa3-AX-ble/117 chloroplast transformants in which the ble cassette has escaped to the nucleus, by screening for zeomycin resistant colonies. ND: experiment not done; bright light: 45 µE/m²/s.
This analysis determined putative pBa3-AX-117 transformants 1, 3 and 6 to contain the ble (117) cassette (Fig. 5.7).

To confirm the PCR analysis and determine whether the introduced cassette was homoplasmic, total DNA from pBa3-AX-117 transformants 1, 2, 3 and 6 was isolated, digested with BamHI, separated by electrophoresis and filter hybridised by Southern blot. This blot was then probed firstly with the ble (117) cassette which hybridised to a 1.7 kbp and a 6.8 kbp band in transformants 1, 3 and 6, confirming that these strains were true transformants (Fig. 5.8a). Secondly, this blot was stripped and re-probed with 1.5 kbp BamHI/EcoRI fragment from pBa3-AX-117 (Fig. 5.2). As in section 5.2.2, the presence of a 1.7 kbp band and absence of a 8.8 kbp band in pBa3-AX-117 transformants 1, 3 and 6 confirmed they were homoplasmic (Fig. 5.8b). In putative transformant number 2, the 8.8 kbp band characteristic of the C575D host strain hybridised to the probe (Fig. 5.8b). Presumably this strain has incorporated the wild-type psaA exon 3 without the adjacent ble (117) cassette and so retains the aadA cassette. The two higher molecular weight bands in transformant 6 are believed to be partially digested DNA.
Figure 5.7 The ble (117) cassette is amplified from genomic DNA of four putative pBa3-AX-117 transformants (1, 2, 3 and 6). Using primers homologous to 5' and 3' RBCS2 regions a 1010 bp PCR product was amplified from transformants 1, 3 and 6. M: DNA size marker; Pl: pBa3-AX-117 plasmid DNA used as template for PCR (positive control); WT: WT CC-1021(mt+) genomic DNA used as template for PCR (negative control). Black bar: exon 3 of psaA.; hatched bars: 5' and 3' elements from RBCS2; stipled bar: ble coding sequence; chequered bars: RBCS2 intron 1 (I).
Figure 5.8 Southern analysis of pBa3-AX-117 transformants (1, 2, 3 and 6), confirms the presence and homoplasmic state of the ble (117) cassette. Panel a: Genomic DNA from transformants digested with BamHI and probed the EcoRI cassette from pBa3-AX-117, demonstrating presence of Ble (117) cassette in transformants 1, 3 and 6. Panel b: Southern blot from panel a re-probed with 1.5 kbp EcoRI/BamHI fragment from pBa3-AX-117, demonstrating homoplasmic state of the ble (117) cassette in transformants 1, 3 and 6. +ve: EcoRI fragment from pBa3-AX-117; WT: Genomic DNA from WT CC-1021(mt+) digested with BamHI.
It was also possible to confirm the homoplasmic state of the pBa3-AX-117 transformants by demonstrating their sensitivity to spectinomycin. If the ble (117) cassette is homoplasmic it should have completely replaced the aadA cassette (conferring spectinomycin resistance, (Goldschmidt-Clermont, 1991a)) present at the same locus in the C575D host. Figure 5.9 shows each pBa3-AX-117 transformant, along with C575D and wild-type CC-1021 (mt+), spotted onto TAP and TAP + spectinomycin (100 µg/ml) plates. Confirming their homoplasmic state, transformants 1, 3 and 6 are unable to grow on spectinomycin containing medium. Whereas, putative transformant number 2 is viable on spectinomycin due to its retention of the aadA cassette.

5.2.7 Zeomycin resistant colonies are not detected with pBa3-AX-117 chloroplast transformants

Using the homoplasmic pBa3-AX-117 transformant number 3, attempts were made to isolate strains where the ble (117) cassette had escaped from the chloroplast to the nucleus. The various methods used to attempt to isolate zeomycin resistant strains are described in Table 5.1. As with the pBa3-AX-ble transformants (section 5.2.5), no zeomycin resistant colonies containing ble (117) in the nucleus were isolated.
Figure 5.9 Phenotypic analysis confirms the homoplasmic state of the *ble* cassette in *ble* (117) transformants. pBa3-AX-117 transformants 1, 3, and 6 are spectinomycin sensitive due to replacement of *aadA* cassette by the *ble* (117) cassette, whereas C575D and transformant 2 remain spectinomycin resistant. WT: WT CC-1021(mt+); CD: C575D photosynthetic mutant host strain; TAP: TAP medium; TAP+Spc100: TAP medium containing spectinomycin at 100 μg/ml.
5.3 Discussion

The goal in this chapter was to use *C. reinhardtii* to design and effect a system that would enable *in vivo* analysis of the movement of DNA from chloroplast to the nuclear genome. An analogous system has already been shown to be effective in demonstrating transfer of genetic material from the mitochondria to the nucleus in *S. cerevisiae* (Thorsness and Fox, 1990). Furthermore, this system allowed isolation of mutant strains affected in genes tentatively involved in mitochondrial fusion and/or division (Thorsness and Fox, 1993; Thorsness *et al.*, 1993).

In order to instigate this system it was decided to use the 'ble' cassette, a dominant selectable marker for nuclear transformation in *C. reinhardtii*. The chimeric ble cassette confers resistance to the phleomycin group of antibiotics, including zeomycin (Stevens *et al.*, 1996). Although other selectable markers for nuclear transformation are available (e.g. Kindle *et al.*, 1989), the ble marker was thought to be most applicable due to its small size (1.5 kb) and the extremely low level of spontaneous resistance to phleomycin in *C. reinhardtii* (<1 x 10-9, Stevens *et al.*, 1996). The ble marker would be introduced into the chloroplast genome and, once it had been confirmed to be in a homoplasmic state, escape of DNA from the chloroplast to the nucleus would be assayed by the isolation of zeomycin resistant colonies.

After transformation of the ble cassette using the vector pBa3-AX-ble (Fig. 5.2), PCR (Fig. 5.3) and then Southern analysis (Fig. 5.4a) demonstrated that the introduced gene was present in the chloroplast genome of all the putative transformants analysed. Similarly, it was shown that the recombinant chloroplast genomes containing the ble cassette had completely replaced the host C575D genome, i.e. had reached homoplasy, in all the transformed strains (Fig. 5.4b).

Prior to assaying for escape of ble to the nucleus in these transformants, several assumptions needed to be proven experimentally. Firstly, it had been assumed that maintenance of ble in the chloroplast would not confer zeomycin resistance in transformed strains. The ble cassette contains nuclear controlling elements, therefore there should have been little or no expression of ble in the chloroplast as it lacked the appropriate promoter, ribosome-binding-site, etc. essential for chloroplast gene expression (Gruissem, 1989; Mayfield *et al.*, 1995). Even if there was a some level of expression, it is highly unlikely that the resulting protein would be able to exit the chloroplast and be transferred in sufficient quantity to the nucleus, where it is required to inhibit the activity of zeomycin (Lown and Purton, unpublished). This first
assumption was confirmed to be correct by the demonstration that pBa3-AX-ble transformants were not viable on zeomycin containing medium (Fig. 5.5). Secondly, it had also been assumed that after propagation of the ble cassette in vivo within a recombinant chloroplast genome, it would still be viable as a dominant selectable marker of nuclear transformation. This assumption was confirmed by PCR amplification of the cassette from two of the homoplasmic pBa3-AX-ble transformants and subsequent use of this DNA to effect nuclear transformations when selecting on zeomycin containing medium (Fig. 5.6).

Once these assumptions had been confirmed, attempts were made to assay the rate of DNA escape from the chloroplast to the nucleus by plating approximately $1 \times 10^9$ pBa3-AX-ble transformant number 4 cells onto zeomycin containing medium. Initially these assays were carried out using zeomycin at 20 μg/ml, a typical concentration used when carrying out nuclear transformations with the ble marker (Lumbreras et al., 1998a). Under these conditions no zeomycin resistant colonies were observed from the ble transformant or wild-type cells (Table 5.1). Several hypotheses could be put forward to explain why strains where ble had escaped from the chloroplast to the nucleus were not detected. Firstly, it was possible that this phenomenon was simply not occurring in the pBa3-AX-ble transformants. Alternatively, escape of the ble gene to the nucleus might not have been occurring frequently enough to be detected, or the conditions used might not have been conducive to detecting such cells. Previous studies have suggested down-regulation or epigenetic silencing can affect the expression of foreign genes introduced into the nuclear DNA in C. reinhardtii (Stevens et al., 1996; Cerutti et al., 1997a; Cerutti et al., 1997b; Lumbreras et al., 1998a). With this in mind, it was decided to increase the sensitivity of the assay by repetition using lower concentrations of zeomycin. The only colonies which appeared under any of these conditions were found to be 'palmellloid' clusters of cells (Table 5.1). Palmellloid colonies were probably viable because cells in the centre of the cluster were protected by cells on the periphery which absorbed the zeomycin.

Assuming that the assay was sensitive enough the next possibility to be addressed was that the transfer of ble to the nucleus was occurring less frequently than can be detected when plating $1 \times 10^9$ cells. Studies in vivo (Thorsness and Fox, 1990; Thorsness and Fox, 1993) and in vitro (Cerutti and Jangendorf, 1995) have demonstrated that extremes in incubation temperature increase the ability of nucleic acids to traverse organelle membranes. It is thought that alteration in temperature compromises the integrity of the organelle membrane by causing deformation or temporary breakage of the lipid bilayer. Therefore, in an attempt to increase the rate of escape of DNA from the
chloroplast a pBa3-AX-ble transformant was grown in liquid culture at 33 °C. Also, by the same supposition a starter culture of pBa3-AX-ble transformant cells was 'heat-shocked' at 42 °C. Increasing the incubation temperature to 33 °C resulted in the appearance of zeomycin resistant colonies to a similar order on both the ble transformant and the wild-type control plate (Table 5.1). Presumably, the increased temperature (C. reinhardtii grow optimally at 25 °C) caused an acceleration in the rate of spontaneous mutation. Also, the culture resulting from the heat-shocked starter did not produce any zeomycin resistant colonies (Table 5.1). Therefore under these conditions, incubating ble transformant cultures at artificially high temperatures appears to have no, or an undetectable effect on the rate of escape of genetic material to the nucleus.

The next possibility to be considered that could be affecting the detectability of DNA escape to the nucleus was the efficiency of the ble marker. The level of expression of foreign genes introduced into the nucleus can be assessed by carrying out a co-transformation without selection for the gene of interest. As integration of the second unselected gene into the nuclear genome is often as high as 80 % (Stevens et al., 1996), the percentage of these co-transformants then expressing the unselected gene gives an unbiased measure of its level of expression. When the pSP108 ble marker was co-transformed with the ARG7 marker only 2.4 % of co-transformants expressed the ble gene (Lumbreras et al., 1998a). Whereas, introduction of two copies of RBCS2 intron 1 into the coding sequence of ble and deletion of part of the RBCS2 5' region increased this efficiency to 25 %, a rate equivalent to the endogenous auxotrophic marker ARG7 (Lumbreras et al., 1998a). Also, enhanced versions of the ble marker increased direct transformation efficiency by up to thirty times in comparison to the original pSP108 marker (Lumbreras et al., 1998a). Therefore, it was decided to introduce an enhanced copy of the ble marker ('ble (117)', Fig. 5.2) containing two copies of the RBCS2 intron 1 (from pSP117, Lumbreras et al., 1998a) into the chloroplast and repeat the assay for its escape to the nuclear genome. This enhanced copy of the ble marker increased nuclear transformation rates by nearly twenty times compared to the original pSP108 marker (Lumbreras et al., 1998a), and so might make it easier to identify cells where it has transferred from the chloroplast to the nucleus. In the same way as the original ble marker (section 5.2.1), homoplasmic pBa3-Ax-117 (Fig. 5.2) chloroplast transformants containing the ble (117) marker were produced (section 5.2.6).

Using pBa3-AX-117 transformant number 3, attempts were made to isolate zeomycin resistant strains where this marker had escaped from the chloroplast to the nucleus. Assays were carried out in the same way as described for the pBa3-AX-ble transformant (section 5.2.5). The initial attempt used zeomycin at 20 μg/ml, but no
zeomycin resistant colonies resulted from this screen (Table 5.1). This screen was then altered by growing the cells at 30 °C, which it was thought may be high enough to compromise the chloroplast membrane structure but not so high as to increase the mutation rate as described previously (see above). In contrast to the pBa3-AX-ble transformant grown at 33 °C (section 5.2.5), no colonies were seen on either the pBa3-AX-117 transformant plate or on the wild-type control plate (Table 5.1). Possibly, DNA mutation rate was not affected at this temperature, but neither was the rate of ble (117) escape to the nucleus.

It was supposed that if, as has been suggested (Thorsness et al., 1993), escape of genetic material from the chloroplast occurs via leakage during organelle division then this may occur at an increased rate during mating. Cytoplasmic fusion of gametes during mating in C. reinhardtii also involves the fusion, within the zygote, of the chloroplast from each gamete. The result of this fusion is destruction of the chloroplast DNA from the mating-type minus gamete (uniparental inheritance), by a mechanism that is not fully understood (section 1.2.2). Leakage of genetic material may be more frequent during fusion of gametic chloroplasts within the zygote. To test this hypothesis, pBa3-AX-117 transformant number 3, which was created in a mating-type plus (mt+) strain, was mated with wild-type CC-12 (mt-) and the plate containing the resultant zygotes overlain with 0.7% TAP agar containing zeomycin at 20 μg/ml. Unfortunately, the high background of unmated gametes rapidly depleted the zeomycin and allowed large patches of growth, inhibiting identification of any zeomycin resistant daughter cell colonies (Table 5.1). Obviously, the protocol for this screen needs modification and so this hypothesis remains untested. Finally, it was thought that rapidly reculturing a pBa3-AX-117 chloroplast transformant over a long time period may increase the chances of isolating strains where ble (117) had escaped to the nucleus. Therefore, a liquid culture of pBa3-AX-117 transformant number 3 was serially recultured once a week for eight weeks, after which time zeomycin resistant colonies were selected on TAP + zeomycin at 15 μg/ml plates. This screen failed to produce zeomycin resistant colonies either in the pBa3-AX-117 chloroplast transformant or in the wild-type control (Table 5.1).
Chapter 6

Discussion
6 DISCUSSION

6.1 Addressing the aim of this thesis

The title of this thesis is 'the introduction and expression of foreign genes in the chloroplast of *C. reinhardtii*'. Each set of experiments described in the results chapters have different specific aims and these are discussed below. However, all three chapters are unified in their aim to introduce and/or express a foreign gene in the chloroplast of *C. reinhardtii*. Modern molecular genetics teamed with well defined model organisms has revolutionised the understanding of many fundamental problems in biology. The long term goal of this, and many other such works, is to use molecular genetic techniques as a means to understand the function of proteins *in vivo*.

In chapter three the aim was to develop a new dominant selectable marker for chloroplast transformation. At present only one such marker is available for chloroplast transformation in *Chlamydomonas*, limiting this plastid's tractability to molecular genetic dissection. A second selectable marker provides the means with which to overcome this limitation.

The aim in chapter four was to use the plastid of *C. reinhardtii* as a model environment in which to express two different foreign genes. Firstly, it was desirable to express the *por* gene encoding NADPH-protochlorophyllide oxidoreductase (POR), from *Synechocystis* sp. PCC 6803. Once successfully expressed the function of the enzyme could be probed by site-directed mutagenesis. Similarly, heterologous expression of the *tufA* gene, encoding elongation factor Tu (EF-Tu), from the plastid of *Plasmodium falciparum* was attempted. Ability (or lack thereof) of the *P. falciparum* EF-Tu to functionally replace its *C. reinhardtii* homologue would lend insight into the phylogenetic relationship of the two plastids.

Finally, the aim of the fifth chapter was to introduce a nuclear transformation marker into the chloroplast genome. Once stably incorporated this vector could be used to screen for transfer of genetic material from the plastid to the nuclear compartment. A process which has played a major role in the evolution of eukaryotic cells.
6.2 Premises of aim

The aims described above are based on several premises which are broadly presented throughout the introduction. A more focused précis of these ideas follows.

*Chlamydomonas* has been developed primarily as a photosynthetic model organism but also as a model for the study of many other biological processes. As discussed in section 1.2.1, many of the genetic, biochemical and physiological characteristics are now well defined in *Chlamydomonas*. This allows one to carry out technically complex experiments while standardising many of the variables which in other organisms would interfere with the interpretation of the results. The benefits of using *Chlamydomonas* as a model organism have already attracted many workers and should soon promote it to the status of other model organisms such as *Escherichia coli*, *Arabidopsis thaliana* and *Saccharomyces cerevisiae*.

As described in section 1.2.1, both the nuclear and chloroplast genomes can be readily transformed in *C. reinhardtii*. In higher plants foreign genes have been transformed into the nuclear genome and the product targeted to the chloroplast by the addition of the appropriate transit peptide (McBride *et al*., 1994; Verwoert *et al*., 1994). Nuclear transformation in *Chlamydomonas* is subject to low levels of homologous recombination such that introduced DNA is mostly incorporated at random (Debuchy *et al*., 1989; Kindle *et al*., 1989; Gumpel *et al*., 1994). In contrast, homologous recombination of introduced DNA occurs readily in the chloroplast genome (e.g. Boynton *et al*., 1988). Therefore by flanking it with the appropriate chloroplast sequence, introduced DNA can be precisely targeted. Meaning that foreign DNA can be inserted intergenically where it will not interfere with the expression of endogenous genes.

A crucial prerequisite for this thesis was the ability to readily transform the chloroplast genome. Although not the only method, it is particle-gun bombardment that has enabled chloroplast transformation to be achieved routinely (section 1.3.1). In all the experiments described in this thesis genetic material was introduced via particle-gun bombardment.

Finally, as discussed in section 1.3.4, there have been several precedents of foreign genes expressed in the chloroplast of *Chlamydomonas* (Goldschmidt-Clermont, 1991a; Sakamoto *et al*., 1993; Cerutti *et al*., 1995). These studies provided a model of the *cis*-acting factors required for efficient expression of a foreign gene in the *Chlamydomonas*
chloroplast. However, all of the factors that affect foreign gene expression are not fully understood.

6.3 Specific conclusions

6.3.1 A novel dominant selectable marker for chloroplast transformation

The use of the aminoglycoside phosphotransferase-encoding \textit{aphA-6} gene from \textit{A. baumannii} as a selectable marker for chloroplast transformation proved very successful. In accordance with current understanding of chloroplast gene expression (section 1.2.4), the \textit{aphA-6} coding sequence was juxtaposed with controlling elements from several highly expressed chloroplast genes. The three promoter elements used conferred varying levels of antibiotic resistance (section 3.2.4). Therefore, as with \textit{aadA} (Goldschmidt-Clermont, 1991a), \textit{aphA-6} can be used as a reporter gene by assaying levels of antibiotic resistance in transformants. The ability to quickly and simply assess \textit{aphA-6} expression should make it a useful tool for studying \textit{Chlamydomonas} chloroplast gene expression. Of the various \textit{aphA-6} expression constructs, \textit{aphA-6} under the control of the 5' UTR from the constitutively expressed gene \textit{psbA} gave the most consistent expression and so this cassette was developed as a dominant selectable marker. Subsequently, it was demonstrated that transformation of the chloroplast genome with this cassette could be selected either on kanamycin or amikacin containing medium. The ability to grow \textit{aphA-6} transformants on two different (aminoglycoside) antibiotics is a very useful characteristic. Firstly, it provides workers with a choice of substrates on which to isolate transformants. Secondly, it allows elimination of colonies spontaneously resistant to one or other of the drugs. On transfer to the alternate antibiotic only true \textit{aphA-6} transformants will be viable. It is highly unlikely that a spontaneously resistant strain will also have spontaneously acquired resistance to the alternate antibiotic.

Proof that \textit{aphA-6} transformants contained the marker within the chloroplast genome, rather than the nuclear genome, was provided by Southern analysis (section 3.2.5). Northern analysis of \textit{aphA-6} transformants demonstrated the presence of transcripts (of ambiguous size) which also hybridised to the \textit{aphA-6} probe (section 3.2.6). Up to this point in this series of experiments the new marker had only been introduced into an intergenic location in the chloroplast genome. Therefore, confirmation of the \textit{aphA-6} cassette's 'raison d'être' was needed i.e. that it could be used to delete a chloroplast gene. To achieve this the cassette was inserted into the PSII gene \textit{psbH}. Strains in
which *psbH* has been insertionally mutagenised are unable to grow photosynthetically due to lack of functional PSII assembly (O'Connor *et al.*, 1998). This phenotype was replicated in transformants containing *psbH* insertionally mutagenised using the *aphA-6* cassette (section 3.2.7). Deletion of *psbH* is proof that the *aphA-6* cassette is a fully functional portable selectable marker.

The development of a second selectable marker for chloroplast transformation in *Chlamydomonas* should enable many experiments that were previously impossible. As mentioned above, the lack of a second marker has limited *Chlamydomonas* chloroplast research during the current decade. Previously, secondary mutations could not be introduced into a strain in which the *aadA* cassette was already resident. This problem is circumvented by use of the *aphA-6* cassette as transformants are selected on kanamycin/amikacin rather than spectinomycin/streptomycin, the substrate of AADA. The expression of individual chloroplast genes is interconnected so that different subunits of the photosynthetic complexes are co-ordinately assembled (section 1.2.4). The lines of communication between different chloroplast genes are complex and probably involve multiple nuclear encoded factors. Whatever the mechanism, the ability to introduce mutations into different genes in the same strain using the *aphA-6* and *aadA* cassettes should further the understanding of this complex problem.

One potential development of the *aphA-6* marker is to use it to analyse the possible existence of a second, nuclear encoded chloroplast RNA polymerase. As described in section 1.1.2.1, deletion of *rpoB* (encoding the β subunit of RNA polymerase) in tobacco caused the abolition of transcription of many chloroplast genes and suggested that the unaffected genes were transcribed from a second nuclear encoded RNA polymerase (Allison *et al.*, 1996). When the same experiment was attempted in *Chlamydomonas* the transformants remained heteroplasmic, suggesting this organism has only one chloroplast RNA polymerase, encoded by the chloroplast genome (Fischer *et al.*, 1996). However the two studies differed in a minor, but possibly crucial manner: in tobacco the selectable marker was driven from a modified rRNA promoter, whereas expression of the marker used in *Chlamydomonas* was driven by the promoter from the photosynthetic gene *atpA*. There is evidence that the nuclear encoded polymerase transcribes mainly genetic system genes while photosynthetic genes are transcribed by the chloroplast encoded polymerase (Allison *et al.*, 1996). This presents a potential conflict in that the gene being deleted by the *aadA* cassette, although possibly non-essential, may be needed for expression of the marker. If this is the case the *rpoB:aadA* deletion could never become homoplasmic. This problem could be resolved by replacing the promoter on the *aadA* or *aphA-6* cassette with a promoter from a non-
photosynthetic gene. Deletion of a rpo gene could then be repeated to determine whether the new marker can completely eliminate the chloroplast encoded RNA polymerase in *Chlamydomonas*.

### 6.3.2 Foreign gene expression of por from *Synechocystis*, and tufA from the plastid of *P. falciparum*

Introduction of these two foreign genes into the chloroplast was attempted because of the ease of use of *C. reinhardtii* for such a purpose (section 6.2). The por gene has been cloned and sequenced from a variety of organisms including pea (Spano *et al.*, 1992), wheat (Teakle and Griffiths, 1993), oats (Darrah *et al.*, 1990), *Chlamydomonas* (Li and Timko, 1996), and *Synechocystis* (Suzuki and Bauer, 1995). However, characterisation of the enzyme has been achieved mainly by biochemical analysis (Teakle and Griffiths, 1993; Dahlin *et al.*, 1995; Griffiths *et al.*, 1996; Martin *et al.*, 1997). Only one study, in which the pea por gene was used to complement protochlorophyllide reduction mutants in *Rhodobacter capsulatus*, has used site-directed-mutagenesis to study POR *in vivo* (Wilks and Timko, 1995). Therefore, a general system in *Chlamydomonas* for introduction and mutagenesis of por from any species would be highly advantageous. This was the rationale behind the rescue of the protochlorophyllide oxidoreductase null mutant, *pc-ly-7*, with the *Synechocystis* por gene.

Using the methods described in sections 4.2.2 - 4.2.3, it was not possible to recover chlorophyll-synthesising, green colonies after transformation with the cyanobacterial por. As a result of this it was decided to determine whether it was at all possible to achieve expression of the foreign por gene in the *Chlamydomonas* chloroplast. psbH, present on the por expression vector was used as a marker to introduce the cyanobacterial gene by rescuing a psbH deletion strain. Western analysis of transformants containing por within the chloroplast genome only detected the ca. 36 kDa *C. reinhardtii* nuclear encoded POR. Further analysis of these transformants is needed to determine whether there is any expression of the ectopic chloroplast encoded por. Possibly, the foreign por gene is being transcribed (and would be detected by northern analysis) but the protein is unstable. *In vitro* analysis has demonstrated that POR without substrates bound, or POR bound to chlorophyllide is susceptible to proteolytic degradation (Reinbothe *et al.*, 1995). Binding of POR to protochlorophyllide alone, or in combination with NADPH stabilised the enzyme. Perhaps in the system described here the cyanobacterial POR, whether because of
topological constraints or incorrect localisation, is unable to bind its substrate and so is rapidly degraded. Whatever, these transformants obviously need further analysis in order to understand whether the *Chlamydomonas* chloroplast is amenable to heterologous expression of POR.

The *tufA* gene from the malarial parasite *P. falciparum* has been isolated relatively recently during a project to completely sequence the plastid genome of this organism (Wilson *et al.*, 1996). Analysis of gene organisation and content, and phylogenetic analysis of the predicted polypeptide sequence of genes encoded by the 35 kbp circle have shown conclusively that it is plastidic in origin (reviewed in Palmer, 1992; McFadden and Waller, 1997). Recent phylogenetic analysis of the *tufA* gene from three different Apicomplexan plastids ('apicoplasts'), showed that they consistently clustered with *tufA* from green algal plastids (Kohler *et al.*, 1997). This result suggested that the Apicomplexa acquired their plastid from a green alga by secondary endosymbiosis. It was hoped that ectopic expression of the Apicomplexan EF-Tu in the 'true' plastid of *C. reinhardtii* would provide experimental evidence as to the degree of divergence of the two organelles, i.e. functional replacement of the *C. reinhardtii* EF-Tu with its *P. falciparum* homologue would suggest that the two plastids are closely related.

Primary transformants were obtained in which the *P. falciparum tufA* gene was occasionally installed in place of the endogenous *tufA* (section 4.2.7). After selection for the *P. falciparum tufA* cassette genotypic analysis was used to discern whether any copies of the *C. reinhardtii tufA* remained (section 4.2.10). This analysis determined that replacement of the chloroplast gene by its *P. falciparum* homologue was not possible. This result suggests that the protein synthetic machinery of the two organelles are not similar enough for an essential component such as EF-Tu to be interchangeable between them. Perhaps then, the chloroplast of *Chlamydomonas* and other green algae are not as closely related to the plastid of the Apicomplexans as has been suggested (Kohler *et al.*, 1997). Phylogenetic analysis of nuclear genes from Apicomplexan species clusters them closest to the dinoflagellates (reviewed in Palmer, 1992). Therefore, the apicoplast may be a derivative of the dinoflagellate plastid rather than the result of a secondary endosymbiotic event between a progenitor Apicomplexan and a green alga. Unfortunately, definitive analysis of this hypothesis awaits the sequencing of dinoflagellate plastid genes.

It was decided to use the *tufA* gene for this study not only for practical reasons, i.e. that it was present in both plastids, but also because of the conservative nature of EF-Tu. EF-Tu is essential for cell viability in *C. reinhardtii* (section 4.2.9), therefore complete replacement of the *C. reinhardtii tufA* gene with its *P. falciparum* counterpart requires
that the heterologous protein be fully functional. The evolutionary bifurcation from a common ancestor of the two plastids may be relatively recent. However, the complex mechanism of action of EF-Tu and the fact that it has several substrates may prohibit its functional transfer to a heterologous system.

Finally, though the foreign tufA gene did not replace its C. reinhardtii homologue, western analysis suggested that it was being expressed. This result is promising in that it shows that it should be possible to use Chlamydomonas as a host for the expression of Plasmodium plastid genes. The plastid of P. falciparum has already been shown to be specifically targeted by certain chemotherapeutic agents (Fichera and Roos, 1997). The results presented here may provide a preliminary model for use of Chlamydomonas as a heterologous system for testing antimalarial drugs.

6.3.3 Introduction of the ble cassette, a nuclear transformation marker, into the chloroplast

The ble cassette was introduced into the chloroplast genome as part of a system for modelling transfer of genetic material from the chloroplast to the nucleus (section 5.1). Inspiration for this system was based on a set of experiments in S. cerevisiae where the nuclear gene URA3 was installed into the mitochondrion of a uracil auxotrophic strain (Thorsness and Fox, 1990). It was then possible to isolate colonies which were viable in the absence of uracil where URA3 had transferred to the nucleus. In C. reinhardtii the ble cassette confers resistance to zeomycin when expressed in the nucleus (Stevens et al., 1996). Therefore, once installed in the chloroplast the aim was to isolate strains in which ble had moved to the nucleus by screening for zeomycin resistant colonies.

The primary aim to install the ble cassette in the chloroplast genome was achieved, as confirmed by Southern analysis (section 5.2.3). A second version of the cassette, with an improved rate of nuclear transformation, was also introduced into the chloroplast genome (section 5.2.6). Unfortunately, it was not possible to isolate strains in which either version of the ble cassette had transferred to the nucleus. Ostensibly, the conclusion to be drawn from this result is that in C. reinhardtii transfer of genetic material from the chloroplast to the nucleus does not take place at a detectable rate. Obviously this has not been true in the past as the chloroplast genome currently contains a small fraction of the number of genes needed to exist as a free-living bacterium. Perhaps then, this mass loss/transfer of genetic material was a unique event that occurred during the original symbiosis of photosynthetic bacterium and host cell. As
discussed in section 1.1.3, several chloroplast genes have been isolated which have relocated to the nucleus relatively recently. Also, transfer of DNA from the mitochondrial to the nuclear compartment has been demonstrated experimentally in *S. cerevisiae* (see above). It should be noted however that in this study the construct introduced into the mitochondrion which escaped to the nucleus contained a replication origin. This may be a crucial difference to the experimental system described here. In the yeast experiments the escaping DNA was able to replicate autonomously within the nucleus, whereas the *ble* cassette would have to incorporate into the nuclear genome to be stably expressed. Theoretically, the rate of escape of genetic material from the chloroplast may be similar to that from the mitochondrion (~2 x 10^{-5} per cell per generation). However, the need for the *ble* cassette to incorporate into the nuclear genome may greatly decrease the actual detectable rate of escape. Although nuclear transformation rates in *C. reinhardtii* have been analysed (section 1.2.1), it is difficult to assess the efficiency of recombination of exogenous DNA once inside the nuclear compartment. If the rate of DNA escape from the chloroplast is assumed to be similar to that from the mitochondrion, a recombination rate of less than 1 x 10^{-4} per cell per generation would make transfer undetectable in this study (<1 x 10^{-9} per cell per generation). The obvious way to remedy this problem is to insert a replication origin into the *ble* cassette. Unfortunately no nuclear replication origins have yet been successfully isolated from *Chlamydomonas*.

Another reason why escape of the *ble* cassette to the nucleus may not have been detected is that it involved an RNA intermediate. The involvement of mRNA in transfer of organelle genes to the nucleus has been suggested by the isolation of edited versions of mitochondrial genes from the nuclear genome (Covello and Gray, 1992; Blanchard and Schmidt, 1995). Perhaps this is also the case for *C. reinhardtii* chloroplast genes and the *ble* cassette is not transferred because it is not transcribed. The addition of a chloroplast promoter upstream of the cassette may alleviate this problem. Also, inclusion of a chloroplast intron in the *ble* coding sequence would confirm the involvement of a mRNA intermediate as this would be spliced from the transcript prior to escape from the chloroplast.

Although escape of the *ble* cassette from the chloroplast was not detected the strains produced provide a useful resource for further experiments. For example, it has been postulated that escape of DNA from organelles occurs by leakage during organelle division (Thorsness and Webber, 1996). *C. reinhardtii* mutants have been isolated that are defective in chloroplast division. Crossing such a mutant to a strain containing the *ble* cassette in the chloroplast may increase the rate of nuclear transfer to a detectable level.
6.4 Overall conclusions and future prospects

The overall aim to introduce and express foreign genes in the *Chlamydomonas* chloroplast has been achieved. The results presented continue to confirm that genetic material from diverse organisms, both prokaryotic and eukaryotic, can be introduced into the chloroplast of *Chlamydomonas*. Although precise introduction of foreign DNA into the *C. reinhardtii* chloroplast genome has proven straightforward, heterologous gene expression has presented more of a challenge. In spite of this, the eubacterial gene *aphA-6* has been functionally expressed to a level where it can be used as a selectable marker for chloroplast transformation. Also, preliminary data suggests that the *tufA* gene from the protist *Plasmodium falciparum* has been successfully expressed. This represents the first example of a eukaryotic gene to be expressed in the *Chlamydomonas* chloroplast.

Foreign gene expression in the *Chlamydomonas* chloroplast is not only used as a means of obtaining heterologous protein. Several studies have used the *E. coli uidA* gene (β-glucuronidase, GUS) as a reporter to probe the involvement of cis-acting factors in chloroplast gene expression (Blowers *et al.*, 1990; Klein *et al.*, 1992; Sakamoto *et al.*, 1993; Salvador *et al.*, 1993b). The application of expression of a foreign gene as a selectable marker has been an essential tool in furthering the understanding of chloroplast molecular genetics. Ideally, the introduction of foreign genetic material into the *Chlamydomonas* chloroplast will advance to a level equivalent to that in *E. coli*. In other words, where DNA can be stably introduced intra- or extrachromosomally with varying, controllable copy numbers. Once in the chloroplast, expression of the introduced DNA would be precisely controlled using appropriate controlling elements or even inducible promoters. The ability to greatly overexpress genes in the *Chlamydomonas* chloroplast might be useful for isolation of large quantities of active protein. Obviously more research is needed to develop the application of this organelle to its full potential. However, the work presented here is further proof of the utility of the *Chlamydomonas* chloroplast as an environment for the expression and analysis of foreign genes.
Appendices
APPENDIX I OLIGONUCLEOTIDE PRIMERS

DNA oligonucleotide primers used for PCR and DNA sequencing are listed. Sequences are shown in the 5'→3' direction. Changes to original sequence to incorporate restriction sites or improve codon usage are shown in uppercase, and the position of primers refers to reference shown.

I.Primers used in chapter 3

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Gene/position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBCX5</td>
<td>gCTTaTcGaCgCgttttaggtattttgaga</td>
<td>rbcL; 823→853</td>
<td>Dron et al., 1982</td>
</tr>
<tr>
<td>RBCX3</td>
<td>ctctacgcGcGcGctacatccgcttttagtag</td>
<td>rbcL; 2896→2864</td>
<td>Dron et al., 1982</td>
</tr>
<tr>
<td>ATPB5</td>
<td>ctctaagaCgCgtacaatgttttg</td>
<td>atpB; -386→-361</td>
<td>Woessner et al., 1986</td>
</tr>
<tr>
<td>ATPB3</td>
<td>ttagcCAtGgtcatatgttaatttttaac</td>
<td>atpB; 15→-12</td>
<td>Woessner et al., 1986</td>
</tr>
<tr>
<td>PSBA5</td>
<td>GGACGcgtcctattttaatactcgaaggagg</td>
<td>psbA; -256→-225</td>
<td>Erickson et al., 1984</td>
</tr>
<tr>
<td>PSBA3</td>
<td>aaCCATGgtcatatgtaatatttaagg</td>
<td>psbA; 12→-19</td>
<td>Erickson et al., 1984</td>
</tr>
<tr>
<td>APHA5</td>
<td>ctCCatggaattAccAaatattatc</td>
<td>aphA-6; 132→157</td>
<td>Martin et al., 1988</td>
</tr>
<tr>
<td>APHA3</td>
<td>gttaactctgAGagtatggatag</td>
<td>aphA-6; 951→923</td>
<td>Martin et al., 1988</td>
</tr>
<tr>
<td>MLU5</td>
<td>gccTcTAGActtccgtaagataacg</td>
<td>psbH; 2733→2759</td>
<td>Johnson and Schmidt, 1993</td>
</tr>
<tr>
<td>MLU3</td>
<td>CCCGGGATCCaagaaaa tgtagctattaacg</td>
<td>psbH; 2788→2760</td>
<td>Johnson and Schmidt, 1993</td>
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### I.ii Primers used in chapter 4

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<tr>
<td>POR51</td>
<td>gaacaacTCatgaaaccacg</td>
<td><em>por</em>; 59-79</td>
<td>Suzuki and Bauer, 1992</td>
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<tr>
<td>POR31</td>
<td>gctgggaatttGtgcACgaacta</td>
<td><em>por</em>; 1044-1022</td>
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<tr>
<td>LPCR5</td>
<td>ctggagcttgtatctgatgg</td>
<td><em>lpcr</em>; 8→27</td>
<td>Li and Timko, 1996</td>
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<tr>
<td>LPCR3</td>
<td>gatggcggtagctttccttgga</td>
<td><em>lpcr</em>; 352→330</td>
<td>Li and Timko, 1996</td>
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<tr>
<td>CRTF55</td>
<td>gttatatatttgacctgtaaatcc</td>
<td><em>psbK</em>; 152→177</td>
<td>Silk <em>et al.</em>, 1990</td>
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<tr>
<td>CRTF53</td>
<td>cttacacagcgactatatgtg</td>
<td><em>tufA</em>; 15→5</td>
<td>Baldauf and Palmer, 1990b</td>
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<tr>
<td>CRTF35</td>
<td>cCATATgagccaaAGAtctgtagg</td>
<td><em>tufA</em>; 1303→1328</td>
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<tr>
<td>CRTF33</td>
<td>gccacgGgatCcaacgtctggttc</td>
<td><em>trnEl</em>; 784→762</td>
<td>O'Neill <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>PFTF5</td>
<td>GGGGCATatgaataaatatatatttaaag</td>
<td><em>tufA</em>; 8747-8769</td>
<td>Wilson <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>PFTF3</td>
<td>AGATCttaatttttatctgttatatatcc</td>
<td><em>tufA</em>; 9979-9954</td>
<td>Wilson <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>CRTFKPN</td>
<td>ccacaataaaataattgctcctc</td>
<td><em>tufA</em>; 1412→1390</td>
<td>Baldauf and Palmer, 1990b</td>
</tr>
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</table>

### I.iii Primers used in chapter 5

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<tr>
<td>PSAA3</td>
<td>caaaggacagtagtagcac</td>
<td><em>psaA3</em></td>
<td>Kueck <em>et al.</em>, 1987</td>
</tr>
<tr>
<td>RBCS23</td>
<td>cgagctcggtacccgctc</td>
<td>RBCS2</td>
<td>Goldschmidt-Clermont and Rahire, 1986</td>
</tr>
</tbody>
</table>
II.i Construction of chloroplast expression vectors

Plasmid p72B (Fig. 3.1a) was obtained from the *Chlamydomonas* Genetics Centre. p72B is simply pUC8 with the 4.4 kbp *EcoRI* fragment (Eco19, Harris, 1989) of the *C. reinhardtii* chloroplast genome inserted into its polylinker. To produce p72B-HS, the *HindIII/SphI* fragment was removed from p-72B by restriction digestion, filling in overhangs with Klenow polymerase I fragment and re-circularisation of the plasmid by ligation. The *rbcL5'/3'* cassette was amplified by PCR from the plasmid rbcX (Goldschmidt-Clermont, 1991a) using primers RBCX5 (incorporating a *MluI* site) and RBCX3 (incorporating a *BssHII* site). This PCR product was digested with *MluI/BssHII* and ligated into the unique *MluI* site in p72B-HS selecting for recombinants with *rbcL5'/3'* in the reverse orientation to *psbH*, thus destroying the proximal *MluI* site. Next the unique *HindIII* site between *rbcL5'* and *rbcL3'* was destroyed by restriction digestion, filling in overhangs with Klenow polymerase I fragment and re-circularisation of the plasmid by ligation. This plasmid was named p72-rbcXII (Fig 3.1a).

To produce the two other chloroplast expression vectors containing 5' elements from *atpB* and *psbA*, firstly the *rbcL5'* fragment was excised from p72-rbcXII with *MluI/NcoI*. A 397 bp fragment from *atpB* (Woessner et al., 1986) containing upstream sequence, the 5'-untranslated leader and the first five codons was amplified by PCR from wild-type total DNA using primers ATPB5/ATPB3. A similar 267 bp fragment from *psbH* (Erickson et al., 1984) was amplified using primers PSBH5/PSBH3. Using the *MluI/NcoI* sites incorporated into both sets of primers, the 5' fragments from both *atpB* and *psbA* were digested and ligated separately into the equivalent sites in the *MluI/NcoI* digested p-72-rbcXII, producing p72-atpB and p72-psbA respectively (Fig 3.1a).

II.ii Construction of *aphA-6* expression cassettes

The coding sequence from *aphA-6* was then amplified by PCR from the plasmid pAT253 (Martin et al., 1988), using primers APHA5/APHA3. Using the *NcoI* site and *PstI* site incorporated into APHA5/APHA3 respectively, the amplified gene was digested and ligated into the multiple cloning region of p72-rbcXII, p72-atpB, and p72-psbA consecutively (Fig. 3.1a).
To facilitate its future cloning the psbA5'::aphA6::rbcL3' cassette was amplified from p72-psbA-aphA-6 (Fig. 3.1a) using primers MLU5/MLU3, which incorporated pertinent restriction sites. This PCR product was then ligated into the EcoRV site within the multiple cloning region of pBluescript SK(+) (Stratagene), thus producing pskKmR (Fig. 3.1b). The chimeric aphA-6 cassette was then excised from pskKmR as a Smal fragment and inserted into the unique BstXI site of p72B (whose overhangs had been filled with Klenow polymerase I fragment). Recombinants with the aphA-6 cassette in the reverse orientation to psbH were selected and this new plasmid named p72KmR, which contains an insertionally mutated copy of psbH (Fig. 3.1b).

II.iii Construction of the tufA transformation cassettes

To obtain sections of DNA flanking the C. reinhardtii tufA gene 730 bp (tfa 5') and 850 bp (tfa 3') fragments were amplified by PCR from C. reinhardtii genomic DNA using primers CRTF55/CRTF53 and CRTF35/CRTF33 respectively. Both PCR products were then ligated separately into pBluescript SK(+) (Stratagene), which had been previously digested with EcoRV, to produce pBStfA5' and pBStfA3'. Using restriction sites introduced into the CRTF35/CRTF33 primers the tfa 3' fragment was excised from pBluescript SK(+) by digestion with Ndel/BamHI and ligated into the equivalent sites in pBStfA5' to produce ptfA5'3' (Fig. 4.11). Next, the P. falciparum tufA gene coding sequence was amplified from the plasmid pPF, obtained from Dr R.J.M. Wilson, using primers PFTFA5 and PFTFA3. The P. falciparum tufA PCR product was ligated into the EcoRV site of pBluescript SK(+) then excised with Ndel/BglII whose sites had been incorporated into the PFTFA5 and PFTFA3 primers. This Ndel/BglII fragment was then ligated into the equivalent sites in ptfA5'3' to produce ptfA5'3'Pf. Finally the aadA cassette was excised as a BamHI fragment from pSK.aadA and cloned into the BglII site of ptfA5'3'Pf, selecting for clones with aadA in the opposite orientation to the P. falciparum tufA gene, to produce pPtfA (Fig. 4.11). (pSK.aadA was produced by Dr S. Purton (University College London) by excising the aadA cassette as an EcoRV/Sall fragment from pUC-atpX-AAD (Goldschmidt-Clermont, 1991a) and ligating it into the Smal/Sall sites of pBluescript SK(-)). Finally, to produce pAtfA the same BamHI cut aadA cassette was ligated into ptfA5'3', so that it was in the same orientation as pPtfA (Fig. 4.11).
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