CHAPTER 4

Tools and Techniques for Chloroplast Transformation of *Chlamydomonas*

Saul Purton*

Abstract

The chloroplast organelle of plant and algal cells contains its own genetic system with a genome of a hundred or so genes. Stable transformation of the chloroplast was first achieved in 1988, using the newly developed biolistic method of DNA delivery to introduce cloned DNA into the genome of the green unicellular alga *Chlamydomonas reinhardtii*. Since that time there have been significant developments in chloroplast genetic engineering using this versatile organism, and it is probable that the next few years will see increasing interest in commercial applications whereby high-value therapeutic proteins and other recombinant products are synthesized in the *Chlamydomonas* chloroplast. In this chapter I review the basic methodology of chloroplast transformation, the current techniques and applications, and the future possibilities for using the *Chlamydomonas* chloroplast as a green organelle factory.

Introduction

The Chloroplast Genome

It is now generally accepted that the chloroplast organelle of plant and algal cells evolved from an oxygenic photosynthetic bacterium that established an endosymbiosis within a nonphotosynthetic eukaryotic host cell over one billion years ago. Since that time, the genome of this incarcerated bacterium has undergone a significant reduction in complexity, either through gene loss or gene transfer to the host nucleus, such that modern-day chloroplasts possess multiple copies of a small (120-200 kb) circular genome (or 'plastome') comprising some 100-250 genes. The majority of these genes encoded either components of the chloroplast's transcription-translation apparatus, or core components of the photosynthetic apparatus. Not only are these gene products homologous to those found in modern-day cyanobacteria, but the arrangement and expression of the genes also reflect the chloroplast's prokaryotic ancestry. Many genes are arranged in cotranscribed operons and the RNA polymerase and the 70S ribosome of the chloroplast are eubacterial rather than eukaryotic in nature.[1] The small size of the plastome, its relative simplicity and the prokaryotic nature of chloroplast gene expression make the chloroplast an attractive target for genetic engineering.

The Chloroplast as a Sub-Cellular Factory

The chloroplast compartment is the site of a number of important biosynthetic pathways and can also serve as a storage organelle. It is able to accumulate significant quantities of both

*Saul Purton—Algal Research Group, Department of Biology, University College London, Gower Street, London, WC1E 6BT, U.K. Email: s.purton@ucl.ac.uk

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soluble proteins and intrinsic membrane proteins (e.g., the rubisco enzyme and the core complexes of the photosynthetic apparatus, respectively), as well as large amounts of other macromolecules including chlorophylls, carotenoids, starch and lipids. The chloroplast is therefore an obvious cellular location for the synthesis and accumulation of valuable recombinant products.[2] Expressing the genes for these recombinant products in the chloroplast, rather than inserting the genes into the nucleus such that the protein is targeted into the chloroplast, is an attractive strategy for several reasons.[3] First, each chloroplast contains as many as a hundred copies of the plastome with genes encoding components of the photosynthetic apparatus expressed at a high level. Consequently, the copy number of a foreign gene (or 'transgene') inserted into the plastome is amplified significantly compared to the same gene inserted into the nuclear genome, and when fused to appropriate cis elements from these photosynthetic genes, the transgene has the potential for high-level expression. Second, chloroplast transformation studies in both algae and plants have shown that DNA integration occurs almost exclusively through homologous recombination. Not only does this allow precise and predictable manipulations of the plastome itself such that specific site-directed changes can be introduced into any chloroplast gene, but foreign DNA can be inserted at any chosen location within the plastome. As a consequence, the genetic engineering of the chloroplast avoids the problems of 'position effects' that are inherent with the random insertion of genes into the nuclear genome, and which affect significantly the level of transgene expression in the nucleus (see Chapter 1). Screening of large numbers of transformant lines is therefore not necessary when inserting transgenes into the chloroplast. Furthermore, integration via homologous recombination avoids the acquisition of undesirable *E. coli* vector sequences within the plastome. Third, studies to-date indicate that transgenes expressed in the chloroplast are not subject to the transcriptional and post-transcriptional gene silencing processes often observed with nuclear transgene expression. Fourth, multiple transgenes can be expressed as a single operon, allowing the coordinated synthesis of enzymes for a particular biosynthetic pathway or subunits of a protein complex. Fifth, the uniparental inheritance of chloroplast genes in many plant and algal species provides a mechanism for transgene containment.

Given these many advantages, it is perhaps not surprising that the manipulation of the plastome of plants and algae for commercial production of recombinant products—often referred to as "transplastomics"—is an increasingly active field.[3,4] A number of recent studies using tobacco chloroplasts have shown that remarkable yields of recombinant protein can be obtained; in some cases the protein accumulating to >25% of the total soluble protein (TSP) within the plant leaves.[3]

The Value of Chlamydomonas reinhardtii

The green unicellular alga *Chlamydomonas reinhardtii* occupies a special place in transgenic chloroplast research. The first demonstration of stable chloroplast transformation was achieved in 1988 using this alga,[5] and three years later the first recombinant protein produced in a chloroplast was by expression of a bacterial gene in the *Chlamydomonas* organelle.[6] Extensive reverse-genetic studies using *Chlamydomonas* over the past 18 years has provided significant insights into chloroplast gene function and expression, and a number of techniques first developed for plastome engineering in *Chlamydomonas* have been adapted for use in tobacco and other higher plants.[3] These include, the use of bacterial markers such as *aadA* and *aphA-6*, the technique of marker recycling and the use of plastome deletion mutants as recipient strains, as discussed below.

Whilst the levels of recombinant protein obtained in transplastomic *Chlamydomonas* have yet to match those obtained with tobacco (Table 1), this freshwater alga has several attributes that make it particularly attractive as a green cell factory. These are discussed in more detail by Fletcher et al in chapter 8, but to summarize: (1) *Chlamydomonas* chloroplast transformation is a simple and well-established technique that can generate transformant lines within 4-6 weeks. (2) The organism is a single cell with a single chloroplast, so any culture is a homogenous

Table 1. Foreign genes expressed in the* C. reinhardtii *chloroplast

Gene	Source Organism	Gene Product	Yield of Recombinant Protein (as % TSP)	Ref.
aadA	eubacteria (R plasmid)	Aminoglycoside adenyl transferase	nd	6
uidA	*Escherichia coli*	β-glucoronidase	nd	27
recA	*Escherichia coli*	RecA protein	nd	18
uidA	*Escherichia coli*	β-glucoronidase	0.08%	43
rluc	*Renilla reniformis*	Luciferase	nd	44
aphA6	*Acinetobacter baumannii*	Aminoglycoside phosphotransferase	nd	23
gfp cDNA	*Aequora victoria*	Green fluorescent protein	~0.05%	28
GFPct	Synthetic gene	Green fluorescent protein	0.5%	29
gds	*Sulfolobus acidocaldarius*	Geranylgeranyl pyrophosphate synthase	0.1%	34
HSV8-lsc	Synthetic gene	Large single-chain antibody	0.5%	8
FMDV.VP1	foot-and-mouth disease virus	Viral protein 1	3%	33
luxCt	Synthetic gene	Bacterial luciferase	nd	30
apcA and *apcB*	*Spirulina maxima*	α and β subunits of allophycocyanin	2-3%	36
nifH	*Klebsiella pneumoniae*	Nitrogenase subunit	nd	35

nd: not determined

collection of cells with all cells actively expressing the transgene. (3) *Chlamydomonas* has a short generation time of less than eight hours under optimum conditions, allowing the rapid production of biomass. (4) Importantly, *Chlamydomonas* can dispense completely with photosynthetic function and can be grown in darkness when supplied with acetate as an exogenous fixed carbon source. This allows the mass cultivation of transgenic lines in traditional fermentors under controlled, sterile and axenic culture conditions. Not only does this provide containment of the genetically modified organism, but also ensures that the production of pharmaceutical products can meet the strict regulations laid down by regulatory bodies such as the U.S. Food and Drug Administration. (5) *Chlamydomonas* is classified as a *GRAS* ('generally regarded as safe') organism with no known viral or bacterial pathogens, and appears to lack the endotoxins that complicate recombinant protein production using *Escherichia coli* and other bacterial platforms. (6) Our own studies have indicated that the 'escape' of chloroplast DNA sequences to the nuclear genome of *Chlamydomonas* is several orders of magnitude less frequent than that observed in tobacco, thereby increasing the containment level of transgenes in the chloroplast.[7]

The potential of the *Chlamydomonas* chloroplast as an expression platform has been elegantly illustrated by the recent work of Mayfield and colleagues who were able to express a fully active human monoclonal antibody in the alga.[8] In the following sections I review the various tools and techniques for chloroplast transformation of *Chlamydomonas* and illustrate its application in both basic research and more recent forays into applied areas of research. For more detailed discussion on the early development of chloroplast transformation in this alga, the reader is directed to three excellent reviews.[9-11]

Delivery of DNA into the Chloroplast Compartment

The most efficient and reliable method for introducing DNA into the chloroplast is microparticle bombardment. This biological-ballistic, or 'biolistic' process involves placing a nutrient agar plate carrying sections of plant tissue or a lawn of algal cells into a vacuum chamber, and bombarding the plate with DNA-coated gold or tungsten microparticles.[12] The kinetic energy of these particles is sufficient to penetrate the cell wall, the plasma membrane and the two membranes surrounding the chloroplast, and deliver multiple copies of the transforming DNA into the organelle. Early biolistic devices used for chloroplast transformation were home-built devices that used gunpowder charges to accelerate the microparticles.[13,14] Nowadays, most *Chlamydomonas* laboratories use the commercial helium-powered device marketed by Bio-Rad. Helium has the advantage of being a cleaner and more controllable propellant, and this together with several other design improvements has resulted in significantly increased transformation rates. The microparticles used in the bombardment process are made of either tungsten or gold, with gold being the more expensive but preferred material for most workers. This preference is because of: (i) the gold particles more uniform size; (ii) the biologically inert nature of gold as compared to tungsten, which can be toxic or mildly mutagenic to some cell types, and (iii) the tendency of tungsten to catalyze the slow degradation of DNA bound to it.

Several workers have investigated alternatives to biolistics as methods for delivering DNA into the *Chlamydomonas* chloroplast. Kindle and colleagues[15] showed that chloroplast transformation can be achieved simply by agitating a suspension of cells, DNA and glass beads using a laboratory vortex. Alternatively, electroporation can be used to generate chloroplast transformants (B. Sears, personal communication). Although the transformation rates using these methods are lower than those obtained with biolistics, and both methods require that a cell-wall-deficient strain is used as the recipient, the much lower costs of the equipment needed make them attractive alternatives to biolistics.

Integration of Transforming DNA

An important finding that came from the first report on chloroplast transformation was that transforming DNA carrying cloned chloroplast sequences integrated into the plastome via homologous recombination between these sequences and the corresponding endogenous sequences.[5] As illustrated in Figure 1, this allows one to carry out precise and premeditated manipulations of the plastome – a process that Rochaix termed 'chloroplast DNA surgery'.[16] In theory, any nonessential endogenous gene can be deleted or modified, with modifications ranging from one or more site-directed changes to the gene sequence, to replacement of promoter or untranslated regions (UTRs), or insertion of novel DNA elements that may influence the gene's expression. Furthermore, foreign genes can be inserted at any chosen position, and in either orientation, within the plastome (Fig. 2). This is in marked contrast to nuclear transformation in *Chlamydomonas*, where transforming DNA integrates at apparently random genomic loci via nonhomologous recombination processes.[17] Consequently, precise engineering of nuclear genes of *Chlamydomonas* is not feasible, and successful expression of foreign genes introduced into the nuclear genome unpredictable (see also Chapters 1 and 7).

Stable integration of foreign DNA into the plastome requires sufficient homologous sequence flanking the DNA to allow two recombination events (the actual mechanism of DNA integration is not known, but is best envisaged as a simple double crossover event as shown in Figs. 1 and 2). What constituent 'sufficient homologous sequence' has not been rigorously examined, although a good rule of thumb is ~1 kb of homology on either side of the DNA to be introduced. This is clearly sufficient since several studies have shown that inter-molecular recombination between incoming DNA and the plastome, or intra-molecular recombination between direct repeats flanking DNA inserted into the plastome can occur with as little as 120

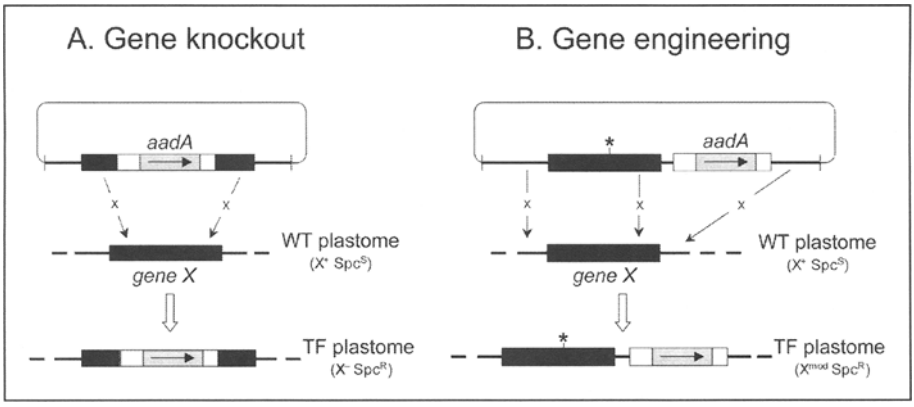

Figure 1. Reverse-genetic analysis of chloroplast gene X. Homologous recombination between chloroplast sequences (thick black lines) on the plasmid and the wildtype (WT) plastome allows disruption (A) or site-directed modifications (B) of the target gene using, for example, the *aadA* cassette[6] to select for spectinomycin (Spc) resistant transformants (TF). In the case of (B), the site-directed change may not be introduced if recombination occurs between the engineered change and *aadA* (broken arrow).

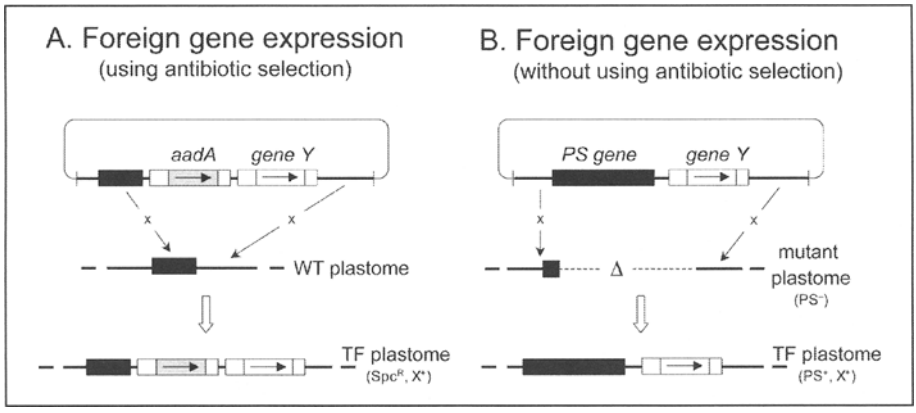

Figure 2. Two different strategies for introducing your foreign gene Y into the plastome. Gene Y fused to endogenous 5' and 3' sequences (open boxes) can be linked either to the *aadA* marker as shown in (A), allowing selection for spectinomycin-resistant transformants (TF), or to an endogenous gene required for photosynthesis (PS) as shown in (B), allowing selection for restored photosynthetic function.

bp and 216 bp, respectively of homologous sequence.[18,19] However, successful recombination using such short elements may be dependent on the sequences chosen.[11]

There are conflicting claims as to whether linear or circular DNA yields better transformation rates (see ref. 10), although any difference is marginal and since the researcher usually analyses only a few transformant colonies from the hundreds obtained in a typical transformation experiment, then most researchers choose the convenience of uncut plasmid DNA, as depicted in Figures 1 and 2.

If the transforming DNA is flanked on one side only with a homologous element then a single crossover takes place and results in the integration of the whole plasmid into the plastome with direct repeats of the element flanking the plasmid sequence. This integration is genetically

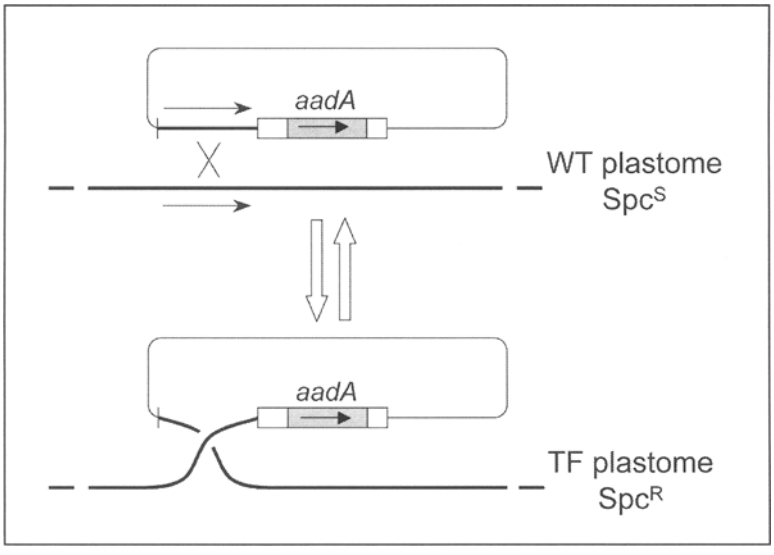

Figure 3. Transformation with a plasmid construct in which foreign DNA such as *aadA* is flanked on one side only with plastome sequence. This leads to a reversible transplastomic state in which the whole plasmid integrates into the plastome but is readily excised through recombination between the resulting direct repeats.

unstable since recombination between the repeats can result in the subsequent excision of the plasmid (Fig. 3). Consequently, the removal of the selective pressure for maintenance of the transforming DNA eventually results in the loss of the DNA since it is unlikely that the free plasmid will be replicated in the chloroplast. In theory, such an approach could be used as an alternative strategy to those developed by Fischer et al[20] (see below) for elimination of a selectable marker from the plastome.

Occasionally, unexpected recombination events can arise when the transforming plasmid contains homologous sequences in addition to the two flanking sequences—for example promoter elements or UTRs from endogenous genes that are used to drive expression of the selectable marker or other foreign genes. Intra- or intermolecular recombination between these sequences and the endogenous gene sequence can result in rearrangements or deletions.[11] Depending on the relative position and orientation of these two copies of the sequence, the result may be one of two states as explained in the next section: a heteroplasmic state in which there is a minor population of fragmented plastomes continuously being generated within the chloroplast, or a homoplasmic state where all plastome copies carry a deletion extending from the insertion site to the endogenous gene.[21]

Polyploidy and the Problems of Heteroplasmy

The plastome of *Chlamydomonas* is highly polyploid with approximately 50-80 copies present within the chloroplast. Transformation therefore invariably results in an initial heteroplasmic state in which only some copies of the plastome within an individual cell have been modified. Growth of transformant lines under selective conditions should eventually result in homoplasmic cells in which all copies of the plastome carry the selected change (Fig. 4). This homoplasmic state is attained typically by taking the lines through several rounds of single colony isolation on selective medium, and once homoplasmy is achieved the transgenic plastome is stable even in the absence of selection. In contrast, the initial heteroplasmic state will persist despite prolonged selective pressure if the engineered change disrupts an essential chloroplast gene. In this

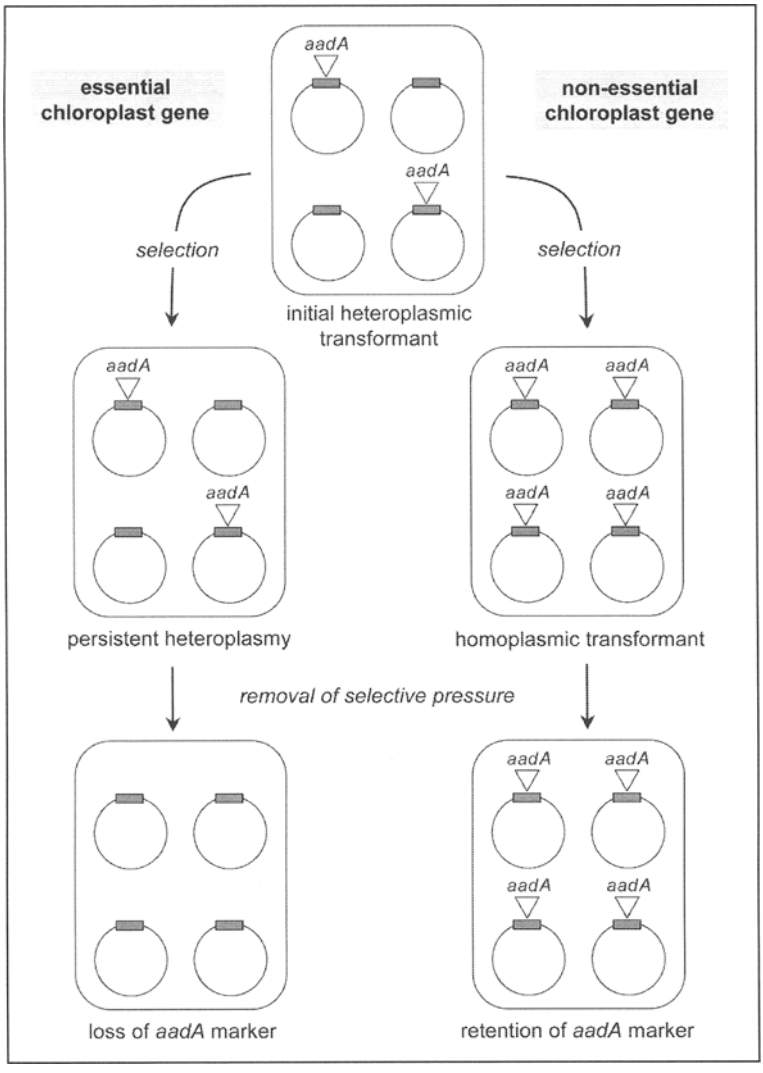

Figure 4. Two possible plastome outcomes to a chloroplast gene knockout experiment using *aadA* depending on whether or not the target gene (shown as a shaded box on the circular plastomes within a single chloroplast) is essential for cell survival.

case, the requirement for retention of the essential gene counters that for the selectable marker and both types of plastome are maintained. Subsequent removal of the selective pressure for the transgenic copy rapidly results in the loss of these plastomes from the chloroplast (Fig. 4).

Heteroplasmy can also prove problematic when recessive mutations are introduced into chloroplast genes, for example site-directed changes that affect the functioning of a photosynthetic gene. Since there is a natural selection against such mutations (unless transformant colonies are grown in the dark or in the presence of inhibitors of photosynthesis), then introducing these changes can prove challenging. Firstly, there is a tendency to recover transformants arising from recombination events that have incorporated the selectable marker into plastome copies, but not the linked site-directed change (see Fig. 1B). Secondly, the change may be

successfully introduced into some copies of the plastome but these are subsequently lost during the rounds of selection since selection favors those plastome copies carrying only the selectable marker. Finally, 'copy correction' mechanisms (either intermolecular recombination or gene conversion) may act during the heteroplasmic state, repairing the mutated gene copies using wild-type copies of the gene. An understanding of these issues is important not just for reverse-genetic studies of chloroplast gene function, but also for the introduction of foreign genes into the plastome. It is likely that many foreign genes will have some level of detrimental effect on the biology of the chloroplast, and can therefore be considered as recessive mutations. Consequently, the same selective pressures will affect the recovery of homoplasmic lines carrying the foreign gene, although in this case copy-correction would involve repair of the locus containing the inserted DNA using copies of the 'empty' wild-type locus.

Two strategies have been developed to overcome these problems. The first involves the treatment of *Chlamydomonas* with 0.5 mM 5-fluordeoxyuridine (FdUrd) prior to transformation.[9] This nucleoside analog appears to be a specific inhibitor of chloroplast DNA replication, reducing the plastome copy number by as much as 10-fold. Since a microparticle entering the chloroplast following bombardment will carry multiple copies of the transforming DNA, then the use of FdUrd significantly increases the chances of the recessive mutation being introduced into all or most plastome copies, and homoplasmy being attained without loss or repair of the mutation. However, FdUrd is also a chemical mutagen, and can lead to unwanted mutations in the plastome. A second strategy, pioneered by Redding and colleagues[22] uses a chloroplast deletion mutant as the recipient strain, and neatly addresses all of the problems discussed above. By ensuring that the homologous DNA carried on the transforming plasmid spans the deletion within the mutant plastome, and that both recessive mutation and the selectable marker are located within the deleted region, then modified plastome copies in the initial heteroplasmic transformants will always have both the selectable marker and the recessive mutation (one such scenario is illustrated in Fig. 2B). Furthermore, no wild-type copies of the mutated locus are present in the chloroplast so elimination via copy correction is not possible.

Selection Strategies

Several different strategies have been developed for the selection of rare transformants amongst the large population of untransformed cells following bombardment of the algal lawn. In the early transformation experiments, selection was based on the rescue of nonphotosynthetic mutants carrying lesions in chloroplast genes encoding key photosynthetic components.[10] For example, the original experiment of Boynton et al involved the bombardment of a mutant line carrying a plastome deletion affecting the *atpB* gene with a plasmid carrying the wild-type gene.[5] Introduction of *atpB* into the plastome of the mutant restored photosynthetic function, and hence the ability to grow on a medium lacking a reduced carbon source. Using this approach, foreign genes can be targeted to a neutral, intergenic region downstream of *atpB* (Fig. 2B), or can be targeted to other loci by cotransformation using the *atpB* plasmid and a second plasmid in which the foreign gene is flanked with the appropriate homologous sequence. The main drawback of this selection strategy is that a specific deletion mutant is required as the recipient rather than any strain carrying a wild-type plastome. There are, however, two specific advantages: firstly, there is strong selection for transformant clones since a wild-type (i.e., photosynthetically competent and light tolerant) phenotype is being restored, and therefore transformants are easily generated and rapidly attain a homoplasmic state. Secondly, foreign DNA can be introduced into the plastome without the use of any bacterial antibiotic-resistance marker. This circumvents one of the major environmental concerns with transplastomic plants and algae - the presence within each cell of multiple copies of bacterial markers and the possibility of their horizontal spread to other organisms in the environment.

An alternative method that allows the use of wild-type recipient strains involves selection for antibiotic resistance using cloned *Chlamydomonas* plastome genes carrying dominant point mutations. For example, mutations that confer resistance to different antibiotics have been

identified in genes encoding the 16S and 23S ribosomal RNAs, the ribosomal protein S12 and elongation factor Tu.[9] As before, the advantage of this strategy is that no bacterial marker is used. However, the disadvantages are that selection on these antibiotics inevitably yields a background of pseudo-transformants (i.e., spontaneous resistance mutants) amongst the transformant population, and the dominant mutations introduced into the chloroplast's translational apparatus may affect the efficiency of protein synthesis - especially when trying to over-express foreign genes.

The most widely used dominant marker is the '*aadA* cassette' developed by Goldschmidt-Clermont.[6] This marker comprises the coding sequence of the bacterial gene *aadA* fused to upstream and downstream elements from *Chlamydomonas* chloroplast genes, and confers resistance to the antibiotics, spectinomycin and streptomycin. More recently, my group has developed a similar marker based on the bacterial *aphA-6* gene that confers resistance to kanamycin and amikacin.[22] As illustrated in Figure 1, these portable cassettes can be targeted to any site on the plastome and used to disrupt or modify endogenous genes, or to introduce foreign genes. However, the limited availability of different dominant markers led Fischer et al to explore different ways of 'recycling' the *aadA* cassette so that serial manipulations of the plastome would be possible using the same marker.[20] By flanking the cassette with direct repeat elements or placing it within an essential gene, Fischer et al showed that the cassette was rapidly lost from the transformed plastome once spectinomycin selection was removed. As discussed above, these "use it, then lose it" strategies also overcome the issue of unwanted bacterial antibiotic markers in the resulting transplastomic lines.

Reverse-Genetic Studies of the *Chlamydomonas* Plastome

The development of a routine and facile method for manipulating the *Chlamydomonas* plastome has allowed a wide range of reverse-genetic studies that have provided fundamental insights into the expression of chloroplast genes and the functioning of their products.[24,25] Some selected examples are as follows: (1) gene disruption studies have identified genes encoding novel components of the photosynthetic complexes, such as the PetL subunit of the cytochrome b_6f complex, and the PsbT and PsbZ subunits of photosystem II.[24] (2) Site-directed changes and nucleotide insertions or deletions around the start codon of chloroplast genes have allowed a dissection of cis-acting sequences required for translation initiation.[26] (3) Site-directed changes to conserved residues of the core subunits of photosystem I (PSI) have identified those residues that modulate the properties of the redox cofactors involved in electron transfer and led to the finding that electron transfer is bi-directional in PSI.[22,25] (4) Reporter genes have been used to investigate the role of the 5' and 3' UTRs of chloroplast genes in mediating RNA stability and translation.[27,28] Furthermore, the recent development of new codon-optimized reporter genes encoding green fluorescent protein[29] or luciferase[30] now opens the door to even more sensitive and sophisticated gene expression studies. (5) Mutational studies of the cytochrome *f* subunit of the cytochrome b_6f complex has identified residues that constitute a translation repression motif involved in the assembly-mediated regulation of cytochrome *f* synthesis.[31] (6) The topology of proteins embedded in the thylakoid membrane has been investigated using *aadA* translational fusions, whereby fusions that place the AAD protein on the stromal side of the membrane yield high levels of spectinomycin resistance whereas those that place it on the luminal side give low resistance levels.[32]

Expression of Foreign Genes in the *Chlamydomonas* Chloroplast

The expression of foreign genes in the *Chlamydomonas* chloroplast is still in its infancy with only a dozen or so reports in the literature (Table 1). Most of these reports represent basic research aimed at developing molecular tools, and include the selectable markers and reporter genes discussed in the previous sections. In each case, the gene construct consists of a promoter, together with 5' and 3' UTRs from endogenous chloroplast genes, fused to coding sequence for the foreign gene. The coding sequence is either obtained directly from bacterial genes (in

the case of *aadA*, *aphA-6* and *uidA*) or is synthesized de novo using the preferred codon usage observed for *Chlamydomonas* chloroplast genes. To-date there has been no reports of successful expression of foreign genes using synthetic promoters or those from other organisms (e.g., from bacterial genes or organellar genes of other eukaryotic species). There have been only a handful of papers describing applied research aimed at producing novel products or introducing novel biosynthetic pathways. As discussed in Chapter 8, Mayfield and colleagues have demonstrated the feasibility of producing soluble, active monoclonal antibodies in *Chlamydomonas*, and their research has illustrated the importance of codon optimization in maximizing transgene expression.[8] Importantly, their studies showed that the single-chain antibody folded correctly and dimerized via a disulfide bridge, but was not subject to unwanted post-translational modifications such as glycosylation within the chloroplast compartment. In a separate study, Sun et al have explored the possibility of vaccine production by expressing a fusion construct comprising the VP1 gene of foot-and-mouth virus fused to the gene for Cholera toxin B.[33] Again, initial studies suggest proper folding of the recombinant protein in the chloroplast.

Promising early work on the manipulation of chloroplast metabolic pathways in *Chlamydomonas* has been reported by several groups. Fukusaki et al successfully expressed an archeal gene encoding a thermostable version of the GGPP synthase enzyme involved in terpenoid biosynthesis,[34] and Cheng et al were able to functionally replace a chloroplast gene involved in chlorophyll biosynthesis with a related gene from *Klebsiella pneumoniae* encoding a component of the nitrogen-fixing enzyme, nitrogenase.[35] Finally, the possibility of expressing multiple foreign genes in *Chlamydomonas*, and thereby constructing novel biosynthetic pathways or multisubunit protein complexes, has been investigated by Su et al who were able to express a two gene operon encoding the alpha and beta subunits of the cyanobacterial light-harvesting protein, allophycocyanin.[36]

Future Prospects

There is currently much interest in transplastomics as a strategy for the commercial production of recombinant macromolecules.[4] Most of this interest has focused on cultivatable plants, not least because of the potential for large-scale production using established agricultural practices. Whilst fermentor-based culturing of green algae such as *Chlamydomonas* can never match field-based cultivation of plants such as tobacco in terms of chloroplast biomass, there is a possible niche market for *Chlamydomonas* in this emerging biotech industry. This niche encompasses high-value recombinant products intended for human use and includes pharmaceutical proteins (e.g., hormones, vaccines and antibodies), nutriceuticals (e.g., speciality carotenoids and long-chain polyunsaturated fatty acids) and other bioactive secondary metabolites.

However, there are several further developments that are required before *Chlamydomonas* can realize its potential. Key amongst these is a significant improvement in recombinant protein yield. Current yields are, at best, one or two percent of TSP (Table 1)—significantly below that of endogenous chloroplast proteins such as the large subunit of the rubisco enzyme or the D1 and D2 proteins of photosystem II. The accumulating mass of published data on chloroplast gene expression in *Chlamydomonas* is highlighting those factors that are important considerations for improving protein levels. Post-transcriptional steps seem to be more critical than gene copy number, transcription rates or transcript levels,[37] and the focus now is on translation initiation, polypeptide synthesis and protein stability. For the first of these, the focus is the cis elements within the 5' UTRs or coding regions of chloroplast genes and how these elements interact with nuclear-encoded trans-acting factors to mediate efficient translation initiation.[38] In terms of efficient translation, the work of Mayfield et al has shown that codon optimization can significantly improve the synthesis of recombinant proteins.[8,29,30] Finally, a clearer understanding of the targets and mechanisms of the different chloroplast proteases may provide opportunities for increased protein accumulation using protease-deficient strains.

In addition to maximizing recombinant protein levels, the commercial production of recombinant proteins in the *Chlamydomonas* chloroplast also requires the development of an inducible system for transgene expression. Currently, all foreign genes expressed in this alga's organelle are driven from endogenous promoters (e.g., those from *rrn16*, *atpA* or *rbcL*) and therefore transgene expression is "on" throughout all the growth phases. What is much more preferable is a system whereby expression is suppressed until late log phase allowing the over-accumulation of the recombinant protein in the cells only once sufficient biomass has been generated. Such an inducible system also allows the production of proteins that have toxic or detrimental effects on the growing cells. Several systems have been developed recently that allow the inducible expression of chloroplast transgenes in tobacco[39,40] and it should be feasible to develop similar strategies for *Chlamydomonas*.

Given such improvements, together with the relative ease with which unicellular algae such as *Chlamydomonas* can be grown heterotrophically on an industrial scale using existing fermentor-based technology,[41] and at minimal cost,[42] it is possible to imagine a future where this alga's chloroplast is a viable and commercially attractive platform for recombinant production, and is able to compete with existing microbial platforms based on bacteria or yeast.

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