Chapter 16

Plastid Transformation in Algae

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Summary

Pioneering work from the late 1980s using the green alga *Chlamydomonas reinhardtii* has paved the way for biolistic chloroplast transformation in general. Since then, the continuous development of a molecular toolkit has made this chlorophyte alga the prime organism for algal transplastomic biotechnological applications. However, comparatively little progress has been made with the stable genetic manipulation of members of other algal groups with the red alga *Porphyridium* UTEX 637 representing a rare exception. In this chapter, we summarize the basic molecular principles of chloroplast transformation in algae as well as current approaches to optimize foreign gene expression in Chlamydomonas.

I. Introduction

Algae represent a diverse group of photosynthetic eukaryotes which are of fundamental ecological importance as primary producers of ca. 50% of the total organic carbon produced on earth per year and consequently a fundamental basis of the food chain. Algae arose during evolution by the uptake of a cyanobacterium by a heterotrophic protist

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host approximately 1.5 billion years ago, a process referred to as endosymbiosis (Gross and Bhattacharya 2009). The engulfed cyanobacterium, probably related to extant *Anabaena* species, then transformed into a cellular organelle, the chloroplast (Deusch et al. 2008). This gave rise to at least three different lineages: the Glaucophyta (glaucophyte algae), the Rhodophyta (red algae) and the Viridiplantae (green algae and land plants). However, the evolutionary spectrum of algal life-forms was further increased due to secondary and even tertiary endosymbiotic events which involved the uptake of green or red eukaryotic algae by other heterotrophic eukaryotic hosts (see Chap. 2). As a consequence, the resulting algal groups contain "complex" plastids which are surrounded by three or four membranes. In contrast, plastids derived from primary endosymbiosis possess only two membranes forming the chloroplast envelope.

Algae have a long-standing tradition as food for humans and animal feed, especially in Asian countries. Todays biotechnological applications involve mainly non-transgenic approaches including the production of polyunsaturated fatty acids, polysaccharides and carotenoids (for review see Hallmann 2007). More recently, microalgae have attracted more attention as a source for the production of renewable energy like biodiesel and hydrogen (Mata et al. 2010; Stephens et al. 2010). Nevertheless with accumulating sequence information from various algal genomes and the parallel development of transformation techniques, a "transgenic century" for algal biotechnology has been initiated. To date ca. 25 algal species have been stably genetically manipulated in their nuclear genomes including green, red and brown algae as well as diatoms and dinoflagellates (for an overview see Walker et al. 2005). Most of them represent unicellular microalgae but also macroalgae like *Laminaria japonica*, *Porphyra miniata* and *Ulva lactuca* have successfully been subjected to stable genetic transformation (Qin et al. 2005). The transformation methods in use mainly include bombardment of algal cells with DNA-coated particles, agitation of algae with glass beads or silicate whiskers in the presence of DNA, electroporation and in rare cases *Agrobacterium tumefaciens* mediated transformation (Walker et al. 2005).

As outlined below, biolistic transformation is the method of choice for generating chloroplast transformants in both algae and land plants. However, with the exception of the green alga *Chlamydomonas reinhardtii* only a very limited number of algal species has been genetically transformed in their plastid genomes (see Sect. IV). As such, *C. reinhardtii* currently still represents a more-or-less stand-alone model system for algal chloroplast transformation.

II. Chloroplast Biology of *C. reinhardtii*

C. reinhardtii is a flagellated unicellular green alga which has a size of 10 μm in diameter. It contains a single cup-shaped chloroplast which accounts for 40% of the total cellular volume. This relatively "big" chloroplast has significantly facilitated chloroplast transformation attempts by using the biolistic approach described below (see Sect. III.A). The chloroplast is surrounded by two envelope membranes and inside the organelle one can identify the organization of the thylakoid membrane system by electron microscopy. In *C. reinhardtii*, as well as in other green algae, plant-like differentiated thylakoid grana regions are lacking but instead thylakoid membranes can be arranged in multiple stacks of 2–10 discs (Harris 2009). Like many algae, *C. reinhardtii* contains a basally located chloroplast pyrenoid whose function is mainly dedicated to carbon concentrating mechanisms and which primarily consists of the CO_2-fixing enzyme ribulose-1,5-bisphosphate carboxylase/

Abbreviations: AHAS – Acetohydroxyacid synthase; CAI – Codon adaption index; CES – Control by epistasy of synthesis; DCMU – 3-(3′ 4′-di-chlorphenyl)-1,1-dimethylurea

oxygenase (Rubisco). Interestingly, the pyrenoid has recently been shown to also serve as the site of thylakoid membrane biogenesis and to play a pivotal role in the spatial organization of chloroplast gene expression (Uniacke and Zerges 2007). Furthermore, motile algae like *C. reinhardtii* can contain an "eye" allowing phototactic movements. One prominent eye constituent is the so-called eyespot, which is located at the periphery of the chloroplast where it associates with the inner chloroplast envelope and thylakoids.

The chloroplast genome of *C. reinhardtii* (203 kbp) is present in ca. 80 copies per cell (Koop et al. 2007). It encodes 109 genes and harbours a high number of short dispersed repeat regions (see Chap. 3). During sexual reproduction, the chloroplast DNA is uniparentally inherited from the mating type + (mt^+) parent. Thus, resembling the situation in vascular plants, an outcrossing of chloroplast transgenes from a mating type – (mt^-) parent is unlikely, increasing the ecological safeness of transplastomic algal lines for biotechnological applications (see also Chap. 18).

The chloroplast gene expression machinery has been intensively studied at the molecular level and crucial regulatory factors and elements regulating this machinery have been dismantled by both genetic and biochemical means (see Sect. V). One remarkable feature of some Chlamydomonas species, including *C. reinhardtii*, is their capacity to grow heterotrophically by consumption of acetate as reduced carbon source. The precise acetate assimilation pathway is still unclear but it is generally assumed that acetate is converted into acetyl-CoA by either acetyl-CoA synthetase activity or by a two-step process catalyzed by acetate kinase and phosphate acetyltransferase (Spalding 2009). Subsequently, most of the acetate assimilation is proposed to occur through the glyoxylate cycle whose subcellular compartmentation is still uncertain (Spalding 2009). However despite these open questions, acetate-containing media allow the maintenance of photosynthetic mutants which still retain their fertility and, thus, can be used for downstream genetic analyses. More important in the course of chloroplast transformation events, these mutants can serve as recipient strains for chloroplast DNA manipulations (Nickelsen and Kück 2000; see Sect. III.D).

III. Transformation Procedures

The principal challenge for chloroplast transformation of both plant and algal cells is the delivery of DNA across the cell wall and at least three membranes, i.e., the plasma membrane and the inner and outer chloroplast envelope membranes. In case of algae harbouring complex plastids, the situation is even more complicated due to the presence of one or two additional chloroplast envelope membranes. Through the development of a so-called particle gun this problem has been overcome. Stable transplastomic lines have been generated for algae and land plants mainly by using this biolistic transformation technique (see below). However, for vascular plants also PEG-treatment of protoplasts has frequently been applied to deliver DNA into the chloroplast compartment of the cell (Koop et al. 2007; see Chap. 18). For *C. reinhardtii*, an alternative, very simple method has been described which is based on the agitation of cell-wall-less strains with glass beads and, therefore, requires no specialized equipment like a particle-gun (Kindle et al. 1991). Usually, this method yields high levels of nuclear transformants, but also chloroplast transformants can be generated to some extent. In contrast to protoplast transformation of plant cells, addition of PEG had no positive effect on chloroplast transformation rates using the glass bead method (Kindle et al. 1991). When this simple method was directly compared to the biolistic technique, however, a drastically (more than tenfold) lower chloroplast transformation efficiency was observed. Therefore nowadays, biolistic transformation is the routinely used method for generating transplastomic lines in *C. reinhardtii*.

A. Biolistic Gene Transfer

In 1988, Boynton and co-workers reported on the first successful transformation of a chloroplast genome. They used *C. reinhardtii* and microparticle bombardment which in parallel had successfully been applied to the transformation of mitochondria from yeast cells (Johnston et al. 1988). The basic steps of the method involve the coating of microparticles with DNA which are then used to bombard an algal cell lawn on an agar plate under vacuum. For acceleration of particles to sufficiently high velocity, initially home-built powder explosion devices were used but these have nowadays been replaced by commercially available helium-powered guns. Alternatively, so-called particle inflow guns have very successfully been used (Finer et al. 1992). Particles are accelerated in a helium stream which is controlled by a timer relay driven solenoid working at moderate helium pressure of eight bar and, thus, causing less damage to algal cells (Nickelsen and Kück 2000). As microprojectiles, both tungsten and gold particles are used with gold being more expensive but also more inert and uniform in size. For chloroplast transformation in *C. reinhardtii*, tungsten particles work sufficiently well.

Once DNA-coated particles hit a cell, they first penetrate through the cell wall/plasma membrane and then are supposed to penetrate the organelle's envelope and deposit the transforming DNA into the chloroplast stroma. How punctured membranes reseal afterwards is completely unknown. Alternatively, one might envisage that DNA is delivered to the cytosol and an unknown mechanism would subsequently transfer this DNA into the organelle (Koop et al. 2007). Irrespective of the incomplete picture of the entire transformation process, the feasibility of the approach is well established and, therefore, the development of the biolistic transformation procedure clearly marks the breakthrough for organelle transgenics.

B. Stable Transformation

Upon reaching the chloroplast, foreign DNA molecules then can integrate into the chloroplast genome. Due to the evolutionary history of chloroplasts as former cyanobacteria, a bacterial recombination system still exists in plastids that mediates integration of DNA via homologous recombination. Therefore, a prerequisite for plastome transgene integration is the presence of flanking homologous regions in the transforming DNA, which is thought to recombine via a double crossover event into the chloroplast genome. Usually, foreign recombinant DNAs should contain homologous flanking regions comprising ca. 1 kbp. However, successful high-frequency integration of DNA at the *psbA* locus from *C. reinhardtii* was also obtained with non-purified PCR fragments containing only 51 bp upstream and 121 bp of homology downstream of the integration site (Dauvillee et al. 2004). Furthermore, recombination between artificially introduced direct repeats of 483 bp – but not of 230 bp – was demonstrated in *C. reinhardtii* chloroplasts (Fischer et al. 1996) suggesting that the size of minimal "recombination platforms" may depend on the structural characteristics of the involved chloroplast genome regions.

As in bacteria, single crossover events due to only one homologous flanking region lead to the integration of the entire plasmid DNA and the generation of direct repeats at the plasmid sequence ends. This, however, creates an unstable situation since the plasmid tends to immediately recombine out after selective pressure is released (Purton 2007).

C. Heteroplasmy and Episomal Maintenance

The recombination event between a copy of the chloroplast genome and the foreign DNA results in a state, termed "heteroplasmic", where only one or few of the 80 genome copies have been altered. However, when transformants are repeatedly transferred to fresh selective medium, eventually, a "homoplasmic" state is accomplished at which all copies of the chloroplast genome contain the transgenic manipulation. In *C. reinhardtii*, homoplasmy is usually achieved after 3–4 weeks with a weekly transfer of transformant colonies. At this point, the transgenic

state is stable and selective pressure can be released (Koop et al. 2007).

A heteroplasmic state is maintained, however, when the genetic alteration affects an essential gene. In this case, about 50% of the cp-genome copies remain wild-type to guarantee survival of cells. As a consequence, subsequent removal of selective conditions leads to a rapid reversion to the homoplasmic wild-type state. Thus, for reverse genetic approaches, a persisting heteroplasmic state is indicative of essential gene functions being compromised (see Chap. 19).

As outlined by Purton (2007), heteroplasmy might also cause problems when recessive mutations are introduced into the chloroplast genome of *C. reinhardtii* via co-integration of a selectable marker. The natural selection against such mutations, for instance in photosynthetic genes, could eventually lead to a low recovery of transformants containing both the site-directed mutation and the selectable marker. This might be due to low incorporation or maintenance of site-directed alterations and/or "copy correction" mechanisms acting on them. Strategies to overcome these problems include the pre-treatment of cells with FUdR (5-fluorodeoxyuridine), an inhibitor of chloroplast DNA replication that leads to reduced chloroplast-genome copy number. Apparently, this reduced copy number facilitates the subsequent segregation process during subculturing of transformants (Goldschmidt-Clermont 1998). Secondly, the use of strains containing chloroplast deletions of the target site avoids the problem of elimination via copy correction by wild-type gene versions (Guergova-Kuras et al. 2001).

Although homologous recombination usually results in stable integration of foreign DNA into the chloroplast genome, exceptions from this rule have been observed. In 1994, Kindle et al. reported on the detection of plasmid-like structures in the chloroplast of *C. reinhardtii* after chloroplast transformation. The episomal elements contained mutated versions of the *atpB* gene and accumulated to ca. 2,000 copies per chloroplast. Genetic crossings revealed that they were uniparentally inherited from the mt^+ parent indicating a chloroplast location. However, subsequent attempts to generate autonomously replicating systems based on these elements failed probably due to a very specialized *atpB*-specific effect (Suzuki et al. 1997). Episomal maintenance of transforming DNA was also observed during transformation of the unicellular alga *Euglena gracilis*, but in this case, copy numbers were drastically reduced as compared to chloroplast genome copy number (Doetsch et al. 2001; see Sect. IV). Taken together, the apparent possibility of creating high-copy-number plasmids within chloroplasts sounds appealing but available data suggest that it will be very difficult to generate chloroplast high-expression systems for transgenic biotechnological applications based on this system.

D. Chloroplast Markers and Marker Recycling

Biolistic chloroplast transformation in *C. reinhardtii* is usually performed by bombardment of a lawn of ca. 1×10^8 algal cells. Usually hundreds of transformants can be generated with one "shot" which are then selected directly on the plate. Alternatively, after bombardment, the cell lawn is transferred to a fresh plate containing the selective medium. In principle, three different selection strategies for transgenic lines have been applied to date. In their first successful attempts, Boynton et al. (1988) complemented an *atpB* deletion mutant by using a wild-type version of the chloroplast *atpB* gene encoding the β subunit of the chloroplast ATP synthase. Consequently, selection for restored photosynthetic activity was performed on minimal medium containing no acetate as reduced carbon source. Similarly, the chloroplast *tscA* gene involved in group II intron trans-splicing of the photosystem I subunit *psaA* mRNA restored photoautotrophic growth of transformants upon introduction into the chloroplast genome of the *tscA* deletion mutant *H13* (Goldschmidt-Clermont et al. 1991). Despite the fact that this approach is limited by the availability of appropriate chloroplast mutants, it has the main advantage that problems of

heteroplasmic states (see Sect. III.C) are minimized. Moreover, no selectable bacterial markers must be co-introduced during transformation avoiding risks of marker spreading into the environment via horizontal gene transfer.

A second strategy described for *C. reinhardtii* is based on the introduction of point mutations into the chloroplast genome that confer resistance to either antibiotics like spectinomycin or herbicides like DCMU (for an overview see Goldschmidt-Clermont 1998). In the red alga *Porphyridium* spec., a mutated version of the chloroplast acetohydroxyacid synthase (AHAS) has successfully been used to select chloroplast transformants based on their resistance against the herbicide sulfometuron methyl (SMM; Lapidot et al. 2002). The advantage of such an approach is that basically any strain can be used as recipient for transformation and that no bacterial marker sequences are involved. However, since resistances occur spontaneously, a background of pseudo-transformants must always be considered which have to be sorted out by molecular analyses.

Most convenient for many applications in basic research is the use of dominant bacterial marker genes fused to regulatory chloroplast 5′ and 3′ regions. The most frequently used one is the *aadA* cassette from *Escherichia coli* which confers resistance to both spectinomycin and streptomycin (Goldschmidt-Clermont 1991). The second marker that is available for chloroplast transformation in *C. reinhardtii* is based on the *aphA-6* gene from *Acinetobacter baumannii* conferring resistance to kanamycin or amikacin (Bateman and Purton 2000). Marker cassettes can be introduced at any site of the chloroplast genome and have been used to inactivate or modify a number of chloroplast genes in *C. reinhardtii* (see Chap. 19). Furthermore, cassette co-integration is the method of choice for the establishment of foreign gene expression in chloroplasts (see Sect. V). During chloroplast transformation of *C. reinhardtii*, high frequencies (ca. 80%) of co-transformation events have been observed when two different markers on separate vectors where transformed (Boynton and Gillham 1993). This offers the possibility of efficient strategies for site-directed mutagenesis even if the marker cassette cannot be integrated close to the mutated site.

Obviously, the number of different markers and, consequently, selection strategies for algal chloroplast transformants are limited. This prevents the manipulation of multiple sites of the chloroplast genome in successive rounds of transformation. One solution to this problem is the use of markers that can be recycled. Fischer et al. (1996) reported on two different approaches for the use of the *aadA* marker for transformant selection and its subsequent removal from the chloroplast genome. The first strategy requires a marker flanked by direct repeats of 483 bp from bacterial plasmid DNA. After co-integration into the chloroplast genome, transformants are selected until homoplasmy is reached and, subsequently, selective pressure is released by cultivation of cells on appropriate (antibiotic-free) medium. Under non-selective conditions, recombination events between the direct repeats result in the excision of the marker located between the repeats. Afterwards, the marker-free strains can be applied to a next round of transformation via the *aadA* cassette (Fischer et al. 1996; Redding et al. 1998). Alternatively, the *aadA* cassette is introduced into an essential gene leading to a heteroplasmic state with regard to the selectable marker. A co-transformed construct creating a mutation of interest in a non-essential gene will reach homoplasmy during the selection period. Upon release of selective pressure, the marker cassette will then be eliminated from its heteroplasmic integration site via copy correction mechanisms or lost by random genome sorting. In summary, a complete molecular toolkit for the genetic manipulation of the chloroplast genome from *C. reinhardtii* is nowadays available and has successfully been applied in both basic and applied science.

IV. Transformed Algae Species

As already mentioned in the introductionary section, the transformation of algal species other than *C. reinhardtii* is still in its infancy. To date, only two additional algae species have been reported to have been successfully transformed. Doetsch and coworkers (2001) subjected the complex plastid of *Euglena gracilis* to transformation with the *aadA* cassette driven by *E. gracilis* control elements from the *psbA* 5′ and 3′ regions. In contrast to *C. reinhardtii,* for which recipient cells are transformed directly on agar plates, Euglena cells had to be spread on filter membranes in form of a mono-layer before bombardment. Apparently, this procedure results in a stabilization of cells against a semirigid backbone during microprojectile entry and, thus, allows the penetration through the protein pellicle surrounding *E. gracilis* cells (Doetsch et al. 2001). Molecular characterization of spectinomycin/streptomycin resistant colonies revealed that the transforming DNA did not integrate into the chloroplast genome, but was maintained as an extrachromosomal copy. Although the copy number was quite low with only 1–2 copies per chloroplast compared to ca. 100–300 copies of the chloroplast genome, this episomal element was maintained during at least 2 years of cultivation on solid medium. Apparently, long-term maintenance was sequence- or gene-dependent, respectively, since constructs containing a complete *psbK* operon were lost after only a few weeks on solid medium, probably due to overexpression problems caused by the introduced genes (Doetsch et al. 2001). Nevertheless despite several unsolved problems with *E. gracilis* chloroplast transformation, these first steps hopefully pave the way for the genetic manipulation of other algae containing complex plastids like brown algae or diatoms (see Chap. 2).

Initial attempts to stably transform the chloroplast genome of the diatom *Phaeodactylum tricornutum* proved to be difficult. Materna et al. (2009) reported on the generation of site-directed plastid mutants of the *psbA* gene encoding the D1 protein of the photosystem II reaction center. Using a commercial particle gun, constructs with mutant variants of codon 264 of the D1 protein leading to herbicide resistance against DCMU were introduced into to *P. tricornutum* cells (see also Chap. 12). However, molecular analyses of resulting DCMU resistant strains revealed that, apparently, the delivered DNA induced elevated mutation rates at the *psbA* locus but no real transformation events (Materna et al. 2009; P. Kroth, Konstanz, personal communication). The molecular basis for this phenomenon is unclear and, therefore, further efforts are required before a reliable chloroplast transformation protocol for diatoms will be available.

Similar to *C. reinhardtii*, the unicellular marine red alga *Porphyridium* UTEX 637 contains primary chloroplasts which are surrounded by only two envelope membranes. Biolistic transformation of this species resulted in stable transformants due to single crossover events that mediated the homologous recombination into the alga's plastid AHAS locus (Lapidot et al. 2002; for selection strategy see Sect. III.D). This resulted in the presence of two AHAS gene copies, one wild-type form and the SMM resistance conferring form. Thus, release of selection pressure is likely to lead to an immediate loss of the introduced DNA via recombination between the two AHAS repeats. Under continuous selective conditions however, the introduced DNA was stable for at least 1 year of cultivation. During the course of their work, the authors noticed that the transformation efficiency drastically increased when cells from dark/light synchronized cultures were used immediately after the dark cycle. As speculated by the authors, this is likely to be an effect of reduced amounts of cell wall polysaccharides at this time point and, consequently, a less solid barrier for the DNA-loaded microprojectiles (Lapidot et al. 2002). Therefore, careful evaluation of chloroplast transformation rates of cells from different

time points of synchronized liquid cultures might represent one promising approach to overcome problems of genetic manipulation of recalcitrant algal species including especially those harbouring complex plastids.

V. Expression of Foreign Genes and Algal Chloroplast Biotechnology

Recent years have seen an increasing interest in using transplastomic approaches for the commercial production of recombinant therapeutic proteins. In algae – mostly in *C. reinhardtii* – substantial progress has been made with regard to the number and yields of recombinant protein production in chloroplasts. As reviewed by Specht et al. (2010), algae have distinct advantages as compared to vascular plants for biotechnological transgenic applications. Usually, they grow in contained bioreactors limiting risks of contaminations of production cultures on the one hand and the environment on the other hand. Transformation protocols are fast and microalgal cells are relatively uniform in size and differentiation status thereby facilitating downstream processing.

One major goal of transplastomic biotechnological applications is the optimization of product yields during the production process, i.e., the increase in stable accumulation of the foreign recombinant protein which is usually measured in relation to total protein amount. Current optimization attempts significantly benefit from the comprehensive knowledge of the molecular principles underlying endogenous chloroplast gene expression in *C. reinhardtii*. Chloroplast gene expression has been shown to be controlled at almost all levels including transcription, RNA metabolism and translation, with the latter one representing in most cases the rate-limiting step in expression of a chloroplast gene (Eberhard et al. 2002). Reverse genetic approaches involving site-directed mutagenesis of the flanking regions of chloroplast genes dramatically accelerated the identification of crucial *cis*-acting elements which are directly involved in the control of gene expression processes (see Chap. 19). The critical elements include promoter structures, which usually are of the bacterial sigma 70 type and contain so-called −10 and −35 elements. However, highly expressed algal genes like *psbD* encoding the D2 protein of photosystem II possess promoters containing only a −10 element (Klinkert et al. 2005). Furthermore, the analysis of plastid reporter gene constructs has demonstrated that the 5′ UTRs of plastid mRNAs play critical roles in RNA stabilization and for translation initiation. These 5′ UTRs serve as recognition sites for trans-acting regulatory protein factors or form structural RNA elements which influence posttranscriptional processes by the formation of barriers against nucleolytic attack or by the control of ribosomal access to mRNAs (Nickelsen et al. 1994; Drager et al. 1998; Bruick and Mayfield 1998; Vaistij et al. 2000; Suay et al. 2005; Klinkert et al. 2006). Complementary biochemical and genetic analyses in *C. reinhardtii* have revealed the nature of many trans-acting factors which exert their function via these cis-elements and, thus, represent the pacemakers for the expression of chloroplast genes and, consequently, also for transgenes (for a recent review see Bohne et al. 2009; Stern et al. 2010). One remarkable general regulatory principle which underlies chloroplast gene expression has been named “control by epistasy of synthesis” (CES). It is valid for the synthesis/assembly of various photosynthetic complexes, i.e., Cytb$_6$f, PSII, PSI, as well as the ATPase in *C. reinhardtii* and the Rubisco enzyme in tobacco (for a recent overview see Choquet and Wollman 2009). Basically, unassembled subunits of multisubunit protein complexes exert a feedback-loop inhibition on their own synthesis via the 5′ untranslated regions of the respective mRNAs. It appears likely that at least some of the abovementioned trans-acting factors are involved in these control circuits.

A. Determinants for the Efficiency of Chloroplast Transgene Expression in C. reinhardtii

Based on the extensive knowledge on the control of endogenous chloroplast gene

expression in *C. reinhardtii*, several transgenic lines have been generated which express foreign reporter genes like *gfp* and *uidA* (encoding the green fluorescent protein and the β-D-glucuronidase, respectively) in the chloroplast under the control of plastid regulatory elements. In a combinatorial approach, several 5′ and 3′ flanking regions from algal chloroplast protein-coding genes were tested (Ishikura et al. 1999; Barnes et al. 2005; Fletcher et al. 2007). Generally, the 5′ regions from the *atpA* and *psbD* genes were found to confer the highest expression rates on transgenes whereas different 3′ regions had only minor impacts. More recently, the *psaA*-exon1 5′ UTR was added to the list of tested regulatory regions and found to confer the highest expression rates amongst the known 5′ regions (Michelet et al. 2010). Nevertheless, all transgene expression rates were found to be significantly lower than those of the highly abundant endogenous algal chloroplast proteins. Moreover, transgene expression in *C. reinhardtii* is usually an order of magnitude lower than that in chloroplasts of higher plants (see Chap. 18). In a systematic evaluation, Surzcycki et al. (2009) defined four main determinants which affect chloroplast transgene expression in *C. reinhardtii*. These include (1) codon optimization, (2) protein toxicity, (3) protease activity and (4) genotypic modifications. Recently, this list was extended by Coragliotti et al. (2010), who showed that translation of recombinant mRNA molecules (5) also affects the accumulation of heterologous proteins.

1. Codon Optimization: Initial work on *C. reinhardtii* had shown that adaption of the codon-usage of a transplastomic *gfp* gene increases the accumulation of its product ca. 80-fold (Franklin et al. 2002). Since then, chloroplast transgenes are routinely designed according to the CAI (Codon Adaption Index) which provides a quantitative method for the prediction of protein expression levels. While codon optimization is usually calculated against a chloroplast codon usage which is derived from a list of all chloroplast genes, Surzcycki et al. (2009) pointed out that it is important to include only highly expressed chloroplast genes into the reference list. When following this rule, the authors obtained the highest expression level for a foreign protein in *C. reinhardtii* chloroplasts, i.e., the VP28 protein of the white spot syndrome virus accumulated to 21% of total cellular protein (TCP).
2. Protein toxicity: Another severe problem of algal transgene expression in chloroplasts is the toxicity of some foreign proteins which per se is not predictable (Surzcycki et al. 2009). However, like in other systems, inducible gene expression systems could solve this problem by expressing the toxic protein only after an appropriate biomass of transgenic lines has been generated (Koop et al. 2007). In *C. reinhardtii*, the *psbA* 5′ region mediates light-dependent regulation of D1 synthesis and, thus, can be used for a controlled onset of the translation of recombinant mRNAs via light (Barnes et al. 2005; Mayfield et al. 2007). However, even in the dark, the *psbA* 5′ region promotes a substantial level of basic gene expression which could lead to accumulation of toxic proteins. A similar, incomplete repression of transgene expression was observed when an artificial *lac* regulation system from *E. coli* was introduced into the chloroplast *rbcL* promoter region of *C. reinhardtii* (Kato et al. 2007).

   A tighter induction system has recently been developed based on the nucleus-encoded Nac2 factor controlling the stabilization of the chloroplast *psbD* mRNA encoding the D2 protein of photosystem II (Boudreau et al. 2000; Surzcycki et al. 2007). Nac2 has been shown to exert its function via the *psbD* 5′ UTR in a concerted manner together with the translational regulator RBP40 (Ossenbühl and Nickelsen 2000; Klinkert et al. 2006; Schwarz et al. 2007). As depicted in Fig. 16.1a, the Nac2 gene has been placed under the control of the copper-sensitive cytochrome c_6 promoter in the nuclear genome of *C. reinhardtii* (Surzcycki et al. 2007). Therefore, the expression of any gene via the *psbD* 5′ UTR is strictly copper-regulated, i.e., under copper-depleted conditions, Nac2 and, consequently, recombinant proteins accumulate while copper-repleted

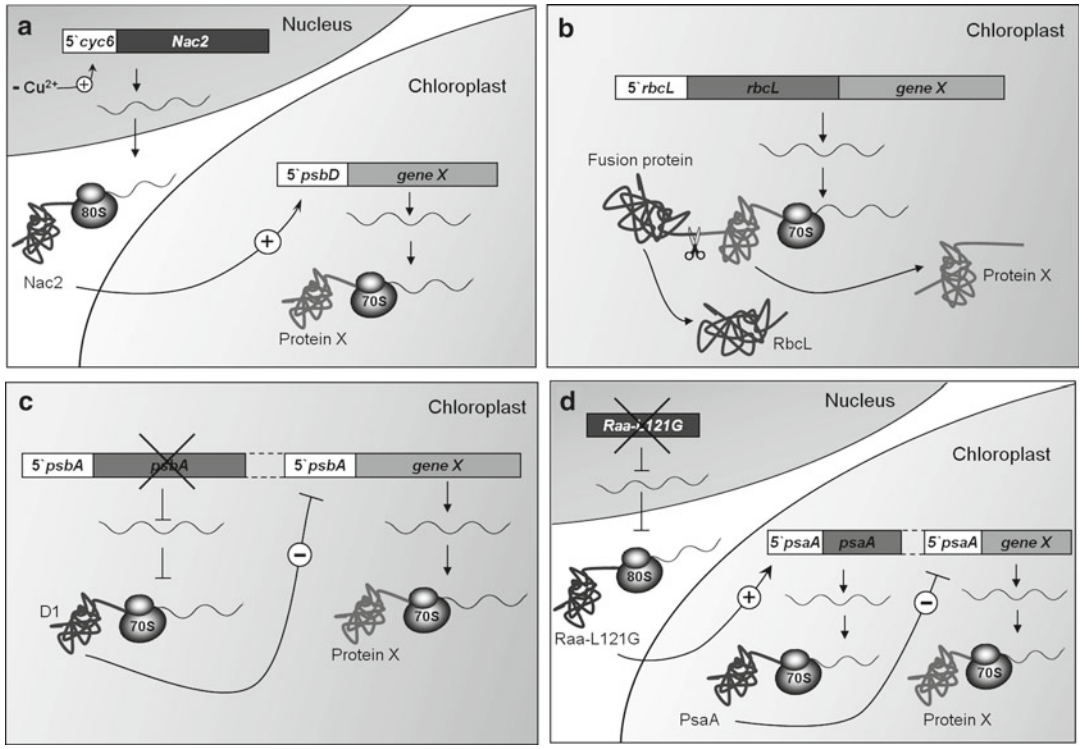

Fig. 16.1. Optimization of foreign gene expression in algal chloroplasts. (**a**) Chloroplast transgene expression is induced by copper depletion-induced expression of the nucleus-encoded Nac2 factor, which is required for stabilization of the transgene mRNA via its *psbD*-derived 5′ UTR (Surzcycki et al. 2007). (**b**) Combination of a foreign gene and the endogenous *rbcL* gene leads to the synthesis of a fusion protein which is processed to yield active RbcL and the recombinant protein (Muto et al. 2009). (**c**) Inactivation of the endogenous chloroplast gene enhances transgene expression driven by the same 5′ UTR due to inactivated negative feedback loops (Manuell et al. 2007). (**d**) Inactivation of negative feedback control by a nucleus-encoded mutation in a gene which is required for the expression of the plastid gene providing the 5′ UTR for transgene expression (Michelet et al. 2010). For further explanations, see text.

medium results in the complete loss of expression from the *psbD* 5′ UTR. To bypass the requirement of endogenous *psbD* gene expression for Nac2 function, the 5′ UTR of the chloroplast-encoded *psbD* gene was replaced with that of the *petA* 5′ UTR. This manipulation then allowed to synthesize PS II and, thus, enabled photoautotrophic growth for optimal biomass production in a Nac2-independent manner in the presence of copper (Surzcycki et al. 2007, 2009). By using this inducible system, the synthesis and accumulation of the otherwise toxic growth promoter DILP-2 in chloroplasts of *C. reinhardtii* was achieved indicating the viability of the system for biotechnological applications (Surzcycki et al. 2009).

3. Protease activity: The stability of recombinant proteins within the chloroplast is an obvious yield-affecting parameter (Mayfield et al. 2007). Nevertheless, relatively limited data are available on the influence of protein degradation on net accumulation of recombinant chloroplast proteins in alga. Recently, a first evaluation revealed a threefold higher recombinant protein stability in *C. reinhardtii* cells which had been treated with the energy uncoupler cyanide m-chlorophenylhydrazone (CCCP) as compared to those which had not

been treated (Surzcycki et al. 2009). This suggests that ATP-dependent proteolytic activities can significantly diminish the levels of foreign proteins in chloroplasts of *C. reinhardtii*. Another recently applied strategy to enhance recombinant protein accumulation in algal chloroplasts – probably due to protein stabilization effects –is the translational fusion of the foreign protein to the large subunit of the Rubisco enzyme, RbcL. Muto et al. (2009) achieved a 33-fold increase of luciferase activity when it was expressed together with RbcL and posttranslationally liberated from the fusion protein via an artificially introduced protease cleavage site from pre-ferredoxin (Fig. 16.1b).

4. Genotypic background/modification: The genetic background of recipient strains for chloroplast genetic manipulation has a significant impact on transgene expression rates. When a *C. reinhardtii* chloroplast transgene is expressed via the *psbA* 5′ region, a tenfold increase in accumulation of recombinant protein is observed in strains lacking the endogenous *psbA* gene (Fig. 16.1c). This phenomenon was attributed to less competition for trans-acting activators of translation and/or less negative feedback control by the abovementioned CES system (Manuell et al. 2007; Rasala et al. 2010; Minai et al. 2006). A similar enhancement of transgene expression driven by the *rbcL* 5′ region has been observed upon deletion of the endogenous *rbcL* gene suggesting that deletion of endogenous chloroplast regulatory regions represents a fruitful general strategy for transplastomic algal biotechnology.

   Besides the chloroplast genome also the nuclear genome has a significant impact on chloroplast gene expression via the abovementioned trans-acting regulatory factors. Michelet et al. (2010) recently showed that transgene expression driven by the *psaA* 5′ region is enhanced in a nuclear mutant background of a factor involved in splicing of the *psaA* mRNA. This phenomenon is likely to be due to two effects, i.e., increased RNA accumulation in the splicing deficient mutant background and the bypass of negative feedback loops caused by unassembled PsaA protein (Fig. 16.1d). However, one major drawback of this approach is the non-photosynthetic phenotype of the producing strain which limits photoautotrophic growth rates.

5. Translation: Recently, the role of translational activity on recombinant chloroplast mRNA templates was analysed in detail. The results suggest that protein synthesis on the level of ribosome association and even more important during translation elongation has severe impacts on heterologous protein accumulation (Coragliotti et al. 2010). Taken together, further understanding of the regulatory principles of chloroplast gene expression will clearly help to optimize biotechnological recombinant protein production strategies.

B. Expressed Transgenes

By using the abovementioned strategies for chloroplast transgene expression in *C. reinhardtii* several recombinant proteins have been produced to date. Early attempts mainly focussed on the expression of marker and reporter genes including the *aadA*, *uidA*, luciferase and *gfp* genes (for a review see Koop et al. 2007). In addition, the *E. coli* RecA protein as well as allophycocyanin from *Spirulina maxima* were successfully expressed in *C. reinhardtii* chloroplasts (Cerutti et al. 1995; Su et al. 2005). The first chloroplast-expressed proteins of pharmaceutical relevance were a human large single-chain antibody, a fusion protein of cholera toxin B subunit and foot and mouth disease VP1 protein (Mayfield et al. 2003; Sun et al. 2003). Since then, several other vaccines and therapeutics have been produced in transplastomic *C. reinhardtii* cells including, for instance, a correctly assembled human monoclonal antibody (Surzcycki et al. 2009; Dreesen et al. 2010; Rasala et al. 2010; Tran et al. 2009; for a comprehensive overview see Specht et al. (2010) and further references therein). Taken together, the available data clearly demonstrate that algal chloroplasts can provide an efficient platform for the production of high value recombinant proteins for human and animal biopharmaceuticals.

VI. Future Perspectives

Recent advances in the genetic manipulation of the chloroplast genome of *C. reinhardtii* promise a bright future for algal biotechnology. The development of a complete molecular toolkit basically allows any alteration of interest to be introduced into the chloroplast genome. Especially, foreign gene expression has been substantially optimized by following various approaches like codon optimization of foreign genes, use of inducible systems, defining optimal genetic backgrounds for gene expression cassettes and stabilization of recombinant proteins via translational fusions to endogenous chloroplast proteins. It is foreseeable, that on-going systematic evaluation of combinations of these and new strategies will further increase the yields of recombinant therapeutic production in chloroplasts into the range of economic sustainability.

The development of transformation protocols for other algae than *C. reinhardtii*, including those harbouring complex plastids, remains a major challenge for algal chloroplast transformation. Especially, some ecologically and/or economically highly relevant groups, like diatoms or brown algae, should be major targets of research efforts in that direction.

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