Chapter 11

Perspective for the Use of Genetic Transformants in Order to Enhance the Synthesis of the Desired Metabolites: Engineering Chloroplasts of Microalgae for the Production of Bioactive Compounds

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Abstract

Eukaryotic microalgae have recently gained particular interest as bioreactors because they provide attractive alternatives to bacterial, yeast, plant and other cell-based systems currently in use. Over the last years there has been considerable progress in genetic engineering technologies for algae. Biotechnology companies start to apply these techniques to alter metabolic pathways and express valuable compounds in different cell compartments. In particular, the eukaryotic unicellular alga *Chlamydomonas reinhardtii* appears to be a most promising cell factory since high amounts of foreign proteins have been expressed in its chloroplast compartment. For this alga the complete nuclear, plastidal and mitochondrial genome sequences have been determined and databases are available for any searching or cloning requirements. Apart from being easily transformable, stable transgenic strains and production volumes in full containment can be obtained within a relatively short time. Furthermore, *C. reinhardtii* is a green alga which belongs to the category of organisms generally recognized as safe (GRAS status). Thus, enhancing food with edible algae like *Chlamydomonas* engineered to (over)produce functional ingredients has the potential to become an important factor in food and feed technologies.

Introduction

Genetic engineering of plants and algae by introducing and controlling foreign genes has been developed to an extent that not only allows pest protection or herbicide tolerance but also provides ample opportunities to improve yields and nutritional contents and to exploit these organisms as bioreactors for the production of high-value compounds. Although initially most efforts have been concentrated on the manipulation of higher plant systems, photosynthetic microalgae have recently gained special attention because a variety of molecular tools including new transformation methods and complete genome sequences are now available for constructing recombinant strains.[1] As compared to higher plants, the use of microalgae as green cell factories assures a significantly faster generation of stable transgenic lines and is often coupled with high yields, lower

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costs and rapid, controllable growth in simple media. Microalgae are not hosts to major pathogens and recombinant strains can be grown in full containment, excluding the risk of contaminating natural populations. Moreover, methods exists which allow to construct transgenic algae without employing antibiotic resistance genes[2-4] thus minimizing any consumer-based concerns regarding the transfer of marker genes from a food product into the cells of the body.

Microalgae have long been used as food or food additives. They comprise a diverse group of prokaryotic and eukaryotic organisms and represent an almost untouched source of foodstuffs, industrial chemicals, therapeutic compounds and even renewable energy in the form of hydrogen gas. For human nutrition, edible microalgae like *Arthrospira* species ("Spirulina") and *Chlorella* are marketed as tablets, capsules and liquid or added to e.g., noodles, breads and candies to improve their nutritive and health values. Other major commercial strains used as food ingredients are *Dunaliella* and *Aphanizomenon flos-aquae*, whose extracts exhibit health promoting effects.[5] The acceptance of new microalga strains by the European Commission for Food Safety has recently been demonstrated with the marine microalga *Odontella aurita* certified in 2002 as novel food by the French company INNOVALG.

While the exploitation of products derived from natural microalgae has a long history, the generation of transgenic microalgae for biotechnological applications has just started to become an attractive system for expressing foreign proteins or other high-value compounds with e.g., antioxidant, colorant, provitamin or therapeutic properties. However, no microalgae are available on the market as genetically modified organisms so far, partly because of public acceptance issues, but also because progress in genetic engineering of microalgae initially was slow. This sluggish development was largely due to the time needed for the development of new transformation techniques, search for suitable promoters or selectable marker genes and often adjustments of reading frames to an unusual codon usage.

Microalgae as Transgenic Bioreactors

An obvious prerequisite for genetic engineering of plants and algae is the ability to transform these organisms with the gene(s) of interest. In the early 1980s there was no method available for generating transformed plants. However, this situation changed significantly when *Agrobacterium*-mediated and direct, i.e., biolistic or PEG-mediated DNA-transfer techniques were established.[6] While *Agrobacterium*-mediated transformation is now the standard method for nuclear transformation, the biolistic approach using accelerated particles (particle gun) turned out to be the preferential procedure for organell transformation. Sophisticated protocols developed along with these techniques now allow transforming all three different plant genomes residing in the nucleus as well as in plastids and mitochondria. The pioneer organism for which all the above mentioned problems were solved first was the unicellular green alga *Chlamydomonas reinhardtii*. This alga has a long history as a powerful model system for diverse areas of plant and also animal research.[7-9] The importance of this small organism like "a plant" has now been very much increased by the development of an extensive molecular toolkit and a draft of the complete genome sequence (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html).[10] Such comprehensive resources are not yet available for other microalgae.

Genetic Engineering of Plant and Microalgal Chloroplasts

The organellar genomes, specifically those of plastids, are particularly attractive for genetic engineering purposes. It is generally agreed that plastids are the result of an endosymbiotic event between a eukaryotic host cell and an ancestor of the cyanobacteria[11] and thus have prokaryotic features. Plastids come in various forms and functions, the most common of which are the photosynthetically active chloroplasts supplying the organism with energy and carbohydrates. Transcription and translation rates in chloroplasts are generally high in order to produce large amounts of the enzyme ribulose bisphosphate carboxylase and to allow a rapid turnover of electron transfer components like the D1 subunit of photosystem II; this core photosynthetic protein gets damaged by reactive oxygen species and must be resynthesized constantly for ensuring uninterrupted electron

flow.[12] Furthermore, chloroplasts are also able to synthesize various plant compounds like amino acids, fatty acids, lipids, plant hormones, nucleotides, vitamins and secondary metabolites. Thus, chloroplasts offer ample opportunities to use this organelle for the expression of foreign proteins and for engineering metabolic pathways.

Not only do chloroplasts naturally produce high amounts of protein, but they are also uniparentally inherited and integrate properly flanked foreign genes via homologous recombination into their plastome. As opposed to plant nuclear genomes, these properties provide distinct advantages of chloroplast transgenes with respect to biosafety and epigenetic stability.[13] Genetic engineering of chloroplasts has made particular progress with genes conferring agronomically valuable traits like e.g., resistance to herbicides,[14,15] to fungal and bacterial deseases[16] or to insects.[17,18] Chloroplasts have also been employed to overproduce biopharmaceuticals like somatotropin or human serum albumin, resulting in an overaccumulation of up to 7% and 11% of total soluble protein in tobacco plants.[19,20] An exceptionally high expression level of up to 45% of the total soluble protein has been obtained by expressing the *Bacillus thuringiensis cry* operon in tobacco plastids, resulting in the formation of protein crystals inside the chloroplast.[21] Furthermore, the engineering of new and also complex metabolic pathways has been demonstrated recently.[22-25]

Chloroplast genetic engineering is currently most advanced in higher plants, particularly in tobacco.[13] Also chloroplasts of edible plants like tomato and some other food crops have been stably transformed.[26-32] While higher plants offer several advantages over expression systems in other organisms, there are distinct drawbacks like e.g., the length of time necessary for their generation or concerns about containment of transgenic plants in the environment even in transplastomic lines.[33-35] Here the use of microalgae like *C. reinhardtii* provides interesting alternatives: from vector construction to lab-scale culture volumes of the transgenic strain it takes about 6 weeks (Fig. 1) and cells can be easily contained in photobioreactors.

C. reinhardtii was the first organism for which stable chloroplast transformation was reported.[36] Boynton and coworkers used a new technique employing cell bombardment with DNA-coated tungsten particles[37] and succeeded in the restoration of photosynthetic growth of an *atpB* deletion mutant of *C. reinhardtii*. While early selection methods used cloned chloroplast genes to rescue photosynthetic mutants or chloroplast gene constructs that confer resistance to herbicides[38] or antibiotics,[39] later on transformant selection was mainly based on the expression of bacterial markers like the *aadA* gene conferring spectinomycin and streptomycin resistance[40] or the *aphA-6* marker conferring kanamycin or amikacin resistance.[41] During preparation of this manuscript there were only two reports available describing stable chloroplast transformation in eukaryotic algae other than *Chlamydomonas*: the unicellular red algae *Porhyridium spp.* was transformed using a mutant

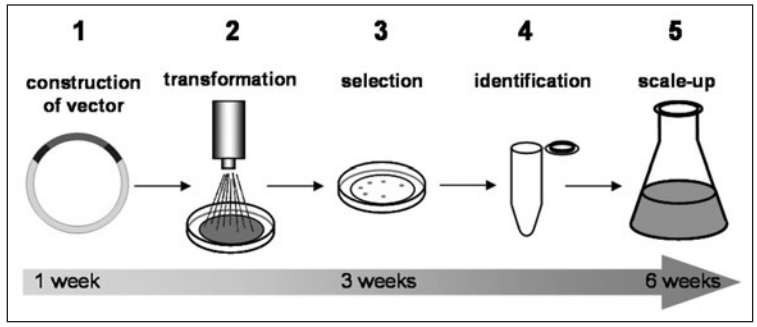

Figure 1. Timeline for the production of foreign proteins in *C. reinhardtii* chloroplasts. 1) Ligation of transgene into a vector containing chloroplast DNA sequences for homologous recombination. 2) Particle gun transformation of vector DNA into cells immobilized on filters. 3) Growth of transformants on selective media. 4) Screening for transgene insertion and protein expression. 5) Cultivation of transformants in larger volumes of liquid media.

form of the gene encoding acetohydroxyacid synthase as a dominant selectable marker[42] and the unicellular protist *Euglena gracilis* using an *aadA* cassette, which persisted in the chloroplast as episomal element without integration into the chloroplast genome.[43]

Expression of Recombinant Proteins in *C. reinhardtii* Chloroplasts

Recent progress in engineering *C. reinhardtii* chloroplasts is the result of 20 years research on developing transformation techniques, improving codon usage and finding efficient promotors and untranslated regions (UTRs) for boosting foreign gene expression. Initially, stable recombinant mRNA accumulation without protein accumulation could be detected in chloroplasts.[44,45] Expression of the *aadA* gene represented the first example of stable, but low foreign protein accumulation as judged only from the appearance of enzymatic activity.[40] Later on the *uidA* and *rluc* genes coding for β-glucuronidase and Renilla luciferase were successfully expressed in *C. reinhardtii* chloroplasts, resulting in correctly folded proteins with the desired reporter properties.[46,47] Although foreign protein expression in these studies was low, the expected products could be detected by Western blot analysis. So far all expressed genes were used as derived from their natural sources without adjusting their codon usage for an optimized expression in *C. reinhardtii* chloroplasts. The importance of codon optimization for enhanced protein production was demonstrated in 2002.[48] In this study it was shown that codon optimization of the *gfp* gene results in a 80-fold increase in green fluorescent protein (GFP) accumulation as compared with the a non-optimized version. Increased protein production could be also observed for other codon-optimized genes, underlining the importance of codon adjustment for high level protein production in plastids.[49,50]

Apart from optimal codon adjustment other factors are known to be crucial for gene expression. The 5′- and 3′-UTR´s are important for mRNA stability[51,52] and transcriptional efficiency is regulated by both chloroplast gene promoters and internal sequences of the 5′-UTR.[53] Containing bacterial like −10 and −35 elements most promoters found in *C. reinhardtii* chloroplast genes resemble bacterial sigma-70-type promoters.[54] More than the transcriptional the translational machinery is limiting chloroplast gene expression.[55] In this context the particular relevance of the 5′-UTR could be demonstrated, whereas the 3′-UTR plays a comparatively smaller role in foreign protein production.[56]

Today several transgenes have been successfully expressed in *C. reinhardtii* chloroplasts (Table 1). Although most of them are intended to be used as reporters and thus serve basic research purposes, now several proteins for pharmaceutical applications appear in the list. A latest and remarkable addition to the record is the expression of a bioactive mammalian protein, whose level is estimated to be above 5% of total cellular protein.[57] In the near future it appears possible that such transgenic Chlamydomonas strains could be ingested orally without the need for extensive purification of the bioactive compound. This perspective is supported by experiments, in which Chlamydomonas cells expressing an epitope of a pathogenic bacterium infecting salmonids were fed to trout. An immune response was observed when transgenic cells were added to the fish food (Patent application US020030022359).[58]

Application to Food Technology

In light of the recent progress in chloroplast genetic engineering described above it seems promising to develop Chlamydomonas-based expression systems to obtain products enriched with proteins or peptides of specific function that could be used e.g., as nutraceutical additives. This may provide a cost-effective means, especially when the whole organism, or a partially processed form of it, can be used as food ingredient without involving costly and complex isolation and purification procedures. As an example, physiologically active peptides derived from plant and animal proteins represent potential health enhancing components for food applications.[70-72] In vitro or in vivo hydrolysis of proteins from these sources and subsequent analyses of peptide fragments for bioactivity show that certain peptide fragments exert a multitude of health effects like antioxidant or antithrombotic activities, cholesterol-lowering abilities or antimicrobial properties, to mention only a few. Bioactive peptides

Table 1. Foreign proteins expressed in* C. reinhardtii *chloroplasts

Year	Proteins	Comments	Refs
1991	Aminoglycoside adenine transferase	Reporter activity, spectinomycin and streptomycin resistance	40
1999	β-Glucuronidase	Reporter activity, conversion of substrates to colored products	46
1999	Renilla luciferase	Reporter activity, luminescence activity	47
2000	Aminoglycoside phosphotransferase	Reporter activity, kanamycin and amikacin resistance	41
2002	Green fluorescent protein	Reporter protein, fluorescent	48
2003	HSV8-lsc	Pharmaceutical activity, first mammalian protein expressed	49
2003	Geranylgeranyl Pyrophosphate Synthase	Prenyltransferase, key enzyme in plant terpenoid biosynthesis	59
2003	Cholera toxin B subunit fused to foot and mouth disease VP1	Pharmaceutical activity, vaccine	60
2004	Bacterial luciferase	Real-time reporter activity, bioluminescence activity	50
2005	HSV8-scFv	Pharmaceutical activity, classic single-chain antibody	61
2005	Allophycocyanin	Fluorescent protein	62
2006	Human metallothionine-2	Pharmaceutical activity, UV protection	63
2006	Firefly luciferase	Real-time reporter activity, bioluminescence activity	64
2006	Human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)	Pharmaceutical activity	65
2007	Lac repressor	Repressor activity, control of transgene expression	66
2007	Classical swine fever virus (CSFV) structural protein E2	Vaccine, immune response activity in animals	67
2008	Human glutamic acid decarboxylase 65 (hGAD65)	Pharmaceutical activity, first full-length autoantigenic protein expressed	68
2008	metallothionein-like gene from *Festuca rubra*	Metal binding protein, heavy metal tolerance induction	69

are already commercially available in functional foods or as food ingredients as e.g., sold by DMV ("C12 Peption" with a Casein-derived dodecapeptide FFVAPFPEVFGK) or Ingredia ("ProDiet F200" with peptide YLGYLEQLLR). A detailed compilation of bioactive peptides in commercial products is given in a recent review by Hartmann and Meisel.[72]

The practicability of expressing bioactive peptides in chloroplasts has already been demonstrated in a study employing the antimicrobial peptide MSI-99.[73] This 22-amino-acid-long peptide is an analog of magainin, which has been isolated from skin secretions of the African clawed frog *Xenopus laevis*. Its activity is thought to arise from the ability to form pores in membranes with negatively charged phospholipid headgroups. This effect is not observed with membranes of plants and animals, which

have no net charge in their outer membrane leaflets.[74] Magainin is effective against diverse bacteria, fungi and protozoa and has anticancer activity.[75] Transgenic tobacco plants expressing a magainin analog in their chloroplasts were indeed protected against different plant pathogens, indicating that in plastids a sufficiently stable peptide can be produced and retains its function, although it is located in a foreign environment. If microalgae like *Chlamydomonas reinhardtii* would express the preferred bioactive peptides in adequate quantities, they could be extremely useful as additives in food industries.

Conclusion

Microalgae have long been used as nutritional supplement or food and feed sources. Recent progress in genetic engineering technologies make eukaryotic microalgae efficient bioreactors for the production of various bioactive compounds. The green alga Chlamydomonas reinhardtii serves as a pioneering model organism because its complete genome sequence is known, transformation procedures are established and extensive molecular toolkits are available. Specifically the chloroplast compartment as a subcellular bioreactor provides distinct advantages with respect to biosafety as well as epigenetic stability and offers new opportunities for the expression of foreign proteins and for engineering metabolic pathways.

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