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Expression and membrane-targeting of an active plant cytochrome P450 in the chloroplast of the green alga *Chlamydomonas reinhardtii*

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ABSTRACT

The unicellular green alga *Chlamydomonas reinhardtii* has potential as a cell factory for the production of recombinant proteins and other compounds, but mainstream adoption has been hindered by a scarcity of genetic tools and a need to identify products that can be generated in a cost-effective manner. A promising strategy is to use algal chloroplasts as a site for synthesis of high value bioactive compounds such as diterpenoids since these are derived from metabolic building blocks that occur naturally within the organelle. However, synthesis of these complex plant metabolites requires the introduction of membrane-associated enzymes including cytochrome P450 enzymes (P450s). Here, we show that a gene (*CYP79A1*) encoding a model P450 can be introduced into the *C. reinhardtii* chloroplast genome using a simple transformation system. The gene is stably expressed and the P450 is efficiently targeted into chloroplast membranes by means of its endogenous N-terminal anchor domain, where it is active and accounts for 0.4% of total cell protein. These results provide proof of concept for the introduction of diterpenoid synthesis pathways into the chloroplast of *C. reinhardtii*.

1. Introduction

The green alga *Chlamydomonas reinhardtii* has served as an important model organism for studies on photosynthesis, chloroplast biology and cell physiology. However, this freshwater alga has also evoked interest as a production platform for recombinant proteins and other products; heterologous genes can be expressed in both the nuclear and chloroplast genomes (Purton et al., 2013; Rasala et al., 2014), and the organism grows rapidly and is relatively inexpensive to culture. These factors raise the possibility that cultures can be grown in large-scale photobioreactors, which significantly reduces the risk of contamination and the escape of genetic modified strains to the environment, and makes rapid scale-up possible. In addition, green algae fall into the GRAS (generally regarded as safe) category, potentially eliminating some downstream processing steps associated with transgenically produced therapeutics (Rasala and Mayfield, 2011; Specht et al., 2010).

The chloroplast of *C. reinhardtii* has been a preferred site for the expression of commercially attractive products since it offers a number of advantages compared to the transformation of the nuclear genome. In the chloroplast, high gene expression levels can be achieved and it is possible to target transgenes to defined sites using homologous recombination. In contrast, genes introduced into the nuclear genome integrate randomly and are therefore prone to position effects and RNA silencing (Day and Goldschmidt-Clermont, 2011).

In recent years several advances have been made in the field of *Chlamydomonas* chloroplast transformation (Purton et al., 2013). Nevertheless, most transformation protocols still rely on antibiotic resistance for selection. A commonly used selectable marker is the *aadA* gene (bacterial aminoglycoside 3″-adenyl transferase), which confers resistance to spectinomycin and streptomycin (Goldschmidt-Clermont, 1991). Alternatively, cloned variants of the endogenous ribosomal RNA genes *rrnS* and *rrnL* can be used as markers. These carry point mutations rendering the ribosomes insensitive to certain damaging antibiotics (Newman et al., 1990). However, such antibiotic selection is prone to high rates of false positives, and in the latter case results in a mutant ribosome in which translation of a highly expressed transgene is potentially compromised. Furthermore, ensuring that transgenic lines are homoplasmic (i.e., that all ~80 copies of the polyploid chloroplast genome carry the introduced transgene) requires multiple rounds of single colony isolation under antibiotic selection. A more elegant method is to rely on the restoration of photosynthetic

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growth using a *Chlamydomonas* chloroplast mutant defective in an essential photosynthesis gene such as *atpB*, *or psbH* (Boynton et al., 1988; Economou et al., 2014). Importantly, the gene of interest remains the only segment of foreign DNA in the transformed genome. A similar strategy has been described earlier by Cheng et al. (2005).

To-date, over 50 different recombinant proteins have been synthesised successfully in the *C. reinhardtii* chloroplast, including markers, reporters, enzymes, and proteins of therapeutic value such as antibodies, hormones and vaccines (Purton et al., 2013). A fully functional human IgG1 monoclonal antibody against anthrax protective antigen 83 was expressed from two separate genes and assembled into a fully active antibody (Tran et al., 2009). In addition, immunotoxins for cancer therapeutics (Tran et al., 2013) as well as vaccine candidates against malaria or foot-and-mouth-disease virus have been produced in the chloroplast (Gregory et al., 2013; Jones et al., 2013). However, most of the recombinant proteins produced, have been soluble proteins, and furthermore have generally been proteins that can be considered 'benign' in that they have no effect on chloroplast metabolism. To-date there has been only a few reported attempts to express membrane-associated proteins, or enzymes that introduce novel metabolic pathways (Blatti et al., 2012; Wu et al., 2010).

As a consequence, *C. reinhardtii* has yet to compete effectively with other, heterotrophic production hosts for the production of recombinant biopharmaceuticals. Here, we have taken the approach that algae may be preferentially suited for the production of plant-specific compounds. One such class of compounds is terpenoids. These are large, structurally-complex plant metabolites that include a range of very high-value examples including paclitaxel, artemisinin and ingenol-3-angelate (Wang et al., 2005). Importantly, their complex structures almost invariably mean that efficient chemical synthesis is extremely difficult, and usually impossible. In principle, it should be possible to introduce novel terpenoid biosynthesis pathways into the plant or algal chloroplast, but this would require re-targeting of the key enzymes in their synthesis, cytochromes P450, into the chloroplasts in an active form. Nielsen et al. (2013) have reported the successful synthesis of Dhurrin in tobacco chloroplasts and showed that synthesis is light-driven using native ferredoxin as the electron donor. Dhurrin is a plant defence compound found in *Sorghum bicolor*. While Dhurrin is not a terpenoid, it is synthesised from tyrosine by the means of two ER localised cytochrome P450 enzymes, CYP79A1 (Sibbesen et al., 1995), CYP71E1 (Kahn et al., 1997) and POR (NADPH cytochrome P450 oxidoreductase), as well as the soluble cytosolic UDP glucosyl transferase UGT85B1 (Jones et al., 1999).

As a first step in introducing such a pathway into a phototrophic microorganism suitable for industrial cultivation (Specht et al., 2010), we report the stable expression of the cytochrome P450 CYP79A1 in the chloroplast of *C. reinhardtii* using a simple transformation method that results in a transgenic line with the CYP79A1 synthetic gene as the only foreign DNA. The enzyme is targeted into the organelle membrane by means of its endogenous N-terminal region, accumulates to ~0.4% of total cellular protein and is shown to be active in the conversion of tyrosine to *p*-hydroxyphenylacetaldoxime. The work paves the way for the introduction of additional components of the Dhurrin pathway.

2. Results and discussion

2.1. Transformation strategy and construction of the chloroplast expression vector

The overall strategy for this study is shown in Fig. 1. In the ER, P450s are located in the ER membrane by means of an N-terminal transmembrane span; they drive reactions using reducing power from NADPH but the immediate electron donor is cytochrome P450 reductase (POR). While the chloroplast does contain imported P450s that are involved in carotenoid biosynthesis (Quinlan et al., 2012) the vast majority are located in the ER and we deemed it important to determine whether such proteins can be expressed in chloroplasts and targeted to membranes in an active form. In this study the aim was to express a model P450, CYP79A1, in *C. reinhardtii* chloroplasts, target the enzyme to the thylakoid membrane and drive the reaction using reduced ferredoxin (Fd). This has been shown to be possible in transiently transfected tobacco chloroplasts (Nielsen et al., 2013) and our aim was to determine whether algal chloroplasts are a viable production base for stable expression of these enzymes.

A recently-developed method for chloroplast transformation (Economou et al., 2014) uses a *C. reinhardtii* strain in which the chloroplast *psbH* gene has been replaced by the *aadA* antibiotic resistance cassette. The vector pASapI is used to introduce the gene of interest and restore photosynthetic growth. The expression cassette of pASapI is comprised of the promoter, the 5′ UTR and the start codon of *atpA*, a multiple cloning site, and the stop codon and 3′ UTR of *rbcL*. Upon homologous recombination the *aadA* cassette is replaced with both a functional copy of *psbH* and the gene

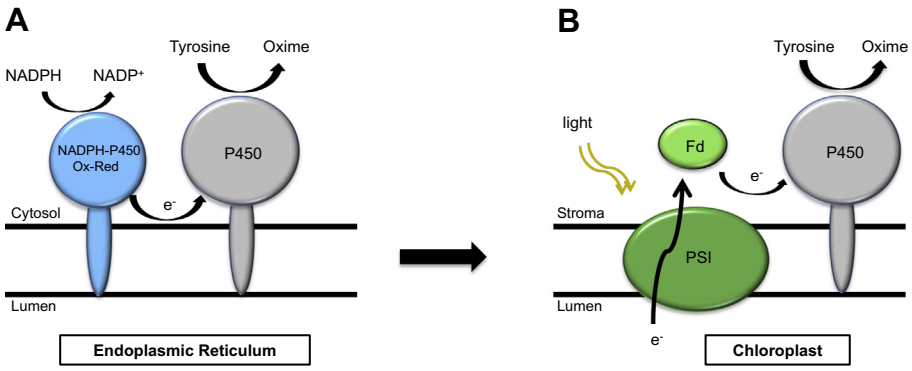

Fig. 1. Strategy for driving cytochrome P450 activity by photosynthetic electron transport. (Left) Normal action of P450s. Typical P450s have an N-terminal transmembrane span that anchors the protein in the ER membrane. Reducing power is provided by NADPH *via* NADPH-cytochrome P450 oxido-reductases (NADPH-P450 ox-red). (Right) Strategy for driving activity by photosynthetic electron transport: the P450 is synthesised in the chloroplast, targeted into the thylakoid membrane and reducing power is provided by Photosystem I (PSI) *via* ferredoxin (Fd).