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Stable expression of a bifunctional diterpene synthase in the chloroplast of *Chlamydomonas reinhardtii*

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Abstract *Chlamydomonas reinhardtii* has been shown to hold significant promise as a production platform for recombinant proteins, but transformation of the nuclear genome is still a non-trivial process due to random gene insertion and frequent silencing. Insertion of transgenes into the chloroplasts is an alternative strategy, and we report here the stable expression of a large (91 kDa) protein in the chloroplast using a recently developed low-cost transformation protocol. Moreover, selection of transformants is based on restoration of prototrophy using an endogenous gene (*psbH*) as the marker, thereby allowing the generation of transgenic lines without the use of antibiotic-resistance genes. Here, we have expressed a bifunctional diterpene synthase in *C. reinhardtii* chloroplasts. Homoplasmic transformants were obtained with the expressed enzyme accounting for 3.7 % of total soluble protein. The enzyme was purified to homogeneity and expression was shown to have a small but reproducible effect on growth rate at the end of log phase growth. These results demonstrate that large recombinant enzymes can be synthesised in the algal chloroplast, and serve to underline its potential as a platform for the biosynthesis of novel metabolites.

Keywords *Chlamydomonas* · Chlorophyta · Chloroplast transformation · Recombinant protein · Diterpene synthase · Glass bead · Endogenous marker

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Introduction

A number of reports have demonstrated the potential of microalgae as biotechnological platforms for recombinant protein production. *Chlamydomonas reinhardtii* has been a popular host strain, because genetic tools are relatively advanced, and recombinant protein targets have included vaccines, antibodies, hormones and immunotoxins (Demurtas et al. 2013; Gregory et al. 2012, 2013; Jones et al. 2013; Soria-Guerra et al. 2014; Tran et al. 2013a, b). However, attempts have also been made to further develop the platform for other applications, such as industrial enzymes (Rasala et al. 2012; Pourmir et al. 2013) and functional food supplements (Campos-Quevedo et al. 2013) or the production of biodegradable plastics (Wang et al. 2010) (reviewed in Purton et al. 2013; Rosales-Mendoza et al. 2012; Specht and Mayfield 2014).

Many such studies have relied on transformation of the nuclear genome for recombinant protein expression, but DNA insertion into the genome is essentially random. Therefore, levels of transgene expression are variable and unpredictable owing to position effects, and frequently unstable because of gene silencing (Rosales-Mendoza et al. 2012). Furthermore, for metabolic engineering strategies where recombinant enzymes are required to localise to the chloroplast, nuclear

transformation requires that cytoplasmically synthesised proteins are targeted into the chloroplast by means of a 'transit peptide'. Insertion of transgenes into the chloroplast genome is thus an attractive alternative approach, particularly since genes can be targeted to specific loci via homologous recombination and high-level and stable expression can be achieved without any evidence for gene silencing (reviewed in Purton et al. 2013; Specht and Mayfield 2014). Genes are typically introduced by particle bombardment and, although chloroplast transformation by agitation with glass beads was reported some time ago (Kindle et al. 1991), very few studies have used this method (Demurtas et al. 2013; Economou et al. 2014).

Recently, a novel method of chloroplast transformation was reported, which combines a rapid and low-cost glass bead-based transformation approach with a novel selection method. In this approach, insertion of the transgene into the chloroplast genome is accompanied by restoration of an intact chloroplast *psbH* gene, and selection is based on restored photoautotrophic growth. The procedure uses a cell-wall mutant recipient strain in which the chloroplast *psbH* gene is disrupted by an *aadA* antibiotic cassette and is incapable of photoautotrophic growth (Economou et al. 2014). The pASapI transformation vector bears a functional copy of *psbH* with the gene of interest inserted into an intergenic region immediately downstream, and integration into the chloroplast genome via homologous recombination simultaneously introduces both *psbH* and the gene of interest. Insertion of the transgene into the chloroplast genome is accompanied by restoration of an intact chloroplast *psbH* gene that encodes an essential subunit of photosystem II (O'Connor et al. 1998) with selection based on restored photoautotrophic growth. Importantly, the *aadA* cassette is removed during this process and is completely absent in transformants that have homoplasmic chloroplast DNA copies. This method thus represents a relatively simple transformation system, in which any gene of interest can be precisely inserted and expressed in a comparably short timeframe.

In this study, we report the expression of a synthetic gene encoding a bifunctional diterpene synthase of 91 kDa. Diterpene synthases are key enzymes in the synthesis of a wide range of terpenoids, many of which have been used for medical and other purposes, and in this study, we expressed *cis*-abienol synthase from Balsam fir. The enzyme catalyses the formation of *cis*-abienol from the abundant precursor molecule geranyl geranyl diphosphate (Zerbe et al. 2012) and *cis*-abienol has potential as a source for the synthesis of Ambrox, an important compound in the perfume industry. We show that the enzyme accumulates in the chloroplast stroma of transformed *Chlamydomonas* strains, accounting for approximately 3.7 % of total soluble protein, and expression has a minor but detectable effect on growth rate at the end of log phase growth. To our knowledge, this is the largest single protein produced to date in the *C. reinhardtii* chloroplast, and indicates that novel metabolic pathways using multi-functional enzymes could be engineered into the organelle.

Materials and methods

Plasmid construction

For plasmid construction, the vector pASapI was used (Economou et al. 2014). A synthetic *TPS4* gene from *Abies balsamea* was designed using the primary sequence reported for recombinant expression in *Escherichia coli* in which the mature sequence, lacking the plant chloroplast transit sequence, is predicted to start at residue 87 (GenBank accession No. AEL99953.1; Zerbe et al. 2012). An HA tag was added at the C-terminus to allow detection of the protein. The gene sequence was codon-optimised for expression in the *Chlamydomonas* chloroplast using the software 'Codon Usage Optimizer' (codonusageoptimizer.org/download) and synthesised de novo by GenScript (USA). The TPS4-HA sequence was cloned into the pASapI vector at the *Sap*I and *Sph*I sites. The plasmid construct, termed pJZ2a, was confirmed by sequencing using the primers atpA.F and rbcL.R (Economou et al. 2014), amplified in *E. coli* DH5α cells and used for *Chlamydomonas* chloroplast transformation.

Cultivation of *Chlamydomonas* strains and transformation

The *C. reinhardtii* strain CC-125 (mt+), used as a wild-type control, was kindly provided by Alison Smith (University of Cambridge, UK). The *C. reinhardtii* strain TN72 (cw15, *psbH*::*aadA*, mt+) used for transformation was created in the Purton lab. All strains were cultivated at 25 °C with approx. 50 μmol photons m^{-2} s^{-1} continuous illumination on agar plates. Liquid cultures were incubated in a Multitron Pro Shaking Incubator (Infors Ltd.) at 110 rpm, 25 °C and approximately 15 μmol photons m^{-2} s^{-1}. Cultures were grown mixotrophically in tris-acetate-phosphate (TAP) medium (Gorman and Levine 1965) with a modified trace metal recipe (Kropat et al. 2011).

A glass bead transformation method as described by Economou et al. (2014) was used for transformation. Mid-log phase cells of the strain TN72 were transformed with the constructs pJZ2a or the pASapI vector lacking a gene insert. For selection of transformants, the minimal medium high salt medium (HSM) (Sueoka 1960) with a modified trace metal recipe (Kropat et al. 2011) was used. After re-streaking the transformant to single colonies six times on HSM, homoplasmy of the cell lines was confirmed by PCR. An overview of the transformation procedure is given in Online Resource 1.

DNA extraction and PCR analysis of transformants

Total genomic DNA of single colonies was extracted using a Chelex 100 Resin (Bio-Rad) using the protocol described in Economou et al. (2014). Homoplasmy was confirmed by PCR with three different primers using the principle described by Economou et al. (2014). For pASapI transformants, the primers used were:

(i) FLANK1 (GTCATTGCGAAAATACTGGTGC). Anneals downstream of the gene of interest (GOI) insertion site, just beyond the 0.8-kb region of homology carried on the pASapI vector
(ii) rbcL.F (CGGATGTAACTCAATCGGTAG). Anneals to the end of the *aadA* cassette inserted within the TN72 genome and gives a 0.85-kb product in conjunction with FLANK1 for the untransformed TN72 genome
(iii) atpA.R (ACGTCCACAGGCGTCGTAAGC). Anneals to the *atpA* promoter/5′UTR element-driving expression of the GOI and gives a 1.20-kb product in conjunction with FLANK1 for the transplastomic genome

A second PCR reaction was run to screen for the loss of the *aadA* cassette upon transformation using the primers atpA.F and rbcL.R (Economou et al. 2014). For all PCR reactions, a standard PCR protocol with Phusion Hot Start Flex Polymerase (New England Biolabs) was used. For the three primer PCRs, an annealing temperature of 65.2 °C was used. In the *aadA* cassette PCR with the primers atpA.F and rbcL.R, the annealing temperature was 62 °C.

Protein expression in *E. coli* and *Chlamydomonas*, SDS-PAGE and Western blot analysis

For the preparation of *Chlamydomonas* lysates, cells were lysed by sonication in lysis buffer (20 mM HEPES pH 7.2, 5 % glycerol, 20 μg mL^{-1} DNAse (Roche), 1/2 tablet EDTA-free protease inhibitor tablet (Roche) per 20 mL). Five equivalents of milligram chlorophyll per litre culture were separated on a 12 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti-HA-antibody (Sigma-Aldrich). The signal was detected with an ECL reagent (Biological Industries) and imaged using a ChemiDoc XRS+ System (Bio-Rad). For expression studies in *E. coli*, pASapI and pJZ2a were transformed into *E. coli* MC4100. The TPS4-HA protein was expressed overnight at 16 °C in TB medium based on a protocol described elsewhere (Keeling et al. 2008). For cell lysis, the *Chlamydomonas* buffer was used, additionally containing 0.1 mg mL^{-1} lysozyme (Sigma-Aldrich). The further procedure was identical as for the algal samples.

TPS4-HA purification

The TPS4-HA protein was purified from 5 L of late log phase *Chlamydomonas* TN72-TPS4 culture. Cells were harvested at 4000 rpm, 4 °C for 20 min in a Beckman Coulter Avanti J-26SXP centrifuge and pellets were resuspended in lysis buffer (see above). Lysates were sonicated and cleared by ultracentrifugation at 70,000 rpm, 4 °C and 30 min (Beckman TL-100 ultracentrifuge). The soluble supernatant was loaded onto five 5-mL Q-Sepharose columns (Q-Sepharose Fast Flow, GE Healthcare) which were washed in 20 mM HEPES pH 7.2, 5 % glycerol, 1 mM $MgCl_2$ and 5 mM dithiothreitol (DTT). The TPS4-HA protein was eluted using two column volumes of the same buffer containing 350 mM NaCl, which was specifically optimised for this protein. The elution fractions were concentrated using Vivaspin 20 columns (GE Healthcare) and subjected to affinity chromatography using 2 mL Pierce Anti-HA Agarose resin (Thermo Scientific) using the manufacturer's protocol. The TPS4-HA protein was eluted with an HA competitor peptide (Thermo Scientific), and purity was confirmed by SDS-PAGE and subsequent Oriole fluorescent gel (Bio-Rad) staining and immunoblotting.

Peptide mass fingerprinting: peptide preparation and analysis

The purified TPS4-HA protein was run on a SDS-PAGE. After Coomassie staining, the TPS4-HA band was excised and dissected into approximately 1 mm-sized pieces. After reduction of disulphide bonds with DTT and alkylation of cysteine residues with chloroacetamide, the sample was digested overnight with trypsin. Generated peptides were extracted and analysed by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The generated mass fingerprint was searched against the SWISS-PROT Database using the MASCOT search engine. The 15 most intense peptide masses were then subjected to further analysis by MS/MS and the obtained data searched against the same data base.

Protein quantitation

Ten millilitres of late log phase cultures of the TN72-TPS4 strain were harvested and lysed. The total soluble protein content of these samples was determined using the *DC* Protein Assay Kit (Bio-Rad) following the manufacturer's instructions. Bovine serum albumin (Bio-Rad) was used as a standard. The same lysate was subjected to SDS-PAGE and Western blot analysis. A recombinant human HA-tagged Ubiquitin protein (Boston Biochem) was loaded in known quantities as a calibration standard. Densitometric analysis was performed using ImageLab 4.1 software (Bio-Rad).

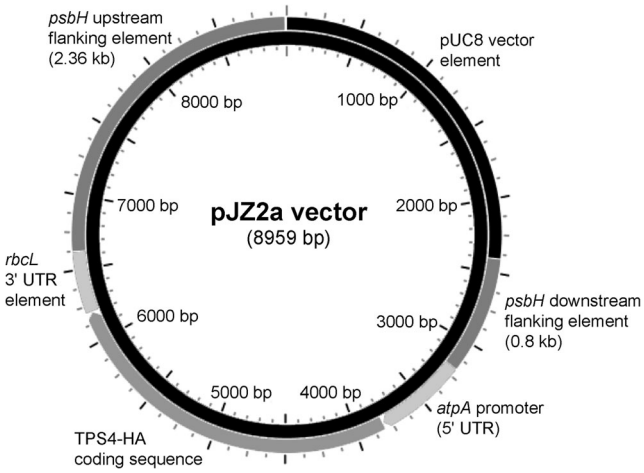

Fig. 1 Schematic map of the plasmid pJZ2a. The pJZ2a vector is based on the plasmid pASapI (Economou et al. 2014). A codon-optimised sequence of the abienol synthase gene *tps4* was cloned into the vector using the *Sap*I and *Sph*I sites. An HA tag coding sequence was appended at the 3′ end of the *tps4* gene. Successful transformation of *Chlamydomonas* is achieved upon homologous recombination of the *psbH* flanking regions. The heterologous gene is driven by the endogenous *atpA* promoter. The *psbH* upstream-flanking element has a functional copy of *psbH*. The pUC8 vector fragment allows replication of the plasmid in *E. coli*. This plasmid map was assembled using PlasMapper Version 2.0 (modified)

Results

Transformation strategy

A synthetic gene encoding the mature sequence of the bifunctional *cis*-abienol synthase TPS4 from the balsam fir tree, *A. balsamea*, was designed based on optimal codon usage in the *C. reinhardtii* chloroplast, and with the 3′ end of the coding sequence extended to encode the hemagglutinin epitope. The gene was inserted into the chloroplast expression vector pASapI, generating construct pJZ2a as shown in Fig. 1, with the *TPS4* gene driven by the chloroplast *atpA* promoter and 5′ untranslated region. A glass bead-based vortexing method was then used to transform the chloroplast genome of the photosynthetic mutant TN72 lacking an intact *psbH* gene as detailed in 'Materials and Methods' and illustrated in Online Resource 1. Following selection for photoautotrophic growth on minimal medium, a transformed line with restored photoautotrophic growth was obtained (termed TN72-TPS4). An additional strain with restored photoautotrophy, TN72-RP, was created for control purposes by transformation using the pASapI vector lacking any insert.

Insertion of the gene into the chloroplast genome, and the resulting loss of the *aadA* cassette, was confirmed by PCR analysis as shown in Fig. 2. Panel a shows PCR reactions carried out using primers that amplify a 1.1-kb region of the '*aadA* cassette' present in the genome of the TN72 recipient strain (Economou et al. 2014). This band is present in the TN72 sample but absent in the TN72-TPS4 transformant and TN72-RP restored wild type, suggesting that the cassette has been lost as a result of transformation. This is further confirmed in panel b where a combination of three PCR primers was used to test each strain for the presence of either type of genome. A primer to the downstream flanking region in combination with a primer to the *aadA* cassette in TN72 gives a 0.85-kb product, whereas the flanking primer together with one to the *atpA* promoter/5′UTR element linked to the gene of interest gives a 1.2-kb product. The results show that the 1.2-kb band is seen for the TN72-RP and TN72-TPS4 transformants, and the 0.85-kb band is seen for the host TN72 strain. Importantly, the absence of the 0.85-kb band in the transformants confirms that the chloroplast DNA copies are homoplasmic, such that all of the *psbH*::*aadA* copies have been replaced with a functional *psbH* together with the transgene cassette. No heteroplasmic cell lines were generated.

Expression of the TPS4 protein

TPS4 expression in the transformant TN72-TPS4 was detected by immunoblot analysis using an anti-HA antibody (Fig. 3). The 'TPS4' lane shows the presence of an approximately 80-kDa band, which is smaller than the predicted size

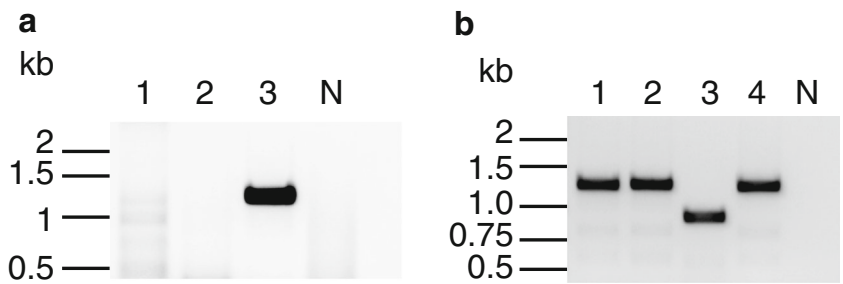

Fig. 2 Transformation results in homoplasmic insertion of the tps4 gene. Total DNA was extracted from TN72-TPS4 and TN72-RP cells and subjected to PCR analysis using primers that amplify a 1.1 kb region including the *aadA* cassette in the host strain (*3*), which was lost upon homologous recombination in the transformants TN72-TPS4 and TN72-RP (*1*, *2*) (**a**). **b** shows the amplification of a 1.2-kb region including the restored *psbH* gene in transformed copies of the chloroplast genome, whereas a 0.85-kb fragment is amplified in untransformed copies of the chloroplast genome. The figure shows PCR data for the TN72-TPS4 and TN72-RP transformants (*1*, *2*), the TN72 host strain (*3*), a positive control (*4*) and a control reaction using H_2O in place of DNA (*N*). Mobilities of the 0.75, 1.0 and 1.5 kb marker bands are indicated

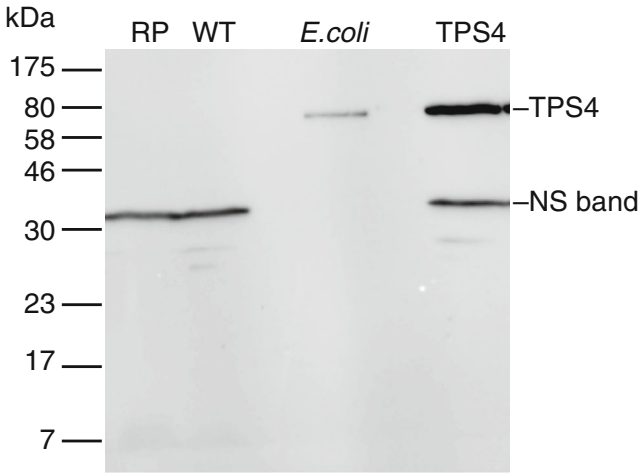

Fig. 3 Abienol synthase (*tps4*) is expressed in *Chlamydomonas reinhardtii* TN72-TPS4 cells. Extracts of *Chlamydomonas* cells, and of the *E. coli* strain expressing pJZ2a, were subjected to immunoblotting using antibodies to the C-terminal HA-tag. The blot shows data for TN72-RP strain (*RP*), the wild-type strain CC-125 (*WT*), *E. coli* host expressing pJZ2a (*E. coli*) and TN72-TPS4 transformant (*TPS4*). The TPS4-HA protein is indicated; *NS* non-specific reacting band. Mobilities of molecular mass markers (in kDa) are indicated on the *left*

of the TPS4-HA protein (around 91 kDa). A protein of the same size is also detected in the *E. coli* strain containing the pJZ2a vector ('*E. coli*' lane), strongly suggesting that the protein runs aberrantly on SDS gels. The protein is absent in samples from CC-125 wild-type cells (WT) or the control transformant generating restored photosynthetic competence (RP). The antibody does react with an endogenous protein of about 30 kDa in all *Chlamydomonas* samples tested, showing that this is a non-specific reaction.

To further confirm that the approximately 80-kDa protein does bear an HA tag, and is therefore TPS4-HA, we purified the protein as shown in Fig. 4. Soluble cell extracts were first subjected to ion exchange chromatography (Fig. 4a) and the panel shows an immunoblot of initial lysate (Lys), wash fractions (W) and the fraction eluted with 350 mM NaCl (E). The data show that the TPS4-HA protein is present in the elution fraction while the non-specifically reacting 30-kDa protein is present in the flow-through and wash fractions. The 350 mM NaCl eluate was then applied to an HA-affinity column (Fig. 4b), and the panel shows an Oriole-stained gel of the flow-through and wash samples, with the purified TPS-HA present in the elution fractions. These data show that this protein can be expressed in *Chlamydomonas* chloroplasts and purified to homogeneity.

Furthermore, the purified TPS4-HA protein was subjected to peptide mass finger print analysis. The protein CAS_ABIBA was identified unambiguously (Score 191 searched against the SWISS-PROT Database, $p=4.3e^{-14}$). The heterologous TPS4-HA protein of this study is identical in its amino acid sequence to CAS_ABIBA (*cis*-abienol synthase from Balsam fir), apart from removal of the N-terminal plastid transit sequence for expression in chloroplasts (shown in Online Resource 2, which also shows the homology exhibited between TPS4 and other diterpene synthases) and addition of a C-terminal HA tag. Details of the mass spectometry analysis are given in Online Resource 3, where coverage of the protein sequence and details of the identified peptides are shown.

Quantitation of protein levels

In order to quantitate the levels of TPS4-HA in the transformant, we carried out densitometric analysis of immunoblots using purified HA-Ubiquitin (containing an identical HA tag to that of TPS4-HA) as a standard for the calibration curve. The expressed TPS4 enzyme was found to represent approximately 3.7 % of total soluble protein. In general, similar expression levels (in terms of percentage of total soluble protein) were obtained for both photoautotrophic growth on minimal medium and mixotrophic growth on

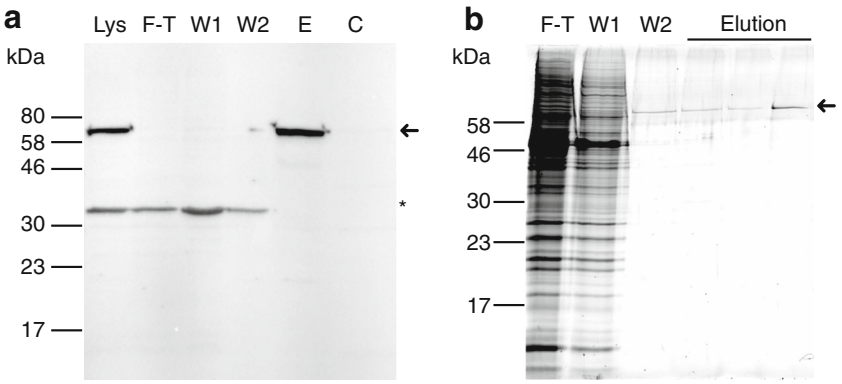

Fig. 4 Purification of TPS4-HA from total soluble protein. **a** Immunoblot analysis of ion exchange chromatography elution fractions; the panel shows results for the total cell lysate (*Lys*), column flow-through (*F-T*), wash fractions (*W1*, *W2*) and the 350-mM NaCl elution fraction (*E*) containing TPS4-HA. Mobilities of molecular mass markers (in kDa) are indicated. **b** Oriole™-stained SDS gel of TPS4-HA affinity purification fractions showing column flow-through (*F-T*), wash fractions (*W1*, *W2*) and elution fractions obtained using the competitor HA-peptide. *Arrows* denote TPS4 protein; an *asterisk* denotes non-specific reacting band in **a**

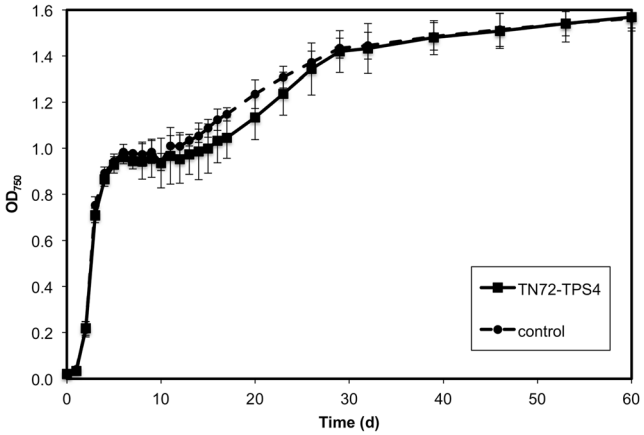

Fig. 5 Expression of the *tps4* transgene has a minor effect on culture growth. The graph shows growth curves for TN72-RP (*circles*, *dashed line*) and TN72-TPS4 cultures (*boxes*, *continuous line*) on TAP medium. The data are averages of three experiments

TAP medium that contains acetate as a source of fixed carbon (data not shown).

Expression of TPS4 has a minor adverse effect on growth rate

TPS4 catalyses the formation of *cis*-abienol using geranylgeranyl diphosphate (GGDP) as substrate (Zerbe et al. 2012), but we were unable to detect *cis*-abienol in the cultures of the transformant strain so it is unclear whether the enzyme is active in the transformed cells. However, expression of the enzyme may be expected to partially deplete the GGDP pool in the chloroplast and therefore affect growth of the strain, and this was tested by directly comparing the growth of TN72-TPS4 and the TN72-RP control strain. The cultures were grown on TAP medium, and the growth curves are shown in Fig. 5. Both exhibit rapid initial log growth characteristics which appear to be identical; thereafter, the cultures enter a steady linear phase which presumably occurs after depletion of the acetate. At this point, growth of the TN72-TPS4 culture is slightly slower than that of the TN72-RP culture, suggesting that TPS4 activity may indeed be depleting the GGDP pool and thereby inhibiting GGDP-dependent metabolic pathways in the chloroplast.

Discussion

A recent study has shown that a relatively simple method can be used to transform the chloroplast genome of *C. reinhardtii* (Economou et al. 2014). In the present study, we sought to test the utility of this method for the expression of heterologous proteins, using a plant diterpene synthase as target protein. For this study, a gene encoding the bifunctional enzyme TPS4 was integrated into the chloroplast genome and the cells were characterised once homoplasmy was achieved.

Immunoblotting showed that the 91 kDa HA-tagged TPS4 protein is stably expressed, and it was furthermore shown that the protein can be purified to homogeneity from cell extracts. MALDI-TOF mass fingerprinting further confirmed the identity of the protein sequence. We calculated that the protein represents 3.7 % of total soluble protein. Together, these data show that the transformation method is suitable for expression at reasonably high levels of a heterologous protein and it should be possible to further enhance expression levels by manipulating promoter regions and other UTR elements. Importantly, the method does not result in the presence of an antibiotic cassette in the chloroplast genome of transformed cells and this may be an advantage in terms of minimising the effort needed to generate and maintain the cultures. The absence of such cassettes is also preferred for commercial production applications.

No clear phenotype was observed for the transformant expressing TPS4 and we did not detect the reaction product, *cis*-abienol (Zerbe et al. 2012), using gas chromatography mass spectrometry. However, this product is not available in purified form for control tests and the compound may well have been masked by other compounds in our tests. We did note that the transformed strain grew slightly more slowly than the mock-transformed control strain, which may be an indirect indication of enzyme activity, but this point remains to be resolved. This enzyme has been studied in *E. coli* but only using in vitro assays (Zerbe et al. 2012), and this is the first report of heterologous expression in photosynthetic hosts; it is therefore difficult to predict the effects of product accumulation. Other studies (Jin et al. 2014) have shown that synthesis of a variety of hydrophobic compounds can adversely affect growth of photosynthetic bacteria, and the observed change in growth of this TPS4 transformant may well reflect this trend.

In summary, the result show that this recently developed transformation protocol is capable of generating strains that stably express a heterologous protein in the chloroplast at levels that are generally comparable with other studies using either nuclear or chloroplast transformation.

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