Metabolic Engineering for Multi-Product Value Enhancement of Engineered Green Algal Cell Chassis

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INTRODUCTION

Recent advances in transgene design have demonstrated the use of *C. reinhardtii* as a photosynthetic cell chassis for sustainable heterologous production of natural products. A domesticated strain (UVM4) with high nuclear genome transgene expression capability was optimized for the capacity to grow on inorganic phosphite and nitrate as sole sources of P and N, respectively, to enable high-cell density cultivation with reduced contamination risk[1]. This strain (UPN) serves as a platform to produce heterologous metabolites in our group[2]. A further goal in the use of this strain was to add value to spent biomass by leveraging *C. reinhardtii*'s generally regarded as safe (GRAS) status to sustainably produce food-grade recombinant proteins (RPs) and high-value pigments, such as the keto-carotenoid astaxanthin. A range of proteins of interest to the food industry were selected, coding sequences optimized for chloroplast expression, and given strep-II tags for Western blot detection / down-stream purification.

The chloroplast of UPN was engineered to encode these RPs by *PsbH* knock-out/ restoration transformation[3] to enable antibiotic-free selection. We present here successful chloroplast genome expression of the ice binding protein *Lp*IBP (PDB: 3ULT) from perennial ryegrass *Lolium perenne*.

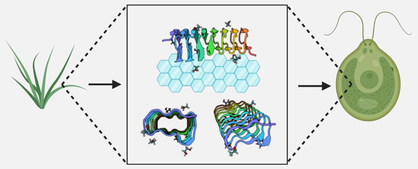

Figure 1: Crystal structure of *Lp*IBP (PDB:3ULT) and illustrated interaction with ice crystals. Expression of recombinant *Lp*IBP in *C. reinhardtii* chloroplast aims to provide a sustainable source of this protein for the food-industry.

Transformants with successful chloroplast RP expression were further engineered to express to ß-carotene hydroxylase (*Cr*CHYB) and ß-carotene ketolase (*Cr*BKT) from the nuclear genome as recently shown[6] to produce astaxanthin (Fig 6.).

DEVELOPMENT OF 'UPN'

The high-density capable strain 'UPN' was generated from cell-wall deficient UVM4 by chloroplast genome transformation with WM88 phosphite NAD^+ oxidoreductase from *Pseudomonas stutzeri* (*ptxD*)[4] allowing conversion of inorganic phosphite (PO_3) into bio-available phosphate (PO_4). Subsequently, NO_3 metabolic capacity was restored by co-transformation of NIT1/NIT2 plasmids[5].

Modified versions of TAP (Tris-acetate-phosphate medium) with equal-molar replacement of phosphate and NH_4Cl with phosphite and $NaNO_3$ were developed for reduced contamination cultivation. For high-density cultivation experiments, 6xP medium[5] was similarly modified by replacing phosphate with equal-molar phosphite solutions. This medium (6xPhi) supports high density growth of UPN in CellDEG photobioreactors.

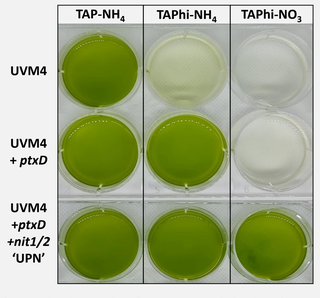

Figure 2: Growth of UVM4 and derived *ptxD* and NIT1/NIT2 strains in modified TAP media.

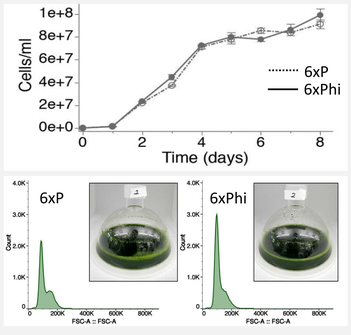

Figure 3: Growth of UPN in CellDEG photobioreactors in 6xP and 6xPhi media. Adapted from[1].

RESULTS

After recovering photoautotrophic capable transformants, confirming integration of *RP* coding sequence into the chloroplast genome and initial growth trials, selected transformants were grown were selected for high-density cultivation in CellDEG photobioreactors with 6xPhi medium.

Cell density was measure daily (Fig 4.). *Lp*IBP transformants showed similar growth to empty vector control suggesting little impact of *Lp*IBP expression on growth.

Western blot quantification of total soluble protein (TSP) fractions show ~7.5% TSP yield of *Lp*IBP (Fig 5.)

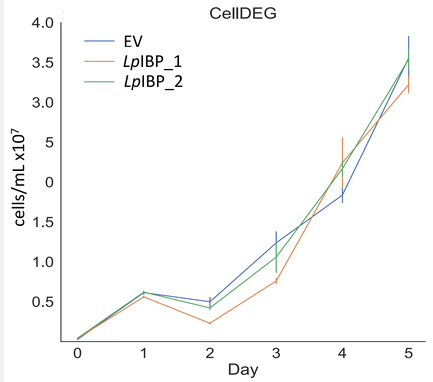

Figure 4: Figure 4: Growth of 2 independent UPN:*Lp*IBP transformants and empty vector control in CellDEG photobioreactors with 6xPhi+5% CO_2. Mean cells/mL ± SEM of technical triplicate measurements from 2 biological replicates of each transformant.

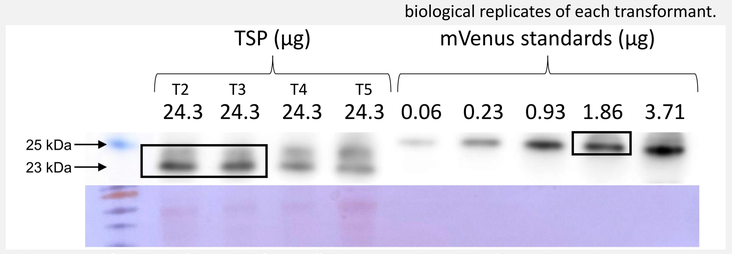

Figure 5: Estimation of *Lp*IBP (23 kDa) %TSP from CellDEG grown UPN:*Lp*IBP cultures (Days 2-5) – compared to purified mVenus (25 kDa) of known concentrations. Anti-strepII-tag Western blot (top), Ponceau-stained membrane (bottom). Signal comparisons allow estimation of *Lp*IBP %TSP

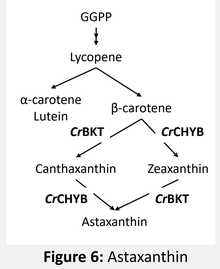

Figure 6: Astaxanthin biosynthesis pathway

Selected transformants showing chloroplast genome expression of RP were transformed with a binary vector encoding chloroplast targeted (CTP) fused version of *Cr*CHYB:mRuby2 and *Cr*BKT:aadA. Preliminary screening of spectinomycin transformants show a brown phenotype typical of ketocarotenoid accumulation (Fig 8.).

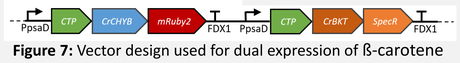

Figure 7: Vector design used for dual expression of ß-carotene hydroxylase (*Cr*CHYB) and ß-carotene ketolase (*Cr*BKT)

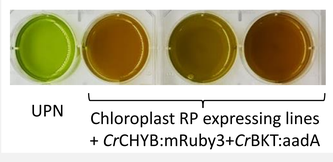

Figure 8: Selected transformants and parental UPN strain grown in TAPhi-NO_3

METHODS

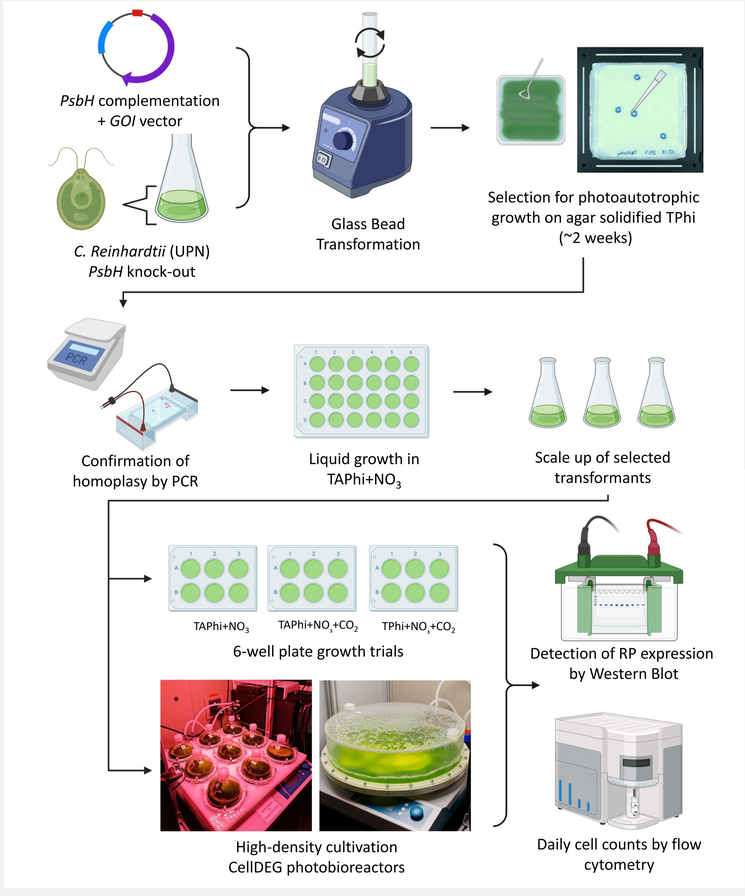

Figure 9: Overview of experimental techniques used for chloroplast genome transformation, photosynthesis restoration selection and growth trials of selected transformants

CONCLUSIONS & FUTURE WORK

The chloroplast of UPN was successfully transformed and expression of recombinant *Lp*IBP detected by Western blot. *psbH* complementation restores photoautotrophic growth allowing culturing in high-density photobioreactors with similar cell densities to empty vector controls.

Co-expression *Cr*CHYB and *Cr*BKT from the nuclear genome in Chloroplast RP expressing transformants display a brown phenotype indicating increased keto-carotenoid/astaxanthin production.

Further work is required to quantify the levels of astaxanthin production and to examine of the effect of chloroplast accumulation of keto-carotenoids on the expression/yield of chloroplast genome encoded RP.

Further screening of transformants with different chloroplast genome expressed RPs and high astaxanthin production is required find an economically attractive product compatible with other metabolic engineering projects in the SSB group.

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