Optimizing high-density phototrophic cultivation of *C. reinhardtii* for chloroplast recombinant protein production

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INTRODUCTION

Recent advances in transgene design and strain domestication have demonstrated the use of *C. reinhardtii* as a photosynthetic cell chassis for sustainable heterologous production of a range 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 risk of contamination[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). A range of proteins of interest to the food industry were selected and coding sequences were optimized for chloroplast expression. A Strep-II tag sequence was added for detection by Western blot and 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*.

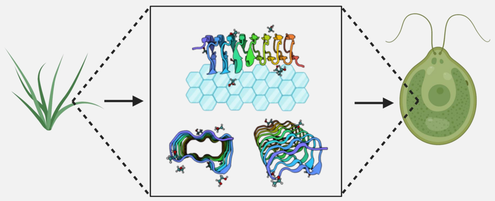


Figure 1: Proposed structure of *Lp*IBP (PDB:3ULT) and interaction with ice during crystal formation to reduce tissue damage. Expression of recombinant *Lp*IBP in *C. reinhardtii* chloroplast aims to provide a sustainable source of this protein for the food-industry.

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[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.

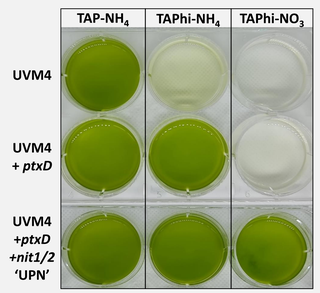


Figure 2: Growth of UVM4 and derived *ptxD* and NIT1/NIT2 expressing strains in modified TAP media.

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.

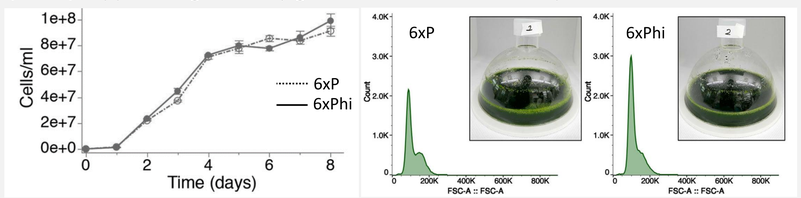


Figure 3: Growth of UPN in CellDEG photobioreactors in 6xP and 6xPhi media. Adapted from[1].

RESULTS

After recovering photoautotrophic capable transformants and confirming integration of *Lp*IBP coding sequence into the chloroplast genome, selected transformants were grown in 6-well plates under different media and CO_2 supply to assess growth compared to empty vector (EV) control (Fig 4).

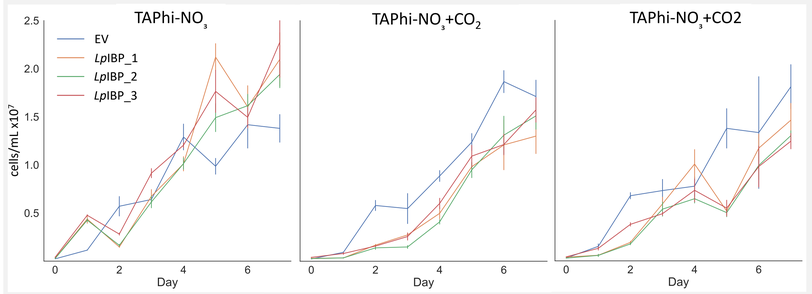
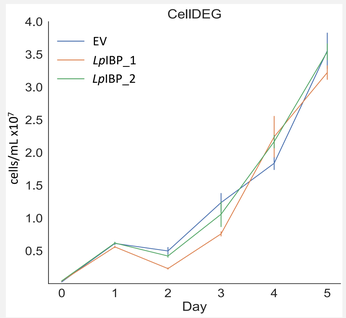


Figure 4: Growth of 3 independent UPN:*Lp*IBP transformants and empty vector control grown in TAPhi (left), TAPhi + 5% CO_2 in air (mid), and acetate free media (TPhi) + 5% CO_2 in air (right). Mean cells/mL ± SEM plotted of 3 biological replicates of each transformant measured (n=3).



*Lp*IBP transformants showed similar growth to empty vector control suggesting little impact of *Lp*IBP expression on growth in small scale cultures. Two transformants were selected for high-density cultivation in CellDEG photobioreactors with 6xPhi medium. Samples of cultures were taken daily for cell number (Fig 5.) and total soluble protein extraction and yield estimates (Fig 6).

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

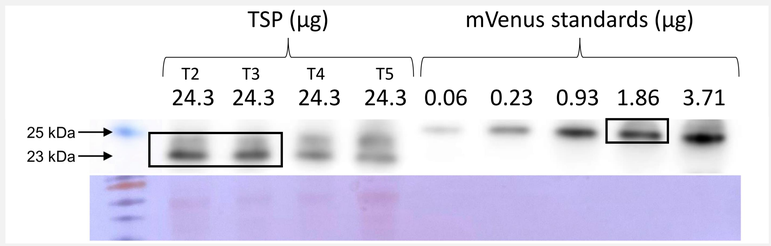


Figure 6: Estimation of *Lp*IBP (23 kDa) yield as a % total soluble protein (TSP) extracted from CellDEG grown UPN:*Lp*IBP cultures (Days 2-5) – compared to *E. coli* expressed fluorescent protein mVenus (25 kDa) of known concentrations. Anti-strepII-tag Western blot (top), ponceau-stained membrane (bottom). Similar chemiluminescence signals allows estimation of *Lp*IBP %TSP yield of ~7.5%.

REFERENCES & CONTACT

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METHODS

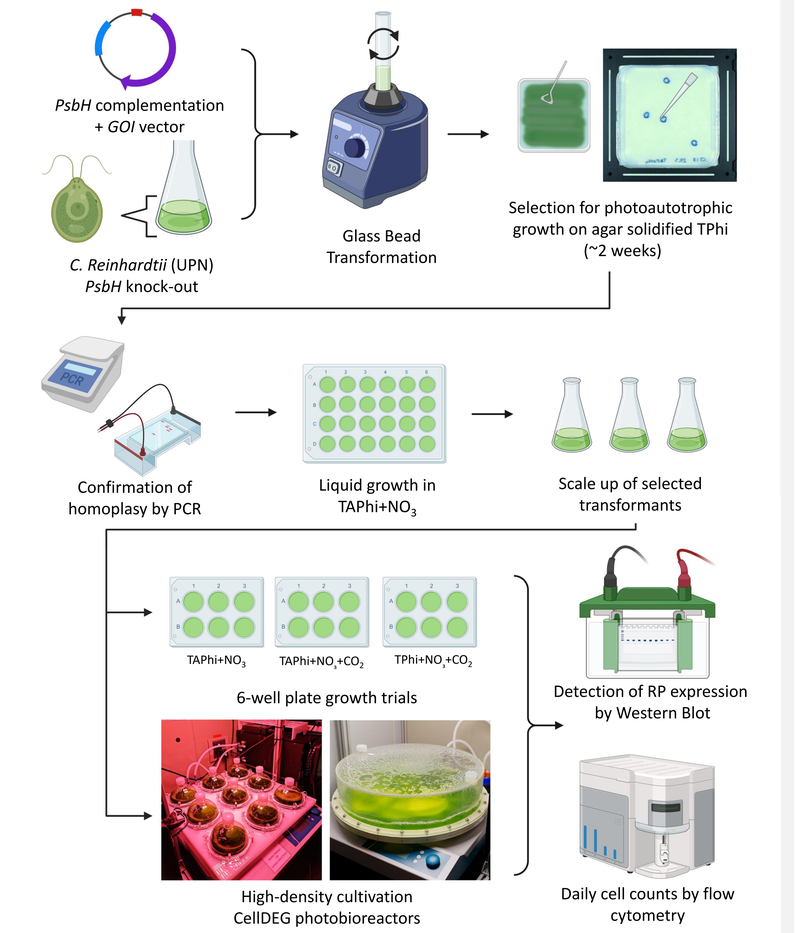

Figure 7: Overview of experimental techniques used for chloroplast genome transformation, photosynthesis restoration selection and growth trials of selected transformants

CONCLUSIONS & FUTURE WORK

The chloroplast of UPN was successfully transformed and expression of recombinant *Lp*IBP detected by anti-Strep-II-tag Western blot. *psbH* complementation restores photoautotrophic growth allowing culturing in high-density photobioreactors with similar cell densities to empty vector controls. Initial estimates are that *Lp*IBP accumulates to ~7.5 % of TSP.

Further optimization of high-density culture conditions (N:P ratio, light regime) for increased culture density, cell protein content and to minimize excessive nutrients supply.

Column purification of RPs to determine RP yield and feasibility of large-scale production for industry uses.

Screening of transformants with different chloroplast genome expressed RPs to find an economically attractive product compatible with other metabolic engineering projects in the SSB group.