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Brief communication

# Optimization of biomass and target protein yield for Phase III clinical trial to evaluate Angiotensin Converting Enzyme 2 expressed in lettuce chloroplasts to reduce SARS-CoV-2 infection and transmission

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The goal of delivering therapeutic proteins in plant cells, free of cold chain and expensive purification processes has been elusive for several decades. Recently, Angiotensin Converting Enzyme 2 (ACE2) expressed in lettuce chloroplasts for delivery via chewing gum (Daniell *et al*., 2022a,b) to prevent infection and transmission of severe acute respiratory syndrome coronavirus (SARS-CoV-2) was approved by the FDA for a Phase I/II clinical trial (IND 154897, NCT05433181, IRB 851459). Further product development, leading to a Phase III clinical trial in preparation for commercial product launch, requires large-scale biomass production under optimal growth conditions, for maximal yield of the target protein drug. Although there are many reports on optimization of lighting, nutrients and spacing to grow lettuce under hydroponic conditions, they focus on biomass accumulation and metabolite levels, except in one report on a vaccine antigen (Lin *et al*., 2013; Okamura *et al*., 2014). Biomass and target protein yield are dramatically lower in lettuce plants grown under hydroponic conditions when compared with the greenhouse (Daniell *et al*., 2020), but there are no reports on the combined optimization of lighting, nutrients and spacing to improve biomass and protein yields in hydroponically grown lettuce plants. Therefore, this study addresses this urgent need in molecular farming.

Methods followed for plant biomass production, including substrate, spacing, nutrients, lighting, photoperiod, temperature, humidity and air flow, and for quantification of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO LS), chlorophyll content, total soluble protein (TSP) and the fusion proteins of the B subunit of cholera toxin (CTB) to Angiotensin-Converting Enzyme 2 (ACE2) and to Angiotensin-(1-7) (ANG-(1-7)) are described in the Appendix S1.

Figure 1a shows representative examples of lettuce plants expressing target protein before or after optimization, and this was accomplished in several steps. Five different growth conditions were explored first in wild-type lettuce (Table S1). Fluorescent lights previously reported for growth of blood clotting factor – FIX lettuce (Su *et al*., 2015) were compared with white light-emitting diodes (LEDs) providing a similar light intensity or ~2–4 times that intensity (Table S1). Similarly, a general-purpose nutrient solution, Peters Professional 20-10-20 (PGP), previously reported for FIX lettuce (Daniell *et al*., 2022a; Su *et al*., 2015) was compared with a nutrient solution optimized for hydroponic applications, Peters Professional Hydroponic Special 5-11-26 (PHS), each delivered along with Calcinit in deionized water (DIW). Plants grown under similar lighting accumulated ~2.4-fold more biomass, when provided with PHS than PGP (Figure 1b). Although this increase in biomass was accompanied by a minor (1.3-fold) decrease in TSP, there was no discernable impact on chlorophyll content or level of RuBisCO LS, encoded by the chloroplast genome (Figure 1b and Figure S1). Also, plants provided the same nutrients and grown under a similar intensity light provided by fluorescent versus LED fixtures showed only minor differences in biomass accumulation, TSP, chlorophyll content and level of RuBisCO LS (Figure 1b and Figure S1). However, doubling and quadrupling the light intensity provided by LED fixtures, while providing the same nutrients, resulted in increases in biomass accumulation of ~2.5–3.4-fold, respectively, increases in chlorophyll content ~1.2 and 1.7-fold, respectively, and increases in the level of RuBisCO LS of ~2.4-fold and 2.7-fold, respectively, while having negligible impact on TSP (Figure 1b and Figure S1). These observations suggest that optimizing nutrients and light intensity may lead not only to higher biomass yields, but also to higher yields of chloroplast expressed recombinant proteins.

Having optimized conditions with wild type lettuce, we compared growth conditions with transplastomic lettuce expressing CTB-ACE2 and CTB-ANG-(1-7) (Figure S2; Table S2). Initially, we confirmed the importance of adequate spacing between plants and sufficient light intensity (Figure 1a). By increasing the spacing between plant stems approximately threefold, to give ~21.5 plants per $m^2$ versus ~193 plants per $m^2$ (threefold difference in linear spacing leads to a ninefold difference in plant density) and the light intensity provided by LED lights approximately 4.5-fold, significant increases were observed in the yields of fresh and dry biomass per plant and also in the accumulation of CTB-ANG-(1-7) (Figure S2, Table S2). Next, LED lighting at the highest light intensity used above (~370 μmol/$m^2$/s) was chosen for three comparative treatments, since that had resulted in optimal biomass generation, chlorophyll content and accumulation of plastid genome encoded RuBisCO LS or CTB-ANG-(1-7) in

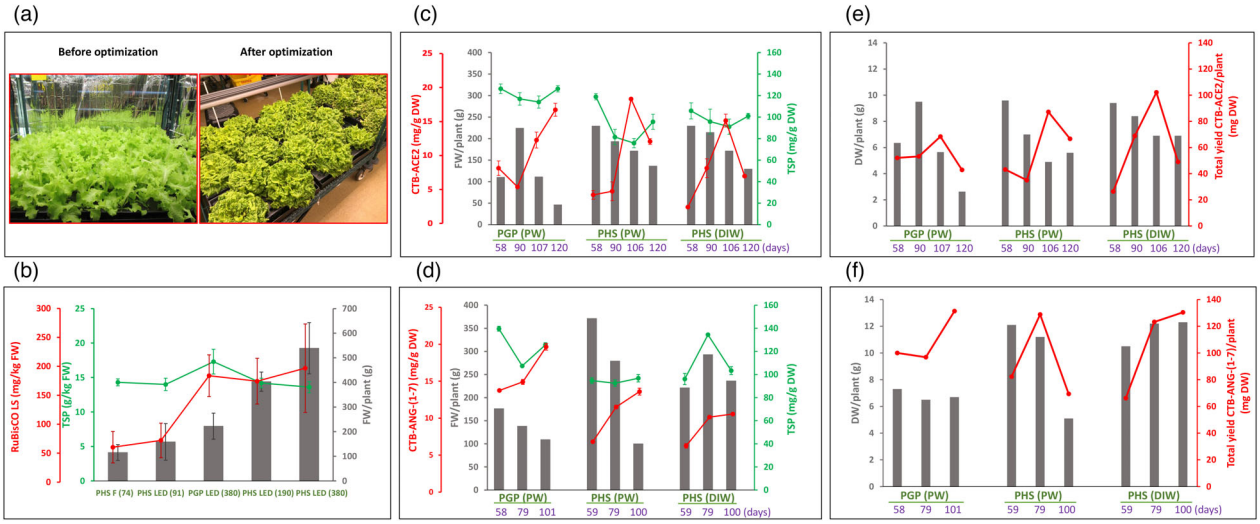


**Figure 1** (a) Representative examples of lettuce plants expressing target protein before or after optimization. For further details, please see Figure S2. (b) FW per plant (g), TSP (g/kg FW) and RuBisCO LS expression (mg/kg FW) for plants grown under different treatments. (c,d) FW per plant (g), TSP (mg/g DW), CTB-ACE2 and CTB-ANG-(1-7) expression (mg/g DW) at different ages (days) for plants grown under alternative conditions. (e,f) DW/plant (g) and total yield of CTB-ACE2 and CTB-ANG-(1-7)/plant (mg DW) at different ages (days) for plants grown under alternative conditions. DW/plant (g) was calculated as the total DW/total number of plants harvested, and total yield of CTB-ACE2 and CTB-ANG-(1-7)/plant (mg DW) was calculated as CTB-ACE2 and CTB-ANG-(1-7) (mg/g DW) expression × DW per plant (g). For panels c–f, plant ages are relative to the date of seeding.

transplastomic lines. Similarly, the wider plant spacing condition that gave improved yields of biomass and target protein was followed. However, the nutrient solution and water source were varied to again compare PHS to PGP and also to compare potable water (PW) to DIW for delivery of PHS, with PGP delivered by PW. High yield of chloroplast expressed proteins depends on the age of the lettuce plants, with older plants showing higher expression (Herzog *et al.*, 2017), and the duration of illumination prior to harvest, with later in the light portion of the day/night cycle being optimal (Ruhlman *et al.*, 2010). Therefore, mature lettuce leaves were repeatedly harvested in the mid-late afternoon throughout the growth cycle of the plants (days 58, 90, 106/107 and 120 post-seeding for CTB-ACE2 and days 58/59, 79 and 100/101 post-seeding for CTB-ANG-(1-7)).

As with wild-type lettuce, transplastomic plants accumulated more biomass when provided with PHS compared with PGP, with similar biomass yields for potable and deionized water (Figure 1c,d). Also, as with wild-type lettuce, transplastomic plants showed a minor decrease in TSP when provided with PHS compared with PGP (Figure 1c,d), with this minor decrease apparent on a dry weight basis and thus not related to water content. For lettuce plants expressing CTB-ACE2, the recombinant protein reached similar levels in plants provided with PHS compared with PGP (Figure 1c), whereas in plants expressing CTB-ANG-(1-7), recombinant protein levels were reduced in plants provided with PHS (Figure 1d). Expression levels are not directly comparable because T1 generation of CTB-Ang (1-7) was engineered two years later than T3 generation of CTB-ACE2. A higher level of CTB-Ang (1-7) expression on a weight basis (mg/g DW) or total yield per plant than CTB-ACE2 may reflect an 8.2-fold difference in molecular weights (~101 kDa versus ~12.4 kDa), requiring greater demand on amino acid and tRNA pools. Most CTB fusion proteins are highly stable as evidenced by higher accumulation in older plants, and therefore, the difference is unlikely related to their stability. For plants provided with PHS, the source of water did not affect recombinant protein accumulation for either CTB-ACE2 or CTB-ANG-(1-7) (Figure 1c, d). CTB-ACE2 accumulation peaked at 106/107 days post-seeding, regardless of the nutrient solution and water source (Figure 1e). CTB-ANG-(1-7) accumulation also peaked late in the growth cycle (Figure 1f).

Irrespective of the higher nitrogen content in PGP (Table S4), the fresh weight of lettuce plants provided this nutrient mix was less than for those provided PHS. Potentially, the increased nitrogen content of PGP may adversely influence plant growth and biomass accumulation. A similar observation was reported in buttercrunch and black seeded Simpson lettuce cultivars, where the highest levels of nitrogen, potassium and calcium significantly reduced plant fresh weight (Sapkota *et al.*, 2019). This negative effect may be attributed to elevated osmotic pressure around the roots. Apart from nitrogen, the much higher levels of magnesium in PHS (Table S4) may be critical for the increased biomass yield when plants are provided with this nutrient mix compared with PGP. Of note, high-level expression of target proteins in transplastomic lines has been shown to reduce the levels of endogenous proteins, especially RuBisCO LS, which could be restored by addition of ammonium nitrate (Bally *et al.*, 2009).

In this study, we found that there was no consistent correlation between TSP and recombinant protein expression. Although, as for wild-type plants (Figure S1), TSP was observed to be slightly higher for plants provided PGP compared with PHS, CTB-ACE2 accumulated to similar levels (Figure 1c) and CTB-ANG-(1-7) to notably lower levels on PHS (Figure 1d). This observation suggests that optimizing growth conditions can focus on achieving the highest yields of biomass and target protein without placing emphasis on the accumulation of TSP.

Notably, for plants provided with PHS, the peak yield for CTB-ACE2 was achieved at 106 days rather than at 120 days for plants provided with PGP, suggesting that higher yields of this recombinant protein could be achieved with an earlier harvest of tissue, so improving the economics of production. In this study, higher light intensity provided by LEDs and a nutrient solution

optimized for hydroponic systems were found to be crucial to achieve high biomass yields. While high light intensity also resulted in increased accumulation of RuBisCO LS and chlorophyll in wild-type plants, the choice of nutrient solution had little impact on their accumulation. However, for transplastomic plants, although the nutrient solution optimized for hydroponic systems again resulted in higher biomass yields, its impact on recombinant protein accumulation differed for different targets. Mass production of therapeutic proteins in transplastomic lettuce under optimal growth conditions for oral delivery would help to reduce protein production and processing costs drastically. This study provides significant progress in optimizing conditions to produce plant biomass containing CTB-ACE2, formulated into chewing gums to reduce viral load and COVID-19 transmission (Daniell *et al*., 2022a,b) or oral CTB-ACE2 and CTB-Ang (1-7) for treatment of cardiopulmonary diseases (Daniell *et al*., 2020).

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## Conflict of interest

The corresponding author (HD) is an inventor on several patents on expression of CTB-ACE2/CTB-Ang1-7 in chloroplasts and therefore declares conflict of interest. University of Pennsylvania has an approved a COI management plan that excludes direct participation in human clinical trials using drug products prepared and characterized in his lab. PK, RM-L, BG and SS work at Fraunhofer USA.

## Author contributions

HD conceived this project from designing chloroplast vectors, creation of transplastomic lines through approval of IND by FDA, interpreted data and wrote several sections of this manuscript. PG and RK quantified CTB-ACE2 and CTB-ANG-(1-7) expression through western blots, prepared figures and wrote corresponding sections. PK, RM-L, BG and SS designed the plant growth conditions, and PK oversaw growth of the plants. RM-L performed wild-type plant analysis. SS contributed to data analysis and interpretation and drafting of the manuscript.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Relationship between chlorophyll content and FW/plant under different growth treatments.

**Figure S2** (a) The Phenotypic comparison of leaves and stems of lettuce plants expressing target proteins. Please note slender stems before optimization and increase in thickness of stems and branches after optimization of plant density, light and nutrient conditions. (b) CTB-ACE2 expression and biomass yield before and after optimization. (c) CTB-Ang (1-7) expression and biomass yield before and after optimization.

**Table S1** Growth conditions for wild type lettuce.

**Table S2** Lettuce Biomass and expression level of CTB-ACE2 and CTB-Ang (1-7) before and after optimization of plant density, lighting and nutrients.

**Table S3** Growth conditions for transplastomic lettuce.

**Table S4** Composition of nutrient contents under alternative growth conditions.

**Appendix S1** Supplementary methods.

 