Algal Research 62 (2022) 102604

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Heterologous expression of phosphite dehydrogenase in the chloroplast or nucleus enables phosphite utilization and genetic selection in *Picochlorum* spp.

Lukas R. Dahlin, Michael T. Guarnieri *

Biosciences Center, National Renewable Energy Laboratory, Golden, CO 80401, United States of America

ARTICLE INFO

Keywords:
Microalgae
Genetic engineering
Picochlorum
Phosphite
Phosphite dehydrogenase
Chloroplast transformation

ABSTRACT

Microalgae present a path to ameliorate problems associated with climate change via capture and reduction of CO_2 to sustainable fuels and chemicals. *Picochlorum* is a genus of algae recently recognized for potential application in these regards due to its high productivity, thermotolerance, and halotolerance. Foundational genetic tools have recently been established in this genus. However, at present, genetic markers are limited, hindering genetic throughput and trait stacking approaches. To expand the suite of genetic tools and markers available for this genus, we sought to heterologously express the phosphite dehydrogenase (*ptxD*) gene from *Pseudomonas stutzeri* WM88 in both the nucleus and chloroplast of *Picochlorum renovo* and *Picochlorum celeri*. The resultant strains allow for utilization of phosphite as a sole phosphorous source and as a nuclear and plastidial selection marker for genetic engineering. Growth analysis indicated comparable growth and composition when transgenic algae were grown in media containing phosphite as a sole phosphorus source, as compared to the conventionally used phosphate. Combined, these results expand the genetic toolbox available to the *Picochlorum* genus and present a potential crop protection and biocontainment strategy.

1. Introduction

Microalgae are amongst the most promising biocatalysts capable of photosynthetically converting carbon dioxide and water to renewable biomass and bioproducts [1]. These organisms have been pursued for production of native metabolites (e.g. astaxanthin, omega-3 fatty acids), and recently for production of non-native metabolites (e.g. bisabolene) and heterologous proteins via genetic engineering, underscoring their potential impact in the emerging bioeconomy [2–4]. Unlike terrestrial crops, microalgae are capable of growing in saline water and on non-arable land, while maintaining approximately an order of magnitude higher areal productivity. Despite the promise of microalgae, successful industrial application is currently limited to relatively high value products (e.g. nutraceuticals), in part due to limited genetic tractability, low productivity, and mass culture contamination events [5,6].

Development and application of genetic tools for trait stacking, targeted productivity improvements, and expansion of microalgal product suites will help to realize the benefits of microalgae as a biotechnological host for lower value commodities, such as fuel and chemical intermediates. Additionally, approaches to prevent the growth of competing algae, microbial contaminants, and predators, which can lead to low productivity and pond crashes, must be established [7].

Researchers have recently developed the use of phosphite, an exceedingly rare phosphorus source in nature, as a dual-pronged fertilizer and weed control system, enabled by expression of the phosphite dehydrogenase (*ptxD*) gene from *Pseudomonas stutzeri* WM88 [8,9]. The *ptxD* gene oxidizes phosphite to metabolically accessible phosphate, via the concurrent reduction of NAD^+ to NADH. Importantly, the *ptxD* gene can also be used as a selectable marker, via cultivation on media with phosphite as a sole phosphorus source [9]. Since its first development in arabidopsis and tobacco, this technology has been further applied to rice, cotton, maize, sorghum, and yeast [10–14]. Recently, phosphite mediated selection and cultivation has been applied in model algal (*Chlamydomonas reinhardtii*) and cyanobacterial systems, where it has proved effective for reduced contamination at up to 1000 L scale cultivation, and as a selectable marker for both nuclear and chloroplast expression [15–24]. However, to date, this strategy has not been deployed in non-model algal systems with traits suitable for economically viable mass-cultivation [16].

Herein, we have developed phosphite utilization in the industrially-

* Corresponding author at: 15013 Denver West Parkway, Golden, CO 80401, United States of America.
E-mail address: michael.guarnieri@nrel.gov (M.T. Guarnieri).

https://doi.org/10.1016/j.algal.2021.102604
Received 10 September 2021; Received in revised form 24 November 2021; Accepted 12 December 2021
Available online 24 January 2022

relevant, emerging model algae, *P. renovo* and *P. celeri* [25,26]. Algae of this genus have recently gained interest for applied biotechnology and basic science pursuits due to their rapid growth, thermotolerance, and ability to thrive at high salinities [25,26,35,27–34]. Phosphite utilization was accomplished via expression of the *ptxD* gene, from either the chloroplast or nuclear genomes. Further, we demonstrate phosphite utilization as a selectable marker, broadening the genetic tools available to this genus, while also imparting potential crop protection and biocontainment traits useful for mass scale cultivation [21].

2. Results and discussion

2.1. Phosphite utilization screening

P. renovo was supplemented with phosphite as the sole phosphorus source on agar plates to assess native phosphite utilization capacity and potential as a selection agent. As shown in Fig. 1, no growth was observed in the phosphite grown culture, and noticeable bleaching occurred when compared to a control with no added phosphorus source, which displayed modest growth, likely due to residual phosphorus stores (Fig. 1). Contrary to prior reports of non-toxicity in other algal systems, distinct phosphite toxicity was observed under the tested conditions [36]. Indeed, phosphite has notable anti-fungal properties, and similar mechanisms of toxicity could be relevant in some algal systems [37]. These results suggested that phosphite could serve as a suitable selection agent for *P. renovo*, using established *ptxD*-mediated strategies [15,16].

2.2. ptxD as a selectable marker

Phosphite utilization in *P. renovo* was established through plastidial or nuclear expression of the *ptxD* gene from *Pseudomonas stutzeri* WM88. $NADP^+$, as opposed to NAD^+, is commonly considered the primary coenzyme in plant chloroplasts. However, the native *P. stutzeri ptxD* gene preferentially utilizes NAD^+ as a cofactor. Leveraging work from prior reports, a mutated *ptxD* capable of $NADP^+$ and NAD^+ utilization was used for plastidial expression [19,38]. This mutated *ptxD* gene was codon optimized and integrated into the tRNA-I, tRNA-A locus of the chloroplast genome, using our previously reported methodology [25]. Chloroplast expression was facilitated by extending the native 16S rRNA operon, a computationally predicted ribosomal binding site (RBS), and a synthetic *E. coli* terminator (Fig. 2a) [39–42]. Positive transformants were readily obtained utilizing our previously established biolistic protocol [25]. In contrast to our previous work, the native promoter element was removed such that transcription occurs via an upstream promoter element in the chloroplast genome. Additionally, the native terminator element was replaced with a synthetic terminator to remove homology to other elements native to the chloroplast genome (Fig. 2a). Combined, these changes, paired with the use of phosphite as a selectable marker, led to the obtainment of homoplasmy of the chloroplast genomes, as evident by the lack of a PCR band corresponding to the wild-type chloroplast genome (Fig. 2b). Homoplasmy and proper insertion was verified using the primers depicted in Fig. 2 and Supplemental Table 1. Notably, colonies of *P. renovo* were apparent 5 days post particle bombardment, as compared to the ~21–30 days previously reported to obtain transgenic *Chlamydomonas* colonies [18,19].

To establish nuclear transformation capacity, the native *Pseudomonas stutzeri ptxD* enzyme was codon optimized for expression from the *P. renovo* nuclear genome, and expressed under the control of a native *P. renovo* elongation factor promoter, as described previously (Fig. 2) [25,28]. Functional expression was achieved via the employ of a 150 base pair promoter, in contrast to our prior nuclear transformation work, wherein a promoter element of 650 base pairs was utilized, thereby simplifying future DNA cloning and synthesis endeavors.

Chloroplast transformation efficiency was similar to our previously established antibiotic-mediated (erythromycin) selection approach, enabling isolation of ~1–3 cfu/transformation [25]. However, nuclear transformation efficiency was markedly reduced by ~10 fold when selection occurs via phosphite, as compared to our previously established phleomycin (*ble*) gene selection marker, potentially due to differential mechanisms of action or residual phosphite toxicity. This reduction in transformation efficiency led to relatively few transgenic colonies per transformation (~3 cfu/transformation, using the methods described herein) for nuclear engineering (Fig. 2a). However, despite low efficiency, we note that no false positive transformants were observed for both nuclear and chloroplast engineering, indicating phosphite is a reliable alternative selection marker for targeted engineering strategies that do not require high efficiency (e.g. single heterologous construct integration events). Optimization of transformation efficiency will be necessary for genetic engineering approaches requiring higher efficiency (e.g. library generation and multiplex engineering), highlighting an area for needed future work in this genus.

2.3. Growth and compositional analyses

In order for phosphite to be effectively utilized in mass cultivation, growth on phosphite must cause minimal to no growth defects. We analyzed growth of these plastidial and nuclear transformants grown on phosphite compared to the wild-type strain grown on phosphate, to determine if any differential was evident. Growth of these transformants on phosphite was equivalent to that of the wild-type strain on phosphate, as measured by optical density curves and endpoint ash-free dry weight (AFDW, ~1.9 g/L) (Fig. 3a, b). Compositional analysis of intracellular lipids and carbohydrates were also equivalent (~12% lipids, ~60% carbohydrates) (Fig. 3c, d), in line with our previously reported stationary phase composition for this alga [25]. Combined, these data show no evident phosphorus stress response or alterations to P-associated metabolism, as indicated by equivalent growth and carbon partitioning for strains cultivated on phosphite, compared to phosphate, as sole phosphorus sources.

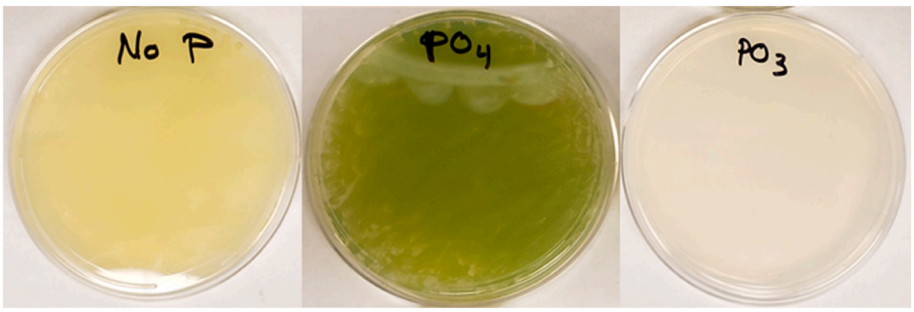

Fig. 1. Plating assay to determine phosphorus source utilization capacity in *P. renovo*. Representative 5-day outgrowth of *P. renovo* with (left to right): no additional phosphorus source, 0.313 mM phosphate addition, and 0.313 mM phosphite addition.

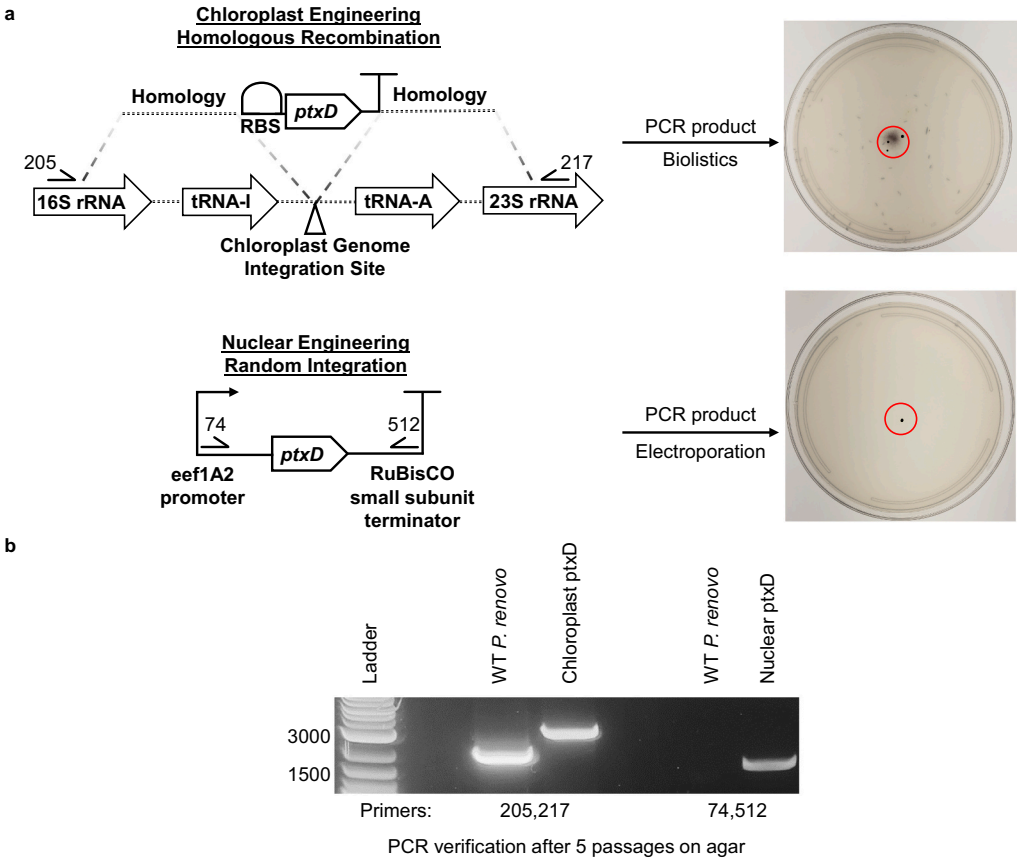

Fig. 2. Overview of *ptxD* expression in *P. renovo*. (a) Left: construct design for homologous recombination into the chloroplast genome, and random integration into the nuclear genome, right: phosphite selection agar plates, highlighting colony formation (encircled in red). (b) PCR verification of transformants, primer binding is noted in panel a. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Expression of phosphite dehydrogenase in Picochlorum celeri

Picochlorum spp. are rapidly gaining interest in the algal community. Thus, we sought to evaluate transferability of phosphite-mediated selection across species of this genus. A query of the *P. celeri* genome indicated the nuclear elongation factor promoter and RuBisCO small subunit terminator utilized above in *P. renovo* had 99% homology (149/150 and 498/504 identical base pairs, respectively). As such, we hypothesized these *P. renovo* genetic elements would also be functional in *P. celeri*. Contrary to prior reports, our electroporation protocol for *P. renovo* readily obtained nuclear transformants in *P. celeri* [30].

The above-described methodology for chloroplast transformation was also paralleled for *P. celeri*. However, as chloroplast integration occurs via homologous recombination and extension of the native 16S rRNA operon, we sought to decrease any complications with disrupting this operon and assembled a plasmid with *P. celeri*-specific homology arms for *ptxD* expression in the chloroplast. Our previously established biolistic protocol for *P. renovo* readily obtained the first reported plastidial transformants for *P. celeri*. Integration of transgenic DNA and homoplasmy was confirmed via PCR (Supplemental Fig. 1).

We next compared growth of these *P. celeri* transformants to the wild-type strain; similar to the results obtained in *P. renovo*, there was minimal biomass accumulation differential between the wild-type strain grown on phosphate and transformants grown on phosphite (Supplemental Fig. 2). *P. celeri* had a comparable composition to that of *P. renovo* for stationary phase cultures, reflective of relatively low lipid content (~13%) and high carbohydrate content (~57%).

To confirm there was no cross contamination between these highly similar *Picochlorum* species, we developed a facile PCR test to differentiate these strains. This methodology amplifies a section of nuclear genomic DNA wherein *P. celeri* possesses an additional ~1.5 kb of DNA in an otherwise homologous region between the two species. PCR primers flanking this region coupled with PCR and gel electrophoresis allows for a rapid distinction between strains (Fig. 4).

3. Concluding remarks

The work reported here expands the genetic toolbox available to the *Picochlorum* genus by establishing the use of phosphite as a selection agent in either the nucleus or chloroplast genomes and developing speciation probes. Importantly, phosphite mediated engineering also enabled the first report of chloroplast transformation in *P. celeri*. This strategy can also facilitate antibiotic-free genetic engineering and/or potential trait stacking in both the nucleus and chloroplast when utilized with previously established selectable markers. Notably, the employ of phosphite as a plastidial engineering tool enabled homoplasmy, which is critical for transgene stability and knockout strategies.

Phosphite-mediated cultivation led to equivalent biomass productivity and composition in two *Picochlorum* species. These data suggest phosphite presents a viable phosphorus source for outdoor cultivation of *Picochlorum* spp. Future work will entail multi scale cultivation and competition experiments, and complementary technoeconomic analyses, to assess the efficacy and economic viability of cultivation processes employing phosphite as a sole phosphorus or intermittent crop protection agent. Further, metagenomic analyses of non-sterile cultures will lend insight into the presence of competing algae and predators capable of growth in phosphite containing media.

In addition to the crop protection benefits conferred by phosphite, this strategy also presents an opportunity for phosphite mediated biocontainment. Prior work in cyanobacteria has established such a system

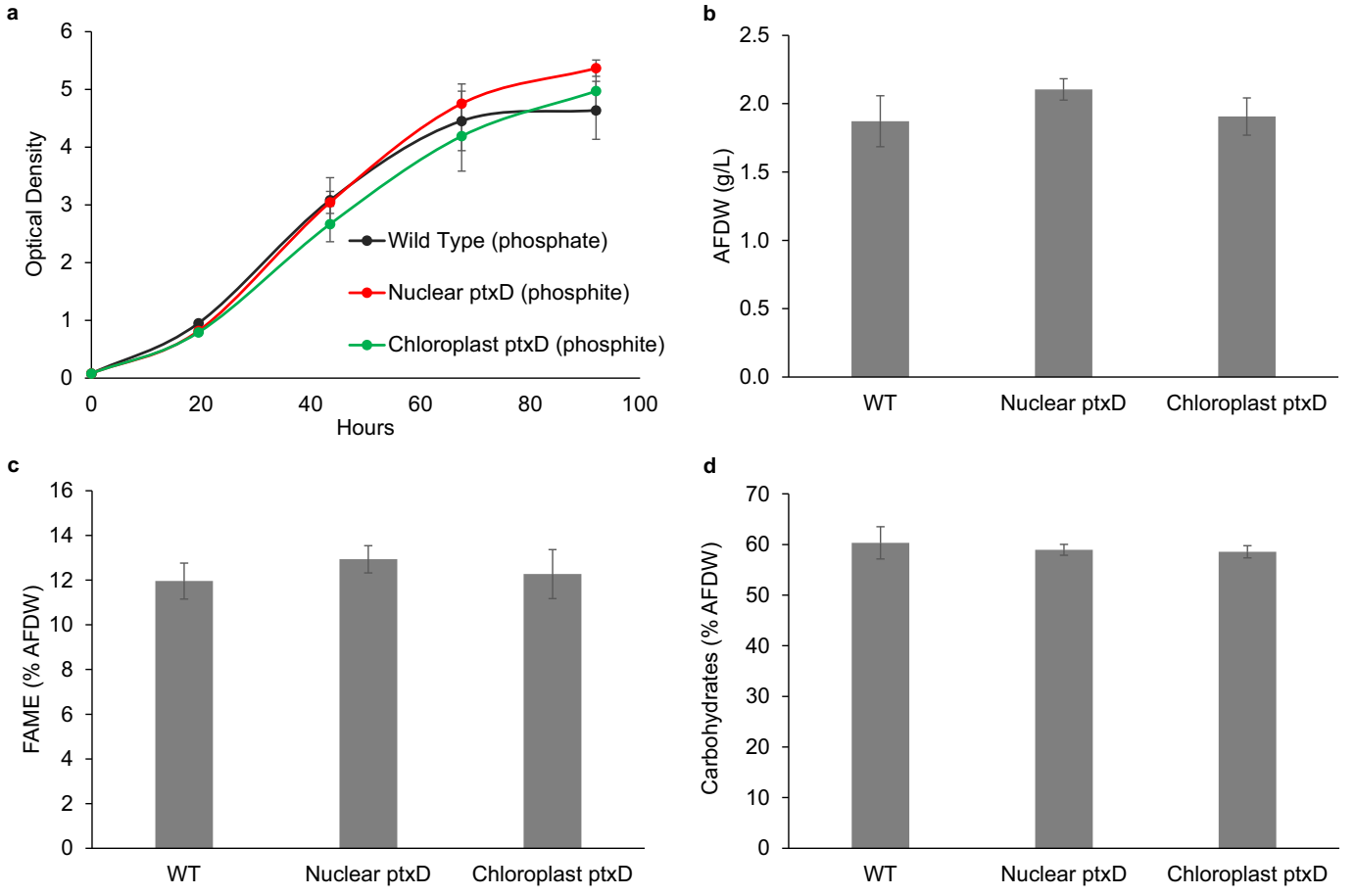

Fig. 3. Growth and compositional analyses of *P. renovo*. (a) Growth analysis of *P. renovo* chloroplast and nuclear transformants expressing *ptxD* grown on phosphite as a sole phosphorus source compared to wild-type grown on phosphate. (b-d) Ash-free dry weight (AFDW), fatty-acid methyl esters (FAME), and carbohydrate analyses corresponding to endpoint biomass of samples in panel a. All data represents an average and standard deviation of 3 biological replicates.

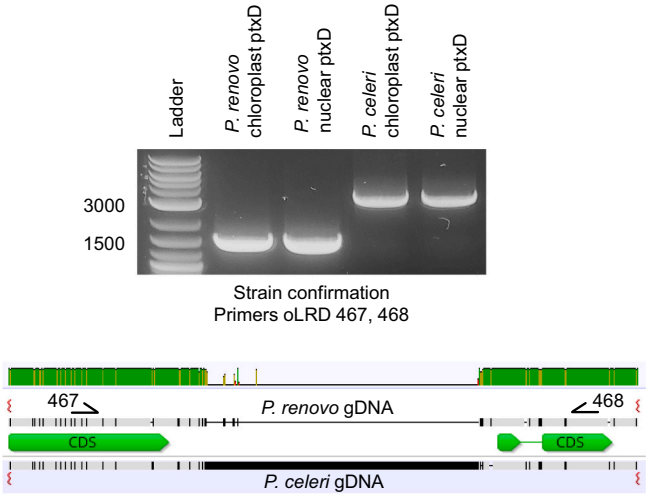

Fig. 4. PCR test utilized to distinguish between *P. renovo* and *P. celeri*. (Top) gel electrophoresis showing efficacy of primer pairs used to distinguish *P. renovo* and *P. celeri*. (Bottom) Sequence alignment of relevant nuclear gDNA amplified and primer binding, forward primer binds to a protein of unknown function, reverse primer binds to the RuBisCO small subunit protein.

wherein *ptxD* and a phosphite-specific transporter (HtxBCDE) are co-expressed, while concurrently knocking out the native phosphate transporters [21,43]. This strategy ensures containment of genetically modified organisms in the natural environment where phosphate is prevalent and phosphite is relatively limited. This, in turn, decreases the potential for gene flow from genetically modified organisms and propagation and/or disruption of native ecosystems, minimizing concern related to regulation of transgenic algae harboring this system.

4. Methods

4.1. Cultivation conditions

Microalgae were cultivated under constant illumination at 280 μmol/m^2/s of light, 2% CO_2, and constant temperature of 33 °C. These conditions were utilized to generate seed cultures for transformation and outgrowth of transformants to generate clonal isolates on agar plates. Media was comprised of a modified f/2 recipe, as described previously [25]. For phosphorus source utilization analyses, agar plates were prepared as described previously, utilizing 8.75 g/L salinity, via dilution of seawater [25]. Sodium phosphite dibasic pentahydrate (Sigma 04283) was utilized as a source of phosphite for this and subsequent experiments. Approximately 4.75 × 10^8 early log phase cells were plated and incubated under the above conditions; plates were imaged after 5 days of growth.

To compare *P. renovo* transformants grown on phosphite to wild-type grown on phosphate, 50 mL of culture, inoculated from a mid-log culture at an optical density (750 nm) of 0.08 was grown in a 125 mL Erlenmeyer flask, and mixed via magnetic stirring. Media composition was as described above, except at a reduced salinity of 8.75 g/L, via dilution of the seawater. Cultures were grown at constant 280 μmol/m^2/

s of light and 1.5% CO_2 in a Percival cultivation chamber (Percival Scientific). Optical density growth curves were generated using a TECAN M plex microplate reader, equipped with cuvette reader functionality (Tecan Group Ltd).

To compare *P. celeri* transformants grown on phosphite to wild-type grown on phosphate, 50 mL of culture, inoculated from a mid-log culture at an optical density of 0.15 was grown in a 250 mL Erlenmeyer flask, mixed on a shaker platform (200 rpm). Media composition was as described above at a salinity of 35 g/L (seawater). These cultures were grown at constant 450 μmol/m^2/s of light and 2% CO_2.

4.2. Construct assembly and transformation

The nuclear construct for *ptxD* expression was assembled via InFusion assembly (Takara Bio) of a pUC19 vector backbone and a synthesized gene fragment containing the *P. renovo* elongation factor promoter, *ptxD* gene from *Pseudomonas stutzeri* WM88, and *P. renovo* RuBisCO small subunit terminator (TWIST biosciences). Following assembly and sequence verification, a linearized PCR product of the *ptxD* gene and associated regulatory elements (Q5 Hot Start High-Fidelity 2× Master Mix, New England Biolabs, M0494L) was generated with primers oLRD74 and oLRD512 for transformation of *P. renovo* and *P. celeri*. Nuclear transformation was performed as described previously, with minor modification [25]. Briefly, ~4.75×10^8 early log phase cells were harvested, washed with 375 mM D-sorbitol and electroporated at 1900 V and a 35 ms time constant with 4 μg of PCR linearized DNA.

The *P. renovo* chloroplast *ptxD* vector was assembled via InFusion assembly of our previously established vector (pLRD037, PCR linearized with oLRD245 and oLRD286) and a synthesized gene fragment of an RBS (ATTGATATTAAGCTCTTTCTAACG), mutated *ptxD* gene (to greatly increase $NADP^+$ utilization), and a synthetic terminator [25,39–41]. The assembled plasmid was sequence verified and a linearized PCR product comprised of the homology arms (to the tRNA-I and tRNA-A region), RBS, *ptxD* gene, and terminator was generated with Q5 polymerase and primers oLRD208 and oLRD209, for biolistic transformation of *P. renovo*.

The *P. celeri* chloroplast *ptxD* vector was assembled via InFusion assembly of homology arms (to the tRNA-I and tRNA-A region) amplified from *P. celeri* gDNA (Lucigen MasterPure Yeast DNA Purification Kit) and a PCR product containing the ribosomal binding site, *ptxD* gene and terminator was amplified with oLRD409 and oLRD253, utilizing the above *P. renovo* construct as a template. The upstream and downstream homology arms were PCR amplified with oLRD480 and oLRD481, and oLRD482 and oLRD483 respectively. This was assembled into a pUC19 backbone to yield an intact plasmid. A PCR product utilizing this new plasmid as a template was generated with primers oLRD496 and oLRD497 for transformation, containing the relevant homology arms, RBS, *ptxD* gene, and terminator.

Chloroplast transformation was performed as described previously [25]. Briefly, DNA (4.5 μL at 1000 ng/μL) was precipitated onto 550 nm gold microcarriers (30 μL, Seashell Technology, now Critter Technology), utilizing $CaCl_2$ (25 μL at 2.5 M) and spermidine (10 μL at 0.1 M). Cells were plated directly onto the above media at a salinity of 8.75 g/L, with phosphite in place of phosphate for media formulation. These cells were bombarded with the DNA coated gold microcarriers utilizing a 1100 psi rupture disk at 25 in of Hg vacuum. Colony PCR, via boiling in Y-PER (ThermoFisher), and gel electrophoresis was performed as reported previously, to verify insertion [25,28].

4.3. Compositional analysis

Compositional analysis for lipids, measured as fatty acid methyl esters (FAME), and carbohydrates, measured as monomeric sugars from hydrolyzed biomass was performed as reported previously [25,44]. Briefly, cells were harvested and following lyophilization were analyzed for residual moisture and ash to calculate ash free dry weight. FAME analysis was performed via transesterification of lyophilized biomass with chloroform and methanol, extracted with hexane and analyzed via gas chromatography and flame ionization detection. Carbohydrates were determined via hydrolysis of lyophilized biomass with sulfuric acid and autoclaving, analysis of the resultant monomeric sugars was performed via high pressure anion exchange liquid chromatography with pulsed amperometric detection.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2021.102604.

CRediT authorship contribution statement

Lukas Dahlin: Conceptualization, Investigation, Formal analysis, Writing- original draft, Writing – review & editing. **Michael Guarnieri**: Conceptualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Dr. Alexander Meyers (NREL) for insightful discussions regarding the use of phosphite as a selection agent. We also thank Stefanie Van Wychen, Alicia Sowell, Hannah Alt, Bonnie Panczak, and Bi Nguyen (all at NREL), for technical assistance on compositional analyses.

This research was supported by the Department of Energy, Office of Energy Efficiency and Renewable Energy (EERE) under Agreement No. 28812 and Office of Science, Office of Biological and Environmental Research, Genomic Science Program under Secure Biosystems Design Science Focus Area IMAGINE BioSecurity: Integrative Modeling and Genome-scale Engineering for Biosystems Security, under contract number DE-AC36-08GO28308. The views and opinions of the authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, expressed or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights.

Statement of informed consent

No conflicts, informed consent, human or animal rights applicable.

Declaration of authors' agreement to authorship and submission

All authors have agreed to authorship and the submission of this manuscript for peer review.

References

[1] Y. Chisti, Biodiesel from microalgae, Biotechnol. Adv. 25 (2007) 294–306.
[2] D.R. Georgianna, M.J. Hannon, M. Marcuschi, S. Wu, K. Botsch, A.J. Lewis, J. Hyun, M. Mendez, S.P. Mayfield, Production of recombinant enzymes in the marine alga Dunaliella tertiolecta, Algal Res. 2 (2013) 2–9.
[3] J. Wichmann, T. Baier, E. Wentnagel, K.J. Lauersen, O. Kruse, Tailored carbon partitioning for phototrophic production of (E)-α-bisabolene from the green microalga Chlamydomonas reinhardtii, Metab. Eng. 45 (2018) 211–222.
[4] B.A. Rasala, M. Muto, P.A. Lee, M. Jager, R.M.F. Cardoso, C.A. Behnke, P. Kirk, C. A. Hokanson, R. Crea, M. Mendez, S.P. Mayfield, Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of Chlamydomonas reinhardtii, Plant Biotechnol. J. 8 (2010) 719–733.
[5] J.C. Quinn, R. Davis, The potentials and challenges of algae based biofuels: a review of the techno-economic, life cycle, and resource assessment modeling, Bioresour. Technol. 184 (2015) 444–452.

[6] R. Davis, A. Aden, P.T. Pienkos, Techno-economic analysis of autotrophic microalgae for fuel production, Appl. Energy 88 (2011) 3524–3531.
[7] R.L. White, R.A. Ryan, Long-term cultivation of algae in open-raceway ponds: lessons from the field, Ind. Biotechnol. 11 (2015) 213–220.
[8] D.L. López-Arredondo, L. Herrera-Estrella, Engineering phosphorus metabolism in plants to produce a dual fertilization and weed control system, Nat. Biotechnol. 30 (2012) 889–893.
[9] D.L. López-Arredondo, L. Herrera-Estrella, A novel dominant selectable system for the selection of transgenic plants under in vitro and greenhouse conditions based on phosphite metabolism, Plant Biotechnol. J. 11 (2013) 516–525.
[10] M. Manna, V.M.M. Achary, T. Islam, P.K. Agrawal, M.K. Reddy, The development of a phosphite-mediated fertilization and weed control system for rice, Sci. Rep. 6 (2016) 1–12.
[11] D. Pandeya D. L. López-Arredondo M. R. Janga L. M. Campbell P. Estrella-Hernández V. Bagavathiannan L. Herrera-Estrella K. S. Rathore Selective fertilization with phosphite allows unhindered growth of cotton plants expressing the ptxD gene while suppressing weeds, doi:10.1073/pnas.1804862115.
[12] H.N. Nahampun, D. López-Arredondo, X. Xu, L. Herrera-Estrella, K. Wang, Assessment of ptxD gene as an alternative selectable marker for Agrobacterium-mediated maize transformation, Plant Cell Rep. 35 (2016) 1121–1132, 2016 355.
[13] K. Kanda, T. Ishida, R. Hirota, S. Ono, K. Motomura, T. Ikeda, K. Kitamura, A. Kuroda, Application of a phosphite dehydrogenase gene as a novel dominant selection marker for yeasts, J. Biotechnol. 182–183 (2014) 68–73.
[14] P. Che, A. Anand, E. Wu, J.D. Sander, M.K. Simon, W. Zhu, A.L. Sigmund, G. Zastrow-Hayes, M. Miller, D. Liu, S.J. Lawit, Z.Y. Zhao, M.C. Albertsen, T. J. Jones, Developing a flexible, high-efficiency agrobacterium-mediated sorghum transformation system with broad application, Plant Biotechnol. J. 16 (2018) 1388–1395.
[15] M.M. Loera-Quezada, M.A. Leyva-González, G. Velázquez-Juárez, L. Sanchez-Calderón, M. Do Nascimento, D. López-Arredondo, L. Herrera-Estrella, A novel genetic engineering platform for the effective management of biological contaminants for the production of microalgae, Plant Biotechnol. J. 14 (2016) 2066–2076.
[16] J.M. Sandoval-Vargas, K.S. Macedo-Osorio, N.V. Durán-Figueroa, C. Garibay-Orijel, J.A. Badillo-Corona, Chloroplast engineering of Chlamydomonas reinhardtii to use phosphite as phosphorus source, Algal Res. 33 (2018) 291–297.
[17] S. Changko, P.D. Rajakumar, R.E.B. Young, S. Purton, The phosphite oxidoreductase gene, ptxD as a bio-contained chloroplast marker and crop-protection tool for algal biotechnology using Chlamydomonas, Appl. Microbiol. Biotechnol. 104 (2020) 675–686.
[18] J.M. Sandoval-Vargas, L.A. Jiménez-Clemente, K.S. Macedo-Osorio, M.C. Oliver-Salvador, L.C. Fernández-Linares, N.V. Durán-Figueroa, J.A. Badillo-Corona, Use of the ptxD gene as a portable selectable marker for chloroplast transformation in Chlamydomonas reinhardtii, Mol. Biotechnol. 61 (2019) 461–468.
[19] E. Cutolo, M. Tosoni, S. Barera, L. Herrera-Estrella, L. Dall'Osto, R. Bassi, A phosphite dehydrogenase variant with promiscuous access to nicotinamide cofactor pools sustains fast phosphite-dependent growth of transplastomic Chlamydomonas reinhardtii, Plants 9 (2020) 473.
[20] D. Polyviou, A. Hitchcock, A.J. Baylay, C.M. Moore, T.S. Bibby, Phosphite utilization by the globally important marine diazotroph Trichodesmium, Environ. Microbiol. Rep. 7 (2015) 824–830.
[21] K. Motomura, K. Sano, S. Watanabe, A. Kanbara, A.H. Gamal Nasser, T. Ikeda, T. Ishida, H. Funabashi, A. Kuroda, R. Hirota, Synthetic phosphorus metabolic pathway for biosafety and contamination management of cyanobacterial cultivation, ACS Synth.Biol. 7 (2018) 2189–2198.
[22] T.T. Selão, A. Włodarczyk, P.J. Nixon, B. Norling, Growth and selection of the cyanobacterium Synechococcus sp. PCC 7002 using alternative nitrogen and phosphorus sources, Metab. Eng. 54 (2019) 255–263.
[23] S.I. González-Morales, N.B. Pacheco-Gutiérrez, C.A. Ramírez-Rodríguez, A.A. Brito-Bello, P. Estrella-Hernández, L. Herrera-Estrella, D.L. López-Arredondo, Metabolic engineering of phosphite metabolism in Synechococcus elongatus PCC 7942 as an effective measure to control biological contaminants in outdoor raceway ponds, Biotechnol. Biofuels 13 (2020) 119.
[24] N. Toda, H. Murakami, A. Kanbara, A. Kuroda, R. Hirota, Phosphite reduces the predation impact of Poterioochromonas malhamensis on cyanobacterial culture, Plants 10 (2021) 1361, 2021, Vol. 10, Page 1361.
[25] L.R. Dahlin, A.T. Gerritsen, C.A. Henard, S. Van Wychen, J.G. Linger, Y. Kunde, B. T. Hovde, S.R. Starkenburg, M.C. Posewitz, M.T. Guarnieri, Development of a high-productivity, halophilic, thermotolerant microalga Picochlorum renovo, Commun. Biol. 2 (2019), https://doi.org/10.1038/s42003-019-0620-2.
[26] J.C. Weissman, M. Likhogrud, D.C. Thomas, W. Fang, D.A.J. Karns, J.W. Chung, R. Nielsen, M.C. Posewitz, High-light selection produces a fast-growing Picochlorum celeri, Algal Res. 36 (2018) 17–28.
[27] W.J. Henley, J.L. Hironaka, L. Guillou, M.A. Buchheim, J.A. Buchheim, M. W. Fawley, K.P. Fawley, Phylogenetic analysis of the 'Nannochloris-like' algae and diagnoses of Picochlorum oklahomensis gen. et sp. nov. (Trebouxiophyceae, Chlorophyta), Phycologia 43 (2004) 641–652.
[28] L.R. Dahlin, M.T. Guarnieri, Development of the high-productivity marine microalga, Picochlorum renovo, as a photosynthetic protein secretion platform, Algal Res. 54 (2021), 102197.
[29] A. Krishnan, M. Cano, T.A. Burch, J.C. Weissman, M.C. Posewitz, Genome editing using Cas9-RNA ribonucleoprotein complexes in the high-productivity marine alga Picochlorum celeri, Algal Res. 49 (2020), 101944.
[30] A. Krishnan, M. Likhogrud, M. Cano, S. Edmundson, J.B. Melanson, M. Huesemann, J. McGowen, J.C. Weissman, M.C. Posewitz, Picochlorum celeri as a model system for robust outdoor algal growth in seawater, Sci. Rep. 11 (2021) 11649.
[31] M. Cano, D.A.J. Karns, J.C. Weissman, M.L. Heinnickel, M.C. Posewitz, Pigment modulation in response to irradiance intensity in the fast-growing alga Picochlorum celeri, Algal Res. 58 (2021), 102370.
[32] F. Foflonker, G. Ananyev, H. Qiu, A. Morrison, B. Palenik, G.C. Dismukes, D. Bhattacharya, The unexpected extremophile: tolerance to fluctuating salinity in the green alga Picochlorum, Algal Res. 16 (2016) 465–472.
[33] F. Foflonker, D. Mollegard, M. Ong, H.S. Yoon, D. Bhattacharya, Genomic analysis of Picochlorum species reveals how microalgae may adapt to variable environments, Mol. Biol. Evol. 35 (2018) 2702–2711.
[34] F. Foflonker, D.C. Price, H. Qiu, B. Palenik, S. Wang, D. Bhattacharya, Genome of the halotolerant green alga Picochlorum sp. reveals strategies for thriving under fluctuating environmental conditions, Environ. Microbiol. 17 (2015) 412–426.
[35] R.J.P. Barten, R.H. Wijffels, M.J. Barbosa, Bioprospecting and characterization of temperature tolerant microalgae from Bonaire, Algal Res. 50 (2020), 102008.
[36] M.M. Loera-Quezada, M.A. Leyva-González, D. López-Arredondo, L. Herrera-Estrella, Phosphite cannot be used as a phosphorus source but is non-toxic for microalgae, Plant Sci. 231 (2015) 124–130.
[37] V.M.M. Achary, B. Ram, M. Manna, D. Datta, A. Bhatt, M.K. Reddy, P.K. Agrawal, Phosphite: a novel P fertilizer for weed management and pathogen control, Plant Biotechnol. J. 15 (2017) 1493–1508.
[38] R. Woodyer, W.A. Van der Donk, H. Zhao, Relaxing the nicotinamide cofactor specificity of phosphite dehydrogenase by rational design, Biochemistry 42 (2003) 11604–11614.
[39] Y.J. Chen, P. Liu, A.A.K. Nielsen, J.A.N. Brophy, K. Clancy, T. Peterson, C.A. Voigt, Characterization of 582 natural and synthetic terminators and quantification of their design constraints, Nat. Methods 10 (2013) 659–664.
[40] T. Tian, H.M. Salis, A predictive biophysical model of translational coupling to coordinate and control protein expression in bacterial operons, Nucleic Acids Res. 43 (2015) 7137–7151.
[41] H.M. Salis, E.A. Mirsky, C.A. Voigt, Automated design of synthetic ribosome binding sites to control protein expression, Nat. Biotechnol. 27 (2009) 946–950, 2009 2710.
[42] D. Verma, H. Daniell, Chloroplast vector systems for biotechnology applications, Plant Physiol. 145 (2007) 1129–1143.
[43] R. Hirota, K. Abe, Z.I. Katsuura, R. Noguchi, S. Moribe, K. Motomura, T. Ishida, M. Alexandrov, H. Funabashi, T. Ikeda, A. Kuroda, A novel biocontainment strategy makes bacterial growth and survival dependent on phosphite, Sci. Rep. 7 (2017) 1–10.
[44] L.R. Dahlin, S. Van Wychen, H.G. Gerken, J. McGowen, P.T. Pienkos, M. C. Posewitz, M.T. Guarnieri, Down-selection and outdoor evaluation of novel, halotolerant algal strains for winter cultivation, Front. Plant Sci. 871 (2018), https://doi.org/10.3389/fpls.2018.01513.