4-2017

Phytohormone signaling in Chlorella sorokiniana: perspectives on the evolution of plant cell-to-cell signaling

Maya Khasin
University of Nebraska-Lincoln, mkhasin2@unl.edu

Phytohormone Signaling in *Chlorella sorokiniana*: Perspectives on the Evolution of Plant Cell-to-Cell Signaling

by

Maya Khasin

A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Biological Sciences

(Genetics, Cellular and Molecular Biology)

Under the Supervision of Professors Kenneth W. Nickerson and Wayne R. Riekhof

Lincoln, Nebraska

April, 2017

Phytohormone Signaling in *Chlorella sorokiniana*: Perspectives on the Evolution of Plant Cell-to-Cell Signaling

Maya Khasin, Ph.D.

University of Nebraska, 2017

Advisers: Kenneth W. Nickerson, Wayne R. Riekhof

Cell-to-cell communication is a key aspect of microbial physiology and population dynamics, and a cornerstone in understanding the evolution of multicellularity. Quorum sensing in bacteria is a canonical example of microbial cell-to-cell signaling, in which bacteria use small molecule signals in order to monitor their population size and modulate their physiology accordingly. We propose that the evolution of plant hormone signaling arose in unicellular green algae, analogously to quorum sensing in bacteria, and that the complexity of these pathways required the recruitment of increasingly specific enzymes to increasingly sophisticated gene networks throughout the course of phytohormone signaling evolution. Using *Chlorella sorokiniana* UTEX 1230 as a model system, we address the evolution of cell-to-cell signaling from the perspective of phytohormone signaling evolution, particularly the evolution of indole-3-acetic acid (IAA) signaling and abscisic acid (ABA) signaling. We demonstrate that key components of these phytohormone signaling pathways are present in *C. sorokiniana*, and that these hormones are present and active in the physiology of these organisms. Indeed, the distribution of early auxin signaling related orthologs in the chlorophytes suggests that some auxin signaling machinery was available early in the evolution of plants. Abscisic acid (ABA) is a phytohormone that has been extensively

characterized in higher plants for its role in stress response. This dissertation demonstrates that ABA is involved in regulating algal stress responses in Chlorella; additionally, the genome contains orthologs to essential genes in higher plants that control ABA biosynthesis, sensing, and degradation. Transcriptomic studies reveal that treatment with ABA induces dramatic changes in gene expression profiles, including transcripts associated with ABA signaling in higher plants. The physiological effects of phytohormones, together with the presence of phytohormone signaling orthologs, suggest that phytohormone signaling evolved as an intercellular stress response signaling molecule in eukaryotic microalgae prior to the evolution of multicellularity and colonization of land.

This dissertation is dedicated to Rand Nashi and Julian K. Jarboe, who have been with me since the beginning and whose strength gives me strength. Thank you.

ACKNOWLEDGEMENTS

I am humbled and grateful for the support I have received from friends, family, and faculty and staff throughout the pursuit of my doctoral degree. I would like to thank my advisors Dr. Kenneth W. Nickerson and Dr. Wayne R. Riekhof. You allowed (and encouraged!) me to pursue a risky project, brainstormed creative solutions in a field new to all three of us, and patiently offered intensely helpful support, advice, and guidance. I consider myself extremely fortunate to have joined a lab that allows and encourages the pursuit of imaginative, unconventional, and challenging projects.

I would like to thank the members of my committee: Dr. Heriberto Cerutti, Dr. Paul Blum, and Dr. James van Etten. Thank you for encouraging creativity and rigor throughout the course of my project.

I would like to thank my undergraduate research advisor, Dr. Michele Klingbeil, from the University of Massachusetts - Amherst, Dr. Michele Klingbeil. Thank you for instilling the principles of detailed, rigorous scientific work, and for preparing me for graduate school.

This dissertation would not have come to fruition without the faculty and support staff at UNL. Dr. Istvan Ladunga, thank you for your nuanced thoughts on bioinformatics that allowed me to extract more precise meaning from my large datasets. Dr. Jean-Jack Riethoven and the other Bioinformatics Core Facility staff, thank you for your help as I learned to navigate databases and use computational tools to guide experimental hypotheses. Dr. Sophie Alvarez and Rebecca R. Cahoon, thank you for your analytical chemistry expertise that allowed me to troubleshoot and analyze challenging samples.

To Mindy Peck, no longer admissions coordinator at UNL but always a friend: your wisdom, your kind words in times of duress, and your friendly smile have helped me endure through difficult times and celebrate triumphant times.

To my friends in graduate school: thank you for being there through strugges, through frustration, and through happiness. Dr. Ruvini Pathirana, Nur Ras Aini Ahmad Hussin, Dr. Sahar Hasim, Dr. Krista Patefield, Dr. Jessica Hargarten, Dr. Kempton Horken, Dr. Drew Brueggeman, Dr. Joseph Msanne, Brenna Zimmer, Samantha Swenson, Shelbi Christgen, Christine Prinsloo, Dr. Aja Hyde, Dr. Caitlin McAtee, Ethan Jensen, Julien Gradnigo, Jennie Catlett, Sean and Erin Carr, Ilonka Zlatar, Dr. Amy Ort, Dr. Danielle Tufts: thank you for being there for me through this journey. To my friends outside of graduate school: members of the music and arts community, particularly Chelsea Egenberger, Monica Maher, Kat Morrow, Rachel Tomlinson Dick, Rebecca Lowry, Andrea Kszystyniak, William Johnson, Daphne Calhoun, Jen Skidmore, Luke Wegener, there are too many to name – thank you for keeping me grounded, for keeping me engaged with the art and culture around me, and thank you for being incredible friends. To lifelong friends Mikaela Ortstein and Matt Drake, Rand Nashi, and Julian K. Jarboe.

To my family: thank you for encouraging a scientific worldview from an early age, for encouraging me to investigate the world around me, and for nurturing my interest in molecular biology. Thank you for your unwavering support throughout my education and throughout my life. You have given me everything. To my in-laws, Linda Urban, Allie Wynn, and Clyde Wynn, thank you for providing a “home away from home” in Nebraska. Finally, and most importantly, I am deeply grateful to my loving wife, Emily

Wynn, whose support, encouragement, and love buoyed me through some of the most difficult times and some of the happiest times in graduate school. Thank you all, thank you so much, thank you for everything.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

A REVIEW OF PHYTOHORMONES AS INTERCELLULAR SIGNALS IN THE CONTEXT OF PLANT EVOLUTION

The classic conceptualization of cell-cell signaling in unicellular organisms is bacterial quorum sensing, in which bacteria use small molecule signals, usually acyl homoserine lactones (AHLs) or peptides, to assess and modulate their population size or physiology using circuitlike gene networks. Originally discovered and characterized in *Vibrio fischeri*, quorum sensing has been discovered in many bacterial species, extensively reviewed by (1). It is rare that an organism exists as a pure culture in nature, and it is in the best interest of microorganisms to structurewa their communities to optimize conditions for themselves and for mutualistic and commensalistic organisms. Symbiotic microbial relationships are well characterized, from polymicrobial communities that colonize the animal gut (Arnold 2016) to intertidal, hypersaline, and hot spring microbial mats (2). Cell-cell communication is central to the organization and maintenance of these highly structured microbial communities: AHLs with photoperiod-dependent side chain length have been extracted from microbial mats in marine stromatolites (3), implying that organisms are able to monitor and manage their environment specific to the time of day. Quorum sensing inhibitory compounds have also been isolated from mixed communities in hypersaline mats (4), inhibiting quorum sensing mediated reporter systems, particularly biofilm formation, in model strains.

This indicates that the species in microbial mats are able to modulate and structure their communities carefully and precisely.

Cross-kingdom communication is also well documented: For instance, both plant growth-promoting and plant pathogenic bacteria contain mechanisms for phytohormone perception and synthesis (5, 6). Additionally, animal hosts' nutritional intake and immune systems interact with and modulate the microbial community (7).

Cyanobacteria, the photosynthetic ancestors of chloroplasts, have previously been demonstrated to utilize phytohormones as a means of communication. While AHL-mediated quorum sensing has been discovered in some cyanobacteria, other cyanobacteria have been found to make plant hormones (8),. While the study of phytohormone mediated function in cyanobacteria is an emerging field, it appears that phytohormones mediate some instances of cell-cell communication. Indeed, *Synechocystis* exhibits phototaxis in the presence of ethylene (9), meeting the definition of quorum sensing in terms of a population-wide response to a stimulus (however, it is unclear whether *Synechocystis* produces endogenous ethylene). **Rice paddies – symbiotic cyanobacteria**

A microbe's ability to modulate and interact with its surrounding biotic community is a powerful adaptation. We propose that microalgal cell-cell signaling arose, analogously to quorum sensing in bacteria, in order to coordinate a population- or community-wide response to environmental stresses, nutrient conditions, population size, and other environmental conditions the population may encounter. Central to this review is the concept that membrane permeability and subsequently the ability to perceive a signal is a key component of cell-cell signaling. In the case of AHLs, QS

molecules generally have an amphipathic structure and diffuse freely across cell membranes in a population. Gram positive bacteria use peptides. In higher organisms, cell to cell communication uses a combination of small molecules (hormones, peptides) and macromolecules (non-cell autonomous proteins, including transcription factors) (10, 11).

Cell-cell signaling: a cornerstone of microbial ecology

Quorum sensing

The central principle of quorum sensing is that a sizeable population of bacteria has a different optimal behavior than a single cell, and quorum sensing allows populations to recognize this and modulate their behavior accordingly. First discovered in Hawaiian squid light organ colonist *Vibrio fischeri,* the canonical exemplar of quorum sensing in Gram negative bacteria is the acyl homoserine lactone (AHL) and the LuxI/LuxR family of proteins. LuxI and LuxR control expression of the luciferase operon (*luxICDABE*). LuxI synthesizes the AHL signal, 3OC6-homoserine lactone, and LuxR is the transcriptional repressor. LuxI is itself a part of the operon, creating a positive feedback system where derepression of LuxR causes increasing amounts of AHL to be produced, and sufficient expression of the operon results in light production.

Since its discovery, quorum sensing has been found and characterized in many diverse systems, from biomedically relevant biofilms like *Pseudomonas, Candida,* and *Staphylococcus* to marine bacteria whose biofilms cause biofouling (1). Gram positive bacteria typically use peptide signals, and one species of Archaea has been found to use AHLs as well (12).

Evidence of AHL-mediated cell-cell signaling in cyanobacteria

Several systems of cell-cell signaling in cyanobacteria have been identified. Sharif et al. were able to identify AHL production in *Gloeothece* PCC 6909 and determine that it acted in an autoinducer-like manner: a C8-AHL was found to accumulate within the culture supernatant only at a critical cell density, and 2D gel electrophoresis analysis indicated that this accumulation effected differential expression of several proteins, including RuBisCo (8). Though the complete function of this AHL autoinducer system has not been reported in great detail, the authors propose that the differential expression demonstrated in the proteome, especially the upregulation of RuBisCo, may indicate an increase in carbon fixation.

In other cases, while AHLs have not been identified as an independent signaling mechanism, they have been found to affect metabolism: AHLs cause nitrogen fixation inhibition in *Anabena* PCC7120 independently of heterocyst formation or differentiation, possibly by acting post-transcriptionally on the nitrogenase (13). QS inhibitory compounds have been characterized from a marine compound library, including peptides, amides, and malingolyde, a lipid, that varyingly inhibited QS and/or growth in reporter strains (14). The same group also identified malyngolide (MAL) in the ethyl acetate:methanol 1:1 fraction of extract from the cyanobacterium *Lyngbya majestica*, which inhibited LasR based AHL reporters (4). Lyngbyoic acid, from the same organism, is another quorum sensing disruptor in *Pseudomonas* (15). In this way, cyanobacteria may have developed quorum sensing and quorum quenching abilities to ensure their survival in microbial mats as previously reported (16).

Cyanobacterial signaling using phytohormones: interspecies and potential intraspecies communication

Previous studies have demonstrated the apparent biosynthesis of phytohormones in some cyanobacterial species, and the perception and signal transduction in other systems. To date, no study has unified these signaling modules in a cohesive system. However, there is compelling evidence for the evolution of phytohormone signaling in cyanobacteria. In the interest of space, we will primarily discuss the phytohormones ethylene, cytokinin, auxin, and abscisic acid, but we anticipate that the perspective below will inform the study of the evolution of the other plant hormones and signaling molecules. Perhaps the most detailed study concerns the functionality of *Synechocystis* ETR1, the canonical ethylene receptor. In *Arabidopsis*, ETR1 encodes a histidine kinase that is part of a two-component system that regulates the ethylene signaling cascade with response regulator ARR2. In an elegant study, Lacey and Binder (2016) demonstrated that *Synechocystis* exhibits phototaxis in the presence of ethylene via a pathway mediated by ETR1 (9). Though no cyanobacteria have yet been reported to make ethylene, this demonstrates the age of the ethylene signaling mechanism, and it is possible that *Synechocystis* uses this pathway in consortia.

ABA has been found to act as a Ca^{2+} agonist in a wide range of systems, from animal smooth muscle to cyanobacteria (17). Huddart et al. discovered that addition of exogenous ABA to cyanobacteria induces increased heterocyst differentiation, a phenomenon replicated by a calcium ionophore (A23187) (18). Cyanobacteria have also

been found to produce ABA under drought and saline stress, suggesting that ABA might be a cell-cell signaling molecule (17) (Zahradničková et al., 1991).

Cytokinin signaling may also trace its origins to a bacterial system. In higher plants, its receptor is a two-component system, typically a bacterial means of signal transduction. The prevailing hypothesis holds that the cytokinin signaling pathway components were acquired during a HGT event during the engulfment event that led to the chloroplast. It was later reported that cytokinins appear enhance transcription in an *in vitro* system containing the DNA and RNA polymerase of *Synechocystis* PCC 6803 (20). Shevchenko et al. (2014) demonstrated zeatin stimulates *in vitro* transcription in this system, in a *Synechocystis* lysate, and in barley lysates, and isolated a 67 kDA cytokinin binding protein that increased the effect on transcription (21). The CHASE domain in cyanobacteria receptors of the AHK family was found to be essential for cytokinin binding. This domain was identified in bacteria and in higher plants; however, not all amino acids essential for cytokinin binding were conserved in the organisms surveyed. However, no cyanobacteria were surveyed in this study (22).

Nostoc PCC 9229 and PCC 268 and *Anabena* spp were hypothesized to make IAA in a Trp-dependent way based on the accumulation of IAA in response to tryptophan addition to the medium (23, 24). However, the potential role of tryptophan utilization as a nitrogen source, thus leading to accumulation of IAA for a different reason, has not been discussed, and radioactive pulse-chase experiments would help clarify this question. Hashtroudi et al. characterized endogenous auxins isolated from (RICE PADDY??) cyanobacteria, identifying IAA, IPA, and IBA in the growth-promoting exudates,. Interestingly, they report a predominance of IBA, compared to

plants, in which IAA is the predominant auxin (25). Ahmed et al. demonstrated that IAA production occurred in rhizospheric cyanobacteria in a circadian-dependent manner (as in plants), and that cyanobacteria colonize roots by forming biofilms, penetrating roots at 5-7 μm (26). If these two factors are connected, it would be extremely suggestive of a quorum sensing mechanism, reminiscent of the lifestyle switch between planktonic and biofilm in species such as *Pseudomonas* and in *Clostridia.* Auxin has an effect on rice field symbionts as well: a concentration of 200μg/mL, IAA was found to be associated with a modest increase in cell yield, but caused growth inhibition higher concentrations. (27). Could it be possible that an overabundance of auxin either acts as or mimics the signal of a saturated culture, inhibiting cyanobacterial growth?

Other cases in which phytohormones have affected cyanobacteria have also been reported, such as the addition of gibberellic acid increasing biomass in *Nostoc* (28). However, while speculation about receptors and physiological effects of phytohormones set up a suggestive story, a complete sensing signal has yet to be described. A time course RNA-Seq experiment exploring the transcriptional effect of hormones would begin to help clarify this question. Limited data on phytohormones in cyanobacteria warrants more study that will hopefully shed more light on the early evolution of phytohormone signaling.

On a final note, Yeh et al. discovered Cph1, a phytochrome two-component light sensory system in the cyanobacterium *Fremyella diplosiphon,* and suggested that due to the ethylene and cytokinin receptors being two component systems, it is worth investigating Cph1 in terms of signal integration (29). Sweere et al. (2001) demonstrated that Cph1 interacts with the cytokinin response regulator ARR4 in *Arabidopsis,*

suggesting that this signal integration may be ancient and indicating that signal integration and transduction mechanisms must coevolve with perception mechanisms (30). To this note, plant hormones are far from the only signaling molecules in photosynthetic organisms: ROS and Ca^{2+} are indispensable small molecule signals, and looking for the evolution of these systems and integration thereof could lead to the discovery or development of useful model systems.

Macromolecular cell-cell signaling: septal junction signaling in filamentous cyanobacteria

Early study of heterocystous cyanobacteria demonstrated that heterocysts were involved in nitrogen fixation, and that a gradient of ammonia determined when the next heterocyst would be formed (31)(32). How did the cells know when and how to limit nitrogen diffusion? The answer lies in septal junction communication. In the *Anabena* PCC 7120 model, cytoplasmic bridges and a continuous periplasm connect cells, concisely reviewed in (33). Though not classical quorum sensing because these septal signals do not diffuse and re-enter, it is reminiscent of plasmodesmal cell-cell signaling as discussed in the evolution of eukaryotic multicellularity. Subsequent studies identified components of the connections and the proteins required for their connections: Omairi-Nasser et al. and Rudolf et al. identified peptidoglycan interacting proteins FraC, FraD and SjcF1, respectively, as essential for channel formation between cells (34, 35). Cell wall remodeling is essential to both filamentous cyanobacteria and to charophytes (36). Though the structures are not homologous, the analogous signaling mechanisms allow for maintenance and proliferation of a multicellular lifestyle.

Cell-cell signaling in basal photosynthetic eukaryotes: an essential component of the emergence of multicellularity

Though the role of plant hormones in eukaryotic microalgae has been explored using several different model systems, a cohesive model integrating phytohormone signaling with plasmodesmal cell-cell signaling, as seen in streptophytes, remains to be developed (37). Prior work in evolutionary cell-cell signaling has focused on the role of adhesins and mobile transcription factors. We propose that the integration of diverse signaling mechanisms, from phytohormone and other small molecule to macromolecular signaling (for instance by mobile transcription factors) is indispensable for the evolution of multicellularity. Furthermore, we propose that these mechanisms evolved and may have begun integrating in as early as the chlorophyte lineages.

Some forms of cell-cell signaling have been characterized in unicellular algae, such as mating factor secretion by *Chlamydomonas* (and matrix polysaccharides in a variety of chlorophytes, as previously reported (38). A study of *Isochrysis zhangjiangensis* quantitated changes in salicylic acid, jasmonic acid, and folic acids upon nitrogen starvation, finding an intracellular increase in SA and JA and a decrease in FA. The same study found that evidence of ROS signaling, another form of small molecule signaling in plants, was also active in the alga (39) 1 μM kinetin and IAA stimulated growth and β-carotene production in *Dunaliella salina* (40). A diverse group of algal growth-promoting bacteria have also been found to produce IAA(41). Based on compelling circumstantial evidence, genomic and physiological, Lu and Xu concisely review the body of evidence for phytohormone signaling in microalgae and suggest that

"phytohormone based communication among aquatic microalgal cells [may] demonstrate a parallel evolution with the quorum sensing by which bacteria use secreted signal molecules to regulate cell population density" and other traits (37). To date, however, no study has explored a potential molecular mechanism for extracellular cell-cell signaling in algae.

When researchers speak about cell-cell communication in the context of multicellularity, they frequently describe mobile transcription factor mediated cell to cell signaling. A number of diffusible transcription factors mediate diverse developmental and other functions in plants, often interacting with phytohormones in the context of cell wall remodeling that mediates permeability to transcription factors, reviewed in detail by (10). In higher plants, the biosynthesis of callose, a major cell wall glucan especially important in plasmodesmal permeability and is regulated by stress signaling, and plant hormones including auxins, salicylic acid, cytokinins, and gibberelins (42). In these higher plants, this leads to the execution of complex developmental programs. The presence of certain embryophyte cell wall components in charophycean green algae suggests that the biosynthetic capabilities were present (though latent) in charophytes and subsequently expanded upon in more complex evolutionary iterations (43). The *Klebsormidium flaccidum* genome encodes nearly complete biosynthetic pathways for IAA, JA, isopentenyl adenine, JA, and salicylic acid, whose presence was confirmed by mass spectrometry. GTG, a putative ABA receptor in *Klebsormidium crenulatum,* is downregulated in response to desiccation stress, and ABA signaling components PP2C and SnRK2 were upregulated. Because of the presence of orthologs to higher plant NCATFs and callose synthase as far back as the chlorophytes, we suggest that this

regulatory integration is an ancient communication strategy that paved the way for land plant evolution.

Whereas choanoflagellates appear to have had their one "big hit" with regard to multicellular lineages, multicellularity has appeared multiple times in diverse iterations, including at least six times in red algae, twice in brown algae, and twice in green algae (44, 45). By surveying diverse multicellular systems, researchers will be able to unify data from different evolutionary strategies and niches into a comprehensive model that speaks to the importance of signal integration in different strategies for multicellularity.

Though chlorophytes, including *Chlorella* species, encode putative orthologs to plant hormone signaling and for callose synthase, molecular work in basal charophytes brings us closer to identifying the emergence of signal integration. A transcriptomic profile of the ethylene response in the basal filamentous charophyte *Spirogyra pratensis* indicated downregulation of photosynthesis, induction of an abiotic stress response, and, central to our model, the modification of the cell wall matrix by expansins and endotransglucosylases/hydrolases (46). In *Klebsormidium crenulatum*, exposure to desiccation stress increases the transcript abundance of ethylene receptor orthlogs ETR and ERS, and differentially regulates several ABA signaling components as described above. A study in *Zygnema* S and E-A and in *Klebsormidium crenulatum, dissectum* (formerly *K. nitens*) found that in callose deposition was unaffected by drought stress in *Zygnema,* and its photosynthetic capacity and recovery from desiccation stress was (potentially causally) inhibited (47, 48). In *Klebsormidium,* callose deposition nearly doubled in response to ethylene stress, correlating to increased recovery from desiccation stress. In the context of the *Spirogyra* data, this seems to

suggest that drought stress may induce an ethylene response, potentially including ABA crosstalk, that alters gene expression and cell wall remodeling much as it does in higher plants. Indeed, 8/14 early (3h) regulated genes in *Spirogyra* had significant BLAST hits to the *Arabidopsis* proteome, suggesting that this pathway is indeed ancient. In the moss *Physcomitrella patens*, a canonical ethylene receptor, an ortholog to which is present in *Spirogyra*, has a dual role in abscisic acid and in ethylene signaling; however, this dual role is absent in higher plants (49). This finding suggests that phytohormone crosstalk is ancient as well, and has adapted and diversified in function throughout the course of evolution.

The study of phytohormone signaling in more developmentally complex charophytes continues to develop. For instance, aromatic and cytokinins and zeatins were not found to influence growth rates in the multicellular red alga *Gracilaria candata*, but were found to influence branching patterns and total soluble proteins, phenomena also regulated by cytokinins in higher plants (Souza 2016). Additionally, PIN2-like mediated polar auxin transport has been identified in *Chara vulgaris* and implicated in spermatogenesis, and predominantly localized to the plasmodesmal junctions (as in higher plants) (50). The authors speculate that the enhanced expression of PIN2-like proteins (PIN2-LPs) and resulting increased levels of auxin "suggest a functional integration among all these structural and regulatory elements might have appeared long before higher plants had evolved."

Multicellularity has undergone many evolutionary iterations in the green lineage. In modern ecosystems, it is important to evaluate findings in the context of the frequent coexistence of cyanobacteria, algae, bryophytes, higher plants, and many other

organisms. Therefore, plant hormones, among other secreted molecules, aid them in communicating their state, comprehending the state of their physical surroundings, and modulating these interactions. This is advantageous to both basic and translational research perspectives: understanding the pathways by which cells communicate can illuminate some of the factors that drive physiology; additionally, modulating or modifying these pathways could modify their physiology. All told, the interplay between phytohormone signaling, cell-cell macromolecular signaling, and the evolution, diversification, and specification of cell wall components is worth exploring in more detail in the chlorophyte and charophyte cyanobacterial lineages. We conclude that expanding the search for both transcription factor and hormone mediated cell-cell signaling genes, and any potential relationships, in the chlorophyte and basal streptophyte lineages will help fortify cell-cell signaling models in the context of the evolution of multicellularity and will additionally aid in the development of attractive model systems for plant hormone signaling and its interactions.

References

1. Waters CM, Bassler BL. 2005. Quorum Sensing : Communication in Bacteria. Annu Rev Cell Dev Biol 21:319–346.
2. Bolhuis H, Cretoiu MS, Stal LJ. 2014. Molecular ecology of microbial mats. FEMS Microbiol Ecol 90:335–350.
3. Decho AW, Visscher PT, Ferry J, Kawaguchi T, He L, Przekop KM, Norman RS, Reid RP. 2009. Autoinducers extracted from microbial mats reveal a surprising diversity of N-acylhomoserine lactones (AHLs) and abundance changes that may relate to diel pH. Environ Microbiol 11:409–420.
4. Abed RMM, Dobretsov S, Al-Fori M, Gunasekera SP, Sudesh K, Paul VJ. 2013. Quorum-sensing inhibitory compounds from extremophilic microorganisms isolated from a hypersaline cyanobacterial mat. J Ind Microbiol Biotechnol 40:759–772.
5. Nafisi M, Fimognari L, Sakuragi Y. 2015. Interplays between the cell wall and phytohormones in interaction between plants and necrotrophic pathogens. Phytochemistry 112:63–71.
6. Zúñiga A, Poupin MJ, Donoso R, Ledger T, Guiliani N, Gutiérrez R a, González B. 2013. Quorum sensing and indole-3-acetic acid degradation play a role in colonization and plant growth promotion of *Arabidopsis thaliana* by *Burkholderia phytofirmans* PsJN. Mol Plant-Microbe Interact 26:546–53.
7. Andrew L. Kau, Philip P. Ahern, Nicholas W. Griffin, Andrew L. Goodman JIG. 2011. Human nutrition, the gut microbiome, and the immune system. Nature 474:327–336.
8. Sharif DI, Gallon J, Smith CJ, Dudley ED. 2008. Quorum sensing in Cyanobacteria: N-octanoyl-homoserine lactone release and response, by the epilithic colonial cyanobacterium Gloeothece PCC6909. ISME J 2:1171–1182.
9. Lacey RF, Binder B. 2016. Ethylene Regulates the Physiology of the Cyanobacterium Synechocystis sp. PCC 6803 via an Ethylene Receptor. Plant Physiol 171:pp.00602.2016.
10. Han X, Kumar D, Chen H, Wu S, Kim JY. 2014. Transcription factor-mediated cell-to-cell signalling in plants. J Exp Bot 65:1737–1749.
11. Han X, Kim JY. 2016. Integrating Hormone- and Micromolecule-Mediated Signaling with Plasmodesmal Communication. Mol Plant 9:46–56.
12. Castro C, Zhang R, Liu J, Bellenberg S, Neu TR, Donati E, Sand W, Vera M. 2016. Biofilm formation and interspecies interactions in mixed cultures of thermo-acidophilic archaea Acidianus spp. and Sulfolobus metallicus. Res Microbiol 167:604–612.
13. Romero M, Muro-Pastor AM, Otero A. 2011. Quorum sensing N-acylhomoserine lactone signals affect nitrogen fixation in the cyanobacterium Anabaena sp. PCC7120. FEMS Microbiol Lett 315:101–108.
14. Dobretsov S, Teplitski M, Alagely A, Gunasekera SP, Paul VJ. 2010. Malyngolide from the cyanobacterium Lyngbya majuscula interferes with quorum

sensing circuitry. Environ Microbiol Rep 2:739–744.
15. Kwan JC, Meickle T, Ladwa D, Teplitski M, Paul V, Luesch H. 2011. Lyngbyoic acid, a “tagged” fatty acid from a marine cyanobacterium, disrupts quorum sensing in Pseudomonas aeruginosa. Mol Biosyst 7:1205–16.
16. Decho AW, Norman RS, Visscher PT. 2010. Quorum sensing in natural environments: emerging views from microbial mats. Trends Microbiol 18:73–80.
17. Minorsky P V. 2002. Abscisic acid: a universal signalling factor? Plant Physiol 128:788–9.
18. Huddart H, Smith RJ, Langton PD, Hetherington AM, Mansfield TA. 1986. Is abscisic acid a universally active calcium agonist? New Phytol 104:161–173.
19. Zahradn’\ičková H, Maršálek B, Polišenská M. 1991. High-performance thin-layer chromatographic and high-performance liquid chromatographic determination of abscisic acid produced by cyanobacteria. J Chromatogr A 555:239–245.
20. Selivankina SY, Zubkova NK, Kupriyanova E V., Lyukevich T V., Kusnetsov V V., Los D a., Kulaeva ON. 2006. Cyanobacteria respond to cytokinin. Russ J Plant Physiol 53:751–755.
21. Shevchenko G V., Karavaiko NN, Selivankina SY, Zubkova NK, Kupriyanova E V., Los D a., Kusnetsov V V., Kulaeva ON. 2014. Possible involvement of cyanobacteria in the formation of plant hormonal system. Russ J Plant Physiol 61:154–159.
22. Heyl A, Wulfetange K, Pils B, Nielsen N, Romanov G a, Schmülling T. 2007. Evolutionary proteomics identifies amino acids essential for ligand-binding of the cytokinin receptor CHASE domain. BMC Evol Biol 7:62.
23. Sergeeva E, Liaimer A, Bergman B. 2002. Evidence for production of the phytohormone indole-3-acetic acid by cyanobacteria. Planta 215:229–238.
24. Prasanna R, Joshi M, Rana A, Nain L. 2010. Modulation of IAA production in cyanobacteria by tryptophan and light. Polish J Microbiol 59:99–105.
25. Hashtroudi MS, Ghassempour A, Riahi H, Shariatmadari Z, Khanjir M. 2013. Endogenous auxins in plant growth-promoting Cyanobacteria-Anabaena vaginicola and Nostoc calcicola. J Appl Phycol 25:379–386.
26. Ahmed M, Stal LJ, Hasnain S. 2014. Biofilm Formation and Indole 3-Acetic Acid Production by Two Rhizospheric Unicellular Cyanobacteria. J Microbiol Biotechnol 24:1015–1025.
27. Jyothi BL. 2016. Study of herbicidal effect 0f 2 , 4-D on growth and cellular metabolites in cyanobacterium Synechococcus aeruginosus Nägeli from rice fields 7:144–146.
28. Mansouri H, Talebizadeh B. 2016. Effect of gibberellic acid on the cyanobacterium nostoc linckia. J Appl Phycol 28:2187–2193.
29. Yeh K. 1997. A Cyanobacterial Phytochrome Two-Component Light Sensory System. Science (80-) 277:1505–1508.
30. Sweere U, Eichenberg K, Lohrmann J, Mira-Rodado V, Bäurle I, Kudla J, Nagy F, Schäfer E, Harter K. 2001. Interaction of the response regulator ARR4 with phytochrome B in modulating red light signaling. Science (80-) 294:1108–1111.
31. Wolk CP, Ernst A, Elhai J. 1994. Heterocyst metabolism and development, p.

769–823. *In* The molecular biology of cyanobacteria. Springer.
32. Ogawa RE, Carr JF. 1969. The influence of nitrogen on heterocyst production in blue-green algae. Limnol Ocean 14:342–351.
33. Haselkorn R. 2008. Cell-cell communication in filamentous cyanobacteria. Mol Microbiol 70:783–785.
34. Omairi-Nasser A, Haselkorn R, Austin J. 2014. Visualization of channels connecting cells in filamentous nitrogen-fixing cyanobacteria. FASEB J 28:3016–3022.
35. Rudolf M, Tetik N, Ramos-Le??n F, Flinner N, Ngo G, Stevanovic M, Burnat M, Pernil R, Flores E, Schleiffa E. 2015. The peptidoglycan-binding protein SjcF1 influences septal junction function and channel formation in the filamentous cyanobacterium Anabaena. MBio 6:1–11.
36. Maldener I, Forchhammer K. 2015. Requirement of Cell Wall Remodeling for Cell – Cell Communication and Cell Differentiation in Filamentous Cyanobacteria of the Order Nostocales 2.
37. Lu Y, Xu J. 2015. Phytohormones in microalgae: A new opportunity for microalgal biotechnology? Trends Plant Sci 20:273–282.
38. Jutur PP, Nesamma AA, Shaikh KM. 2016. Algae-Derived Marine Oligosaccharides and Their Biological Applications. Front Mar Sci 3:83.
39. Wu S, Meng Y, Cao X, Xue S. 2016. Regulatory mechanisms of oxidative species and phytohormones in marine microalgae Isochrysis zhangjiangensis under nitrogen deficiency. Algal Res 17:321–329.
40. Mousavi P, Morowvat MH, Montazeri-Najafabady N, Abolhassanzadeh Z, Mohagheghzadeh A, Hamidi M, Niazi A, Ghasemi Y. 2016. Investigating the effects of phytohormones on growth and $β$-carotene production in a naturally isolates stain of Dunaliella salina.
41. Bagwell CE, Piskorska M, Soule T, Petelos A, Yeager CM. 2014. A diverse assemblage of indole-3-acetic acid producing bacteria associate with unicellular green algae. Appl Biochem Biotechnol 173:1977–1984.
42. Zavaliev R, Ueki S, Epel BL, Citovsky V. 2011. Biology of callose ($β$-1, 3-glucan) turnover at plasmodesmata. Protoplasma 248:117–130.
43. Sørensen I, Pettolino FA, Bacic A, Ralph J, Lu F, O'Neill MA, Fei Z, Rose JKC, Domozych DS, Willats WGT. 2011. The charophycean green algae provide insights into the early origins of plant cell walls. Plant J 68:201–211.
44. Umen JG. 2014. Green Algae and the Origins of Multicellularity in the Plant Kingdom. Cold Spring Harb Perspect Biol 6.
45. Domozych DS, Domozych CE. 2014. Multicellularity in green algae: upsizing in a walled complex. Front Plant Sci 5:1–8.
46. Van de Poel B, Cooper ED, Van Der Straeten D, Chang C, Delwiche CF. 2016. Transcriptome profiling of the green alga *Spirogyra pratensis* (Charophyta) suggests an ancestral role for ethylene in cell wall metabolism, photosynthesis, and abiotic stress responses. Plant Physiol 172:pp.00299.2016.
47. Herburger K, Holzinger A. 2015. Localization and quantification of callose in the streptophyte green algae Zygnema and Klebsormidium: correlation with desiccation tolerance. Plant Cell Physiol pcv139.

48. Herburger K, Karsten U, Holzinger A. 2015. Entransia and Hormidiella, sister lineages of Klebsormidium (Streptophyta), respond differently to light, temperature, and desiccation stress. Protoplasma.
49. Yasumura Y, Pierik R, Kelly S, Sakuta M, Voesenek LACJ, Harberd NP. 2015. An ancestral role for Constitutive Triple Response 1 (CTR1) proteins in both ethylene and abscisic acid signaling. Plant Physiol pp--00233.
50. Żabka A, Polit JT, Winnicki K, Paciorek P, Juszczak J, Nowak M, Maszewski J. 2016. PIN2-like proteins may contribute to the regulation of morphogenetic processes during spermatogenesis in Chara vulgaris. Plant Cell Rep 35:1655–1669.

CHAPTER 2

AUXIN SIGNALING RELATED GENES IN *CHLORELLA SOROKINIANA* UTEX 1230 INDICATE THE EARLY EMERGENCE OF AUXIN SIGNALING

Abstract

Indole-3-acetic acid is a ubiquitous small molecule found in all domains of life. It is the predominant and most active auxin in seed plants, where it coordinates a variety of complex growth and development processes. The molecular origin of auxin signaling in algae remains a matter of some controversy. In order to clarify the evolutionary context of algal auxin signaling, we undertook genomic and transcriptomic studies to assess auxin signaling potential in emerging model chlorophyte *Chlorella sorokiniana* UTEX 1230. *C. sorokiniana* produces the auxin indole-3-acetic acid (IAA), detectable in both the pellet and the supernatant (~ 1 μM), and encodes orthologs to genes related to the auxin response in higher plants. These include candidate genes for auxin biosynthesis, transport, sensing, and signal transduction. Candidate orthologs for the canonical AUX/IAA signaling pathway were not found; however, auxin-binding protein 1 (ABP1), an alternate auxin receptor, is present and highly conserved at essential auxin binding and zinc coordinating residues. Additionally, candidate orthologs for PIN proteins, responsible for intercellular, polar auxin transport in higher plants, were not found, but PILs (PIN-Like) proteins, a recently discovered family that mediates intracellular auxin transport, were found. The distribution of early auxin signaling orthologs in the chlorophytes suggests that some auxin signaling machinery was available early in the evolution of plants. Understanding the simplified auxin pathways in chlorophytes could aid in modeling phytohormone signaling and crosstalk in seed

plants, and in understanding the diversification and integration of developmental signals in higher plants.

Introduction

Auxin in seed plants. Auxin is a phytohormone that contributes to the execution of nearly all complex growth and development processes, including gravitropism, phototropism, and cell expansion and differentiation in plants. Indole-3-acetic acid (IAA) is the most potent and best-studied auxin in higher plants, and in *Arabidopsis thaliana* it is predominantly synthesized from tryptophan via the two-step TAA/YUC pathway: Tryptophan Transaminase of Arabidopsis 1 (TAA) converts tryptophan to indole-3-pyruvic acid (IPyA), and YUC, a family of flavin-dependent monooxygenases, converts IPyA to indole-3-acetic acid (IAA)(1). Tryptophan-independent auxin biosynthesis has also been reported to proceed via a cytosol-localized indole synthase, but the rest of the pathway remains undefined (2).

Polar auxin transport proceeds through tissues via PIN proteins (3), whereas intracellular auxin transport between organelles proceeds through PILs (PIN-like proteins)(4). In its protonated state, auxin can freely diffuse into the cell, where the more neutral pH cytoplasmic results in its deprotonation; therefore, the intracellular concentration of auxin is mediated primarily by efflux proteins of the ATP binding cassette family (specifically, ABCB4) and an auxin-specific amino acid permease like protein, AUX1(5)(6).

Three protein types acting in sequence mediate auxin perception in seed plants: TRANSCRIPT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX PROTEINS

(TIR1/AFB), subunits of a SCF-type E3 ubiquitin ligase and Auxin/INDOLE ACETIC ACID (Aux/IAA) transcriptional repressors. Auxin coordinates the interaction between TIR1/AFB and Aux/IAA proteins, targeting Aux/IAA proteins for 26S proteasome mediated degradation, which in turn derepresses the transcription of auxin response factors (ARFs) (reviewed by Wang and Estelle 2014). An additional auxin sensing pathway is mediated by ABP1, a cupin domain containing protein which effects rapid, nontranscriptional auxin responses including ion fluxes at the plasma membrane (8)(7).

The emerging field of molecular-based studies for plant hormone signaling in algae has revealed a surprisingly sophisticated repertoire of plant hormone signaling orthologs (9). The presence of auxin in the charophytes and chlorophytes has been widely reported; however, the specific roles and origins of auxin signaling in these algae remain obscure and a matter of dispute (10)(11)(12).

We used an *in silico* approach to identify putative orthologs for auxin-active genes in *Chlorella sorokiniana* UTEX 1230 and analyzed their evolutionary relationships with orthologs from other sequenced algae and plants). In particular, *C. sorokiniana* encodes an ortholog for auxin-binding protein 1 (ABP1) in which the auxin binding residues, as well as the zinc coordinating residues essential for auxin binding, as identified in corn (13), are conserved. Additionally, the presence of IAA within the cell pellet and secretion into the culture supernatant indicates a functional IAA biosynthesis pathway (14).

Materials and Methods

We used an *in silico* approach in order to identify putative orthologs for auxin signaling in *Chlorella* and in other chlorophytes. Putative auxin signaling orthologs and outgroups were selected from representative chlorophytes, charophytes, and plants, including seed plants, ferns, and mosses. Reciprocal BLAST searches on the genome revealed candidate genes, aligned with Clustal Omega (except for ABP1, which was aligned with Expresso) (15) and verified by phylogenetic analysis using MEGA 6.06 using the LG+I substitution model (16). Outgroups were selected based on the similarity of broad protein structure or function.

Phylogenetic analysis

ABP1. ABP1 was identified in the genome via reciprocal BLAST search with the *Z. mays* ortholog of ABP1 (13). The outgroup, GLP1, was selected due to its structural similarity as a comparatively short (145 AA) protein with a cupin domain(17).

AUX1. AUX1 is an amino acid permease like transporter specific for auxin transport (18). The outgroup, ANT1, is an aromatic amino acid transporter in plants (19). Amino acid permeases tend to be highly conserved, and these substrates are of similar structure (small, neutral aromatic molecules).

ABCB4. ABCB4 is an ATP binding cassette transporter (5)(6). The outgroup is annotated in *Arabidopsis* as a *p*-coumaric acid transporter; it was chosen because it transports a small, aromatic molecule (as is IAA) and contains a highly conserved ATP-binding cassette domain (20).

PILS. PILS (PIN-like proteins) are recently discovered proteins, similar to but distinct from the PIN polar auxin transporters in plants (4). In order to identify the

distinct lineages of PIN and PILS proteins, PILS proteins from representative plants were aligned with PIN proteins (3).

IBR5. Recently identified in *Arabidopsis* as a receptor specific for indole-3-butyric acid (21), IBR5 has orthologs that can be identified across plants and algae. MKP2 was chosen as an outgroup because both proteins contain MAP kinase phosphatase domains that are involved in complex plant hormone signaling networks (22).

Structural modeling

CsABP1 was structurally aligned to the PDB structure of *Zea mays* ABP1 bound with napthaleneacetic acid (NAA), PDB accession 1LRH using SWISSMODEL on default settings (23). This alignment was further investigated in PyMol v. 3.2, which was used to investigate the conservation of the auxin binding and zinc coordinating residues in CsABP1.

IAA quantitation

C. sorokinana UTEX 1230 cells were obtained from the University of Texas Culture Collection. 50 mL cultures were inoculated at an initial cell density of 5×10^6 cells/mL in Bold's Basal Medium (24). After two days of growth at a 16h light: 8h dark photoperiod, cells were harvested. The supernatant was dried down in a rotary evaporator (Buchi Rotavapor R-215, New Castle, DE) and resuspended in 1 mL methanol. The pellets were extracted with a solution of 80% acetonitrile/1% glacial acetic acid, which was evaporated under nitrogen (N-Evap 112 Nitrogen Evaporator, Organomotion Associates Inc., Berlin, MA) and resuspended in 1 mL methanol. IAA

was quantified via LC/MS/MS as described in (25). In place of a deuterated standard, we used a standard curve of IAA ranging from 1 pM to 1 µM.

Results and Discussion

Conservation of higher plant tryptophan dependent IAA biosynthesis, transport, and signal transduction pathways in *Chlorella sorokiniana*

Genome mining has revealed that *C. sorokiniana* contains orthologs to higher plant IAA biosynthetic enzymes (Figure 1, Table 1). In contrast to higher plants, a tryptophan specific transaminase was not identified; however, three putative general amino acid transaminases were discovered (Table 1). This finding of a limited number of general amino acid transaminases agrees with prior knowledge that most microbial transaminases are relatively nonspecific in their activity. It also suggests that the transaminases proliferated and became more specified in their function throughout higher plant evolution. Similarly, *C. sorokiniana* encodes genes for the formation of IAA by three different routes (Table 1): CsAMI1 for indole-3-acetamide hydrolase (26), CsYUC for indole-3-pyruvate monooxygenase (27), and CsAAO1 for indole3-acetaldehyde oxidase. The amino acid sequence of CsYUC is closest to *Arabidopsis* YUC5 and YUC7, which are expressed in roots (27), though no reciprocal BLAST hit was found. *Arabidopsis* TAA does not return a transaminase specific for tryptophan: the three putative orthologs listed in Table 1 are hits for the general amino acid transaminase family, as suggested by their expect values. However, most microorganisms have a limited number of amino acid transaminases able to interact with multiple amino acids. For instance, *E. coli* has four major transaminases, each of which can interact with 3-6

amino acids (28). Similarly, *S. cerevisiae* has four multisubstrate transaminases which participate in fusel alcohol formation via the Ehrlich pathway with two of these transaminases: Aro8p and Aro9p, neither of which have specific orthologs in *C. sorokiniana*, being broad substrate specificity transaminases for the aromatic amino acids (29). *Arabidopsis* encodes eleven YUC family flavin containing monooxygenases whose expression is tissue dependent, reiterating the theme of proliferation and recruitment of enzymes to specific pathways and locations throughout the course of plant evolution. It does not contain any PIN orthologs.

Conservation of IAA transporters

Arabidopsis contains at least four classes of auxin transporters (Figure 1, Table 1): PIN proteins, which are efflux transporters that mediate polar auxin transport (3); PILS (PIN-like) transporters, which control intracellular auxin gradients (4); ATP binding cassette (ABCB) transporters whose directionality depends on the concentration of auxin (5), and AUX1/Like-AUX1 (LAX) transporters (18), which are plasma membrane-localized auxin permeases that resemble aromatic amino acid permeases. *C. sorokiniana* encodes at least one putative ortholog for three of the four families of transporters: PILS, AUX1, and ABCB type transporters (Figures 2-4, Table 1).

The redundancy of auxin related genes in the *Arabidopsis* genome suggests a multitude of functions that can be finely modulated and thus, it is possible that the single auxin related genes in *C. sorokiniana* control a wide range of physiological processes. The presence of PILs-like intracellular orthologs and the absence of PIN orthologs, which mediate extracellular polar auxin transport, are consistent with a unicellular lifestyle.

The later appearance of PIN proteins in plants and in algae with differentiated organs is consistent with their participation in activities related to a multicellular lifestyle.

ABP1 mediated signal transduction

In higher plants, auxin is perceived by at least two types of receptors: the SCF/TIR1/AFB co-receptors, responsible for transcriptional responses to auxin, and ABP1, a cupin domain containing protein whose signaling primarily effects rapid, nontranscriptional auxin responses such as cell expansion and ion fluxes at the plasma membrane (7). *C. sorokiniana* does not contain orthologs to the SCF/TIR1/AFB co-receptors nor to the auxin responsive transcription factor (ARF). However, it does contain a highly conserved ortholog to ABP1 (Table 1, Figure 5).

ABP1 is a putative auxin receptor first discovered in cell fractions from maize coleoptiles (30). It was characterized as an auxin receptor in the early 1990s, and the protein structure was solved in 2001. Woo et al. (2001) crystallized ABP1 with and without the synthetic auxin napthaleneacetic acid (NAA) (13). The structure determined that the auxin binding region features a cluster of hydrophobic amino acids that bind the aromatic ring(s) of auxin, as well as three histidines and a glutamic acid which coordinate a zinc ion which interacts with the carboxylate moiety of IAA. We identify ABP1 as the putative auxin receptor in *Chlorella* species. Structure-based sequence alignment by Expresso (15) followed by pairwise structural alignment by SWISSMODEL (23) (Figure 6) revealed 42% identity and a high degree of structural similarity between *Z. mays* ABP1 and *C. sorokiniana* ABP1.

The auxin binding pocket is almost completely conserved, except for a phenylalanine to methionine substitution and a glutamic acid to serine substitution. Additionally, the histidines and glutamic acid residues coordinating the zinc atom were completely conserved (Figure 6). This high degree of conservation in key residues suggests a functional role for the ABP1 ortholog in *Chlorella*. In *Arabidopsis*, auxin-bound ABP1 is essential for the activation of Rho-dependent GTPases (ROPs), mediated by plasma membrane associated transmembrane kinases (TMKs) (31). *C. sorokiniana* encodes both ROP and TMK orthologs for this signaling pathway (Table 1, Figure 1). Additionally, *C. sorokiniana* encodes an ortholog for IBR5, a recently identified TIR-interacting indole butyric acid receptor, but putative functions and interactions in *C. sorokiniana* remain cryptic (21).

The presence of ABP1 and IBR5 and the absence of AUX/IAA signaling components suggests that non-AUX/IAA mediated signaling pathways in plants may have originated in algae, and that their function broadened and diversified as plants colonized land.

Synthesis and secretion of IAA into the medium

C. sorokiniana produces approximately 2 ng of IAA in the pellet and secretes 9 ng into 50 mL of the culture supernatant, resulting in a supernatant concentration of approximately 1 nM (Figure 5).

In *Arabidopsis*, the TIR1/AFB receptors perceive IAA at a K_d of 84 nM (32), and purified *Z. mays* ABP1 has a K_d of 100 nM for IAA (33). However, *C. sorokiniana* has been reported to grow as a biofilm in biofuel and waste remediation biotechnology, and we have observed biofilm growth in glass Erlenmeyer growth flasks, in agreement with

prior reports (34, 35). In a biofilm context, the effective concentration of IAA locally between cells would be much higher.

The conservation of many auxin signaling related orthologs in *Chlorella* suggests that some genes required for auxin signaling were present in early diverging chlorophytes, and the production and secretion of IAA into the medium implies that they may have been recruited for auxin signaling in this lineage, as well. The development of *Chlorella* as a unicellular model system for auxin signaling could help illuminate the specific role of chlorophytes in phytohormone signaling evolution and to investigate the the role of specific early emerging pathways in plant hormone signaling.

Evolutionary implications

Our research provides compelling evidence that the molecular precursors necessary for some primitive forms of auxin signaling are present in *Chlorella*, though the specific physiological effects of auxin on algae remain to be determined. This research also clarifies the role of auxin signaling evolution in the chlorophyte more closely related to the charophyte lineage, compared to the *Chlamydomonales* (36)(37). The absence of the PIN proteins in the unicellular *C. sorokiniana* suggests that PIN-like proteins (PILs) may predate the PIN proteins which are characteristic of multicellular auxin transport.

The molecular investigation into the role of auxin signaling in algae is nuanced and unprecedented; however, insight into auxin signaling in algae could lead to a convenient unicellular model system for plants, especially in order to tease apart ABP1-

mediated and AUX/IAA mediated signaling. We anticipate that our work will provide the groundwork for exploring the physiological role of auxin in algae.

Functional category	Gene name (AT)	*Arabidopsis* accession	*Chlorella* accession	Percent identity	Expect	Specific function
Receptor	ABP1	AT4G02980	sca021.g104100.t1*	39	9e-35	Receptor
Amino acid transaminases	TAA1	AT1G70560	sca134.g100350.t1◊ sca110.g101700.t1◊ sca003.g105450.t7◊	25	7e-04	*C. sorokiniana* general transaminases
Biosynthesis	YUC	AT2G33230 (YUC7)	sca096.g103150.t1•	27	3e-14	Indole-3-pyruvate monooxygenase
	AMI1	AT1G08980	sca003.g101900.t1*	39	3e-75	Indole-3-acetamide hydrolase
	AAO1	AT5G20960	sca130.g100900.t1	29	4e-119	Indole-3-acetaldehyde oxidase
Transport	PILS	AT1G71090 (PILS2)	sca099.g100650.t1*	32	2e-21	PIN-like intracellular auxin transporters
	ABCB4	AT2G47000	sca090.g105800.t1*	39	0.0	Auxin-specific ABC-type transporters
	AUX1	AT2G38120	sca028.g105050.t1*	34	1e-21	Aromatic amino acid permease like auxin transporter
Signal transduction	TMK1	AT1G66150	sca128.g101450.t1	26	2e-35	Activates Rho-like GTPase signaling
	ROP2	AT1G20090	sca139.g104200.t1	32	4e-22	Rho-like GTPase signaling

Table 1. The *Chlorella sorokiniana* genome encodes putative orthologs for the biosynthesis, transport, sensing, and signal transduction of auxin. *Arabidopsis* queries were used to identify BLASTP hits in the draft *C. sorokiniana* genome. Reciprocal hits are denoted with an asterisk (*). Three newly discovered putative transaminases, not specific to tryptophan, are denoted by the diamond (◊).AtYUC and the putative CsYUC genes, denoted with a bullet (•), are not perfectly reciprocal hits, even though AtYUC queries return CsYUC and CsYUC queries against *Arabidopsis* return AtYUC among the top 10 hits.

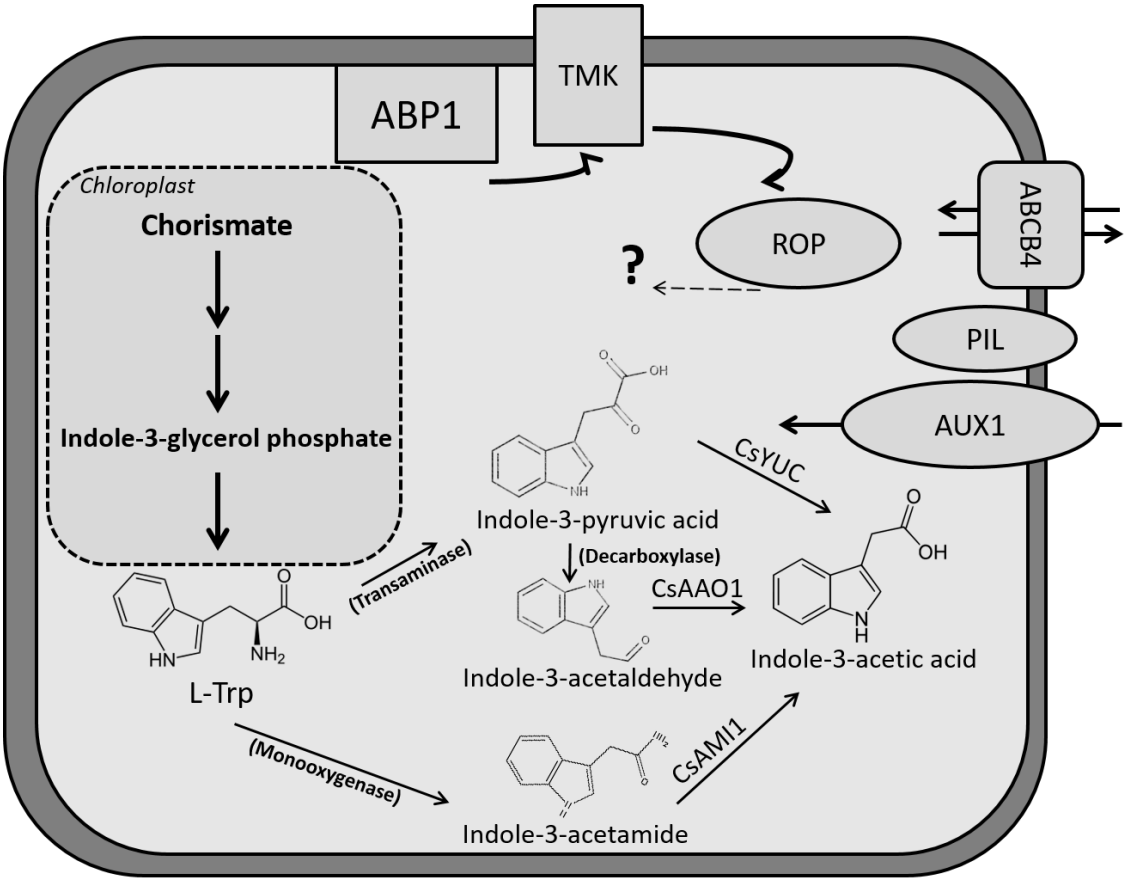


Figure 1. A model for auxin biosynthesis, transport, and signaling in *Chlorella*. 1. The indole-3-pyruvic acid (IPyA) pathway: A tryptophan transaminase first converts Trp to IPyA and flavin monooxygenase YUC converts IPyA to indole-3-acetic acid (IAA). 2. The indole-3-acetamide (IAM) pathway: Primarily a pathway in phytopathogenic bacteria, IAM has been detected in all plants tested; furthermore, plants encode an amidase that converts IAM to IAA. 3. The indole-3-acetaldehyde (IAD) pathway. In the IAD pathway, indole-3-acetaldehyde oxidase converts IAD to IAA.

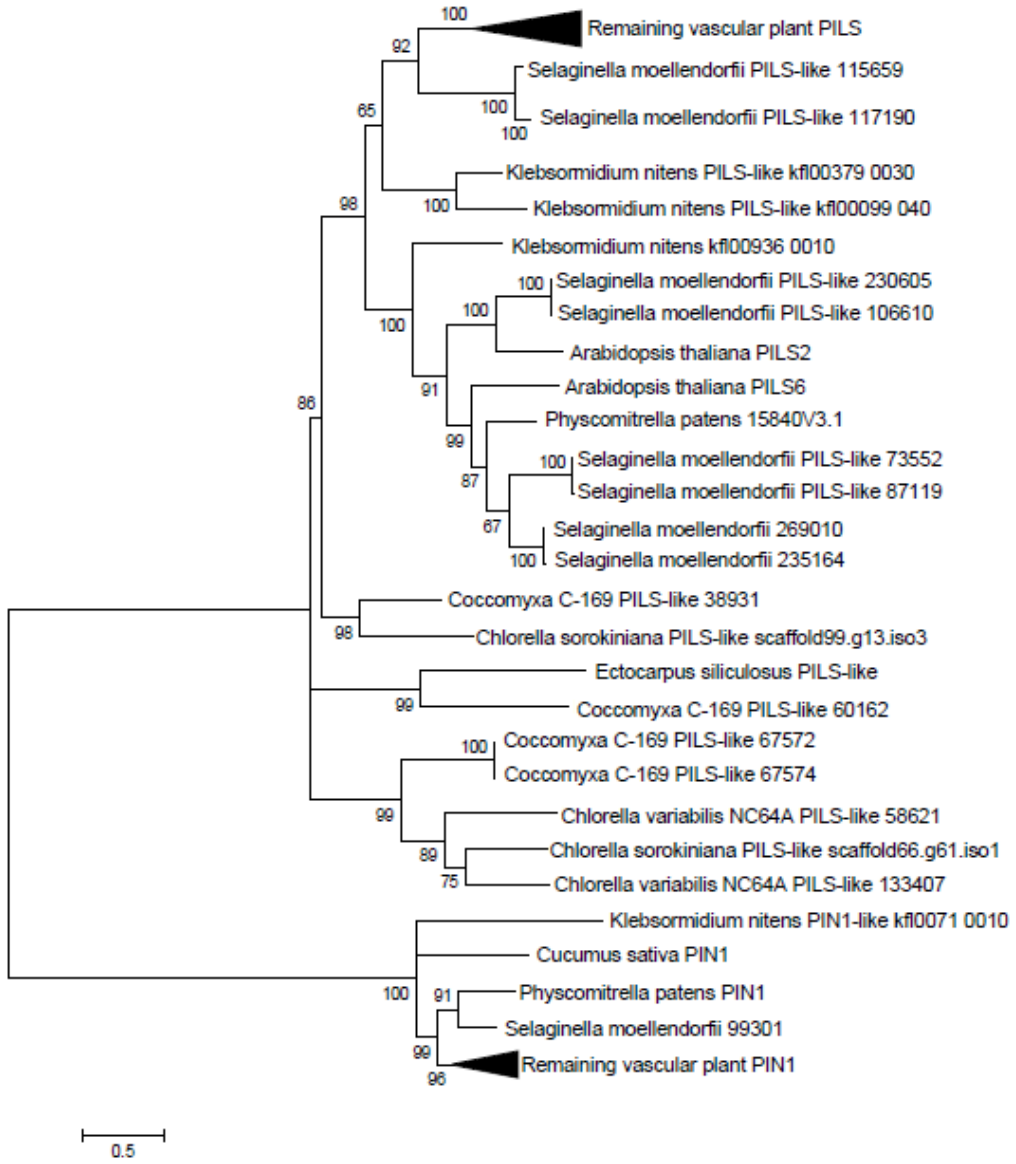


Figure 2. Phylogenetic analysis indicates sequence conservation of PILS. PILS (PIN-like) transporters, similar to but distinct from PIN proteins, mediate intracellular auxin transport. Putative orthologs and outgroups were identified by reciprocal BLAST searches, aligned with Clustal Omega and analyzed using the LG+I substitution model in MEGA 6.06.

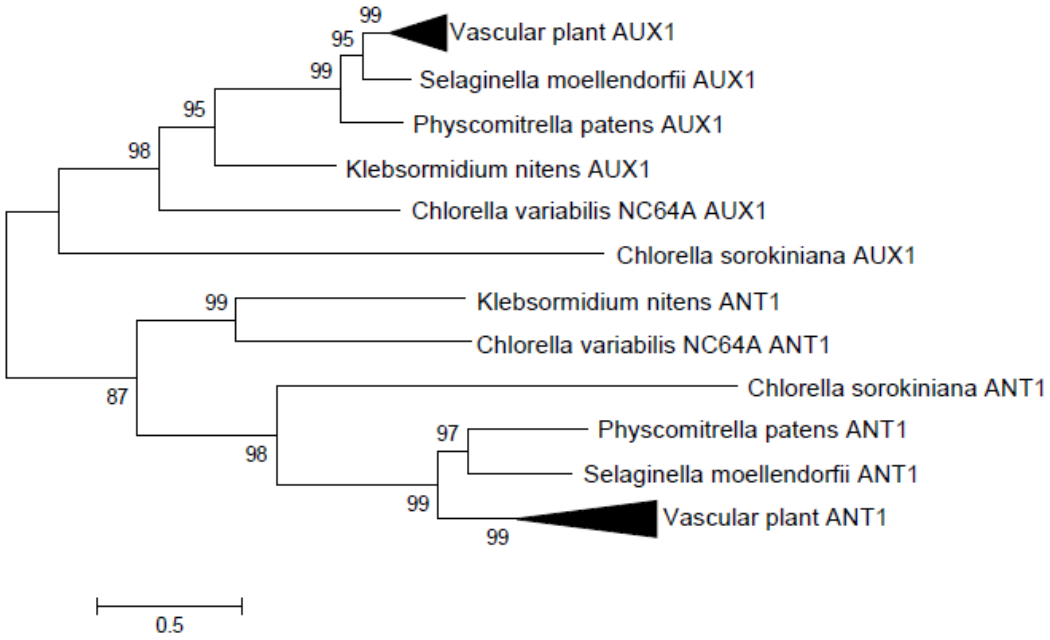


Figure 3. Phylogenetic analysis indicates sequence conservation of AUX1 .AUX1, an AAP-like transporter specific for auxin transport, is compared with ANT1, an aromatic amino acid transporter in plants. Putative orthologs and outgroups were identified by reciprocal BLAST searches, aligned with Clustal Omega and analyzed using the LG+I substitution model in MEGA 6.06.

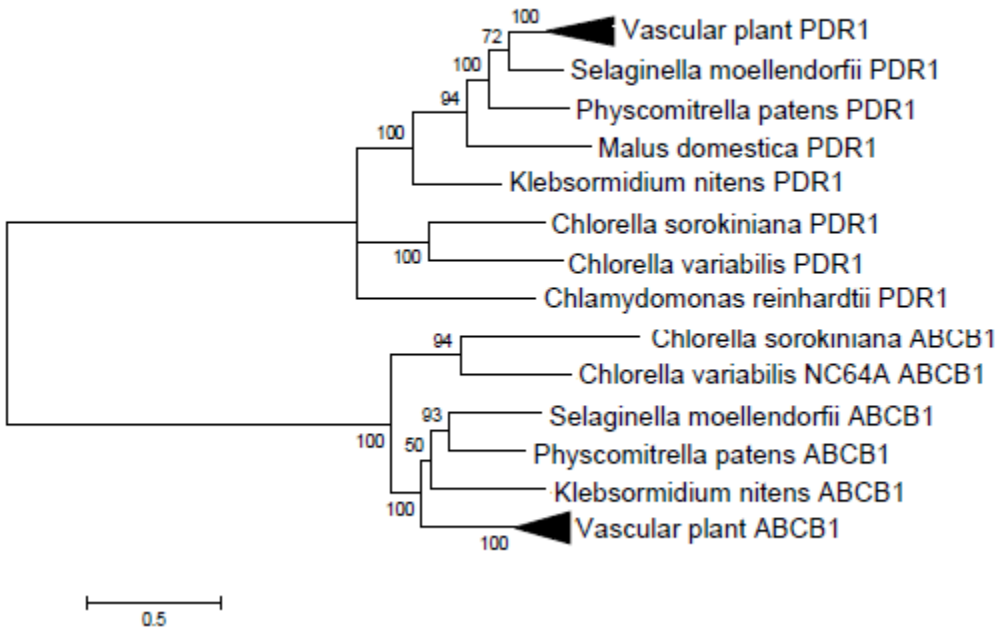


Figure 4. Phylogenetic analysis indicates sequence conservation of ABCB4., an ABCB transporter, is compared with a *p*-coumaric acid transporter which also transports a small organic molecule. Putative orthologs and outgroups were identified by reciprocal BLAST searches, aligned with Clustal Omega and analyzed using the LG+I substitution model in MEGA 6.06.

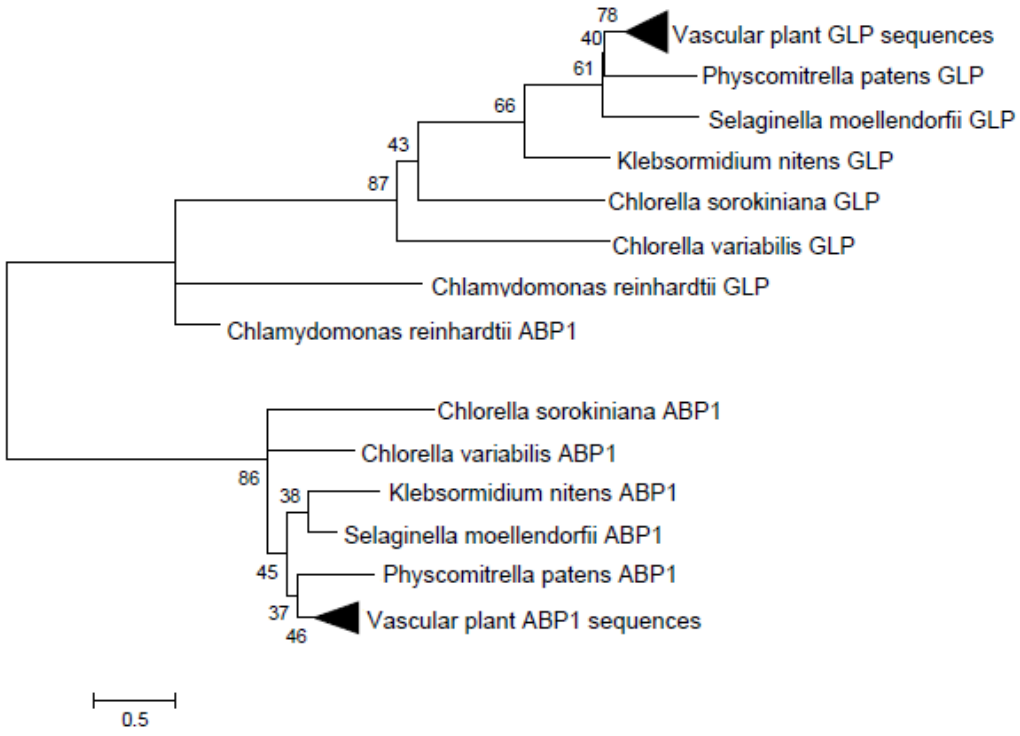


Figure 5. Phylogenetic analysis indicates sequence conservation of ABP1. ABP1, a putative IAA receptor, compared with GLP1, a similarly short (145 AA) cupn domain protein. Putative ABP1 ortholog and outgroups were identified by reciprocal BLAST searches, aligned with Expresso and analyzed using the LG+I substitution model in MEGA 6.06.

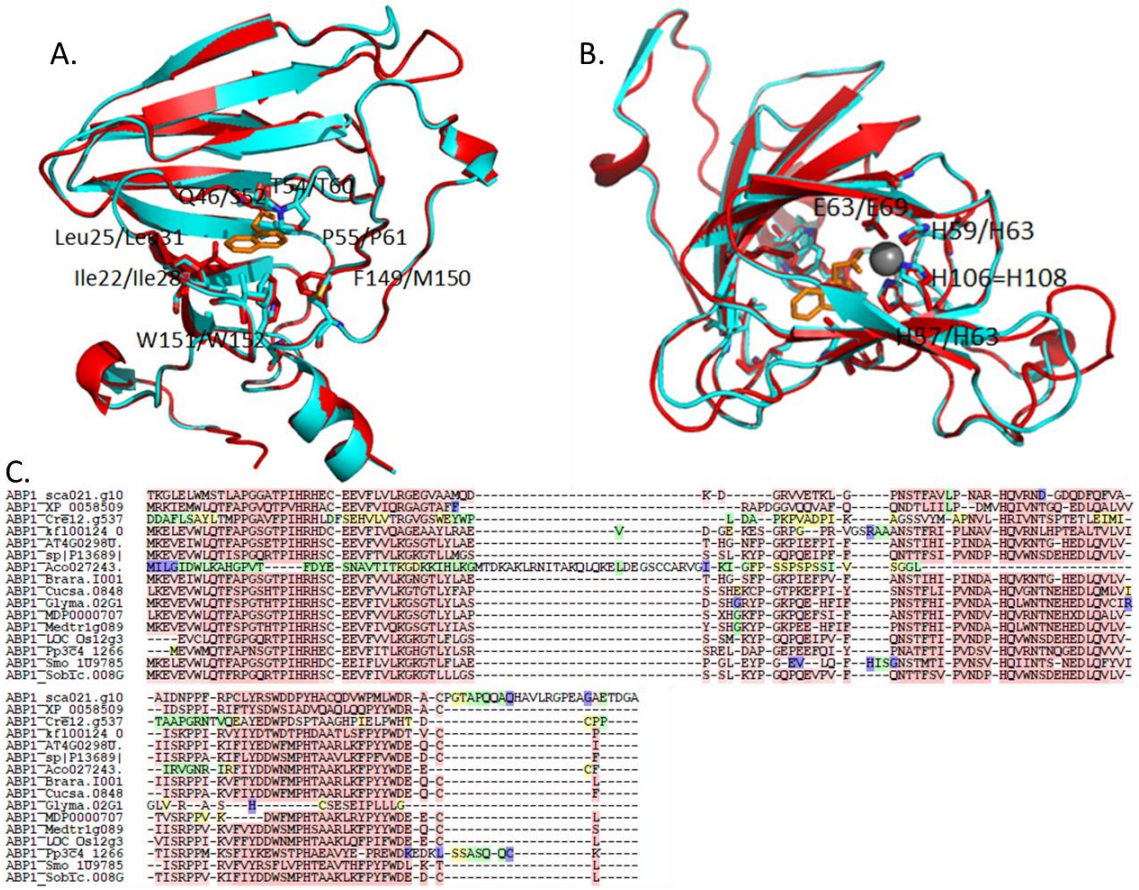


Figure 6. Multiple sequence and structural alignment reveal a high degree of structural conservation between *Z. mays* ABP1 and *C. sorokiniana* ABP1. A SWISSMODEL structural alignment on automated mode aligned CsABP1 with the ZmaABP1 crystal structure co-crystallized with the synthetic auxin naphthalenacetic acid (NAA). The resulting model revealed almost complete conservation of the auxin binding pocket **(A)**, and complete conservation of the histidines and and glutamic acid residues that coordinate the zinc atom **(B)** that stabilizes the carboxylate moiety of IAA/NAA. **(C)** An EXPRESSO structure-based multiple sequence alignment from representative plants and algae highlights the evolutionary conservation of the hydrophobic auxin binding pocket in ABP1 sequences. There is 42% sequence identity between *Z. mays* ABP1 and *C. sorokiniana* ABP1 over the cupin domain.

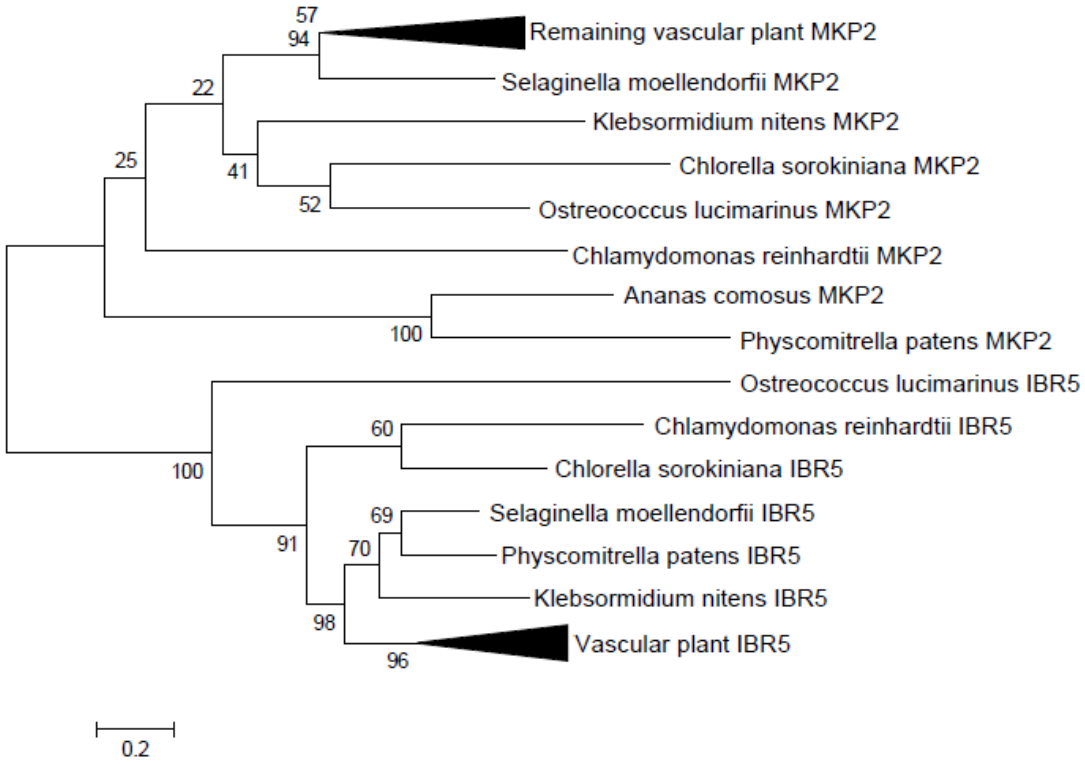


Figure 7. Phylogenetic analysis indicates sequence conservationof IBR5. IBR5, identified as an indole-3-butyric acid specific receptor, is compared with MKP2, which also contains a MAP kinase phosphatase domain and participates in phytohormone signaling pathways. Putative auxin signaling orthologs and outgroups were identified by reciprocal BLAST searches, aligned with Clustal Omega (except for ABP1, aligned with Expresso) and analyzed using the LG+I substitution model in MEGA 6.06.

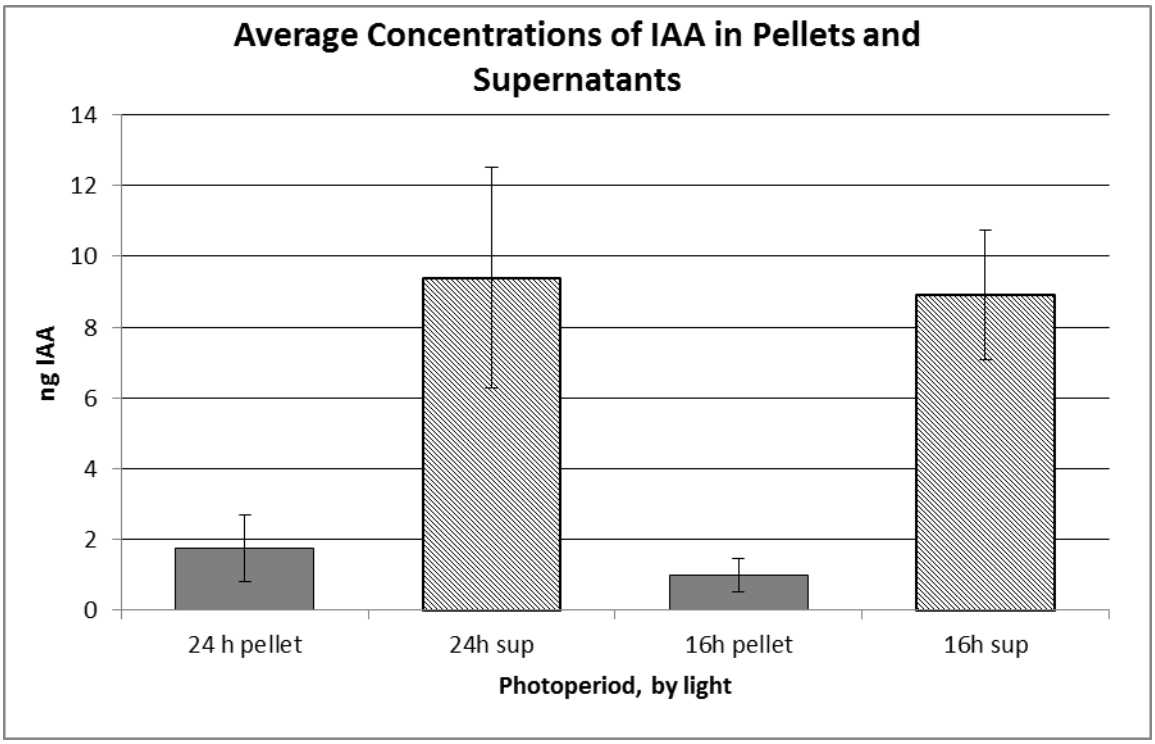


Figure 8. IAA production in *Chlorella sorokiniana*. *Chlorella sorokiniana* produces IAA within the cell pellet and secretes it into the culture medium. While under 2 ng is retained within the cell pellet, 8-9 ng was secreted into the culture medium. The ratio of secreted vs. pellet contained auxin may point to a cell-cell signaling role.

References

1. Kriechbaumer V, Botchway SW, Hawes C. 2016. Localization and interactions between Arabidopsis auxin biosynthetic enzymes in the TAA/YUC-dependent pathway. J Exp Bot 67:4195–4207.
2. Wang B, Chu J, Yu T, Xu Q, Sun X, Yuan J, Xiong G, Wang G, Wang Y, Li J. 2015. Tryptophan-independent auxin biosynthesis contributes to early embryogenesis in Arabidopsis. Proc Natl Acad Sci 112:4821–4826.
3. Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml J, Heidstra R, Aida M, Palme K, Scheres B. 2005. The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. Nature 433:39–44.
4. Barbez E, Kubeš M, Rolčík J, Béziat C, Pěnčík A, Wang B, Rosquete MR, Zhu J, Dobrev PI, Lee Y, Zažímalovà E, Petrášek J, Geisler M, Friml J, Kleine-Vehn J. 2012. A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants. Nature 485:119–122.
5. Kubeš M, Yang H, Richter GL, Cheng Y, Młodzińska E, Wang X, Blakeslee JJ, Carraro N, Petrášek J, Zažímalová E, Hoyerová K, Peer WA, Murphy AS. 2012. The Arabidopsis concentration-dependent influx/efflux transporter ABCB4 regulates cellular auxin levels in the root epidermis. Plant J 69:640–654.
6. Yang H, Murphy AS. 2009. Functional expression and characterization of Arabidopsis ABCB, AUX 1 and PIN auxin transporters in Schizosaccharomyces pombe. Plant J 59:179–191.
7. Wang R, Estelle M. 2014. Diversity and specificity: Auxin perception and signaling through the TIR1/AFB pathway. Curr Opin Plant Biol 21:51–58.
8. Gao, Y., Zhang, Y., Zhang, D., Dai, X., Estelle M and Z. 2015. Auxin binding protein 1 (ABP1) is not required for either auxin signaling or Arabidopsis development. Proc Natl Acad Sci USA 112:2275–2280.
9. Lu Y, Xu J. 2015. Phytohormones in microalgae: A new opportunity for microalgal biotechnology? Trends Plant Sci 20:273–282.
10. Lau S, Shao N, Bock R, Jürgens G, De Smet I. 2009. Auxin signaling in algal lineages: fact or myth? Trends Plant Sci 14:182–188.
11. Yue J, Hu X, Huang J. 2014. Origin of plant auxin biosynthesis. Trends Plant Sci 19:764–770.
12. Turnaev II, Gunbin K V., Afonnikov DA. 2015. Plant auxin biosynthesis did not originate in charophytes. Trends Plant Sci 20:463–465.
13. Woo EJ, Marshall J, Bauly J, Chen JG, Venis M, Napier RM, Pickersgill RW. 2002. Crystal structure of auxin-binding protein 1 in complex with auxin. EMBO J 21:2877–2885.
14. Zhao Y. 2012. Auxin biosynthesis: A simple two-step pathway converts tryptophan to indole-3-Acetic acid in plants. Mol Plant 5:334–338.
15. Armougom F, Moretti S, Poirot O, Audic S, Dumas P, Schaeli B, Keduas V, Notredame C. 2006. Expresso: Automatic incorporation of structural information in multiple sequence alignments using 3D-Coffee. Nucleic Acids Res 34:604–608.
16. Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary

Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599.
17. Wang X, Zhang H, Gao Y, Sun G, Zhang W, Qiu L. 2014. A comprehensive analysis of the cupin gene family in soybean (Glycine max). PLoS One 9.
18. Yang Y, Hammes UZ, Taylor CG, Schachtman DP, Nielsen E, Chen L, Ortiz-Lopez A, Jung A, Bush DR. 2006. High-Affinity Auxin Transport by the AUX1 Influx Carrier Protein. Plant Physiol 125:1123–1127.
19. Chen L, Ortiz-Lopez A, Jung A, Bush DR. 2001. ANT1, an aromatic and neutral amino acid transporter in Arabidopsis. Plant Physiol 125:1813–20.
20. Cerutti T, Delatorre CA. 2013. Nitrogen and phosphorus interaction and cytokinin: Responses of the primary root of Arabidopsis thaliana and the pdr1 mutant. Plant Sci 198:91–97.
21. Strader LC, Monroe-Augustus M, Bartel B. 2008. The IBR5 phosphatase promotes Arabidopsis auxin responses through a novel mechanism distinct from TIR1-mediated repressor degradation. BMC Plant Biol 8:41.
22. Jin SL, Ellis BE. 2007. Arabidopsis MAPK phosphatase 2 (MKP2) positively regulates oxidative stress tolerance and inactivates the MPK3 and MPK6 MAPKs. J Biol Chem 282:25020–25029.
23. Schwede T, Kopp J, Guex N, Peitsch MC. 2003. SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res 31:3381–3385.
24. Allen MM. 1968. Simple conditions for growth of unicellular blue-green algae on plates. J Phycol 4:1–4.
25. Pan X, Welti R, Wang X. 2010. Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography-mass spectrometry. Nat Protoc 5:986–992.
26. Mano Y, Nemoto K, Suzuki M, Seki H, Fujii I, Muranaka T. 2010. The AMI1 gene family: Indole-3-acetamide hydrolase functions in auxin biosynthesis in plants. J Exp Bot 61:25–32.
27. Won C, Shen X, Mashiguchi K, Zheng Z, Dai X, Cheng Y, Kasahara H, Kamiya Y, Chory J, Zhao Y. 2011. Conversion of tryptophan to indole-3-acetic acid by tryptophan aminotransferases in Arabidopsis and YUCCAs in Arabidopsis. Proc Natl Acad Sci 108:18518–18523.
28. Moat AG, Foster JW, Spector MP. 2002. Fermentation pathways. Microb Physiol Fourth Ed 412–433.
29. Hazelwood LA, Daran J-M, van Maris AJA, Pronk JT, Dickinson JR. 2008. The Ehrlich pathway for fusel alcohol production: a century of research on Saccharomyces cerevisiae metabolism. Appl Environ Microbiol 74:2259–2266.
30. Dohrmann U, Hertel R, Kowalik H. 1978. Properties of auxin binding sites in different subcellular fractions from maize coleoptiles. Planta 140:97–106.
31. Xu T, Dai N, Chen J, Nagawa S, Cao M, Li H, Zhou Z, Chen X, De Rycke R, Rakusová H, Wang W, Jones AM, Friml J, Patterson SE, Bleecker AB, Yang Z. 2014. Cell surface ABP1-TMK auxin-sensing complex activates ROP GTPase signaling. Science 343:1025–8.
32. Dharmasiri N, Dharmasiri S, Estelle M. 2005. The F-box protein TIR1 is an auxin receptor. Nature 435:441–5.
33. Hesse T, Feldwisch J, Balshüsemann D, Bauw G, Puype M, Vandekerckhove J,

Löbler M, Klämbt D, Schell J, Palme K. 1989. Molecular cloning and structural analysis of a gene from Zea mays (L.) coding for a putative receptor for the plant hormone auxin. EMBO J 8:2453–61.

34. Berner F, Heimann K, Sheehan M. 2015. Microalgal biofilms for biomass production. J Appl Phycol 27:1793–1804.
35. Blanken W, Janssen M, Cuaresma M, Libor Z, Bhaiji T, Wijffels RH. 2014. Biofilm growth of Chlorella sorokiniana in a rotating biological contactor based photobioreactor. Biotechnol Bioeng 111:2436–2445.
36. Nishida I, Murata N. 1996. Chilling sensitivity in plants and cyanobacteria: the crucial contribution of membrane lipids. Annu Rev Plant Physiol Plant Mol Biol 47:541–568.
37. Leliaert F, Smith DR, Moreau H, Herron MD, Verbruggen H, Delwiche CF, De Clerck O. 2012. Phylogeny and Molecular Evolution of the Green Algae. CRC Crit Rev Plant Sci 31:1–46.

CHAPTER 3

SYNTHESIS, SECRETION, AND PERCEPTION OF ABSCISIC ACID REGULATE STRESS RESPONSES IN *CHLORELLA SOROKINIANA*

Abstract

Abscisic acid (ABA) is a phytohormone that has been extensively characterized in higher plants for its roles in seed and bud dormancy, leaf abscission, and stress responses. Genomic studies have identified orthologs for ABA-related genes throughout the Viridiplantae, including in unicellular algae, however the role of ABA in algal physiology has not been characterized, and the existence of such a role has been a matter of dispute. In this study, we demonstrate that ABA is involved in regulating algal stress responses. *Chlorella sorokiniana* strain UTEX 1230 contains genes orthologous to those of higher plants which are essential for ABA biosynthesis, sensing, and degradation. RNAseq-based transcriptomic studies reveal that treatment with ABA induces dramatic changes in gene expression profiles, including the induction of a subset of genes involved in DNA replication and repair, a phenomenon which has been demonstrated in higher plants. Pretreatment of *C. sorokiniana* cultures with ABA exerts a protective effect on cell viability in response to ultraviolet radiation. Additionally, *C. sorokiniana* produces and secretes biologically relevant amounts of ABA and the oxylipin 12-oxo-phytodienoic acid into the growth medium in response to osmotic stress and oxidative stressors. Taken together, these phenomena suggest that ABA signaling evolved as an intercellular stress response signaling molecule in eukaryotic microalgae prior to the evolution of multicellularity and colonization of land.

Introduction

Plants respond to abiotic stresses in multiple and nuanced ways, involving carefully regulated and interconnected signaling pathways. In particular, response to saline and drought stress – stressors characterized by lowered water availability and desiccation– is mediated by a number of small-molecule signals and many interacting signal transduction pathways. These signals and pathways include reactive oxygen species (ROS), salt overly sensitive genes (SOS), 12-oxophytodienoic acid (OPDA), jasmonic acid (JA), and abscisic acid (ABA) (1). ABA is a molecule found throughout all domains of life except Archaea (2). In plants, it functions as a stress signaling molecule originally discovered and named for its role in leaf abscission, which is integral to their response to many types of abiotic stresses related to water availability, including drought/desiccation, salinity, and cold stress (3).

Orthologs to genes involved in plant hormone signaling pathways, including ABA signaling, have been reported in algal genomes, but at present little functional information is available about the role of ABA in algae. Orthologs to genes implicated in ABA signaling have been observed in *Chlorella variabilis* NC64A(4), the streptophyte *Klebsormidium flaccidum*(5), the picoeukaryotic marine alga *Ostreococcus tauri*(6), and the model chlorophyte *Chlamydomonas reinhardtii* (2)(7)**.** Though putative plant hormone related orthologs, including ABA related genes, have been described in algae (2), the specific functions of ABA and its physiological and evolutionary significance have remained unclear. ABA itself has been identified in extracts of algae from a number of eukaryotic lineages, and has also been identified in cyanobacteria, some of which induce the formation of heterocysts in response to exogenous ABA (8),

(9). *Dunaliella* species have been demonstrated to accumulate ABA and secrete it into the growth medium upon saline or alkaline shock (8). These experiments implicated ABA signaling in β-carotene accumulation and salt stress tolerance (10), leading to the proposition that microalgal ABA signaling may share some functions with this pathway in higher plants.

In higher plants, ABA signaling is mediated by PYR1/PYL/RCAR (11), a series of soluble ABA-binding proteins that inactivate SNF1-related protein kinases (SnRK2s). Additional ABA perception functions are mediated by chloroplast localized magnesium chelatase subunit H (ChlH), a member of the porphyrin biosynthesis pathway with a role in plastid to nucleus retrograde signaling (12), and GTG1, a GPCR-like transmembrane G protein (13).

ABA signaling in higher plants also integrates with other stress-related signaling pathways, including reactive oxygen species (ROS), salt overly sensitive (SOS) signaling, as well as the lipid-derived phytohormones 12-oxo-phytodienoic acid (OPDA) and jasomonic acid (JA) (14). Specifically, the saline stress response in higher plants involves SOS and ROS signaling, both of which interact with each other and with ABA mediated pathways (1),(15). Transcriptomic analyses in *Arabidopsis* have revealed ABA dependent and independent gene expression in response to salt and drought stress (16); additionally, genotoxic stress, DNA damage repair, and ROS signaling may have ABA independent components (17)(18). There are ABA dependent and independent features of both these signaling pathways in *Arabidopsis* (19).

In this study, we demonstrate that *C. sorokiniana* produces ABA and physiologically related compounds during osmotic stress; furthermore, 290 transcripts

are differentially expressed in response to treatment with ABA. A large portion of the upregulated genes are involved in DNA replication and repair, but ABA treatment induces no loss in cell viability, growth rate, or cell yield. Pretreatment of cultures with ABA was found to increase tolerance of cultures to UV irradiation. We further present comparative genomic evidence for the presence and function of ABA and related signaling genes in *Chlorella sorokiniana*. Our work thus integrates multiple approaches to demonstrate that a plant hormone acts an intercellular signaling molecule in unicellular eukaryotic algae. More specifically, we propose that the role of ABA as a stress response phytohormone predates and may have had a pivotal role in the evolution of multicellularity and in the colonization of land.

Results

Chlorella sorokiniana encodes orthologs to phytohormone-mediated stress signaling pathways.

The *Chlorella sorokiniana* strain UTEX-1230 was identified as a promising biomass and waste-remediation organism in a large scale screening study conducted by a consortium of algal biologists at the University of Nebraska (20–22). Its genome was subsequently sequenced, and a combination of *in silico* gene-prediction programs and RNA sequencing was used to annotate predicted genes, open reading frames, and protein sequences. The sequencing and annotation of this strain will be described in detail in a subsequent publication, (Cerutti et al., in preparation), however a current gene catalog including protein sequences used in our analysis, has been deposited in Genbank.

To identify and catalog phytohormone related genes, reciprocal BLAST searches were performed using experimentally verified protein sequences from seed plants to

search the predicted *C. sorokiniana* proteome. We initially analyzed and cataloged genes related to auxin, cytokinin, ethylene, JA, and ABA signaling, and in this study we focus on genes mediating the synthesis and signaling functions of ABA and 12-OPDA, a bioactive precursor of JA (23).

C. sorokiniana genes related to ABA biosynthesis, sensing, and degradation are listed in Table 1, along with their predicted function. The ABA related genes we identified are closely related to ABA synthesis and signaling genes in higher plants, although the canonical seed-plant ABA receptor family (PYR/PYL/RCAR) along with some of the associated signaling components were absent. Phylogenetic analysis (Fig. 1) demonstrates that these genes constitute lineages that are more closely related to plant ABA signaling genes than they are to paralogous genes containing similar domains. Though no PYR1/PYL/RCAR like proteins are evident in the *C. sorokiniana* genome, GTG1 and CHLH orthologs are present (Fig. 1.) Furthermore, *Chlorella sorokiniana* encodes the ABA biosynthetic and degradation pathway, as well as several putative ABA transporters (Table 1; Fig. 1). The presence of these genes suggests that *C. sorokiniana* encodes the components necessary to synthesize, secrete and perceive ABA.

In *Arabidopsis*, both salinity and genotoxic stress signaling pathways are interconnected by MAP kinases and associated phosphatases. Specifically, MAP kinase phosphatase 1 (MKP1) is implicated in both salt and genotoxic stress signaling pathways (24). Mutant *mkp1* plants are resistant to saline but more highly sensitive to genotoxic stress. The genome of *C. sorokiniana* contains an ortholog to MKP1 and other genes in

these pathways (Table 2), suggesting the functional connections between these different stress signaling modes are ancient and conserved from unicellular algae to seed plants.

Abscisic acid is synthesized and secreted by *Chlorella sorokiniana* during saline and oxidative stress.

Plants produce ABA in response to water availability stressors, including cold, salinity, and drought. In order to determine whether *C. sorokiniana*, a freshwater alga, makes abscisic acid under similar conditions, we exposed cultures to 600 mM NaCl (the osmotic strength of seawater). Significant quantities of ABA were present in both the cell pellet and the supernatant (~10 and 20 ng/50 ml culture, respectively), indicating the active synthesis and secretion of ABA upon osmotic stress (Fig. 2). Additionally, the plant stress hormone OPDA was produced and secreted in significant quantities (~2.5 nMol/50 ml culture) in the stressed cultures, but essentially absent from the unstressed controls (Fig. 3).

Treatment with 1 mM H_2O_2 or 5 µM paraquat also resulted in modest increases in the production of ABA in the cell pellet and supernatant (Fig. 2). In seed plants, singlet oxygen stress (stimulated by application of paraquat) directly results in the activation of ABA signaling pathways, while H_2O_2 signaling occurs downstream of ABA signaling and participates in the signal transduction cascade that leads to stomatal closure, as reviewed in (25). In *Chlorella*, both of these stressors resulted in modest induction (~2-fold) of ABA production, suggesting altered mechanisms of cross-pathway regulation between oxidative stress and salt signaling pathways in this alga relative to seed plants.

Abscisic acid results in the differential regulation of 290 transcripts in *C. sorokiniana*.

Treatment of *C. sorokiniana* cultures with 100 µM ABA for 48 h resulted in 219 upregulated and 71 downregulated transcripts. These transcripts were differentially expressed at least two-fold, with a q-value of < 0.05 as determined by CuffDiff2 (26). A large fraction of the upregulated genes control DNA replication and repair (Fig. 4). Notably, DNA pol III, BRCA1, translesion DNA polymerase κ, and the MCM8/MCM9 orthologs participating in homologous recombination repair are upregulated as shown in Fig 5A (27). A summary of representative genes is presented in Table 3, and a complete list of differentially regulated genes is provided as a Supplementary Data file.

In seed plants, ABA retards growth by initiating stomatal closure via H_2O_2 production, thereby decreasing carbon dioxide accumulation; additionally, ABA represses photosynthesis. Accordingly, we observed a decreased abundance of transcripts associated with photosynthesis, including carbonic anhydrase, phytoene desaturase, and chlorophyll biosynthesis associated genes including magnesium chelatase subunit ChlH (12). ChlH also acts as a chloroplastic ABA receptor in some plants: in *Arabidopsis*, it specifically binds ABA and regulates a cascade of ABA signaling genes including RD29A, MYB2, and MYC2 among others (28). It is important to note that, unlike in higher plants, we observed no decrease in cell number or biomass yield in response to ABA treatment under our growth conditions.

Central metabolism enzymes, including PGK, GAPDH, PRK, and malate synthase, were downregulated by ABA. In *Arabidopsis*, mutant lines with specific defects in glycolysis have altered ABA signal transduction: *Arabidopsis* plants deficient

in plastidial glyceraldehyde-3-phosphate dehydrogenase (GAPCp) are ABA insensitive in stomatal closure, growth, and germination assays (29). Muñoz-Bertomeu et al. demonstrate that the disruption of sugar and serine homeostasis causes ABA insensitivity in *gapcp1gapcp2* plants, and supplementing sugar and serine restored and even augmented ABA sensitivity (18).

In addition to direct effects on carbon fluxes and energy capture, altered expression of glycolysis and photosynthesis related genes have been shown to alter the production of ROS, suggesting that ROS signaling is integrated with primary cellular metabolism(15, 30). Crosstalk between ROS and ABA signaling has been demonstrated in plants, where it is influenced by altered metabolic fluxes. Our results sugest that ROS production and scavenging are also altered, with coordinate effects on ABA signaling (24).

Given that a major stress response of many algae is the accumulation and storage of fatty acids in lipid droplets (31), it was surprising that fatty acid biosynthetic genes were largely downregulated by ABA. These genes, including 3-oxoacyl ACP synthase, acyl carrier protein, biotin carboxyl carrier protein, and biotin carboxylase. These components contribute to both fatty acid synthase and acetyl-CoA carboxylase functions in chloroplasts, and suggest the likelihood of additional alterations in central metabolic pathways upon treatment with ABA. GPI ethanolamine phosphate transferase 3, participating in GPI anchor biosynthesis, was one of very few lipid-active genes to be upregulated by ABA, perhaps indicating an alteration in the abundance or type of GPI-anchored proteins displayed at the cell surface. Overall, carbon flow appears to be

shunted away from lipid biosynthesis, at least as judged by steady-state levels of associated transcripts.

Several putative ABA signal transduction genes are differentially expressed upon exposure to ABA. These include, as mentioned above: CHLH, a chloroplastic ABA receptor repressed by ABA, and a MAP3K/NPK1 ortholog which is upregulated by ABA. Notably, the *Arabidopsis* NPK1 ortholog ANP1 induces MPK3 and MPK6 expression via H_2O_2, so that the MPK3 and MPK6 can then participate in genotoxic stress tolerance. MKP1 also interacts with both MPK3 and MPK6 e to mediate salt tolerance. In *Arabidopsis* MKP1 is postulated to be post translationally regulated (24), and the same may be true for *Chlorella*.

ABA pretreatment induces UV tolerance.

Due to the upregulation of DNA replication and repair mechanisms (Figs. 4 and 5A), we suspected that ABA may have a role in priming cells for DNA repair. We investigated this possibility by inducing DNA damage in cells with a dose of short-wave UV irradiation (18 mJ, 270 nm). For untreated cells, this dose reduced the colony forming units (i.e. viable cells) to roughly 8% of the untreated control, representing 92% killing. In contrast, the survival of cells pretreated with 10 µM ABA nearly doubled over the untreated control, to ~15% (Fig. 5B). This result directly demonstrates that treatment with exogenous ABA primes *Chlorella* cells for DNA damage, and that the mechanism by which it does so is very likely to be the via the induction of DNA damage response genes as shown in our transcriptome analysis (Fig. 4).

Discussion

This work demonstrates that ABA is produced, secreted, and sensed by *Chlorella sorokiniana*. Though plant hormone signaling has been proposed for some green algae based on reports of its production and presence of related gene orthologs, our study is the first to integrate genomic, transcriptomic, biochemical, and physiological evidence of algal cell to cell communication using a phytohormone. In higher plants, exposure to exogenous ABA causes plants to reduce photosynthesis and respiration. Conversely, external effects on ABA in algae have been reported to be minimal (8), consistent with our observations: *Chlorella* cells treated with up to 100 µM ABA did not experience a reduction in growth rate or cell yield.

Phylogenetic analysis (Fig. 1) suggests that the genes listed in Tables 1-4 are orthologous to ABA synthesis and perception genes in plants, indicating that the

development of ABA signaling may have been essential for the colonization of land. ABA provides a mechanism for plants and algae to cope with the effects of salt and desiccation stress, both of which are amplified on land. Phytohormones have been implicated in the desiccation tolerance of *Klebsormidium* species, which are filamentous charophyte algae that are thought of as a bridge between unicellular and multicellular plants. ABA related genes in *K. flacidum* were upregulated in response to desiccation, though the hormone itself was not quantified in the study (32). Holzinger and Becker noted, as we do, that our respective organisms contain almost complete ABA signaling pathways except for the PYR/PYL/RCAR type receptors, AREB family transcription factors, and S-type anion channel SLAC1 that is required for stomatal function in higher plants (33). Transcriptomic studies in *Klebsormidium* have revealed a land plant-like defense reaction against desiccation. Indeed, the presence of certain components of these pathways suggests that the use of ABA as a stress signaling molecule by plants occurred early in the evolutionary history of plants – at least as early as the unicellular ancestors of charophytes and chlorophytes – and that it is regulated by ancient gene families which have since differentiated and specify multiple stress and developmental functions in higher plants (34), (32).

Components of ABA signaling, including stomatal closure regulating protein SnRK2 are present in *Chlorella*, and appear to be differentially expressed upon treatment with ABA. *Chlorella* also contains a putative ortholog to MAP kinase phosphatase 1 (MKP1), which participates in salt stress adaptation and tolerance in *Arabidopsis* (24). Considering our inherent experimental limitations, it is possible that it is regulated at an earlier stage after ABA application than what this study measured, and

that this study therefore missed the signaling pathways that occur early in *Chlorella* ABA signal transduction.

Previous studies have demonstrated that genotoxic stresses are linked to salinity and ABA signaling in *Arabidopsis* (35). Saha et al. (36) suggested that the ROS accumulation under NaCl stress contributed to an increase in DNA damage, interconnecting the two stressors with ABA signaling. The production of ABA in response to oxidative stress bolsters this model: salt and oxidative stresses both activate ABA signaling, possibly in addition to ABA-independent oxidative stress response and salt stress response pathways. We propose that, in Chlorella, salt stress triggers a signaling cascade that activates ABA, resulting in protection from DNA damage, whereas UV-induced DNA damage itself does not trigger ABA production.

As the interrelation of abiotic stressors and signaling pathways is an emerging field in plant stress biology, we propose that *Chlorella* will provide a convenient unicellular model system for elucidating the interrelationship of salt and oxidative stresses, DNA damage, and ABA signaling in plant cells. This study also demonstrates that ABA signaling can occur without PYR/PYL/RCAR signaling as it is currently understood, and we propose that the study of an organism with a well-developed ABA response, but missing PYR/PYL/RCAR, could offer advantages to the study of these ancestral modes of ABA perception and signaling, and will help foster a better understanding of ABA signaling in a functional and evolutionary context. In this vein, we note that *Chlorella* encodes an ortholog to ABI2 (ABA insensitive 2), a protein phosphatase regulated by SOS2 that physically interacts with PYR1 in *Arabidopsis* (Park 2009)*;* this ABI2 ortholog is differentially regulated by ABA in *Chlorella* (Table

2), suggesting that PYR/PYL/RCAR-related components are present, but that their functions were recruited for ABA signaling at a later stage in the evolution of higher plants. This observation suggests further interactions between the SOS and ABA pathways, and provides a putative connection between ABA and SOS signaling and the evolution of the PYR/PYL/RCAR family of receptors.

In this study, we demonstrate that ABA has a physiological role in the chlorophyte alga *Chlorella sorokiniana*, and we demonstrate its role as a cell-cell stress signaling molecule in this organism. ABA primes cells to resist DNA damage by upregulating DNA replication and repair related transcripts. The use of ABA as a signaling molecule and the interrelation of drought and UV stress suggest that ABA was a critical step in the evolutionary advancement of land plants.

Materials and Methods

Culture conditions: An agar slant of *Chlorella sorokiniana* UTEX 1230 culture was obtained from the University of Texas culture collection on proteose peptone media. Long term cultures were maintained on TAP agar plates. Starter cultures for all experiments were allowed to grow to saturation in Bold's Basal Media (BBM) prior to dilution for commencement of experiments. All experiments were conducted with at least three biological replicates; each biological replicate contained two technical replicates.

Salt Stress: A saturated starter culture was used to start four flasks per experiment (two each of a control and a salt stressed flask). 250-mL flasks containing 50 mL of culture at 5×10^6 cells/mL were set up for salt stress. After 48 hours of growth (approximately 2.5 doublings), with an average cell density of $3\text{-}4\times10^7$ cells/mL, cells to be treated were

centrifuged at 2,500 x *g* for five minutes and resuspended in BBM containing 600 mM NaCl, chosen because it is approximately the salt concentration of seawater. Cells were harvested after 16 hours of salt treatment and processed immediately for compound quantification.

Oxidative stress: A saturated starter culture was used to start four flasks per experiment (two each of a control and a salt stressed flask). 250-mL flasks containing 50 mL of culture at 5×10^6 cells/mL were set up for salt stress. After 48 hours of growth (approximately 2.5 doublings), with an average cell density of $3\text{-}4\times10^7$ cells/mL, 5 µM paraquat (from a 5 mM stock in water) and 1 mM H_2O_2 (from a 1M stock) were added. Cells and supernatants were harvested after 16 hours of oxidative stress treatment.

Sample preparation for analysis by LC/MS:

Supernatants.

ABA quantitation: Supernatants were harvested by centrifugation and vacuum filtered through a 0.22 µm filter. A deuterated standard (d6-ABA, [2H6](+)-cis,trans-ABSCISIC ACID, OlChemIM, Olomouc, Czech Republic) was added at 1 ng/mL. The supernatant was passed through a Supelco Discovery C18 reverse phase column (Cat. no. 52604-U) according to the manufacturer's protocol, and eluted in a buffer of 50% acetonitrile/1% acetic acid. Upon elution, samples were immediately stored at -80ºC until vacuum concentration and analysis by LC/MS/MS

Extraction of cell pellets. Pellets of $1\text{-}2\times10^9$ cells were extracted by agitation in 10 mL of an 80% acetonitrile/1% acetic acid solvent overnight at 4ºC. After centrifugation to remove cell debris, the extract was evaporated under N_2 gas in an N-Evap™ 112 (Organomation Associates, Inc.) and resuspended in ~20 mL of water. This preparation

was subsequently passed over a C18 reverse phase column and eluted as described above.

LC/MS/MS quantitation parameters: For LC separation, ZORBAX Eclipse Plus C18 column (2.1 mm × 100 mm, Agilent) was used flowing at 0.45 mL/min at 40ºC. The LC system is interfaced with a Sciex QTRAP 6500+ mass spectrometer equipped with a TurboIonSpray (TIS) electrospray ion source. The hormones ABA, SA, JA, JA-Ile, and OPDA were analyzed in negative ion mode using the following source parameters: curtain gas, 20 arbitrary units (a.u.); source gas 1, 50 a.u.; source gas 2, 50 a.u.; collision activated dissociation, high; interface heater, on; temperature, 500 ºC; ionspray voltage, –4500. The LC gradient was from 80% solvent A (0.1% [v/v] acetic acid in Milli-Q water) to 50% A in 0.5 min, then to 100% solvent B (90% acetonitrile [v/v] with 0.1% acetic acid [v/v]) in 3.5 min, then hold for another 3.5 min at 100% B. The LC was finally ramped back to initial conditions in 0.5 min and re-equilibrated for both methods for 30 min. Both quadrupoles (Q1 and Q3) were set to unit resolution. Analyst software (version 1.6.3) was used to control sample acquisition and data analysis. The QTRAP 6500+ mass spectrometer was tuned and calibrated according to the manufacturer's recommendations. All hormones were detected using MRM transitions that were previously optimized using a standard and a deuterium-labeled standard. For quantification, a series of standard samples containing different concentrations of unlabeled hormones was prepared. The peak area in samples was first normalized in the same way as used for the standard samples and then quantified according to the standard curve.

OPDA quantitation: Supernatants were harvested as described above, then evaporated under reduced pressure with gentle heating in a rotary evaporator (Rotovapor R-215, Büchi). In order to recover secreted metabolites, 2 ml methanol was then swirled around the flask and transferred to a 1.5 mL conical tube. The tube was centrifuged to remove any insoluble carryover, and stored at -20ºC until LC/MS/MS as described in (37).

UV Irradiation: A saturated starter culture was diluted to 5×10^6 cells/mL. Two flasks were set up for treatment with 10 µM ABA dissolved in methanol from a 10 mM stock at a 1:1000 dilution. The control included methanol at a 1:1000 dilution. Methanol was not found to affect any growth properties at this concentration. After two days of growth, at an average cell density of $3\text{-}4*10^7$ cells/mL, cells were diluted to $1*10^6$ cells/mL, and 10 mL of this low density cell mixture was transferred to 5 mL sterile petri dishes, resulting in a thin layer that would avoid shading from subsequent UV exposure. Cells were exposed to UV treatment in a UV Stratalinker (model, part number)

Phylogenetics: Phylogenetic trees were constructed using either TCoffee (ref) or Clustal-Omega (ref). Maximum likelihood trees were generated and visualized using the LG model with Mega 6.06. The orthologs of each gene were identified by BLAST searches of NCBI and other genome-specific databases, and were identified from the following species (abbreviations correspond to those presented in Fig. 1.): *Arabidopsis thaliana*, At; *Brassica rapa*, Brapa*; Coccomyxa*, "Coccomyxa, C169"; Chlamydomonas reinhardtii, Cre; Chlorella sorokiniana, "Cs, Cso"; Cucumus sativa, *Csativa; Citrus*

cinensis, Csinensis*; Glycine max, "Gmax, Glymax"; Galdieria* sulphuraria, Gsulphuraria; Klebsormidium flaccidum, "Kflaccidum, Kfl"; *Medicago trunculata*, "Medicago, Mtru"; *Ostreococcus lucimarinus*, "Olucimarinus, Olu"; *Oryza sativa*, Osativa; *Physcomitrella patens*, Ppatens; *Populus trichocarpa*, Ptrichocarpa; *Sorghum bicolor*, "Sbicolor, Sobic"; *Solanum lycopersicum*, "Slycopersicum, Solyc"; *Selaginella moellendorfii*, Smo/Smoellendorfii; *Volvox carteri*, Vcarteri; *Zea mays*, Zmays

Sample preparation for transcriptomic analysis:

Cells were inoculated into a 50 mL starter culture of BBM and allowed to grow to saturation, about 5 to 6 days. Cells were then subcultured at a density of 5×10^6 cells in 125 mL media in a 1L flask. Cells were grown for 48 hours in continuous light to a density of 3-4 $\times 10^7$, with 100 µM ABA in methanol and with an ABA-free methanol control, in duplicate. 100 mL of cells was harvested and frozen in liquid nitrogen and stored at -80ºC until isolation. RNA was isolated using the TriZOL method as described in the manufacturer's protocol.

Transcriptomic analysis:

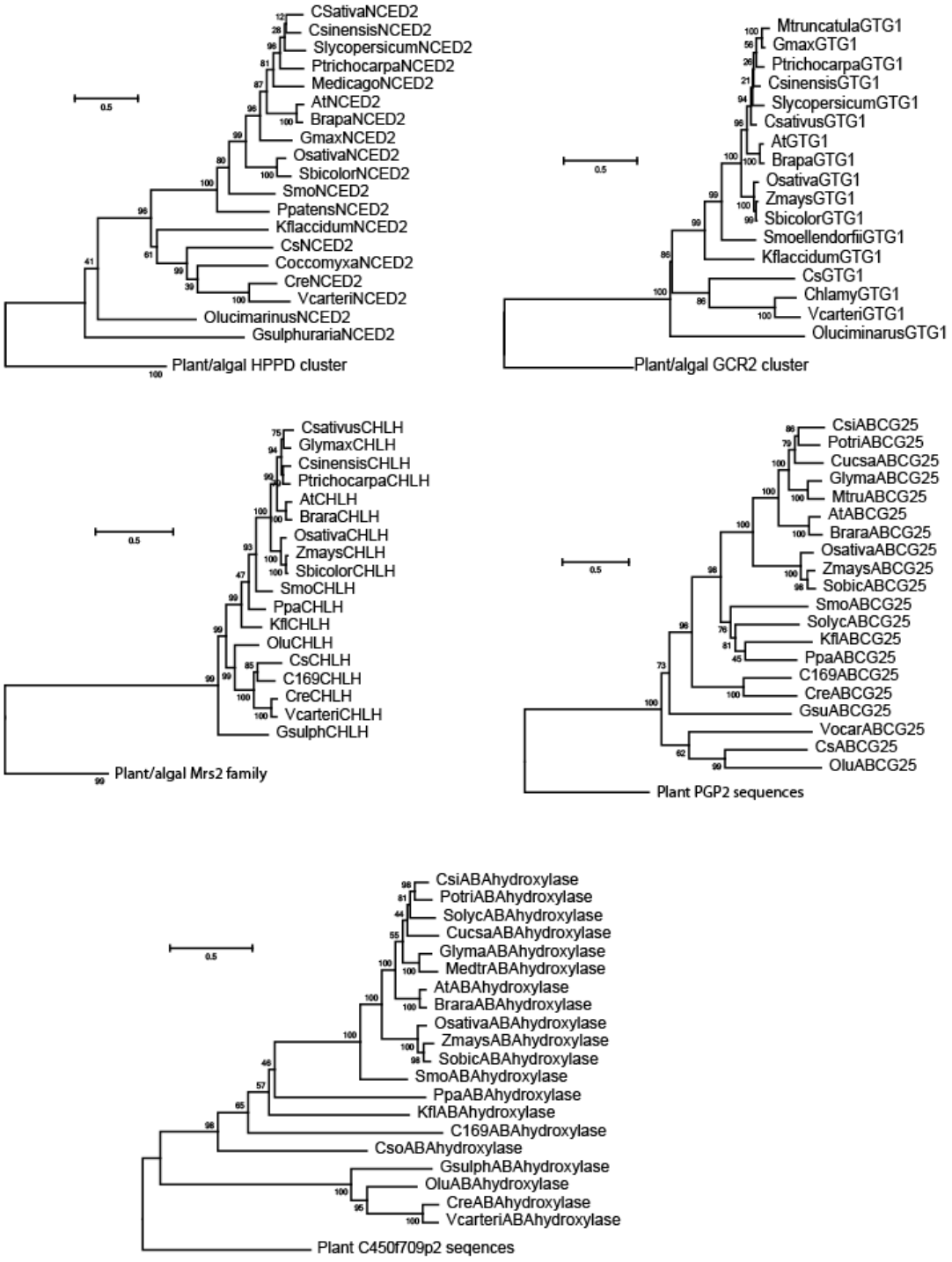
RNA was sequenced at Cofactor Genomics (St. Louis, MO) on the Illumina Genome Analyzer 2000 platform with two biological replicates per condition, as follows:

Control 1: 37,320,193 50 bp single end reads

Control 2: 47,652,886 50 bp single end reads

Abscisic acid 1: 60,393,257 50 bp single end reads

Abscisic acid 2: 53,749,656 50 bp single end reads



Khasin et al. 2017, Figure 1

Figure 1. Phylogenetic analysis of ABA related genes. Phylogenetic trees were constructed for A: 9-cis-epoxycarotenoid dioxygenase NCED2 (biosynthetic commitment step), B: ABA receptor GPCR-type G protein 1 (GTG1), C: Alternative ABA receptor magnesium-chelatase H subunit (CHLH), D: ABA efflux transporter ABCG25, and, E: ABA inactivation enzyme ABA hydroxylase (v. a closely related Cp450 redox domain containing protein). The amino acid substitution model selected was LG+G. Phylogenetic analysis using maximum likelihood was performed in MEGA ver 6 with 500 bootstraps.

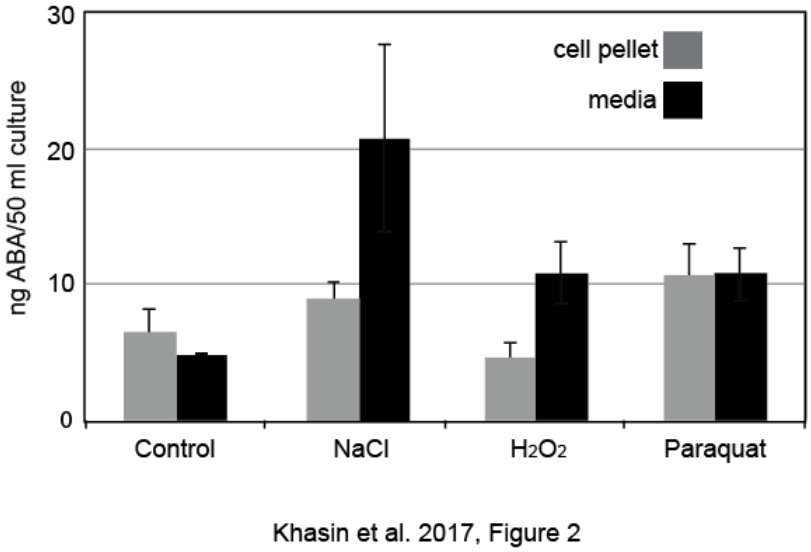
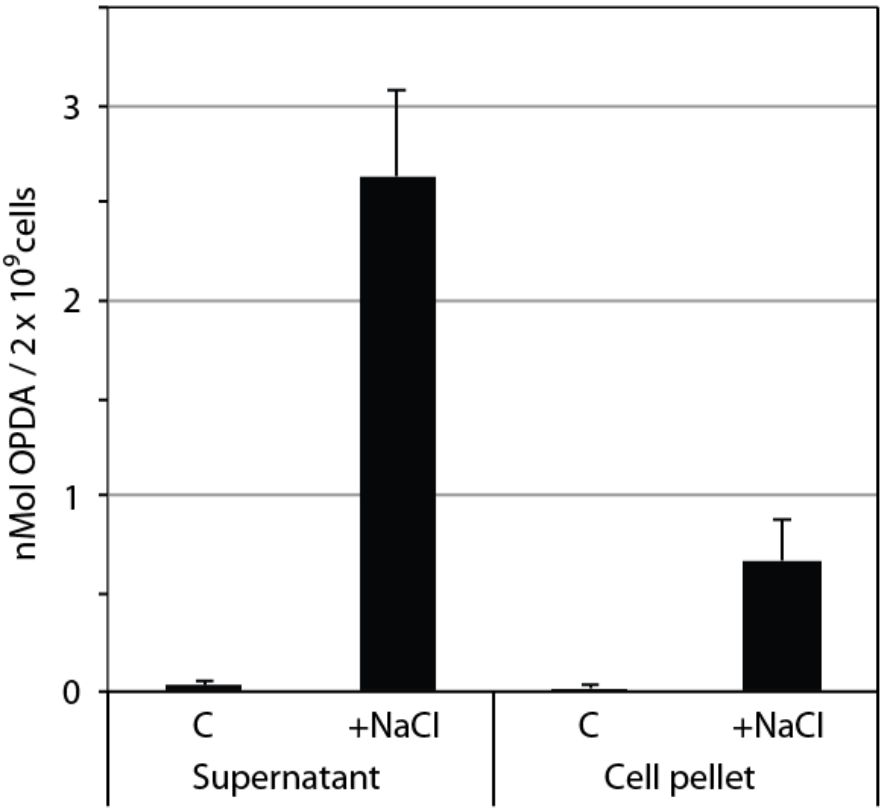
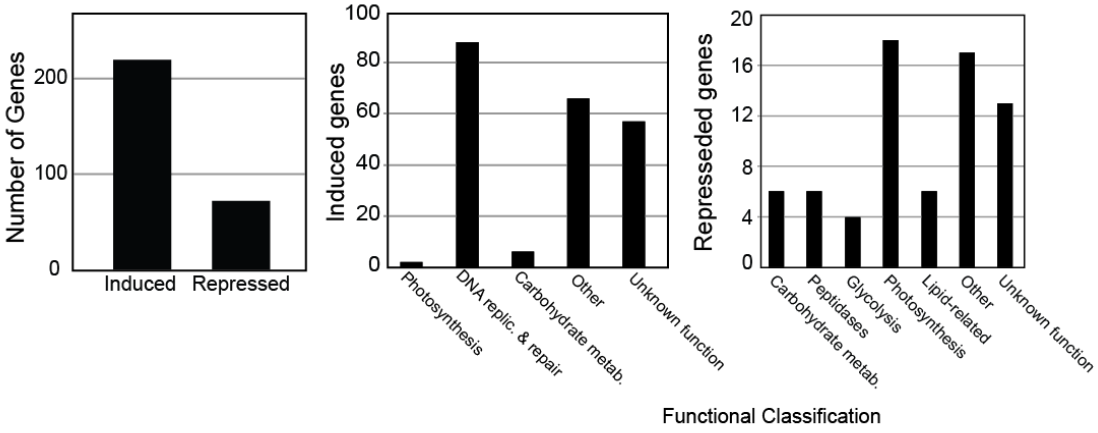


Figure 2. *Chlorella sorokiniana* cells produce and secrete ABA. In response to 600 mM salt stress and oxidative stresses, *Chlorella* secretes almost twice as much ABA into the supernatant as in the pellet, except for paraquat, which remains stable. Error bars represent standard error of biological replicates conducted in duplicate. Cell pellets, n =2, culture media, n = 3.



Khasin et al. 2017, Figure 3

Figure 3. OPDA, a stress phytohormone, is produced in response to salt shock. Cultures were treated with 600 mM NaCl as described for Figure 2, and OPDA quantified by LC/MS/MS as described in Materials and Methods. Error bars represent the S.E.M. of 3 independent biological replicates conducted in duplicate.



Khasin et al. 2017, Figure 4

Figure 4. Differential expression of genes upon ABA treatment. A: 290 total genes are differentially regulated by at least twofold: 71 are downregulated, and 219 are upregulated. B. DNA replication and repair related genes make up the largest category of upregulated genes with predicted functions . C. Genes related to photosynthesis and glycolysis are downregulated.

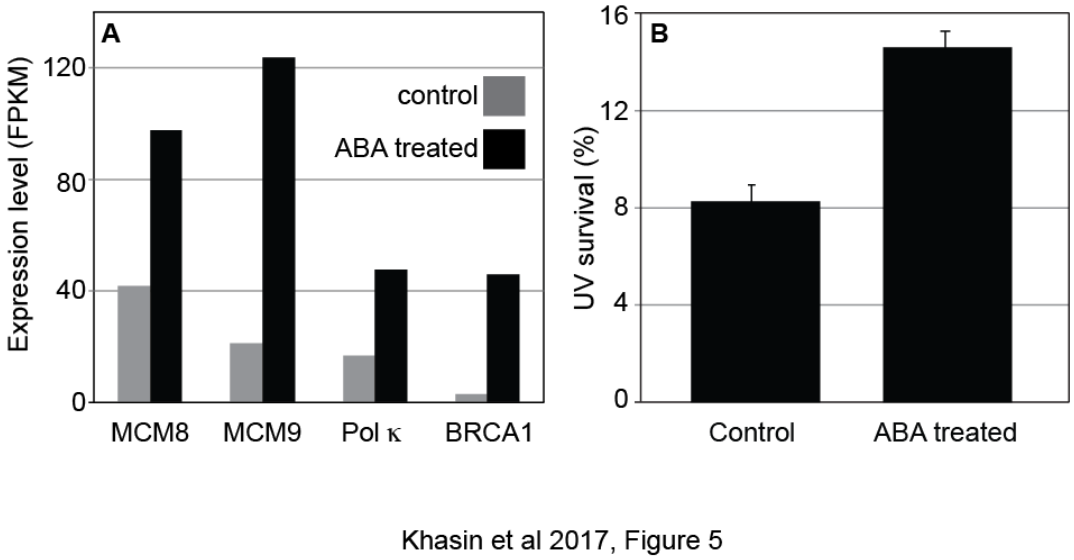
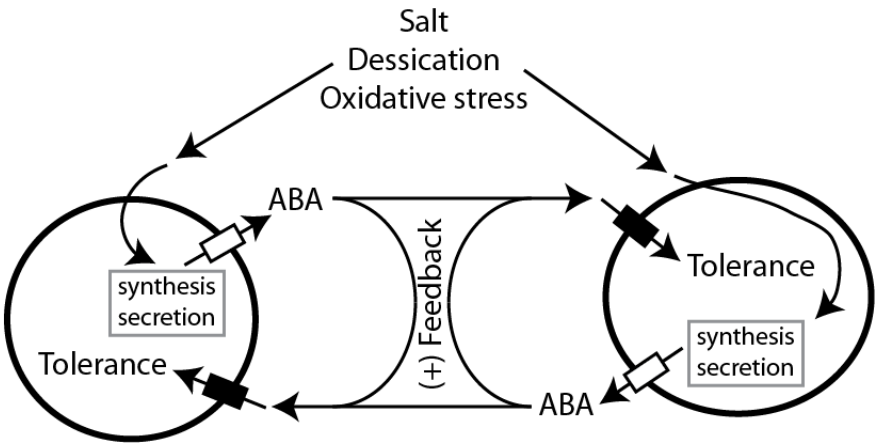


Figure 5. Abscisic acid pretreatment enhances tolerance to UV irradiation. Cells pretreated for 48 hours with 10 µM ABA were found to have a survival rate of 15%, compared to an 8.3% survival rate for the untreated cells. N = 4, *p*= 0.0004, computed using the student's T test for equal variances.



Khasin et al. 2017, Figure 6

Figure 6. A general model for ABA synthesis and signaling in *Chlorella sorokiniana*. We propose a framework in which abiotic stressors induce the synthesis and secretion of ABA and other potential signaling molecules, leading to gene expression changes associated with stress tolerance. Importantly, the concentration of these molecules will be proportional to the population density, allowing for signal amplification and coordination of population-level stress responses.

Gene name and function	Arabidopsis accession	*Chlorella* ortholog	Expect
GTG1 – alternative ABA receptor	AT1G64990	sca012.g110550.t1	6e-95
NCED2 – ABA biosynthesis, 9-cis-epoxycarotenoid dioxygenase	AT4G18350	sca015.g107700.t1 sca146.g104150.t1	2e-65 2e-22
abscisic acid 8'-hydroxylase 3; ABA inactivation enzyme	AT5G45340	sca221.g100650.t2	2e-42
ABCG25, ABA efflux transporter	AT1G71960	sca052.g100950.t3	1e-102

Table 1. The *Chlorella sorokiniana* genome encodes orthologs to ABA biosynthesis, perception, transport, and degradation. Representative members of the respective pathways were used to conduct reciprocal BLAST searches. It was found that *C. sorokiniana* contains at least one ortholog to each pathway.

Gene	***Arabidopsis* accession**	***Chlorella* accession**	**Expect**	**Function**
SOS2 (SNRK3)	AT5G35410	sca154.g100300.t1	3e-119	Ser/Thr kinase, regulated by ABI2, SOS3, Ca^{2+}
SOS3	AT5G24270	sca106.g102500.t1	3e-56	Calcium sensor; activates SOS2
SOS1 (ATNHX7)	AT2G01980	sca004.g105100.t1	2e-80	Na^{+}/H^{+} antiporter, regulated by SOS2 and SOS3
SOS4	AT5G37850	sca081.g103450.t1	7e-105	Pyridoxal kinase
MAPKKK (NPK1)	AT3G06030	sca015.g103350.t1	3e-109	Activates oxidative signal cascade; *Arabidopsis* transgenics show higher photosynthetic rates; tolerance to cold, heat, salinity

Table 2. Stress signal transduction genes in *C. sorokiniana*. The SOS pathway is found in *Chlorella,* suggesting that it is an ancient pathway that has since differentiated and specialized in higher plants. Additionally, CsMAPKKK/CsNPK1 are significantly ($2.52\log_2$, $q = 0.027$) upregulated by treatment with ABA (Table 3).

Central metabolism genes upregulated by ABA

Gene	*Chlorella* accession	log_2-fold-change	Pathway/function
Malate synthase	sca085.g100800.t1	3.65	**Glyoxylate bypass**
Pyruvate ferredoxin/flavodoxin oxidoreductase	sca098.g101900.t1	2.78	**Glycolysis**
PEP carboxykinase	sca027.g100650.t1	2.71	**Gluconeogenesis**

Central metabolism downregulated by ABA

Gene	*Chlorella* accession	log_2-fold-change	Pathway/function
PUMP2	sca004.g110000.t1	-3.25	mitochondrial transporter proteins that create proton leaks across the inner mitochondrial membrane
PGK	sca089.g101600.t1	-3.87	Glycolysis
GAPDH	sca161.g101550.t2	-2.86	Glycolysis
PRK	sca102.g100400.t1	-2.31	Calvin cycle

Photosynthesis genes downregulated by ABA

Gene	*Chlorella* accession	log_2-fold-change	Pathway/function
Carbonic anhydrase	sca004.g108450.t1	-3.79	Carbon concentration/photosynthesis
Phytoene desaturase	sca153.g100750.t1	-3.15	Chlorophyll biosynthesis/photosynthesis
Rubisco large subunit methyltrasferase	sca221.g102000.t1	-2.57	Photosynthesis
Photoysystem II stability/assembly factor HCF136	sca134.g104050.t1	-2.82	Photosynthesis
Divinyl chlorophyllide A 8-vinyl-reductase	sca034.g104550.t1	-2.38	Photosynthesis/chlorophyll biosynthesis
Uroporphyrinogen-III decarboxylase	sca067.g100650.t1	-2.3237	Photosynthesis/chlorophyll biosynthesis
Coproporphyrinogen III oxidase	sca077.g103150.t1	-2.18	Chlorophyll/heme biosynthesis

Table 3 (continued on next page). Representative members of gene ontology classes that are altered by ABA exposure.

DNA replication and repair proteins upregulated by ABA

Gene	*Chlorella* accession	log_2-fold-change	Pathway/function
DNA helicase MCM8	sca081.g104500.t1	3.71	**Recombination repair**, with MCM9
DNA helicase MCM9	sca104.g106050.t1	3.37	**Recombination repair**, with MCM8
DNA Pol α	sca079.g105350.t1	3.47	**Okazaki fragment synthesis**
DNA Pol κ	sca106.g112250.t2	2.48	**DNA repair: translesion synthesis**
BRCA1-like	sca206.g103000.t1	3.69	**Double strand break repair**, homologous recombination

Table 3 (cont.) Representative members of gene ontology classes that are altered by ABA exposure.

References

1. Mahajan S, Pandey GK, Tuteja N. 2008. Calcium- and salt-stress signaling in plants: Shedding light on SOS pathway. Arch Biochem Biophys 471:146–158.
2. Hauser F, Waadt R, Schroeder JI. 2011. Evolution of abscisic acid synthesis and signaling mechanisms. Curr Biol 21:R346–R355.
3. Huang GT, Ma SL, Bai LP, Zhang L, Ma H, Jia P, Liu J, Zhong M, Guo ZF. 2012. Signal transduction during cold, salt, and drought stresses in plants. Mol Biol Rep 39:969–987.
4. Blanc G, Duncan G, Agarkova I, Borodovsky M, Gurnon J, Kuo A, Lindquist E, Lucas S, Pangilinan J, Polle J, Salamov A, Terry A, Yamada T, Dunigan DD, Grigoriev I V, Claverie J, Etten JL Van. 2010. The Chlorella variabilis NC64A Genome Reveals Adaptation to Photosymbiosis , Coevolution with Viruses , and Cryptic Sex 22:2943–2955.
5. Hori K, Maruyama F, Fujisawa T, Togashi T, Yamamoto N, Seo M, Sato S, Yamada T, Mori H, Tajima N, Moriyama T, Ikeuchi M, Watanabe M, Wada H, Kobayashi K, Saito M, Masuda T. 2014. factors for plant terrestrial adaptation. Nat Commun 5:1–9.
6. Worden AZ, Robbens S, Derelle E, Ferraz C, Rombauts S, Rouze P, Degroeve S, Echeynie S, Cooke R, Saeys Y, Wuyts J, Jabbari K, Bowler C, Panaud O, Delseny M, Demaille J, Peer Y Van De, Piganeau G, Baets B De. 2006. Genome analysis of the smallest free-living eukaryote Ostreococcus tauri unveils many unique features 103.
7. Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, Witman GB, Terry A, Salamov A, Fritz-laylin LK, Maréchal-drouard L, Marshall WF, Qu L, Nelson DR, Sanderfoot AA, Spalding MH, Kapitonov V V, Ren Q, Ferris P, Lindquist E, Shapiro H, Lucas SM, Grimwood J. 2007. The Chlamydomonas Genome Reveals the Evolution of Key Animal and Plant Functions 318.
8. Hirsch, R, Hartung W, Gimmler H. 1989. Abscisic acid content of algae under stress. Bot Acta 102:326–334.
9. Hartung W. 2010. The evolution of abscisic acid (ABA) and ABA function in lower plants, fungi and lichen. Funct Plant Biol 37:806–812.
10. Cowan a K, Rose PD. 1991. Abscisic Acid Metabolism in Salt-Stressed Cells of Dunaliella salina: Possible Interrelationship with -Carotene Accumulation. Plant Physiol 97:798–803.
11. Park S, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TF, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Desveaux D, Rodriguez PL, McCourt P, Zhu J-K, Schroeder JI, Volkmann BF, Cutler SR. 2009. Abscisic Acid Inhibits Type 2C. Science (80-) 324:1068–1069.
12. Wu F-Q, Xin Q, Cao Z, Liu Z-Q, Du S-Y, Mei C, Zhao C-X, Wang X-F, Shang Y, Jiang T, Zhang X-F, Yan L, Zhao R, Cui Z-N, Liu R, Sun H-L, Yang X-L, Su Z, Zhang D-P. 2009. The Magnesium-Chelatase H Subunit Binds Abscisic Acid and Functions in Abscisic Acid Signaling: New Evidence in Arabidopsis. Plant Physiol 150:1940–1954.
13. Pandey S, Nelson DC, Assmann SM. 2009. Two Novel GPCR-Type G Proteins Are Abscisic Acid Receptors in Arabidopsis. Cell 136:136–148.
14. Fischer BB, Ledford HK, Wakao S, Huang SG, Casero D, Pellegrini M, Merchant SS, Koller A, Eggen RIL, Niyogi KK. 2012. PNAS Plus: SINGLET OXYGEN RESISTANT 1 links reactive electrophile signaling to singlet oxygen acclimation in Chlamydomonas reinhardtii. Proc Natl Acad Sci 109:E1302–E1311.
15. Cho D, Shin D, Jeon BW, Kwak JM. 2009. ROS-mediated ABA signaling. J Plant Biol 52:102–113.
16. Wei JZ, Tirajoh A, Effendy J, Plant AL. 2000. Characterization of salt-induced changes

in gene expression in tomato (Lycopersicon esculentum) roots and the role played by abscisic acid. Plant Sci 159:135–148.
17. Balestrazzi A, Confalonieri M, Macovei A, Donà M, Carbonera D. 2011. Genotoxic stress and DNA repair in plants: Emerging functions and tools for improving crop productivity. Plant Cell Rep 30:287–295.
18. Zhu J-K, Xiong L, Zhu J-K. 2002. Molecular and genetic aspects of plant responses to osmotic stress. Plant, Cell Environ 25:131–139.
19. Matsui A, Ishida J, Morosawa T, Mochizuki Y, Kaminuma E, Endo TA, Okamoto M, Nambara E, Nakajima M, Kawashima M, Satou M, Kim JM, Kobayashi N, Toyoda T, Shinozaki K, Seki M. 2008. Arabidopsis transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. Plant Cell Physiol 49:1135–1149.
20. Kobayashi N, Noel EA, Barnes A, Watson A, Rosenberg JN, Erickson G, Oyler GA. 2013. Bioresource Technology Characterization of three Chlorella sorokiniana strains in anaerobic digested effluent from cattle manure. Bioresour Technol 150:377–386.
21. Rosenberg JN, Kobayashi N, Barnes A, Noel EA, Betenbaugh MJ, Oyler GA. 2014. Comparative Analyses of Three Chlorella Species in Response to Light and Sugar Reveal Distinctive Lipid Accumulation Patterns in the Microalga C . sorokiniana 9.
22. Kobayashi N, Noel EA, Barnes A, Black P, Oyler GA. 2013. Rapid Detection and Quantification of Triacylglycerol by HPLC – ELSD in Chlamydomonas reinhardtii and Chlorella Strains 1035–1049.
23. Taki N, Sasaki-sekimoto Y, Obayashi T, Kikuta A, Kobayashi K. 2005. 12-Oxo-Phytodienoic Acid Triggers Expression of a Distinct Set of Genes and Plays a Role in Wound-Induced Gene Expression in Arabidopsis 1 [w] 139:1268–1283.
24. Ulm R, Ichimura K, Mizoguchi T, Peck SC, Zhu T, Wang X, Shinozaki K, Paszkowski J. 2002. Distinct regulation of salinity and genotoxic stress responses by Arabidopsis MAP kinase phosphatase 1. EMBO J 21:6483–6493.
25. Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ. 2006. ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H2O2 synthesis. Plant J 45:113–122.
26. Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L. 2013. Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat Biotechnol 31:46–53.
27. Nishimura K, Ishiai M, Horikawa K, Fukagawa T, Takata M, Takisawa H, Kanemaki MT. 2012. Mcm8 and Mcm9 Form a Complex that Functions in Homologous Recombination Repair Induced by DNA Interstrand Crosslinks. Mol Cell 47:511–522.
28. Shen Y-Y, Wang X-F, Wu F-Q, Du S-Y, Cao Z, Shang Y, Wang X-L, Peng C-C, Yu X-C, Zhu S-Y, Fan R-C, Xu Y-H, Zhang D-P. 2006. The Mg-chelatase H subunit is an abscisic acid receptor. Nature 443:823–826.
29. Muñoz-Bertomeu J, Cascales-Miñana B, Alaiz M, Segura J, Ros R. 2010. A critical role of plastidial glycolytic glyceraldehyde-3-phosphate dehydrogenase in the control of plant metabolism and development. Plant Signal behav 5:67–9.
30. Apel K, Hirt H. 2004. REACTIVE OXYGEN SPECIES: Metabolism, Oxidative Stress, and Signal Transduction. Annu Rev Plant Biol 55:373–399.
31. Msanne J, Xu D, Konda AR, Casas-Mollano JA, Awada T, Cahoon EB, Cerutti H. 2012. Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae Chlamydomonas reinhardtii and Coccomyxa sp. C-169. Phytochemistry 75:50–59.
32. Holzinger A, Becker B. 2015. Desiccation tolerance in the streptophyte green alga

Klebsormidium: The role of phytohormones. Commun Integr Biol 8:1–4.
33. Lind C, Dreyer I, López-sanjurjo EJ, Meyer K Von, Kohchi T, Lang D, Zhao Y, Kreuzer I, Al- KAS, Ronne H, Reski R, Zhu J, Geiger D, Hedrich R. 2015. Stomatal Guard Cells Co - opted an Ancient ABA - Dependent Desiccation Survival System to Regulate Stomatal Closure 928–935.
34. Holzinger A, Kaplan F, Blaas K, Zechmann B, Komsic-Buchmann K, Becker B. 2014. Transcriptomics of desiccation tolerance in the streptophyte green alga Klebsormidium reveal a land plant-like defense reaction. PLoS One 9.
35. Albinsky D. 1999. Plant responses to genotroxic stress are linked to an ABA/salinity signaling pathway. Plant J 17:73–82.
36. Saha P, Mukherjee A, Biswas AK. 2014. Modulation of NaCl induced DNA damage and oxidative stress in mungbean by pretreatment with sublethal dose. Biol Plant 59:139–146.
37. Pan X, Welti R, Wang X. 2010. Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography-mass spectrometry. Nat Protoc 5:986–992.

APPENDIX A

MOLECULAR BIOLOGY TOOL DEVELOPMENT IN *CHLORELLA* SPECIES: TRANSIENT INTEGRATION, TRANSIENT EXPRESSION, AND BEYOND

Abstract

The biotechnological potential of algae is immense: algae are used in the manufacture of high-value products for cosmetics, as unicellular models for photosynthesis, and have been explored as potential biofuels feedstocks. Among the chief advantages of algae for biofuels are that algae do not compete for arable land, they quickly accumulate biomass, and, from a biofuels perspective, they produce a high percentage of lipids per unit biomass that can be easily converted to fatty acid methyl esters for biodiesel. In order for more nuanced control of these microbial factories, *Chlorella vulgaris* UTEX 259 and 395, genetic systems were established to hasten a nuanced understanding of *Chlorella* biology, from feedstock production to a biophysical model for photosynthesis. We predicted that strong *cis*-regulatory elements from *Chlorella* species and from the *Chlorella*-infecting virus PBCV-1 will be able to control positive selection markers and reporter constructs in *Chlorella* species, paving the way for genetic tool development in this previously intractable species. This project had the added goal of optimization of *Chlorella* vectors with regard to other *cis*-regulatory elements, such as the number and type of intron, as well as the role of a native terminator. The development of a transformant library will enable mutant screens for desirable characteristics, including increased oil production. This appendix describes the current attempt taken at this juncture in algal molecular biology studies.

Introduction

As the global demand for transportation fuels increases, the necessity for an alternative to the finite fossil fuel reserves becomes inescapable. Among the chief advantages of algae for biofuels are that algae do not compete for arable land, they quickly accumulate biomass, and they produce a high percentage of lipids per unit biomass that can be easily converted for fatty acid methyl esters for biodiesel (1). Developing an alternative fuel and energy source is one of the most critical problems facing our generation of researchers.

Molecular biology in *Chlorella* has been limited by the lack of a nuclear transformation system, whereas a robust array of nuclear genetic tools exists in *Chlamydomonas reinhardtii,* a model green alga. In particular, whereas chloroplast and mitochondrial recombination is homologous, nuclear recombination is typically non-homologous. Cell line engineering is therefore partially limited by non-homologous recombination, with many tools being developed to circumvent the disadvantages of non-homologous recombination within the nucleus. Several factors can influence the stability of *C. reinhardtii* nuclear transformants. *C. reinhardtii* has a GC-rich genome. Codon optimization is therefore critical for the efficient expression of transgenes in C. reinhardtii ; non-codon optimized genes are more likely to be silenced (2).

Materials and Methods

Construct design

Chlorella vulgaris UTEX 395 and *Chlorella vulgaris* UTEX 259 were sequenced at the National Renewable Energy Laboratory (Michael Guarnieri, pers. com., 2013)

using paired-end reads and the University of California – Sand Diego (Stephen Mayfield, pers. com., 2013) using single-end reads, respectively, and annotated with AUGUSTUS. Transcriptomics studies on *Chlorella vulgaris* UTEX 259 identified five strongly and constitutively expressed genes: RuBisCO subunits *rbcs1* and *rbcs2*, ubiquitin, photosystem I reaction center subunit II *psaD*, and alpha tubulin.

Our approach to developing molecular biology tools in *Chlorella* draws on precedent in *Chlamydomonas* and previous attempts in *Chlorella* (2, 3). "First generation" *Chlorella* constructs contained respective promoters and 5' UTRs from *rbcs1*, *rbcs2*, *psaD*, and ubiquitin, the intron from *Chlamydomonas rbcs2*, the first two codons from the corresponding gene, and the 3' UTR from *Chlamydomonas psaD* (Fig 1). However, these constructs were not able to transform *Chlorella*, suggesting a strong specific barrier in between *Chlamydomonas* and *Chlorella*. We therefore identified the corresponding orthologs to these five highly expressed genes in *Chlorella* (Table 1) and used native *cis*-regulatory elements corresponding to those promoters to design five constructs, except for the intron, which was from *Chlorella* alpha tubulin (Fig. 3). Additionally, we codon optimized the reporter construct, Sh *ble*::*gfp*, a Zeocin® (Invivogen, San Diego, CA) binding protein conferring resistance and GFP fusion protein (codon optimized for *Chlorella* by Genscript, Piscataway, NJ). The region 600 bp upstream of the 3' UTR of the corresponding gene was chosen as the promoter-containing region.

Transformation protocol

Cells were inoculated at a density of 5×10^6 cells/mL grown in TAP medium + ampicillin to late log phase ($1\text{-}3 \times 10^8$ cells/mL). 15 mL of cells (per transformation)

were harvested at 2500 rpm for 10 min at room temperature. The cells were resuspended in 250 µL TAP + 60 mM sucrose at room temperature and transferred to a 0.4 cm electroporation cuvette. 0.5-2.0 µg linearized plasmid DNA was added and the mixture was incubated at room temperature for 5 min. The cuvette was then electroporated on a Bio-Rad Gene Pulser II (Cat. no. 165-2105, Bio-Rad, Hercules, California) at 0.6 kV, 50 µF capacitance, and infinite resistance. The cells were immediately transferred to 60 x 15 mm petri dishes containing 5 mL TAP + sucrose recovered overnight on a lighted shaker at 100 rpm. After recovery, cells were plated on 200 µg/mL Zeocin® selection plates (~250 µL/plate).

Results and Discussion

Through PCR (Fig. 3) and through fluorescence microscopy (Fig. 4), several putative transformants were identified. However, PCR screening revealed that many transformants were partial, and PCR does not reveal a difference between integration and extrachromosomal maintenance, and several transformants indicated a complete construct but not a partial one, indicating potential nonspecific binding of primers. Additionally, initially fluorescent clones soon lost fluorescence. A putative insertional mutant displayed slow growth and a light green phenotype (Fig. 5), indicating a putative disruption of the photosynthetic apparatus; however, an off target effect from the high concentrations Zeocin® required for *Chlorella* selection cannot be ruled out, as Zeocin® is itself an intercalating agent and a mutagen. These transient and partial expression effects indicate either partial integration or extrachromosomal maintenance, and transient expression, respectively, indicating remaining barriers to integration and

protein expression in *Chlorella*. For instance, it is possible that 600 bp upstream was neither a complete nor correct promoter choice.

Southern blot analysis indicated that while *Chlorella* is able to maintain constructs, it appears that the construct is maintained separately from the chromosome (Horken and Cerutti, pers. comm., 2014); this offers a plausible explanation for the loss of fluorescence in putative transformants. Future work on this endeavor may elucidate physiological barriers to transformation (such as the presence or absence of polyamines, the physiological state of the cell, etc.) in order to obtain a reproducible transformation protocol for *Chlorella*. This will additionally further a more robust understanding of codon usage and *cis*-regulatory element usage between *Chlorella* and *Chlamydomonas*, and allow for the screening of desirable characteristics, such as oil or high value product production (4).

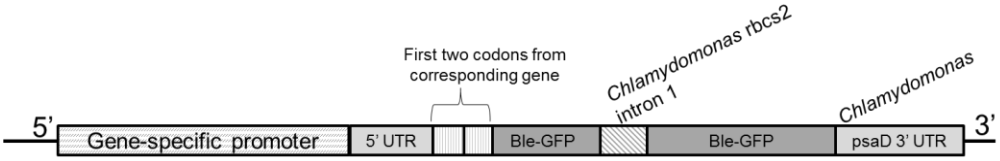


Figure 1. "First generation" *Chlorella* transformation constructs contained a promoter corresponding to a 5' UTR and the first two codons for rbcs1, rbs2, psaD, ubiquitin, and alpha tubulin orthologs in *Chlorella* NC64A, a Sh *ble*::*gfp* Zeocin® resistance gene codon optimized for *Chlamydomonas* and containing the intron from *Chlamydomonas rbcs2*, and terminating with the *psaD* 3' UTR from *Chlamydomonas*.

Figure 2. Second-generation *Chlorella* constructs contained a species-specific promoter for *rbcs1, rbcs2, psaD*, ubiquitin, or alpha tubulin from the respective *Chlorella vulgaris* 259 or 395, and UTRs and the first intron for alpha tubulin.

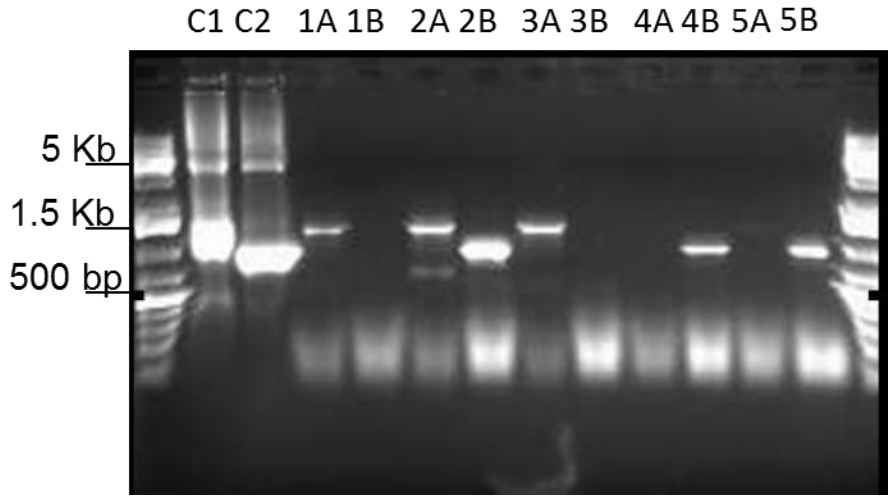


Figure 3. A preliminary PCR screen identifies putative transformants of Chlorella vulgaris UTEX 259 for the psaD::Bleo-GFP construct. The first lane of each construct amplifies the full insert (predicted size: ~1.5 kb) and the second amplifies a truncated insert (predicted size: ~1 kb). Lanes: +C, PCR directly from the transforming construct; in subsequent lanes, A represents the complete construct and B represents a partial construct.

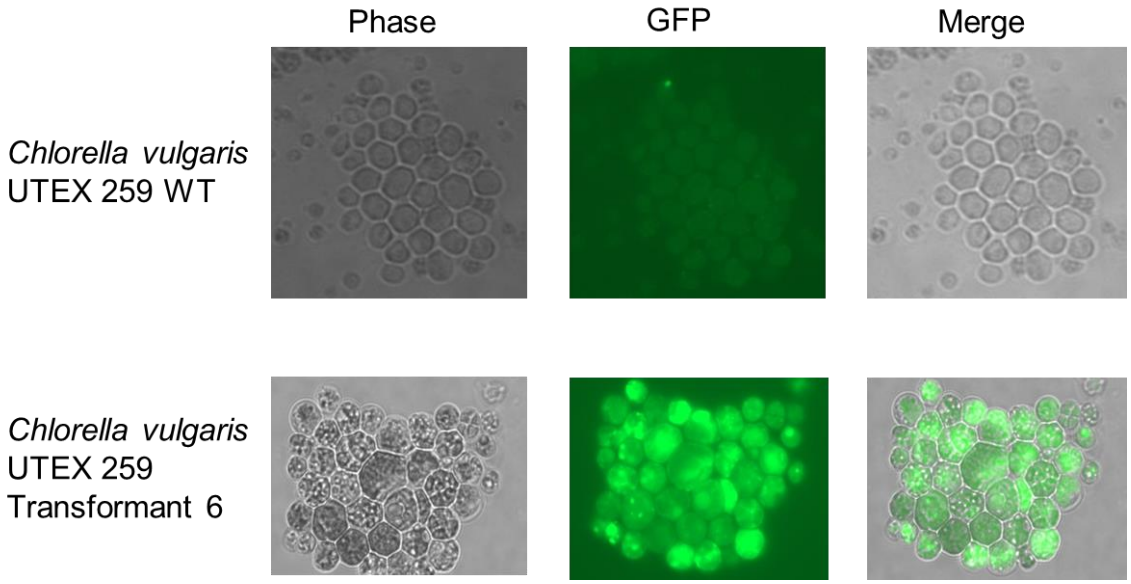


Figure 4. A fluorescence micrograph demonstrates green fluorescence in a putative transformant above the wild-type. The putative transformant was transformed with *psaD*::*ble-gfp*.

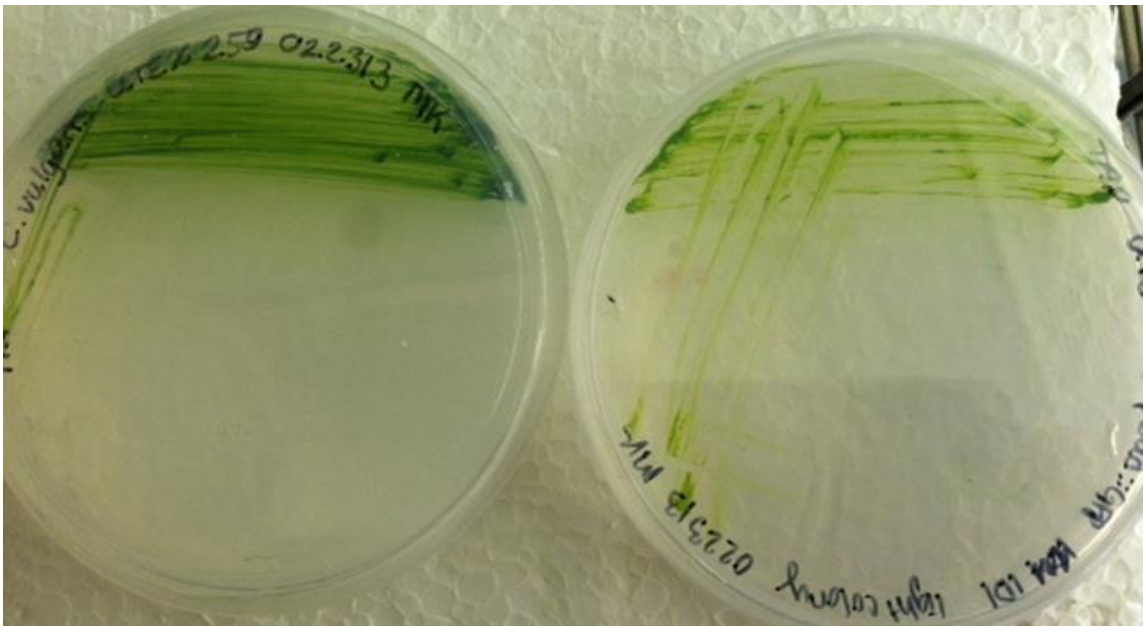


Figure 5. Insertional mutant 4 (right), in which PCR screening identified a putative partial insertion, compared to wild-type Chlorella vulgaris UTEX 259 (left). The insertional mutant is lighter in color than the wild-type, suggesting a potential mutation in a gene encoding the photosynthetic apparatus.

References

1. Menetrez MY. 2012. An overview of algae biofuel production and potential environmental impact. Environ Sci Technol 46:7073–7085.
2. Shimogawara K, Fujiwara S, Grossman A, Usuda H. 1998. High-efficiency transformation of Chlamydomonas reinhardtii by electroporation. Genetics 148:1821–1828.
3. van Overbeek LS, Saikkonen K. 2016. Impact of Bacterial-Fungal Interactions on the Colonization of the Endosphere. Trends Plant Sci 21:230–242.
4. Cagnon C, Mirabella B, Nguyen HM, Beyly-Adriano A, Bouvet S, Cuiné S, Beisson F, Peltier G, Li-Beisson Y. 2013. Development of a forward genetic screen to isolate oil mutants in the green microalga Chlamydomonas reinhardtii. Biotechnol Biofuels 6:178.

APPENDIX B

DETERGENT RESISTANCE AND PBCV-1 INFECTIVITY IN *CHLORELLA VARIABILIS* NC64A

Abstract

Developing an alternative fuel and energy source is one of the most critical problems facing our generation of researchers. In order for an algal system to be viable for fuel production, it is important that it be able to grow in the presence of pollutants. *Chlorella variabilis* NC64A, an endosymbiont to *Paramecium bursaria* and host to giant chlorovirus PBCV-1, has been reported to resist lysis in up to 1% sodium dodecyl sulfate (SDS), but growth data in the presence of SDS has not been reported. The growth of *Chlorella variabilis* NC64A was measured presence of the detergent SDS, an anioinic detergent; Triton X-100, a neutral detergent, and cetylpyridinium chloride (CPC). Under increased magnesium concentration, SDS resistance was increased by a factor of at least two, measured in maximum cell yield. Cell wall composition not only mediates detergent resistance, but also attachment of large DNA virus PBCV-1. It was found that increased magnesium had a protective effect against infection by PBCV-1, and that increased sulfate had a deleterious effect. This appendix discusses the commonalities between detergent resistance and resistance to viral infectivity.

Introduction

Detergents are common environmental pollutants that have multiple deleterious effects on biological systems. An ideal candidate for algal biotechnology would be able to grow in the presence of these common pollutants, especially in common algal uses, such as wastewater remediation. *Chlorella variabilis* NC64A, is a unicellular microalga,

endosymbiont of *Paramecium* and host to chlorovirus PBCV-1, and has previously been reported to resist lysis at up to 1% SDS in a protein extraction protocol (Van Etten et al., 1983; Dunigan 2011, personal communication). Other *Chlorella* species, such as *Chlorella vulgaris* are highly susceptible to detergent, experiencing a decrease in photosynthetic capacity. Due to the high degree of detergent resistance in *Chlorella*, the effects of anionic, neutral, and cationic detergents on growth rate and cell yield were investigated. The effect of media composition on surface polymers being well documented in microbial physiology led us into an investigation on the effect of media composition on PBCV-1 attachment, as the receptor remains unidentified.(3, 4). It was found that certain media compositions, particularly increased magnesium, appeared to have a protective effect both against detergent and viral infection.

Materials and Methods

Detergent resistance growth assay

Cells were grown to mid-log phase (~8×10^6 – 1.0×10^7 cells/mL) in a 12h light/12h dark cycle, spun down, washed 1X with MBBM, and resuspended at a concentration of 1-2 $\times 10^6$ cells/mL into a 10 mL culture (5). Detergents were diluted from a 10% w/v liquid stock. Cells were counted directly in a hemocytometer once per day.

Media modifications

30% S: MBBM was made up without peptone and without suflate solutions. We refer to this as 0% sulfate MBBM. 7 mL of 0% sulfate MBBM was added to 3% normal MBBM.

1X seawater sulfate refers to MBBM + 8.55 g Na_2SO_4/L

5X seawater sulfate refers to MBBM + 42.75 g Na_2SO_4/L

5X Mg^{2+} refers to MBBM + 0.5 mM $MgCl_2$.

10X Mg^{2+} refers to MBBM + 1mM $MgCl_2$.

PBCV-1 infectivity:

Cells were grown to mid-log phase (~$8 \times 10^6 – 1.0 \times 10^7$ cells/mL) in a 12h light/12h dark cycle, spun down, washed 1X with MBBM, and resuspended at a concentration of 1.0 × 107 cells/mL. Cells were infected with PBCV-1 at a multiplicty of infection (MOI) of 5 for 30 min. After each respective protocol, plaque assays were plated onto fresh NC64A cells.

Attachment assay: 1.5 mL of cells were pelleted. The supernatant was removed, serially diluted, and plated. This assay measures viral particles remaining in the supernatant.

Burst size assay: 10 mL of cells were pelleted, washed with MBBM, diluted 5000-fold, and incubated a total of 9h. This determines how many viral particles the host cells release upon lysis.

Infective centers assay: At 2h post infection, cells were pelleted and washed 1X with MBBM. This assay measures the amount of cells in the suspension that are able to release viral particles (6).

Results and Discussion

Detergent susceptibility and resistance in Chlorella

Detergents are common environmental pollutants that have multiple deleterious effects on biological systems (7). Detergents impeded the growth of *Chlorella* across the

board; however, the cationic detergent cetylpyridinium chloride (CPC) exhibited the most deleterious effect, exhibiting lysis and cell death at concentrations as low as 0.001% Cells were more tolerant of neutral detergent Triton X-100, tolerating up to 0.001% detergent, and most tolerant of SDS, tolerating up to 0.0075% detergent (Figure 1). A side by side growth curve comparison of the detergents in 0.006% detergent indicates that 0.006% is tolerated, but inhibitory of growth rate, but nevertheless, growth does occur. Lysis does not occur at 0.006% Triton X-100, however, growth does not occur at this concentration. Lysis was observed within two days of inoculation with CPC (Figure 2).

Nutrient modification

Gram positive bacteria are the canonical example for the influence of media on cell wall composition: the availability of magnesium and phosphate impacts the production of teichoic acid, which decrease the susceptibility of the cell to lysis (8). When magnesium is rate limiting, more teichoic acids are synthesized; when phosphate is rate limiting, fewer teichoic acids are synthesized . Altering nutrient concentrations affects detergent resistance in *Chlorella* NC64A. Increasing the concentration of magnesium fivefold restored the terminal cell yield nearly to that of cells grown without detergent, and above cells grown in unmodified MBBM with 0.001% SDS. The addition of sulfate to seawater levels and to 5x seawater levels resulted in a decrease in cell numbers from inoculation; however, this could be a result of off target effects caused by the amount of salt added to the media in order to gain this sulfate concentration, as described in the results (Figures 3 and 4).

PBCV-1 infectivity assays

Attachment assay. Reduced sulfate conditions and increased magnesium concentrations both resulted in fewer viral particles being taken out of solution (Figure 5A), possibly due to weaker interactions between the viral particles and the cell wall.

Infective center assay. Chlorella grown in 0.005% SDS and grown in reduced (30%) sulfate lowered the plaquing efficiency fourfold (Fig. 5B) in the infective center assay, indicating that the proportion of cells in the cell suspension that are able to release viral particles have decreased.

Burst size assay. Cells grown in 5X magnesium and cells grown in 30% sulfate had a smaller burst size compared to the control and with cells grown in sulfate at the concentration of seawater, indicating fewer viruses released from a host cell upon lysis.

The commonalities between detergent resistance and viral resistance, specifically, the roles of magnesium and sulfate, warrant consideration as they indicate that the cell wall components responsible for detergent resistance and virus resistance may be related. Increasing magnesium concentration has a protective effect on both SDS and against viral infectivity, burst size, and attachment; increasing sulfate concentration had the reverse effect. The preliminary burst size, attachment, and infective center assays indicate that sulfate plays a role in the attachment of PBCV-1, possibly in the form of a sulfated polymer.

If algal biofuels are to be viable, industrial algae must be able to grow in the presence of detergents and other contaminants found in wastewater. We aim to further analyze and optimize nutrient conditions to increase detergent resistance in economically important algae and to further characterize the mechanisms of detergent

resistance. Nutrient modification studies may identify media compositions that result in hardier growth, increasing the utility of algae in difficult environments, such as in wastewater treatment plants. Additionally, altering media nutrient concentrations will further help us characterize and identify the receptor of unique large DNA chlorovirus PBCV-1, whose receptor eludes discovery.

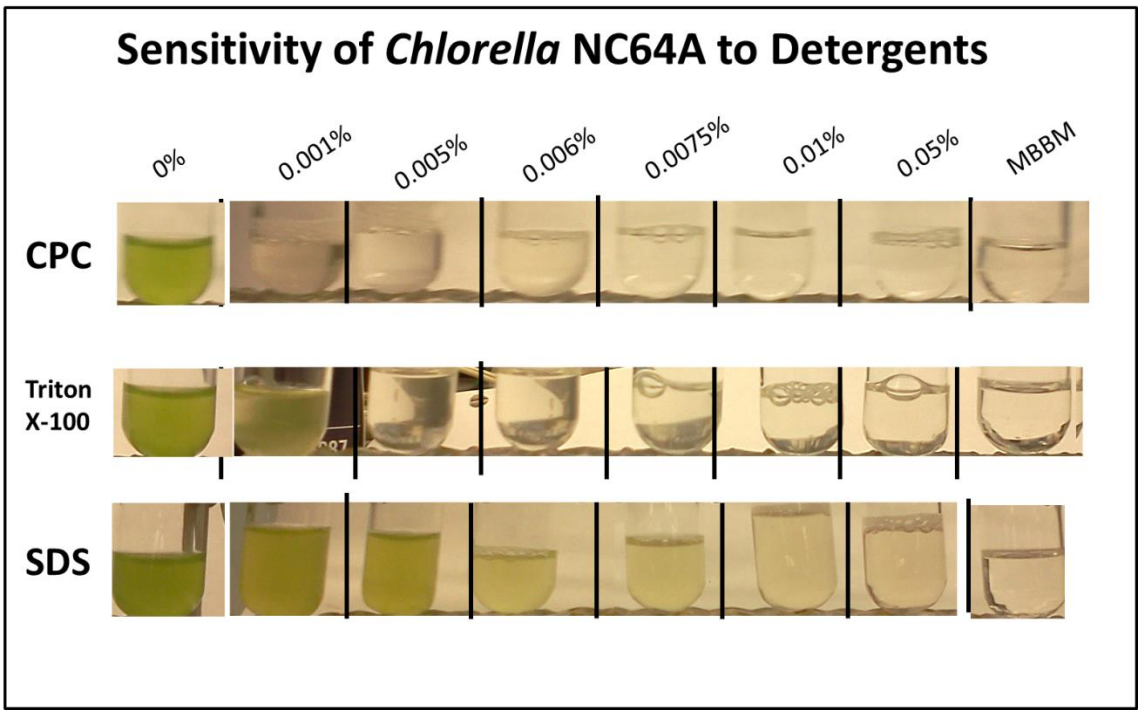


Figure 1. Growing *Chlorella* NC64A in the presence of detergents affects maximum cell yield. *Chlorella* NC64A cells were grown for ten days in MBBM from a starting inoculum of ~2.5 × 10^6 cells/mL in the presence of CPC (cetylpyridinium chloride), a cationic detergent; SDS (sodium dodecyl sulfate), an anionic detergent; uninoculated algal media provided for comparison (far right).

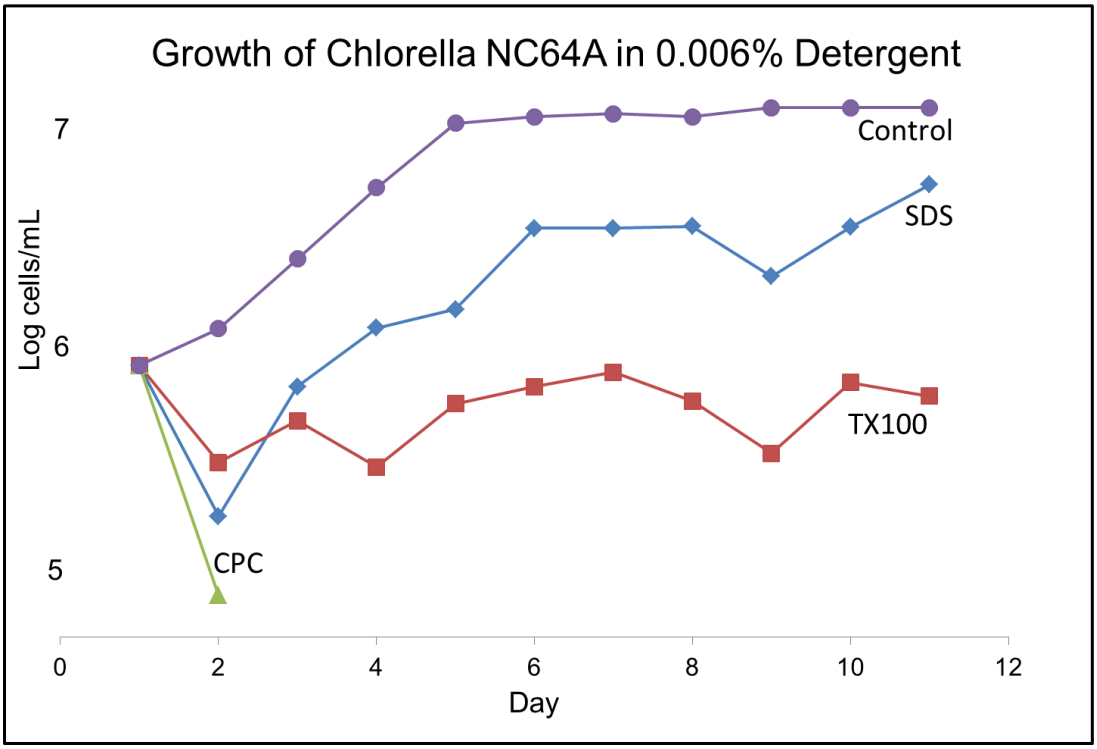


Figure 2. Growth rate of *Chlorella* NC64A in the presence of 0.006% detergents affects growth rate. *Chlorella* NC64A cells were grown for ten days from a starting inoculum of ~1.0×10^6 cells/mL. Cells were more tolerant of anionic SDS than neutral TritonX-100 or cationic CPC. Lysis was observed within two days of inoculation with CPC. SDS inoculation resulted in a slower growth rate and a lower maximum cell yield compared to the detergent-free control.

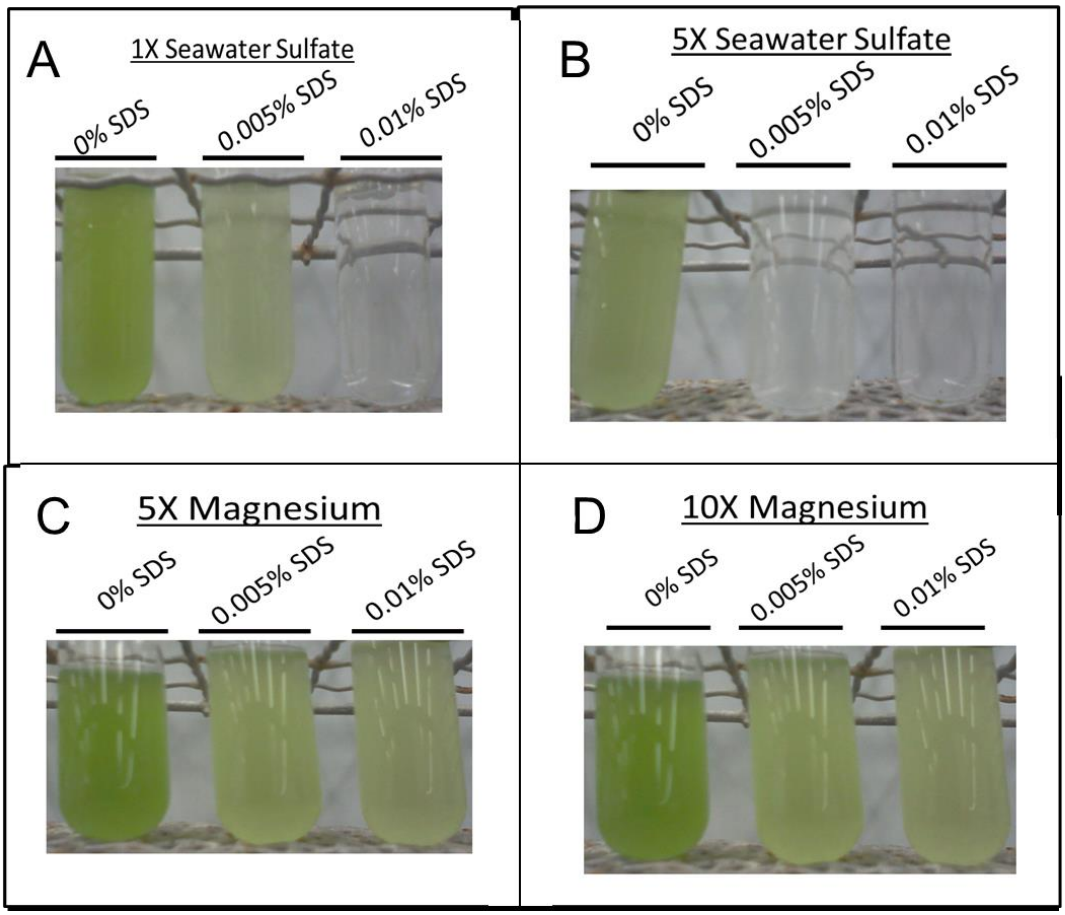


Figure 3. Altering nutrient concentrations affects detergent resistance in *Chlorella* NC64A. Cells were grown for ten days from an inoculum of ~2.5×10^6 cells/mL in MBBM which was altered as labeled. **A.** Increasing the sulfate concentration to the level of seawater decreased the maximum cell yield as SDS concentration increased. **B.** Increasing the sulfate concentration to five times that of seawater further decreased the maximum cell yield (beyond that of unmodified MBBM, Fig 2). **C**. Increasing the magnesium concentration to five times the amount present in the original media increased the maximum cell yield as SDS concentration increased. Cells grown in unmodified MBBM did not tolerate SDS concentrations of 0.01% (Fig 1). D. Increasing the magnesium concentration to ten times the amount present in the media also increased maximum cell yield.

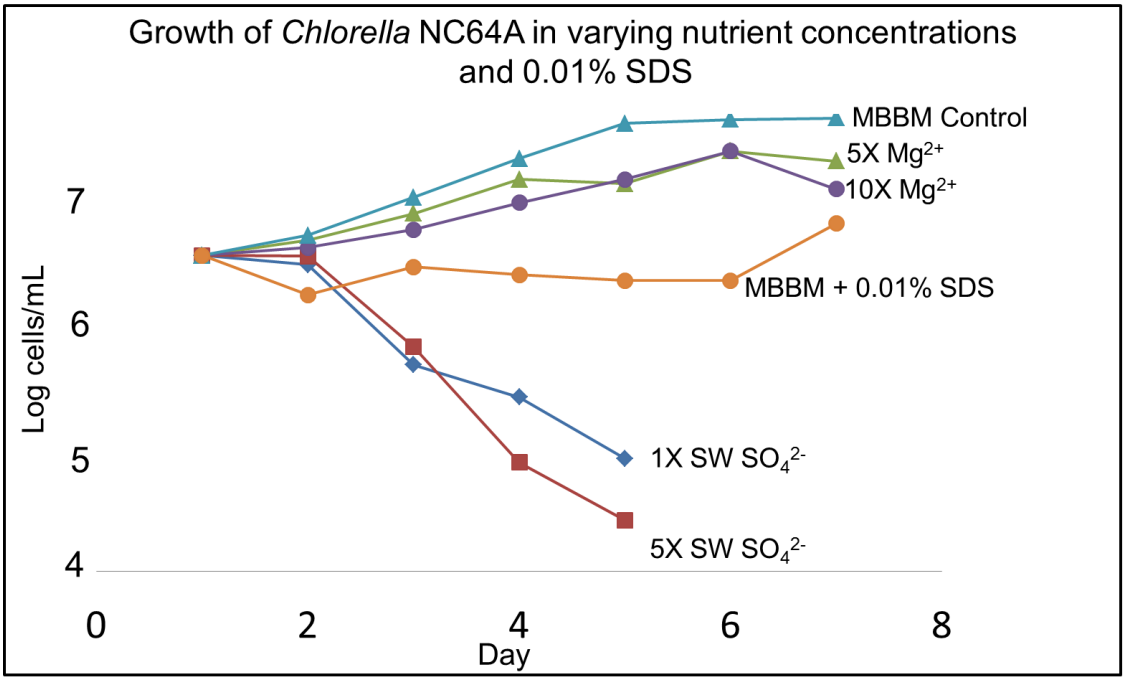


Figure 4 (right). Growth of *Chlorella* NC64A at varying nutrient concentrations in 0.01% SDS. Magnesium has a protective effect against lysis by SDS; sulfate has a deleterious effect on survival in SDS. Abbreviations: MBBM control, a control growth curve of *Chlorella* NC64A with neither detergent added nor media modified; 5X and 10 Mg^{2+}, five and ten times the amount of Mg (II) present in normal MBBM, respectively; NC64A 0.01% SDS, the growth curve of *Chlorella* NC64A with SDS added to a concentration of 0.01% and no media modifications; SW SO_4^{2-}, the amount of sulfate present in artificial seawater media; 5X SW SO_4^{2-}, five times the amount of sulfate present in artificial seawater media.

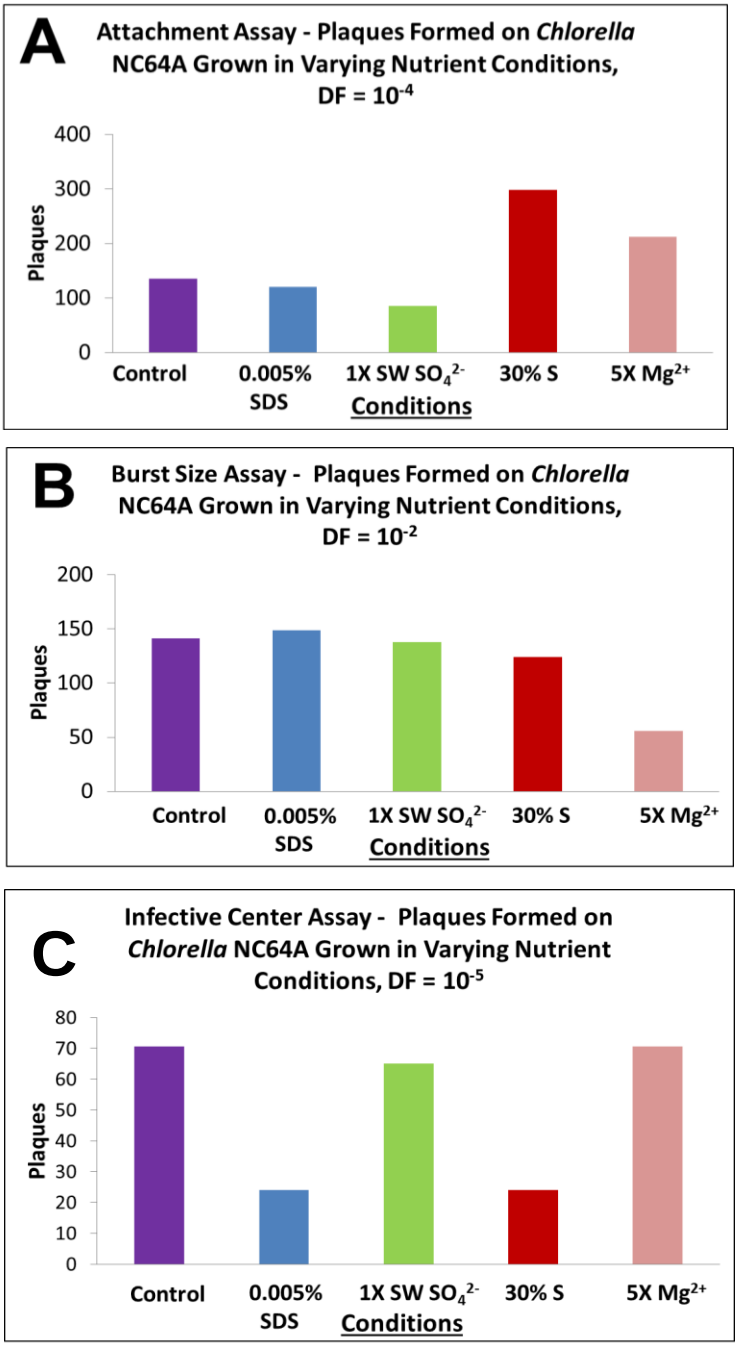


Figure 5. Representative results of viral attachment assays.
A: Attachment assay. An attachment assay measures the amount of infectious viral particles remaining in the supernatant after infection. Cells grown in higher sulfate concentrations have higher numbers of attached viral particles (fewer remaining in the supernatant); cells grown in reduced sulfate and increased magnesium have lower numbers of attached viral particles (more available in the supernatant).
B: Burst size assay. The burst size assay measures the amount of viruses released from a host cell upon lytic release. Cells grown in 5X magnesium and 30% sulfate had a smaller burst size compared to the control and to cells grown in 1X SW sulfate.
C: Infective center Assay. *Chlorella* grown in 0.005% SDS and grown in reduced (30%) sulfate lowered the plaquing ssay, indicating that the proportion of cells in the cell suspension that are able to release viral particles have decreased.

References

1. Van Etten JL, Burbank DE, Xia Y, Meints RH. 1983. Growth cycle of a virus, PBCV-1, that infects Chlorella-like algae. Virology 126:117–125.
2. Amantonico A, Urban PL, Zenobi R. 2010. Analytical techniques for single-cell metabolomics: State of the art and trends. Anal Bioanal Chem 398:2493–2504.
3. Meints RH, Burbank DE, Van Etten JL, Lamport DTA. 1988. Properties of the Chlorella receptor for the virus PBCV-11. Virology 164:15–21.
4. Mceldowney S, Fletcher M. 1986. Effect of growth conditions and surface characteristics of aquatic bacteria on their attachment to solid surfaces. Microbiology 132:513–523.
5. Nichols HW, Bold HC. 1965. Trichosarcina polymorpha gen. et sp. nov. J Phycol 1:34–38.
6. Van Etten JL, Burbank DE, Kuczmarski D, Meints RH. 1983. Virus infection of culturable Chlorella-like algae and development of a plaque assay. Science (80-) 219:994–996.
7. Corre G, Templier J, Largeau C, Rousseau B, Berkaloff C. 1996. Influence of cell wall composition on the resistance of two Chlorella species (Chlorophyta) to detergents1. J Phycol 32:584–590.
8. Lambert PA, Hancock IC, Baddiley J. 1975. The interaction of magnesium ions with teichoic acid. Biochem J 149:519–524.