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Iterative screening of an evolutionary engineered *Desmodesmus* generates robust field strains with pesticide tolerance

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ABSTRACT

Microalgae hold remarkable promise to produce food, fuel, and nutraceuticals. To satisfy industrial economics, microalgae are commonly cultivated outdoors in open ponds that are subject to invasions by a suite of pests including predators, competitors, and parasites. Although pests can be mitigated and/or controlled by biocides or pesticides, therapeutic doses of these chemicals often adversely affect the productivity and yield of the target crop. To combat this tradeoff imposed by pesticide treatment, if pesticides are to be used, robust strains with increased pesticide tolerance must be developed and validated for growth at commercial scales. In this study, we evolutionarily engineered strains of the green algae *Desmodesmus armatus* to tolerate treatment with Omega 500F®, a broad-spectrum agricultural fungicide that is used to treat pest invasions in open, outdoor microalgal ponds. An algal clone library generated through UV mutagenesis was screened for strains that exhibited the greatest growth rates under batch and semi-continuous culture in the lab with and without exposure to Omega. We then screened strains at increasing spatial (i.e., from 96-well plates to 33,000 L raceways) and temporal (i.e., from 5 days to months) scales to select for those strains that were most robust. Our process resulted in the cultivation of two robust field strains that demonstrated tolerance to repetitive dosing with Omega without apparent trade-offs in productivity. This work highlights the utility of non-GM methods, specifically UV mutagenesis, to improve cultivation strains for the production of biomass and bioproducts from microalgae. Moreover, it demonstrates the importance of iterative validation steps in facilitating a successful lab to field transition of engineered strains.

1. Introduction

With largely untapped taxonomic and phenotypic diversity, microalgae hold remarkable promise to produce food, fuel, and nutraceuticals at industrial scales, meeting growing societal demands for these commodities. Microalgae exhibit high doubling rates and can be grown on non-arable land in natural or artificial ponds, generating high yields and reducing economic competition with traditional agricultural systems [1]. Microalgae are also particularly amenable to laboratory evolution due to their rapid doubling rates and unicellular form [2,3]. To satisfy industrial economics, microalgae are commonly cultivated outdoors in open raceway ponds [4–6]. Unfortunately, with this approach, predators, competitors, and parasites can quickly invade ponds and decrease productivity and yield or decimate entire crops [7–9]. Pest pressure in ponds can be controlled biologically (e.g., by introducing herbivores; [10]), mechanically (e.g., filtering the pond culture; [11]), or chemically. In the latter case, chemicals may be introduced either to change environmental conditions such that pests are not favored [12,13] or to directly kill pests through the application of a biocide or pesticide [14–16]. Although pest pressure can be effectively mitigated by biocide and pesticide treatment, such treatment may come at a cost to algal fitness in the form of lower productivity/biomass and/or altered environmental tolerances [17,18]. Development and validation of robust algal field strains with increased tolerance or resistance to

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pesticides is one solution to maintaining microalgal productivity and minimizing losses due to pests.

Approaches to improve or select for specific physiological traits in algae can rely on classical strain improvement, evolutionary engineering, and genetic modification (GM) [19–23]. As consumer preferences trend towards non-GM products [24], classical strain improvement and evolutionary engineering offer viable routes to improve strains without transgenic modification. These approaches do not require *a priori* knowledge of complete biological pathways, which are often unavailable for newly cultivated algal strains, and may be more tractable due to the unique challenges of DNA transformation in algae [25].

In this study, we engineer microalgae strains in the laboratory for tolerance to the Syngenta-manufactured fungicide Omega 500F® (hereafter "Omega"), a broad-spectrum agricultural fungicide with a single active ingredient, fluazinam (3-chloro-*N*-[3-chloro:-2,6-dinitro-4-trifluoromethyl)phenyl]-5-trifluoromethyl-2-pyridinarriine). Although Omega is typically used in terrestrial systems to control disease within legume vegetables, onions, and potatoes, it can also be used microalgal cultivation systems [26]. Here, we focus our engineering efforts on the green alga *Desmodesmus armatus*, an oleaginous species within the family Scenedesmaceae that holds potential for the production of biofuel, as well as other high-value products.

2. Methods

2.1. Approach

To generate robust algal strains for outdoor and commercial cultivation, we isolated a native algal strain from the environment, generated a clone library via UV-mutagenesis, and screened clones at increasing spatial (i.e., from 96-well plates to outdoor raceways) and temporal (i.e., from 5 days to months) scales (Fig. 1). Our iterative screening process resulted in the selection of two Omega-resistant clones from a library of 94 clones, and ultimately allowed us to validate strain performance at the spatial scale of 33,000 L raceways and temporal scale of months. Below, we describe each of these process steps in detail.

2.2. Strain isolation, maintenance, and initial characterization

During 2009, we used traditional cell-culture techniques to isolate a freshwater green alga from an evaporation pond at Sapphire Energy, Inc. in Las Cruces, NM (Fig. 1A). Via microscopy, we initially identified this strain as a member of the family Scenedesmaceae. We subsequently identified it as *Desmodesmus armatus* via sequencing and comparison of the ITS1&2 regions. The strain was established in culture and maintained in medium described in McBride et al. [16]. To the base freshwater medium, nitrogen (N, as urea, nitrate and ammonium), phosphorus (P, as phosphate) and iron (Fe, as Dissolvine® and ferric sulfate) were added to target concentrations of 150 ppm, 80 ppm and 0.5 ppm, respectively. Trace elements (cobalt, molybdenum, manganese and zinc) were also added to the media to maintain these elements in non-limiting concentrations. This medium was used during all experiments and field trials, and Bacto-agar was added to create solid plates, as needed. For maintenance, the strain was cultured semi-continuously in polycarbonate Erylenmeyer flasks kept on shaker tables set at a rate high enough to keep cultures suspended. Shakers were housed within climate controlled boxes with 5% CO_2 and a temperature range of 26–28 °C. Light was supplied continuously by 32 W GE T8 Starcoat cool white fluorescent bulbs at ~80 μEin $m^{-2} s^{-1}$. For long term storage, the strain was maintained on agar plates in a lower temperature (20 °C) incubator.

To characterize the WT strain's tolerance to Omega, we exposed triplicate cultures in 5 mL polypropylene tubes to Omega at fluazinam concentrations from 1 to 210 ppm (i.e., 1, 2.1, 4.6, 10, 21, 46, 100, 210 ppm). Omega doses were considered inhibitory if cultures lost pigmentation and did not generate colonies after plating on solid growth medium.

2.3. Generation of the clone library

To create a library of strains that could be screened for Omega resistance, we induced genomic mutations in the WT isolate by exposing it to ultraviolet (UV) radiation (Fig. 1A). Briefly, a WT culture was concentrated to an optical density at 750 nm (OD_{750}) of 1.4. Fifteen milliliter aliquots of this culture were placed into 150 × 15 mm petri dishes and were exposed to total doses of UV (254 nm) radiation of 50 mJ, 100 mJ, 150 mJ and 200 mJ using the "ENERGY" mode of a Spectrolinker XL-1000 UV Crosslinker (Spectronics Corporation, Westbury NY). The proportion of each culture that was killed by the UV treatments was estimated by counting the number of surviving colonies from subsamples plated on agar growth medium. Cultures in which cell viability declined ≥90% with UV exposure were allowed to recover in flasks in liquid medium to an $OD_{750} \geq 0.2$. After recovery, the cultures exhibited detectable chlorophyll fluorescence and quantum yields (i.e., F_v/F_m) ≥ 0.7. For these and subsequent measurements, optical density at 750 nm (OD_{750}) and chlorophyll fluorescence (excitation at 430 nm, emission at 685 nm, 5 mm path length) were measured on 200 μL aliquots (diluted 10× for chlorophyll fluorescence) using a Spectramax M2 microtiter plate reader (Molecular Devices, Sunnyvale CA). For photosynthetic or quantum yield, measurements of Fv/Fm were taken in triplicate with a Walz mini-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) using samples that had been dark-acclimated for 5 to 10 min.

The surviving cultures were pooled to serve as the mutagenized library for Omega screening (Fig. 1A). The mutagenized library and a WT control were incubated in 125 mL polycarbonate Erlenmeyer flasks containing the minimum inhibitory concentration of fluazinam (determined to be ~100 ppm) for six days. At the end of this treatment, 1 mL culture aliquots were spread on biocide-free agar plates and incubated for two weeks under the conditions described above except that light was supplied at 50 μEin $m^{-2} s^{-1}$. The surviving clones were isolated and maintained for subsequent assays (Fig. 1A).

2.4. Laboratory-scale screening of the clone library

2.4.1. Batch culture with and without Omega

We used high-throughput assays to screen the clones in media with and without added Omega (Fig. 1B). The assay without Omega allowed us to detect trade-offs between Omega-tolerance and normal growth rate, whereas the assay with Omega allowed us to test clones under the stress of the fungicide. The former screening was necessary before testing cultures in outdoor cultivation ponds, as a strain with a reduced growth rate without fungicide pressure would be undesirable given only intermittent fungicide treatment of ponds in practice. For each assay, 200 μL of each culture at a starting OD_{750} of 0.05 was added to triplicate wells of 96 well plates (Corning, Corning NY). The plates were covered with polydimethylsiloxane lids or rayon acrylate sealing tape and maintained under the culture conditions described above (see Section 2.2 Strain isolation and maintenance) except that light was supplied at 130 μEin $m^{-2} s^{-1}$ for the assay without Omega. We tracked OD_{750} every 6 h for five to six days. Growth rate (r) was calculated from optical density data using a logistic function [46,47]. We used these data to select clones for further screening with Omega.

2.4.2. Semi-continuous culture with Omega

The next round of screenings was designed to test responses of clones to Omega, first at a constant dose and then with repeated and increasing doses of Omega (Fig. 1C). These screenings were longer (i.e., 12 days) with slightly greater culture volume (i.e., 1 mL) using a 96-deep well plate format. Clones were again inoculated into triplicate