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Evaluation of novel starch-deficient mutants of *Chlorella sorokiniana* for hyper-accumulation of lipids

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ABSTRACT

When green algae are exposed to physiological stresses such as nutrient deprivation, growth is arrested and the cells channel fixed carbon instead into storage compounds, accumulating first starch granules and then lipid bodies containing triacylglycerides. In recent years there has been significant interest in the commercial exploitation of algal lipids as a sustainable source of biodiesel. Since starch and lipid biosynthesis involves the same C3 precursor pool, it has been proposed that mutations blocking starch accumulation should result in increased lipid yields, and indeed several studies have supported this. The fast-growing, thermotolerant alga *Chlorella sorokiniana* represents an attractive strain for industrial cultivation. We have therefore generated and characterized starch-deficient mutants of *C. sorokiniana* and determined whether lipid levels are increased in these strains under stress conditions. One mutant (ST68) is shown to lack isoamylase, whilst two others (ST3 and ST12) are defective in starch phosphorylase. However, we find no significant change in the accumulation or profile of fatty acids in these mutants compared to the wild-type, suggesting that a failure to accumulate starch per se is not sufficient for the hyper-accumulation of lipid, and that more subtle regulatory steps underlie the partitioning of carbon to the two storage products.

1. Introduction

The unsustainable use of our finite reserves of fossil fuels, and the issues of producing renewable fuels from crop plants given the limitations on available agricultural land, have resulted in major interest in using microalgae as an alternative feedstock for biofuel production [1,2]. Some microalgal species are particularly attractive as a source of lipid-derived biodiesel given their high growth rates, efficient solar conversion, and tolerance to a wide range of environmental conditions — together with their rich diversity of lipids and ability to accumulate storage lipids to high levels [3,4]. Accumulation of these neutral lipids occurs under stress conditions such as deprivation of key nutrients (e.g., nitrogen), with the lipids mainly in the form of triacylglycerides (TAGs) that accumulate as lipid bodies within the cell. The extraction and transesterification of the TAGs yield fatty acid methyl esters (FAMEs) that can be used as biodiesel or further processed into bio-jet fuel [5].

In green algae, stress conditions also trigger the accumulation of starch granules in the cells, with starch accumulation preceding the accumulation of lipid bodies following the onset of stress [6,7]. It is generally assumed that the starch and TAGs serve as electron sinks under conditions where photosynthesis, or metabolism of an exogenous carbon source, is still active but growth is limited [5]. Prolonged stress ultimately results in the breakdown of the photosynthetic membrane and the loss of chlorophyll pigmentation [6]. The maximization of TAG productivity in microalgae therefore requires consideration of both the restricted growth rate under particular stress conditions and the cellular TAG content. Since both starch and TAGs share common precursors in the form of the C3 metabolite pool [8] then it has been proposed that TAG content could be increased by blocking or reducing starch biosynthesis, and thus partitioning carbon towards TAGs.

Several studies have looked at the relationship between TAGs and starch in *Chlamydomonas reinhardtii*; a model alga where starch accumulation has been extensively studied and well-characterized mutants are available [9]. Studies of the sta6 mutant, which accumulates no starch due to a mutation in the small subunit of ADP-glucose pyrophosphorylase (AGPase), have all shown a marked increase in lipid accumulation under nitrogen deprivation when compared to wild type strains [10–15]. Analysis of other *C. reinhardtii* starch-deficient mutants (i.e., sta1, sta7 and sta11) also indicated a correlation between the amount of starch accumulated under stress conditions and the TAG levels obtained [13,15]. However, Siaut et al. [7] have questioned these correlations given that they found significant variations in lipid levels among laboratory wild-type strains. They could find no significant

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difference when comparing sta1, sta6 and sta7 to the presumed parental strain. Nonetheless, studies of starch mutants of other green algal species have also reported hyper-accumulation of lipids when compared to their parental wild-type. de Jaeger et al. [8] found that starchless mutants of the oleaginous species, *Scenedesmus obliquus* showed a clear increase in TAG content compared to the WT without compromising biomass productivity. Similarly, a starchless mutant of *Chlorella pyrenoidosa* showed significant hyper-accumulation of lipid [16], suggesting that the selection for starch mutants of industrially-relevant microalgal species is one strategy towards their "domestication" for mass cultivation [17].

Members of the genus *Chlorella* represent particularly attractive species for such mass cultivation given that they are already cultivated commercially for the health food and cosmetics markets [18], and show key attributes for biodiesel production in terms of robust cultivation in open pond systems and biomass recovery [19,20]. One species that is particularly suited for industrial cultivation is *Chlorella sorokiniana* [21]. This freshwater species has remarkably short doubling times of only a few hours [22,23]. It grows optimally at elevated temperatures of 35–40 °C; can tolerate temperatures as high as 46.5 °C and light intensities over 1700 μmol/m^2/s, and exhibits high biomass productivity [24,25].

Here we report the isolation and biochemical analysis of starch-deficient mutants of *C. sorokiniana*, including mutants defective in isoamylase and starch phosphorylase. Significantly, we find that these mutants show no increase in TAGs or changes in fatty acid profile, suggesting that the re-engineering of carbon partitioning to favor TAG production is not achieved simply by reducing starch biosynthesis, or that such a strategy is not applicable to all industrial species.

2. Materials and methods

2.1. Strains and culture conditions

C. sorokiniana UTEX1230 was obtained from the University of Texas culture collection. Strains were maintained on tris-acetate-phosphate (TAP) agar plates at 25 °C under constant light [26]. Liquid cultures were grown under constant light (~35 μmol/m^2/s) and agitation (120 rpm) at 25 °C. For induction of starch and triacylglyceride accumulation following nitrogen depletion, the NH_4Cl in the TAP medium was either reduced to 1/10th of normal (termed TAP-1/10N): final NH_4Cl concentration of 0.74 mM) or omitted completely (TAP-N).

2.2. Isolation of starch mutants

Mutants were isolated following the method described for *C. reinhardtii* [9]. Cells were subject to ultraviolet irradiation to survival rate of 10% and colonies appearing after seven days of growth on solid TAP-1/10N medium were stained directly with iodine vapor. Colonies appearing less stained and not displaying the typical dark blue/purple color, were recovered and restained with iodine to confirm the color change.

2.3. Quantification of starch

C. sorokiniana was cultivated for five days in 1 L acetate medium with (TAP) or without (TAP-N) nitrogen. The cells were pelleted, washed in water and kept at −80 °C until use. Cells were lysed by passage twice through a French press at 10,000 psi (with complete breakage confirmed by microscopy), and then centrifuged at 3000 *g* for 20 min at 4 °C. The supernatant was used for measuring total protein using a protein assay kit (Bio-Rad). Starch was extracted from the remaining pellet according to the methods detailed in Delrue et al. [27], using a commercial kit (Enzytec™ kit E1268). Total starch was calculated and expressed as mg starch/mg protein or μg starch/mg cell dry weight. Water soluble polysaccharides (WSP) from the supernatant were also assayed using the Enzytec™ kit.

2.4. Sepharose CL-2B gel permeation chromatography

Amylose and amylopectin were separated by gel permeation chromatography on a sepharose CL-2B column equilibrated in 10 mM NaOH as described in Delrue et al. [27]. The optical density of the iodine–polysaccharide complex for each fraction was measured at λ_{max} (maximal absorbance wavelength) after adding iodine solution (1% KI, 0.1% I_2 w/v) at a dilution of 1:5. The remaining fractions corresponding to the amylopectin were combined and kept at −20 °C until further analysis of chain length distribution by ion exchange chromatography.

2.5. Analysis of water soluble polysaccharides

Water soluble polysaccharides (WSPs) were extracted from the remaining supernatant with chloroform:methanol according to the methods described in Dauvillée et al. [28]. After the removal of the solvent, the dried sample was re-suspended in 10% DMSO (v/v) and loaded on a TSK HW50 gel permeation column, and eluted with 10% DMSO in 500 μL fractions. Each fraction was assayed for total sugars using phenol–sulfuric acid. From each fraction 20 μL was mixed with 20 μL of 5% phenol in a 96-well plate and placed on ice, before addition of 100 μL of concentrated sulfuric acid. The plate was then incubated at 80 °C for 30 min and the absorbance measured at 490 nm. Additionally each fraction was stained by adding iodine solution and the optical density measured as described for fractions separated by CL-2B. Fractions staining red with iodine were combined and kept at −20 °C until further analysis of chain length distribution.

2.6. Chain length distribution

To remove NaOH in amylopectin fractions, as well as DMSO from the WSP fractions recovered from the TSK column, samples were subject to dialysis for 2 h in H_2O. The solution was then lyophilized and the

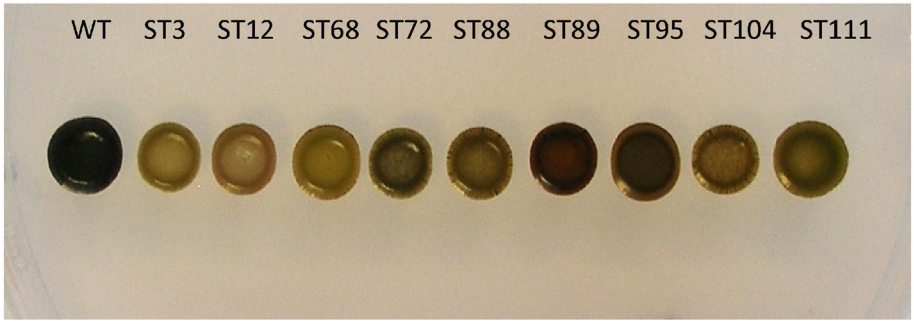

Fig. 1. Iodine staining of nine selected mutants isolated after UV irradiation demonstrates a reduction in starch in each strain.