Phosphoproteome of the Oleaginous Green Alga, *Chlorella vulgaris* UTEX 395, under Nitrogen-Replete and -Deplete Conditions

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The unicellular green alga, *Chlorella vulgaris* UTEX 395, represents a promising biocatalyst for renewable biofuel production due to its relatively rapid growth rate and high lipid accumulation capacity (Guarnieri et al., 2011, 2012; Gerken et al., 2013; Griffiths et al., 2014; Zuniga et al., 2016). Prior analyses have unveiled the global proteome dynamics of *C. vulgaris* following nitrogen depletion, which induces a high lipid accumulation phenotype (Guarnieri et al., 2011, 2013). More recently, we have reported a draft genome, genome-scale model, and nitrosoproteome for this alga (Zuniga et al., 2016; Henard et al., 2017) providing further insight into lipid biosynthetic-, nutrient response-, and post-transcriptional-regulatory mechanisms. To further our understanding of these regulatory mechanisms and expand the knowledge base surrounding this organism, comparative phosphoproteomic analyses were conducted under nitrogen-replete and -deplete conditions to identify differentially phosphorylated proteins that will aid in the evaluation of the potential role of phosphoregulation in lipogenesis.

**METHODS**

**Algal Cultivation**

*Chlorella vulgaris* UTEX 395 was cultivated in biological triplicate, as described previously (Guarnieri et al., 2011, 2013). Briefly, algae were inoculated at OD750 = 0.05 in 1 L Roux bottles using modified Bold’s Basal Media (mBBM). Cultures were maintained at 25°C ± 1°C, with continuous (24 hr) white fluorescent light illumination (200 μE m⁻² s⁻¹). Cultures were supplemented with 2% CO₂/air and mixed with a magnetic stir bar at 500 rpm. 50 mL of nitrogen-replete cell culture was harvested at OD750 = 2, centrifuged for 5 min at 5000 × g, and immediately quenched on liquid nitrogen.

To induce nitrogen deprivation, the remaining cell culture was centrifuged for 5 min at 5000 × g, washed once in nitrogen-free mBBM, and resuspended in nitrogen-free mBBM for continued growth to OD750 = 4, followed by harvest, as described above. Cell pellets were immediately quenched in liquid nitrogen.

**Protein Isolation**

Cell pellets were thawed and solubilized on ice in 2 mL of lysis buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM DTT, 10% glycerol, supplemented with 1 × PhosSTOP Phosphatase Inhibitor Cocktail (Roche Diagnostics Corporation)]. The cells were then sonicated on ice at 4°C, at 90% power setting for 30 s × 6 cycles, with a 1 min cool-down period between sonication cycles using a Braun Sonic-L.

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ultrasonicator. Lysates were cleared via two cycles of centrifugation at 16,000 × g at 4°C for 30 min, and the supernatants were isolated for use in subsequent phosphoproteomic analysis.

Peptide Digestion and Phosphopeptide Enrichment

500 μg of lysate material was digested in solution with the following concentration of buffer and enzymes: 100 mM DTT, 20 mM HEPES, 100 mM iodoacetamide, 25 ng/μL trypsin (final E:S ratio of 1:20), and 20.5% TFA. 200 μg of peptide were desalted using solid phase extraction on a Waters C18 Sep Pak 1cc cartridge (part no. WAT054960) as follows: column was activated with 1 mL 100% acetonitrile followed by 1 mL 50% acetonitrile, and equilibrated with 3 × 1 mL 0.1% TFA. The sample was loaded on column and washed 2x with 1 mL 0.1% TFA, followed by elution with 0.5 mL 60% acetonitrile, 0.1% TFA. The eluate was dried to completion and phosphopeptides were enriched using the GL Sciences TiO2 kit (5010-21312), following manufacturer’s instructions. Samples were dried to completion and resuspended in 120 μL 0.1% TFA.

Phosphoproteomic Analysis

Each enriched sample was analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. The acquisition order was randomized. In each case, peptides were loaded on a trapping column and eluted over a 75 μm analytical column at 350 nL/min; both columns were packed with Jupiter Proteo resin (Phenomenex), employing a 2 h gradient, as follows: buffer A—0.1% formic acid in water; buffer B—0.1% formic acid in acetonitrile; t0 = 2%B, t1min = 5% B, t95 = 25% B, t110 = 35% B, t112 = 90% B, t113 = 2%B, t120 = 2%B. The mass spectrometer was operated in data-dependent mode, with MS performed in the Orbitrap at 70,000 FWHM resolutions and MS/MS performed using HCD and product ions detected in the mass spectrometer was operated in data-dependent mode, with B, t110 = Griffiths, M. J., van Hille, R. P., and Harrison, S. T. L. (2014). The effect of nitrogen

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