

TOR inhibition drives accumulation of amino acids through transcriptional activation in algae

Melissa Roth

mroth@berkeley.edu

University of California Berkeley <https://orcid.org/0000-0001-7389-5982>

Shivani Upadhyaya

University of California Berkeley

Suzanne Kosina

Lawrence Berkeley National Laboratory <https://orcid.org/0000-0003-2885-1248>

Samuel Purvine

Pacific Northwest National Laboratory

Kim Hixson

Institute of Biological Chemistry

Athena Schepmoes

PNNL

Ritesh Mewalal

DOE Joint Genome Institute, Lawrence Berkeley National Laboratory <https://orcid.org/0000-0002-0153-2434>

Yu Zhang

U.S. Department of Energy Joint Genome Institute <https://orcid.org/0000-0003-4495-3447>

Ronan O'Malley

The University of Chicago <https://orcid.org/0000-0002-4907-0339>

Leo Baumgart

U.S. Department of Energy Joint Genome Institute <https://orcid.org/0000-0002-2773-5897>

Ian Blaby

U.S. Department of Energy Joint Genome Institute <https://orcid.org/0000-0002-1631-3154>

Mary Lipton

Pacific Northwest National Laboratory

Trent Northen

Joint BioEnergy Institute <https://orcid.org/0000-0001-5903-1870>

Krishna Niyogi

University of California, Berkeley <https://orcid.org/0000-0001-7229-2071>

Article

Keywords:

Posted Date: October 14th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11051110/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: **Yes** there is potential Competing Interest. Trent R.Northen. is a founder of two non-profits, Prosper Soils and Bioaligned Labs with prior approval from LBNL. None of the other authors declare any conflicts of interest.

TOR inhibition drives accumulation of amino acids through transcriptional activation in algae

Shivani Upadhyaya^{*1}, Suzanne M. Kosina², Samuel O. Purvine³, Kim Hixson^{3,4}, Athena Schepmoes³, Ritesh Mewalal⁵, Yu Zhang⁵, Ronan O'Malley^{5,6}, Leo A. Baumgart⁵, Ian K. Blaby^{5,2}, Mary S. Lipton³, Trent R. Northen², Krishna K. Niyogi^{1,7,8}, Melissa S. Roth^{*1}

¹ Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720

² Environmental Genomics and Systems Biology Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

³ Earth and Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA 99352

⁴ Institute of Biological Chemistry, Washington State University, Pullman, WA 99164

⁵ U.S. Department of Energy, Joint Genome Institute, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

⁶ Department of Human Genetics, University of Chicago, Chicago, IL 60637

⁷ Howard Hughes Medical Institute, University of California, Berkeley, CA 94720

⁸ Molecular Biophysics and Integrated Bioimaging Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

*Corresponding authors: Shivani Upadhyaya, Melissa S. Roth

Email: shivani.u@berkeley.edu, mroth@berkeley.edu

Abstract

Cellular homeostasis, the balance between energy production and breakdown, is fundamental to all forms of life. The conserved, ancient target of rapamycin (TOR) kinase is a central metabolic regulator in eukaryotes that integrates carbon and nitrogen to maintain homeostasis and promote growth and development through protein synthesis. While TOR regulatory mechanisms of amino acid accumulation are well known in yeast and mammals, they remain unknown in photosynthetic organisms. Here, we developed the unicellular green alga *Chromochloris zofingiensis* as a simple model system for understanding TOR function. Multiomics experiments showed that TOR inhibition leads to an increase in amino acid levels. We observed upregulation of select amino acid biosynthesis pathways at the transcript and protein levels as potential mechanisms driving the increase in amino acids. Transcriptomics and proteomics experiments identified a basic helix-loop-helix ACT domain containing (bHLH^{ACT+}) transcription factor with rapid upregulation during TOR inhibition. DAP-seq analysis demonstrated that the bHLH^{ACT+} can bind directly to the promoters of amino acid biosynthesis genes, potentially regulating transcription in response to TOR inhibition. We found high conservation of the bHLH^{ACT+}-binding motif in the genomes of other green algae, suggesting a putative conserved regulatory mechanism for amino acid biosynthesis across the chlorophyte lineage. Altogether, our findings elucidate the transcriptional regulation of amino acid metabolism and explain how TOR regulates nitrogen metabolism to support growth and development in photosynthetic organisms.

Introduction

Energy and nutrient signaling pathways are fundamental regulatory networks controlling growth and development. Integrating multiple nutrient and energy sources, particularly carbon and nitrogen, is essential for maintaining cellular homeostasis. Target of rapamycin (TOR) kinase is a central, conserved metabolic regulator that plays key roles in coordinating carbon and nitrogen sensing and metabolism with signaling pathways that drive growth and development in eukaryotes¹⁻³. Dysfunctional TOR kinase leads to disease, and nonfunctional TOR kinase is lethal⁴. TOR kinase functions as two main complexes, TORC1 and TORC2, in eukaryotes. Both TOR complexes are found in yeast and mammals, whereas photosynthetic organisms only have TORC1. TORC1 is comprised of the TOR kinase, RAPTOR (regulatory associated protein of TOR), and LST8 (Lethal with Sec Thirteen 8)⁴⁻⁷. In yeast, plants, and mammals, TORC1 senses carbon as sugars and nitrogen as amino acids to promote growth, and TOR inhibition suppresses growth and causes amino acid accumulation^{1,8,9}.

Tightly regulated TOR activity is critical because prolonged TOR inactivation is associated with immunosuppression and metabolic dysregulation in mammals⁴. In yeast and mammals, the mechanism of TOR inhibition-mediated amino acid accumulation involves TOR inhibition or amino acid starvation-induced general control nonderepressible 2 (Gcn2)-mediated translational activation of the general control nonderepressible 4/Activation Transcription Factor 4 (Gcn4/Atf4), a transcription factor (TF) that upregulates expression of amino acid biosynthesis genes¹⁰. This feedback loop ensures reactivation of TOR kinase once the inhibition or nutrient deprivation ends⁴. While photosynthetic organisms have homologs of Gcn2, the downstream Gcn4 TF is missing. Consequently, the mechanism regulating TOR reactivation in photosynthetic organisms remains unknown.

Studying TOR signaling in plants is complex, because they use light, organic carbon (e.g. glucose, sucrose) and inorganic nitrogen (e.g. NO_3^- , NH_4^+) sources, each capable of activating TOR¹¹⁻¹⁴. In the model plant *Arabidopsis thaliana*, the complexity is further highlighted by the different activation mechanisms of TOR in shoot and root meristems^{13,15}. Layers of tissue-specific regulation have prevented complete characterization of the upstream and downstream components of TOR signaling in photosynthetic organisms.

Green algae are at the evolutionary base of Viridiplantae and offer single-cell systems for studying TOR signaling in photosynthetic organisms. The TORC1 components are conserved in algae including the model green alga *Chlamydomonas reinhardtii* (Chlorophyceae), where acetate availability and nitrogen status were shown to modulate TOR kinase activity^{16,17}. *C. reinhardtii* can be used to study carbon and nitrogen signaling in relation to TOR kinase, and recent studies have documented that both photosynthetic CO_2 fixation and TOR inhibition lead to amino acid accumulation^{18,19}. In *C. reinhardtii*, TOR inhibition triggers de novo amino acid synthesis by increasing the activities of glutamine synthetase and glutamate synthase¹⁹, whereas in *A. thaliana* and the red alga *Cyanidioschyzon meroeae*, TOR inhibition leads to upregulation in gene expression of glutamine synthetase, ammonium and nitrate transporters, and nitrate reductase, thereby suggesting a transcriptional regulation of nitrogen metabolism^{20,21}.

The unicellular green alga *Chromochloris zofingiensis* (Chlorophyceae) is an emerging model organism for photosynthesis, metabolism, and energy sensing and signaling²²⁻²⁸. *C. zofingiensis* readily metabolizes exogenous glucose, a known activator of TOR in plants, yeast, mammalian cells, and other eukaryotes^{1,4,8}. Glucose is a significant driver of fast growth and high biomass in *C. zofingiensis* (23-25, 28). In contrast, *C. reinhardtii* cannot utilize glucose. Because *C. zofingiensis* accumulates biofuel precursors such as triacylglycerols (TAGs) and the high-value antioxidant astaxanthin while increasing biomass, this alga and its biology are relevant for commercialization^{23,25,27,28}. When *C. zofingiensis* consumes glucose under iron-limiting conditions, it switches off photosynthesis and accumulates TAGs via hexokinase1 (HXK1)²³⁻²⁵. Nitrogen deprivation also suppresses photosynthesis while TAGs accumulate in *C. zofingiensis*²⁶. However, nitrogen deprivation-dependent TAG accumulation is accompanied by decreased biomass productivity²⁹, making it distinct from glucose-induced TAG accumulation, which occurs alongside increased biomass productivity. The differences in biomass productivity suggest that these two inputs drive lipid accumulation through distinct physiological mechanisms^{23,30}, and nitrogen deprivation is a well-established inhibitor of TOR in eukaryotes^{3-8,10,13,15,21}.

Here, we test whether glucose interacts with TOR 1) through a canonical activation model to promote growth, 2) through a noncanonical inhibitory model where glucose can inhibit TOR activity, or 3) whether glucose signaling operates independently of TOR signaling in *C. zofingiensis*. To evaluate these possibilities, we use growth (biomass), amino acid accumulation, and downstream phosphorylation outputs as key readouts of TOR activity. We show that while TOR inhibition prevents glucose-dependent biomass accumulation, glucose and TOR signaling act independently specifically in relation to amino acid biosynthesis. We also provide evidence that inhibition of TOR increases amino acid biosynthesis through transcriptional activation. Furthermore, we reveal bHLH^{ACT+} as a key TF with conserved DNA binding sites in the promoters of amino acid biosynthesis genes as a possible mechanism of transcriptional regulation in photosynthetic organisms. We present *C. zofingiensis* as a single-cell system for elucidating fundamental mechanisms related to glucose and TOR signaling.

Results

TOR Inhibition Prevents Glucose-driven Growth.

The TOR signaling pathway regulates growth in response to environmental cues in eukaryotes⁴, and TOR inhibition is a critical strategy to determine key players and elucidate the pathway¹¹⁻¹³. To inhibit TOR in *C. zofingiensis*, we used the kinase inhibitor AZD8055 (AZD), the common ATP-competitive active site TOR inhibitor⁷. We first determined that 1 μ M AZD was the appropriate concentration for *C. zofingiensis*, as it prevented growth on plates (Supplementary Fig. 1a). The AZD concentration used in this study is the same as in previous studies with *C. reinhardtii*^{20,31-33}.

To disentangle glucose-TOR signaling in *C. zofingiensis*, wild-type (WT) photoautotrophic *C. zofingiensis* cells were treated with TOR inhibition by 1 μ M AZD, 100 mM glucose addition, and providing both 100 mM glucose + 1 μ M AZD (glucose+AZD) for 96 hours under continuous illumination (100 μ mol photons $\text{m}^{-2} \text{s}^{-1}$). TOR inhibition independently and in combination with glucose, led to decreased

photosynthesis and prevented glucose-dependent increases in cell density and culture volumetric biomass (Fig. 1a-c, Supplementary Fig. 1e). Glucose addition alone switched off photosynthesis and increased cell growth and biomass. Control cells remained green while treated cells changed colors and decreased total chlorophyll (Fig. 1b, Supplementary Fig. 1b). The treated cultures turned varying shades of orange due to the accumulation of the ketocarotenoid astaxanthin, and free and esterified astaxanthin peaks were detected by HPLC in AZD, glucose, and glucose+AZD treatments (Supplementary Fig. 1c-d). Together, these results demonstrate that TOR inhibition prevents glucose-driven biomass accumulation, indicating that TOR inhibition overrides or precludes glucose signaling.

Distinct Global Regulation of TOR Inhibition and Glucose Responses.

To decipher the independent and synergistic signaling of TOR inhibition and glucose in *C. zofingiensis*, we performed transcriptomics, tandem mass tag (TMT) proteomics, and metabolomics experiments with the following treatments: TOR inhibition by 1 μ M AZD, 15 mM glucose addition, the combined effect of 15 mM glucose + 1 μ M AZD, and 15 mM sorbitol addition (as an osmotic control) at 1 h and 24 h time points under continuous illumination (100 μ mol photons $\text{m}^{-2} \text{s}^{-1}$) (Supplementary Fig. 1f). Principal component analyses (PCA) of the omics datasets showed that TOR inhibition and glucose addition, both independently and synergistically, significantly altered the transcriptome, proteome, and metabolome particularly by 24 h (Fig. 1d). While variance in the transcriptome PC1 was driven by glucose+AZD at 24 h, the proteome PC2 variance was driven primarily by glucose at 24 h. These results indicate that AZD and glucose have distinct global transcriptomic and metabolic responses.

TOR Inhibition Induces Changes in Phosphorylation of ATG13, EIF4G, and LARP1 Independent of Hexokinase.

To confirm AZD-mediated inhibition of TOR in *C. zofingiensis*, we performed phosphoproteomics of TOR inhibition using the same treatments (1 μ M AZD, 15 mM glucose addition, 15 mM glucose + 1 μ M AZD, and 15 mM sorbitol-treated WT cultures at 1 h and 24 h) for both WT and the *hvk1-1* mutant. The *hvk1-1* mutant was generated by forward genetics, has a strong HXK1 knockdown phenotype, prevents glucose signaling mediated repression of photosynthesis and lipid accumulation, and has been used to study glucose signaling in *C. zofingiensis*^{24,25}.

We extracted a statistically significant dataset of 347 differentially phosphorylated peptides using moderated *t*-statistics (Fig. 2a, $P \leq 0.05$, Supplementary Data 1–2, 4545 phosphopeptides). To verify benchmark phosphopeptides affected by TOR inhibition, we identified 14 *C. zofingiensis* orthologs of 16 known TOR targets found in the literature from plants⁹. Of the 14 identified *C. zofingiensis* orthologs, 12 phosphopeptides mapped to seven conserved TOR targets and were detected across both WT and *hvk1-1*. Upon TOR inhibition by AZD or glucose+AZD, changes in phosphopeptides of the autophagy initiating complex protein 13 (ATG13), the protein translation-associated eukaryotic initiation factor (EIF4G), and the La-related protein 1 (LARP1) were statistically significant at either 1 h or 24 h in both WT and *hvk1-1* (Fig. 2b; $P \leq 0.05$, moderated *t*-statistics). Both ATG13 and LARP1 phosphopeptides downregulated while EIF4G levels were upregulated upon AZD and glucose+AZD treatments, consistent with their

responses observed in the plant *A. thaliana* upon TOR inhibition^{12,34}. However, the precise mechanism of regulation by TOR remains unexplored. These *C. zofingiensis* phosphopeptides are conserved across *C. reinhardtii*, the distantly related green alga *Auxenochlorella protothecoides* x *symbiontica* UTEX 250-A, and *A. thaliana* (Supplementary Fig. 2a).

The broader benchmark set included well-established TOR target proteins, the ribosomal S6 kinase (S6K), Yet Another Kinase 1 (YAK1), the regulatory-associated protein of TOR (RAPTOR), and the transcription factor E2F3^{12,34-40}. The phosphopeptide abundances for the broader benchmark TOR proteins were identified and reported for both genotypes (Supplementary Fig. 2b, Supplementary Fig. 3). Only two benchmark TOR targets, LARP1 and ATG13 showed a significant downregulation upon TOR inhibition with or without glucose. Importantly, glucose addition did not change the phosphorylation status of any benchmark TOR targets in WT or *hxx1-1*.

Overall, the phosphoproteomic analysis shows changes in phosphorylation of conserved benchmark TOR targets ATG13, EIF4G, and LARP1 with AZD treatment, thereby indicating inhibition of TOR in *C. zofingiensis* in the AZD and glucose+AZD treatments.

TOR Inhibition Triggers Upregulation of Amino Acid Biosynthesis Pathways.

To determine the relationship between transcription and translation of genes, we integrated the 24 h transcriptomic and proteomic datasets using Z-scores (Fig. 3, Supplementary Data 3–5). The glucose-treated samples showed the highest positive correlation ($R^2 = 0.48$), and similar to glucose+AZD samples ($R^2 = 0.43$) while AZD-treated samples had the lowest correlation ($R^2 = 0.26$), suggesting an inhibition of translation during TOR inhibition. Although there was an overall dampening of translation during TOR inhibition in AZD treatment conditions, an upregulation of transcripts of various amino acid biosynthesis pathways in AZD in both AZD and glucose+AZD (Fig. 3, Supplementary Data 6).

De novo amino acid biosynthesis relies on precursors derived from the central metabolic pathways of glycolysis and the tricarboxylic acid (TCA) cycle (Fig. 4a). While the pathways listed in Fig. 4a are not comprehensive, key precursors and fueling reactions for de novo amino acid biosynthesis include pyruvate for biosynthesis of Ala, Leu and Val; α -ketoglutarate for the synthesis of Glu, Pro, Arg and Gln; and oxaloacetate for the synthesis of Asp and related amino acids^{41,42}. Phosphoenolpyruvate, a glycolytic intermediate, acts as a precursor for aromatic amino acids, while the pentose phosphate pathway intermediates, ribose 5-phosphate and erythrose 4-phosphate, act as precursors for the synthesis of His and aromatic amino acids, respectively. Inorganic nitrogen is also assimilated into Glu- and Gln-derived amino acids⁴².

To understand TOR-dependent regulation of amino acid biosynthesis, gene and protein expression of amino acid biosynthetic pathways were explored in WT during TOR inhibition by 1 μ M AZD, 15 mM glucose addition, and 15 mM glucose + 1 μ M AZD treatment at 1 and 24 h at their transcript and protein levels (Fig. 4, Supplementary Fig. 4, Supplementary Data 7). We observed a strong upregulation at the transcript level but selective upregulation at the protein levels during TOR inhibition for WT cells. Interestingly, glucose addition also upregulates transcript and protein levels but only by

24 h, which likely provides the precursors for protein biosynthesis (Supplementary Fig. 4).

To test whether glucose based amino acid biosynthesis is TOR-dependent or mediated by HXK1, we conducted transcriptomics and proteomics with the *C. zofingiensis hxx1-1* mutant²⁴. We used the same treatments as were used with WT: 1 h and 24 h TOR inhibition by 1 μ M AZD, 15 mM glucose addition, and 15 mM glucose + 1 μ M AZD for *hxx1-1*. Transcript and protein expression analyses revealed that while TOR inhibition upregulated amino acid biosynthetic pathways in *hxx1-1* similarly to WT, the glucose-dependent upregulation observed in WT at 24 h was absent in *hxx1-1* (Supplementary Fig. 5, Supplementary Data 1, 8). These results indicate that glucose stimulation of amino acid biosynthesis requires HXK1 and is independent of TOR inhibition, consistent with previous results (Fig. 2b). Additionally, these results establish that upregulation of amino acid biosynthesis pathways is in response to TOR inhibition, independent of glucose.

TOR Inhibition Triggers Accumulation of Amino Acids.

To determine if the upregulation of amino acid biosynthesis pathways also led to amino acid accumulation, we performed untargeted metabolomics by HILIC-Z mass spectrometry of TOR inhibition by 1 μ M AZD, 15mM glucose addition, 15mM glucose + 1 μ M AZD, and 15 mM sorbitol treated cells and conducted *k*-means clustering with five clusters on the putatively annotated features (Supplementary Fig. 6, Supplementary Data 9–10). Remarkably, we observed an increase in amino acids solely during TOR inhibition in cluster 4, evident as early as 1 h and consistently observed at 24 h (Supplementary Fig. 6, Supplementary Data 9-10).

To validate the untargeted metabolomics, we performed targeted metabolite analysis during control, AZD, glucose and glucose+AZD (Fig. 4b, Supplementary Data 11–13). Glucose addition led to the accumulation of select amino acids including Phe, Ala, Leu, Gln and Asn by 24 h in WT cells but not in *hxx1-1*, establishing its dependence on glucose-HXK1 signaling (Fig. 4b, $P \leq 0.01$, Student's *t*-test, Supplementary Data 12). TOR inhibition resulted in a significant accumulation of Phe, Leu, Ile, Trp, Met, Pro, Val, Tyr, Ala, Thr, Ser, Gln, Asn, His, and Lys in WT and *hxx1-1* at both 1 h and 24 h, consistent with their transcript induction and demonstrating that amino acid accumulation is a direct consequence of TOR inhibition and independent of the glucose-HXK1 signaling axis (Fig. 4b, $P \leq 0.01$, Student's *t*-test, Supplementary Figs. 4–5). Specifically, enzymes involved in the biosynthesis of Asp, Asn, Glu and Gln derived, and TCA cycle derived were upregulated at the transcript and protein levels in AZD and glucose+AZD by 24 h (Supplementary Fig. 4-5). However, some amino acids such as Leu and Val accumulated in TOR-inhibited samples by 1 h (Fig. 4b), their enzymes are only upregulated by 24 h (Supplementary Fig. 4-5), suggesting that the translation of their biosynthetic enzymes is sufficient to promote accumulation or the involvement of either translation inhibition or protein catabolism, possibly via the proteasomal pathway.

To explore alternative mechanisms of amino acid accumulation, we conducted a 1 h experiment of 35 μ M cycloheximide (CHX) treatment to inhibit cytoplasmic protein synthesis, 10 μ M MG-132 treatment to prevent proteasome-dependent protein degradation, and a combination of TOR inhibition by 1 μ M AZD + 35 μ M CHX or 10 μ M

MG-132 (Fig. 4c, Supplementary Data 14). We observed that both translation inhibition (CHX) and TOR inhibition (AZD) caused substantial increases in accumulation of free amino acid levels, with combined treatment leading to a further elevation (Fig. 4c, Supplementary Data 14). However, in this experiment, TOR inhibition specifically increased Ala, Gln, Glu, Arg, Gly, Asn, His, Lys, and Ser levels in comparison to CHX treatment, suggesting a specific upregulation for these amino acids (Fig. 4c, $P \leq 0.01$, Dunnett's test, Supplementary Data 14).

To distinguish TOR specific de novo accumulation from additive effects of protein turnover, we compared AZD with AZD+CHX treatments. Amino acids such as Leu, Ile, Tyr, Met, Val, Thr, Ser, Asn, His, Arg, and Lys showed a significant accumulation between AZD and AZD+CHX treatments consistent with an additive effect of TOR inhibition and protein turnover on their accumulation (Fig. 4c, red asterisk, $P \leq 0.05$, ANOVA, Tukey test, Supplementary Data 15). In comparison, amino acids including Phe, Pro, Ala, Thr, Gly, Gln, and Glu showed no significant difference between AZD and AZD+CHX treatments despite significant differences to the control, suggesting that their accumulations result from de novo biosynthesis rather than protein turnover as early as 1 h (Fig. 4c, green asterisk, $P \geq 0.05$, ANOVA, Tukey test, Supplementary Data 15). Proteasome inhibition with MG-132 alone had limited impact on the log₂ fold change of intensities of amino acid levels. However, the combination of MG-132 with AZD resulted in an increase in all amino acids except Gly, equivalent to AZD treatment alone (Fig. 4b, $P \leq 0.01$, ANOVA, Tukey test, Supplementary Data 13). A comparison between AZD and AZD+MG-132 showed that only the amino acids Leu and Ile were significantly higher in AZD+MG-132, suggesting an additive effect by TOR inhibition and proteasomal degradation in their accumulation (Fig. 4c, blue asterisk, $P \leq 0.05$, ANOVA, Tukey test, Supplementary Data 15). Overall, these results show that TOR inhibition increases amino acid biosynthesis pathways at the transcript level, coinciding with de novo accumulation of specific amino acids by 1 h.

A bHLH^{ACT+} Transcription Factor Binds to Amino Acid Biosynthesis Genes.

To identify TFs underlying the possible transcriptional upregulation of amino acids in *C. zofingiensis*, we performed *k*-means clustering of differentially expressed transcripts and reported 6 clusters that were enriched with GO terms (Supplementary Fig. 7, $P \leq 0.01$, 8,500 transcripts from DESeq2 analysis, Supplementary Data 16-18). Cluster 4 represents TOR associated genes. We observed all differentially expressed TFs under TOR inhibition in transcriptomic clusters 1 and 5 (Supplementary Fig. 7). From these clusters, we identified 48 TFs that were upregulated at either 1 h or 24 h following AZD or glucose+AZD treatment in WT and *hxx1-1* (Supplementary Figs. 8-9, Supplementary Data 19).

To map genome-wide TF-DNA interactions and identify putative target promoters, we performed DNA affinity purification sequencing (DAP-seq) for the 48 TFs. Successful DAP-seq was obtained for 24 TFs (fraction of reads in peaks ≥ 0.05). Pathway enrichment on their target gene sets led to identification of four TFs associated with amino acid biosynthesis pathways (Fig. 5, Supplementary Data 20-21): an APETALA2 domain TF (AP2: Cz01g34320), two basic helix loop helix domain containing TFs harboring the aspartate kinase, chorismate mutase, and TyrA (ACT)-like domains (bHLH^{ACT+}: Cz04g01220 and Cz10g09160) and a basic leucine zipper domain

containing TF (bZIP: Cz04g21220). All four TFs increased in abundance during TOR inhibition at both transcript and protein levels in WT and *hxx1-1* (Fig. 5a). De novo motif discovery of the most enriched peaks bound by the respective TFs revealed that the most significant motifs were 8-11 nucleotides long (Fig. 5b).

Comparative analyses of TF binding across related species have been previously used to identify conserved transcription factor binding sites (TFBSs) and to gain insight into their *cis*-regulatory evolution across lineages⁵⁴. To assess TFBS conservation across divergent species, we performed a multiplexed DNA affinity purification (multiDAP) experiment in which we tested the binding of the four *C. zofingiensis* TFs to a distantly related chlorophyte, the Trebouxiophyte alga *Auxenochlorella* UTEX 250-A, which diverged from the *C. zofingiensis* lineage >800 million years ago⁴³. For all four TFs, we recovered nearly identical consensus motifs from the genomic libraries of the two species (Fig. 5b). However, since this primarily reflects intrinsic binding specificity of the TFs, we next assessed the evidence of functional conservation of TFBSs and their associated target genes.

MultiDAP has been used to demonstrate that TFBSs conserved across species are enriched in *cis*-regulatory elements (CREs), and conserved TF-target gene sets across species highlight functional binding sites. Therefore, to identify conserved functional targets, we performed pathway enrichment analysis on the TF-target gene sets from both species (Fig. 5c, Supplementary Data 20). Focusing specifically on amino acid biosynthesis and related metabolic pathways, we found that only the bHLH^{ACT+} TF Cz04g01220 showed consistent enrichment across multiple amino acid biosynthesis pathways in both *C. zofingiensis* and UTEX 250-A (Fig. 5c). Given this cross-species conservation, we focused the analysis on identifying the core set of target genes within the amino acid biosynthesis pathways shared between the two species.

To identify the conserved TF-target gene set across *C. zofingiensis* and UTEX 250-A, we first extracted all the TF-target genes enriched in the amino acid biosynthesis pathways from both species (Fig. 5c). Next, we mapped orthologs from *C. zofingiensis* to UTEX 250-A and extracted their corresponding *C. zofingiensis* IDs. We then extracted only the gene sets that were common in the two species, which included a core set of 48 genes with orthologs in UTEX 250-A. To further test whether their binding led to changes in gene expression, we examined the abundances of the 48 genes in WT and *hxx1-1* under TOR inhibition by 1 μ M AZD, 15 mM glucose addition, and combined 15 mM glucose + 1 μ M AZD (Supplementary Fig. 8-9). Although TFBSs can be present throughout the genome, including promoters, gene bodies and other intra/intergenic regions, we focused on TFBSs 2000 bp upstream and 500 bp downstream of the protein start codon⁴⁴. We categorized these genes into central metabolism pathways including glycolysis and TCA cycle, which can produce precursors for amino acid biosynthesis followed by genes associated with Gly/Ser biosynthesis and Ala, Asp, Gln, Glu, and Arg biosynthesis. The transcription of these genes is likely promoted by the bHLH^{ACT+} TF Cz04g01220 upon TOR inhibition (Fig. 6, Supplementary Fig. 11-12).

bHLH^{ACT+} Transcription Factor Binding Motif is Conserved in Amino Acid Biosynthesis Genes in Green Algae and Plants.

The bHLH family, including the bHLH^{ACT+} TFs recognize the core DNA sequence motif known as the palindromic G-box (5'-CACGTG-3')⁴⁵. We found that the bHLH^{ACT+} TF binding motif CACGTG in amino acid biosynthesis gene promoters (Fig. 5b). To test the evolutionary conservation of this motif in target gene promoters across various green algae and plants, we identified orthologs of targets of the core set of 19 genes previously shown to be bound by the bHLH^{ACT+} TF Cz04g01220 (Fig. 6), and scanned their promoters (2000 bp upstream and 500 bp downstream from the protein start site) for the presence of the G-box motif (Supplementary Data 22). In addition to *C. zofingiensis*, we included the green algae *C. reinhardtii*, *Volvox carteri* (Chlorophyceae), UTEX 250-A, and *Monoraphidium neglectum* (Chlorophyceae) as well as the plant genomes of *A. thaliana* and *Oryza sativa*. Therefore, the sampling spans major species within the Viridiplantae and covers a broad evolutionary distance.

We calculated the frequency of occurrence of G-boxes across whole genomes and within the promoters of the core set of biosynthesis genes (Table 1). G-box motifs were present in more than 65% of target gene promoters in algae, while the frequency of occurrence reduced to less than 50% in higher plants. Motif enrichment analysis relative to genomic background revealed that chlorophyte algae except *C. reinhardtii* show significant motif enrichment of the G-box in the core set of amino acid biosynthesis genes (Fig. 7). Overall, these data show high conservation of the G-boxes in amino acid biosynthesis gene promoters across green algae, indicating a conserved role of bHLH in regulating these metabolic pathways.

Discussion

The TOR pathway integrates different nutrient signals including glucose to promote growth and metabolism in photosynthetic eukaryotes. Here, we carefully dissected TOR-specific responses from the glucose-TOR-based signaling response in the green alga *C. zofingiensis*. Using AZD as a TOR kinase inhibitor, we found TOR inhibition prevents growth and suppresses photosynthesis in *C. zofingiensis* (Fig. 1a-c), consistent with previous studies in *C. reinhardtii* and *A. thaliana*^{32,34,35}. Exogenous glucose in *C. zofingiensis* promoted growth and biomass accumulation as reported previously^{23-25,28}. In contrast, TOR inhibition prevented growth and biomass accumulation, suggesting that TOR signaling is responsible for high biomass accumulation in *C. zofingiensis*. Moreover, TOR inhibition, with and without glucose, also led to the accumulation of ketocarotenoid astaxanthin in free and esterified forms (Supplementary Fig. 1b-d), as has been previously observed with glucose^{23-25,27,28,30,46}, highlighting a previously unrecognized role of TOR in ketocarotenoid accumulation.

Using a global multiomics analysis (transcriptome, proteome, and metabolome), we further disentangled glucose-TOR signaling in *C. zofingiensis*. The TOR inhibition proteome clustered near the control despite the transcriptome showing significant variance for TOR inhibition treatments (Fig. 1d). These results suggest modulation of the proteome that is not reflected in the transcriptome. TOR is a known regulator of global translation in algae, plants, yeast, and mammals and regulates the translation machinery via phosphorylation at multiple levels³⁶. Thus, TOR inhibition can lead to a decrease in overall translation and dampening of the proteome. As such, the TOR inhibition and glucose transcriptome showed the greatest variation from the control, yet the proteome was substantially more similar to the control than the glucose proteome.

TOR activity is marked by the phosphorylation of specific downstream targets, primarily associated with protein biosynthesis and autophagy^{9,36}. The TOR-LARP1 signaling plays an important role in ribosome biogenesis, auxin signaling, and plant development promoting protein biosynthesis^{35,37,40}. Moreover, TOR inhibition-dependent dephosphorylation of ATG13 also activates autophagy³⁷. The phosphoproteome analyses further validated conserved TOR targets of ATG13, EIF4G and LARP1 and identified 347 significant TOR-dependent phosphosites (Fig. 2, Supplementary Fig. 2). These findings establish the conservation of known TOR targets in *C. zofingiensis*.

Glucose and sucrose are established upstream activators of TOR kinase in *A. thaliana* and increase the phosphorylation of benchmark TOR targets of LARP1, ATG13 etc.,^{12,34}. However, glucose addition in both *C. zofingiensis* WT and *hvk1-1* did not increase the phosphorylation of ATG13, LARP1 or S6K (Fig. 2b, Supplementary Fig. 2), suggesting that glucose signaling in this alga operates through a TOR-independent mechanism. Similar to WT, *hvk1-1* also shows a change in phosphorylation of TOR targets upon TOR inhibition. Thus, we conclude that TOR inhibition is independent of the glucose-HXK1 axis. Furthermore, the observation that TOR inhibition still alters the phosphorylation of canonical TOR targets in *hvk1-1* demonstrates that TOR activity itself remains intact and responsive, and that TOR inhibition operates independently of the glucose-HXK1 signaling axis. Glucose alone did not activate TOR further compared to control, but TOR activity is still required as its inhibition prevents glucose-driven biomass accumulation in glucose+AZD (Fig. 1a-c, Fig. 2b).

The multiomics datasets also revealed a TOR inhibition-specific upregulation of amino acid biosynthesis at the transcript and metabolite levels, independent of glucose signaling (Fig. 4b). The increase in amino acids during TOR inhibition is consistent with previous studies in *C. reinhardtii* and *A. thaliana*^{19,21}. Moreover, the inhibitor-based experiments blocking protein translation and proteasome degradation could identify TOR inhibition-dependent accumulation of specific amino acids: Phe, Pro, Ala, Thr, Gly, Gln, and Glu (Fig. 4c). Other mechanisms including increased nitrogen assimilation and autophagy induced upon TOR inhibition can also contribute to accumulation of these amino acids^{19,21} and require further investigation.

Transcriptional upregulation of specific nitrogen assimilation genes and glutamine synthetase upon TOR inhibition has been reported in algae and plants^{20,21}. However, the basis of this upregulation remains unknown. Because the transcriptome showed an upregulation of amino acid biosynthesis genes, we propose transcriptional regulation of these genes upon TOR inhibition. We provide a list of 48 TOR inhibition responsive TFs that can putatively induce transcription of the amino acid biosynthesis pathways (Supplementary Fig. 8-9). From this list, we identified four TFs, two bHLH^{ACT+}, an AP2, and a bZIP, with target genes defined by DAP-seq that are enriched in biosynthesis of amino acids pathways (Fig. 5).

While this study and previous studies⁴⁷⁻⁴⁹ suggest that bHLH^{ACT+/ACT-} TFs interact with TOR and/or its associated signaling pathways, further experiments are needed to dissect the mechanism. We identified a bHLH^{ACT+} (Cz04g01220) and found that TOR inhibition leads to an increase in its transcript and protein abundance (Fig. 5a) and leads to increased amino acid levels (Fig. 4). The ACT domain (60-80 amino acids) is also present in multiple amino acid biosynthetic enzymes and acts as a small metabolite sensor^{51,52,53}. In mammalian cells, the cytosolic protein CASTOR1 acts as a cellular

arginine sensor and contains ACT domains^{54,55}. Binding of arginine to CASTOR1 activates mTORC1 signaling. The bHLH associated ACT domains in photosynthetic eukaryotes form a monophyletic group and likely share a common ancestor with the bacterial glutamate/glutamine sensing uridylyl-transferase (GlnD), which is a small molecule sensor^{50,51,56}. However, only ~30% of flowering plant bHLHs and 52% of green algae (chlorophytes) possess the ACT domain⁵⁰. Importantly, dimerization of ACT domains provides the ability for bHLH^{ACT+} to dimerize and can negatively affect DNA binding⁵⁰. The ACT domain in the *C. reinhardtii* α -amylase2 (AMA2) is specifically bound by Gln, which leads to the activation of its amylase activity, thereby establishing the ACT domain as a Gln sensor⁵⁷. Gln levels increase within 5 min of TOR inhibition by rapamycin, likely due to an increase in nitrogen assimilation¹⁹. Hence, we hypothesize that such binding by Gln to the bHLH^{ACT+} could act as a trigger to activate transcription of amino acid biosynthesis genes, but elucidation of this mechanism requires further research. Crosstalk between glucose and TOR signaling can also upregulate amino acids, perhaps via the bHLH^{ACT+} TF, and future studies can address this aspect as well. Overall, these results indicate that the bHLH^{ACT+} TF (Cz04g01220) specifically binds to the promoters of amino acid biosynthesis genes to upregulate their transcription upon TOR inhibition.

Lastly, the bHLH^{ACT+} TF (Cz04g01220) binds to amino acid biosynthesis genes in two distantly related genomes, suggesting an evolutionarily conserved enrichment of TFBSs in these genes (Fig. 6). We uncovered the evolutionary conservation of the bHLH binding site (CACGTG motif) upstream of a specific set of amino acid biosynthesis genes across many distantly related chlorophyte algae. We did not observe conservation in land plants, suggesting a putative green algal-specific mechanism to regulate TOR inhibition-dependent amino acid accumulation (Table 1), but further testing is required.

In conclusion, we define a TOR inhibition-specific pathway regulating amino acid accumulation that is independent of glucose-TOR signaling in the green alga *C. zofingiensis* (Fig. 7). The DAP-seq experiment revealed that a bHLH^{ACT+} TF binds amino acid biosynthesis genes and may upregulate their transcription during TOR inhibition. While previous studies showed amino acid accumulation upon TOR inhibition in photosynthetic organisms^{19,21}, we propose that previously unrecognized transcriptional regulation of amino acid biosynthesis genes leads to amino acid accumulation in TOR-inhibited cells. We also show that TOR inhibition-dependent transcriptional reprogramming of amino acid biosynthesis is independent of HXK1. Furthermore, this transcriptional regulation appears conserved across distantly related chlorophyte algae. Understanding the control and regulation of amino acid biosynthesis and nitrogen metabolism is crucial, as these processes maintain growth and plant homeostasis. We conclude that regulation of nitrogen metabolism by TOR, an ancient and conserved protein, may help photosynthetic organisms maintain their carbon and nitrogen balance to support their growth and development, and insights into these pathways enhance the broader understanding of nutrient regulation and may offer strategies for improving biomass and yield.

Methods

Chromochloris zofingiensis SAG211-14 cultures²² were grown in *Chromochloris* Optimized Ratio of Elements (CORE) medium²⁸ under continuous 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light. Growth and photosynthesis metrics (cell density, F_v/F_m) were measured alongside omics to correlate physiological responses with molecular changes under varying nutrient and stress conditions. Samples were collected at 1 h and 24 h for multiomics analyses. Transcriptomics utilized RNA-Seq via Illumina sequencing, quantified with Salmon and DESeq2. Proteomics and phosphoproteomics involved TMT isobaric labeling, phosphopeptide enrichment, and Orbitrap mass spectrometry. Metabolomics employed LC/MS with GNPS annotation. multiDAP-seq identified transcription factor-DNA interactions through in vitro binding assays and motif analysis. Data integration included GO and pathway enrichment, k -means clustering, and orthology comparisons to identify conserved regulatory motifs. A detailed description of all methods and materials used in this study is provided in Supplementary Methods.

Funding sources

This work was supported by the U.S. Department of Energy (DOE), Office of Science, Office of Biological and Environmental Research Award DESC0018301 (M.S.L, T.R.N., K.K.N, and M.S.R). Transcriptomics, proteomics, and phosphoproteomics were performed under the Facilities Integrating Collaborations for User Science (FICUS) Program (DOI: 10.46936/fics.proj.2017.49960/ 60000021, to K.K.N and M.S.R) and used resources at the Joint Genome Institute (JGI) and the Environmental Molecular Sciences Laboratory (EMSL), which are DOE Office of Science User Facilities. Both facilities are sponsored by the Biological and Environmental Research program. Metabolomics analyses were supported by the U.S. Department of Energy, Office of Science, Office of Biological & Environmental Research, Genomic Sciences Program and used resources of the National Energy Research Scientific Computing Center, a Department of Energy Office of Science User Facility operated under contract number DE-AC02-05CH11231 to Lawrence Berkeley National Laboratory. DAP-seq and metabolomics were performed under a JGI Community Science Program (CSP) (<https://doi.org/10.46936/10.25585/60008114>, to M.S.R and K.K.N). This material was partially supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, under Award Number DE-SC0023027 (M.S.L, T.R.N., K.K.N, and M.S.R). This article is subject to HHMI's Open Access to Publications policy. HHMI lab heads have previously granted a nonexclusive CC BY 4.0 license to the public and a sublicensable license to HHMI in their research articles. Pursuant to those licenses, the author-accepted manuscript of this article can be made freely available under a CC BY 4.0 license immediately upon publication. K.K.N. is an investigator of the Howard Hughes Medical Institute.

Author Contributions:

S.U. and M.S.R. designed research; S.U., S.M.K., A.S., R.M., Y.Z., and M.S.R. performed research; S.U., S.M.K., S.O.P., K.H., R.O.M., L.B., I.K.B., M.S.L., T.R.N., K.K.N., and M.S.R. analyzed data; S.U. and M.S.R. wrote the paper with contributions from all authors.

Competing Interest Statement: T.R.N. is a founder of two non-profits, Prosper Soils and Bioaligned Labs with prior approval from LBNL. None of the other authors declare any conflicts of interest.

Data availability

LC-MS/MS raw data have been deposited at GNPS2 https://gnps2.org/result?task=5517e1d004334a4b88f2a79a5bb0f602&viewname=filesuimary&resultdisplay_type=task. Proteomics raw data have been deposited at MassIVE <http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD078428>. RNA-Seq and DAP-seq data are available from the NCBI Gene Expression Omnibus (accession no. GSE309591). Source data are provided with this paper. TOR Multiomics explorer webpage generated to mine the multiomics dataset: https://shivaniu.shinyapps.io/Shivani_TOR_phosphoproteomics/

References

1. Wu, Y. et al. Integration of nutrient, energy, light, and hormone signalling via TOR in plants. *J. Exp. Bot.* **70**, 2227–2238 (2019).
2. Robaglia, C., Thomas, M. & Meyer, C. Sensing nutrient and energy status by SnRK1 and TOR kinases. *Curr. Opin. Plant Biol.* **15**, 301–307 (2012).
3. Burkart, G. M. & Brandizzi, F. A tour of TOR complex signaling in plants. *Trends Biochem. Sci.* **46**, 417–428 (2021).
4. Wullschleger, S., Loewith, R. & Hall, M. N. TOR signaling in growth and metabolism. *Cell* **124**, 471–484 (2006).
5. Brunkard, J. O. Exaptive evolution of target of rapamycin signaling in multicellular eukaryotes. *Dev. Cell* **54**, 142–155 (2020).
6. Mallén-Ponce, M. J., Pérez-Pérez, M. E. & Crespo, J. L. Deciphering the function and evolution of the target of rapamycin signaling pathway in microalgae. *J. Exp. Bot.* **73**, 6993–7005 (2022).
7. Montané, M.-H. & Menand, B. TOR inhibitors: from mammalian outcomes to pharmacogenetics in plants and algae. *J. Exp. Bot.* **70**, 2297–2312 (2019).
8. González, A. & Hall, M. N. Nutrient sensing and TOR signaling in yeast and mammals. *EMBO J.* **36**, 397–408 (2017).
9. Li, L., Liu, K. & Sheen, J. Dynamic nutrient signaling networks in plants. *Annu. Rev. Cell Dev. Biol.* **37**, 341–367 (2021).
10. Hinnebusch, A. G. Translational regulation of GCN4 and the general amino acid control of yeast. *Annu. Rev. Microbiol.* **59**, 407–450 (2005).
11. Xiong, Y. et al. Glucose–TOR signalling reprograms the transcriptome and activates meristems. *Nature* **496**, 181–186 (2013).

12. Van Leene, J. et al. Capturing the phosphorylation and protein interaction landscape of the plant TOR kinase. *Nat. Plants* **5**, 316–327 (2019).
13. Liu, Y. et al. Diverse nitrogen signals activate convergent ROP2-TOR signaling in Arabidopsis. *Dev. Cell* **56**, 1283–1295.e5 (2021).
14. O’Leary, B. M., Oh, G. G. K., Lee, C. P. & Millar, A. H. Metabolite regulatory interactions control plant respiratory metabolism via target of rapamycin (TOR) kinase activation. *Plant Cell* **32**, 666–682 (2020).
15. Li, X. et al. Differential TOR activation and cell proliferation in Arabidopsis root and shoot apices. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 2765–2770 (2017).
16. Pérez-Pérez, M., Couso, I. & Crespo, J. The TOR signaling network in the model unicellular green alga *Chlamydomonas reinhardtii*. *Biomolecules* **7**, 54 (2017).
17. Upadhyaya, S., Agrawal, S., Gorakshakar, A. & Rao, B. J. TOR kinase activity in *Chlamydomonas reinhardtii* is modulated by cellular metabolic states. *FEBS Lett.* **594**, 3122–3141 (2020).
18. Mallén-Ponce, M. J., Pérez-Pérez, M. E. & Crespo, J. L. Photosynthetic assimilation of CO₂ regulates TOR activity. *Proc. Natl. Acad. Sci. U.S.A.* **119**, e2115261119 (2022).
19. Mubeen, U., Jüppner, J., Alpers, J., Hinch, D. K. & Giavalisco, P. Target of rapamycin inhibition in *Chlamydomonas reinhardtii* triggers de novo amino acid synthesis by enhancing nitrogen assimilation. *Plant Cell* **30**, 2240–2254 (2018).
20. Imamura, S. et al. Target of rapamycin (TOR) plays a critical role in triacylglycerol accumulation in microalgae. *Plant Mol. Biol.* **89**, 309–318 (2015).
21. Ingargiola, C. et al. The Arabidopsis Target of Rapamycin kinase regulates ammonium assimilation and glutamine metabolism. *Plant Physiol.* **192**, 2943–2957 (2023).
22. Roth, M. S. et al. Chromosome-level genome assembly and transcriptome of the green alga *Chromochloris zofingiensis* illuminates astaxanthin production. *Proc. Natl. Acad. Sci. U.S.A.* **114**, E4296–E4305 (2017).
23. Roth, M. S. et al. Regulation of oxygenic photosynthesis during trophic transitions in the green alga *Chromochloris zofingiensis*. *Plant Cell* **31**, 579–601 (2019).
24. Roth, M. S., Westcott, D. J., Iwai, M. & Niyogi, K. K. Hexokinase is necessary for glucose-mediated photosynthesis repression and lipid accumulation in a green alga. *Commun. Biol.* **2**, 347 (2019).
25. Jeffers, T. L. et al. Iron rescues glucose-mediated photosynthesis repression during lipid accumulation in the green alga *Chromochloris zofingiensis*. *Nat. Commun.* **15**, 6046 (2024).

26. Liu, J. et al. Multiomics analysis reveals a distinct mechanism of oleaginousness in the emerging model alga *Chromochloris zofingiensis*. *Plant J.* **98**, 1060–1077 (2019).
27. Jeffers, T. L. & Roth, M. S. Revealing mechanisms of algal astaxanthin production and bioengineering potential using multiomics. in *Global Perspectives on Astaxanthin* 181–208 (2021).
28. Jeffers, T. L. et al. An algal nutrient-replete, optimized medium for fast growth and high triacylglycerol accumulation. *Plant Direct* **9**, e70106 (2025).
29. Mulders, K. J. M. et al. Nitrogen-depleted *Chlorella zofingiensis* produces astaxanthin, ketolutein and their fatty acid esters: a carotenoid metabolism study. *J. Appl. Phycol.* **27**, 125–140 (2015).
30. Meagher, M. et al. Genome-scale metabolic model accurately predicts fermentation of glucose by *Chromochloris zofingiensis*. *Algal Res.* **84**, 103805 (2024).
31. Montané, M.-H. & Menand, B. TOR inhibitors: from mammalian outcomes to pharmacogenetics in plants and algae. *J. Exp. Bot.* **70**, 2297–2312 (2019).
32. Upadhyaya, S. & Rao, B. J. Reciprocal regulation of photosynthesis and mitochondrial respiration by TOR kinase in *Chlamydomonas reinhardtii*. *Plant Direct* **3**, e00184 (2019).
33. Werth, E. G. et al. Investigating the effect of target of rapamycin kinase inhibition on the *Chlamydomonas reinhardtii* phosphoproteome: from known homologs to new targets. *New Phytol.* **221**, 247–260 (2019).
34. Scarpin, M. R., Leiboff, S. & Brunkard, J. O. Parallel global profiling of plant TOR dynamics reveals a conserved role for LARP1 in translation. *eLife* **9**, e58795 (2020).
35. Dobrenel, T. et al. The Arabidopsis TOR kinase specifically regulates the expression of nuclear genes coding for plastidic ribosomal proteins and the phosphorylation of the cytosolic ribosomal protein S6. *Front. Plant Sci.* **7**, 1611 (2016).
36. Scarpin, M. R., Simmons, C. H. & Brunkard, J. O. Translating across kingdoms: target of rapamycin promotes protein synthesis through conserved and divergent pathways in plants. *J. Exp. Bot.* **73**, 7016–7025 (2022).
37. Battaglion, S., Benjamin, D., Wälchli, M., Maier, T. & Hall, M. N. mTOR substrate phosphorylation in growth control. *Cell* **185**, 1814–1836 (2022).
38. Barrada, A. et al. A TOR-YAK1 signaling axis controls cell cycle, meristem activity and plant growth in Arabidopsis. *Development* **146**, dev171298 (2019).
39. Perdoux, R. et al. A drug-resistant mutation in plant target of rapamycin validates the specificity of ATP-competitive TOR inhibitors in vivo. *Plant J.* **117**, 1344–1355 (2024).

40. Xiong, Y. & Sheen, J. Rapamycin and glucose-target of rapamycin (TOR) protein signaling in plants. *J. Biol. Chem.* **287**, 2836–2842 (2012).
41. Akashi, H. & Gojobori, T. Metabolic efficiency and amino acid composition in the proteomes of *Escherichia coli* and *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 3695–3700 (2002).
42. Buchanan, B. B., Gruissem, W. & Jones, R. L. *Biochemistry and Molecular Biology of Plants* (Wiley, 2015).
43. Craig, R. J. et al. Targeted genetic manipulation and yeast-like evolutionary genomics in the green alga *Auxenochlorella*. *Plant Cell* **37**, koaf259 (2025).
44. Baumgart, L. A. et al. Recruitment, rewiring and deep conservation in flowering plant gene regulation. *Nat. Plants* **11**, 1514–1527 (2025).
45. Toledo-Ortiz, G., Huq, E. & Quail, P. H. The Arabidopsis basic/helix-loop-helix transcription factor family. *Plant Cell* **15**, 1749–1770 (2003).
46. Liu, J. et al. *Chlorella zofingiensis* as an alternative microalgal producer of astaxanthin: biology and industrial potential. *Mar. Drugs* **12**, 3487–3515 (2014).
47. Gaudinier, A. et al. Transcriptional regulation of nitrogen-associated metabolism and growth. *Nature* **563**, 259–264 (2018).
48. Sanagi, M. et al. Low nitrogen conditions accelerate flowering by modulating the phosphorylation state of FLOWERING BHLH 4 in Arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* **118**, e2022942118 (2021).
49. Persyn, F. et al. A nitrogen-specific interactome analysis sheds light on the role of the SnRK1 and TOR kinases in plant nitrogen signaling. *Mol. Cell. Proteomics* **23**, 100842 (2024).
50. Lee, Y. S., Shiu, S.-H. & Grotewold, E. Evolution and diversification of the ACT-like domain associated with plant basic helix–loop–helix transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* **120**, e2219469120 (2023).
51. Ettema, T. J. G., Brinkman, A. B., Tani, T. H., Rafferty, J. B. & Van der Oost, J. A novel ligand-binding domain involved in regulation of amino acid metabolism in prokaryotes. *J. Biol. Chem.* **277**, 37464–37468 (2002).
52. Lang, E. J. M., Cross, P. J., Mittelstädt, G., Jameson, G. B. & Parker, E. J. Allosteric ACTion: the varied ACT domains regulating enzymes of amino-acid metabolism. *Curr. Opin. Struct. Biol.* **29**, 102–111 (2014).
53. Chipman, D. The ACT domain family. *Curr. Opin. Struct. Biol.* **11**, 694–700 (2001).

54. Xia, J., Wang, R., Zhang, T. & Ding, J. Structural insight into the arginine-binding specificity of CASTOR1 in amino acid-dependent mTORC1 signaling. *Cell Discov.* **2**, 16035 (2016).
55. Ke, Y. et al. Animal amino acid sensor — a review. *Anim. Biosci.* **38**, 198–208 (2025).
56. Zhang, Y., Pohlmann, E. L., Serate, J., Conrad, M. C. & Roberts, G. P. Mutagenesis and functional characterization of the four domains of GlnD, a bifunctional nitrogen sensor protein. *J. Bacteriol.* **192**, 2711–2721 (2010).
57. Scholtysek, L., Poetsch, A., Hofmann, E. & Hemschemeier, A. The activation of *Chlamydomonas reinhardtii* alpha amylase 2 by glutamine requires its N-terminal aspartate kinase–chorismate mutase–tyrA (ACT) domain. *Plant Direct* **8**, e609 (2024).

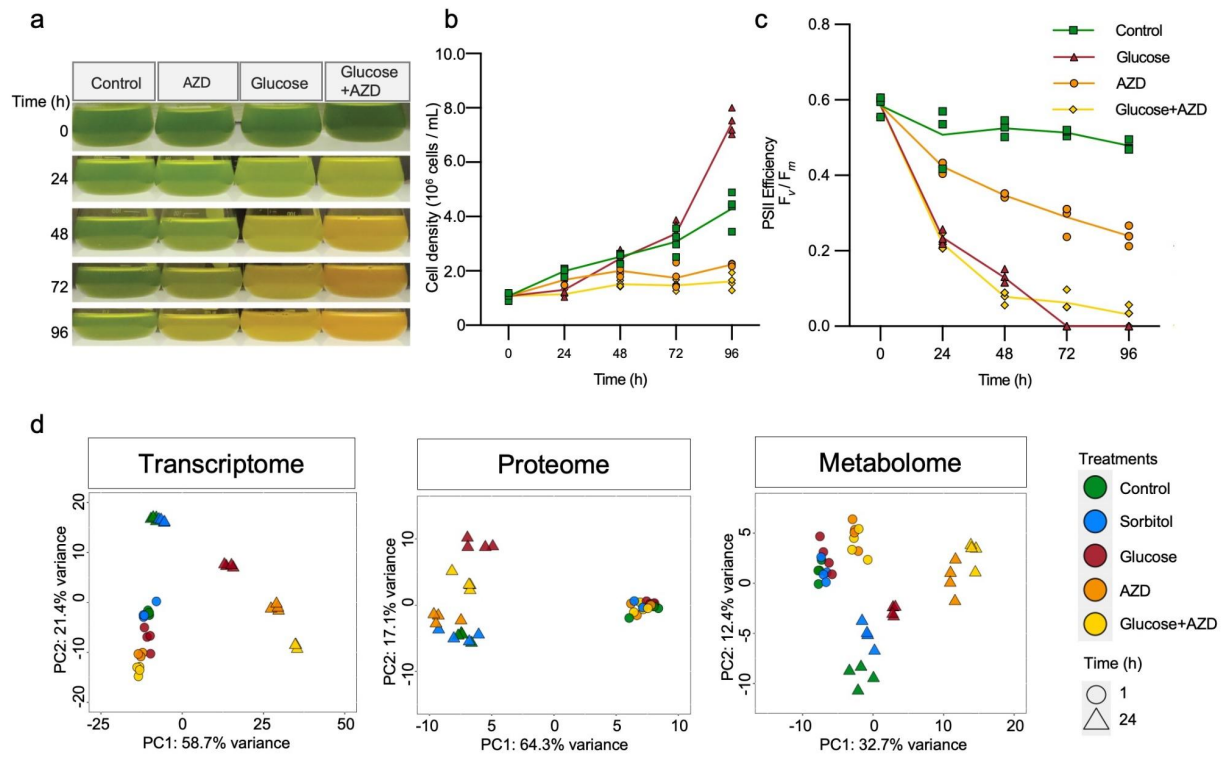


Fig. 1. TOR inhibition prevents glucose-driven growth and suppresses photosynthesis in the green alga *C. zofingiensis*. (a) Representative *C. zofingiensis* (WT) cultures during the 96 h experiment with the following treatments: control, TOR inhibition by 1 μ M AZD8055, 100 mM glucose addition, and 100 mM glucose + 1 μ M AZD treatment. (b) Cell density and (c) maximum PSII photosynthetic efficiency (F_v/F_m). For (B) and (C), datapoints represent biological replicates with lines representing means; $n = 3-4$. (d) Principal Component Analyses (PCA) of WT transcriptome, proteome, and metabolome with treatments: control, 1 μ M AZD, 15 mM glucose, 15 mM glucose + 1 μ M AZD, and 15 mM sorbitol as an osmotic control. For the transcriptome, normalized $\log_2$ counts of 500 genes with highest variance were considered. For the proteome and the metabolome, $\log_2$ intensity values of proteins or metabolites with highest variance were considered. Data represent biological replicates; $n = 3-4$.

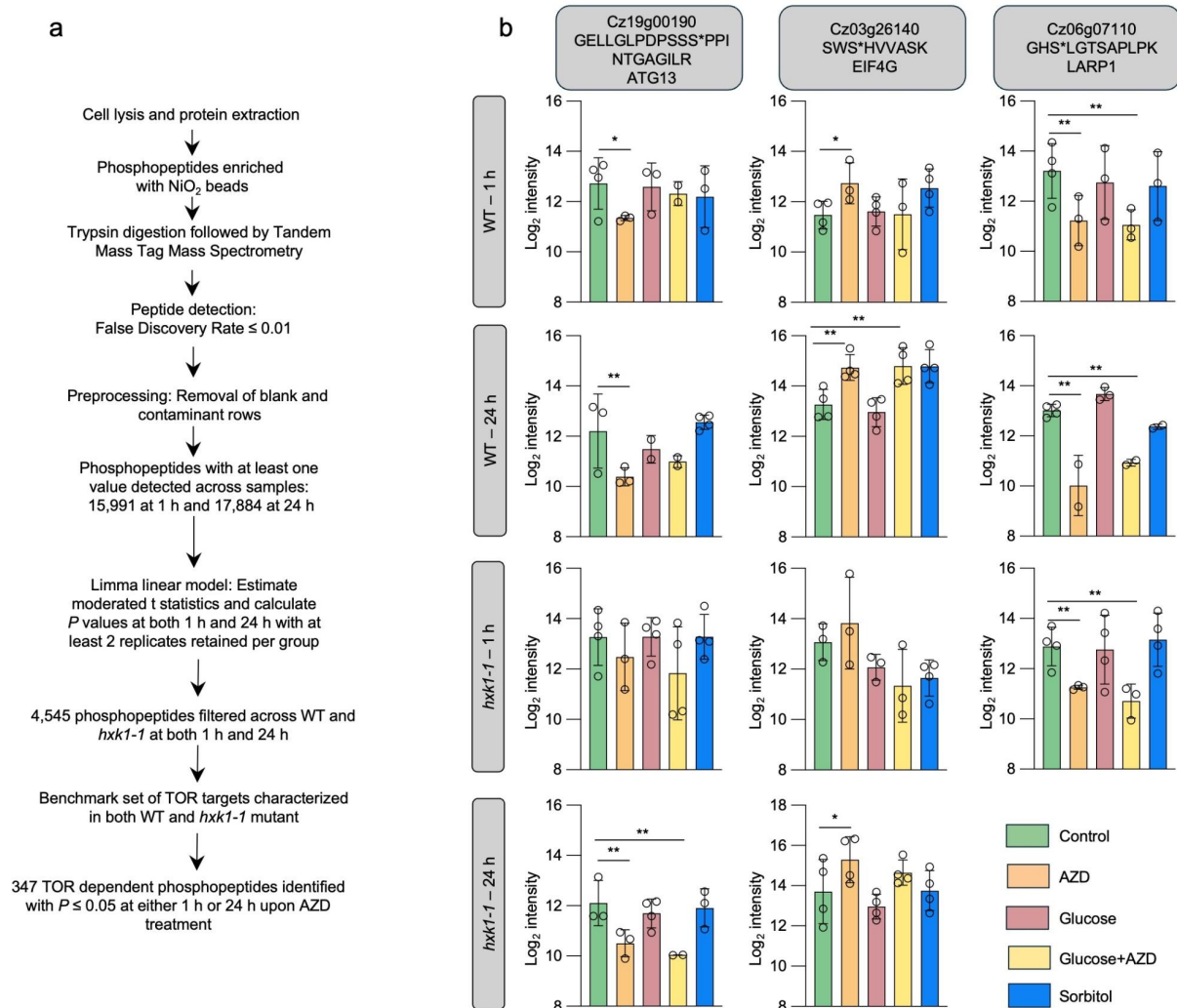


Fig. 2. Phosphoproteomics of TOR-inhibited *C. zofingiensis*. (a) Schematic overview of the TOR phosphoproteomics workflow resulting in identification of 347 AZD sensitive sites. (b) Log₂ intensities of phosphosites for conserved TOR targets ATG13, EIF4G, and LARP1 during TOR inhibition by 1 μ M AZD treatment, 15 mM glucose addition, combined 15 mM glucose + 1 μ M AZD treatment and sorbitol for WT and *hxx1-1*. The *hxx1-1* 24 h LARP1 did not pass filtering criteria. Data represent means $\pm$ SD; *n* = 2-4 biological replicates; **P* ≤ 0.05, ***P* ≤ 0.01 (moderated *t*-statistics).

Figure 3

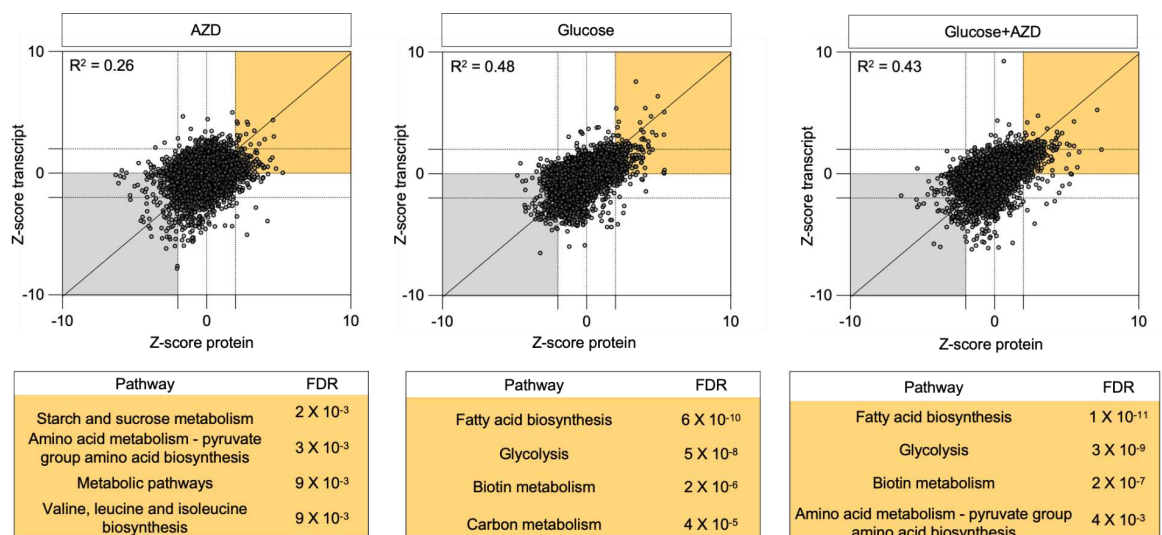


Fig. 3. Integrated transcriptomics and proteomics reveals TOR inhibition-dependent upregulation of amino acid pathways. Scatterplot representation of correlation of transcript and protein expression ratios. *C. zofingiensis* WT transcript and protein Z-scores of 24 h TOR inhibition by 1 μ M AZD, 15 mM glucose addition, and 15 mM glucose + 1 μ M AZD treatment ($n = 2-4$ biological replicates; Supplementary Data 4-6). The scatterplots are divided into four sectors with upregulated transcript (orange) and downregulated transcript (gray). Supplementary Data 6 contains the complete list of pathways.

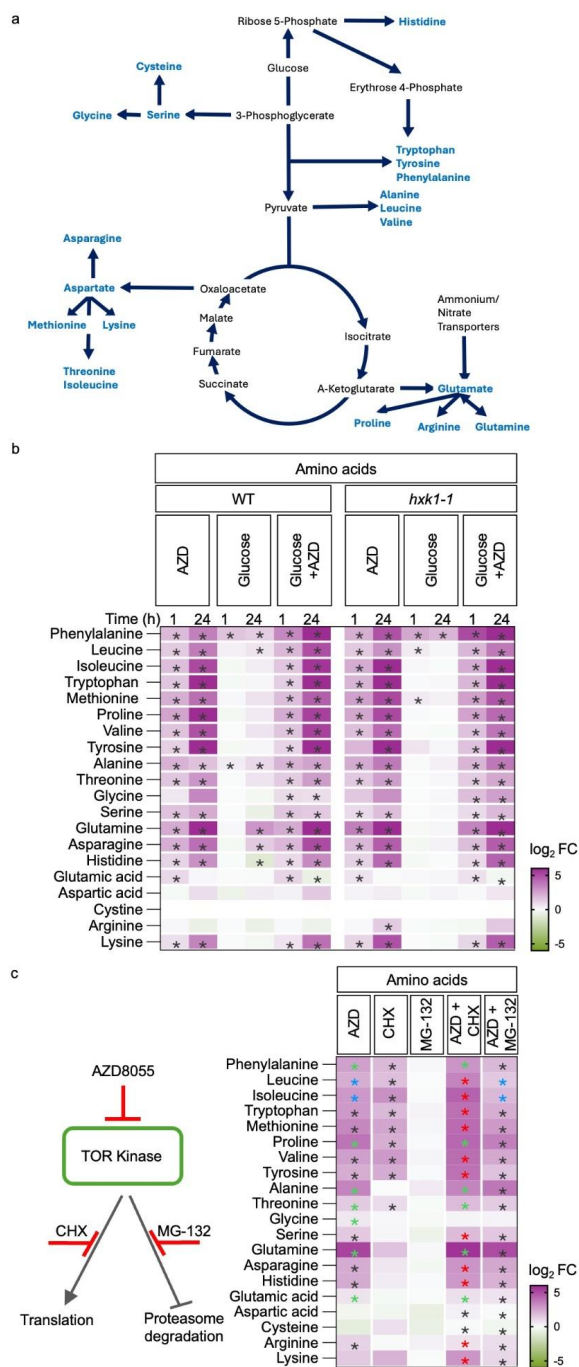


Fig. 4. TOR inhibition increases amino acids independent of hexokinase1 (HXK1). (a) Representation of amino acid biosynthesis precursors and pathways. Light blue represents amino acids and black represents the carbon skeletons used for biosynthesis. (b) Heatmap of $\log_2$ fold change (FC) of amino acid intensities from *C. zofingiensis* WT and *hxx1-1* mutant during TOR inhibition by 1 μ M AZD treatment, 15 mM glucose addition, and combined 15 mM glucose + 1 μ M AZD treatment. Data represent means; $n = 3-4$ biological replicates (Supplementary Data 13); asterisk represents $P \leq 0.01$ (Student's *t*-test). (c) Heatmap of $\log_2$ FC of amino acid intensities under conditions of TOR inhibition by 1 μ M AZD, 35 μ M cycloheximide (CHX), 10 μ M MG-132, 1 μ M AZD + 35 μ M CHX, and 1 μ M AZD + 10 μ M MG-132 after 1 h

of treatment. Data represent means; $n = 3$ biological replicates (Supplementary Data 14); asterisk represents significant differences between all treatments compared to control ($P \leq 0.01$, one-way ANOVA, Dunnett's test); red asterisk represents significant differences between AZD and AZD+CHX ($P \leq 0.01$, ANOVA, Tukey test); blue asterisk represents significant differences between AZD and AZD+MG-132 ($P \leq 0.01$, ANOVA, Tukey test); green asterisk represents no significant differences between AZD and AZD+CHX ($P \geq 0.05$, ANOVA, Tukey test), despite significance as compared to controls ($P \leq 0.01$, one-way ANOVA, Dunnett's test).

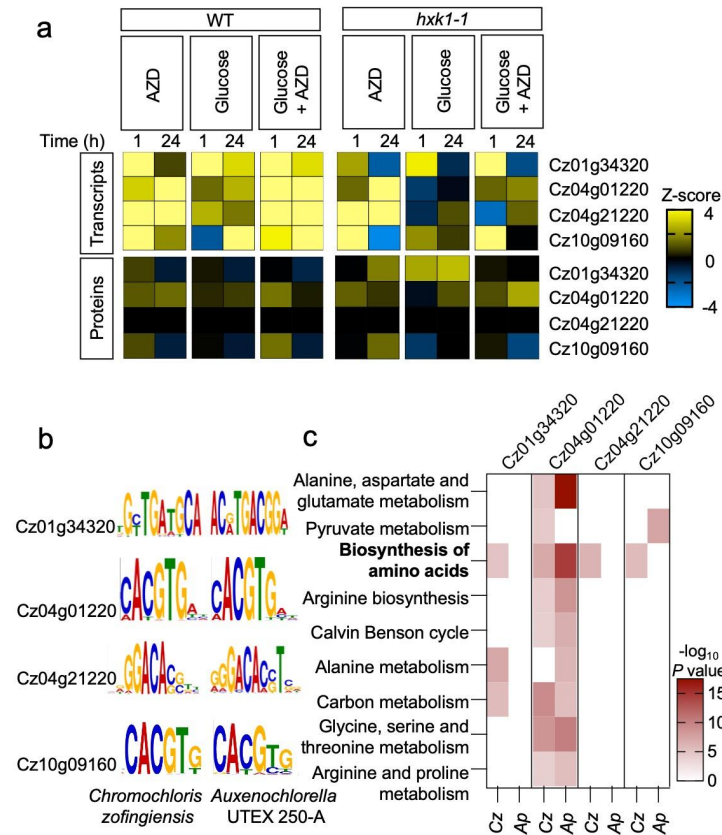


Fig. 5. The bHLH^{ACT+} family transcription factor (Cz04g01220) associates with amino acid biosynthesis pathways in two distantly related algae. (a) Heatmap of Z-scores of transcript abundances and protein abundances of transcription factors (TFs), Cz01g34320 (AP2 family), Cz04g01220 (bHLH^{ACT+} family), Cz04g21220 (bZIP family), and Cz10g09160 (bHLH^{ACT+} family) during TOR inhibition by 1 μ M AZD treatment, 15 mM glucose addition, and combined 15 mM glucose + 1 μ M AZD treatment in *C. zofingiensis* WT and *hxx1-1*. Data represent means; $n = 2-4$ biological replicates. (b) DAP-seq based motif identification for the TFs in (a) to the genomes of two algal species: *C. zofingiensis* and *Auxenochlorella* UTEX 250-A. (c) Heatmap displaying the $-\log_{10} P$ value for enriched pathways of the target genes associated with the TFs in the two genomes. Cz, *C. zofingiensis*; Ap, *Auxenochlorella* UTEX 250-A.

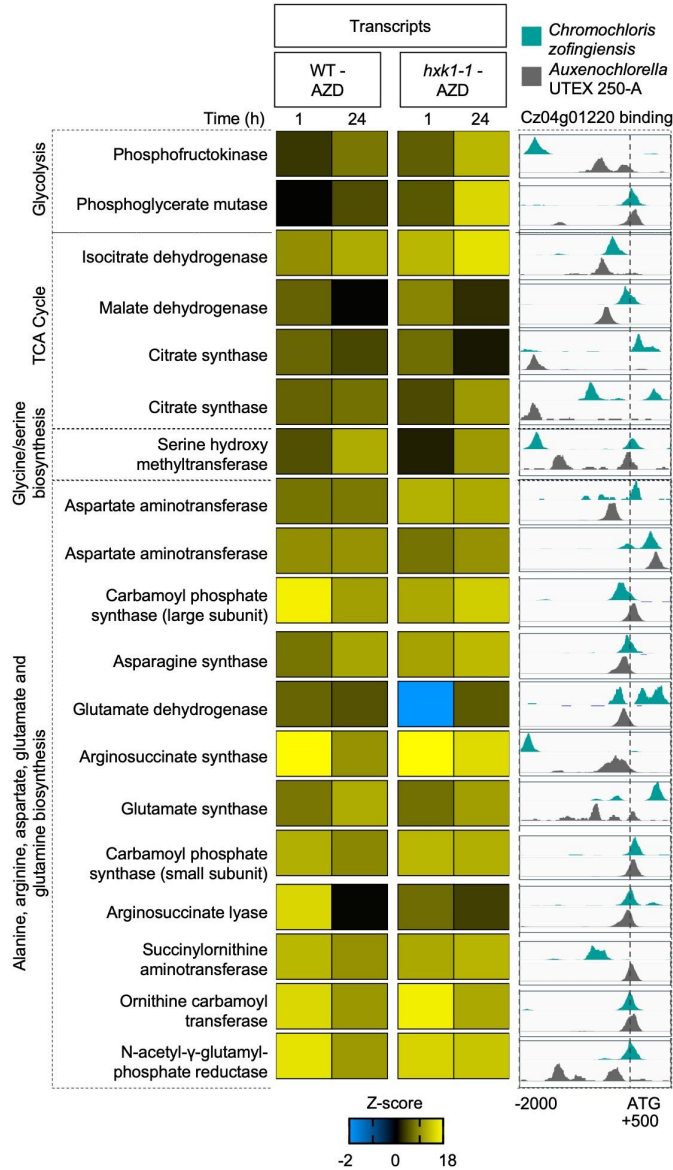


Fig. 6. Binding of the bHLH^{ACT+} transcription factor (Cz04g01220) to amino acid biosynthesis genes potentially regulates transcription. Heatmap of Z-scores of transcript abundances of amino acid biosynthesis genes during TOR inhibition by 1 μ M AZD treatment, 15 mM glucose addition, and combined 15 mM glucose + 1 μ M AZD treatment in *C. zofingiensis* WT and *hxx1-1*. Data represent means; $n = 2-4$ biological replicates (Supplementary Data 20). DAP-seq peaks of *C. zofingiensis* (cyan) and *Auxenochlorella* UTEX 250-A (gray) beside the corresponding gene. Genome region between -2000 bp upstream and +500 bp of the protein start site (ATG) used for representation. Dotted line represents the protein start site. Gene IDs in Supplementary Data 20.

	<i>C. zofingiensis</i>	<i>C. reinhardtii</i>	<i>V. carteri</i>	<i>Auxenochlorella</i> UTEX 250-A	<i>M. neglectum</i>	<i>A. thaliana</i>	<i>O. sativa</i>
Genome wide promoters with G-box (%)	43.7	57.1	42.3	38.6	46.6	29.2	31.9
Selected a.a. biosynthesis genes bound by bHLH	18	nd	nd	18	nd	nd	nd
Selected biosynthesis genes with G-box	16/19	13/18	13/19	16/18	15/18	7/27	11/25
Biosynthesis gene promoters with G-box (%)	84.2	72.2	68.4	88.8	83.3	25.9	44
Odds ratio (target/genome)	10.1	2.0	3.0	12.7	5.7	0.8	1.7
Fisher's exact test P value	***	ns	*	****	**	ns	ns
Number of bHLH genes without ACT domain	3	2	2	2	2	90	175
Number of bHLH with ACT domain	2	7	1	1	1	61	14
Total bHLH genes	5	9	3	3	3	151	189

Table 1. Comparative analysis of G-box enrichment in promoters of amino acid (a.a.) biosynthesis genes for selected species of Viridiplantae. nd, not determined; ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

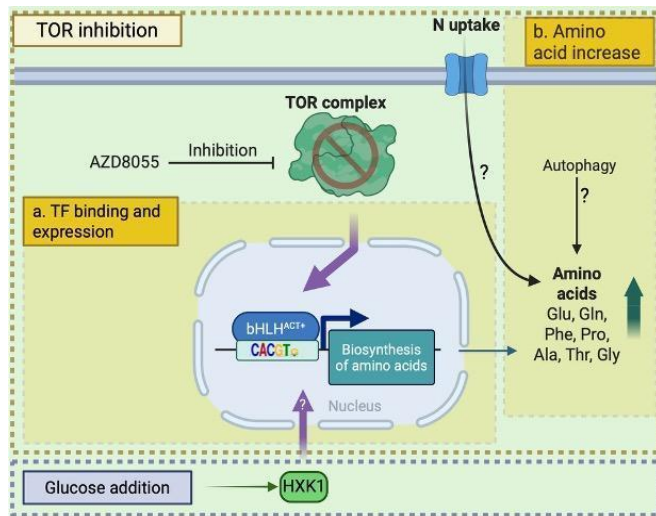


Fig. 7. Summary of the mechanism of TOR inhibition-dependent accumulation of amino acids in green alga *C. zofingiensis*. TOR inhibition by AZD induces the expression of a transcription factor bHLH^{ACT+}, which binds to amino acid biosynthesis and nitrogen metabolism genes, to potentially regulate their transcription. This process, which is independent of glucose-TOR signaling, leads to an accumulation of amino acids during TOR inhibition. Glucose addition via hexokinase1 (HXK1) also upregulates amino acid biosynthesis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [DatasetS10.xlsx](#)
- [DatasetS14.xlsx](#)
- [DatasetS4.xlsx](#)
- [DatasetS18.xlsx](#)
- [DatasetS20.xlsx](#)
- [DatasetS11.xlsx](#)
- [DatasetS9.xlsx](#)
- [DatasetS21.xlsx](#)
- [DatasetS6.xlsx](#)
- [SINatComms.docx](#)
- [DatasetS16.xlsx](#)
- [DatasetS7.xlsx](#)
- [DatasetS15.xlsx](#)
- [DatasetS2.xlsx](#)
- [DatasetS1.xlsx](#)
- [DatasetS3.xlsx](#)
- [DatasetS17.xlsx](#)
- [DatasetS12.xlsx](#)
- [DatasetS19.xlsx](#)
- [DatasetS8.xlsx](#)
- [DatasetS5.xlsx](#)
- [DatasetS13.xlsx](#)
- [DatasetS22.xlsx](#)