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Patterns of Evolutionary Conservation and Divergence in the Short-Term Hyposalinity Stress Response of a Euryhaline Diatom, Skeletonema marinoi

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Patterns of Evolutionary Conservation and Divergence in the Short-Term Hyposalinity Stress Response of a Euryhaline Diatom, *Skeletonema marinoi*

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Abstract

Survival under fluctuating environmental conditions, such as those increasing in frequency and magnitude under environmental change, requires a successful response to stress. Interspecific differences in stress responses may result in differential survival of species, even within a lineage. Diatoms may constitute one such lineage, as salinity tolerance among extant species is diverse, and the observation of frequent historic habitat transitions between marine and freshwater environments indicates that diatoms successfully mitigated (low) salinity stress in the past, followed by adaptation and diversification over evolutionary time scales. To understand to what extent the diatom hypoosmotic stress response consists of conserved and variable elements, we used RNA sequencing during an 8 hour time series to characterize the short-term stress response to hyposalinity of the ancestrally marine, euryhaline, diatom *Skeletonema marinoi*, and compared it to its distant relative *C. cryptica* which shows a broader salinity tolerance. Our data show that upon exposure to low salinity, *S. marinoi* mounts a rapid response to manage osmotic and oxidative stress (15–30 min), after which the diatom transitions into an energy-intensive recovery phase (2–4 h). By 8 hours, *S. marinoi* approaches acclimation to low salinity. Comparison with *C. cryptica* showed that chloroplastic K+ efflux and a broad response to oxidative stress constitute conserved mechanisms related to an euryhaline lifestyle across the diatom lineage. However, our data also suggested that *S. marinoi* is less adept in mitigating low salinity stress than *C. cryptica*, which unlike *S. marinoi* tolerates freshwaters and shows distinct mechanisms for initial survival and long-term growth in low salinity. Altogether, our study highlights that the cellular mechanisms behind low salinity tolerance in diatoms include elements that are both conserved and variable across the diatom lineage. Given the crucial ecological roles of diatoms in aquatic food webs, understanding the

various strategies of marine diatoms to overcome freshening of their environments is central to predicting the impacts of climate change on coastal primary production.

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Introduction

To survive in a fluctuating environment, organisms must successfully respond to abrupt shifts in environmental conditions. Such shifts can be regular, such as tidal patterns and seasonal temperature or precipitation changes, but can also occur irregularly, including droughts, flooding, and fires. In either case, the capability of a species to respond to a rapid environmental shift is vital for the survival of that species in an environment where such shifts occur. Due to anthropogenic climate change, environmental shifts, such as those caused by severe weather events, are becoming increasingly frequent and more severe in magnitude (IPCC, 2012). In addition, ecosystems are affected by permanent changes, such as the dilution of oceanic topwaters (IPCC, 2014) and increased ocean temperatures (Bates et al., 2008). Consequently, many species are facing novel challenges imposed by environmental shifts of unprecedented frequency, magnitude, and duration (Basto et al., 2018; Duke et al., 2017; Heron et al., 2016; Smale & Wernberg, 2013). Accordingly, recent research has sought to understand whether species will adapt to the increased frequency, severity, or permanence of environmental shifts, such as increased temperatures (Anderson & Song, 2020; Hoffmann & Sgró, 2011; Pistevos et al., 2011).

The ability of species to adapt to abrupt changes in their environment rests ultimately on their ability to initially survive the environmental shift via a stress response. When an organism experiences an environmental perturbation, a stress response attempts to restore homeostasis (Borowitzka, 2018). Certain aspects of stress responses are widely conserved across species. For example, heat shock proteins are involved in the stress responses across eukaryotes and prokaryotes (Lindquist & Craig, 1988). In addition, both polyamines and antioxidants are commonly involved in the responses to oxidative stress caused by reactive oxygen species (ROS) in aerobic species across the tree of life (Rhee et al., 2007; Scandalios, 2002). However, lineage-specific stress responses have also been identified, including in yeasts (Brion et al., 2016) and red algae (Khoa et al., 2021). The implications of such lineage-specific responses are

especially important, as these differences may enable differential success of species under changing conditions, result in differing adaptability of such species to climate change, and contribute to the ability of a species to colonize a new environment.

Diatoms serve as the base of many aquatic food webs and are involved in the global cycling of nutrients including nitrogen, silica, carbon, and oxygen (Armbrust, 2009; Sarthou et al., 2005). These photosynthetic microeukaryotes are distributed across the globe in marine, freshwater and soil environments. This includes areas that experience large fluctuations in salinity, such as coastal and estuarine regions which are generally inhabited by diatoms with broad salinity tolerances (euryhaline taxa) (Round et al., 1990; Thomson & Manoylov, 2017). Indeed, for the diatom lineage, salinity has been a particularly important source of species diversification, as evidenced by repeated transitions between marine and freshwater environments, followed by bursts of diversification in freshwaters, across the diatom clade (Alverson et al., 2007; Nakov et al., 2019). Such habitat transitions initially require colonists to successfully mitigate osmotic stress during the first minutes to hours of exposure. Following initial survival of the stressor, an organism may return to a new homeostasis (acclimation). Finally, when an organism persists in the new environment over evolutionary time scales, it can lead to adaptation (Borowitzka, 2018).

Methods

2.1 Diatom culture conditions. We maintained cells of *Skeletonema marinoi* strain AJA304-19 isolated from the North Sea near Gothenburg, Sweden for 3 years in artificial seawater (ASW) at its native salinity of 24 grams salt per liter water (i.e., ASW 24) (see Pinseel et al., 2022 for details on the medium). Cells were grown in a Percival incubator at 15℃ and 21.5 µmol photons/m^2/s irradiance under a 16:8 light:dark cycle.

2.2 Experimental design and preparation. The goal of the study was to understand the short-term response to acute hypoosmotic stress in a predominantly marine diatom that also occurs throughout a range, salinity 5 - 24, in the Baltic Sea. Given that the native salinity of the strain used is salinity 24, ASW 8 represents a survivable hypoosmotic condition that the strain could be exposed to in the Baltic, unlike ASW 0, which killed treated cells within 24 hours. Thus, we transferred cells from ASW 24 to ASW 8 to mimic an abrupt hypoosmotic shift, collecting cells at one negative control and seven time series collections spanning 0 minutes to 8 hours to capture the short-term stress response. This was done in triplicate, resulting in 24 samples for RNA-seq analysis. Prior to the experiment, cells were inoculated in triplicate into 1 L flasks under the maintenance conditions described above, and monitored until exponential growth was reached. Cell density of each culture was estimated with a Fluid Imaging Technologies Benchtop B3 Series FlowCAM® cytometer and used to calculate the volume needed from each replicate to inoculate each experimental vessel with $3x10^6$ cells. The required volume of cells from each flask was transferred into sterile 50 mL Falcon tubes. All tubes were centrifuged at 800 rcf for 3 min at 4 ℃ to concentrate the cells. We transferred concentrated cell pellets to 50 mL Falcon tubes containing ASW 24 control media or low-salinity ASW 8 stress media, resulting in a final volume of 40 mL per tube. This ensured all treatments received the same handling.

2.3 Time series collections. Each of the 7 treatment tubes for each replicate received the same experimental treatment but was collected at a distinct post-inoculation time point: 0 min, 15 min, 30 min, 1 h, 2 h, 4 h, and 8 h. The control treatment was collected alongside the 0 min

experimental treatment. At each time point, we collected the cells by gentle centrifugation for 3 min at 400 rcf and 4 ℃, removal of the supernatant media by pipetting, and flash-freezing of the cell pellets in liquid nitrogen before storage at –80 ℃. Following inoculation, cells from the control and t=0 min were collected instantly. The remaining tubes were incubated at 15 ℃ and 20 µmol photons/m^2/s irradiance under gentle agitation with a Boekel Scientific adjustable speed wave rocker until harvesting at the predefined time-points. Cells were kept under constant illumination for the duration of the experiment.

2.4 RNA extraction and library building. We extracted RNA and prepared Illumina libraries in four batches. Samples were randomized among extraction and library prep batches to avoid batch effects (Supp Table #). RNA was extracted with an RNeasy Plant Mini Kit (QIAGEN, The Netherlands) and quantified with a Qubit 2.0 Fluorometer (Invitrogen, USA). We determined RNA quality using an Agilent Technologies 2200 TapeStation (Agilent Technologies, USA) and made RNA libraries with the KAPA mRNA HyperPrep kit (KAPA Biosystems, USA). Indexed libraries were sent to the Biological Sciences Core Facility at the University of Chicago for quantification, pooling, and sequencing with an Illumina HiSeq 4000 instrument. Indexed libraries were multiplexed and run together on a single lane.

2.5 Data preprocessing, transcriptome assembly, and gene annotation. A total of 696,475,182 100 bp paired-end reads were recovered and run through the FastQC software program (v0.11.5) for quality assessment (Andrews, 2010). Index sequences and low-quality reads were trimmed using kTrim (v1.1.0, specified parameters: -t 15 -m 0.5) and reanalyzed with FastQC for further quality assessment (Sun, 2020). We mapped trimmed reads to the *S. marinoi* genome (v1.1) using STAR (v2.7.3a, default settings accounting for intron size, --alignIntronMin 4 --alignIntronMax 17105) (Dobin et al., 2013). Read counts for all genes and exons were determined using HTSeq (v0.11.3) in *union* mode (Anders et al., 2015). In some cases, we augmented gene annotations with local searches to the SwissProt database (last updated Aug 2019) using DIAMOND BLASTP (--sensitive, e-value ≤ 1e-6, return max. 1 target

per query) and the Uniprot database (last updated June 2019) using NCBI BLAST+ (v2.6.0+, e-value ≤ 1e-6, return max. 1 target per query) (Buchfink et al., 2015; Camacho et al., 2009). KEGG pathways were annotated using the KofamKOALA web server on 4 Aug 2020 (Aramaki et al., 2020). We determined protein localization to the cytoplasm, chloroplast, or mitochondria using SignalP-3.0, ASAFind, HECTAR (v1.3), TargetP-2.0, and MitoProt (Almagro Armenteros et al., 2019; Bendtsen et al., 2004; Claros, 1995; Gruber et al., 2015; Gschloessl et al., 2008).

2.6 RNA-seq analysis. Transcript counts were imported into R (v4.0.2; R Core Team, 2020) alongside the annotated genome and annotation supplements. Only genes with ith at least 1 count per million (CPM) in 3+ samples were retained. We used edgeR (v3.30.3) to adjust for variation in library size and composition using the trimmed mean of M-values (TMM) method and then to fit a quasi-negative binomial general linear model (GLM) for each gene (Lund et al., 2012; Robinson et al., 2010). stageR (v1.10.0) was used to identify differentially expressed genes in each contrast between an experimental timepoint and time 0 (t=0 min) at ASW 8 with a false discovery rate (FDR) of 0.01 (Van den Berge et al., 2017). Genes were considered significantly differentially expressed if p-value ≤ 0.01in 2+ consecutive timepoints. We visualized genome-wide differences in gene expression among time points through ordination of samples with multidimensional scaling (MDS) as implemented in limma (v3.44.3) through the plotMDS function (Ritchie et al., 2015) based on the top 500 log2-fold-changes between each pair of samples. Cluster (v3.0, using hierarchical clustering of centroids, Pearson's correlation for similarity matrix) and Java Treeview were used to sort genes with similar expression patterns into 7 manually determined clusters (de Hoon et al., 2004; Saldanha, 2004). We performed Gene Ontology (GO) term enrichment using topGO (v2.40.0) and summary on the significant results (p-value = 0.05) using REVIGO (accessed 22 Nov 2021, similarity cutoff = 0.5, other settings default) to identify functional similarities within clusters and time points (Alexa & Rahnenfuhrer, 2019; Supek et al., 2011).

Results

3.1 Growth response of *S. marinoi* to hyposalinity stress

Skeletonema marinoi does not show measurable growth within 2 h of hyposalinity stress (Fig 1). Stressed cells at 2 h exhibited decreased fluorescence relative to freshly inoculated stressed cells (0 min). From 2 to 4 h, the growth rates of the stressed cultures started to recover, but remained lower than those of unstressed cells. Beyond 4 h, growth rates were similar in stressed and unstressed cells. Both stressed and unstressed cells resumed exponential growth by 12 h. After 7 d, fluorescence values of the cultures in low and high salinity were no longer significantly different.

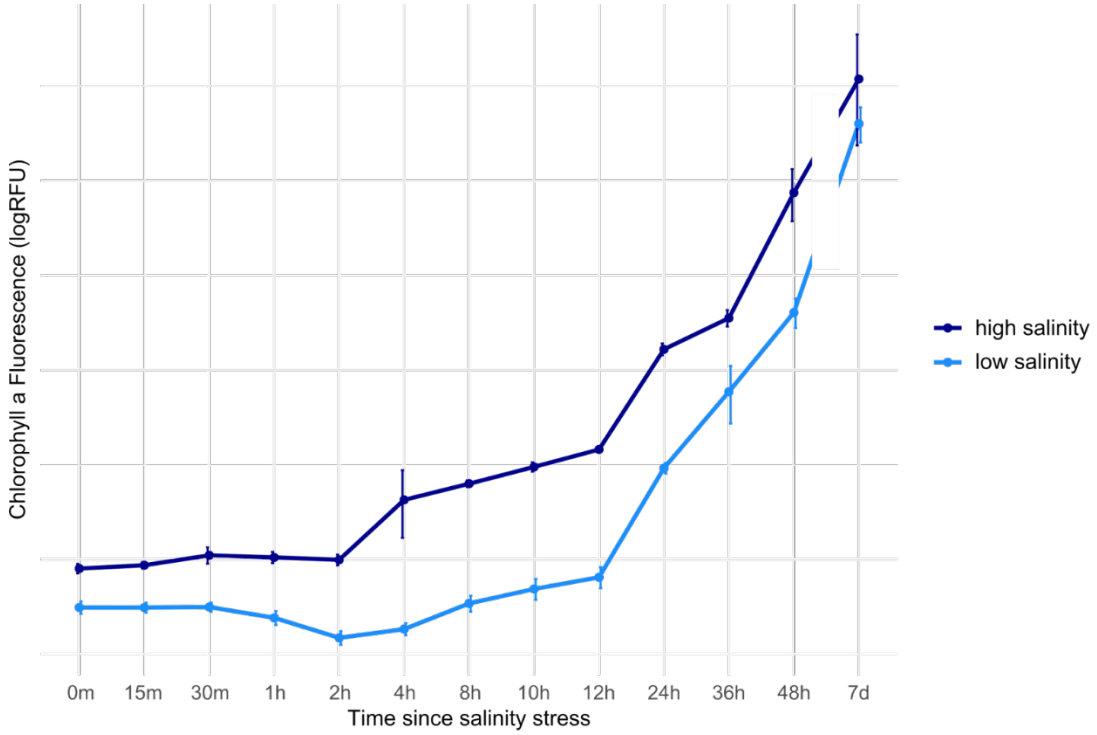


Fig 1. Chlorophyll *a* fluorescence (log2 RFU) as a proxy for growth in *S. marinoi* over 7 days. Growth is shown for both high (24) and low (8) salinity. Error bars indicate the standard deviation of biological triplicates.

3.2 Gene expression response of *S. marinoi* to hyposalinity stress

Within the seven sampled time points between 0 min and 8 h, a total of 17 989 genes were expressed across stressed and unstressed cells, constituting 80% of the 22 440 annotated

genes in the *S. marinoi* genome (v1.1). Among the expressed genes, 14 860 were differentially expressed in at least one time point, and 10 050 of these were differentially expressed in two or more consecutive time points. The peak transcriptomic response, as measured by the total number of differentially expressed genes at each time point, occurred at 2 h following stress exposure (8 086 genes) (Fig. 2A). By comparison, 5 590 genes were differentially expressed 15 min post-exposure, and at 8 h 4 437 genes were differentially expressed.

We used multidimensional scaling (MDS) to visualize differences among sampled time points based on the top 500 differentially expressed genes. The MDS showed clear separation of time points and tight clustering of replicates at each time point, which confirmed that our experiment captured differences in transcriptomic change across the time series and that gene expression was highly similar among technical replicates. Furthermore, the relative proximity of the 0 min and 8 h time points in ordination space indicate that the gene expression profiles at the extreme time points were more similar to one another than to the intermediate time points (Fig 2B).

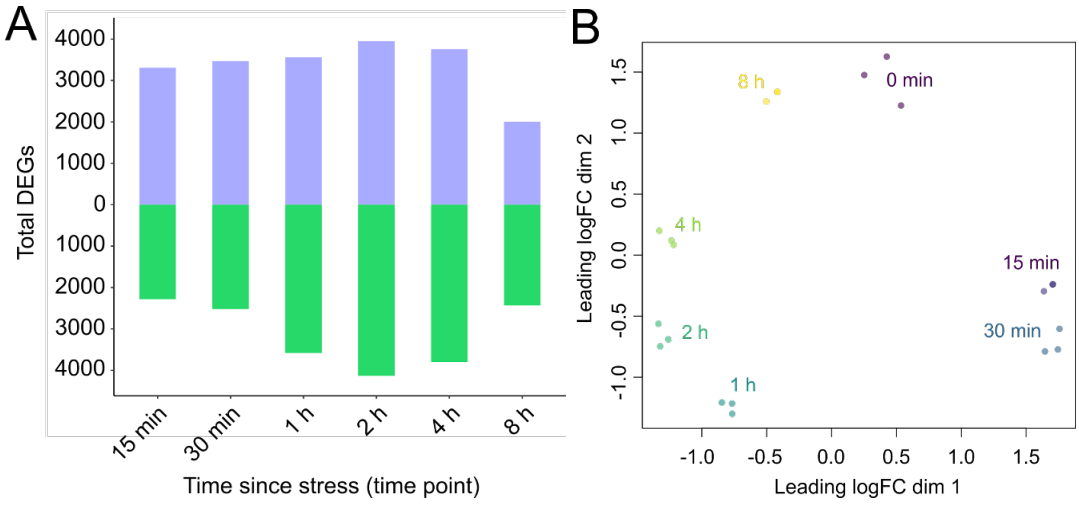
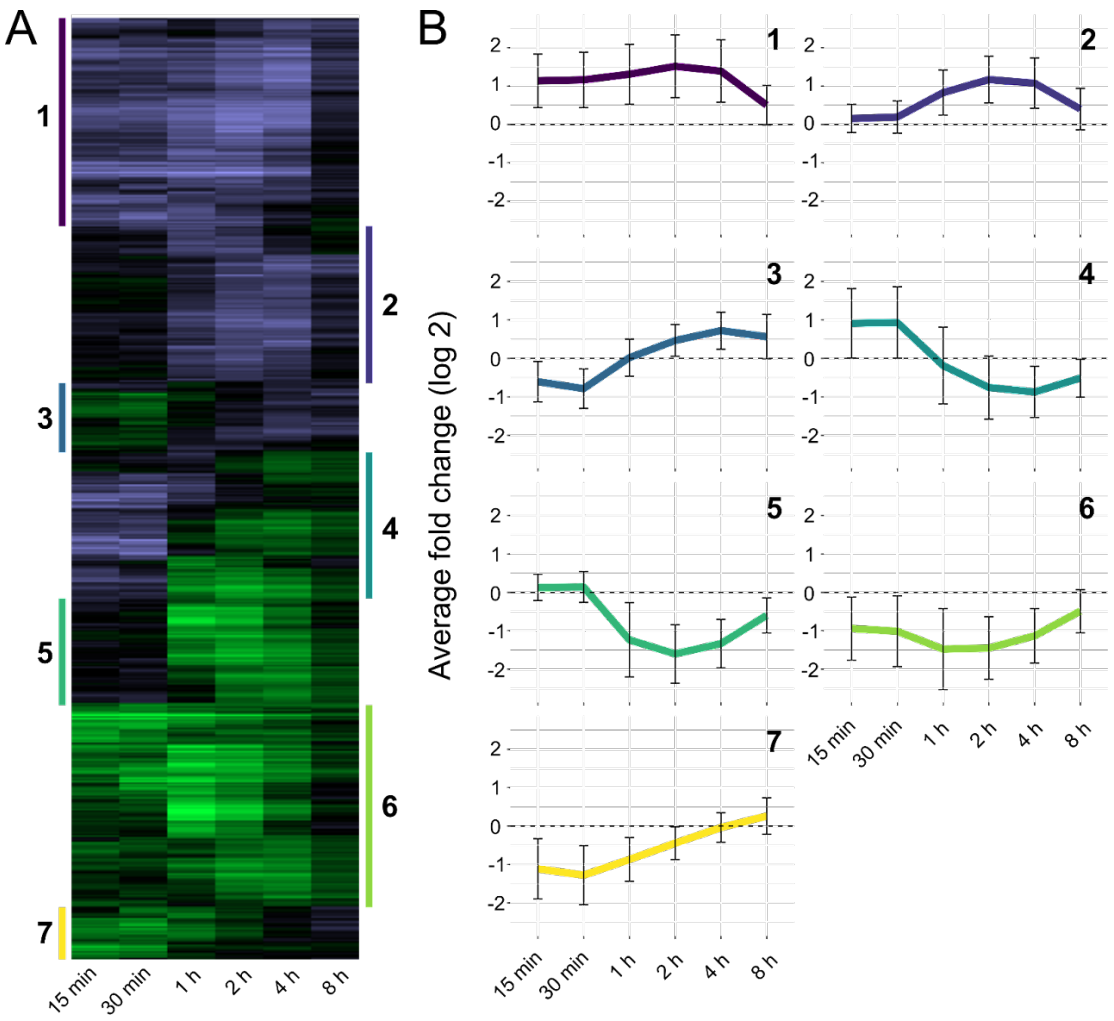


Fig 2. **A.** Number of genes that were differentially expressed at each time point. Differentially expressed genes with LFC > 0 at a given time point are upregulated (purple). Differentially expressed genes with LFC < 0 at a time point are downregulated (green). **B.** Multidimensional scaling (MDS) plot, showing that gene expression at each time point is distinct from all other time points. Distances between the samples represent logFC changes in the top 500 genes for each pairwise comparison.

3.3 Expression and enrichment of major metabolic processes

We grouped differentially expressed genes with similar expression patterns into seven clusters and performed GO enrichment on each cluster to reveal coordinated differential expression of genes underlying important cellular processes (Fig 3). GO enrichment of clusters 1 and 2, which mostly contained genes with expression ranging from unchanged to upregulated over the time series, indicated that predominantly upregulated processes included the tricarboxylic acid (TCA) cycle, translation, and redox homeostasis. Cluster 3, which included genes that were downregulated for the first 1–2 h and upregulated thereafter, was enriched for genes involved in light-harvesting photosynthesis, ubiquitin-dependent proteolysis, and DNA replication. In contrast, genes in cluster 4 showed roughly the opposite pattern of cluster 3, with upregulation for the first 30 min to 2 h followed by downregulation at later time points. Cluster 4 was enriched for genes involved in transcription factor activity and serine-type endopeptidase activity. Clusters 5 and 6, with gene expression ranging from unaltered to downregulated across the time series, were consistent with an overall downregulation of redox processes (cluster 6), serine-type endopeptidases (cluster 5), and endopeptidase inhibition (cluster 6). At last, cluster 7 consisted of genes that were initially downregulated before reaching baseline expression or upregulation two or more hours after stress exposure. This cluster was enriched for processes involved in proteasome function, light-harvesting photosynthesis, recombination, and storage molecule biosynthesis.



C

cluster	# genes	enriched process	associated GO terms
1	2224	translation, TCA cycle	**GO:0006364**, **GO:0006099**, **GO:0006414**
2	1778	redox homeostasis, protein folding	**GO:0045454**, *GO:0006457*
3	614	photosynthesis, DNA replication, protein catabolism	**GO:0009765**, *GO:0031297*, *GO:0006275*, **GO:0006511**
4	1643	transcription factor activity, protein catabolism	**GO:0003700**, **GO:0004252**
5	1057	protein catabolism	**GO:0016567**, **GO:0004252**
6	2174	oxidoreductase activity, proteolysis inhibition	**GO:0016491**, **GO:0004867**
7	560	photosynthesis, recombination, protein catabolism, storage molecule biosynthesis	**GO:0009765**, **GO:0006310**, **GO:0036402**, **GO:0003843**, **GO:0003989**

Fig 3. **A.** Heatmap showing all 10 050 differentially expressed genes, sorted by similar gene expression. Genes were further classified into seven clusters of alike gene expression patterns across the time series. Cluster numbers are indicated at the sides of the heatmap. **B.** Average expression (as logFC) of genes assigned to each cluster. Error bars indicate standard deviation. **C.** Table showing cluster-specific information, including number of genes in each cluster, enriched cellular processes, and specific GO terms related to the aforementioned processes. GO terms in bold and italic indicate enrichment at p-value < 0.001 and< 0.01, respectively.

3.4 Rapid mitigation of hypoosmotic and oxidative stresses

Marine diatoms exposed to low salinity experience osmotic pressure due to the decreased osmolality of their environment, which can cause cell volume shifts and cell damage (Kirst, 1990), and mitigate this through strategies to restore osmotic balance. These can include ion import/export (Helliwell et al., 2021) and removal of osmolytes (Schobert, 1980). The latter are compatible solutes that are accumulated within cells to maintain isotonicity with the environment (Burg & Ferraris, 2008). Osmotic stress may also increase levels of oxidative stress-causing reactive oxygen species (ROS) (Dring, 2005; Kumar et al., 2010), managed by molecules including polyamines and antioxidant proteins (Das & Roychoudhury, 2014; Liu et al., 2015). In our experiment, slightly less than half (11/26) of the identified transporters of Na+ and/or K+ were upregulated from 15–30 min (Fig S1). Transporters with the strongest upregulation during the first 30 min included two chloroplastic K+ efflux antiporters. Although not involved in ion transport, five putative ABC superfamily amino acid transporters showed strong coregulation under osmotic stress, with all five upregulated from 15–30 min and then downregulated at 1 h and beyond (Fig S2).

Given their roles in maintaining osmotic balance under hypersaline conditions, we investigated the regulation of biosynthesis and degradation pathways for a variety of diatom osmolytes, including dimethylsulfoniopropionate (DMSP) (Lyon et al., 2016), betaine (Kageyama et al., 2018), taurine (Jackson et al., 1992), ectoine (Fenizia et al., 2020), and proline (Krell et al., 2007). Two methyltransferase genes involved in DMSP and/or glycine betaine biosynthesis were immediately downregulated at the start of our experiment (Fig 4). Similarly, two genes putatively involved in ectoine biosynthesis (Pinseel et al., 2022) were downregulated at most time points, with peak differential expression between 15 min and 1 h. Glutamate decarboxylase, involved in taurine biosynthesis, was also downregulated through 1 h while two taurine degradation genes were upregulated, on average, from 15–30 min. Regulation of proline biosynthesis varied by pathway, with mild upregulation of proline synthesis from

glutamate, opposite expression patterns by two homologs of ornithine cyclodeaminase involved in single step proline biosynthesis, and downregulation of proline synthesis from ornithine.

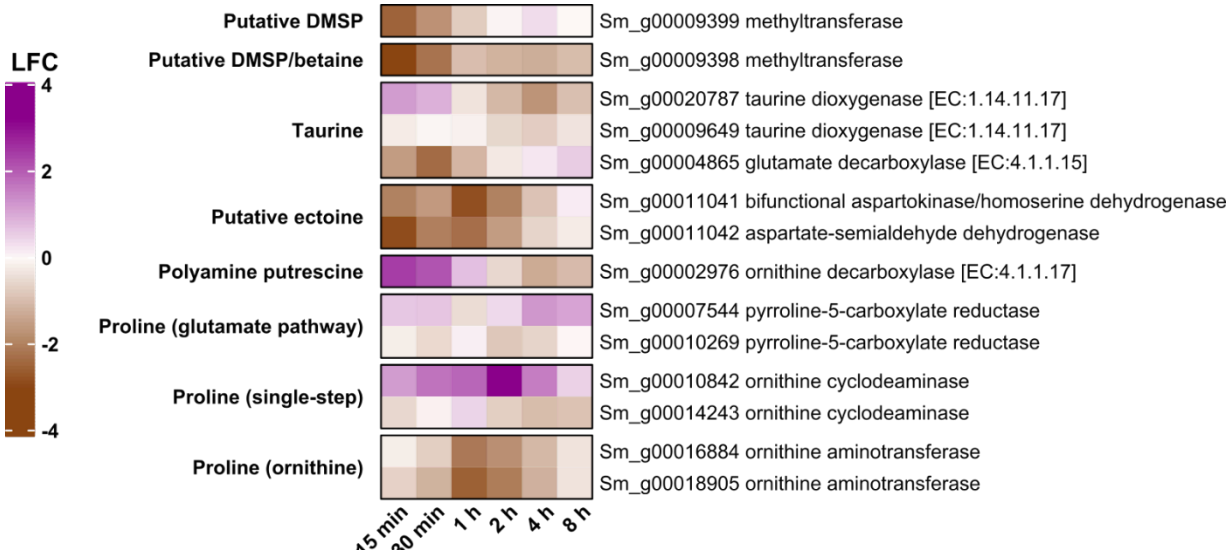


Fig 4. Heatmap showing the expression of differentially expressed genes involved in the biosynthesis and degradation of osmolytes over the 8 hour time series. All shown genes were significant in two or more consecutive time points.

Biosynthesis of the polyamine putrescine from L-ornithine was induced in the first three time points, after which it was downregulated from 2 h onwards (Fig 4). Superoxide dismutases (SOD), ROS-scavengers that convert superoxide anions to peroxide (Fukai & Ushio-Fukai, 2011), were, on average, upregulated at 15–30 min and 8 h (Fig S3). Expression of peroxiredoxins, which convert peroxide to water (Fukai & Ushi-Fukai, 2011), appeared tied to cellular compartment, with most upregulated peroxiredoxins localized to the chloroplast. Nineteen antioxidant thioredoxins were also identified among the significant differentially expressed genes in *S. marinoi*. Based on GO annotation, four out of five downregulated thioredoxin genes were cytosolic, whereas most of the upregulated thioredoxins were likely associated with organelles, including the chloroplasts, mitochondria, and endoplasmic reticulum. On average, thioredoxin reductases, required for replenishing thioredoxin activity (Arnér & Holmgren, 2000), were upregulated from 1–4 h. Genes involved in the xanthophyll cycle, a process involved in photoprotection by preventing ROS-producing overexcitation of pigments

(Goss & Jakob, 2010; Latowski et al., 2011), varied in expression. Specifically, one copy of violaxanthin de-epoxidase was upregulated in all time points except 8 h. Finally, genes annotated as heat shock factors and proteins, which are involved in many stress responses (Guo et al., 2016), were predominantly upregulated from 15–30 min and downregulated at 1 h and beyond (Fig S4).

3.5 Multipart regulation of photosynthesis

Under hyposalinity stress, *S. marinoi* regulated photosynthetic activity in two consecutive phases. This was indicated by the enrichment of light-harvesting photosynthetic processes among two groups of similarly-expressed genes (Fig 3), mainly consisting of binding proteins for light-harvesting pigments. The first group of these genes became upregulated by 2 h and the second group by 8 h (Fig S5). There was also a minority of genes associated with pigment binding that were upregulated at all times. Similarly, genes involved in chlorophyll biosynthesis tended either towards immediate, lasting upregulation, or upregulation at later time points (Fig S6). The first set of chlorophyll biosynthesis genes were largely upregulated at 15 min through 8 h and support the production of the pigment biliverdin. A majority of genes in the second set became upregulated by 4 h and are involved in the biosynthesis of chlorophyll.

Regulation of the Calvin cycle during hyposalinity stress occurred in three distinct phases. First, following weak differential expression of the Calvin-cycle genes in the first 15 min, *S. marinoi* repressed the dark reactions of photosynthesis from 30 min to 1 h. Second, as stress was mitigated at later time points, the Calvin cycle became most strongly upregulated at 4 h (Fig 5). Third, by 8 h almost all the differentially expressed Calvin-cycle genes trended back towards baseline expression, suggesting that *S. marinoi* began to approach a new equilibrium, a hallmark of acclimation (Borowitzka, 2018).

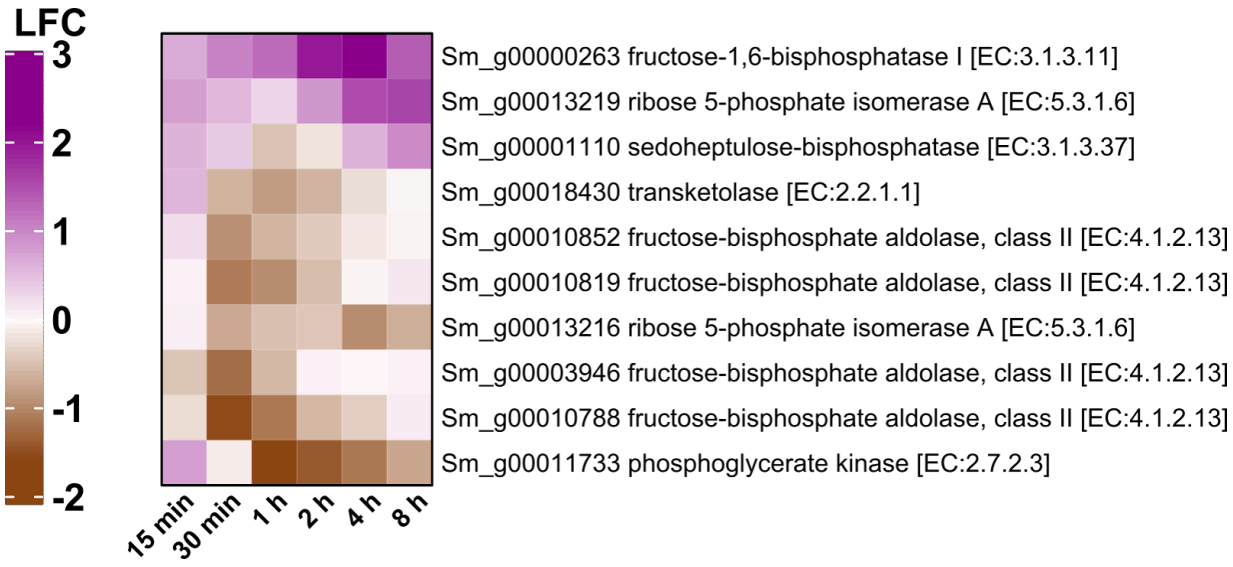


Fig 5. Heatmap showing the expression of differentially expressed genes involved in the Calvin cycle over the 8 hour time series. All shown genes were significant in two or more consecutive time points. In case a gene functions in both the cytosol and chloroplast, only homologs localized to the chloroplast were included.

3.6 Carbohydrate metabolism

Our data suggest that *S. marinoi* repressed the energy-consuming processes of gluconeogenesis and storage molecule biosynthesis during acute hyposalinity stress. This is evidenced by the expression patterns of key enzymes in these pathways (Fig 6A,B). Repression of the key irreversible step of gluconeogenesis indicated that gluconeogenesis was immediately downregulated under stress. This occurred alongside downregulation of the biosynthesis of fatty acids and the storage polysaccharide chrysolaminarin (beta-1,3-D-glucan). In contrast, two out of three irreversible steps of glycolysis displayed the opposite pattern (i.e., upregulation across the time series), whereas the third (glucokinase), which catalyzes the first step of glycolysis, only became upregulated after 1 h. Altogether, this indicates that glycolysis was generally upregulated throughout our time series, but the activity of the pathway was limited during the first hour of the stress response.

This delay in the upregulation of carbohydrate metabolism was also evident in the expression of the TCA cycle and storage molecule degradation genes. Despite downregulation

of the TCA-associated transcription factor *bZIP14* from 2–8 h, most TCA-cycle genes were upregulated across the time series, with peak expression between 2 h and 4 h (Fig 6C) (Matthijs et al., 2017). This corresponds to the upregulation of glucokinase in glycolysis. Similarly, two of three identified glucanases (chrysolaminarin degradation genes) were upregulated most strongly between 1 and 4 h. However, the third glucanase displayed the opposite pattern as it was downregulated at all time points with peak repression from 1 h to 4 h.

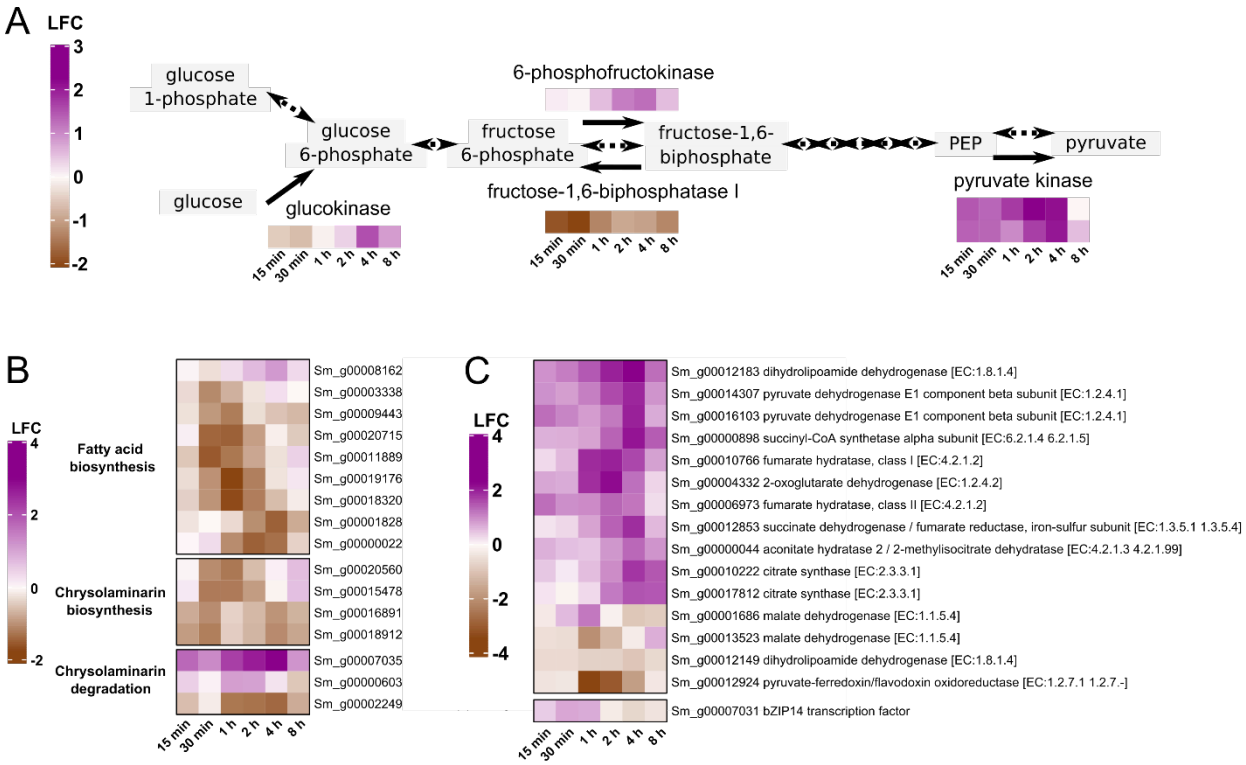


Fig 6. Expression of differentially expressed genes involved in carbohydrate metabolism and energy storage over the 8 hour time series. All shown genes were significant in two or more consecutive time points. **A.** Heatmaps of the irreversible steps of glycolysis/gluconeogenesis. Each row is a single gene at each time point. **B.** Heatmap showing expression of differentially expressed genes involved in biosynthesis and degradation of storage molecules. **C.** Heatmap showing expression of differentially expressed genes involved in the TCA cycle, including the *bZIP14* transcription factor which regulates the TCA cycle.

3.7 Protein synthesis and degradation

Following the transient induction of ABC transporters, polyamine biosynthesis, and heat shock factor/protein activity within 1 h, protein biosynthesis and carbohydrate metabolism increased in parallel, but many genes related to translation were also upregulated in early time

points. Most genes in the tRNA biosynthesis pathway were upregulated across the entire time series, with over half of the 31 differentially expressed tRNA synthetases experiencing peak upregulation between 2 h and 4 h (Fig S7). 30 of these genes also showed decreased upregulation, or even mild downregulation, at 8 h relative to the peak upregulation at earlier time points. Other genes associated with translation by GO annotation were also primarily upregulated, including 91 ribosomal proteins, 41 of which were highly upregulated (LFC > 1) at both 15 min and 4 h. Consistent upregulation of translation across the time series is also supported by the expression of amino acid transporters. Roughly three-quarters of identified non-ABC amino acid transporters were upregulated in most time points (Fig S2), presumably providing fodder for aminoacylation and thus translation.

Our data indicate that genes involved in nitrogen metabolism were more commonly upregulated than downregulated. Specifically, the highest LFC values were associated with upregulated genes (Fig S8). Pathways that contained mostly downregulated genes were largely characterized by a nitrogen flux away from amino acids or to forms not conducive for incorporation of nitrogen into amino acids. In contrast, predominantly upregulated pathways related primarily to the assimilation of nitrogen. Specifically, transport and assimilation of ammonium in the chloroplast and nitrate/nitrite was generally upregulated. However, at 8 h, genes involved in nitrate/nitrite transport and assimilation were not differentially expressed. Genes of the anabolic part of the ornithine-urea cycle, which incorporates nitrogen in the mitochondria and cytosol under nitrogen-replete conditions (Smith et al., 2019), were, on average, upregulated from 15 min to 1 h. Both single-step proline biosynthesis and synthesis of proline from glutamate displayed both up- and downregulation across homologs. However, in each case, the average gene expression was positive, suggesting both proline biosynthesis pathways were upregulated alongside other nitrogen assimilation pathways.

Finally, protein degradation activity was split into two general patterns of expression (Fig S9). Proteasome activity was enriched among genes that were downregulated until 2 or 8 h,

reflecting an initial repression of protein catabolism. In contrast, serine-type endopeptidase activity was enriched among genes that were not differentially expressed or upregulated in the first 30 min of stress exposure before becoming downregulated at 1 h. In addition, serine-type endopeptidase inhibition was downregulated across all time points.

Discussion

With our analysis of the *S. marinoi* transcriptome under short-term hyposalinity stress, we characterized the progression of the stress response and identified the cellular processes involved therein. However, a potential mismatch exists between the transcriptome, which we captured in our time series experiment, and the true response of the cell. Mapped transcripts may not represent complete genes, expressed gene transcripts may not be translated immediately or at all, and post-translational modifications may alter protein function. Each of these reflects a possible disconnect between the transcriptome and the true cellular response. We have highlighted this disparity where it is particularly relevant (e.g., contradictory responses), but otherwise we have assumed that differential expression of genes reflects the true regulation of cellular processes. Further studies on this response using proteomics or metabolomics are needed to fully understand the exact dynamics of the short-term response of *S. marinoi* to low salinity.

4.1 The response of *S. marinoi* to acute hyposalinity stress

The decreased growth experienced by cells transferred to hyposaline conditions, compared to cells kept in high salinity, suggests that the rapid shift from high (24) to low (8) salinity is stressful for *S. marinoi* (Fig 1). However, the detrimental effect of this stressor is largely managed within eight hours by a combination of the immediate regulation of processes to mitigate the experienced stress followed by the induction of processes to enable resumption of growth. Finally, by eight hours of stress exposure, gene expression in *S. marinoi* substantially decreased in magnitude, indicating the onset of a new steady state as the cells began to acclimate to hyposaline conditions (Fig 2). Accordingly, the short-term response of *S. marinoi* can be split into four temporal phases, summarized below (Fig. 7).

The first phase (‘stress mitigation’) occurred within 30 minutes of stress exposure and was characterized by regulation of specific processes to manage ion, osmolyte, and ROS balance. The second phase (‘transition’) encompassed only one time point (t = 1 h). It

represents a transitional zone between the first and the third phase, as the expression of various cellular processes at 1 h did not consistently match the expression of these processes during either phase. The third phase (‘recovery’) spanned two to four hours after the onset of hyposalinity stress, and included the largest number of differentially expressed genes, many of which were involved in energy production and protein synthesis. During this phase, many differentially expressed genes involved in the mitigation of oxidative and osmotic stress in the first phase experienced alterations in the direction or significance of expression. The fourth phase (‘pre-acclimation’) was identified at the ‘t = 8 h’ time point, where the magnitude of up- and downregulation of most genes and pathways was reduced. This suggests *S. marinoi* was beginning to approach acclimation. Regulation of differentially expressed processes within and across these four phases gives insight into the cellular mechanisms behind the euryhaline lifestyle of *S. marinoi*.

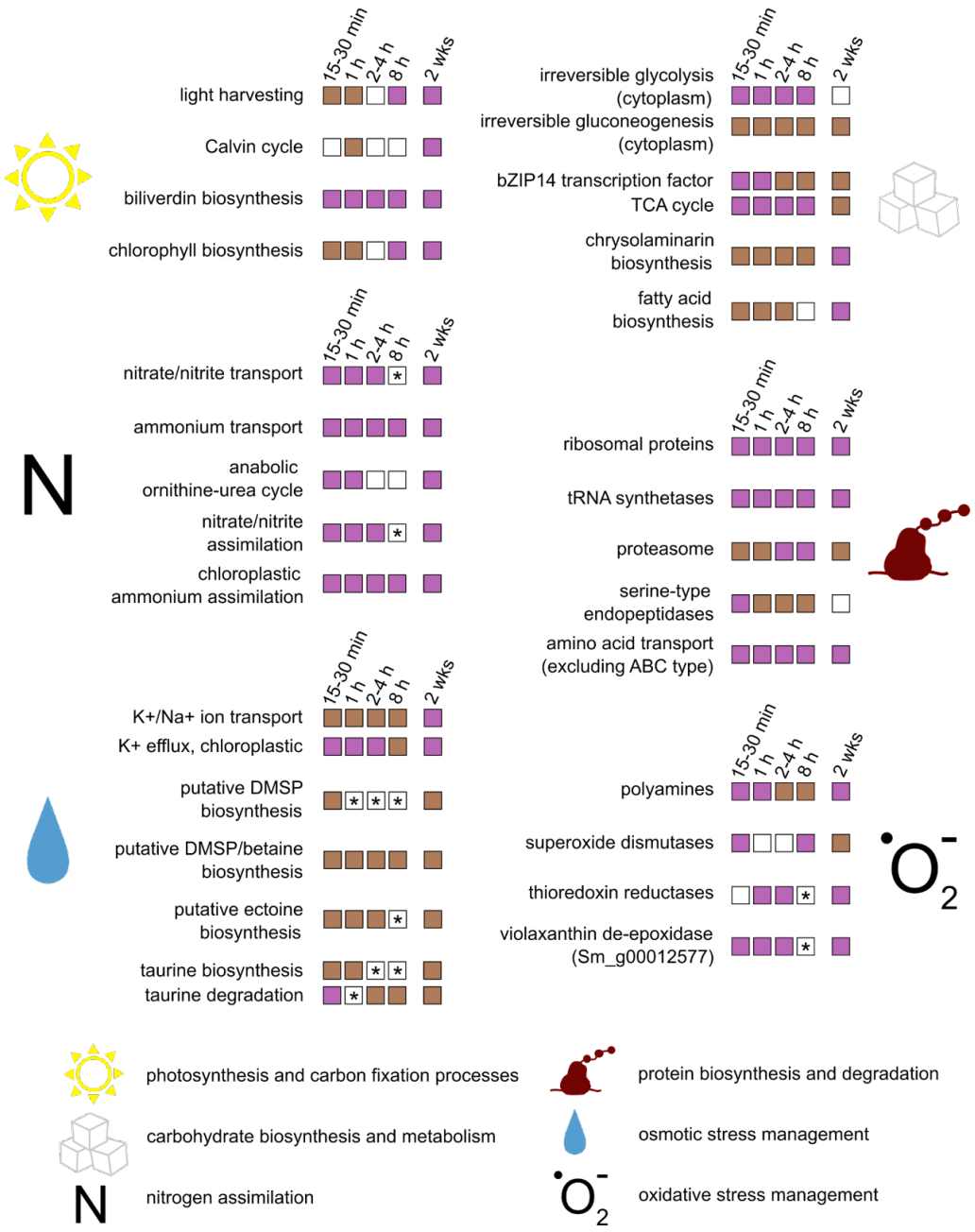


Fig 7. Overview of differentially expressed genes involved in major processes regulated under acute and long-term exposure to low salinity. The colored tiles represent the four phases of short-term expression: stress mitigation (15–30 min), transition (1 h), recovery (2–4 h), and pre-acclimation (8 h), as well as expression after acclimation at two weeks, obtained from Pinseel et al. (2022). Vertical spacing of tile rows indicates association between processes (e.g., the *bZIP14* transcription factor regulates the TCA cycle). Tile color was determined based on the proportion of genes that were significantly up- and downregulated at each time point(s) for the corresponding process. Specifically, brown tiles indicate > 60% downregulated genes, purple tiles indicate > 60% upregulated genes, and white tiles indicate >= 40% and <= 60% up- and downregulated genes. Tiles are highlighted with an asterisk for time points at which no genes were differentially expressed for a given process.

4.1.1 Stress mitigation phase, 15–30 min

Several major cellular pathways were induced or repressed within 15–30 min following stress exposure. First, our data indicate that immediately following exposure to low salinities, *S. marinoi* increases proteolysis of proteins which are possibly damaged by the stressor. This is evidenced by the upregulation of serine-type endopeptidases, which are thought to be involved in degradation of stress-damaged proteins, a response commonly seen in macrobiota under stress (Kidrič et al., 2014; Park & Kwak, 2020). In addition, heat shock proteins were also upregulated. These molecular chaperones help maintain protein homeostasis by directing damaged or misfolded proteins to proteases (Mogk et al., 2011). The upregulation of general protein synthesis and nitrogen assimilation processes also suggests (damaged) proteins are being replaced at an increased rate.

Within 30 min, *S. marinoi* adjusts the osmotic balance of the cytosol and chloroplasts for low salinity, by downregulation of osmolyte biosynthesis, and up- and downregulation of ion transporters. However, several differentially expressed genes involved herein remain differentially expressed beyond 30 min, which suggests some genes involved in osmotic stress mitigation are required for continued survival in low salinity. Three genes with average upregulation continuing beyond 30 min were chloroplastic K+ efflux antiporters, which prevent chloroplast swelling in plants (Kunz et al., 2014). Some genes for biosynthesis of the osmolytes DMSP/betaine, ectoine (possibly, see Pinseel et al., 2022), and taurine also remained downregulated beyond 30 min, as expected considering their primary role in hyperosmotic conditions. Genes that were differentially expressed exclusively during the mitigation phase included five amino acid ABC transporters. Although their role in the stress response is unknown, algal ABC transporters are involved in detoxification and are upregulated in response to heavy metal and allelopathic chemical stress (Gu et al., 2019; Zheng et al., 2021) or play a role in expelling organic anions and lipophilic organic molecules (Scherer et al., 2008). It is possible that the here differentially expressed ABC transporters also participate in substrate

efflux. Thus, their upregulation may reflect the transient removal of amino acids from the cytosol at the onset of stress, suggesting they served as osmolytes at higher salinities, as seen in other diatom species (Jackson et al., 1992; Scholz & Liebezeit, 2012).

Oxidative stress management at the onset of stress was apparent both in the downregulation of photosynthesis and the upregulation of polyamine production and antioxidants. The downregulation of photosynthesis is thought to reduce oxidative stress by decreasing light absorption and was evidenced by decreased expression of chlorophyll biosynthesis and light harvesting complex binding genes, responses that have also been observed in rice seedlings experiencing water stress (Dalal & Tripathy, 2018). Simultaneously, one copy of a violaxanthin de-epoxidase gene, involved in the energy-dissipating xanthophyll cycle (Goss & Jakob, 2010), is upregulated, presumably also to minimize ROS production. Additionally, biosynthesis of the pigment biliverdin, capable of scavenging oxygen radicals (Stocker et al., 1990), also appears to be upregulated in response to oxidative stress. Upregulation of polyamine biosynthesis likely serves a role in both ROS management (Liu et al., 2015) and osmotic balance (Chen et al., 2018). Thus, given the immediate upregulation of putrescine biosynthesis, SODs, and several chloroplastic thioredoxins, mitigation of oxidative stress appears to be concurrent with the osmotic stress response under hyposalinity stress. This coexpression of stress response genes may be due to shared upstream signaling networks (Stuecker et al., 2018), suggesting the observed oxidative stress response may simply be coregulated with salinity stress rather than indicative of ROS imbalance. However, oxidative stress induced by hyposalinity has been reported in other algae (Downs et al., 2009; Rugiu et al., 2020). This suggests that the apparent ROS response reflects an active stressor—one which continues beyond the first 30 minutes of stress exposure, as evidenced by the continuing upregulation of organelle-associated thioredoxins and peroxiredoxins and upregulation of thioredoxin reductases from 1–4 h.

In parallel with managing hypoosmotic and oxidative stress, *S. marinoi* pauses other cellular activities, such as glycolysis, DNA replication and recombination, chlorophyll biosynthesis, the Calvin cycle, storage molecule biosynthesis, and proteolysis by the proteasome. Some of these processes (e.g., storage molecule biosynthesis, DNA replication/recombination, and proteasome activity) are energy-intensive, which suggests that under hypoosmotic stress cells divert energy away from these processes and towards ones involved in stress mitigation. In contrast, the pause of glycolysis and the Calvin cycle appears to reflect a transient decrease in overall energy production, perhaps related to the mitigation of oxidative stress, which involved reducing photosynthetic light harvesting and thus ROS generation. However, this observation is complicated by the upregulation at 15–30 min of *bZIP14*, a transcription factor responsible for inducing transcription of TCA cycle genes (Matthijs et al., 2017). This suggests an increased demand for TCA cycle genes and metabolites, at odds with the downregulation of other energy-producing processes. Given the apparent presence of oxidative stress, this upregulation of TCA cycle genes may indicate replenishment of stress-damaged TCA cycle enzymes, several of which are particularly vulnerable to ROS (Noster et al., 2019).

4.1.2 Transition phase, 1 h

Major changes in expression occurred between 30 min and 2 h. Specifically, several upregulated genes became downregulated and vice versa, several differentially expressed genes returned to baseline expression, and some processes that were not differentially expressed during the first phase became differentially expressed in either direction. Our expression data indicate that this transition between the 30 min and 2 h time point happened gradually, as expression levels at 1 h show a mix of both patterns. As such, the 1 h time point represents a transition zone between the earlier and later time points in our experiment. When relevant elsewhere, we mention gene and pathway expression at 1 h.

4.1.3 Recovery phase, 2–4 h

After mitigating the initial osmotic and oxidative stresses, *S. marinoi* resumes photosynthesis, appears to increase energy production from carbohydrate metabolism, supported by the degradation of storage molecules, and directs this energy to protein synthesis and cell division. Specifically, after 1 h, several processes and genes involved in the immediate response to oxidative and osmotic stress were no longer differentially expressed or became downregulated. This includes photosynthesis-associated processes, components of the ROS and damaged protein responses, and the *bZIP14* transcription factor. Simultaneously, processes paused during stress mitigation were resumed, including proteasome activity and glycolysis. Most cellular processes that are generally upregulated throughout the entire time series reached peak upregulation during 2–4 h, including protein biosynthesis and the TCA cycle. These changes in gene expression coincide with growth resumption by 4 h, suggesting a 'burst' of transcriptional activity enables the recovery of *S. marinoi* from acute salinity stress.

Among the genes that were up- and downregulated during the recovery phase, multiple seemingly contradictory expression patterns are apparent. For example, most genes of the TCA cycle reach peak differential expression from 2–4 h, but the *bZIP14* transcription factor that modulates transcription of TCA cycle genes is downregulated. This suggests that the observed upregulation of *bZIP14* from 15 min to 1 h may have induced the peak differential expression of TCA cycle genes from 2–4 h. Similarly, the downregulation of this transcription factor from 2–4 h is expected to result in the downregulation of the TCA cycle at later time points. Indeed, when acclimated to low salinities (8) during two weeks, *S. marinoi* continues to downregulate *bZIP14* (Pinseel et al., 2022). Initial upregulation of the TCA cycle in our data may be explained by non-*bZIP14* effectors of TCA cycle genes. Such effectors induced TCA cycle genes before *bZIP14* became induced in *Phaeodactylum tricornutum* under nitrogen starvation (Matthijs et al., 2017).

We observed variable regulation of osmotic or oxidative stress response genes. Several osmotic stress-related genes, such as K+ efflux antiporters and some osmolyte biosynthesis

genes, remained differentially expressed during recovery, while other osmolyte biosynthesis and degradation genes show altered expression (Fig. 7). Similarly, most cell compartment-associated peroxiredoxins and thioredoxins remained upregulated at 2–4 h, whereas SODs, polyamines, and photosynthesis processes showed altered expression. Additionally, thioredoxin reductases were induced from 1–4 h and not before, unlike the other antioxidant genes differentially expressed during rapid stress mitigation. Together, these observations likely reflect optimization of the stress response by enabling simultaneous mitigation of the initial stress and resumption of growth.

4.1.4 Pre-acclimation phase, 8 h

During the pre-acclimation phase cells show signs of acclimating to low salinity. At 8 h, a majority of the differentially expressed processes were returning towards baseline levels, which could correspond to gene expression leveling off as a new homeostasis (i.e., acclimation) is reached (Borowitzka, 2018). However, ten generations is conventionally considered sufficient time for acclimation to occur (Nunn et al., 2013). This is far more than is possible within 8 h for *S. marinoi*, which divides approximately twice a day at 15℃ (Olofsson et al., 2022). That being said, the novel upregulation of several cellular processes at 8 h that were all downregulated in earlier time points indicates which pathways may remain upregulated in cells acclimated to low salinity. These include the biosynthesis of storage molecules, specifically chrysolaminarin and fatty acids. Diatoms accumulate storage molecules under various stresses (Vårum & Myklestad, 1984), a process that was observed to begin within 12 hours of stress and persist over at least 8 days in *Phaeodactylum tricornutum* (Jiang & Gao, 2004; Sayanova et al., 2017). This suggests storage molecule accumulation occurs in diatoms acclimated to some stressors. In addition, we found that both DNA recombination and proteasome activity were upregulated at 8 h (Fig S9A, S10). These processes play a role in mitotic cell division in diatoms (Bulankova et al., 2021; Vierstra, 1996), and damage repair (Chatterjee & Walker, 2017; Rousseau & Bertolotti, 2018).

In addition, we found that the average expression of SOD genes increased again at 8 h. Additionally, light-harvesting photosynthetic processes were upregulated at 8 h, potentially increasing cellular ROS. Indeed, in the brown alga *Fucus vesiculosus*, ROS-managing genes were induced during gradual acclimation to hyposalinity (Rugiu et al., 2020), indicating that acclimated cells continue to experience increased oxidative stress. At last, we found that the biosynthesis of chlorophyll was also upregulated at 8 h. Such increase in chlorophyll production under salinity stress has not been commonly observed. At extreme salinities, both high and low, the marine diatoms *P. tricornutum* and *Chaetoceros gracilis* displayed decreased chlorophyll content (Liang et al., 2014), while two other marine diatoms, *Thalassiosira decipiens* and *Cyclotella* sp., showed no relationship between per cell chlorophyll content and salinity (McLachlan, 1961). In general, changes in pigment concentrations in diatoms were noted during light, nutrient, and heavy metal stresses (Kuczynska et al., 2015). Thus, the increased chlorophyll biosynthesis of *S. marinoi* at low salinity appears to be a relatively unique response. This response might have industrial and commercial interest (Kuczynska et al., 2015).

4.1.5 Synthesis

Overall, the trends of gene expression across eight hours of low salinity exposure in *S. marinoi* reveal a coordinated, multiphasic response to stress. Initially, *S. marinoi* responds to hypoosmotic stress by mitigating intracellular contributors to stress, including ROS and the concentration of ions and osmolytes. Subsequently, *S. marinoi* increases energy production and protein synthesis as growth is resumed, while fine-tuning the response to continued low salinity stress. Finally, by 8 h, *S. marinoi* begins to approach acclimation, characterized by an increase in the biosynthesis of storage molecules and chlorophyll and upregulation of DNA recombination and proteolysis activity.

4.2 Comparing the short-term stress response and long-term acclimation of S. marinoi to low salinity

In a previous experiment, the acclimation of *S. marinoi* to low salinity has been characterized by exposing eight strains from across the Baltic salinity cline to low salinity (8) for two weeks (Pinseel et al., 2022). This allowed us to compare the short-term stress response, including the 'pre-acclimation' state (8 h of stress), to the long-term acclimated response (Fig 7). This comparison revealed that most processes that were differentially expressed during the pre-acclimation phase remained differentially expressed after long-term acclimation. However, several processes do not follow this pattern, but instead experience similar differential expression early in the short-term response and after acclimation (2 weeks), but without differential expression, or with differential expression in the opposite direction, at in-between time point(s). This suggests that the pre-acclimation phase of *S. marinoi* in low salinity may represent an 'overcorrection' for many pathways. Such overcorrection has previously been observed in growth rates of a colonial cnidarian, where treatment with various stressors, including low salinity, caused oscillations in growth rates as response mechanisms overshot homeostasis, prompting mitigation of the overcorrection and another oscillation (Stebbing, 1981).

First, the expression of photosynthesis and associated processes was similar at 8 h and two weeks. After two weeks in low salinity, light harvesting, Calvin cycle, and pigment biosynthesis processes were all upregulated. Although not all of these processes were upregulated at 8 h, the general expression trend across our time series indicated that long-term upregulation for all photosynthesis-related processes was a component of the acclimated response. Similarly, biosynthesis of storage molecules was upregulated both at 8 h and two weeks. Gluconeogenesis is downregulated in low salinity, regardless of stress duration. The irreversible steps of glycolysis, though upregulated at all short-term time points, are less upregulated, on average, at 8 h than during the recovery phase, which is not incongruent with

the average near-baseline expression of this pathway in acclimated cells. The TCA cycle shows a similar pattern, supporting our conclusion that expression of the *bZIP14* transcription factor, downregulated from 2–8 h, has a delayed impact on expression of TCA cycle genes. *bZIP14* is also downregulated at two weeks, indicating that despite the transient upregulation during the short-term stress response, the TCA cycle, and thus oxidative respiration, is downregulated under hyposalinity stress.

The general upregulation of nitrogen assimilation processes in the short-term response is also maintained in acclimated cells. Even assimilation pathways not differentially expressed at 8 h return to upregulation at two weeks, supporting the characterization of the 8 h time point as an overcorrection phase. Presumably supported by upregulated nitrogen assimilation, regulation of protein synthesis is also similar in the short-term and after long-term acclimation. Although the magnitude of expression at 8 h for genes associated with protein synthesis was less than during the recovery phase, the continued upregulation at 8 h is mirrored in the upregulation of these genes in acclimated cells. The regulation of serine-type endopeptidases after acclimation is similarly coherent given the short-term expression. By 8 h, most of these genes exhibited near-baseline expression, presumably as their repression during the transition and recovery phases eased, and on average, at two weeks, these genes are not differentially expressed. In contrast, differential expression of proteasome genes in the short and long term is markedly different. From 4–8 h, proteasome genes are strongly upregulated, not displaying the 2–4 h ‘burst’ and ‘overcorrection’ pattern of many differentially expressed genes. However, despite the strong upregulation of these genes at 8 h, a pattern which correctly suggested upregulation during acclimation for genes involved in photosynthetic processes, proteasome activity is downregulated at acclimation.

By definition, acclimated cells have achieved a new homeostasis under the ‘stress’ condition and can be considered to no longer be stressed, even if their growth remains below that of cells in other conditions (Borowitzka, 2018). Considering this, the downregulation of the

proteasome at two weeks of low salinity indicates that the new homeostasis achieved by acclimated cells manages ROS levels without requiring increased damage repair by the proteasome, and so the proteasome genes are downregulated. The expression of other oxidative stress-related genes at two weeks supports the conclusion that increased proteasome activity is not needed to handle ROS-produced damage at acclimation. Several groups of genes associated with ROS management, such as thioredoxin reductases, the putrescine biosynthesis pathway, and a violaxanthin de-epoxidase became upregulated again in acclimated cells. This represents a potential mechanism for managing ROS after acclimation that could prevent damage to cellular components and allow the observed downregulation of proteasome genes. However, the superoxide dismutases did not show this pattern and were instead downregulated at two weeks. All differentially expressed SOD genes at two weeks were associated with the Fe-Mn family of superoxide dismutases, which typically occur in the mitochondria (Kwasigroch et al., 2008). Thus, the downregulation of SODs at two weeks may reflect the decreased oxidative respiration suggested by decreased TCA cycle activity.

Expression of the non-SOD oxidative stress-response genes at 8 h and two weeks also supports the characterization of the 8 h time point as an overcorrection phase. By 8 h, the expression of other major ROS-managing genes was decreased relative to earlier time points, with some genes no longer differentially expressed or downregulated. However, ROS-response genes are upregulated at long-term acclimation, indicating a continued greater need for ROS management, relative to unstressed cells. Accordingly, the temporary decreased expression of these genes at 8 h appears to be an overcorrection stemming from earlier high differential expression of these same genes, during the stress mitigation and/or recovery phases.

Most genes involved in the short-term mitigation of osmotic stress also appear to display an overcorrection response at 8 h. All osmolyte biosynthesis genes downregulated during the stress mitigation phase are less strongly differentially expressed by 8 h, but all are downregulated again at long-term acclimation. This indicates the weakened or absent

differential expression at 8 h was an overcorrection for the strong downregulation at 15–30 min. The average expression of K+ efflux transporters, involved in osmotic balance of the chloroplast, also shows this response, being upregulated at all phases except the 8 h overcorrection phase.

In contrast, the expression of ion transport differs under short-term stress and at long-term acclimation. On average, transporters of K+ and/or Na+ are downregulated at all short-term time points and upregulated at two weeks. Lessened differential expression at 8 h suggests the beginnings of acclimation were visible at 8 h, but overall, the role of ion transport in long-term salinity management was not mirrored under short-term stress. There are some indications that diatoms utilize different osmolytes under short- and long- term salinity stresses (Nakov et al., 2020), but though some studies do not mention significant differential expression of ion transporters (Lyon et al., 2011), a contrasting response of ion transporters in short- and long- term salinity stress has not been previously reported in diatoms. Thus, it remains unclear why *S. marinoi* involves most ion transporters only in long-term acclimation.

Although the explanation for some patterns of short- and long-term expression remain unknown, a potential mechanism for the ‘overcorrection’ response visible between 8 h and two weeks is apparent. A possible general mechanism involves highly upregulated processes producing a surplus of metabolites during the recovery phase, leading to decreased expression of associated genes by feedback inhibition. When the surplus of metabolites is depleted, the continued exposure to low salinity reinduces these processes, as observed after acclimation. For example, products of nitrogen assimilation decreased the expression of nitrate assimilation genes within 4–6 hours of nitrate supplementation in barley (Vidmar et al., 2000). Similarly, in the green alga *Chlamydomonas reinhardtii*, the ratio of nitrate to ammonium in cells determined whether nitrate assimilation genes were induced (more nitrate) or repressed (more ammonium) (Llamas et al., 2002; Sanz-Luque et al., 2015). Additionally, genes involved in the biosynthesis of metabolites such as putrescine and ectoine are similarly transcriptionally regulated. In

Escherichia coli, transcription of ornithine decarboxylase is inhibited by polyamines such as putrescine, which it produces (Huang et al., 1990; Panagiotidis et al., 1994). Although the transcriptional regulation of these genes have not been studied in diatoms, the observed response of these and other genes in *S. marinoi* suggests similar regulatory processes could be occurring under hyposalinity stress.

Overall, gene expression at two weeks indicates the new homeostasis reached by *S. marinoi* has more elements in common with the recovery phase, in terms of which pathways are differentially expressed, than the prestressed state. This reflects the lingering effects of suboptimal salinity despite the successful mitigation of stress. This confirms that the transition from salinity 24 to 8 is a substantial stressor for this diatom, despite the ability of *S. marinoi* to survive in salinities ranging from full seawater to near-freshwater in the Baltic Sea (Sjöqvist et al., 2015). Furthermore, *S. marinoi* cannot survive freshwater conditions (salinity 0), unlike some other euryhaline diatom species such as *Cyclotella cryptica*. This suggests that the mechanisms observed here for mitigating acute, and later on chronic, hyposalinity stress are near the limits of what *S. marinoi* can accomplish under hyposalinity stress.

It has to be noted that *S. marinoi* is an ancestrally marine diatom (Alverson, 2014; Nakov et al., 2018). This long evolutionary history in marine conditions suggests that the strategies to manage hypoosmotic stress used by *S. marinoi* are recently acquired, rather than ancestral legacies to an euryhaline lifestyle. To further investigate this, we compared our data with a similar salinity experiment on the euryhaline diatom *Cyclotella cryptica*, which was exposed to freshwater conditions at salinity 0 during a comparable time frame. In contrast to *S. marinoi*, *C. cryptica* belongs to a clade of diatoms which experienced repeated habitat transitions between freshwater and marine environments and contains species that are both salinity generalists and specialists (Alverson, 2014). The most common recent ancestor of *S. marinoi* and *C. cryptica* lived ca. 70 Ma and probably transitioned from a marine to freshwater lifestyle in the *Cyclotella* clade. To compare both species, we focus on overarching gene expression strategies,

similarities and differences in expression of key pathways, and comparisons of the short-term and acclimated responses.

4.3 Evolutionary conservation of the low salinity stress response in diatoms

Another euryhaline diatom, *Cyclotella cryptica*, can grow across a broader salinity (0-56) range than *S. marinoi* (2.5-35) (Balzano et al., 2011; Liu & Hellebust, 1976; Reimann et al., 1963). The short-term transcriptomic response of *C. cryptica* was characterized by our lab in a similar experiment as performed here for *S. marinoi*, via differential expression analysis of gene counts over time (Downey et al., 2022). Aside from a minor variation in experiment duration (an additional 10 h sampling) and the use of freshwater as the stress condition for *C. cryptica*, these two experiments were set up identically and performed in parallel. Thus, exposure to low salinity (8) for *S. marinoi* and freshwater (0) for *C. cryptica* represent comparable stressors, as both result in a short-term stress response of similar duration followed eventually by acclimation, rather than cell death.

In both experiments, the duration of the time series was chosen to capture the short-term stress response up to the onset of acclimation, as was evidenced by similarities in gene expression to the prestressed state ('t = 0 min'). This occurred within 8 h for *S. marinoi* and 10 h for *C. cryptica*. The short-term stress responses were also compared to the acclimated response for each species: at two weeks for *S. marinoi*,and three months for *C. cryptica*. Although the timing of the collection of acclimated data are different, the nature of the acclimated state, where cells are at homeostasis under the 'new' condition (Borowitzka, 2018), allows us to compare general similarities and differences in the short-term versus acclimated responses across species.

4.3.1 Evolutionary conservation of mechanisms for euryhalinity

Despite a similar duration of the short-term stress responses in both species, the overall pattern of gene expression differed substantially. Among these differences, several findings provide potential insights into the broader salinity tolerance of *C. cryptica* and other euryhaline

species capable of surviving freshwaters. First, relative to the multiphase response of *S. marinoi*, the stress response of *C. cryptica* is condensed. This is suggestive of accelerated response to low salinity, which may be a general feature of broadly euryhaline diatoms. In *C. cryptica*, the stress mitigation response, spanning 30 min to 1 h, was followed by a return to baseline expression by 4 h for most of the involved processes, whereas in *S. marinoi*, baseline gene expression for genes differentially expressed during stress mitigation was not widely resumed even within 8 h. Additionally, peak gene expression occurred during the recovery phase in *S. marinoi*, whereas for *C. cryptica* peak expression happened during initial stress mitigation. This further suggests that a rapid, strong stress response is required for a marine species to tolerate freshwater.

Second, protein synthesis and photosynthesis pathways were regulated differently during stress mitigation in each species. Our data suggests that *C. cryptica* experiences less osmotic stress than *S. marinoi*, contributing to its heightened tolerance of low salinity. In *S. marinoi*, genes involved in protein synthesis, protein degradation, and ROS management were upregulated across the time series, suggesting replacement of oxidative stress -damaged proteins may have been necessary. In contrast, *C. cryptica* repressed genes associated with ribosome biogenesis during stress mitigation, presumably in concordance with halted cell division as seen in *Saccharomyces cerevisiae* (Gasch et al., 2000) instead of in response to cellular damage. *S. marinoi* and *C. cryptica* also differ in their regulation of light-harvesting genes involved in photosynthesis. Most differentially expressed genes for light harvesting complex proteins were upregulated during stress mitigation in *C. cryptica*, suggesting indirect quenching of overexcited chlorophyll, a potential method for preventing ROS generation (Latowski et al., 2011), was involved in mitigating oxidative stress. Although some identified light harvesting complex genes in *S. marinoi* were similarly upregulated during stress mitigation, a majority were downregulated (Fig S5), suggesting this species attempted to limit ROS by decreasing photosynthetic light harvesting altogether, as in some plant species (Dalal &

Tripathy, 2018). This may indicate the oxidative stress induced by low salinity in *S. marinoi* is too severe to be managed by increased photoprotection mechanisms alone, or it may reflect a differing capability of the two species to respond to oxidative stress, further explaining the differences in euryhalinity.

Third, differences in direct osmotic stress mitigation strategies may also explain the decreased euryhalinity of *S. marinoi* relative to *C. cryptica*. In *S. marinoi*, the primary mechanism of restoring cytoplasmic osmotic balance in low salinity appears to be a decrease in osmolyte levels, whereas *C. cryptica* transports K+ in and Na+ and H+ out of the cytosol to restore osmotic balance. This suggests that although osmoregulation by modulating osmolyte concentrations can contribute to euryhalinity, on its own, it is insufficient for survival in freshwater. Thus, a short-term response to hyposalinity via ion transport may be required for euryhaline diatoms to persist in freshwater.

While the diverged responses of these two species suggest strategies and processes that may be required for an euryhaline species to cross the marine-freshwater salinity barrier, a few key similarities between species give potential insights into the overall basis of euryhalinity in diatoms. In both *S. marinoi* and *C. cryptica*, K+ efflux antiporter genes are upregulated during stress mitigation to maintain osmotic balance in the chloroplast. This similarity, contrasted with the broad differences in other aspects of this response across species, suggests upregulation of chloroplastic K+ efflux antiporters at low salinity may be a requirement for euryhalinity in diatoms. Additionally, the stress responses of both species involve preventing ROS generation and mitigating damage by generated ROS. As well as regulating light-harvesting genes as described above, both species downregulate aspects of carbohydrate metabolism and thus energy production during stress mitigation, possibly to reduce ROS generation. Accordingly, both chloroplastic K+ efflux antiport and management of oxidative stress appear to be requirements for euryhalinity.

Altogether, the similarities and differences of short-term stress responses in these two euryhaline diatoms have implications for the colonization of new environments, such as freshwaters, by diatom species. First, managing chloroplastic ion balance and oxidative stress appear to be prerequisites for tolerating low salinity, indicating that a successful colonist must induce these responses. These conserved responses are probably also required in species adapted to freshwater, including freshwater specialists, considering that oxidative stress co-occurs with many other stresses (Demidchik, 2015) and that chloroplastic K+ efflux is required for proper chloroplast morphology in unstressed plants (Kunz et al., 2014). Second, successful freshwater colonists will probably have more features in common with the stress response of *C. cryptica*, such as a swift stress response, involvement of ion transporters, and effective mitigation of oxidative stress that rapidly lessens its impact.

4.3.2 Diverged mechanisms for long-term acclimation

Comparisons between the short-term stress response and long-term acclimation of each species also reveal substantial differences in the stress management strategies of *S. marinoi* and *C. cryptica*. In *C. cryptica*, most differentially expressed genes and pathways during short-term stress were not differentially expressed after acclimation. Further, of the relatively few genes that were differentially expressed during the short-term stress response and long-term acclimation, the majority experienced opposite directions of differential expression. Accordingly, the acute response of *C. cryptica* is highly distinct from the acclimated state. In contrast, many genes and processes differentially expressed under short-term stress in *S. marinoi* were also differentially expressed after acclimation. This suggests that for *S. marinoi*, unlike *C. cryptica*, most aspects of the short-term response to hyposalinity stress are also utilized in acclimated cells to maintain viability in low salinity. This observation may indicate that the *S. marinoi* genome does not include other functional mechanisms for survival in low salinity, unlike *C. cryptica*, which appears to have differing mechanisms for initial survival and long-term growth in freshwater.

However, a few categories of genes in *S. marinoi* also showed incongruent regulation between the short-term time series and after acclimation, suggesting some key differences in the transcriptomes of stress-responding and acclimated cells. Most notably, the overall trend of ion transport regulation in *S. marinoi* was opposite in short-term stress and after acclimation—these transporters were downregulated through 8 h but upregulated at two weeks. Given the involvement of such transporters in the short-term hyposalinity response of *C. cryptica*, we considered that upregulation of ion transport may be a requirement for survival in freshwater for euryhaline diatoms. If this is the case, the absence of this response in *S. marinoi* may explain, at least in part, the inability of the species to tolerate freshwater. Considering this, the upregulation of these genes in *S. marinoi* acclimated to low salinity suggests such acclimated cells may be able to survive in freshwater environments. If this is the case, it would suggest that upregulation of existing ion transport systems is what is required for 'full' euryhalinity, and that *S. marinoi* acclimated to high salinity cannot tolerate freshwater because it simply does not upregulate ion transport genes rapidly enough to survive an abrupt shift.

However, the natural range of *S. marinoi* does not include freshwater environments, despite strains acclimated to low salinities having been identified in nature (Sjöqvist et al., 2015). This suggests that one or more additional mechanisms may be enabling the survival of *C. cryptica* in freshwater. Considering the differences we have noted between the short-term stress responses of *C. cryptica* and *S. marinoi*, these potential mechanisms may relate to a variety of processes. These may include tolerance and management of oxidative stress, expression of energy-producing processes such as the TCA cycle under early stress, and of course, ion transport itself, which may consist of genes with kinetics more favorable for export/import of appropriate ions in *C. cryptica*.

Conclusion

In this study, we characterized the stress response of *S. marinoi* and compared the single set of mechanisms used by this species to survive in low salinity to the suite of mechanisms used by another euryhaline diatom with a broader salinity tolerance, *C. cryptica*. In doing so, we identified candidate mechanisms for conveying euryhalinity in diatoms, namely upregulation of chloroplastic K+ efflux transporters and activation of oxidative stress management, and determined potential characteristics of successful freshwater colonists, including rapid upregulation of ion transport and effective oxidative stress mitigation. Considering that anthropogenic climate change will continue to expose populations to novel and intensifying environmental shifts, such as freshening water, this identification of potential mechanisms of euryhalinity may provide insight into differential survival of species in the future. For example, a marine specialist species without functional K+ efflux transporters in the chloroplast would likely not survive inundation of freshwater, as is occurring in polar regions, into its native range, perhaps leading to a range shift or local extinction, instead of acclimation with the possibility of eventual adaptation. Given the crucial roles of diatoms in marine food webs and harmful algal blooms (Armbrust, 2009; McKibben et al., 2017), understanding which species will tolerate salinity shifts is vital for predicting impacts of climate change on ecosystems and human activities (Berdalet et al., 2015). Overall, further research into the mechanisms of initial survival under hyposalinity stress in diatoms can reveal the underpinnings of an euryhaline lifestyle and the multiple salinity transitions in the diatom clade while progressing our knowledge of how climate change will affect key ecosystem services of our oceans.

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Appendix

6.1 Figures

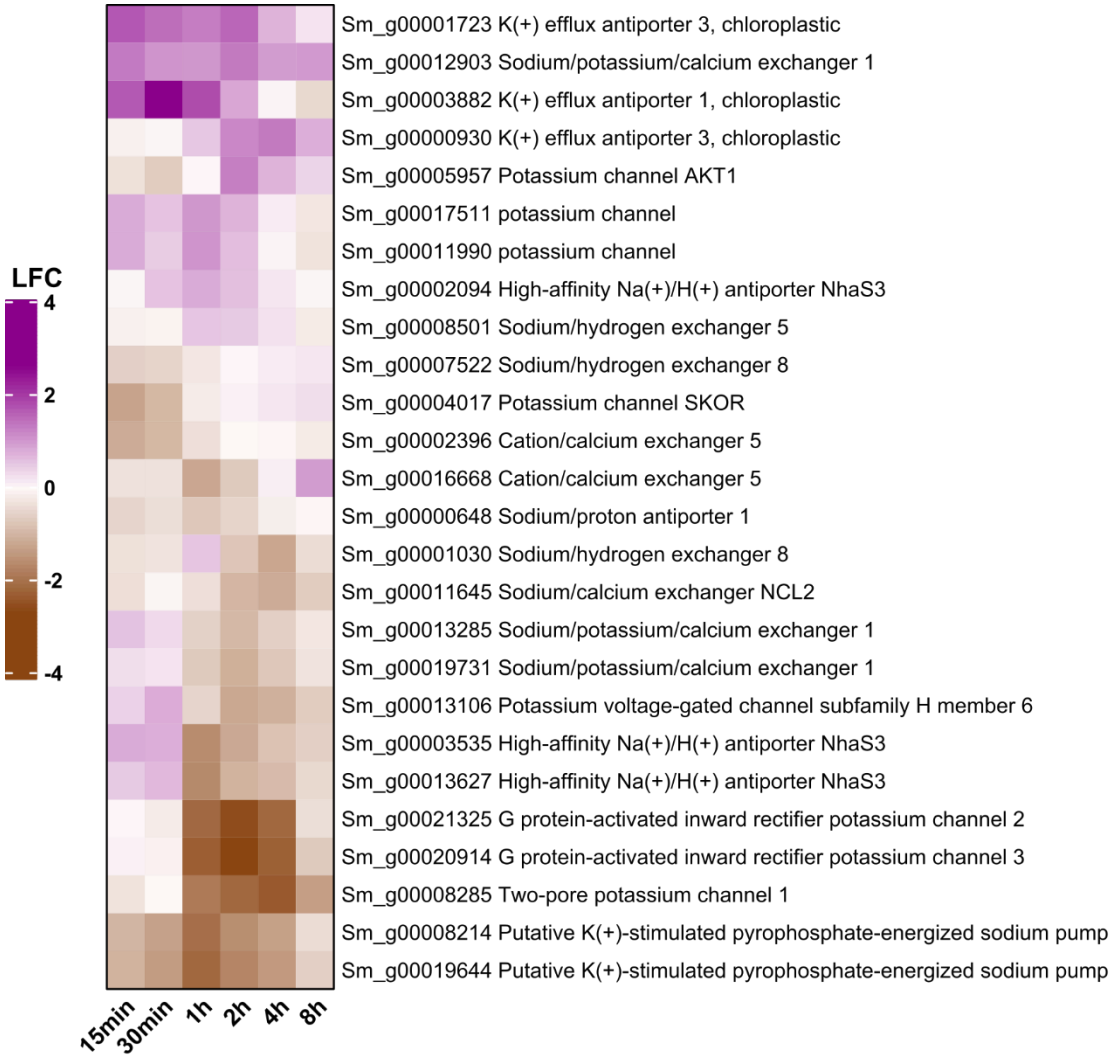

Fig S1. Heatmap showing the expression of differentially expressed genes involved in K+ and Na+ ion transport over the 8 hour time series. All shown genes were significant in two or more consecutive time points.

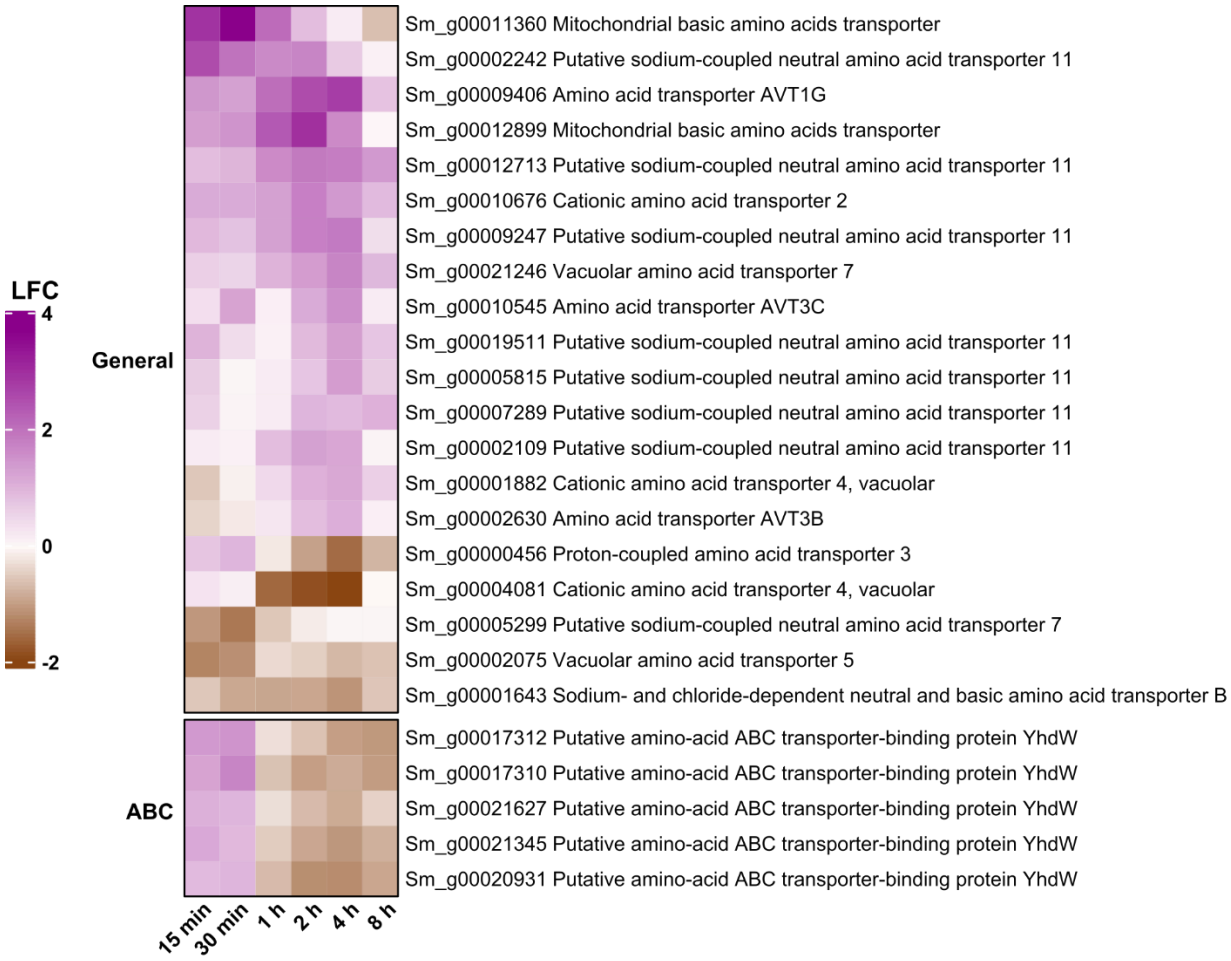

Fig S2. Heatmap showing the expression of differentially expressed genes involved in amino acid transport over the 8 hour time series. All shown genes were significant in two or more consecutive time points.

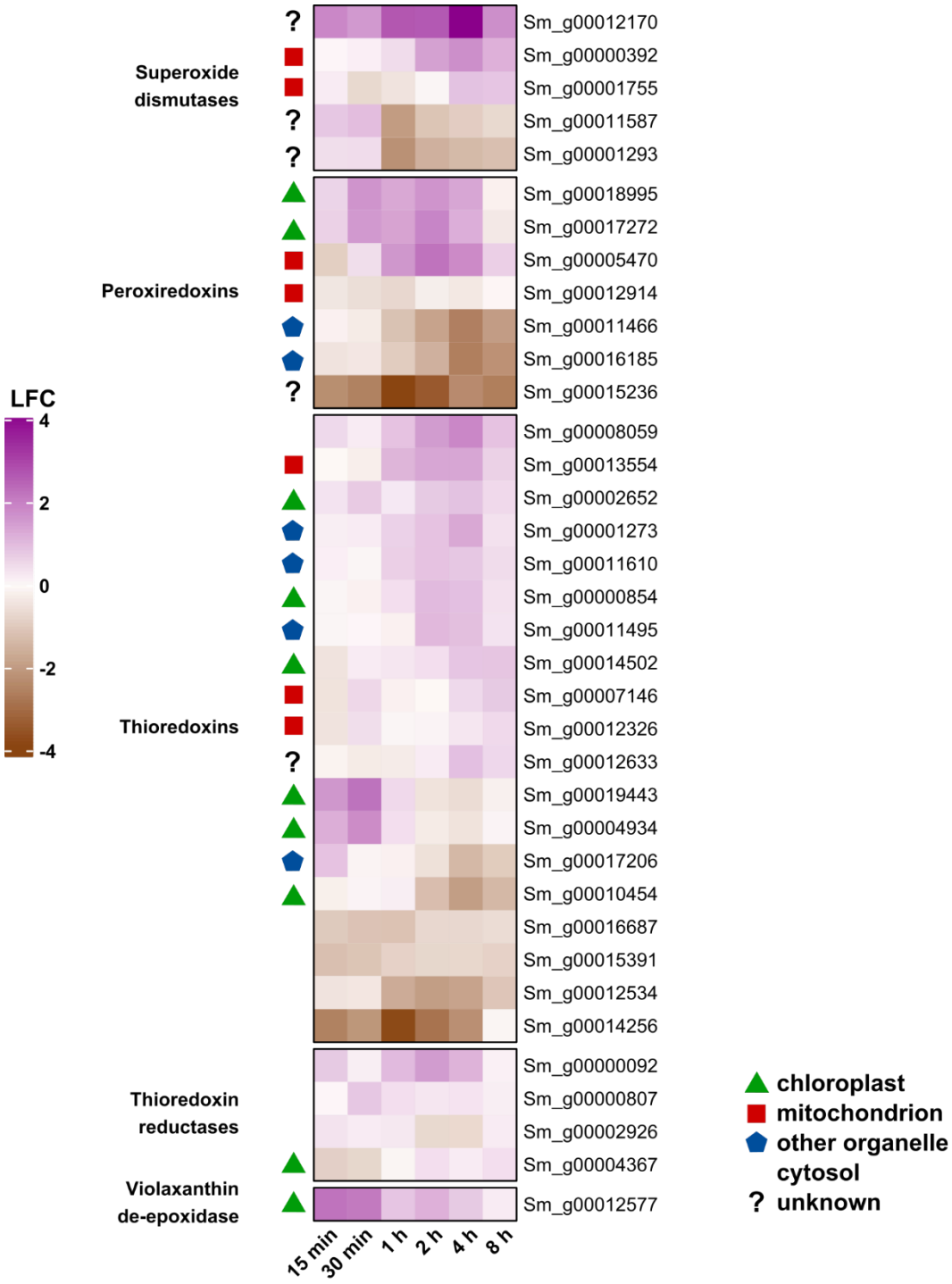

Fig S3. Heatmap showing the expression of ROS mitigation-associated differentially expressed genes over the 8 hour time series. Localization is indicated to the left of each gene. All shown genes were significant in two or more consecutive time points.

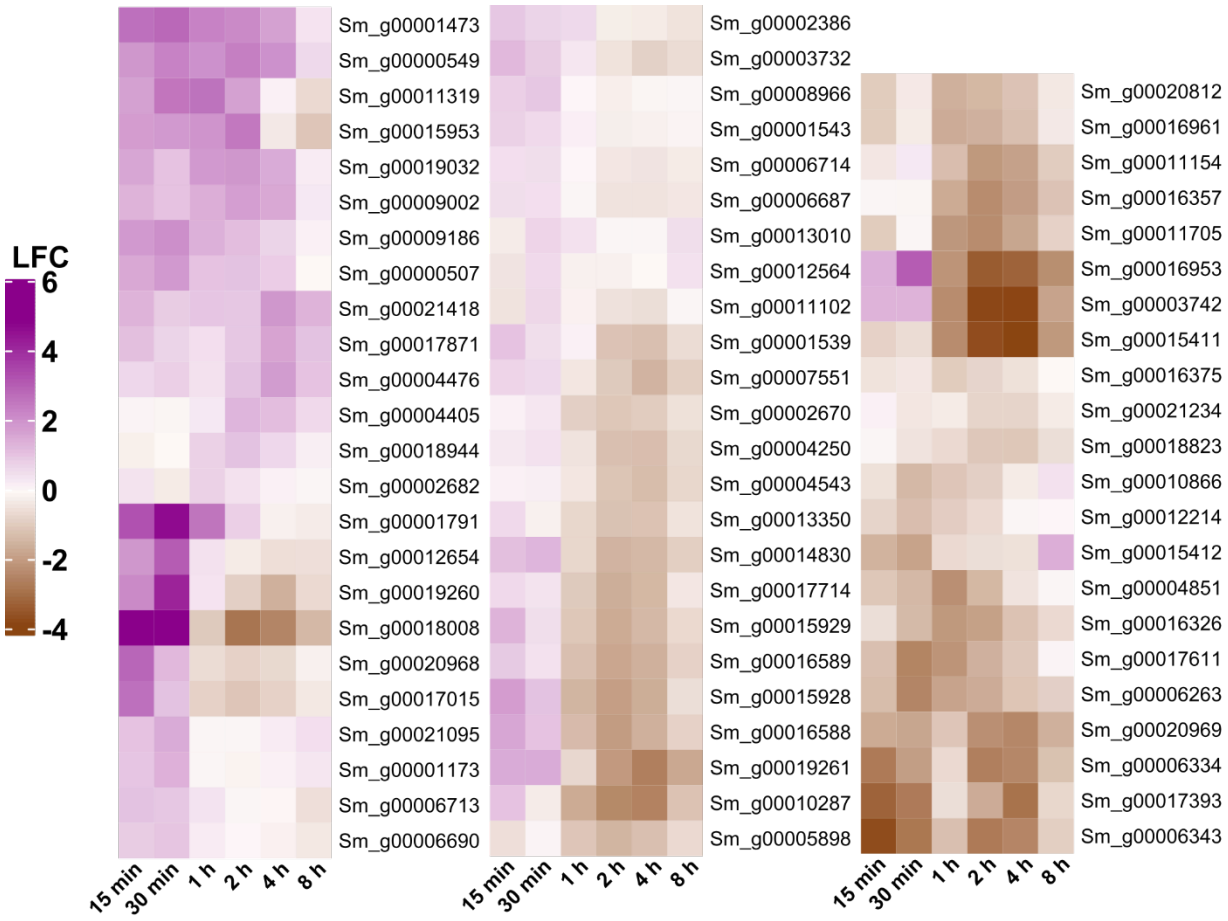

Fig S4. Heatmap showing the expression of differentially expressed genes annotated as heat shock factors/proteins over the 8 hour time series. All shown genes were significant in two or more consecutive time points.

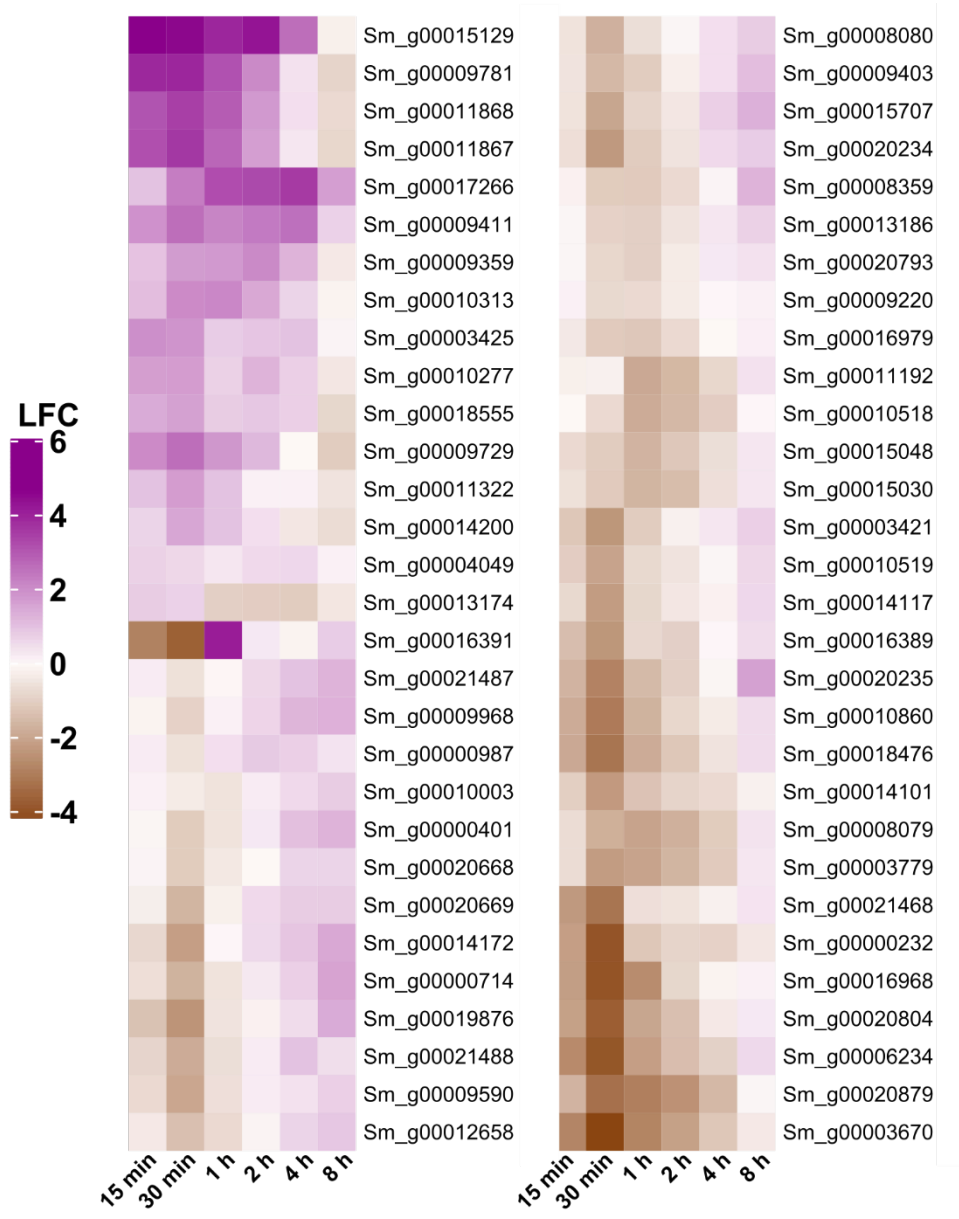

Fig S5. Heatmap showing the expression of differentially expressed genes associated with GO:0009765 (photosynthesis, light harvesting) over the 8 hour time series. All shown genes were significant in two or more consecutive time points.

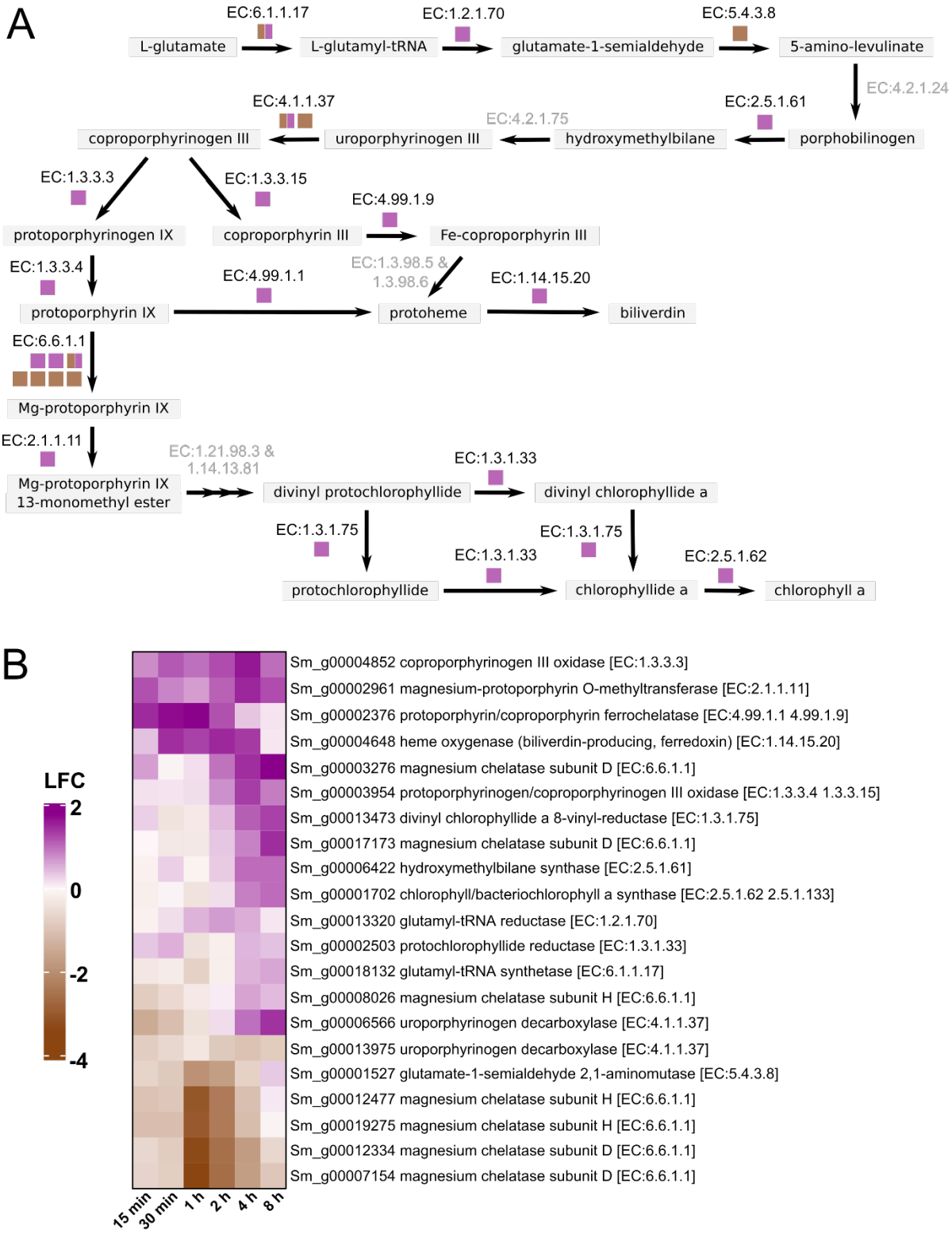

Fig S6. **A.** Pathway and **B.** Heatmap showing the expression of differentially expressed genes involved in chlorophyll biosynthesis over the 8 hour time series. All shown genes were significant in two or more consecutive time points.

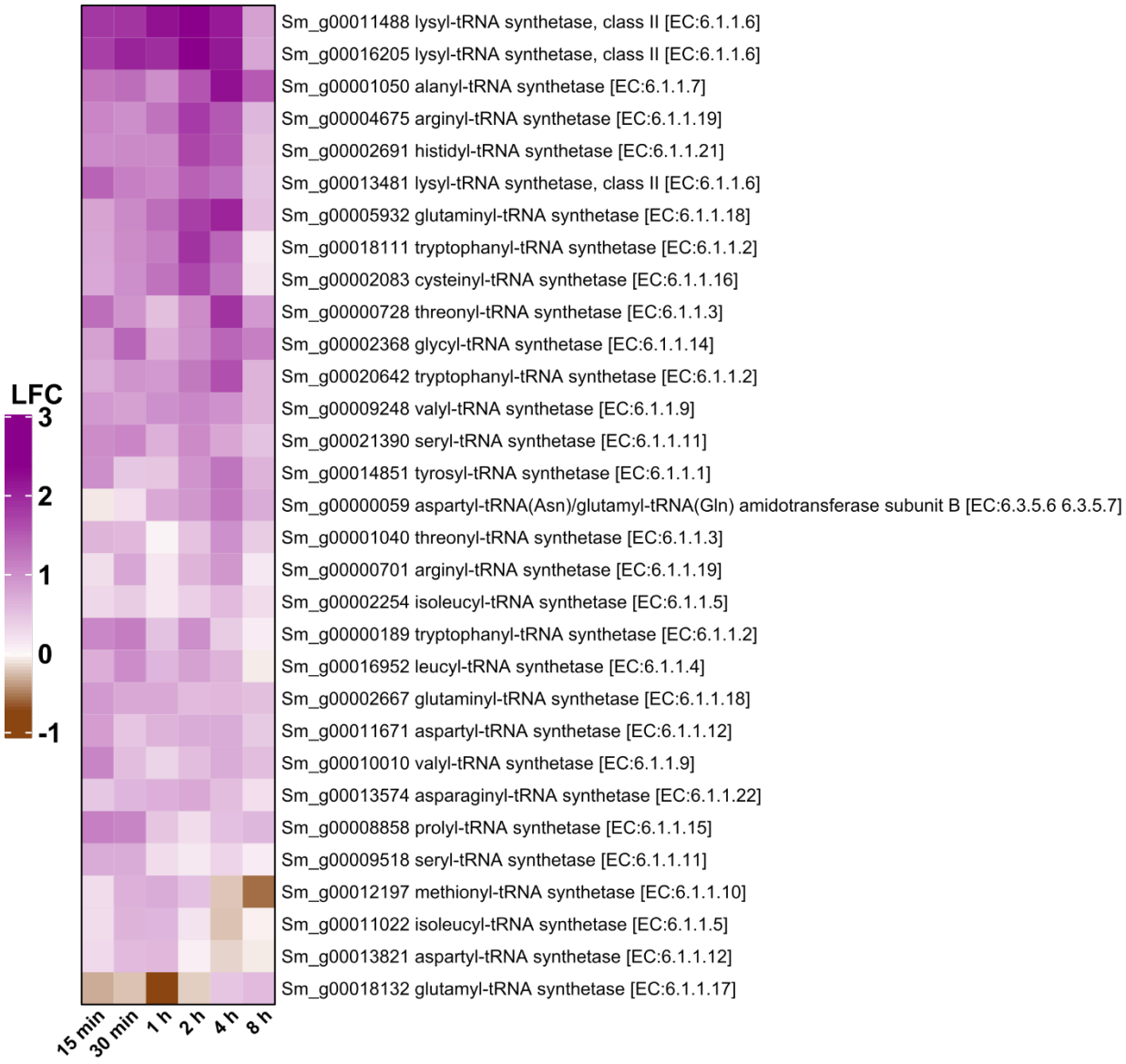

Fig S7. Heatmap showing the expression of differentially expressed genes involved in tRNA biosynthesis over the 8 hour time series. All shown genes were significant in two or more consecutive time points.

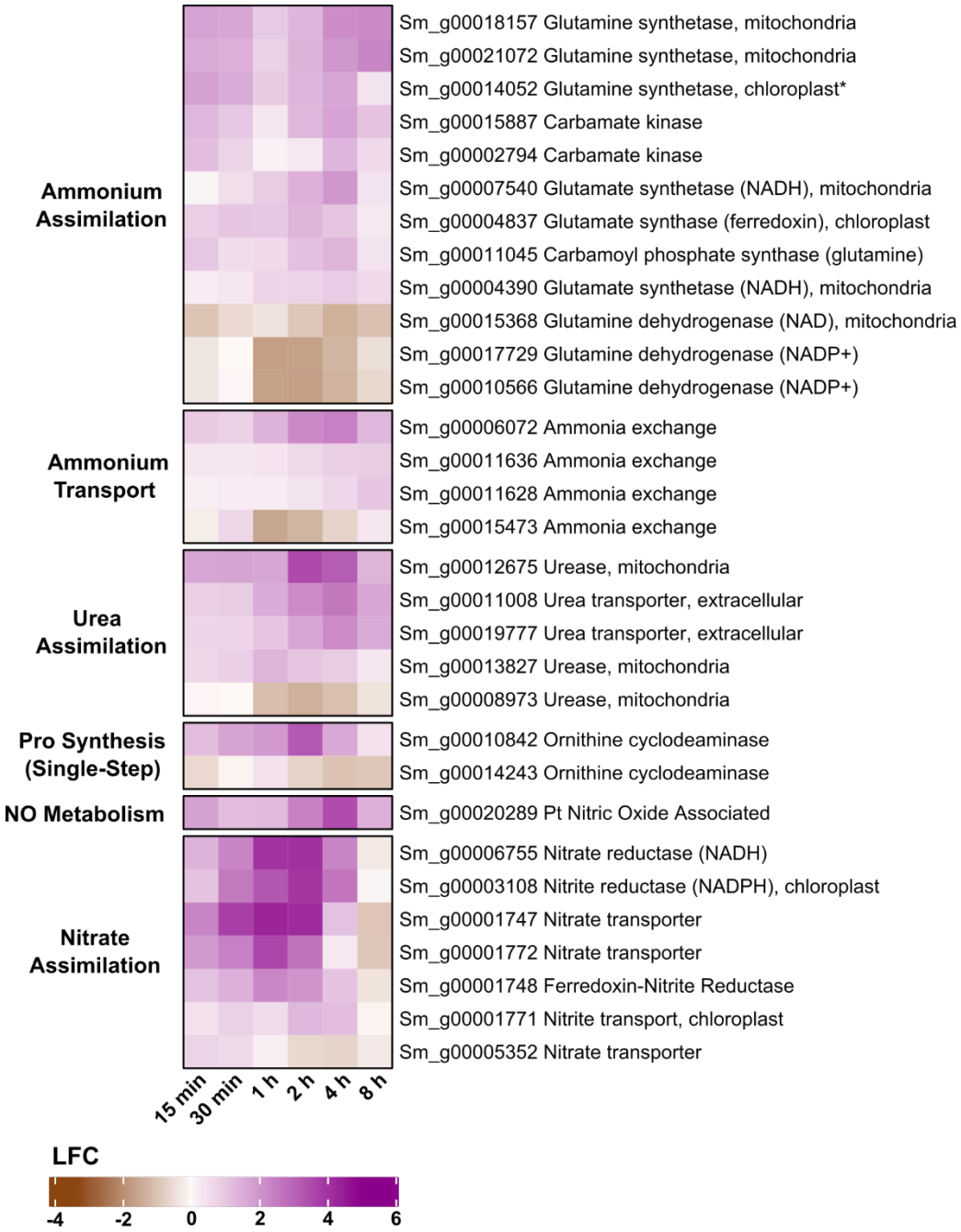

Fig S8. Heatmap showing the expression of differentially expressed genes associated with nitrogen assimilation over the 8 hour time series. All shown genes were significant in two or more consecutive time points. Continued on the next page.

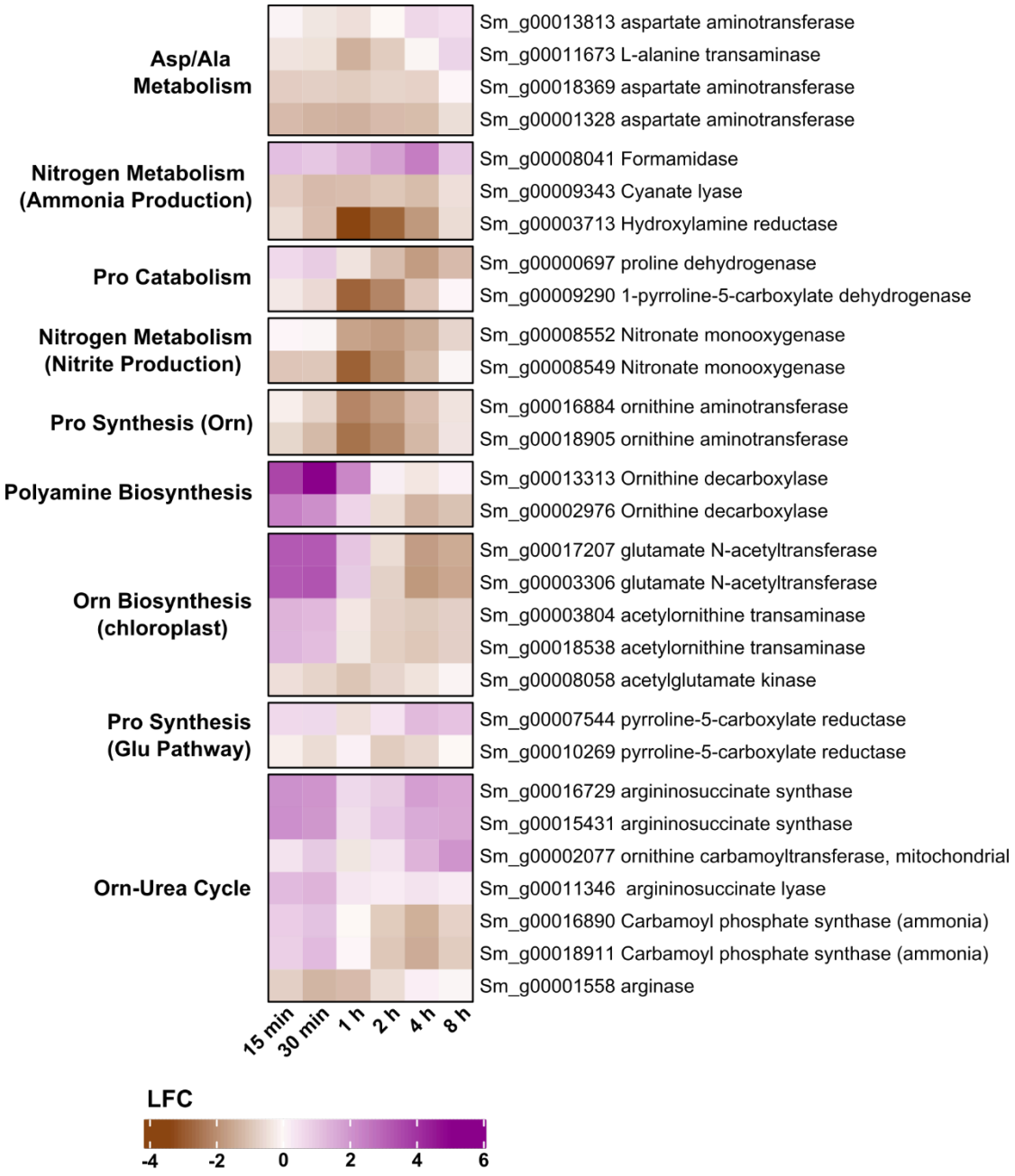

Fig S8 (Cont.). Continued from the previous page. Heatmap showing the expression of differentially expressed genes associated with nitrogen assimilation over the 8 hour time series. All shown genes were significant in two or more consecutive time points.

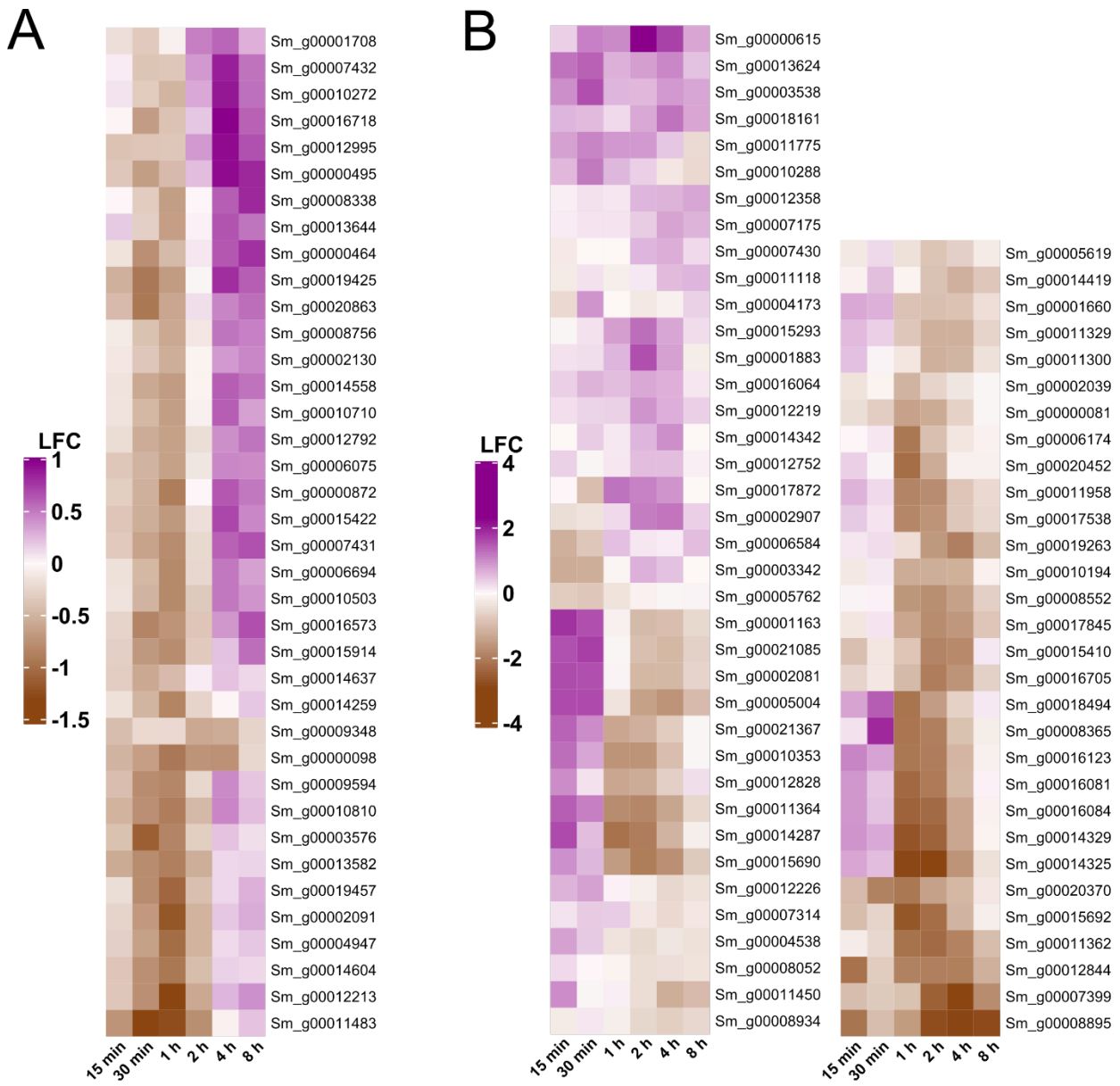

Fig S9. Heatmap showing the expression of differentially expressed genes associated with **A.** proteasome activity and **B.** serine-type endopeptidase activity over the 8 hour time series. All shown genes were significant in two or more consecutive time points.

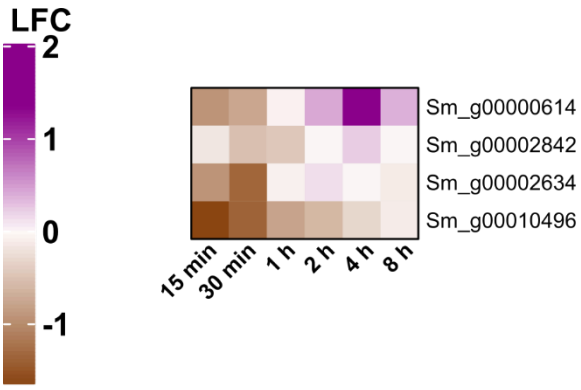

Fig S10. Heatmap showing the expression of differentially expressed genes involved in DNA recombination based on GO term annotation over the 8 hour time series. All shown genes were significant in two or more consecutive time points.