Gene expression response of the alga *Fucus virsoides* (Fucales, Ochrophyta) to glyphosate solution exposure☆

Marco Gerdol [a], Andrea Visintin [a], Sara Kaleb [a], Francesca Spazzali [a], Alberto Pallavicini [a, b, c], Annalisa Falace [a, b, c, *]

[a] *Department of Life Sciences, University of Trieste, 34127, Trieste, Italy*
[b] *CoNISMa, Piazzale Flaminio 9, 00196, Roma, Italy*
[c] *Istituto Nazionale di Oceanografia e di Geofisica Sperimentale – OGS, Trieste, Italy*

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ABSTRACT

Fucus virsoides is an ecologically important canopy-forming brown algae endemic to the Adriatic Sea. Once widespread in marine coastal areas, this species underwent a rapid population decline and is now confined to small residual areas. Although the reasons behind this progressive disappearance are still a matter of debate, *F. virsoides* may suffer, like other macroalgae, from the potential toxic effects of glyphosate-based herbicides.

Here, through a transcriptomic approach, we investigate the molecular basis of the high susceptibility of this species to glyphosate solution, previously observed at the morphological and eco-physiological levels. By simulating runoff event in a factorial experiment, we exposed *F. virsoides* to glyphosate (Roundup® 2.0), either alone or in association with nutrient enrichment, highlighting significant alterations of gene expression profiles that were already visible after three days of exposure. In particular, glyphosate exposure determined the near-complete expression shutdown of several genes involved in photosynthesis, protein synthesis and stress response molecular pathways. Curiously, these detrimental effects were partially mitigated by nutrient supplementation, which may explain the survival of relict population in confined areas with high nutrient inputs.

1. Introduction

The increase in pressures associated with human activities (i.e. agriculture, urbanization, industry), whose effects on land and ocean are exacerbated by climate change, is leading to an unprecedented alarming erosion of marine biodiversity worldwide (Halpern et al., 2015; O'Hara et al., 2019). Shallow marine ecosystems are particularly threatened by these factors, which may severely affect the distribution of coastal species and habitats (Coll et al., 2010). Among land-based human activities, the use of chemicals (i.e. herbicides and fertilizers) in agriculture represents a serious hazard for seagrass meadows and macroalgae, with potential cascade effects on the functioning of associated ecosystems (Felline et al., 2019).

Glyphosate (N-[phosphonomethyl] glycine), the active component of several commercial formulations of glyphosate-based herbicides (GBHs), has become the most widely used broad-spectrum non-selective herbicide since the 1970s (Myers et al., 2016), but its worldwide use underwent a significant increase after the introduction of glyphosate-tolerant crops in 1996 (Benbrook, 2016; Siehl et al., 1997; Duke, 2018). Glyphosate exerts its herbicidal effect on plants through the disruption of the shikimic acid pathway, which inhibits the enzymatic function of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase and consequently halts the synthesis of aromatic amino acids, that are crucial for ribosome activity and plant growth (Siehl et al., 1997; Komives and Schroder, 2016; Wang et al., 2016a,b).

Due to the pervasive applications of GBHs in agriculture, forestry, gardening, and invasive species management, glyphosate is currently nearly ubiquitous in the environment, with local hotspots of high contamination (Maggi et al., 2020). Glyphosate and its primary degradation product derived by microbial biochemical processes (Maycock et al., 2010), i.e. aminomethylphosphonic acid

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* Corresponding author. Department of Life Sciences, University of Trieste, 34127, Trieste, Italy.
E-mail address: falace@units.it (A. Falace).

(AMPA), frequently simultaneously occur in both terrestrial and aquatic environments (Mamy et al., 2005; Borggaard and Gimsing, 2008). AMPA, which can be further degraded to carbon dioxide (Cole et al., 1999), is a phytotoxin that can exacerbate the detrimental effects of glyphosate by affecting chlorophyll biosynthesis and causing further plant growth reduction (Reddy et al., 2004; Serra et al., 2013; Gomes et al., 2014).

The transportation of glyphosate and AMPA from land to the aquatic environment occurs by different means, i.e. leaching of polluted urban surfaces, outfalls, washout from the ground, runoff, wastewater treatments, erosion, and drainage (Solomon and Thompson, 2003; Sasal et al., 2015). However, both the timing and the modality of degradation of these compounds in natural aquatic environments, likely controlled by many variables (i.e. nutrients, light, and pH), have not been fully elucidated to date (Hennion and Barcelo, 1997; Mercurio et al., 2014).

Glyphosate and AMPA have been detected in streams, lakes, estuaries, and marine coastal ecosystems, where they can persist, either in suspension in the water column or in association with sediments, for several weeks (National Registration Authority, 1996; Degenhardt et al., 2012), with potential ecotoxicological effects (Botta et al., 2009; Zgheib et al., 2011; Annett et al., 2014; Skeff et al., 2015; Komives and Schroder, 2016; Bonansea et al., 2018; Castro Berman et al., 2018). The long-distance transportation of glyphosate and AMPA in seawater is facilitated by sediment- and particulate-binding (Pandey et al., 2019), which may greatly enhance the half-life of these compounds if chelating cations are present (Tsui and Chu, 2008). Although the metabolic fate of glyphosate in water sediments remains uncertain (Wang et al., 2016a,b), the potential delayed remobilization of AMPA, linked with the rapid biodegradation of glyphosate, poses a significant hazard to aquatic organisms (Kästner et al., 2014).

Only sparse data are currently available regarding the concentrations reached by glyphosate and its derivatives in marine ecosystems, since in-field measurements are not included in routine monitoring campaigns due to their high costs, complexity and lack of national and international regulations concerning threshold alert levels of concentration (Mercurio et al., 2014). Currently available data have reported variable glyphosate concentrations in seawater samples collected in coastal zones, which often exceed both the short- and the long-term predicted no-effect concentrations (PNECs) identified by the UK Water Framework Directive for saltwater species (i.e. 196 and 398 μg L^{-1}, respectively) (Maycock et al., 2010). Such concentrations range from 10 to 1000 μg L^{-1} in the Northern Adriatic Sea (Milan et al., 2018), from 28 to 1690 ng L^{-1} in the Baltic Sea (Skeff et al., 2015), from 13.6 to 1377 μg L^{-1} in the Western Pacific (Wang et al., 2016a,b) and reach a maximum concentration of 1.2 μg L^{-1} Atlantic Ocean (Burgeot et al., 2008).

In parallel with concerns about the detrimental effects of glyphosate on human health and terrestrial fauna (Myers et al., 2016), this compound has long been assumed to be toxicant for a broad range of non-target aquatic animals (de Brito Rodrigues et al., 2019). These include fishes (e.g. Cattaneo et al., 2011; Roy et al., 2016; Giaquinto et al., 2017), amphibians (e.g. Lajmanovich et al., 2003; Güngördü, 2013; Costa and Nomura, 2016), crustaceans (e.g. Mensah et al., 2011; von Fumetti and Blaurock, 2018; Pala, 2019), and molluscs (e.g. Sandrini et al., 2013; Matozzo et al., 2018, 2019).

Herbicides, as emergent marine pollutants, may be important drivers of the observed loss of valuable marine macroalgal forests (i.e. Fucales) in the Mediterranean Sea (Felline et al., 2019). Nevertheless, very limited information are presently available about the effects of glyphosate on the marine flora (Burridge and Gorski, 1998; Pang et al., 2012; Kittle and McDermid, 2016; Falace et al., 2018; Felline et al., 2019). The few studies carried out so far, that took into account different commercial formulations, exposure timings (i.e. from 5 min to 6 days) and concentrations (i.e. from 883 μg L^{-1} to 300 mg L^{-1}), have suggested the existence of a dependency between the morphological structure of the tested species and the effects of glyphosate. Indeed, filamentous or monostratified thalli (e.g. *Neosiphonia savatieri*, *Ulva intestinalis*, *Rhizoclonium riparium*) and early life stages appear to be more vulnerable than algae with larger size and thicker cortical layers (Burridge and Gorski, 1998; Pang et al., 2012; Kittle and McDermid, 2016).

Fucus virsoides, the only representative of the genus in the Mediterranean, is a brown canopy-forming species endemic to the Adriatic Sea (Orlando-Bonaca et al., 2013), that has a crucial role in creating habitat and nursery for the associated biota, supporting the biodiversity and functioning of intertidal rocky shores. Similar to other Fucales (i.e., *Cystoseira* s.l), *F. virsoides* has suffered an alarming decline/disappearance during recent years and its residual populations are currently confined to very small areas, compared with the past range of distribution (Orlando-Bonaca et al., 2013; Battelli, 2016). Due to its endangered status, *F. virsoides* has been included in the Red List of Threatened Species (IUCN, 2016) and is subject to international conservation measures (e.g. Barcelona Convention UNEP). Although *F. virsoides* has a complex anatomical structure, experimental evidence indicates that this species is highly susceptible to glyphosate. Indeed, low concentrations of exposure lead to visible damage (i.e. chlorosis) (Falace et al., 2018; Felline et al., 2019), with negative and irreversible toxic effects even after short-term experiments (i.e. 24 h).

Identifying the land-sea interactions that may lead to marine ecosystems changes and elucidating the ability of species to cope with terrestrial pollution are priorities for the adoption of adequate legislative instruments for marine coastal management and for the development of efficient monitoring programs (Friberg et al., 2011).

In this study, we investigated the molecular genetic mechanisms underlying the previously reported high susceptibility of *F. virsoides* (Falace et al., 2018) to a six-day exposure to a commercial glyphosate preparation (i.e., Roundup®), in factorial combination with nutrient enrichment, using an RNA-seq approach. The experimental design enabled us to assess the interaction between herbicides and nutrients, which can co-occur in polluted coastal areas, and to build the first comprehensive transcriptomic resource available for this species.

2. Materials and methods

2.1. Laboratory experimental setting

In the present study, we analyzed the same biological samples collected within the frame of a previously published study, for which fronds photosynthetic efficiency data are available (Falace et al., 2018). We here summarize the key aspects of the experimental design. For further methodological details, the reader is referred to the study by Falace et al. (2018).

Healthy apical fronds of *F. virsoides* were collected in October 2017 in the Gulf of Trieste, Northern Adriatic (45° 46′ 25.67″ N; 13° 31' 52.31" E), and transported under dark condition at 8 °C to the laboratory, within 1 h of collection. Temperature (19 °C) and photoperiod (11 Light:13 Dark) were selected according to natural conditions at the sampling site. Light irradiance, provided by LED lamps (AM366 Sicce USA Inc., Knox-ville, USA), was set at 100 μmol photons $m^{-2}s^{-1}$.

The experiment was designed to test the effects of the commercial formulation of glyphosate (Roundup® Power 2.0 MONSANTO Europe S.A.) on *F. virsoides* gene expression, after continuous exposure for one (D1), three (D3) and six (D6) days.

The following experimental conditions were considered: C (control medium), G (glyphosate addition only), N (nutrient addition only), and N + G (nutrient and glyphosate addition). The control medium (C) was filtered seawater (0.22-μm filter membrane) collected at the *F. virsoides* sampling site [17.5 μg/L N−NO_3; 0.63 μg L^{-1} P-PO_4]. The glyphosate-containing medium (G) was prepared by mixing commercial Roundup® (i.e. glyphosate acid in the form of potassium salt) with the control medium to a final concentration of 2.5 mg L^{-1} Roundup. The concentration of glyphosate potassium salt in the G medium was 882.5 μg L^{-1}, which is comparable to levels estimated for the ambient seawater in the Northern Adriatic (Milan et al., 2018). The remaining volume included surfactants (i.e., high affinity ethoxylated heteramines, representing the 6% of the formulation) and water. The nutrient-enriched medium (N), corresponding to the higher mean annual values registered in the Gulf of Trieste (ARPA FVG, 2009−2014), was prepared by adding $NaNO_3$ and $Na_2HPO_4 \bullet 12H_2O$ (Sigma-Aldrich) to the control medium to attain a concentration of 364 μg/L N−NO_3 and 1.395 μg L^{-1} P-PO_4.

Each experimental condition was replicated in three independent aquaria (5 L), containing 40 apical fronds each. The position of the aquaria in the cultivation room with regulated temperature and light was randomized. The medium was renewed every day in each aquarium to minimize nutrient and herbicide limitations or degradation. Constant aeration was provided using air pumps to maintain homogenous experimental conditions over time.

2.2. RNA extraction and library preparation

Total RNA was extracted from 36 samples, which included three replicates for each treatment group at each experimental time point (see Table 1). Extractions were performed with an E.Z.N.A.® Plant RNA Kit (Omega Biotek, Norcross, GA, USA), from homogenized *F. virsoides* apical fronds (~50 mg), according to the manufacturer's instructions. RNA concentration and quality were assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

The SENSE mRNA-seq Library Prep KitV2 (Lexogen, Vienna, Austria) was used for the preparation of cDNA libraries, following the manufacturer's protocol, which allows selection of only polyadenylated mRNAs by means of oligo d(T) magnetic beads. cDNA libraries were sent to Admera Health LLC (South Plainfield, NJ, USA), where they were sequenced on a single lane of an Illumina HiSeq 2500 platform (Illumina, Davies, CA, USA). Sequencing was performed in paired-end (PE) mode, with 151 cycles, aiming at a target sequencing depth of 8−12 Mln paired-end reads for each sample (4−6 Mln reads for each direction).

Table 1
Experimental design and samples. Sample codes match the files name of the sequence datasets stored in the SRA database. Note that for each time point and experimental condition, three biological replicates were sampled. These are indicated by the suffix "1", "2" and "3", placed after the code indicating the treatment ("C", "G", "N" and N + G″).

Treatment	Day	Sample codes		
Control (C)	1	C1_D1	C2_D1	C3_D1
	3	C1_D3	C2_D3	C3_D3
	6	C1_D6	C2_D6	C3_D6
Glyphosate (Roundup®) (G)	1	G1_D1	G2_D1	G3_D1
	3	G1_D3	G2_D3	G3_D3
	6	G1_D6	G2_D6	G3_D6
Nutrients (N)	1	N1_D1	N2_D1	N3_D1
	3	N1_D3	N2_D3	N3_D3
	6	N1_D6	N2_D6	N3_D6
Nutrients + Glyphosate (N + G)	1	N1+G _D1	N2+G _D1	N3+G _D1
	3	N1+G _D3	N2+G _D3	N3+G _D3
	6	N1+G _D6	N2+G _D6	N3+G _D6

2.3. De novo transcriptome assembly and annotation

The CLC Genomics Workbench ver. 11.0 (Qiagen Aarhus, Denmark) was used to perform the trimming procedure, which enabled the removal of adapters (i.e. short sequence stretches which flank the DNA insert and include the sequencing primers binding sites and library barcodes) and low-quality bases (the quality score threshold was set to 0.01). Residual reads shorter than 75 bp were also discarded. The *de novo* transcriptome assembly was obtained with Trinity ver. 2.4.0 using default parameters, setting the minimum allowed contig length to 200 nt (Grabherr et al., 2011). The quality of the initial draft assembly was further improved by identifying and removing contigs (i.e. the assembled consensus cDNA sequences) resulting from exogenous contamination or poorly represented RNAs. First, we ran the EvidentialGene tr2aacds ver. 2017.12.21 tool (Gilbert, 2016) to reduce the redundancy of the transcriptome assembly. Then, contigs corresponding to mtRNAs and cpRNAs were identified with BLASTn (Altschul et al., 1990) using the available mitochondrial and plastidial genomes of the congeneric species *F. vesiculosus* (NC_016735.1 and NC_007683.1). Then, the cleaned sequencing reads were mapped to the reference transcriptome with the following parameters: mismatch cost 2, insertion cost 3, deletion cost 3, length fraction 0.75, and similarity fraction 0.98. Contigs achieving very low sequencing coverage, i.e., cumulatively accounting for the lowest 10th percentile of mapped reads, were flagged as potential exogenous contaminants or as RNAs with limited biological significance and therefore discarded. The quality of the transcriptome was evaluated with BUSCO ver. 3.0.2 (Simão et al., 2015), verifying the presence and completeness of the orthologous sequences included in OrthoDB v. 10.1 Eukaryote dataset (Kriventseva et al., 2019, p. 10). The transcriptome was functionally annotated using Trinotate 3.1.1 (Bryant et al., 2017).

2.4. Differential gene expression analysis

Clean reads obtained for each sample were mapped against the reference transcriptome with the CLC Genomic Workbench with the following parameters: mismatch cost 2, insertion cost 3, deletion cost 3, length fraction 0.75, and similarity fraction 0.98; expression values were calculated based on the Transcript Per Million (TPM) metric (Wagner et al., 2012). Subsequently, differentially expressed genes (DEGs) were identified by comparing paired control and treated samples with a generalized linear model (GLM)-based analysis. DEGs were identified based on the following criteria: |fold change| > 2 and false discovery rate (FDR)-corrected p-value ≤ 0.05. TPM gene expression levels of selected DEGs were extracted for graphical representation in heat maps, created by hierarchical clustering of the genes, based on Euclidean distance.

DEGs were subjected to gene set enrichment analyses based on hypergeometric tests on Gene Ontology (GO) annotations (Falcon and Gentleman, 2008) with the following thresholds: FDR-corrected p-value≤0.05, number of DEGs per GO > 3.

3. Results

3.1. De novo transcriptome assembly and annotation

The raw sequencing output, deposited under the BioProject accession ID PRJNA524465 and consisting of approximately 8.5×10^8 reads, was reduced to approximately 4.3×10^8 reads following the trimming procedure. The *de novo* assembled non-redundant reference transcriptome comprised 86,413 contigs,

with mean and median length of 550.59 and 384 nucleotides, respectively. The assessment of the quality of the assembly revealed a high level of completeness with the detection of 87.2% eukaryote BUSCOs and a satisfactory degree of integrity of the assembled transcripts and with a higher representation of complete (i.e., encoding full-length proteins) than fragmented mRNAs (54.5% vs 32.7%, respectively). A total of 12,904 contigs were annotated with at least one Pfam conserved domain, 14,127 with GO cellular component terms, 15,160 with GO molecular function terms and 14,084 with GO biological process terms. Overall, 18,637 contigs (21.6% of the total) could be annotated.

3.2. Differential gene expression analysis

The gene expression analysis, which compared treated samples with untreated paired controls, revealed largely different trends of differential gene expression for the three treatments applied. In general, the alterations induced by nutrients (N) were of a limited entity, both in terms of upregulation and downregulation at all time points, even though a time-dependent effect was detectable (8, 26 and 59 DEGs were detected at D1, D3, and D6, respectively; see Fig. 1). As discussed below, exposure to a nutrient-enriched medium had, however, a positive time-dependent effect on the expression of several gene networks which, in most cases did not reach the threshold-fold change value of significance.

Comparatively, the two treatments that included glyphosate led to a substantial alteration of gene expression, even though the differences with N were negligible at D1, where a small number of DEGs was found in both the N + G (4 DEGs) and G (10 DEGs) groups. The G treatment had a greater effect at later time points, as evidenced by the differential expression of 354 genes at D3 and 563 genes at D6. Comparatively, the N + G treatment had a lower impact on gene expression, with 193 and 147 DEGs being observed at D3 and D6, respectively.

The glyphosate treatment mostly resulted in downregulation, as clearly evidenced by the disproportion between negatively and positively regulated genes (287 vs 67 at D3 and 537 vs 26 at D6). On the other hand, the alterations due to the N + G treatment were more balanced (118 up- and 75 and downregulated genes at D3, 50 up- and 97 and downregulated genes at D6). The vast majority of the DEGs that displayed a downregulation trend in response to N + G were shared with G, both at D3 (62 out of 75) and D6 (91 out of 97) (with 27 shared by all four samples), but were not affected by N (data not shown). On the other hand, a considerably lower overlap could be observed for the upregulated DEGs, with only a single shared gene among the four samples exposed to glyphosate (Fig. 1). Nutrient supplementation seemed to have a somewhat higher impact on upregulation, as evidenced by the significant overlap observed between the upregulated DEGs responsive to N + G at D3 and D6, compared with the complete lack of overlap between those downregulated in the same samples.

3.3. Functional enrichment of DEGs

To investigate the biological function and involvement in functional pathways, all sets of DEGs in response to each treatment (N, G, and N + G) and experimental time point (D1, D3, and D6) were tested for the enrichment of Gene Ontology (GO) annotations. The overrepresented GO terms that identified, as supported by enrichment FDR-corrected p-values < 0.05, are shown in Fig. 2.

Consistent with the low number of DEGs identified in response to the N treatment at all time points, no enriched GO term could be detected.

Conversely, 26 and 22 GO terms were linked with the DEGs found in response to G at D3 and D6, respectively. These annotations refer to different biological processes, molecular functions, and cellular components, which can be grouped into two broad functional categories. The first category includes GO terms related to photosynthetic processes, with particular reference to the GO terms "photosynthesis", "light-harvesting" (GO:0009765, GO:0009768, and GO:0009645), and associated processes ("protein-chromophore linkage", GO:0018,298 and "one-carbon metabolic process", GO:0006730, associated carbonic anhydrase). Although both "photosystem I" (GO:0009522) and "photosystem II" (GO:0009523) were significantly altered, the latter was the most strongly affected (Fig. 2). Several plastidial components (e.g., the "chloroplast thylakoid membrane", GO:0009535), as well as "chlorophyll-binding" (GO:0016,168) and "response to low light intensity stimulus" (GO:0009645), were severely affected. As will be discussed in detail below, these alterations were linked to a

	Time point		
	D1	D3	D6
N	8 (0↑, 8↓)	26 (14↑, 12↓)	59 (39↑, 20↓)
N+G	4 (0↑, 4↓)	193 (118↑, 75↓)	147 (50↑, 97↓)
G	10 (5↑, 5↓)	354 (67↑, 287↓)	563 (26↑, 537↓)

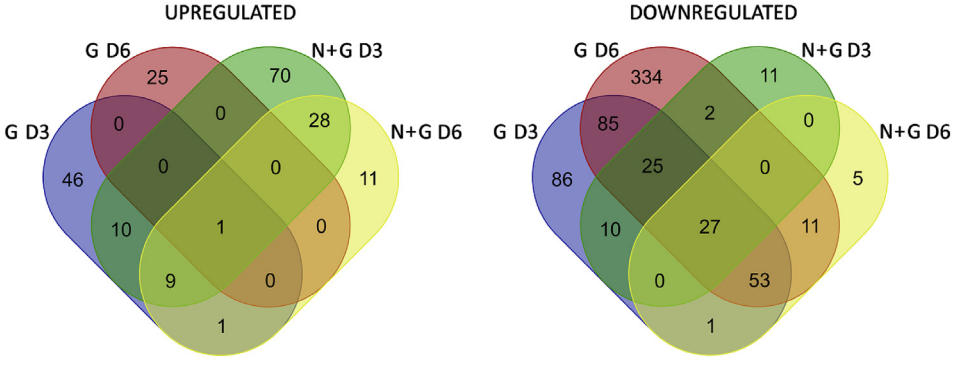

Fig. 1. Number of DEGs identified. Up- and downregulated genes are shown in red and blue, respectively. See Table 1 for abbreviations. The two Venn diagrams display the overlap among the up-and downregulated DEGs identified at D3 and D6 in the G and N + G experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

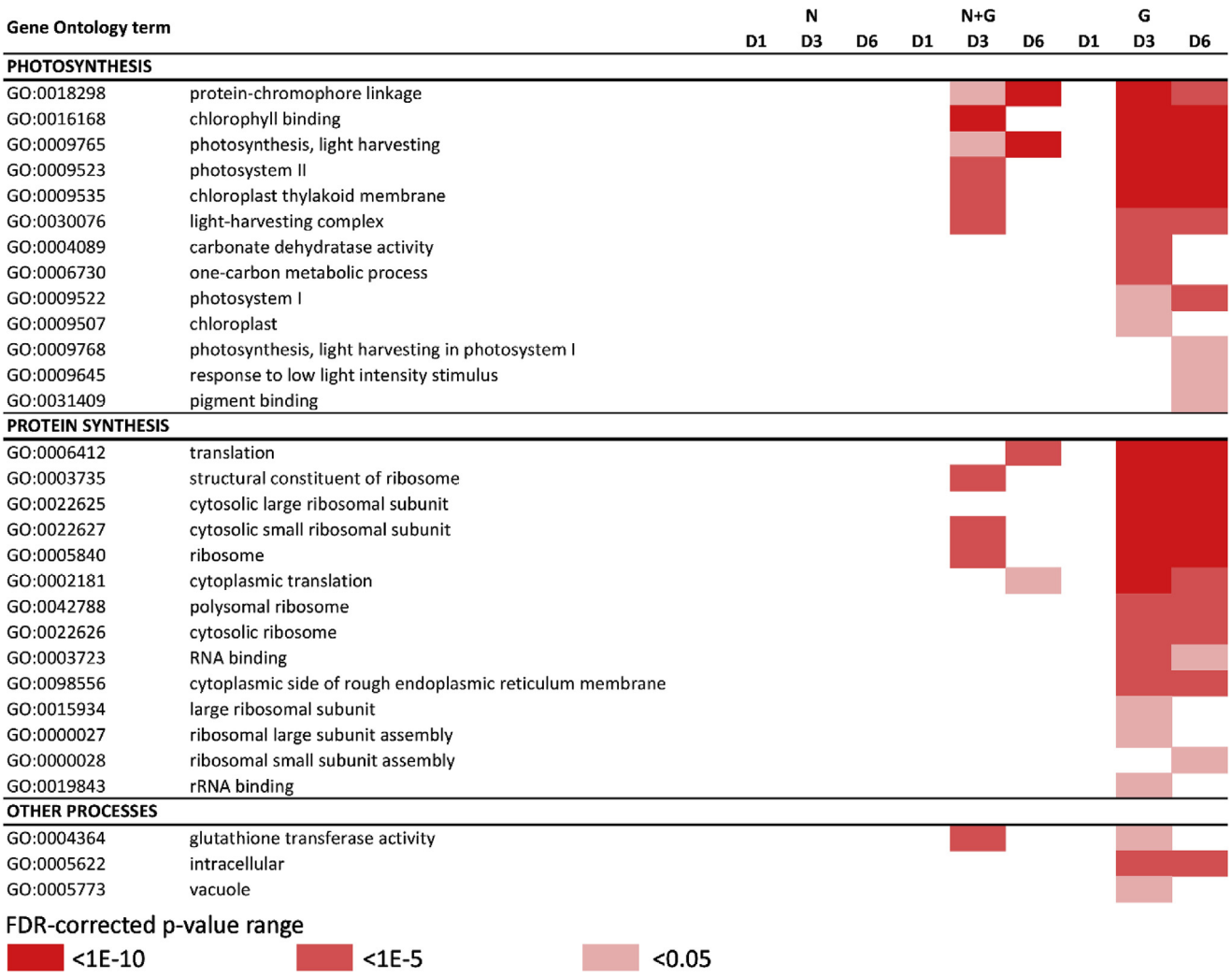

Fig. 2. Summary of the enriched Gene Ontology terms among the DEGs detected in Glyphosate (G), Nutrients (N) and Glyphosate + Nutrient (N + G) treatments. Gene Ontology annotations have been subdivided into three broad categories, i.e., photosynthesis, protein synthesis and other processes. The detailed enrichment p-values are provided in Table 1S (see Supplementary material).

marked downregulation of several genes associated with photosynthesis-related processes or encoding protein components of the photosystems.

The second broad category of DEGs identified in response to the G treatment includes those involved in protein translation (GO:0006412 and GO:0002181) and related processes (i.e., the assembly of ribosomal units, GO:0000027 and GO:0000028) (Fig. 2). Both ribosome subunits (GO:0022,625 and GO:002262) and associated components (GO:0003735) were strongly affected by downregulation, mirroring the situation observed for photosynthesis-related genes. Only a small number of other GO terms were significantly enriched in response to G, most notably "glutathione transferase activity" (GO:0004364), only mildly affected at D3, and "intracellular" (GO:0005622).

In accordance with the weaker effect of the exposure to N + G compared with G and with the significant overlap among the DEGs found in response to both treatments (see Fig. 1), the enriched GO terms associated with the N + G treatment at both D3 and D6 were similar to those described above for G (Fig. 2). Overall, the perturbation was minor, and often, the fold change and p-values associated with altered genes did not achieve the threshold of significance, suggesting a mitigating effect of nutrient supplementation towards the detrimental effects of glyphosate exposure.

3.4. Detailed overview on the expression of photosynthesis-related genes

The regulation of genes involved in the processes mentioned above was then further characterized to elucidate the molecular mechanisms activated or repressed in response to glyphosate exposure in the presence or absence of nutrient supplementation.

As shown in Fig. 3, all the photosynthesis-related genes followed a similar trend of expression with highly limited or nearly negligible alterations at D1 and highly significant perturbations at D3 and D6. Glyphosate exposure strongly repressed the expression of many structural components of the photosystems of brown algae, including chlorophyll- and fucoxanthin-binding proteins (Alberte et al., 1981; Caron et al., 2001). Moreover, the expression of several essential enzymes involved in carbon interconversion (i.e., carbonic anhydrases) (Badger and Price, 1994) and energy production (e.g., GAPDH) (Marri et al., 2005) was also strongly repressed, consistent with the data reported in Fig. 2. On average, the expression levels of photosynthesis-related genes decreased by

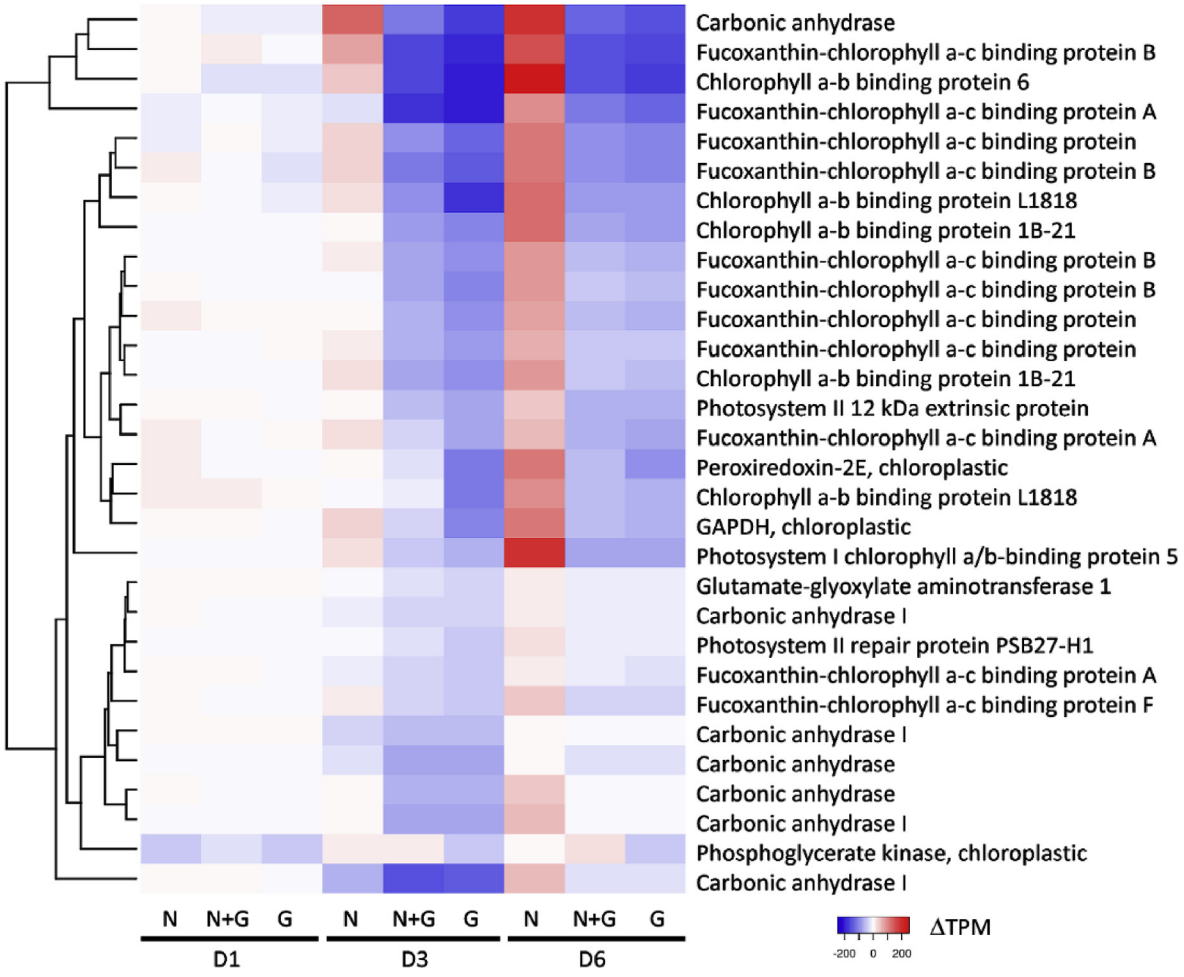

Fig. 3. Heat maps showing the expression trends of the DEGs related to photosynthesis (see Fig. 2). The order of the DEGs on the Y axis is based on hierarchical clustering and Euclidean distance of the ΔTPM obtained through the comparison between control and treated samples. For simplicity, only the DEGs with a ΔTPM >30 were included in this graph.

~83% at D3 and ~95% at D6. Although this marked downregulation was also evident in the N + G treatments, it was of a minor entity, i.e., ~46% at D3 and ~73% at D6, indicating a partial mitigating action of nutrient addition.

These observations can be explained by the positive effect that exposure to nutrient-only medium (N) had on the regulation of the same processes in *F. virsoides*. N treatment led to an average increase in gene expression by ~6% at D3 and ~45% at D6 (Fig. 3). Despite the presence of a clear upregulation trend, the majority of such genes did not reach the FC threshold (i.e., 2), explaining the lack of detected enriched GO annotations in the N treatment (Fig. 2).

All treatments displayed a strong time dependency with minimal effects on the expression profiles of photosynthesis-related genes at D1 (as suggested by the very low number of DEGs, see Fig. 1) and a significant impact at D3 followed by a further shutdown (in N + G and G) or potentiation (in N) at D6.

3.5. Detailed overview on the expression of protein synthesis-related genes

The protein synthesis-related genes displayed a general expression trend that was highly similar to that followed by photosynthesis-related genes (Fig. 4). Glyphosate exposure led to a decrease in the expression of all protein translation-related genes by ~72% at D3 and ~87% at D6. As in the case of photosynthesis, this repression was partially mitigated by exposure to nutrient-enriched medium (N + G), with decreases on the order of ~32% and ~51% at D3 and D6, respectively. These observations concerning the mitigating effect of N were further supported by the finding that this treatment had a highly significant, time-dependent impact on the expression of protein synthesis-related genes, with an average increase of ~6% at D3 and 57% at D6.

The vast majority of the downregulated genes in the glyphosate samples were structural components of the large and small ribosome subunits (Fig. 4), confirming the results of the hypergeometric tests (Fig. 2). However, other important related genes were also markedly affected. These genes include the two eukaryotic translation initiation factors 4 E and 5 (Duncan et al., 1987; Das et al., 2001) and the dbp2 helicase (involved in ribosome biogenesis) (Martin et al., 2013). In parallel with a decrease in the expression of genes linked with protein synthesis-related processes, some key genes involved in mRNA and miRNA production and maturation were also strongly repressed. These genes included elongation factor 1 alpha (Sasikumar et al., 2012), poly(A)-binding protein (fundamental in pre-mRNA maturation) (Mangus et al., 2004), mago nashi homologue 2 and NHP2-like protein 1 (both components of the spliceosome complex) (Nottrott et al., 1999; Singh et al., 2013), and ribonuclease III/Drosha (fundamental for miRNA biogenesis) (O'Brien et al., 2018).

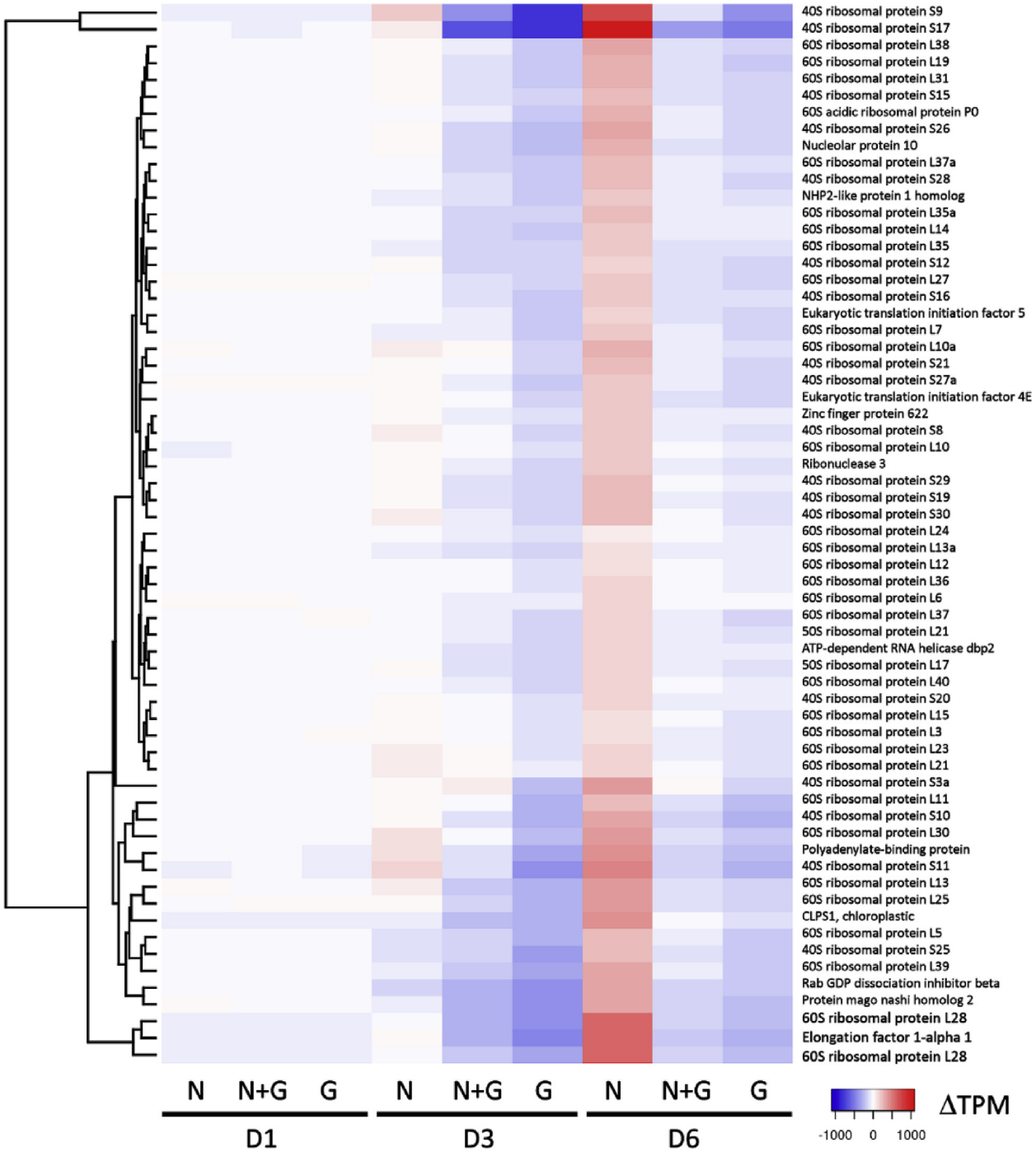

Fig. 4. Heat maps showing the expression trends of the DEGs related to protein synthesis (see Fig. 2). The order of the DEGs on the Y axis is based on hierarchical clustering and Euclidean distance of the ΔTPM obtained through the comparison between control and treated samples. For simplicity, only the DEGs with a ΔTPM >80 were included in this graph.

3.6. *Other relevant differentially expressed genes*

Although the overwhelming majority of the DEGs were linked to the two major broad categories outlined in the previous sections (see Fig. 2), several other cases are worth mentioning due to their possible impact on the physiological response of *F. virsoides* to glyphosate exposure.

The vast majority of these DEGs displayed very similar expression trends compared with those involved in photosynthesis and protein synthesis, with a significant downregulation in G treatments (even though the time-dependent effect was often weak), somewhat mitigated by the positive effect of nutrient availability in N + G (Fig. 5). Despite not being formally associated with the gene ontology terms reported in Fig. 2, several DEGs were implicated either in translation or in photosynthesis processes. For example, the Clp protease, the prefoldin subunit 6, and the peptidyl-prolyl cis-trans isomerase H have a role in aiding the folding of newly synthesized proteins (Gottesman et al., 1990; Horowitz et al., 1997; Vainberg et al., 1998). In addition, the downregulation of the DNA methyltransferase 1-associated protein

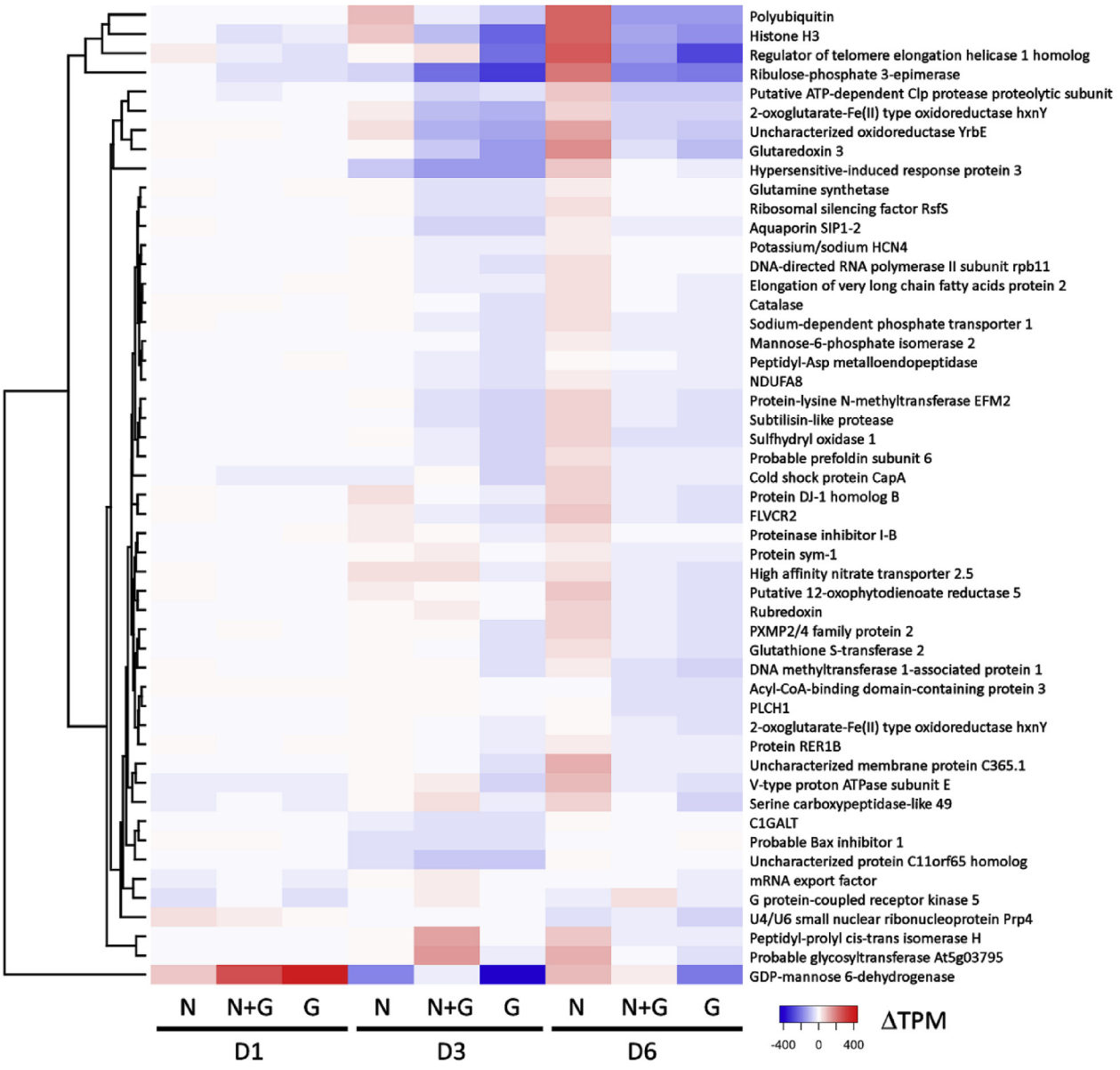

Fig. 5. Heat maps showing the expression trends of the DEGs not linked to photosynthesis and protein synthesis (i.e. other processes, see Fig. 2). The order of the DEGs on the Y axis is based on hierarchical clustering and Euclidean distance of the ΔTPM obtained through the comparison between control and treated samples. For simplicity, only the DEGs with a ΔTPM >30 bearing an annotation were included in this graph.

1, splicing factor ribonucleoprotein Prp4, and RNA polymerase II subunit revealed a partial impairment of mRNA synthesis.

Similarly, several other genes involved in chloroplast biogenesis (i.e., DJ-1 homologue B) and energy metabolism, probably linked with the reduced efficiency of the photosynthesis process, were also repressed. These genes include ribulose-phosphate 3-epimerase, part of the Calvin cycle (Nowitzki et al., 1995), mannose-6-phosphate isomerase 2, a fundamental regulator of glycolysis (Gao et al., 2005), V-type proton ATPase subunit E, and NDUFA8, both of which are involved in mitochondrial respiration (Emahazion et al., 1998; Lu et al., 2001). Other proteins, such as glutamine synthetase and the high-affinity nitrate transporter 2.5, are involved in ammonia assimilation.

Other downregulated genes suggest a reduction in the rate of DNA replication and cell division, i.e., glutaredoxin (essential for nucleotide biosynthesis) (Holmgren, 1979), the regulator of telomere elongation helicase 1 (fundamental for the DNA repair process) (Youds et al., 2010), and the mRNA export factor (a key factor in mitotic spindle formation) (Blower et al., 2005). Several enzymes involved in redox homeostasis, such as sulfhydryl oxidase 1, catalase, rubredoxin, microsomal glutathione S-transferase 3, and a 2-oxoglutarate-Fe(II)-type oxidoreductase, were significantly downregulated in glyphosate-exposed fronds. However, few contigs annotated as glutathione S-transferase were markedly upregulated in N + G, indicating the functional impairment of this biological process (Fig. 2). One of the most heavily responsive DEGs was GDP-mannose 6-dehydrogenase, a key enzyme in the biosynthesis of alginate (Tenhaken et al., 2011), which underwent extreme fluctuations in response to all treatments and was markedly repressed in G.

Finally, another effect of glyphosate exposure was the downregulation of a small number of typical stress-induced proteins with protective functions, including the hypersensitive-induced response protein 3 (Li et al., 2019, p. 1), a subtilisin-like protease

(Figueiredo et al., 2014), the cold shock protein CapA (Berger et al., 1997, p. 55), and the sym1 protein (Trott and Morano, 2004).

4. Discussion

To the best of our knowledge, we report the first functionally annotated transcriptome for *F. virsoides*, which represents a valuable addition to the limited amount of omic resources available for the Phaeophyceae (Cock et al., 2010). This resource can be employed as a reference for gene expression studies, as well as a catalogue of the mRNA expressed in this species.

We exploited this resource to conduct a differential gene expression analyses to investigate the effects of the exposure to nutrients, glyphosate, and their combination (N, G, and N + G) on *F. virsoides*, in comparison with paired untreated controls, highlighting different temporal patterns of gene expression for the three treatments. This approach was used to fill the gap of knowledge that exists concerning the regulatory mechanisms involved in glyphosate stress processes in marine flora, and macroalgae in particular, which remain completely unknown, despite the broad literature available on this subject for terrestrial plants (e.g., Funke et al., 2006; Zhu et al., 2008; Piasecki et al., 2019b).

We need to remark that the RNA-seq approach we used can only provide a snapshot of the levels of mRNA expressed at a given time point and that such changes are not necessarily coupled with alterations of comparable magnitude at the proteomic and metabolomics levels, which can be affected by other factors that cannot be directly measured with this approach. Nevertheless, gene expression profiles can provide a valuable support to the observations about the time-dependent decline in the photosynthetic efficiency of glyphosate-exposed *F. virsoides* thalli collected, for the very same biological samples here subjected to RNA-seq, in a previous study (Falace et al., 2018).

We identified just a few DEGs after one day of exposure under all experimental conditions, which are likely of limited biological interest due to their minor expression shifts and almost non-existent overlap with subsequent time points. However, these subtle changes do not imply that a short-term exposure to glyphosate is not harmful to *F. virsoides*, as relevant physiological alterations may have been already triggered, but not visible yet at the transcriptional level at this time point. In support of this consideration, we demonstrated in a previous study that the exposure of *F. virsoides* for 24 h the same concentration of glyphosate used in this study (with and without nutrient addition) was sufficient to produce a significant reduction of photosynthetic efficiency, causing irreversible damage and ultimately leading to the death of thalli (Falace et al., 2018).

The nutrient-enriched treatment (N), implemented in this study to mimic the significant nutrients inputs that characterize the marine coastal waters, salt marshes and estuarine zones where *F. virsoides* lives (Orlando-Bonaca et al., 2013), only led to minor alterations of gene expression. The few DEGs detected were not associated with over-represented GOs, confirming the scarce effects exerted on *Fucus* by nutrient enrichment alone, previously evidenced by a meta-analysis (Wahl et al., 2011).

Glyphosate treatment (G) mostly affected gene downregulation, with an increasing trend of DEGs from the third day to the sixth day of exposure. An in-depth analysis of the most significantly enriched functional annotation associated with the DEGs revealed that the glyphosate-only treatment (G) led to a significant down-regulation of genes involved in several key biological processes, i.e. photosynthesis, protein synthesis, energetic metabolism, stress response pathways, membrane and cytoskeleton structure. However, when glyphosate was administered in combination with nutrients (N + G), its impact on transcription was softened, but not completely reverted, both in terms of number of DEGs and strength of repression. It is worth noting the remnant populations of *F. virsoides* are restricted to areas close to freshwater inputs (i.e., rivers) or to the most confined parts of the lagoons (i.e., Venice and Grado lagoons), which are characterized by high concentrations of nutrients. The partial mitigation of the most severe effects of glyphosate on downregulation in the N + G experimental group suggests that the availability of nutrients may assist *F. virsoides* in overcoming biotic/abiotic stresses.

Glyphosate exposure led to a near-complete shutdown of the expression of several genes involved in photosynthesis and its related processes (e.g., light harvesting, response to low light intensity stimulus, chlorophyll binding) after six days. In particular, we observed a strong repression of the expression of carbonic anhydrases and light-harvesting proteins (i.e., chlorophyll *a*/c- and fucoxanthin-binding proteins). These molecular data support the negative impact of glyphosate previously reported at the morphological (i.e., chlorosis) and eco-physiological levels (i.e., photosynthetic efficiency measured on the very same biological samples analyzed in this study) in *Fucus* (Falace et al., 2018) and other macroalgal species (Pang et al., 2012; Kittle and McDermid, 2016). Moreover, these findings are in line with the well-documented decline in the photosynthetic rate (i.e., Mateos-Naranjo et al., 2009; Vivancos et al., 2011; Yanniccari et al., 2012; Zobiole et al., 2012) and the inhibition of chlorophyll synthesis (Zobiole et al., 2011; Huang et al., 2012; Gomes et al., 2016) documented in vascular plants in response to glyphosate exposure.

In *Ectocarpus* and *Fucus vesciculosus*, photoinhibition and reduction of photosynthetic rates are employed as energy-saving mechanisms, to lower the cost of acclimation in response to abiotic stress, as this can redirect more energy to stress-response processes (Dittami et al., 2009; Rugiu et al., 2020). On the other hand, the inhibition of the shikimate cycle, recently demonstrated in the thalli of *F. virsoides* exposed to glyphosate (Felline et al., 2019), and the consequent prevention of plastoquinone synthesis, have been implicated in the reduction of carotenoid content in glyphosate-sensitive plants (Sandmann et al., 2006). This factor, together with an increased production and accumulation of reactive oxygen species (ROS) (Schönbrunn et al., 2001), may have a negative impact on photosynthesis (Gomes et al., 2017). Most likely, the lowered photoprotection capability, triggering damage to Photosystem II, can be held accountable for the reduced maximal PSII photochemical yield (i.e. a proxy of PSII integrity) recorded by Falace et al. (2018) and Felline et al. (2019) in *F. virsoides*. It is important to remark that a nearly immediate reduction in photosynthetic efficiency was measured in the same biological samples analyzed in this study in response to short-term (24 h) exposure to glyphosate. This decrease in photosynthetic efficiency peaked at −95% after six days, mirroring the timing we report in this study for the shutdown of the expression of several photosynthesis-related genes. These toxic effects were irreversible and led to the death of this seaweed within approximately one week (Falace et al., 2018).

The second major network of genes negatively impacted by glyphosate involved in protein synthesis and related processes. As for the photosynthesis-related genes, the strong down-regulation was mitigated when glyphosate was administered in combination with nutrients (N + G). The majority of downregulated genes encoded structural components of the ribosome, or proteins involved in ribosome biogenesis/assembly, translation (e.g., the folding of newly synthesized proteins), transcription, and post-transcriptional processes. As observed in terrestrial plants (Komives and Schroder, 2016; Wang et al., 2016a,b), *F. virsoides* exposed to glyphosate may suffer from the depletion of the aromatic amino acid pool (i.e., L-phenylalanine, L-tyrosine, and L-

tryptophan) due to the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase and the interruption of the shikimate pathway (Felline et al., 2019). This depletion may lead to reduced synthesis of protein and secondary compounds (i.e., auxins and polyphenols) necessary for growth maintenance (Sáenz et al., 1997; Siehl et al., 1997).

Consistently with the previously evidenced progressive decline in the physiological conditions of *F. virsoides* exposed to glyphosate (Falace et al., 2018), the expression of genes encoding components of cell wall and cytoskeleton (i.e., actin, tubulin, cellulose synthase, and GDP-mannose dehydrogenase) was also impaired in the response to treatments with glyphosate (G). The production of ROS and the related oxidative stress can alter actin and tubulin synthesis, thereby affecting the strength of the cytoskeleton and the cell volume (Bagniewska-Zadworna, 2008; Livanos et al., 2014). In particular, the expression of GDP-mannose dehydrogenase, which is involved in the synthesis of alginates (Zhang et al., 2016), the main polysaccharides of the brown algal cell wall, was severely altered in all treatments. Metabolomic analyses carried out on thalli exposed to different concentrations of glyphosate revealed an accumulation of glyphosate in *F. virsoides* (Felline et al., 2019), which may be consistent with structural alterations of this important barrier offered by this polysaccharide against the surrounding milieu, particularly in the osmotic environment (Rugiu et al., 2020).

In terrestrial plants, the blockage of the shikimic acid pathway, which induces ROS overproduction (Schönbrunn et al., 2001), leads to the accumulation of shikimic acid and reducing power (NADPH + H) (Ahsan et al., 2008; Maroli et al., 2015; Piasecki et al., 2019a). Indeed, glyphosate resistance in plants is linked to the capability to avoid oxidative stress through the production of antioxidants (Maroli et al., 2015). Only a small number of known stress response genes were upregulated in *F. virsoides*, even though they displayed an unusual expression pattern, with the highest fold-change values being attained at day three in response to the treatment with glyphosate added with nutrients (N + G). The most notable ones encoded proteins belonging to the "glutathione S-transferase" family, a class of detoxifying enzymes against oxidative stress, that metabolizes several xenobiotics (i.e., herbicides, heavy metals) through conjugation, converting them into substances of low toxicity (Cataneo et al., 2003; Miteva et al., 2003).

Since we used the commercial Roundup formulation, one of the limitations of this study is that it is not possible to separate the toxic effects of glyphosate from those exerted by the surfactant (or from their combination). Although surfactants are formally declared inert (i.e. they are not the active compound of herbicides), their formulation is classified as reserved for regulatory purposes (Mesnage et al., 2015). Several studies have previously shown that they can enhance the toxicity of glyphosate (Hanana et al., 2012; de Liz Oliveira Cavalli et al., 2013; Wagner et al., 2013; Cattani et al., 2014; de Brito Rodrigues et al., 2017), or even be more toxic than glyphosate itself (Williams et al., 2000; Bradberry et al., 2004). Since negative effects of herbicide surfactants previously reported in microalgae (Hampel et al., 2001), we cannot exclude that the surfactants included in the Roundup formulation are toxic to *F. virsoides*.

5. Conclusion

With this study, we provide molecular support to the observations gathered by two previous studies, which highlighted the high susceptibility of *F. virsoides* to glyphosate exposure on the morphological, eco-physiological and metabolomics levels (Falace et al., 2018; Felline et al., 2019). The strong decrease in photosynthetic efficiency and the metabolic impairment observed in these studies find a possible molecular explanation in the strong down-regulation of genes involved in photosynthesis and protein synthesis related processes, whose expression often decreased by 90/95%. Although the precise causes behind the progressive disappearance of *F. virsoides* in the Northern Adriatic Sea are still unclear, the widespread use of glyphosate in agriculture and the well-documented influx of associated toxic compounds in shallow marine coastal waters are thought to represent significant threats for macroalgal communities. Interestingly, we observed that nutrient supplementation had a somewhat mitigating effect on the most severe effects of glyphosate on gene downregulation, which may explain why the relict populations of *F. virsoides* are usually confined to areas characterized by high concentrations of nutrients.

Although this study supports the hypothesis that macroalgal communities are affected by agricultural land use, further studies will be required to address the fate of glyphosate in the marine environment and to dissect the possible role of other factors in the rapid decline in *F. virsoides* populations.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Credit author statement

Marco Gerdol: Methodology, Formal analysis, Visualization, Writing - original draft, Andrea Visintin: Investigation, Formal analysis, Sara Kaleb Conceptualization, Methodology, Investigation, Francesca Spazzali Investigation, Alberto Pallavicini: Methodology, Writing - original draft, Annalisa Falace Conceptualization, Methodology, Investigation, Writing - original draft, Funding acquisition

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