



Transcriptional analysis reveals induction of systemic resistance in tomato treated with *Chlorella* microalgae

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ABSTRACT

The application of algae-based products has been reported to promote plant growth and yield of tomato plants, especially by enhancing flowering. However, how microalgae (MA) affect plants at the molecular level remains elusive. The aim of this study was to elucidate the effects of live microalgae application on plant photosynthesis and the transcriptome of the unopened flower buds of tomato plants. Microalgae increased leaf temperature differential in tomato leaves but hardly affected photosynthesis. Contrary to our expectations, RNA-seq data revealed remarkable differential expression of several genes participating in responses to abiotic stresses but only a few genes involved in flowering or pollen/ spore development. Late Embryogenesis Abundant (LEA) proteins (mostly dehydrins), oleosins, ethylene, and abscisic-related genes, and Nascent Polypeptide-Associated Complex (NAC)-domain-containing proteins were upregulated. Genes involved in carbohydrate metabolism were also differentially expressed; glycolysis-related genes were upregulated, while those involved in sugar transport were downregulated. The only upregulated gene implicated in the induction of flowering was *Solyc07g006500.3.1* (encoding trehalose-6-phosphate synthase enzyme TPS1). Overall, microalgae treatment led to an empirical upregulation of genes involved in jasmonic acid, abscisic acid, and ethylene pathways which are all essential for abiotic stress response. This study shows that microalgae treatment primed plants by inducing the expression of genes involved in response to abiotic stress, especially cold and water stress, as well as pathogen attack.

1. Introduction

Using microorganisms as plant biostimulants is gradually becoming feasible, with numerous studies demonstrating their potential to promote growth across diverse plant species. Biostimulants are substances of biological origin whose application to plants or rhizosphere stimulates the natural processes that enhance nutrient uptake and efficiency, tolerance to stresses, crop quality, and yield [1]. Therefore, biostimulants are eco-friendly and excellent substitutes for chemical fertilizers/pesticides that negatively affect the environment.

Among the microorganisms found to be natural biostimulants, microalgae (MA) stand out as an excellent candidate because of its wide biotechnological application. For example, microalgae can be cultivated in biological wastewater, promoting the renewable use of natural resources [2]. Such wastewater treatment plants can simultaneously produce bio-ammonia [3] or bio-hydrogen when algae strains are mixed

with their bacteria partner [4]. For instance, Low et al. [5] reported a design incorporating microalgae to take up nutrients, especially phosphorous, from palm oil mill effluent (POME) and produce biofuel. The microalgae biomass from such systems can be harvested for extraction of other valuable products. However, farmers can still use recycled water directly for crop irrigation or hydroponic farming. Thus, incorporating microalgae in biological wastewater treatment would allow cyclic production that could sustainably meet global food and energy demands while reducing the environmental pollution.

When biostimulants are applied to plants or growth media, growth promotion usually manifests in the form of increased vegetative growth, early flowering, improved crop quality, and increased yield [6–8]. Typically, the growth promotion is linked to the microorganism's capacity to improve a plant's nutrient use efficiency and soil porosity, thus influencing water availability and uptake, soil microbial communities, and protecting plants from disease-causing pathogens. Microalgae act in

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various ways; cyanobacteria fix nitrogen, while eukaryotic MA strains photosynthesize and release exopolysaccharides, thus increasing organic carbon content in soils. Thus, MA promotes plant growth by creating a conducive environment with sufficient nutrients.

In addition to growth promotion, microorganisms also trigger systemic-induced resistance in plants. Induced resistance refers to an enhanced defensive capability that a plant develops with appropriate stimulation [9]. Microorganisms, including bacteria, fungi, and viruses, have been found to trigger systemic resistance. For example, Ilham and colleagues reported that seed treatment of *Arabidopsis thaliana* with *Bacillus amyloliquefaciens* and *Trichoderma harzianum* also induced systemic resistance [53]. In two separate studies, *Bacillus subtilis* and *Bacillus cereus* AR156 applied via the soil drench method also had similar results in tomato and *A. thaliana*, respectively [54,55].

Recent studies have also shown the ability of MA and MA-derived substances to induce systemic resistance when different modes of application were used. Polysaccharides extracted from different cyanobacteria strains induced systemic resistance in tomatoes when sprayed on leaves [10], while sonicated extracts of various MA enabled tomato seedlings to cope with bacterial canker disease [11]. Suspensions of *Chlorella fusca* caused cytological changes such as accumulation of vesicles and thickening of cell walls in leaves of cucumbers when sprayed prior to infection with *Colletotrichum orbiculare* [12]. All these studies showed the activation of the salicylic, jasmonic, and ethylene defense mechanisms. These studies employed biochemical methods to measure key markers of the salicylic acid pathway as well as antioxidant enzymes such as peroxidases and, only one study used multi-omics approaches [13].

Growth promotion can be observed phenotypically through the assessment of plant height, diameter, leaf number, flower number, and fruit number, among other parameters. Induced resistance can be detected by challenging the plants with stress or pathogen infection, followed by disease index assessment and determination of infection markers. However, induced resistance can also be assessed using transcriptomic, proteomic, and metabolomics approaches. De novo data of transcriptome studies can then be used in designing experiments to gain deeper insights into the signaling pathways underlying upregulated defense-related genes and pathways. Most transcriptome studies on plant biostimulants investigate the leaf [14], and little is known about the flower bud transcriptome. This study targeting the unopened flowers could help explain why biostimulants promote early flowering resulting in early fruit harvest of treated plants compared to the untreated controls [15]. We applied live cells of eukaryotic MA on tomato plants via the soil drench method and then performed whole transcriptome sequencing of the unopened flower buds. The main objective was to reveal the effect of microalga application on plants at the molecular level by identifying plant genes with specifically altered expression levels. Since we had observed algae-induced early flowering in our previous studies [16], we hypothesized that MA might cause the upregulation of flowering genes in tomato plants. We also analyzed the photosynthetic performance of plants to complement our transcription studies as recommended for such studies. To the best of our knowledge, this is the first whole transcriptome study of the unopened flower buds from plants treated with algae biostimulants.

2. Materials and methods

2.1. Plant establishment and treatment

Solanum lycopersicum L. seeds of Vilma variety purchased from a retailer in Szeged, Hungary, were used for the studies. Plants were surface sterilized with 10 % hypochlorite solution for 5 min and then thoroughly washed with sterile distilled water (DW). The seeds were allowed to imbibe water for about 2 h. The seeds were then sown in a 12-well germination box containing moist soil and vermiculate in the ratio of 2:1. After germination, the seedlings were maintained in the

greenhouse on this platform for 2 weeks and respective treatments administered to the soil on weekly basis. Seedlings were then transplanted into 3 L pots (2 seedlings per pot) containing moist soil and vermiculate (2:1) which was moistened with a fertilizing solution, Solution 1, diluted 40 times. Solution 1 was prepared as follows: First, the following macronutrient stock solutions were prepared separately: 20.2 g/L KNO₃, 73 g/L CaCl₂ × 2H₂O, 24.6 g/L MgSO₄, 43.5 g/L K₂SO₄, 8.2 g/L Fe-Na-EDTA and 27.2 g/L KH₂PO₄ and 0.05 M H₃BO₃. Secondly, a microelement stock solution was prepared by adding 6.2 g MnSO₄, 10 g KCl, 1 g ZnSO₄ × 7H₂O, 1 g (NH₄)₂Mo₇O₂₄ × 4H₂O, 0.5 g CuSO₄ and 0.5 mL H₂SO₄ into water and topping it up to 1 L. The stock solutions and 800 mL distilled water (DW) were autoclaved separately. Finally, 25 mL of each of the macronutrient solutions and 1.35 mL micronutrient stock solution were added into 800 mL of sterile water to make Sol 1 stock solution. Plants were grown in the greenhouse at 24 °C to 26 °C and with a 16-h photoperiod. Each treatment had a total of 6 plants: 2 plants per pot, 3 pots per treatment. In total, there were 6 pots arranged in a randomized block design and the position on the bench constantly changed to ensure uniform exposure to environmental factors. Thus, each treatment had 6 biological replicates. Weekly treatment continued after transplantation until collection of unopened flower buds (about 3 weeks after transplantation and 5 weeks after initial planting). The treatment was therefore applied 5 times. The experiment was repeated two times. Transcriptome analysis was conducted with samples from the first experiment while validation with qPCR was done with samples from the two independent experiments.

2.2. Preparation of the algae for plant treatment

Under aseptic conditions, a sterile rod was used to scrub the surface of a fully grown *Chlorella* sp. MACC-360 lawn from a Tris-Acetate-Phosphate (TAP) [56], pH 7 agar plate. The rod was then dipped in a 50 mL Erlenmeyer flask containing 15 mL of TAP media. The flask was then placed in an algal growth chamber with the following conditions: 25 °C temperature, 16/8-h light/dark cycle, white light, and 180 rpm shaker speed. 5 mL of the culture was transferred after five days into a 100 mL Erlenmeyer flask containing 50 mL TAP media and placed in the growth chamber. The culture was allowed to grow for seven days. On the seventh day, 5 mL of the culture was transferred into a new conical flask containing 50 mL of TAP medium to initiate the next application's culture. 50 mL were used to prepare the algae treatment.

To prepare the soil drench treatment, 50 mL culture suspension (cells with their growth media) was diluted with sterile DW to a final volume of 1 L. This corresponds to a concentration of approximately 1 g/L wet biomass based on our previous method [16]. For the algae treatment, each pot received 300 mL of the algae suspension while the control pots received 300 mL of the DW.

2.3. Photosynthetic parameters analysis

Fluorescence-based measurement of photosynthetic parameters was done on plant leaves on a weekly basis with the Multispeq hand-held device [57]. This data was recorded for 5 weeks beginning at the first week after transplantation.

2.4. RNA extraction and sequencing

At the onset of flowering (3 weeks after transplanting), unopened flower buds were collected in triplicates. Sterile forceps and scalpels were used to cut out the unopened flower buds on the 5th week of plant growth since germination. The samples were immediately frozen in liquid nitrogen before RNA extraction with the Qiagen RNeasy plant kit (Qiagen). The genomic DNA was removed with Thermo Fisher's DNase1 according to manufacturer's protocol (Thermo Fisher). RNA concentration and purity was determined with Nanodrop and gel electrophoresis on 1 % agarose gel.

For RNA sequencing, an equal amount of RNA was added from each of the replicates to make a single pooled sample for each treatment. For each sample, 1 µg of total RNA was reverse-transcribed for 60 min at 42 °C and for 10 min at 75 °C in a 20 µL reaction volume using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA products were diluted 1:10 in RNase-free water (Lonza, Verviers, Belgium) and the RNA quality was assessed using an RNA ScreenTape on a TapeStation 4150 (Agilent Technologies), and the quantity determined with Qubit RNA Assay. In vitro fragment libraries were prepared using the TruSeq RNA Library Prep Kit v2 from Illumina; the libraries were sequenced on an Illumina NextSeq1000 NGS platform to generate 150 nt paired-end reads. All reads were uploaded to the National Center for Biotechnology Information (NCBI), Sequence Read Archive (SRA) database (PRJNA880331).

2.5. Differential expression analysis

Sequenced reads were first processed using rcorrector [58], a kmer-based error correction method for RNAseq data. The error-corrected reads were then trimmed using Trimmomatic v0.39 [59] with adapter sequences and keeping a quality score of 25 over a 5 base pairs (bp) sliding window. Reads shorter than 50 bp were discarded from further analysis. The trimmed reads were then mapped to reference *S. lycopersicum* transcripts v3.0 downloaded from ENSEMBL using Kallisto v0.46.1 [60]. Differential analysis on Kallisto quantified transcripts were carried out using generalized fold change (GFOLD) [61].

2.6. Gene ontology and functional enrichment analysis

Differentially expressed genes (DEGs) with a ± 1.5-fold change were subjected to functional and ontology enrichment studies to explore the functions of the genes. The upregulated and downregulated gene lists were analyzed separately.

Gene enrichment studies were done in Shiny GO version 0.76.3 with default settings [17]. The species selected was *S. lycopersicum*. All the transcripts identified in our RNA sequencing data were uploaded as the background unlike in other analysis platforms which used all genes in *S. lycopersicum*. This step was important to uncover pathways of a gene list with a few genes.

Functional profiling analysis was conducted in g: Profiler with default settings [62]. The Database for Annotation, Visualization and Integrated Discovery (DAVID) website was used to produce functional annotation clusters for both gene lists as well as identification of associated pathways [63,64].

2.7. Real time quantitative polymerase chain reaction (RT-qPCR)

Transcripts were randomly chosen to validate transcriptome data and primers designed with Primer Quest (Supplementary data, Sheet 1, Table 1). The RT-qPCR reactions were carried out in the CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). The PCR reactions were conducted in Hard-Shell® 384-well plates (thin-wall, skirted, clear/white; Bio-Rad, Cat. no: HSP3805); 3 µL of a master mix containing 0.15 µL forward primer, 0.15 µL reverse primer and 3 µL Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific) was aliquoted to the wells. Then, 3 µL cDNA of each sample was added to make 6 µL PCR mixture per well. For amplification, a standard two-step thermal cycling profile was used (35 s at 95 °C and 1 min at 58 °C) during 40 cycles, after a 2 min preheating step at 50 °C and 7 min at 95 °C. Finally, a dissociation stage was added with 65 °C to 95 °C at increment of 0.5 °C for 0.05 s. Data analysis was performed using Bio-Rad CFX Maestro (Bio-Rad) software and Microsoft Excel 2016. The relative mRNA levels normalized to the average of Actin and mRNAs expression was calculated using the $(2)^{-\Delta\Delta Ct}$ method. The mRNA level of the control/water-treated samples was used as control

Table 1
Emergence of the first flower since the Day of Planting (DAP).

| Time in 2021 | Treatment | | <i>Chlorella</i> sp. MACC-360 | |
|------------------|-------------|-------------|-------------------------------|-------------|
| | DW/Control | | First flower opening | DAP |
| 07 June - 14 Sep | 20-Jul | 43 | 15-Jul | 38 |
| 14 June-14 Sep | 30-Jul | 46 | 23-Jul | 39 |
| | Average DAP | 44.5 ± 2.12 | | 38.5 ± 0.71 |

(relative mRNA level: 1). All tested amplification efficiencies were in a narrow range and were not used in the data normalization. Data were averaged from two independent biological experiments with three technical replicates for each gene/sample combination.

3. Results and discussion

3.1. Flowering and photosynthetic performance

MA application hastened flowering in tomato plants. In two independent greenhouse experiments conducted at different times of the year, the first flower always emerged in the algae-treated plants at least 5 days before the control regime (Table 1).

For this reason, the number of flowers open at a given stage could not reflect the actual differences between treatments. However, it was evident that MA-treated plants were ahead in flowering, as depicted in Fig. 1, where all the flowers in the control (Fig. 1A) were freshly opened while some of the flowers in MA-treated plants were already withering (red arrows in Fig. 1B).

Moreover, both individual plants in the MA-treated pot had flowered by the 5th week of growth while, only one in the control pot had flowered. These trends were observed in all the pots in independent experiments and corresponded to the high standard deviation captured in Table 2. The standard deviation was higher in MA-treated plants due to the non-uniform initial flower opening but lower in the control plants as few flowers were open at any time for all plants during the initial flowering period. The flowering kinetics of plants treated with MA showed that MA caused enhanced flowering, although there was no significant difference in the number of open flowers at the scoring time [16]. Table 2 shows the raw statistics of the open flowering data.

Application of the MA did not significantly affect photosynthetic performance. Regardless, it increased leaf temperature differential (LTD), the ratio between leaf surface temperature and the environmental/ambient temperature (Fig. 2 a-d). Although it is not statistically significant, MA slightly enhanced photosynthesis by increasing photosystem II (PS II) quantum yield and decreasing both regulated (PhiNPQ) and non-regulated energy loss (PhiNO) (Fig. 2c). Soil Plant Analysis Development (SPAD) indicates a plant's nitrogen status and relative chlorophyll. In contrast to previous reports of increased SPAD with biostimulants application [14], no significant effect of *Chlorella* sp. MACC-360 on SPAD was observed in this study.

The differences in photosynthetic performance between algae-treated plants and control plants were subtle. This result could be because the plants were not under stress and their growth was optimal. However, the difference in LTD shows that the algae-treated plants were unaffected by environmental temperature instabilities. Increased LTD means an increased ability to withstand abiotic stresses such as water, as reported in tomato plants [18]. MA treatment affected LTD in maize which also had advanced root development relative to the control [19].



Fig. 1. Pictures of the plants in the second week of flowering. A. Control/distilled water (DW) and B. Treated with *Chlorella* sp. MACC-360. Red arrows show flowers that are withering already. The white arrow shows an open flower in one of the plants of the *Chlorella* sp. MACC-360 pot. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2

Raw statistics of the flowering data.

| Date of scoring | DW/control | | | <i>Chlorella</i> sp. MACC-360 | | |
|-----------------|------------|-----|----|-------------------------------|-----|----|
| | Mean | SD | N | Mean | SD | N |
| 26-Jul-2021 | 0.2 | 0.6 | 10 | 2.0 | 2.6 | 10 |
| 28-Jul-2021 | 0.6 | 1.3 | 10 | 3.7 | 3.3 | 10 |
| 30-Jul-2021 | 1.0 | 1.9 | 10 | 2.9 | 2.7 | 10 |
| 1-Aug-2021 | 1.4 | 1.8 | 10 | 3.5 | 3.0 | 10 |

Open flowers per day in plants with and without *Chlorella* sp. MACC-360. Values represent the mean per plant \pm standard deviation (SD). N = 10 plants.

3.2. Flower bud transcriptome results

3.2.1. Read correction, trimming, and mapping against reference

Overall, about 90 % of the sequence reads mapped back to the *S. lycopersicum* reference transcripts (Supplementary data, Sheet 1,

Table 2).

3.2.2. Gene enrichment studies: upregulated genes

Only 103 tomato genes had a ± 1.5 -fold change. In total, 12 GO biological (GO: BP) terms were significantly enriched; they clustered into two broad categories, cold-related and transcription-related terms. Response to cold was the most significant term. No molecular (MF) or cellular component (CC) terms were significantly enriched (Fig. 3).

Functional profiling in g: Profiler identified reproduction-related terms, one Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway term, and the cold stress-related terms (Table 3). Response to cold and cold acclimation remained the most significant terms.

The 4 genes involved in “response to cold acclimation” were one oleosin protein (Oleosin 18.2 kDa (LOC101263398)) and three dehydrins, namely; Embryogenic cell protein 40 (LOC101259487), dehydrin Ras-related (RAB)-18 protein and tomato abscisic acid and environmental stress-inducible protein (TAS)14 (Table 3). NAP2, a NAC domain-containing protein, was also involved in all the responses except

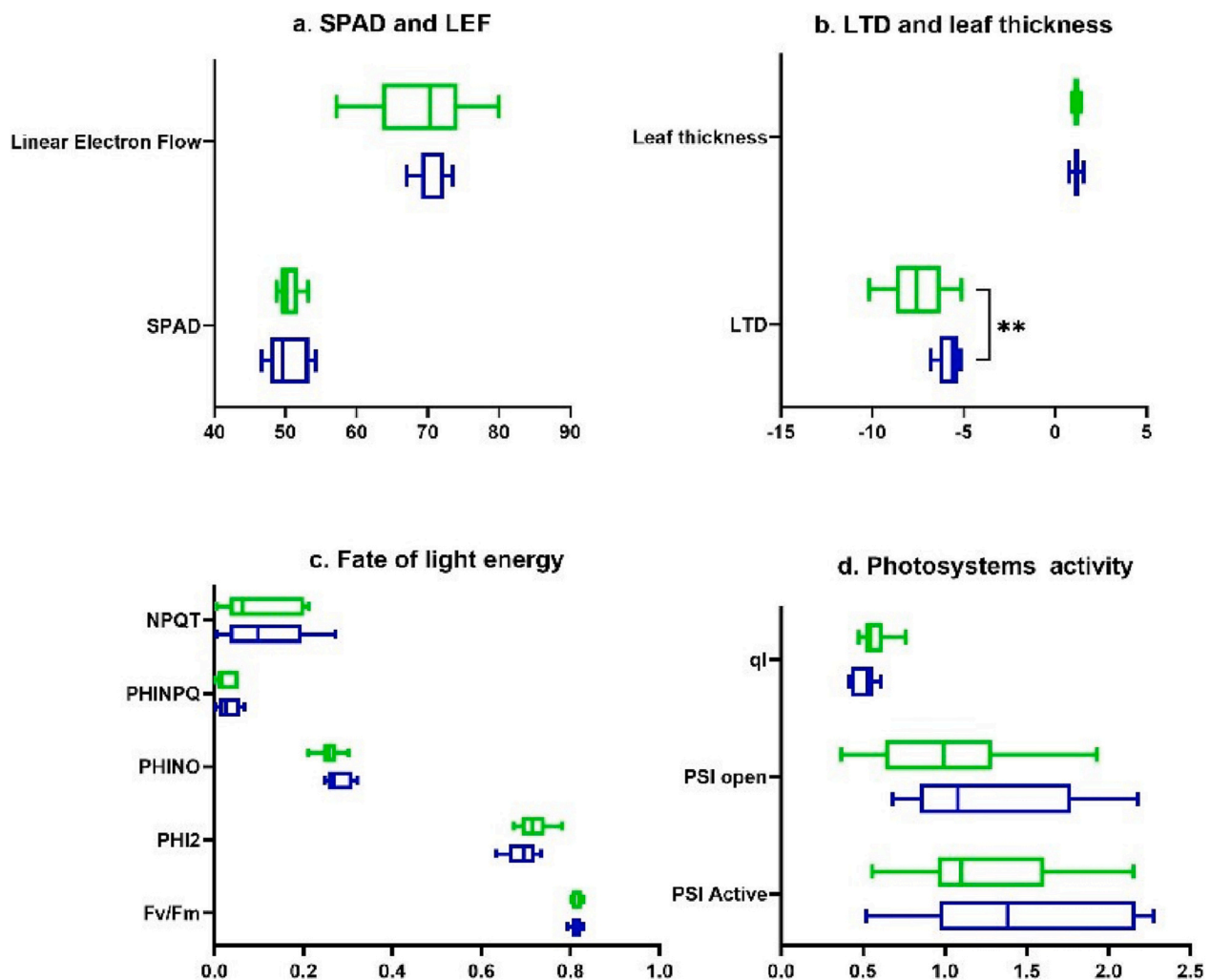


Fig. 2. Boxplots of photosynthetic parameters reflecting plant's status; a) Soil plant analysis development (SPAD) value, an indicator of plant nitrogen status and relative chlorophyll and linear electron flow (LEF), b) Leaf temperature differential (LTD), the ratio between temperature leaf temperature and the environmental temperature, and Leaf thickness is the thickness of the leaf section clamped by the Multispeq device, c) Fv/Fm-maximum quantum yield, PS II quantum yield/ratio of incoming light (excited electrons) used in photochemistry/photosynthesis, PhiNO- ratio of incoming light (excited electrons) that is lost in non-regulated processes and can cause photodamage, PhiNPQ-ratio of incoming light (excited electrons) lost through regulated non-photochemical quenching and NPQT-Non-photochemical quenching. The two whiskers show the minimum and maximum values; the box shows the interquartile region where most values fall, and the central line indicates the median. DW is distilled water control, and *Chlorella* sp. MACC-360 is the microalgae (MA) treatment. Asterisks indicate significant differences between treatments based on student t-test performed in Graphpad prism 8.

to cold and temperature stimuli.

Functional annotation clustering in DAVID returned four main gene clusters (Table 4). The cluster with the highest enrichment score was abundant of NAC-domain-containing proteins. In addition to the seven NAC proteins, APETALA2/ Ethylene-responsive factor (AP2/ERF) domain-containing protein and jasmonic acid 2 belonged to this cluster. The second cluster contained proteins involved in response to abscisic acid and cold acclimation including, dehydrins, LEA proteins, vacuolar protein sorting-associated (Vps54) domain-containing protein, and a ninja family protein. The third cluster significantly associated with the term 'sequence-specific binding protein', implying a role in transcription. It comprised four homeobox-leucine zippers, two NAC-domain, and a delay in germination (DOG)1 domain-containing protein. The last cluster with the least enrichment score had no significant term associated with it. However, it inclusively consisted of adenosine triphosphate (ATP)-binding and nucleotide-binding proteins, some of which participate in glycolysis, for example, ATP-dependent 6-phosphofructokinase 6-like protein.

LEA proteins, including dehydrins, play different roles in plants. They are highly abundant in the final seed formation stage during the

natural seed desiccation process. In vegetative organs, they accumulate during water scarcity. Therefore, they are involved in tolerance to drought. The structure of dehydrins allows them to dissolve at high temperatures and bind to proteins and acidic phospholipids, thus protecting lipid membranes against peroxidation [65]. They are also thought to act as chaperons or emulsifiers ([20]. TAS14, in particular, was found to accumulate in response to environmental stress [66] or abscisic acid [65]. Overexpression of TAS14 in drought-stressed plants revealed improvement in plant biomass and greater and earlier accumulation of abscisic acid [67].

Dehydrins are also necessary for the protection of gametophytic material in the pollen within unopened flowers. Their presence allows the pollen to remain viable despite undergoing harsh environmental conditions during water, animal/insect, and wind dispersal. The accumulation of dehydrins ensures that upon rehydration, the pollen is viable. Accumulation of dehydrin proteins has often been reported in seeds. Nevertheless, Taylor reported that conditions in mature tomato pollen triggered the expression of a LEA protein involved in water stress responses [68]. Transcripts that encode TAS14 and RAB 18 were up-regulated in tomato and *A. thaliana* plants treated from *Ascophyllum*

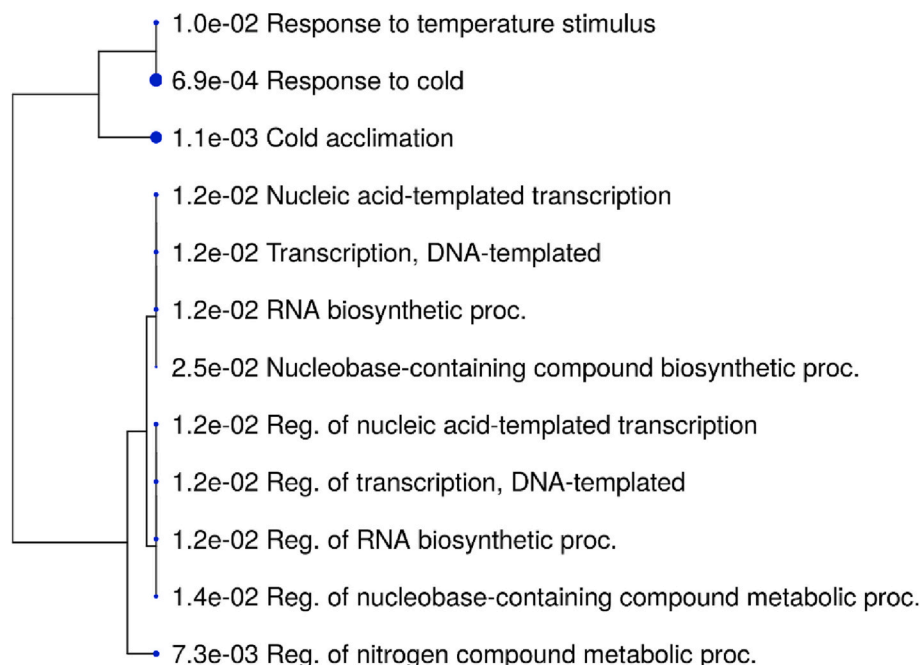


Fig. 3. A hierarchical clustering tree summarizes the correlation among significant Biological process (BP) pathways in the enrichment list of the differentially expressed genes (DEGs) upregulated in the unopened flower buds of *Chlorella* sp. MACC-360-treated plants relative to those of control plants. Pathways with many shared genes are clustered together. Bigger dots indicate more significant *P*-values.

nodosum extracts [21]. In *A. thaliana*, overexpression of many dehydrins improved freezing stress [69]. The upregulation of multiple dehydrins in MA-treated plants explains why cold acclimation and response to cold were the most significant terms in this study.

Oleosins are amphipathic structural proteins that are present in seeds and florets. They play a structural role in stabilizing the lipid body by preventing the coalescence of the oil body during desiccation. Oleosins are present in the pollen surface in the lipoidal substance referred to as the tryphine or pollen coat [70]. They enable pollen to stick together and on the surface of pollinators, thus facilitating pollen dispersal. In addition, they assist with pollen-stigma attachment and aid in water uptake required for pollen germination [22]). Thus, upregulation of the oleosin genes either shows enhanced pollen transmission and successful germination or advanced pollen development in floral buds of MA-treated plants relative to those of control plants. It could also show enhanced membrane stability due to the prevention of oil body coalescence during cold stress.

MA caused the upregulation of eight NAC-domain-containing proteins. The NAC-domain is an N-terminal module of about 160 amino acids in the No apical meristem (NAM) family proteins which are plant-specific transcriptional regulators [23]. NAM proteins participate in developmental processes such as shoot apical meristem, floral organ, and lateral shoot development. Moreover, they play a crucial role in plant hormonal control and defense. Usually, diverse C-terminal transcriptional activation domains occur alongside the NAC domain [23]. The NAC domain has been confirmed to be a DNA-binding domain (DBD) as well as a dimerization domain [24,25]. Based on their universal structure, it is assumed that the NAC domain causes dimerization through conserved interactions such as those involving salt bridges and binds to DNA through the numerous positive charges in the NAC dimer face [26].

The upregulation of genes encoding NAP in conjunction with enriched terms for abscisic acid homeostasis indicate increased senescence. Its clustering with jasmonic acid and involvement in response to abscisic acid suggests a role linked to plant hormone regulation. Kim and colleagues reported the increased leaf senescence in rice mediated by a NAC and abscisic biosynthesis pathway [71]. Upregulation of abscisic

acid homeostasis also indicates an enhanced ability to withstand adverse environmental conditions such as water deprivation. Interestingly, abscisic acid-responding genes, and dehydrin genes are highly expressed in mature ovaries and quickly reduce after pollination [72]. MA application seems to induce genes associated with flower development and abiotic stress. These results correspond to reports of flowering, abiotic stress, and herbivory-related genes sharing similar regulatory networks [73–75].

In addition to the NAM proteins, dehydrins, and oleosins, a soluble N-ethylmaleimide attachment receptor (SNARE) protein was associated with cold acclimation. SNARE protein family comprises membrane-associated proteins with an α -helical coiled-coil domain known as the SNARE motif [27]. The SNARE motif allows the fusion of two membranes, an essential phenomenon for exocytosis. SNAREs occur in distinct membrane compartments of the secretory and endocytic trafficking pathway, ensuring the specificity of intracellular membrane fusion. There are three SNARE motif categories, namely: syntaxin 1a (t-SNARE), VAMP-2 (v-SNARE), and the N- and C-terminal motifs. The v-SNAREs are localized on the vesicles, while the t-SNAREs are in the target membranes. Syntaxin 112 upregulated in this study bears the t-SNARE and participates in vesicular transport. Notably, a Vps4 motif-bearing protein that binds to syntaxin was upregulated. The combination of these two proteins could be trafficking compounds such as lipids or oil bodies to the cell membrane, thus allowing tolerance to cold stress. The homolog of this gene in *A. thaliana* was found to play a role in cold tolerance, although this role was negative [28].

Cytochrome P450 86A1 (CYP86A1) and Fatty alcohol: caffeoyl-CoA acyltransferase (LOC101244975) were associated with cutin, suberin, and wax biosynthesis KEGG pathway term (Table 3). The superfamily of cytochrome P450 enzymes comprises heme-containing mono-oxygenases present in all kingdoms. In plants, they participate in the biosynthesis of hormones, fatty acids, and defensive compounds [29]. In a comprehensive review, Kandel and colleagues reported that CYP86A1 participates in fatty acid degradation by hydrolyzing long fatty acids at the terminal methyl (ω -position) [29]. ω -hydrolyses are utilized for the synthesis of cutin and suberin. The elevated production of cutin could explain the thickening of leaves in MA-treated plants. Increased cutin,

Table 3
Functional profiles of upregulated genes from g; Profiler.

| Gene ID | Gene description | Pathway/mechanism |
|-----------------------------------|--|---|
| Solyc02g084850.3.1 ^{a,b} | TAS14 peptide (AA 1130) (TAS14) | Dehydrin located in the cytosol; Binds to membranes phospholipids protecting membranes during stress |
| Solyc03g112440.1.1 ^a | Oleosin 18.2 kDa (LOC101263398) | Oleosin located in the membranes; Oil-body biogenesis; Oil body coalescence prevention |
| Solyc01g109920.2.1 ^{a,b} | Embryonic cell protein 40 (LOC101259487) | LEA protein; Dehydrin located in cytosol; Protein chaperons and emulsifiers |
| Solyc02g084840.3.1 ^{a,b} | Dehydrin (A0A3Q7F9F5_SOLLIC) | Dehydrin located in cytosol; Binds to membrane phospholipids protecting membranes during stress |
| Solyc02g085090.1.1 ^a | Syntaxin-112 (LOC104645739) | soluble N-ethylmaleimide attachment protein receptor (SNARE) vesicular transport; Intracellular protein transport, exocytosis |
| Solyc08g013830.1.1 ^c | Fatty alcohol: caffeoyl-CoA acyltransferase (LOC101244975) | Transferase; Cutin, suberin, and wax biosynthesis |
| Solyc06g076800.3.1 ^c | Cytochrome P450 86A1 (LOC101259447) | Cytochrome P450 family protein; Located in the membrane; Fatty acid degradation; Cutin, suberin, and wax biosynthesis |
| Solyc04g005610.3.1 ^{d,g} | NAC domain-containing protein 2 (NAP2) | NAM protein; Located in the nucleus; Regulation of transcription; Developmental process, defense, plant hormonal control |
| Solyc05g007770.3.1 ^{d,g} | NAC domain-containing protein 1 (NAP1) | Regulation of transcription; Developmental process, defense, plant hormonal control |

Superscripts on gene IDs indicate the GO term ID, name, and *P*-value reflecting the significant association between the gene and the GO term where; a = GO:0009409; Response to cold (*P* value = 0.0010). b = GO:000963; Cold acclimation (*P* value = 0.0019). c = KEGG:00073; Cutin, suberin and wax biosynthesis (*P* value = 0.0288). d = GO:0030582; Reproductive fruiting body development (*P* value = 0.0051). e = GO:0031155; Regulation of reproductive fruiting body development (*P* value = 0.0051). f = GO:0075259; Regulation of spore-bearing organ development (*P* value = 0.0051). g = GO:0075260; Regulation of spore-bearing organ development (*P* value = 0.0051).

suberin, or wax production enables the fortification of the cell walls to offer physical protection and resist infection by biotrophic pathogens [76]. Upregulation of the wax biosynthesis has been reported to occur with the overexpression of AP2/ERF type transcription factors from *Medicago truncatula* in *M. sativa*, whereby the plants became tolerant to drought stress [77]. In our study, similar transcription factors were found to be upregulated. Moreover, members of the cytochrome P450 family participate in cutin synthesis plant development, reproduction, and detoxification. For example, in *A. thaliana*, CYP86A2, which shares 73 % amino acid sequence with CYP86A1, was found to repress bacterial type III genes enabling plants to withstand confrontation with *Pseudomonas syringae* [30]. CYP86A1 might play a similar role in plant-pathogen interaction [29].

Fatty alcohol: caffeoyl-CoA acyltransferase, on the other hand,

Table 4
Functionally annotated clusters of the upregulated genes from DAVID.

| Transcript ID | Gene name |
|---|---|
| Cluster 1, Enrichment score = 3.51, Significant term = NAC domain | |
| Solyc03g119580.1.1 | AP2/ERF domain-containing protein (A0A3Q7FWB6_SOLLIC) |
| Solyc06g063380.1.1 | NAC domain-containing protein 52-like (LOC112941642) |
| Solyc03g114260.1.1 | NAC domain-containing protein (A0A3Q7GFY9_SOLLIC) |
| Solyc08g028850.1.1 | NAC domain-containing protein (A0A3Q7IH21_SOLLIC) |
| Solyc05g007770.3.1 | NAC domain-containing protein 1 (NAP1) |
| Solyc04g005610.3.1 | NAC domain-containing protein 2 (NAP2) |
| Solyc07g066330.3.1 | NAC domain-containing protein 21/22 (LOC101261342) |
| Solyc02g088180.3.1 | NAC2-domain-containing protein (NAC2) |
| Solyc12g013620.2.1 | jasmonic acid 2 (JA2) |
| Cluster 2, Enrichment score = 2.75, Significant term = Response to abscisic acid and cold acclimation | |
| Solyc02g084840.3.1 | Dehydrin (A0A3Q7F9F5_SOLLIC) |
| Solyc04g005610.3.1 | NAC domain-containing protein 2 (NAP2) |
| Solyc09g008620.2.1 | Polyadenylate-binding protein (A0A3Q7HYP3_SOLLIC) |
| Solyc02g084850.3.1 | TAS14 peptide (AA 1–130) (TAS14) |
| Solyc02g030105.1.1 | Vps54 domain-containing protein (A0A3Q7EWI9_SOLLIC) |
| Solyc01g109920.2.1 | embryonic cell protein 40 (LOC101259487) |
| Solyc04g005380.3.1 | ninja-family protein AFP3 (LOC101268860) |
| Solyc07g006500.3.1 | trehalose-6-phosphate synthase (TPS1) |
| Cluster 3, Enrichment score = 2.19, Significant term = Sequence-specific DNA binding | |
| Solyc09g005610.3.1 | DOG1 domain-containing protein (A0A3Q7HXN2_SOLLIC) |
| Solyc05g007770.3.1 | NAC domain-containing protein 1 (NAP1) |
| Solyc04g005610.3.1 | NAC domain-containing protein 2 (NAP2) |
| Solyc01g096320.3.1 | homeobox-leucine zipper protein ATHB-12 (LOC101262661) |
| Solyc06g053220.3.1 | homeobox-leucine zipper protein ATHB-12 (LOC101264731) |
| Solyc02g085630.3.1 | homeobox-leucine zipper protein ATHB-40 (LOC101251349) |
| Solyc03g082550.3.1 | homeobox-leucine zipper protein ATHB-7 (LOC101245037) |
| Cluster 4, Enrichment score = 0.11, No significant term | |
| Solyc07g062630.1.1 | ABC transporter G family member 19 (ABCG19) |
| Solyc04g072580.1.1 | ATP-dependent 6-phosphofructokinase 6-like (LOC101259840) |
| Solyc03g121780.1.1 | Protein kinase domain-containing protein (K4BMX7_SOLLIC) |
| Solyc12g098560.2.1 | X8 domain-containing protein (A0A3Q7JEE6_SOLLIC) |

Four clusters labelled from the most enriched to the least enriched. The enrichment score is based on the EASE scores/*P*-value at 0.05 of each term member; the higher the score the more enriched the cluster.

participates in the biosynthesis of unsaturated fatty acids by catalyzing the conversion of feruloyl-CoA to 16-Feruloyloxypalmitic acid. The phenylpropanoid biosynthesis is linked to feruloyl-CoA by an unknown mechanism. The reduction of feruloyl-CoA due to the upregulation of Fatty alcohol: caffeoyl-CoA acyltransferase consequently affects the phenylpropanoid pathway, whose products are secondary metabolites that participate in stress response and defense [31].

MA affected the transcription of genes involved in carbohydrate metabolism with a notable upregulation of genes of the glycolysis cycle. For instance, the uncharacterized transcript *Solyc04g080540.2.1* is localized in the nucleus [32] and associated with anaerobic respiration (Supplementary data, Sheet 2). Other upregulated transcripts involved in carbohydrate metabolism were UDP-glycosyltransferase 91C1 (*Solyc02g043550.3*), UDP glucose epimerase (*Solyc07g043550.3*) and ATP-dependent 6-phosphofructokinase (*Solyc04g072580.1*) (Supplementary data Sheet 2). ATP-dependent 6-phosphofructokinase catalyzes the reaction ATP + D-fructose-6-phosphate = ADP + D-fructose 1,6-bisphosphate; Glucose-6-phosphate isomerase 1 further processes D-fructose 1,6-bisphosphate, which then enters the pentose phosphate pathway, that has an indirect link to carbon fixation via pyruvate (KEGG pathways).

Carbohydrate metabolism is crucial in stress responses because the different sugars (fructose, glucose, trehalose) control ionic balance, act as signaling molecules, detoxify reactive oxygen species (ROS), and maintain cell turgor, thus enabling plants to withstand stress [33].

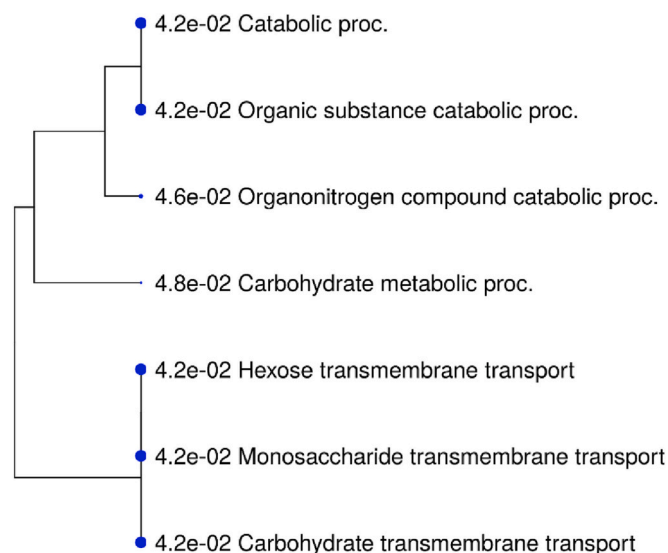


Fig. 4. A hierarchical clustering tree summarizes the correlation among significant Biological process (BP) pathways in the enrichment list of the differentially expressed genes (DEGs) downregulated in the unopened flower buds of *Chlorella* sp. MACC-360-treated plants relative to those of control plants. Pathways with many shared genes are clustered together. Bigger dots indicate more significant *P*-values.

Several studies have reported that carbohydrate metabolism positively regulates the expression of defense-related genes [34]. Increased glycolysis implies increased respiration and more available energy for the growth and synthesis of cellular components in MA-treated plants. The enhanced gene regulation/expression requires much more energy than basic processes, and it seems that MA application enhances these processes (glycolysis and gene expression).

TPS1 gene is involved in carbohydrate metabolism, but it also clustered with the genes involved in response to abscisic acid and cold. This gene is involved in generating trehalose, a disaccharide sugar molecule. In plants, trehalose plays essential roles in embryo formation and

flowering, regulation of carbon metabolism, photosynthesis, and plant-microorganism interactions [35]. In *A. thaliana*, *TPS* promotes the signal for flowering pathways [36]. *TPS* in tomato plants also has additional roles in stress amelioration due to its role in starch accumulation. Lyu and colleagues overexpressed a yeast *TPS* gene in tomato plants leading to a transgenic plants with enhanced abiotic stress tolerance and showing greater photosynthetic rate under salt stress [83]. *TPS* has been found to induce flowering, and *A. thaliana* with a defective *TPS* gene showed a late flowering phenotype [37]. Upregulation of this gene in MA-treated plants correlates with the early flowering phenotype reported in our previous study [16]. Biostimulants have been shown to promote flowering in several studies, although the flowering genes reported in these studies, such as the single flowering truss (*SFT*) and *Falsiflora* (*FA*) [38,39] were not detected in our studies. These differences might indicate the importance of sampling time or the diverse actions of various biostimulants.

In addition to the genes significantly associated with the enriched GO terms discussed above, transcription factors and plant defense-related genes were identified (Supplementary data, Sheet 2). These included *Solyc04g072460.3.1* transcription factor *TGA* (*TGACG-Binding*)1 (LOC101265431) involved in plant defense and *Solyc04g071770.3.1* ethylene-responsive transcription factor *ERF84* (*ERF84*) involved in plant hormonal signal transduction. Other genes associated with plant defense were *Solyc03g119580.1.1* *AP2/ERF* domain-containing protein, *Solyc12g014420.2.1* a glucan endo-1,3-beta-glucosidase, *Solyc12g098560.2.1* X8 domain-containing protein and *Solyc04g071000.1.1* a clade XIII lectin receptor kinase involved in biotic stress (e.g., bacteria and oomycetes). While these results show that these transcripts are involved in defense, it should be noted that they are also involved in other processes. For instance, *AP2/ERF* is involved in tomato flower pedicel abscission, while *TGAs* in soybean are involved in symbiotic nodulation and response to nitrogen availability [40,41]. Upregulation of such genes in MA-treated plants could indicate an advanced development with respect to flowering relative to control plants. Elevated transcription of most of these genes has been found in plants treated with different biostimulants such as beneficial bacteria, beneficial fungi, algal extracts, chitosans, botanical extracts, protein hydrolysates, and fulvic and humic acids [21].

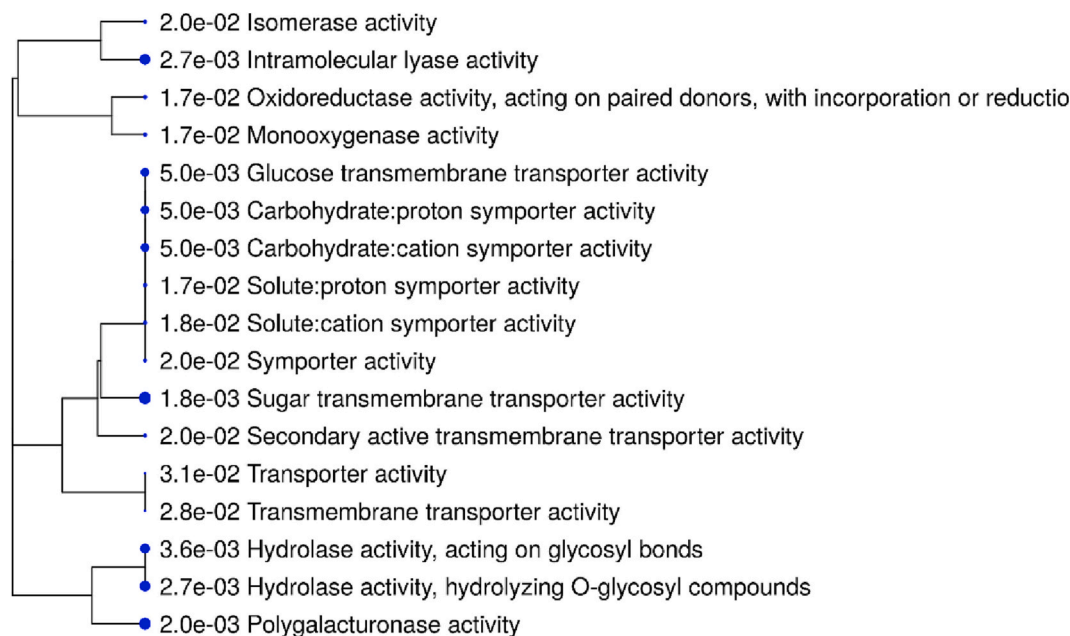


Fig. 5. A hierarchical clustering tree summarizes the correlation among significant Molecular function (MF) pathways in the enrichment list of the differentially expressed genes (DEGs) downregulated in the unopened flower buds of *Chlorella* sp. MACC-360-treated plants relative to those of the control plants. Pathways with many shared genes are clustered together. Bigger dots indicate more significant *P*-values.

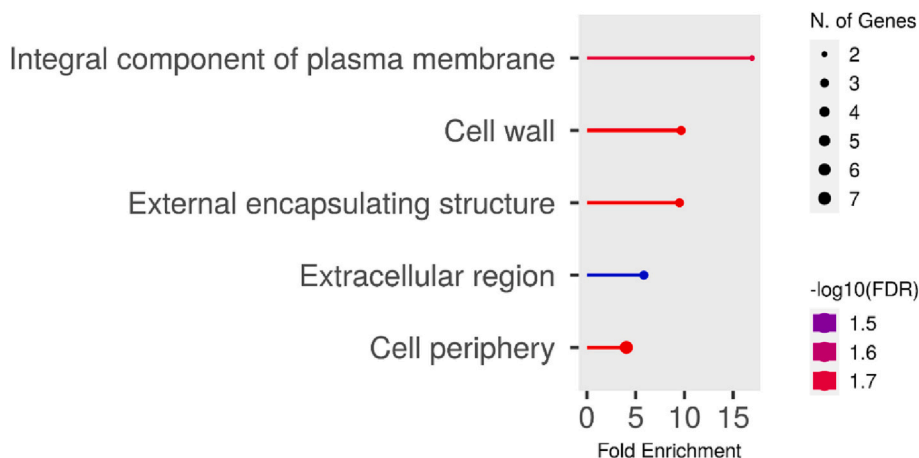


Fig. 6. Cell Component (CC) terms associated with the differentially expressed genes (DEGs) downregulated in the unopened flower buds of *Chlorella* sp. MACC-360-treated plants relative to those of control plants on the y-axis and Fold enrichment on the x-axis: Circle size indicates the number of genes involved, large size more genes. The color indicates significance; light color shows high significance, while dark color shows low significance.

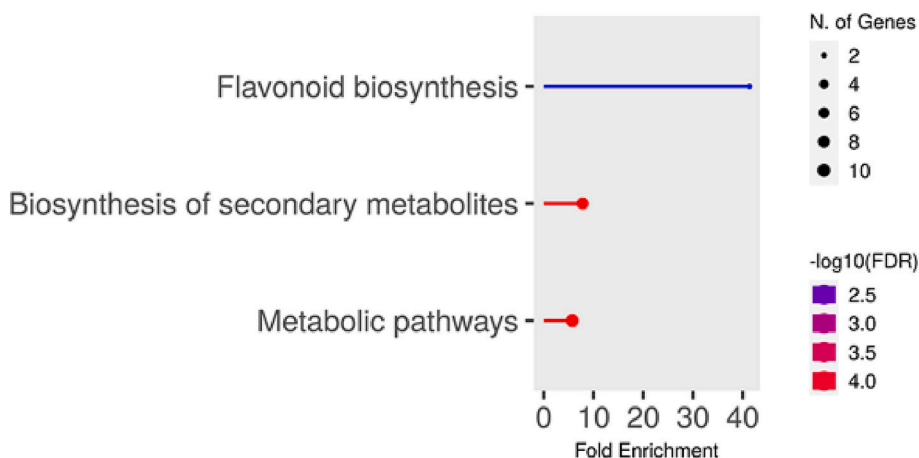


Fig. 7. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway terms associated with the differentially expressed genes (DEGs) downregulated in the unopened flower buds of *Chlorella* sp. MACC-360-treated plants relative to those of control plants on the y-axis and Fold enrichment on the x-axis: Circle size indicates the number of genes involved in the pathway, the bigger the circle, the more the genes. The color indicates significance; light color shows high significance, while dark color shows low significance.

3.2.3. Gene enrichment studies: downregulated genes

Only 50 downregulated genes had a fold change above 1.5. Analysis in Shiny GO with default settings and the background as all the transcripts obtained from the RNA-sequence data in our study revealed significantly enriched terms in all the ontology categories, as shown in Figs. 4–7. Functional analysis in g: Profiler identified similar terms, as shown in Table 5, which lists the specific gene names. Functional clustering in DAVID gave only one list of glycosyl hydrolase enzymes.

The downregulated genes in this study are strongly associated with catabolic process and carbohydrate transmembrane transport (Fig. 4). At the molecular function level, sugar transmembrane transporter, polygalacturonase, and intramolecular activity were the most significant functions (Fig. 5). Genes expressing proteins associated with the plasma membrane were the most downregulated, with a fold enrichment of 15, while those in the cell periphery were less affected (Fig. 6). Most downregulated genes were components of the plasma membrane or cell wall. Some of these genes belong to the flavonoid biosynthesis pathway (two), metabolic pathways (ten), and the biosynthesis of secondary metabolites (eight) (Fig. 7).

The downregulated genes associated with the term carbohydrate metabolism are glycosidase/hydrolase enzymes (Fig. 5 and Table 5). Glycosyl hydrolase enzymes hydrolyze the glycosidic bonds between two or more carbohydrates and between a carbohydrate and a non-carbohydrate moiety. The three glycosyl enzymes downregulated in this study belong to the Glycoside hydrolase family 28 (G28), whose

activities are well characterized; For example, polygalacturonase randomly hydrolysis 1,4- α -D-galactosiduronic linkages in pectate and other galacturonans [42,43]. It participates in fruit ripening via the cell wall metabolic processes. The microbial version of these enzymes plays a critical role in plant-pathogen interactions whereby they cause maceration and soft-rotting of plant tissue in plants infected with *Ralstonia solanacearum*, *Erwinia carotovora* and *Aspergillus niger* [42,43]. In general, enzymes with the pectate lyase fold act as virulence factors. They act on pectin or pectate (demethylated pectin), a cell wall component. Therefore, the downregulation of this category of genes implies a reduction in hydrolysis of oligosaccharides and accumulation of large sugar molecules in cells, especially in the cell walls. In conjunction with reduced catabolic processes, these processes facilitate the maintenance of cell wall integrity, which is necessary to serve as the first line of defense in a pathogenic attack.

Sugar transporters are membrane proteins that bind and transport compounds such as carbohydrates, acids and organic alcohols in prokaryotes and eukaryotes [44]. The SWEET sugar transporter family comprises specific sugar efflux transporters required for plant nectar production, pollen, and seed development. In most organisms, they transport the glucose sugar molecule. In rice, bacterial pathogens exploit two specific homologs for virulence [45]. This phenomenon occurs when bacterial effectors bind directly to the SWEET promoter. In *Arabidopsis*, SWEET is essential for pollen viability whereas, in *M. truncatula*, they are involved in nodulation [46]. Thus, the downregulation of

Table 5

Downregulated genes associated with significant GO terms in g: Profiler.

| Gene ID | Gene description | Pathway/mechanism |
|---------------------------------|--|---|
| Solyc12g099070.1.1 ^a | Inositol transporter 4-like (LOC101246700) | Sugar/inositol transporter; Membrane protein; Phosphate transport |
| Solyc09g074530.3.1 ^a | Bidirectional sugar transporter NEC1(LOC101259076) | SWEET sugar transporter; Glycoside hydrolase; Membrane protein, sugar transport |
| Solyc08g080300.1.1 ^a | Sugar transport protein 1-like (LOC101266251) | Sugar/inositol transporter; Membrane protein; Phosphate transport; sugar transport |
| Solyc01g087280.1.1 ^b | Polygalacturonase-like (LOC101263946) | Glycoside hydrolase Family 28 (GH28); Cell wall component, secreted; Pentose and glucuronate interconversions; Metabolic pathways |
| Solyc07g044870.3.1 ^b | Polygalacturonase (A0A3Q7I6W1_SOLLC) | Glycoside hydrolase, GH28; Cell wall, secreted; glycosidase, hydrolase |
| Solyc07g056290.2.1 ^b | Exopolygalacturonase-like (A0A3Q7HDN8_SOLLC) CYTH domain-containing protein (A0A3Q7HT17_SOLLC) | Glycoside hydrolase, GH28; Pectate-lyase fold, cell wall component |

Superscripts on gene IDs indicate the GO term ID, name, and *P* value reflecting the significant association between the gene and the Go term where; a = GO:0051119; Sugar transmembrane transporter activity (*P* value = 0.0318). b = GO:0004650; Polygalacturonase activity (*P* value = 0.0353).

SWEET in plants treated with MA also offers protection against bacterial species whose effectors might bind to the SWEET promoter. In general, the downregulation of sugar transporters may be a strategy to increase sugar molecules in the cytosol, whereby they may directly act as osmolytes or signaling molecules to transcribe defense molecules. They also serve as the building blocks synthesizing these defense molecules [47].

Two downregulated genes belonged to the flavonoid biosynthesis pathway, while about ten belonged to the metabolic pathways and biosynthesis of secondary metabolite. The latter two pathways are close/linked to each other. CHS1 catalyzes the reactions converting cinnamoyl-CoA, p-Coumaroyl-CoA, and Caffeoyl-CoA into their subsequent products, which end up in the flavanol or flavanone biosynthesis or anthocyanin biosynthesis sub-pathways of the flavonoid biosynthesis pathway (Fig. 8). Downregulation of these genes restricts the pathway activities to the reactions leading to the formation of lignin precursors, not anthocyanin. Lignin is essential in fortifying of the cell walls to make them impenetrable by pathogens and resistant to digestion by pathogen-related enzymes.

These results show the influence of MA on phenylpropanoid/flavonoid biosynthesis pathways. Upregulation of both phenylalanine ammonia-lyase (PAL) and Chalcone isomerase (CHI) genes leads to the accumulation of phenolic compounds which serve as signaling molecules to attract beneficial microbes to plants [78]. In this study, PAL is upregulated (Supplementary data, Sheet 2), while CHI is downregulated. Redirection of this pathway leads to the accumulation of metabolites, including flavonoids, phenolic compounds, lignins, phytoalexins, and shikimic acids [10]. However, it should be noted that the downregulation of CHI does not necessarily imply a hindrance to flavonoid biosynthesis, which is responsible for floral pigmentation in plants. Downregulation of chalcone genes was found to be critical for the development of the green wild-type stigma color. However, the yellow color in mutants did not seem to affect pollination [79].

An example of a downregulated transcript belonging to the

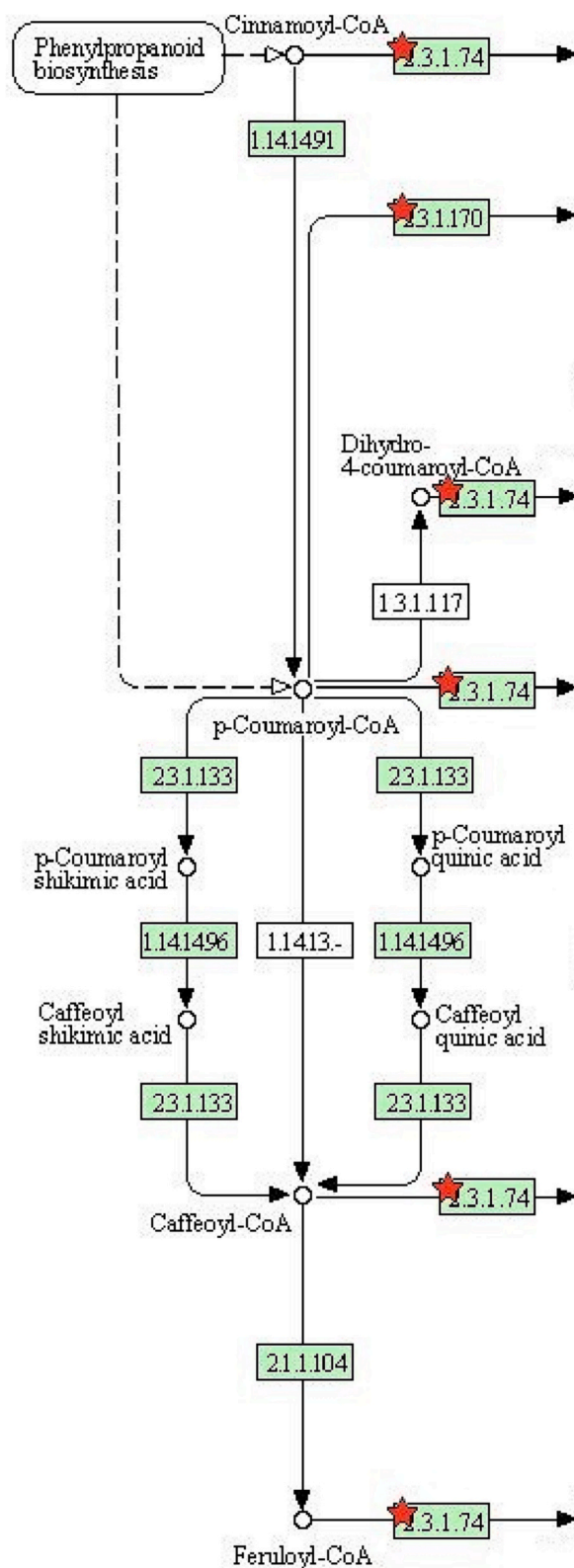


Fig. 8. Part of the Flavonoid biosynthesis pathway obtained from the KEGG pathways linked to DAVID. Red stars indicate the position of the differentially expressed genes (DEGs) downregulated in the unopened flower buds of *Chlor-ella* sp. MACC-360-treated plants relative to those of control plants. The two genes are Chalcone synthase (CHS1 = 2.3.1.74) and Chalcone isomerase (CHI2 = 2.3.1.170). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

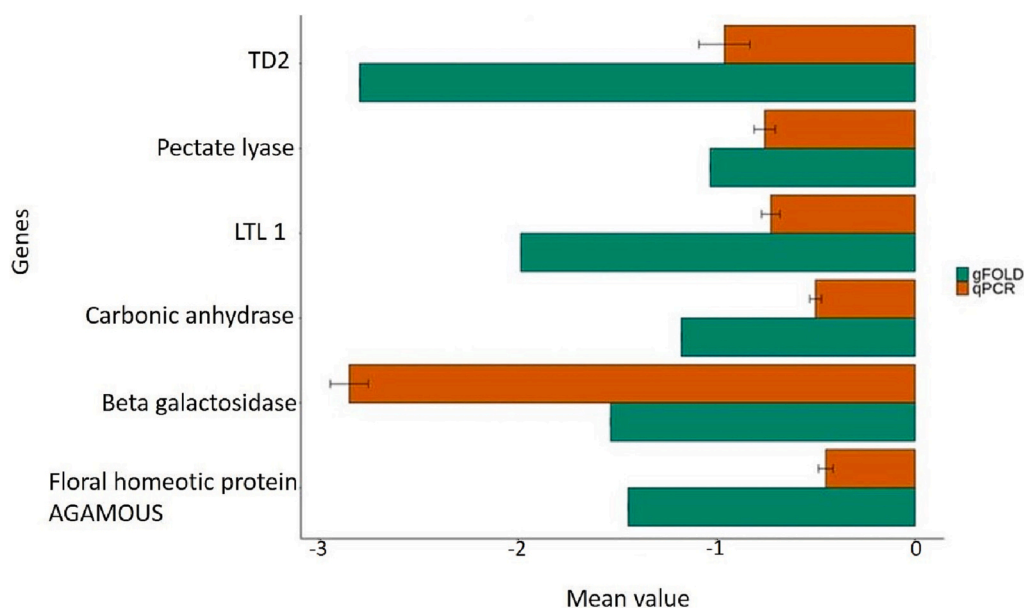


Fig. 9. Validation of transcriptomic data with qPCR of randomly selected differentially expressed genes (DEGs) downregulated in the unopened flower buds of *Chlorella* sp. MACC-360-treated plants relative to those of control plants. The x-axis shows the mean relative expression of genes from two independent biological replicates relative to the control (green-colored bars represent g FOLD values based on transcription data from the first experiment, while orange-colored bars represent relative expression values based on qPCR analysis with samples from two independent experiments). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

metabolic pathways is proline dehydrogenase 2 (Supplementary data, Sheet 2). It participates in the arginine/ proline metabolism by oxidizing proline to glutamate; the possible consequence of the downregulation of this gene is the accumulation of proline in MA-treated plants [80]. The breakdown of proline is linked to H_2O_2 production, which activates antioxidant signaling pathways. Thus, downregulation of the proline dehydrogenase 2 leads to the accumulation of a proline pool, which can either act as an osmolyte to help withstand stress or as a trigger of H_2O_2 synthesis which consequently triggers the onset of a robust antioxidant activity when necessary. Several authors have opined that proline metabolism provides stress protection through the maintenance of the oxidation-reduction photosynthesis reactions (nicotinamide adenine dinucleotide phosphate/nicotinamide adenine dinucleotide (NADPH/NADP)) balance, glutathione (GSH) levels and drives the oxidative burst of hypersensitive response during pathogen infection [81,82].

3.2.4. RT-qPCR for transcription validation

The selected genes for validation of transcriptomic results were confirmed to be downregulated indeed (Fig. 9). Among these genes, threonine dehydrogenase 2 (TD2) dehydrogenates threonine into 2-amino-3-ketobutyrate. This gene's downregulation leads to an accumulation of threonine and other amino acids synthesized upstream of threonine. Chen and coworkers reported that TD2 played a role in response against herbivory by lepidopteran larvae [48].

Nevertheless, downregulation of this gene could have occurred in conjunction with other genes involved in floral development, given that MA-treated plants were ahead in the floral development process. This observation aligns with recent studies that found that herbivory-induced defense responses and flower development shared a common phytohormonal, metabolic, and molecular regulatory machinery [49].

Beta-galactosidases, polygalactose-like, and pectate lyase enzymes belong to the glycosidase hydrolase protein group. They have been reported to play diverse roles in early flower and fruit development and ripening [50] as well as abscission. In *S. lycopersicum*, there is limited literature about the function of these enzymes in flower buds. However, silencing some beta-galactosidase [51] and pectate lyase genes [52] increased fruit firmness without necessarily affecting the ripening process. This phenomenon implies that the downregulation of these genes, which begins as early as the flower bud stage, could increase fruit firmness in MA-treated plants, a trait desirable to prolong the fruit shelf-life. Further studies are required to confirm whether an MA application will also enhance fruit firmness. Yang et al. [52] even reported reduced

tomato fruit susceptibility to the grey mold upon silencing of a beta-galactosidase gene [52].

4. Conclusion

To conclude, this study has provided a brief glimpse into the utility of MA application as a biostimulant. The differential regulation of genes involved in carbohydrate metabolism, amino acid metabolism and hormone signaling and responses to abiotic stress response indicates that MA application modulates plant metabolic processes and primes plants for defense against abiotic and biotic stress.

The MA influence on defense-related and flowering gene expression indicates that similar signaling mechanisms might induce these genes. An essential flowering-related gene identified in this study was *TPS1* which also plays a crucial role in sugar metabolism. Other genes involved in flowering and defense belong to the jasmonic acid signaling pathways. The upregulation of these genes in MA-treated plants could be one of the contributing factors to the early flowering phenotype.

Overall, the tested MA biostimulant enhanced processes that supply more energy to plant cells of MA-treated plants than those of control plants. Cells can use the energy for cell division and gene expression. A soil drench application of MA led to an empirical induction of systemic resistance genes in unopened flower bud tissues. This suggests that even a soil treatment with MA may exert a global effect on the plant host. In the future, studies of root tissue and rhizosphere will be done to get a better insight into the molecular effects of MA on plants.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2023.103106>.

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CRedit authorship contribution statement

Margaret Mukami Gitau: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Prateek Shetty:** Data

curation, Formal analysis, Writing – review & editing. **Gergely Maróti:** Conceptualization, Funding acquisition, Resources, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

All relevant data can be found within the manuscript, its supporting material and the NCBI SRA database under the project number PRJNA880331.

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