

Extracellular Vesicles: A Novel Messenger of Unicellular Microalgae Communication?

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Research

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Abstract

Background

Extracellular vesicles (EVs) are nanoparticles with membrane structures secreted by cells that play a role in the transfer of proteins, lipids, small RNAs, lncRNAs and DNA. Thus, EVs mediate mammalian cell-to-cell communication and have potential applications in the diagnosis and treatment of diseases. However, these studies have been primarily focused on the microenvironmental fluids between mammalian cells. Microalgae are single-celled organisms living in natural and dynamic aquatic environments. Whether microalgae can secrete EVs and adapt to changing environments via EV-mediated communication between cells is still unclear.

Results

We demonstrated that EVs are widely present in microalgae and have surprisingly rich contents of miRNAs and proteins. The differential expression of miRNAs and proteins was correlated with different cell growth stages and abiotic stressors. Our preliminary data suggested that *Chlamydomonas* EVs significantly affected the growth of the cyanobacterium *Synechocystis* in full BG11 medium. However, incubating EVs isolated from *Chlamydomonas* with *Synechocystis* cells showed that EVs themselves did not promote cell growth in nitrogen depleted BG11 medium. In this case, EVs appear to function primarily via information sensing and message delivery between cells under nutrient stress conditions. More detailed studies need to be conducted to revise our current perspective on the distribution of nutrients in aquatic environments and how EVs may affect microbial communications and interactions.

Conclusions

These findings suggest that EVs may play a critically important role in information exchange between microalgal cells and, in turn, adaptation to changing aquatic environments.

Background

Extracellular vesicles (EVs or exosomes) are small vesicles approximately 30–150 nm in diameter with a lipid bilayer membrane [1, 2]. In 1987, these vesicles were shown to be released from the cytoplasmic membrane during multivesicular fusion and during the *in vitro* culturing of sheep reticulocytes [3]. Subsequent research has found that most types of cells secrete exosomes and that they are found in various biological fluids, such as blood, saliva and breast milk [4].

EVs were originally thought to be a type of cellular waste, specifically waste transporters. However, in 2002, EVs were determined to also mediate cell-to-cell communication and regulate immune responses in dendritic cells [5]. EVs secreted by immune cells can be incubated with antigen-presenting cells to activate T cells and promote adaptive immunity [6].

Detailed research on EVs has revealed that EVs are rich in cellular substances, such as proteins, lipids, mRNAs and microRNAs (miRNAs). These substances can be transferred to recipient cells and thereby mediate intercellular communication [7]. In addition, the detection of DNA in EVs has provided us with a novel understanding of their properties [8]. To date, many studies have demonstrated the critical role that EV biomarkers can play in disease development and diagnostics and treatment [9]. For example, several reviews have summarized the role of EVs in cardiovascular disease [10], and how the microRNAs in EVs can promote the production of neurotoxins in neurological diseases [11].

However, current research on EVs has been primarily concentrated on mammalian cells, especially the fluids of human cells, including blood [12], milk [13], amniotic [14], bronchoalveolar fluid [15], saliva [16], semen [17] and even fecal matter [18]. Although carrot cells were discovered to secrete EVs as early as 1967 [19], little research on EVs in plant cells has been conducted, and only recently have laboratories begun to conduct basic studies. In 2009, ultracentrifugation was used to separate vesicles 50–200 nm in size from the extracellular fluid in sunflower (*Helianthus annuus*) seeds, which were thought to play a role in intercellular communication between plant cells [20]. By 2017, EVs had been isolated from the apoplastic fluids of *Arabidopsis* leaves, and proteomics showed that these EVs were highly enriched with proteins involved in biotic and abiotic stress responses [2]. Together, these studies suggest that these EVs may play an important role in the plant immune response and act as intercellular messengers in plant cells.

In aquatic environments, population composition and ecosystem function can be affected by communication between microorganisms. Communication signals include specialized metabolites used as infochemicals, quorum sensing and other molecules that can carry information on cell density and the presence of biotic and abiotic stress [21]. Recently, EVs have been discovered to provide a new mode of communication across all domains of life. Studies have suggested that EVs have the potential to regulate host-to-virus dynamics [22] and influence the partitioning of carbon during autotrophic and heterotrophic bacterial interactions [23]. Similarly, the unicellular microalga *Chlamydomonas* produces ciliary ectosomes that act as carriers of the proteolytic enzyme necessary for the liberation of daughter cells from mitosis. Thus, algal cilia have been suggested to be a source of bioactive, extracellular membrane vesicles. However, unlike EVs in animals and higher plants, whether all unicellular microalgae secrete EVs and whether their EVs function during cell growth and under stress remain unclear.

OMICS, high-throughput techniques that detect whole-cell molecules (such as RNA, proteins and metabolites), provide a more complete picture of the regulatory mechanisms underlying biological activities. RNomics and proteomics have already shed light on the contents and potential functional mechanisms of EVs in animal fluids, cells and organisms. EVs have been shown to mediate intercellular communication through various small non-coding RNAs and mRNAs. These molecules remain intact even after entering the cell via EVs. Furthermore, some microRNAs in EVs with important functions exert their effects after entering the recipient cell through EVs²²⁻²⁴ [24-26]. If EVs are secreted by different types of cells or cells of the same type under different states, the contents and types of RNA contained therein would vary and could thus be used as markers for diagnosing diseases [25, 27]. Thus, we believe that the

study of EV miRNAs and lncRNAs in unicellular microalgae EVs is particularly important. Proteomics is a promising high-throughput approach for detecting and screening potential biomarkers and can also be used for the early diagnosis of important diseases. In addition, current research is mostly focused on individual molecules; in contrast, the study of the overall nature of the nucleic acids and proteins transported by EVs is rare. RNomics and proteomics analyses of EVs will enhance our understanding of communication among unicellular microalgae.

Here, we isolated nanometer-sized particle precipitates with membrane structures from the cell culture media of a cyanobacterium *Synechocystis sp.* PCC6803. This media contained a model green microalga, *C. reinhardtii* CC124, and two industry microalgal strains, *Euglena gracilis* and *Haematococcus pluvialis*. Pellets from each microalgal species were verified as EVs using classical identification methods. We used *C. reinhardtii* to address the following questions: *i*) What do the EVs contain? *ii*) Do the contents of EVs change during cell growth and stress? and *iii*) Can some microRNAs and proteins in EVs act as biomarkers for cell growth and stress indicators in liquid culture media? To answer these questions, we conducted RNomics and proteomics analyses to study EVs in *C. reinhardtii* at different stages of cell growth and under different types of abiotic stress.

Results And Discussion

EVs detected in selected microalgae cell culture media

Generally, exosomes produced in mammalian cells are isolated from cell culture fluids and other biological fluids. Common methods of separation include ultracentrifugation, gradient ultracentrifugation, co-precipitation, size-exclusion chromatography and field-flow fractionation [28]. Here, we used differential ultracentrifugation, which is the most widely used method. Coupled with filtration, we harvested small particles (small EVs) from all of the selected microalgae culture media, including *Synechocystis*, *Chlamydomonas*, *Euglena gracilis* and *Haematococcus*. To verify the presence of EVs in the resuspension, we used negative staining and TEM. The resuspension contained many distinct membrane structures with small clumps of particles approximately 110–120 nm in diameter, the characteristic size range of exosomes and small microvesicles (Fig. 1a,b). We were also able to determine the size range of microalgal EVs using dynamic light scattering. There were some differences in structure and size among EVs from different microalgae. *Synechocystis* EVs had particle diameters of approximately 24–450 nm (Fig. 1b) with a major peak diameter of approximately 128.2 nm. The average particle size was 94.68 nm with a particle distribution coefficient of 0.259 (Supplemental Table S1). Similarly, EVs from *Chlamydomonas* had particle diameters of approximately 37–710 nm (Fig. 1b) with a main peak diameter of 175.5 nm. The average particle diameter was 120.1 nm with a particle distribution coefficient of 0.276. These results indicated that the membrane structure of the vesicle-like sample can be regularly separated from the algal culture medium.

Microalgae EVs can be transferred between cells

When studying EVs secreted by mammalian cells, specific protein markers, such as the four transmembrane proteins (CD9, CD63, CD81, etc.), are often used to identify and label small extracellular vesicles [29]. Unfortunately, no specific proteins have been reported in microalgal EVs. Considering that EVs have a lipid bilayer membrane structure, PKH67 lipophilic dyes were used to label EVs by most researchers. We first incubated the PKH67 lipophilic dye with EVs extracted from microalgae and then added the labeled EVs to *Chlamydomonas* cells for co-culturing. If cells took up EV-PKH67 through phagocytosis, green dots would be visible inside and outside the cell with confocal microscopy because PKH67 emits a green fluorescence under the excitation of 488-nm light. In this study, small dots of PKH67 were regularly observed inside microalgal cells. DAPI is often used in EV-tracking research to locate the nucleus. Because microalgal cells contain chlorophyll and because chlorophyll fluorescence is red under the excitation of light, we can employ self-chlorophyll to locate microalgal cells and the potential positions of the labeled EVs. Our observations suggest that microalgal EVs could be absorbed by recipient cells; thus, the contents inside EVs could be transferred between cells (Fig. 2).

Interestingly, EVs are capable of being absorbed by receptor microalgal cells given that they have cell walls, unlike human and animal cells. The cilia of the model green microalga *Chlamydomonas* have been documented to be involved in the secretion of bioactive, extracellular membrane vesicles [30]. We propose that a similar pathway involving secretion and uptake of EVs may take place in green microalgae and cyanobacteria.

Small non-coding RNAs in microalgal EVs

Samples, including EVs (EV) and host cells (Cell), at different cell growth stages and treatments, such as logarithmic (Log) and platform (Pla) stages, biotic stress, nitrogen depletion for 3 days (N-) and nitrogen recovery for 3 days after N- treatment (NR), were collected and analyzed. Mapped small RNA in microalgal EVs were analyzed separately for biotype, and the total number of reads for ribosomal RNA, tRNA, miRNA, piwi-RNA, Y RNA and protein-coding mRNA are shown in Table 1. We detected a substantial fraction of EV RNA that was <100 nt in size. In addition, we detected various classes of small non-coding RNA within the <100 nt size range, including rRNA (74.5% \pm 12%), microRNA (3.7% \pm 1% of the RNA in the 20–40 nt fraction) as well as abundant tRNA (3.6% \pm 1%), snRNA, snoRNA and Y RNAs, at all stages of EVs. The differential composition of non-coding RNAs was observed between EV and cell samples. Most small RNA in EVs was mapped to known RNA sequences, a much smaller percentage (17.8% \pm 8.3%) compared with non-mapped RNA (65.4 \pm 12.5%). However, there were significantly higher percentages of known RNAs (74%) in EVs relative to host cells (~20%). Similarly, there was an average of only 3.7% of miRNAs detected in EVs compared with 7.6% in host cells. Similarly, there was an average of 3.5% and 7.1% of tRNAs in EVs and host cells, respectively. Thus, EVs isolated from culture medium were primarily secreted by microalgal cells instead of simply being derived from cell lysis or breakage.

Highly reproducible data were obtained in EV triplicates, and a significantly different composition of small RNAs was observed between EVs at different stages (Table 1). For nitrogen stress, similar differential expression patterns of small RNAs were observed between EVs and host cells. For instance,

percentages of miRNA, snRNA and other RNAs increased in nitrogen-depleted samples, N-EV and N-Cell, whereas rRNAs were significantly reduced in samples from both of these groups. These results show that EVs carry several small RNA biotypes, which could potentially have regulatory functions when delivered to target cells.

The functional annotation of genetic variants of small RNAs was also extensively investigated. Different distributions of small RNAs were recognized in EVs and host cells at different stages, especially under the abiotic stress of nitrogen depletion (Table 2). Small RNAs were mostly mapped to three regions: UTR (~40%), intergenic (~36%) and upstream and downstream (~15%). This pattern contrasts with the more diverse distribution observed in host cells: UTR (~34%), intronic (21%), upstream and downstream (~17%), exonic (~15%) and intergenic (~12%) (Table 2). Among EV samples at different stages, less small RNAs mapped to the UTR (mean 26.8% versus 36–38%) and intergenic (mean 37.8% vs. ~40%) regions than to intronic (10.9% vs 4.8–4.9%), exonic (5.6% vs 2.7–3.4%) and splicing (0.037% vs 0.01–0.0003%) regions relative to N-EV samples (Table 2). We propose that increased mapping changes in coding and splicing regions of genes may employ small RNAs in response to the stress caused by nutrient limitation, similar to the host cell's response to nutrient stress. More detailed studies of these mapped genes are needed to determine how EVs function during stress.

EVs carry non-coding RNA molecules and some of these RNAs function as regulatory RNAs in human body fluids, such as semen [17]. In semen, these non-coding RNAs exert their main functional consequences on immune cells in the mucosa [17]. High cell densities, such as in algal blooms, may provide conditions where communication via EVs is favorable, similar to the highly dense microbial communities originating from aggregated cells [21-23]. Given the enriched and diverse non-coding RNAs that are associated with cell growth and nutrient depletion detected in microalgae EVs, additional studies of microalgae EVs should enhance our understanding of the role that EV RNA-mediated regulation plays in the sensation of cell density and nutrient contents in aquatic environments—and more generally—in microalgal growth and nutrient starvation.

EV miRNA profiling of *Chlamydomonas*

EVs contain miRNA, and this miRNA can be delivered to recipient cells to regulate physiological processes in mammalian cells and the human body. The expression profile of EV miRNAs varies depending on cell type and external conditions. To characterize miRNA profiles and their potential correlation with cell growth and abiotic stress, we used Illumina HiSeq 2500 high-throughput sequencing to study EV miRNA expression in the *Chlamydomonas* culture medium at different growth stages. We used the miRNAs derived from host cells as controls.

The presence of miRNA in EVs from certain bodily fluids has been widely documented [31-33]. miRNAs are small non-coding RNAs that play a role in post-transcriptional tuning of gene expression by suppressing translation or by degrading mRNA. A single miRNA can regulate a large number of genes and multiple miRNAs can regulate the same gene with additive or synergistic effects [34]. A recent study

has shown that miRNAs in *Chlamydomonas* are not phylogenetically conserved and play a role in responses to nutrient deprivation [35].

We detected 64 known miRNAs that could be found in the miRbase database. Based on the presence of a signature hairpin stem-loop structure in miRNA precursors, the miReep2 software was used to calculate the secondary structure and seed region of the sequence by intercepting a certain length of the sRNA alignment. These calculations were then used to predict the potential 84 new miRNAs that existed in EVs.

Surprisingly, less than 7.51% of common miRNA reads were shared between EV and host cells at different growth and stress conditions (Table 3). Significantly higher reads were detected in EV samples, such as Log, N-, NR and Pla, with averages of 74.05%, 72.05, 68.24 and 49.41%, respectively. These results suggested that miRNAs were highly enriched in microalgal EVs and were secreted by cells but that little miRNA was shared with EVs inside host cells. These EVs may therefore be transferred to other cells. Interestingly, the miRNA contents in EV decreased during cell growth from the Log to the Pla stage, while those in host cells increased over the same set of stages (from means of 74.05% vs. 21.19% to 49.51% vs. 42.99%).

A total of 16 miRNAs (cre-miR1144b, cre-miR1147.1, cre-miR1149.1, cre-miR1149.2, cre-miR1150.3, cre-miR1155, cre-miR1156.1, cre-miR1156.2, cre-miR1160. 2, cre-miR1164, cre-miR1166.1, cre-miR1172.1, cre-miR1172.2, cre-miR909.1, cre-miR910 and cre-miR912) detected in EVs were known to respond to sulfur deprivation [36]. Among them, cre-miR1147.1 targets the COP signalosome subunit 5 and participates in proteolysis and peptidase regulation; targets adenylate/guanylate kinase and participates in purine metabolism. cre-miR1150.3 targets Cytochrome P450 CYP3/CYP5/CYP6/CYP9 subfamilies, plays a regulatory role in photosynthesis by targeting phosphatidylinositol 3-kinase related protein kinase and is involved in inositol metabolism. cre-miR1166.1 targets phosphatidylglycerol phosphate synthase; plays a regulatory role in photosynthesis, ribosome biogenesis and pescadillo-like protein; and participates in DNA binding, RNA binding and transcription and translation processes. cre-miR909.1, which targets pheophorbide oxygenase, is involved in photosynthesis. cre-miR1156.2, which targets N-acetyltransferase, is involved in amino acid metabolism. These miRNAs are involved in the regulation of gene expression at the transcriptional and post-transcriptional levels in response to sulfur-deficiency stress in *Chlamydomonas* cells [35]. Thus, we speculate that EVs contained these miRNAs under nitrogen depletion, which is reminiscent of the nutrient stressors experienced by *C. reinhardtii*. miRNAs in *Chlamydomonas* cells might play a large modulatory role in gene expression regulation under diverse trophic states. Thus, the first *C. reinhardtii* cells to respond release exosomes that carry nitrogen-responsive miRNAs to other *C. reinhardtii* cells, allowing these miRNAs to participate in the regulation of physiological characteristics in response to environmental stressors.

Proteome analyses of microalgal EVs

Proteomic profiles of microalgal EVs were determined in each sample using LC-MS/MS. Overall, 10,812 unique EV proteins were identified, of which 2,078 and 1,788 proteins were present and quantifiable in all samples (Table 4). Four hundred forty-two proteins were differentially expressed in NR-EV compared with

the control Pla-EV ($p < 0.05$), of which 238 proteins were down-regulated and 204 proteins were up-regulated at the 1.5-fold threshold. Similarly, 480 differentially expressed EV proteins were detected in Log-EV and the control, with 200 and 280 proteins up- and down-regulated, respectively (Table 5).

The 10 most abundant proteins in microalgal EVs were flagella membrane glycoproteins (2), associated proteins (4), mastigoneme-like flagellar protein, fibrocystin-like protein, cytoplasmic dynein 1b and one unknown protein. An ectosome from *Chlamydomonas* has been shown to be derived from their ciliary as ciliary EV [37, 38]. Although lots of flagella-associated membrane proteins were detected in our proteome data from all samples, three ESCRT proteins [36], A8IXRO, A8IAJ1 and A0A2K3E4X9, were also observed in the EVs protein list. For the isolation method, we added a step with a 0.22- μ M membrane to filter out larger particles in the medium. Similarly sized particles were also isolated using the same isolation technique in *Synechocystis* with no ciliary. Based on these results, we proposed that particles detected in our study were small EVs that perhaps included some ciliary EVs rather than ciliary EVs only.

The proteins of EVs can be derived from mitochondria, nucleus, cytoplasm and plasma membrane from mammalian cells. Thus, we investigated the cellular location of EV proteins in microalgae. Subcellular analyses based on differential expression of EV proteins indicated that microalgal EV proteins were most correlated with the chloroplast (37.08–39.59% in growth and stress, respectively), cytoplasm (22.5–22.62%), plasma membrane (13.12–10.41%), nucleus (11.46–8.82%), mitochondria (6.25–8.6%), extracellular (3.96–2.94%), other (2.92–3.39%) and vacuolar membrane (2.71–3.62%) (Table 6).

Under nitrogen starvation, *Chlamydomonas* cells sense the nutrient stress and response on a large scale leading to thousands of differentially expressed genes and proteins, the down-regulation of carbon assimilation and chlorophyll biosynthesis and an increase in nitrogen metabolism and lipid biosynthesis [39]. Differentially expressed EV proteins showed enrichment for signaling pathways and responses to cell growth and nutrient stress in *Chlamydomonas*. Enrichment analysis for gene ontology (GO) terms related to 1) biological processes, 2) cellular components and 3) molecular functions, as well as pathway analysis, was performed to gain insight into the functionality of EV proteins at different cell growth stages and under nutrient stress.

A total of 54 GO terms related to biological processes were significantly enriched among EV proteins in Log-EV, which indicated that these GO terms were correlated with cell growth. Top enriched GO terms included multiple biological processes, primarily transport, including “transport along microtubule”, microtubule-based (protein) transport, and response to water deprivation, chitin metabolism, cilium assembly and organization, with ribosomal biogenesis and ribonucleoprotein complex assembly; molecular functions such as “carbonate dehydratase activity,” “1,3-beta-D-glucan synthase activity,” “structural constituent of ribosome” and “chitinase activity;” cellular components such as proteasome core complex, alpha-subunit complex, intraciliary transport particle, small nucleolar ribonucleoprotein complex, ribosome and ribosome subunits and chloroplast envelope (Fig. 3). KEGG enriched pathways in cell growth related to EVs were cre03010 Ribosome and cre00195 photosynthesis. These results appear to reflect growth characteristics, such as the regulation of cell division, cell wall construction and protein

biosynthesis in general. In addition, these processes were present in the Log-EVs rather than in carbon assimilation processes in N-EVs.

Nitrogen stress is a common strategy used to stimulate lipid accumulation in microalgae, a biofuel feedstock of topical interest. We observed completely different responses from EVs to nitrogen stress during cell growth. A total of 57 GO terms related to nitrogen depletion were significantly enriched among EV proteins in N-EV, which indicated that these GO terms were correlated with host cell nitrogen stress in this study. Top enriched GO terms included multiple cellular components that were unexpectedly located in mitochondria and chloroplast, such as mitochondria ribosome, matrix, chloroplast membrane, photosystem I, II stroma and thylakoid (Fig. 4). Among biological processes, major terms included high light intensity, photosynthesis, polysaccharide biosynthesis, glucan metabolic process, carbohydrate biosynthetic process. Molecular functions tended to involve ammonia ligase and peptidase activities, such as “glutamate-ammonia ligase activity,” “glucose-1-phosphate adenylytransferase activity,” “ATP-dependent peptidase activity” and “metalloendopeptidase activity” (Fig. 4). These results appear to reflect photosystem damage and changes in carbohydrate metabolism in general. Additionally, these responses were more prevalent in the nitrogen response rather than in cell growth processes.

KEGG enriched pathways in nitrogen stress related to EVs were cre00730 Thiamine metabolism, cre00040 pentose and glucuronate interconversions, cre00520 amino sugar and nucleotide sugar metabolism, cre00196 photosynthesis antenna proteins, cre00670 one carbon pool by folate and cre00195 photosynthesis. Nutrient deprivation causes significant stress to the unicellular microalga, *Chlamydomonas*, which responds by significantly altering its metabolic program. Following N deprivation, the accumulation of starch and triacylglycerols is significantly altered after massive reprogramming of cellular metabolism. A proteomic-based profiling of *Chlamydomonas* grown under N-starvation identified 259 proteins from lipid droplets. Among these, only 16 proteins were predicted to be involved in lipid metabolism [40]. Similarly, in N-EVs, few proteins involved in lipid metabolism were detected, despite nitrogen starvation being the most widely used method for inducing lipid accumulation.

Candidate EV biomarkers for microalgal cell growth and nitrogen depletion were also investigated. The top six most discriminating cell growth up-regulated EV proteins (cell wall protein A0A2K3DE90, FAP133, pesticidal crystal cry8Ba, unknown, glycoside hydrolase and endoglucanase A0A2K3CQY0) and down-regulated (3 histone proteins: H3, H2B and H4; low CO₂-inducible protein; extracellular matrix protein; and bifunctional sensory photoreceptor) were screened out based on the proteomic data (Supplemental material table S1). Cell wall proteins were connected to cell growth and division, and histone proteins located in the nucleus were regulated and maintained several cell cycles in eukaryotic cells.

The top six overrepresented nitrogen stress EV proteins included up-regulated proteins (A8I8Z1-6,7-dimethyl-8-ribityllumazine synthase, A0A2K3CUR4 glutamate receptor 3.5-like isoform X2, A0A2K3CYA4-unknown, Q9AXF6-Chlorophyll a-b binding protein, A0A2K3D4Y6-CPD photolyase class II and A0A2K3DZ68-flagella associated protein) and down-regulated proteins (histone H3, A8ILD5, formin-like protein 3, 30S ribosomal protein S11, chloroplastic and Peptidyl-prolyl cis-trans isomerase)

(Supplemental material table S1). Nitrogen stress is known to lead to the breakdown of chlorophyll, the major content of the photosystem, and the down-regulation of several genes involved in central metabolism, including those of the ribosome and chloroplast. Because they contain high contents of lipids, proteins and nucleic acids, EVs are a crucial source of organic carbon and nitrogen for heterotrophic bacteria [22]. Our preliminary data suggested that that EVs are widely present in microalgae and have surprisingly rich contents of miRNAs and proteins. Furthermore the *Chlamydomonas* EVs significantly affected the growth of the cyanobacterium *Synechocystis* in full BG11 medium. However, incubating EVs isolated from *Chlamydomonas* with *Synechocystis* cells In this case, EVs appear to function primarily via information sensing and message delivery between cells under nutrient stress conditions (Fig. 5). showed that EVs themselves did not promote cell growth in nitrogen depleted BG11 medium. More detailed studies need to be conducted to revise our current perspective on the distribution of nutrients in aquatic environments and how EVs may affect microbial communications and interaction.

Conclusions

Our results concluded that this exosome, mainly inside different microalgae media. Furthermore this exosome, which can go from cell to cell, as well as like other exosomes, contains a lot of biological information, such as proteins and amino acids.

Materials And Methods

Strains and culture growth

Synechocystis sp PCC6803, *Chlamydomonas reinhardtii* strain CC124, *Euglena gracilis* Z and *Haematococcus pluvialis* were the species examined in this study. *Synechocystis* cells were cultured in BG11 medium [41] and under continuous cool-white light conditions at 30°C with a light intensity of 150–160 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. EV isolations from the culture medium were performed with Platform period cultures. *Chlamydomonas* cells were cultured in normal TAP medium⁴² and incubated at 25°C under continuous light, with a light intensity 180–200 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. The EV isolation experiments were performed at the logarithmic (sample “Log”, 1.0×10^7 cells mL^{-1}), nitrogen depletion (sample “N-”, 1.5×10^7 cells mL^{-1} cells after culture for 3 days in the TAP-N culture medium, using NaCl instead of NH_4Cl), nitrogen recovery (sample “NR”, 1.5×10^7 cells mL^{-1} cells re-added N- cells with full TAP medium and cultured for another 3 days) and platform stages (sample “Pla”, 2.5×10^7 cells mL^{-1} cells from Log continuous culture in TAP medium after 8 days). For the microRNAsome, the samples Log, N- and NR were collected, and for proteome samples, Log, N- and Pla were collected for further investigation.

E. gracilis cells were cultured in EG medium and under continuous cool-white light conditions at 22°C with a light intensity of 110–120 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. The experiments were performed with Platform period cultures ($\sim 1.0 \times 10^6$ cells mL^{-1}).

H. pluvialis cells were cultured in ESP medium and under continuous cool-white light conditions at 24°C with a light intensity of 40–60 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. The experiments were performed with logarithmic phase cultures ($\sim 1.0 \times 10^5$ cells mL^{-1}).

EV Isolation

EVs from the microalgae were isolated via the ultracentrifuge method [42].

Dynamic Light Scattering

Light-scattering analyses were performed using a ZETASIZER Nano Series-Nano-ZS (Malvern) as described previously [28].

EV Labeling with PKH67 and Cell Uptake Assay

EVs were labeled with PKH67 using the PKH67 Kit (SIGMA USA) per the manufacturer's protocol. The resuspended and labeled EV was then added into 3 mL of *Chlamydomonas* cells under continuous light with 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for co-cultivation overnight at 28°C under Confocal Microscopy with a 100x oil mirror (Leica TCS-SP8, Leica Microsystems, Japan).

TEM

For TEM, an aliquot sample (25 μL of EVs resuspended in PBS buffer) was applied to an ordinary carbon coated 200 mesh copper grid (Beijing Zhongjingkeyi Technology) for 5 min. The sample was blotted, stained with 2% Phosphotungstic Acid for 1 min and then allowed to dry. Samples were then examined using a JEM-1230 transmission electron microscope (NIPPON TEKNO, Japan) operating at 200 KV.

Isolation of EV RNA and RNA analysis

EVs from green microalga *Chlamydomonas* culture medium were enriched by differential centrifugation [28]. EV RNA was isolated using the RNAiso Plus kit (#9109 TaKaRa) per the manufacturer's protocol, with some modifications including the addition of glycogen in the sixth step. After the RNAiso plus reagent was added and thoroughly mixed during RNA isolation, 25 fmol of *C. elegans* cel-mir-39 RNA was added to each sample as a spike-in control [43]. Qubit and Agilent 2200 Bioanalyzer were used to determine the concentration of EV RNAs and for quality control.

We performed polyadenylation and reverse transcription using the S-Poly(T)Plus method following as previously described [44]. Based on our EV microRNAsome, 21 miRNAs were randomly chosen to validate the RNA-seq data following a previously described method [44].

Total RNAs from EVs were used for miRNA library preparation and sequencing, which were performed at RiboBio (Guangzhou, China). Total RNA samples were briefly fractionated on a polyacrylamide gel (Invitrogen), and only small RNAs ranging from 18 to 30 nts were used for library preparation. Small

RNAs were reverse transcribed and amplified using PCR. The PCR products were sequenced using the Illumina HiSeq 2500 platform.

Comparative proteomic analyses

Protein extraction, trypsin digestion, iTRAQ labeling, LC-MS/MS analysis, database search, bioinformatic methods including GO Annotation, KEGG pathway annotation, subcellular localization, enrichment of Gene Ontology, pathway analysis, protein domain analysis, enrichment-based clustering and protein-protein interaction were conducted following previously described methods [45, 46].

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Proteomic raw data were deposited in PRIDE with ID number PXD016525 and NCBI BioProject dataset accession: PRJNA592842

Competing interests

The authors declare that they have no competing interests

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Author Contributions: Liqing Zhao and Jiangxin Wang were design the experimental work. Rongfang Zhu perform the experimental work. Yerong Liu, Chenchen Liu, and Yiting Song were extracted and analyzed the data. Yixuang Luo and Anping Lei were helps in search strategies and to draft the manuscript. All authors read and approved the final manuscript. Correspondence and Requests for materials should be addressed to: jxwang@szu.edu.cn

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Tables

Table 1 Small RNAs observed between EVs and host cells at different stages. EV1, EV2, and EV3 are triplicates of biological repeats of sRNomics. Cell samples are sRNAs isolated from host cells.

| | LogEV1 | LogEV2 | LogEV3 | N-EV1 | N-EV2 | N-EV3 | NREV1 | NREV2 | NREV3 | LogCell | N-Cell | NRCCell | PlaCell |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|---------|--------|---------|---------|
| miRNA | 1.62% | 1.70% | 1.78% | 6.88% | 6.35% | 7.50% | 3.51% | 1.78% | 2.32% | 5.01% | 11.21% | 6.70% | 5.86% |
| tRNA | 2.83% | 5.92% | 3.68% | 3.81% | 4.72% | 4.05% | 2.89% | 1.71% | 2.44% | 10.79% | 1.18% | 9.38% | 10.45% |
| rRNA | 85.79% | 81.81% | 74.49% | 65.62% | 60.26% | 55.98% | 78.95% | 84.76% | 82.48% | 28.88% | 7.88% | 21.98% | 28.99% |
| snRNA | 0.19% | 0.15% | 0.25% | 0.76% | 0.74% | 0.68% | 0.07% | 0.12% | 0.13% | 0.05% | 0.24% | 0.09% | 0.07% |
| snoRNA | 0.01% | 0.01% | 0.01% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% | 0.04% | 0.04% | 0.06% | 0.06% |
| Y_RNA | 0.04% | 0.05% | 0.09% | 0.12% | 0.09% | 0.11% | 0.06% | 0.06% | 0.06% | 0.07% | 0.04% | 0.06% | 0.05% |
| Other | 9.52% | 10.36% | 19.70% | 22.81% | 27.83% | 31.69% | 14.52% | 11.56% | 12.58% | 55.16% | 79.41% | 61.73% | 54.51% |

Table 2 Mapped Reads Percentages of sRNA in different genetic regions in EVs and host cells at different cell treatments.

| Region | LogEV | N-EV | NR-EV | PlaEV | LogCell | N-Cell | NRCCell | PlaCell |
|---------------------|---------|-------|-------|-------|---------|--------|---------|---------|
| all | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| upstream/downstream | 15.36 | 18.71 | 12.52 | 23.87 | 10.26 | 17.18 | 18.81 | 17.66 |
| exonic | 2.73 | 5.58 | 3.38 | 5.32 | 16.21 | 15.09 | 13.71 | 11.37 |
| intergenic | 36.43 | 26.84 | 38.67 | 23.7 | 15.72 | 6.25 | 12.93 | 16.22 |
| intronic | 4.86 | 10.95 | 4.95 | 16.59 | 21.64 | 26.96 | 20.4 | 17.65 |
| splicing | 0.00033 | 0.037 | 0.013 | 0.02 | 0.12 | 0.23 | 0.13 | 0.11 |
| UTR | 40.62 | 37.88 | 40.46 | 30.5 | 36.06 | 34.3 | 34.03 | 37.01 |

Table 3 Shared and unique miRNAs detected in EVs and host cells.

| miRNA % | Log | N | NR | Pla |
|---------|-------|-------|-------|-------|
| common | 4.76 | 5.72 | 5.35 | 7.51 |
| EV | 74.05 | 72.05 | 68.24 | 49.51 |
| Cell | 21.19 | 22.23 | 26.41 | 42.99 |

Table 4 Proteome of total EVs with MS/MS spectrum database search analysis summary

| Total spectrum | Matched spectrum | Peptides | Unique peptides | Identified proteins | Quantifiable proteins |
|----------------|------------------|----------|-----------------|---------------------|-----------------------|
| 101,127 | 18,251 | 11,079 | 10,812 | 2,078 | 1,788 |

Table 5 Differentially expressed protein summary (Filtered with threshold value of expression fold change and P value < 0.05)

| Compare group | Regulated type | fold change >1.2 | fold change >1.3 | fold change >1.5 | fold change >2 |
|---------------|----------------|------------------|------------------|------------------|----------------|
| N-/Pla | up-regulated | 366 | 332 | 238 | 105 |
| | down-regulated | 366 | 313 | 204 | 74 |
| Log/Pla | up-regulated | 290 | 253 | 200 | 85 |
| | down-regulated | 446 | 385 | 280 | 90 |

Table 6 Subcellular locations of EV DEG proteins, against cell growth (LogEV vs PlaEV) and nutrient stress (N-EV vs PlaEV).

| Subcellular Location | LogEV vs PlaEV | N-EV vs PlaEV | Identified |
|---------------------------------------|----------------|---------------|------------|
| endoplasmic reticulum,plasma membrane | 1 | 1 | 2 |
| Golgi apparatus | 1 | 2 | 5 |
| endoplasmic reticulum | 8 | 5 | 29 |
| peroxisome | 2 | 1 | 9 |
| vacuolar membrane | 13 | 16 | 49 |
| cytoplasm | 108 | 100 | 520 |
| extracellular | 19 | 13 | 50 |
| chloroplast,mitochondria | 0 | 2 | 3 |
| cytoplasm,nucleus | 1 | 1 | 9 |
| cytoskeleton | 1 | 3 | 7 |
| nucleus | 55 | 39 | 246 |
| chloroplast | 178 | 175 | 783 |
| plasma membrane | 63 | 46 | 201 |
| mitochondria | 30 | 38 | 164 |

Figures

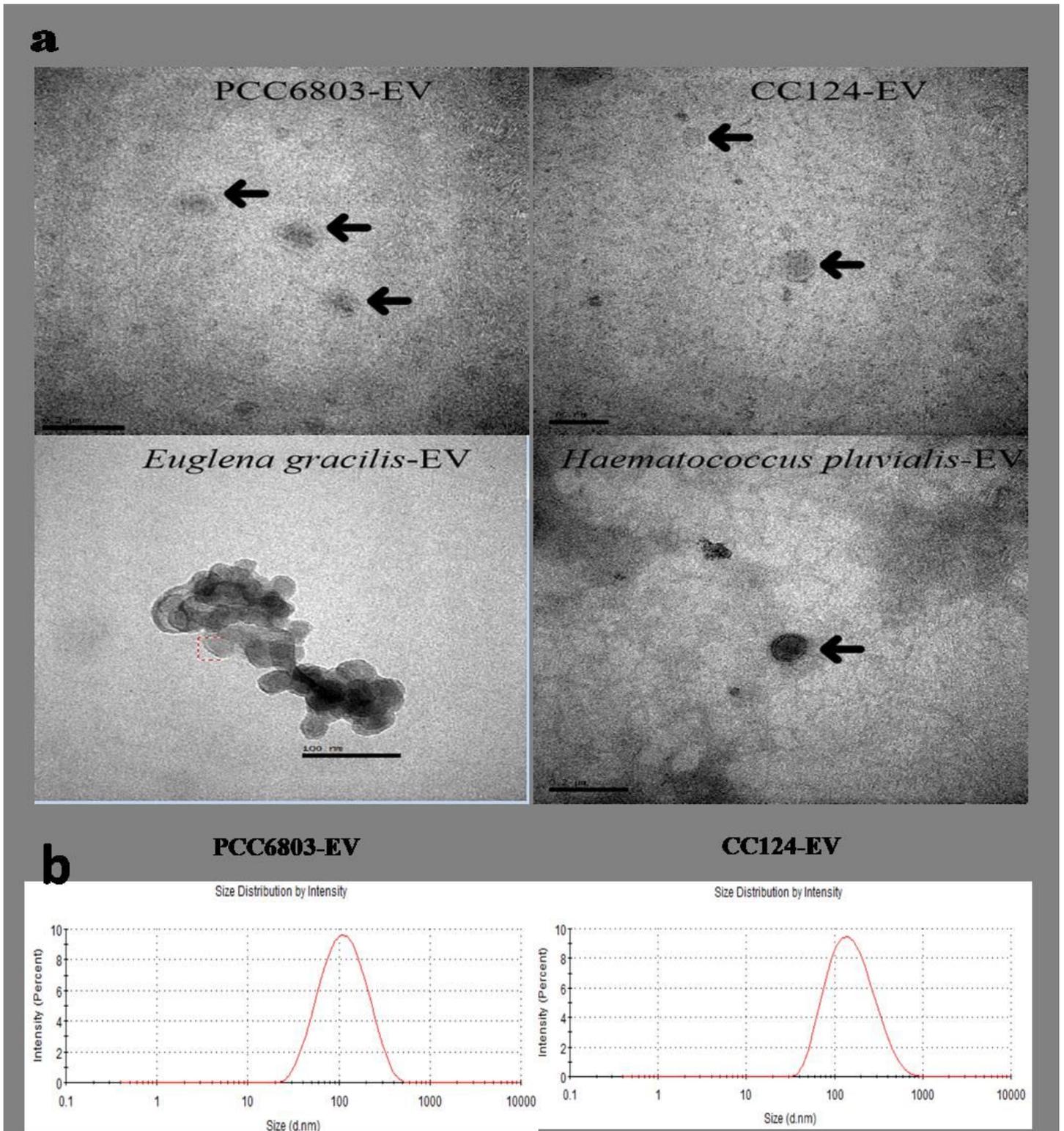


Figure 1

Small Extracellular vesicles isolated from several microalgae culture media. A, representative electron micrograph of small Extracellular vesicles isolated from cells medium revealing the typical morphology and size. B, represent PCC6803 Extracellular vesicles and Chlamydomonas CC124-EV size distribution of particles isolated with Dynamic Light Scattering.

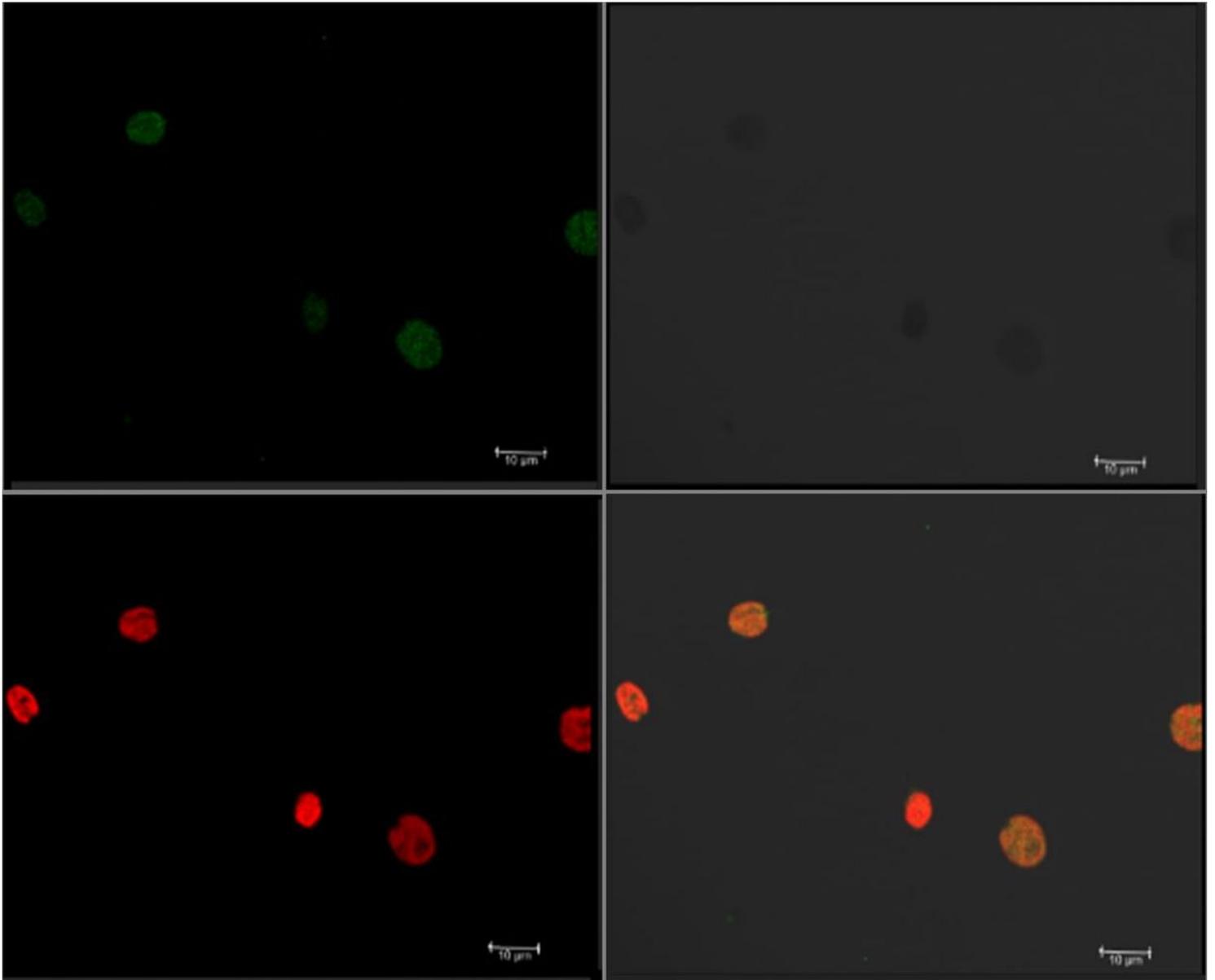


Figure 2

Co-culture of PKH67-labeled EVs with CC124 algal cells. Representative Confocal microscopy images showing the internalization of PKH67-labeled EVs (green) by *Chlamydomonas* CC124 cells.

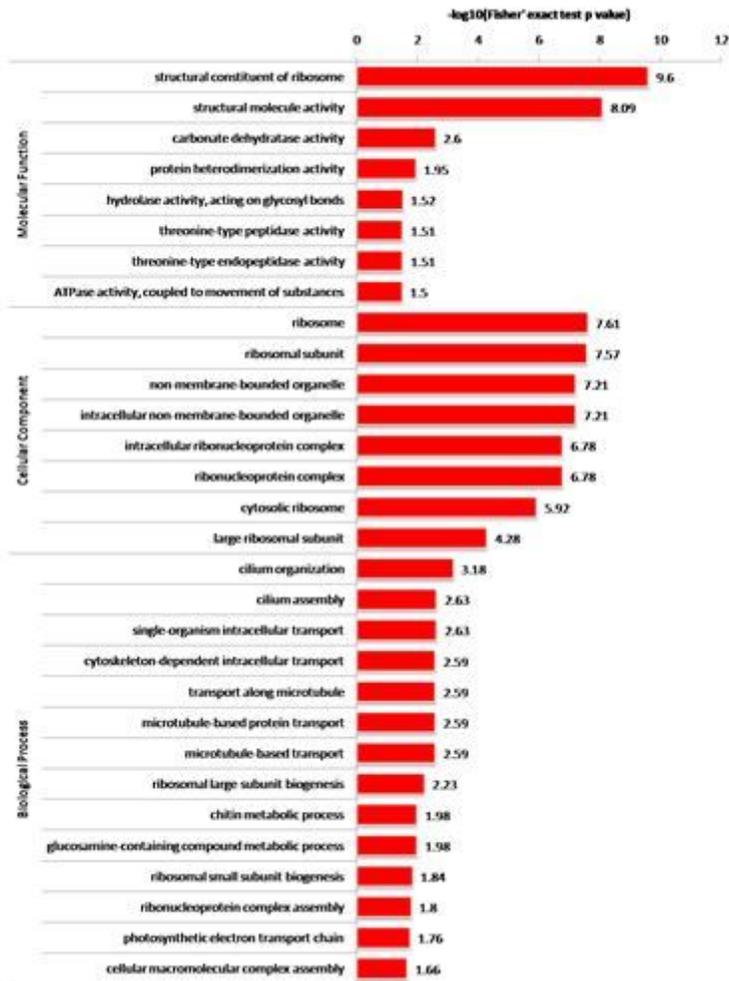


Figure 3

GO pathway enrichments of LogEV vs PlaEV showing the EV proteins and pathways related to cell growth in microalgae.

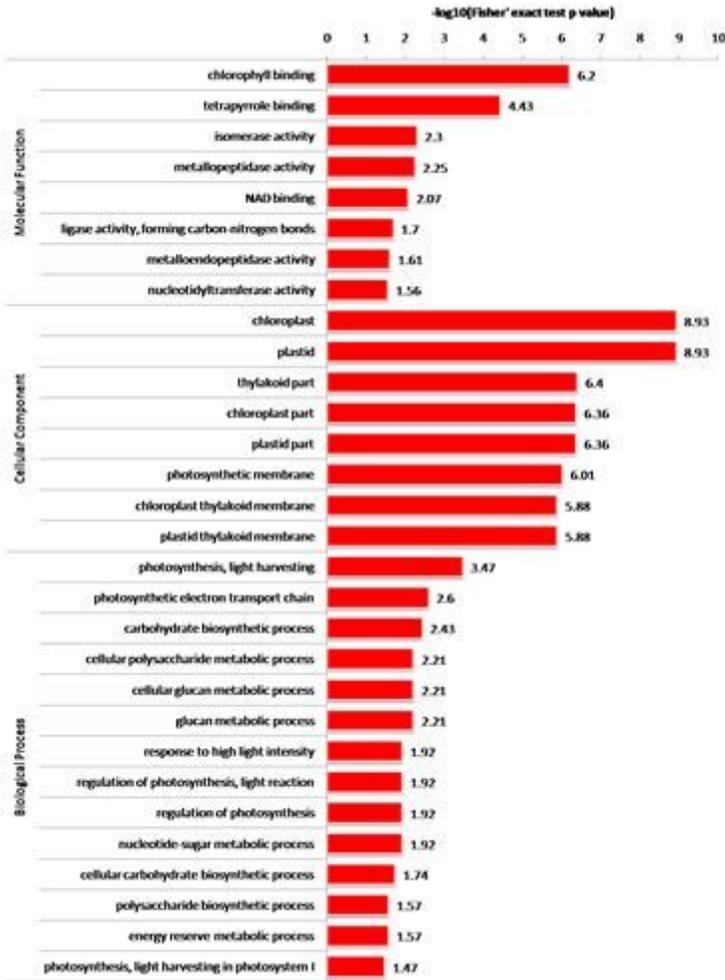


Figure 4

GO pathway enrichments of N-EV vs PlaEV showing the EV proteins and pathways related to nutrient stresses in microalgae.

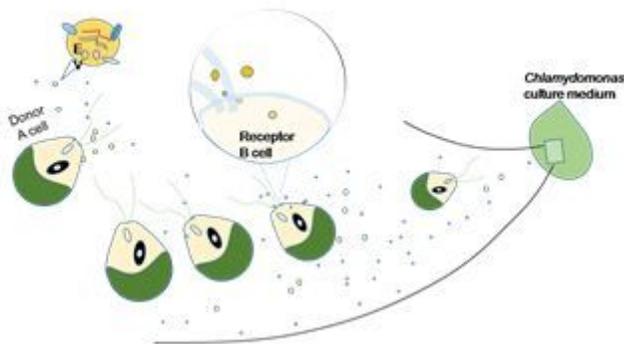


Figure 5

A propose for the cell-cell communications via EVs in microalgae culture medium.

Supplementary Files

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