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Coordinated alterations in gene expression and metabolomic profiles of *Chlamydomonas reinhardtii* during batch autotrophic culturing

Roman Puzanskiy, Daria Romanyuk, and Maria Shishova

Department of Plant Physiology and Biochemistry, Faculty of Biology,
Saint Petersburg State University, Universitetskaya nab., 7–9, Saint Petersburg, 199034,
Russian Federation

Address correspondence and requests for materials to Maria Shishova, mshishova@mail.ru

Abstract

Chlamydomonas reinhardtii was grown under autotrophic batch culturing, which is known to be a widely applicable method for both fundamental research and applied purposes. This type of cultivation results in elevation of cell density and fast exhaustion of nutrient resources. We expected the revealed metabolic adaptation to be triggered at the transcriptional level. This investigation focuses on analyzing expression of the genes encoding enzymes involved in primary metabolism and plastid transporters during the exponential phase of *C. reinhardtii* autotrophic batch culture. About two-thirds of the tested genes demonstrated differential expression during algae growth. Patterns of expression were clustered into 5 groups. Most of the genes were gathered in two large clusters, characterized by peaks of expression at early or later exponential growth (EG). Genes which showed maximal expression in early EG were *OMT1*, *HXK1*, *AMYB1*, *ACK1*,*2*, *CHLREDRAFT_123419*, *APE2*, *PCK1*, *CHLREDRAFT_195672*, *CIS2*, *TPT2* and *ACLA1.* Among the genes with maximal expression in later EG were *SBE3*, *TPIC*, *CHLREDRAFT_137300*, *CHLREDRAFT_111372*, *PPT1* and *CHLREDRAFT_122970*. There were no genes detected with maximal expression at the cessation of proliferation. PCA showed that the expression profiles in the beginning EG were similar, and profiles changed drastically in the middle of exponential growth. PLS-DA revealed the difference between the beginning of EG and later periods linked to PC1 (44%), between late EG and early stationary linked to PC2 (23%) and finally between two points at the beginning of growth linked to PC3 (10%). Mapping of genes and metabolites according to their correlation revealed a graph with two clusters. The first, smaller cluster contains genes that encode plastid exporters, enzymes of starch and carbohydrates metabolism. The expression level of these genes peaked later in EG. These genes are mainly associated with metabolites such as carbohydrates, acylglycerols and fatty acids metabolism. The second cluster is larger and more diverse. It combines genes with maximum expression in the beginning of EG. The core of this cluster is formed by genes encoding enzymes of fatty acids synthesis, energy and plastic pathways, and plastid transporters. This cluster included the majority of amino acids, carboxylic acids and many fatty acids.

Keywords: gene expression, primary metabolism, autotrophic growth, plastid transporter, *Chlamydomonas reinhardtii*, batch culture, PCA, PLS, MEBA, exponential growth.

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Author's information: Roman Puzanskiy, Junior Researcher, orcid.org/0000-0002-5862-2676; Daria Romanyuk, Ph.D., Researcher, orcid.org/0000-0001-9576-1256; Maria Shishova, Professor, Dr. Sci., orcid.org/0000-0003-3657-2986

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Introduction

Batch culturing is a common method of growing microalgae. The essence of this method is cultivation in a certain volume of incubation media, which is not renewed and not supplied with nutrients. The advantages of this culture are universality, simplicity, reliability and scalability (Perez-Garcia and Bashan, 2015).

Since batch culture is almost a closed system, dramatic changes in the medium occur during the algae growth. Due to the increase in culture cell thick-

ness, it becomes less light permeable, which leads to a decrease in the density of the PAR by several times (Krishnan et al., 2015). Mineral nutrients assimilation during cell growth causes media depletion, which significantly modulates the physiological activity of cells. For example, nitrogen and phosphorus deficiency causes intensive physiological alterations in *Chlamydomonas reinhardtii* cells (Guerrini et al., 1971; Lee et al., 2012; Zalutskaya et al., 2015). Due to the elevation of cell density and intensive microalgae metabolism, the content of C_i and O_2 in the media varies. Under autotrophic conditions, the oxygen content generally increases, and the C_i content decreases (Kim et al., 2013; Smith et al., 2015). Frequently, the growth of the microalgae population in culture is also accompanied by pH alteration in the media. One of the reasons for acidification is that photosynthesis assimilates CO_2 and produces hydroxyl ions, which leads to a pH shift (Mandalam and Pallson, 1995; Kim et al., 2013). On the other hand, the absorption of ammonium might promote acidification of the media (Pratt and Fong, 1940).

Changes in the media during culture growth correlate with the dynamics of a number of physiological processes. The activity of both photosynthesis and respiration decreases with ageing of the microalgae culture (Emerson and Lewis, 1939; Sargent, 1940; Pratt, 1943; Kulandaivelu and Senger, 1976; Fan et al., 2014). Maximum photosynthetic activity usually occurs in the first half or middle of the exponential growth phase (Winokur, 1949; Samuelsson and Oquist, 1977; Orus et al., 1991; Yang et al., 2000; Smith et al., 2015). Our previous studies showed that under autotrophic conditions, respiration and photosynthesis intensity slow down in the middle of the *C. reinhardtii* culture EG (Puzanskiy et al., 2015a). Photosynthesis activity coincides with pigment accumulation during batch culture development (Puzanskiy et al., 2011; 2015a; 2017). Since the trophic conditions are changed along with the culture growth, the rate of degradation might slow down and even stop while photosynthesis occurs. This leads to an increase in carbon deposition. In *C. reinhardtii* cells, starch and triacylglycerols (TAGs) are the main reserve compounds. Starch is synthesized faster and more intensely than TAG under stress, and is easily mobilized after returning to normal conditions (Siaut et al., 2011). Similarly, starch accumulation is more intense at the initial period of development of the *Chlorella vulgaris* culture, whereas accumulation of TAG occurs more slowly and later (Adesanya et al, 2014). Under autotrophic conditions, *C. reinhardtii* does not accumulate neutral lipids during logarithmic growth, but increases its amount within a special period of the stationary phase, named the lipid accumulation phase (LAP), and forms lipid bodies (Lv et al., 2013). Significant changes occur during the development in the metabolome of both mixotrophic (Puzanskiy et al., 2017) and autotrophic (Puzanskiy et al., 2015a; b) *C. reinhardtii* cultures.

The basis of physiological rearrangements is commonly associated with alterations in cell metabolism via a shift in the activity of biochemical pathways. One of the possible mechanisms to regulate enzyme activity is to change its amount via alteration in mRNA concentration. This supposition is confirmed by modulation of genes expression during the development of a batch culture. For example, under autotrophic conditions, the expression of genes encoding the subunits of Rubisco in *Chlorella* is higher in the exponential growth stage (Wan et al., 2011; Fan et al., 2014). For the autotrophic *C. reinhardtii* culture, it was shown that in the beginning the expression level of the gene encoding citrate synthase decreases, and then grows gradually (Deng et al., 2013). Moreover, metabolic rearrangements that occurred with the increase of cell density during *C. reinhardtii* autotrophic culturing and the transition to lipid storage were accompanied by a change in the expression of more than 2500 genes (Lv et al., 2013). Recent metabolic studies have shown a strong link between the development of culture and the profile of sugars (Puzanskiy et al., 2015a; b). Plastid transporters play a crucial role in the metabolism of autotrophic cells, implementing a key role in the export of photoassimilates and maintaining the energy balance between the cytosol and plastid (Johnson and Alric, 2013). Moreover, the *C. reinhardtii* cell contains enzymes of the ACK-PAT and ACS systems, playing an important role in anaerobic metabolism and assimilation of acetate (Terashima et al., 2010; Atteia et al., 2013, Yang et al., 2014).

Thus, significant changes in trophic conditions and the metabolic rearrangements associated with them occur during the development of the batch microalgae culture. This could be based on modulation of the expression of genes encoding enzymes of central metabolism, especially respiration, photosynthesis, lipid and starch storage.

Previously, expression of these central metabolism genes was analyzed during the development of myxotrophic culture (Puzanskiy et al., 2018), whereas the expression of these genes under autotrophic conditions was not determined. Therefore, the main goal of the present study was to analyze the level of transcripts of 32 genes (listed in Table 1) which encode the enzymes of the Calvin cycle, glycolysis and processes of carbon deposition, carbohydrate metabolism, acetyl group metabolism and plastid transporters. In order to shed light on the link between transcriptional and metabolomic profiles, a correlation analysis of the expression levels of genes of interest with the level of metabolites was performed. This work was mostly focused on the exponential growth phase analysis in order to reveal the mechanisms of maintaining cell density elevation under fast and intensive condition changes.

Table 1. Analysed genes and their products

gene	product	gene	product
ACK1	acetate kinase	*CIS2*	citrate synthase
ACK2	acetate kinase	*FBA3*	fructose-1,6-bisphosphate aldolase
ACLA1	ATP citrate lyase, subunit A	*HXK1*	hexokinase
ACLB1	ATP citrate lyase, subunit B	*HXT1*	hexose transporter
ACS1	acetyl CoA synthetase	*LCI20*	2-oxoglutarate/malate translocator
ACS2	acetyl CoA synthetase	*MEX1*	maltose exporter-like protein
AGA1	alpha-galactosidase	*OMT1*	oxoglutarate:malate antiporter
AMYB1	beta-amylase	*PCK1*	phosphoenolpyruvate carboxykinase
APE2	triose phosphate translocator	*PPT1*	phosphate/phosphoenolpyruvate translocator
BCC1	acetyl-CoA biotin carboxyl carrier	*RBCS1*	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit 1, chloroplast precursor
CHLREDRAFT_111372	PfkB-type carbohydrate kinase	*SBE3*	starch branching enzyme
CHLREDRAFT_122970	acetyl-CoA carboxylase, biotin carboxylase subunit	*STA11*	4-alpha-glucanotransferase
CHLREDRAFT_123419	3-oxoacyl-[acyl-carrier-protein] synthase III	*TAL2*	transaldolase
CHLREDRAFT_137300	starch phosphorylase	*TPIC*	triose phosphate isomerase
CHLREDRAFT_184945	acetyl-CoA carboxylase carboxyl transferase subunit alpha	*TPT2*	triose phosphate/phosphate translocator
CHLREDRAFT_195672	alpha,alpha-trehalase	*TRK1*	transketolase

Materials and Methods

STRAINS AND CELL CULTURING

Chlamydomonas reinhardtii P. A. Dangeard strain CC-124 wild-type was obtained from the collection of the Department of Genetics and Biotechnology, St. Petersburg State University. The culture was maintained under constant illumination with cold-white lamps with 3000 lux intensity in liquid TAP (Tris Acetate Phosphate) medium (Gorman and Levine, 1965). Sampling was carried out four times (Fig. 1). The cell density of the culture was determined by cell counting in the Goryaev chamber.

RNA EXTRACTION

10^7 cells were harvested by centrifugation at 3000 g for 5 min. Cells were resuspended in 1 ml of PureZOL reagent (PureZOL™, Bio-Rad, USA) (Chomczynski, 1987). The homogenate was frozen and stored at –80° C; after thawing, the samples were incubated at room temperature for 5–10 minutes and centrifuged at 12000 g for 10 minutes at 4° C. Then, 0.2 ml of chloroform was added to the homogenate, vigorously stirred for 15–20 seconds and then incubated 5 minutes at room temperature with occasional stirring and centrifuged at 4° C for 15 minutes at 12500 g. After centrifugation, 0.5 ml of isopropanol was added to aqueous phase and incubated for 10 minutes at room temperature, with subsequent centrifugation for 10 minutes at 4° C at 12500 g. To wash the pallet, 1 ml of 75% ethanol was added, vortexed and centrifuged for 5 minutes at 4° C and 12500 g. Finally, the pallet was dried and dissolved in DEPC water. RNA concentration and purity (260/280 nm ratio) was determined using the spectrophotometer NanoDrop 1000 (Thermo Fisher Scientific, USA). The samples were stored at –80° C.

C-DNA SYNTHESIS

The RNA samples were treated with RNase-Free DNase (Thermo Fisher Scientific, USA) according to the manufacturer's instructions in order to remove genomic DNA. The cDNA synthesis was performed using Olygo(dT) primers (Beagle, Russia) and M-MuLV reverse transcriptase (Thermo Fisher Scientific, USA) according to manufacturer's instructions, additionally using the RNase inhibitor RNase (Thermo Scientific RiboLock RNase Inhibitor). The samples were stored at –80° C.

REAL TIME QUANTITATIVE QRT-PCR

Primers were designed using the Beacon-Designer 8 program (Sigma Aldrich) with NCBI RefSeq database (O'Leary et al., 2015) as a source of *C. reinhardtii* genome sequence (Merchant et al., 2007). The length of the primers was about 20 nucleotides, the length of the amplicon was 80–250 nucleotides, and the predicted melting point was about 60° C. The verification of primers was performed by setting up real-time quantitative PCR reactions followed by analysis of the reaction products accumulation curves and melting curves, as well as the length of the synthesized fragments by polyacrylamide gel electrophoresis. qRT-PCR reactions were performed in the presence of SYBR Green I ("Synthol", Russia) on the CFX96 (Real Time PCR Detection System, BioRad, USA) in the following mode: 95° C — 15 s, 60° C — 50 s, 45 cycles.

MATHEMATICAL PROCESSING

Experiments were performed in three biological and three analytical replicates. Gene expression ratios were normalized against *CBLP (RACK1)* and *RPL19* genes. *CBLP* encodes a polypeptide similar to the G protein beta subunit-like polypeptide (Schloss, 1990) and is used as a reference gene in a large number of studies (Pootakham et al., 2010; Schmollinger et al., 2013; Maikova et al., 2016). *RPL19* encodes the ribosomal protein L19, and expression of this gene has been shown to be constitutive under stress conditions (Liu et al., 2012). Additionally, constitutive expression of this gene during the development of *C. reinhardtii* culture under autotrophic conditions has been tested and confirmed. Relative expression was calculated as the geometric mean of relative expressions by two reference genes using the formula $2^{\text{mean}(\Delta Ct CBLP,\ \Delta Ct RPL19)}$.

The analysis was conducted using R 3.3.The analysis was carried out using (R Core Team, 2016). ANOVA for replicates was performed. Hierarchical cluster analysis was executed using as a distance **1-r**, where r is the Pearson correlation coefficient. The Ward method was used for clustering (Murtagh et al., 2014). Tree pruning was performed on the basis of the scree plot analysis, which characterized distance between clusters at each agglomeration step.

The data for multivariate analysis has been standardized. The PCA was performed using the pcaMethods package (Stacklies et al., 2007). PLS-DA was conducted in the ropls package (Thevenot et al., 2015). The reliability of the model was estimated from the values of R^2Y and Q^2Y and the influence of permutation. MEBA was implicated in the timecourse package, and the relationship of the variable with time was evaluated by the value of the Hotelling test T2 (Tai and Speed, 2006, 2007).

METABOLIC DATA AND MAPPING

Metabolite profiles at the same time points, which were used for the expression test, were obtained according to the methodology of O. Fiehn (2008) with changes (details are published in Puzanskiy et al., 2015a). Raw data on metabolites concentration per cell (Puzansky et al., 2015b) were logarithmic, and then Pearson correlation coefficients were recalculated between metabolites content and the level of gene expression. In total, nine pairs of data sets from three independent cultures from the metabolomics experiment and from three independent cultures from the gene expression experiment were used for correlation analysis. Mean values and confidence intervals of Pearson coefficient were determined. Only pairs of gene–gene and gene–metabolite correlation links with a coefficient significantly higher than 0.7 were chosen for further testing. The Cytoscape program (Shannon et al., 2003) was used for graph construction where the nodes correspond to genes and metabolites, and the edges had strong correlation links (r> 0.7). Organic layout was used for the graph building.

Results

DYNAMICS OF MRNA CONTENT OF CENTRAL METABOLISM GENES DURING CULTURE DEVELOPMENT

Samples were collected during *C. reinhardtii* autotrophic culture development: three times during the exponential growth phase, and at the beginning of the stationary phase (Fig. 1). The analysis of accumulation of 32 gene transcripts (Table 1, 2, Fig. 2) revealed significant altera-

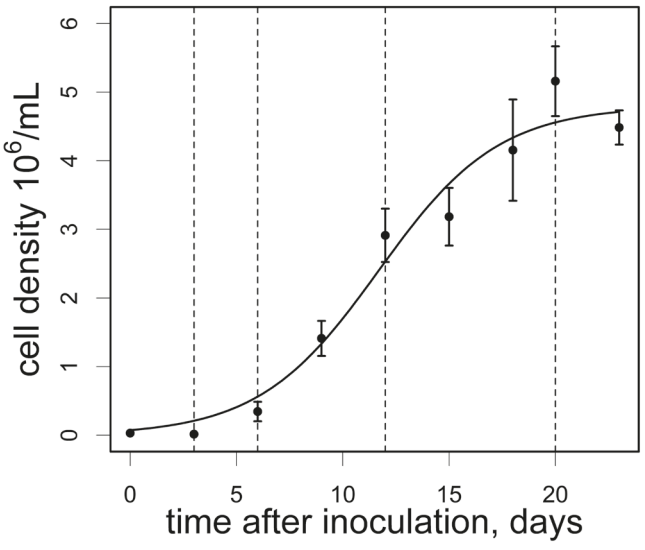

Fig. 1. Cell density (10^6 cells per mL) of *Chlamydomonas reinhardtii* cc-124 batch culture under autotrophic conditions (TM media, 3000 lux continuous light), bars are standard deviation (SD), the curve is an approximation to logistic function $CD=4.79/(1+e^{(0.355\text{-}4.14\cdot t)})$ (CD –cell density (10^6/mL), t — time after inoculation (days). Vertical dashed lines mark points of sampling.

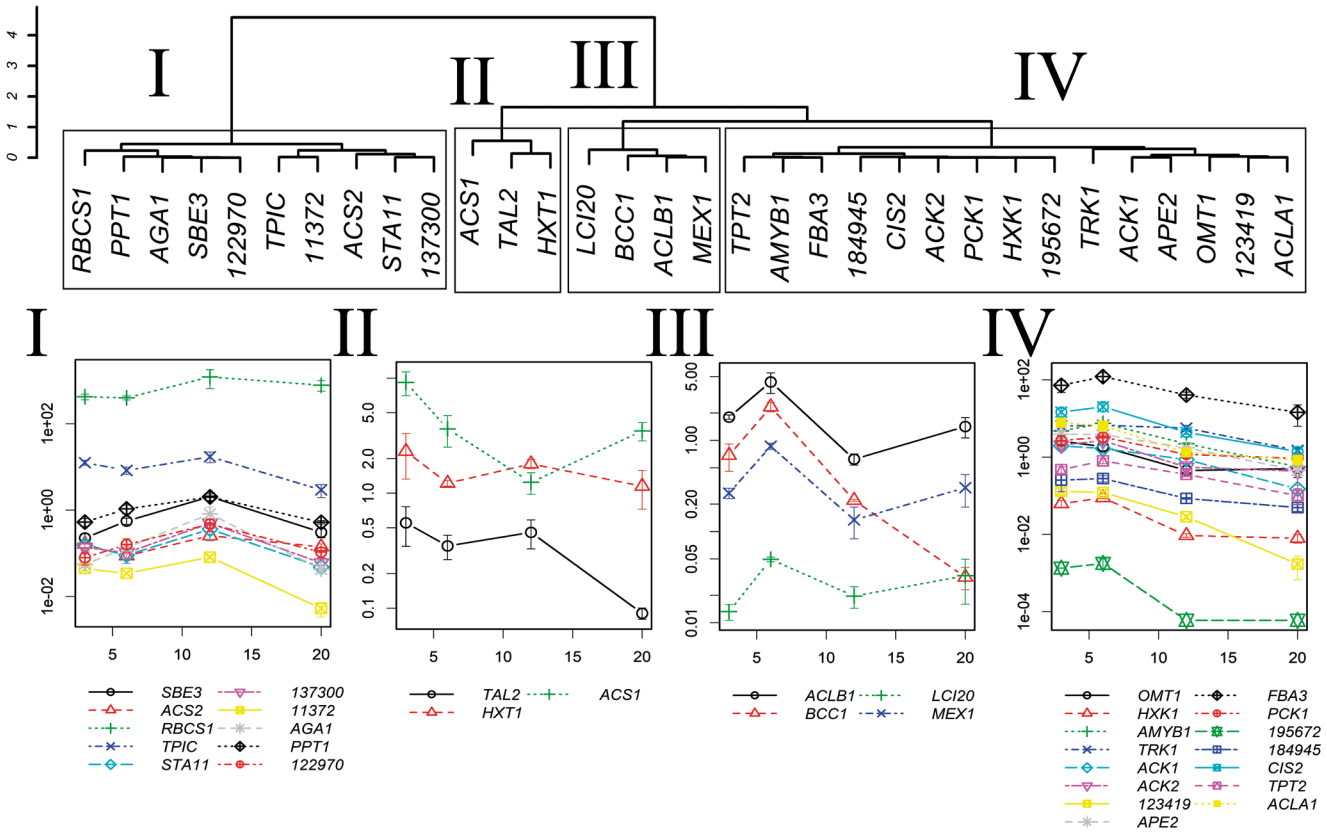

Fig. 2. Dendrogram of hierarchical clustering (Ward method) of the genes encoding primary metabolism enzymes and plastid transporters of *Chlamydomonas reinhardtii* cc-124, Pearson correlations of their relative expression levels as a measure of distance (1-r) were used, the boxes mark revealed clusters. Below are plots representing the dynamics of the relative expression levels (a. u.) (logarithmical), genes grouped by revealed clusters.

tions in the expression of genes of interest. According to the results of variance analysis, 18 of 32 genes had significant (FDR <0.05) differences in the expression level at the tested time points of culturing. Additionally, six genes demonstrated changes at the level of statistical trend (0.5<FDR<0.1). From the *post hoc* Tukey test (Table 2) it was concluded that changes in gene expression were uneven. Data comparison at the third and sixth days of culture development showed that at the early EG phase only four genes — *BCC1*, *MEX1*, *LCI20* and *ACS1* — had changes in the expression level with a Tukey p<0.05 and one gene — *ACLB1* — with p<0.1. The majority of genes (Table 2) demonstrated significant differences between the 6th and 12th days, which corresponded to the "equator intersection" of the exponential phase. A small number of changes also occurred at the late EG phase (12–20 days). Only six genes showed differences with Tukey p<0.05.

MEBA OF GENES EXPRESSION PROFILES DURING CULTURE DEVELOPMENT

One of the most effective methods for analyzing the dynamics of a large number of parameters in time is the Multivariate Empirical Bayesian Analysis (MEBA). Its speciality is to take covariance of variables in several investigated objects (in our case, independent *C. reinhardtii* cultures). It makes it possible to level out the differences between cultures and to determine more accurately the influence of time on the analyzed variables. The link between features and time was evaluated using Hotelling-T^2 statistics value (Xia et al., 2015). Table 2 shows values of T^2. The highest values of T^2 were demonstrated by genes *BCC1, CHLREDRAFT_111372, AMYB1, HXK1, APE2, CHLREDRAFT_123419* and *ACK1.*

PATTERNS OF GENE EXPRESSION

To reveal common trends in gene expression profiles and to detect gene expression patterns, a correlation analysis was followed by hierarchical clustering using the distance **1-r** –, where r is the Pearson coefficient. As a result, four patterns of expression were estimated for genes of interest (Fig. 2). Genes of the first group showed an increase in the expression level during culture growth, especially in the middle of exponential growth, and a decrease at the end of the EG stage. This group included 10 genes, among which were *SBE3*, *TPIC*, *CHLREDRAFT_137300*, *CHLREDRAFT_111372*, *PPT1* and *CHLREDRAFT_122970*. The alteration in the expression of these genes was also reliable, accord-

Table 2. Characterization of the dynamics of the gene expression level during growth of the autotrophic *Chlamydomonas reinhardtii* batch culture

Gene	Fold changes of the expression levels						ANOVA (for repeated measures)								MEBA	PLS-DA			
									Tukey test										
	3d/6d	3d/12d	6d/12d	3d/20d	6d/20d	12d/20d	p	FDR	3d-6d	3d-12d	6d-12d	3d-20d	6d-20d	12d-20d	T2	VIP	p1	p2	p3
ACK1	1,43	2,74	1,96	**13,56**	**13,52**	6,92	**0,047**	0,069	0,878	0,116	0,281	**0,015**	**0,033**	0,353	87,4	0,8	0,147	0,247	0,033
ACK2	0,86	6,46	**6,28**	8,27	**14,85**	3,77	**0,016**	**0,03**	0,616	0,084	**0,02**	0,064	**0,016**	0,995	75,5	0,85	-0,133	0,202	0,092
ACLA1	1,54	6,64	5,5	9,36	8,13	1,89	**0,022**	**0,039**	0,946	0,111	0,214	0,087	0,167	0,997	34,7	0,94	0,202	0,148	0,18
ACLB1	0,47	3	**6,88**	1,54	**3,19**	0,49	**0,01**	**0,024**	0,051	0,453	**0,009**	0,953	**0,028**	0,719	33,2	0,61	-0,149	0,091	-0,013
ACS1	**3,71**	**8,85**	2,79	**3,12**	0,99	0,36	**0,036**	0,057	**0,042**	**0,008**	0,471	**0,038**	1	0,516	25,3	0,81	0,251	0,058	-0,001
ACS2	1,79	0,71	0,38	1,31	0,69	1,82	0,075	0,092	0,651	0,504	0,128	0,983	0,834	0,345	17,3	0,94	0,225	0,08	0,085
AGA1	0,45	0,15	0,26	1,5	3,25	17,36	0,052	0,073	0,986	0,072	0,107	1	0,979	0,067	12,1	1,28	0,077	-0,096	-0,408
AMYB1	0,7	2,11	**3,44**	**9,66**	**15,57**	4,47	**0,01**	**0,024**	0,147	0,191	**0,011**	**0,032**	**0,003**	0,479	109,7	1,07	-0,028	0,307	0,108
APE2	1,15	**2,26**	2,19	**8,96**	**10,09**	4,35	**0,012**	**0,024**	0,998	**0,044**	0,054	**0,005**	**0,006**	0,25	100,8	0,93	0,23	0,16	0,109
BCC1	**0,33**	3,07	**10,66**	21,29	**90,06**	7,94	**0,001**	**0,005**	**0,001**	0,262	**0**	0,096	**0**	0,835	84	0,86	0,216	0,105	-0,11
CHLREDRAFT_111372	1,3	**0,54**	**0,42**	**10,41**	**8,18**	**19,35**	**0**	**0,003**	0,538	**0,008**	**0,002**	**0,006**	**0,025**	**0**	86,8	1,06	0,181	-0,072	-0,267
CHLREDRAFT_122970	0,57	**0,17**	**0,35**	0,77	1,42	**5,4**	**0,001**	**0,005**	0,532	**0,002**	**0,006**	0,959	0,79	**0,003**	47,8	1,08	0,228	0,056	0,247
CHLREDRAFT_123419	1,14	**4,99**	**4,3**	**149,45**	**139,86**	32,02	**0,004**	**0,018**	0,951	**0,005**	**0,009**	**0,002**	**0,002**	0,502	89,1	1,18	0,228	0,061	-0,243
CHLREDRAFT_137300	1,35	**0,29**	**0,24**	2,31	1,78	**8,43**	**0,008**	**0,022**	0,983	**0,037**	**0,025**	0,851	0,969	**0,016**	23,7	0,88	0,251	0,036	-0,062
CHLREDRAFT_184945	0,89	2,89	3,2	5,85	6,41	1,89	0,101	0,12	0,993	0,358	0,266	0,229	0,168	0,979	22,6	0,91	0,237	0,125	-0,067

CHLREDRAFT_195672	0,93	Inf	Inf	Inf	Inf	NA	**0,006**	**0,019**	0,659	**0,041**	**0,012**	**0,041**	**0,012**	1	52,5	1,22	-0,147	0,285	-0,162
CIS2	0,79	3,24	**4,68**	**11,44**	**14,66**	3,32	**0,012**	**0,024**	0,461	0,096	**0,017**	**0,036**	**0,007**	0,831	47,1	0,75	0,234	0,092	0,001
FBA3	0,76	1,88	3,36	6,31	**16,14**	4,78	0,064	0,086	0,415	0,726	0,12	0,307	**0,043**	0,816	51,9	1,3	0,197	-0,061	-0,321
HXK1	0,71	**6,66**	**9,79**	**7,98**	**12,78**	1,33	**0,001**	**0,005**	0,116	**0,006**	**0,001**	**0,005**	**0,001**	0,999	106,4	0,98	0,091	0,26	0,206
HXT1	1,94	1,23	0,69	2,06	1,32	1,86	0,316	0,316	0,497	0,893	0,857	0,451	1	0,813	12,8	0,82	0,224	0,014	-0,024
LCI20	**0,27**	0,76	2,88	0,86	2,69	1	0,074	0,092	**0,046**	0,922	0,094	0,318	0,427	0,602	30,5	0,67	0,181	0,041	-0,014
MEX1	**0,3**	2,34	**7,91**	1,27	**4,1**	0,76	**0,002**	**0,011**	**0,008**	0,694	**0,003**	0,986	**0,012**	0,518	61	0,87	-0,094	0,271	0,004
OMT1	1,64	**5,98**	4,12	**6,65**	6,25	1,39	**0,012**	**0,024**	0,387	**0,01**	0,065	**0,011**	0,076	0,999	74,6	1,04	-0,133	0,301	-0,025
PCK1	0,82	2,26	3	2,98	**3,61**	1,28	**0,033**	0,056	0,757	0,173	0,053	0,106	**0,033**	0,975	66	1,2	-0,062	0,319	0,024
PPT1	0,52	**0,26**	0,54	1,03	2,16	**3,93**	**0,005**	**0,019**	0,399	**0,012**	0,081	1	0,39	**0,012**	34,2	0,82	0,24	0,103	-0,03
RBCS1	1,05	0,48	0,46	0,58	0,56	1,87	0,295	0,305	1	0,379	0,358	0,859	0,835	0,775	16	0,95	-0,12	0,246	-0,122
SBE3	0,39	**0,11**	**0,28**	0,74	1,93	**7,85**	**0**	**0**	0,158	**0**	**0**	0,927	0,322	**0**	64,2	1,21	-0,079	0,266	-0,196
STA11	2,35	0,64	0,33	3,19	1,79	7,38	0,141	0,155	0,927	0,292	0,142	0,752	0,976	0,088	16	0,93	0,045	0,163	0,302
TAL2	1,96	1,16	0,94	5,79	4,04	4,89	0,137	0,155	0,71	0,954	0,936	0,167	0,562	0,306	35,1	1,39	0,104	-0,101	0,382
TPIC	1,67	0,84	0,56	5,86	3,4	**6,72**	**0,04**	0,061	0,739	0,681	0,233	0,188	0,585	**0,048**	41,6	1	0,193	0,14	-0,2
TPT2	0,59	1,41	2,3	7,09	**11,08**	4,46	**0,006**	**0,019**	0,185	0,84	0,069	0,126	**0,009**	0,336	40,9	1,18	-0,142	0,276	-0,171
TRK1	1,08	1,03	1,3	4,21	4,73	3,91	0,15	0,16	0,998	0,985	0,956	0,202	0,166	0,301	68,7	0,89	0,207	0,038	0,192

ing to the results of ANOVA, and was characterized by high T^2 values (Table 2). The second group included only three genes. Reliable differences were shown only by *ACS1*. The expression of this gene decreased during the culture growth. The pattern of gene expression of the third cluster was characterized by a peak of expression in the second quarter of the growth period (day 6). It included genes such as *ACLB1, BCC1, LCI20* and *MEX1.* The fourth cluster combined the largest (15) number of genes. The expression intensity of these genes decreased during the growth of the culture either from three days or from six days. This group included the following genes: *HXK1, AMYB1, OMT1, CHLREDRAFT_123419, ACK1,2, APE2, PCK1, CHLREDRAFT_195672, CIS2, TPT2, ACLA1* and *FBA3*. Changes in the expression of these genes were significant in ANOVA data, and relatively high T^2 values were characteristic for the dynamics of expression of these genes (Table 2).

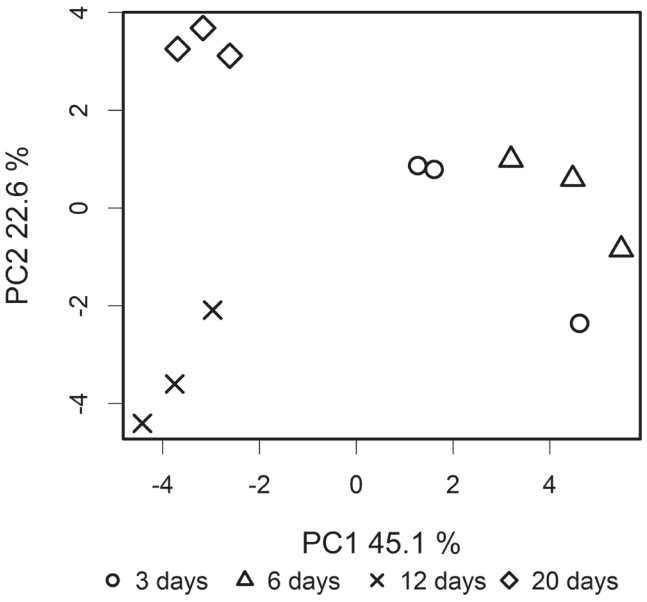

Fig. 3. PCA score plot obtained from the analysis of expression profiles of the autotrophic *Chlamydomonas reinhardtii* cc-124.

PCA

To establish the similarity of transcription profiles at different time points of culture development, the method of unsupervised analysis, PCA, was used. Figure 2 shows the graphs of the score matrix PC1 and PC2, which are explained by 44% and 23.0%. As shown in Figure 3, the samples are grouped according to the time of culture growth. This indicates a significant link between the growth of culture and the expression pattern of genes encoding enzymes of central *C. reinhardtii* metabolism. As can be seen from the chart of the score matrix, the expression profiles of cultures at the beginning of exponential growth differ from those in the second half of growth in PC1 space (44%). The culture profiles in the second half of growth and at the end differ only in the PC2 space (23%).

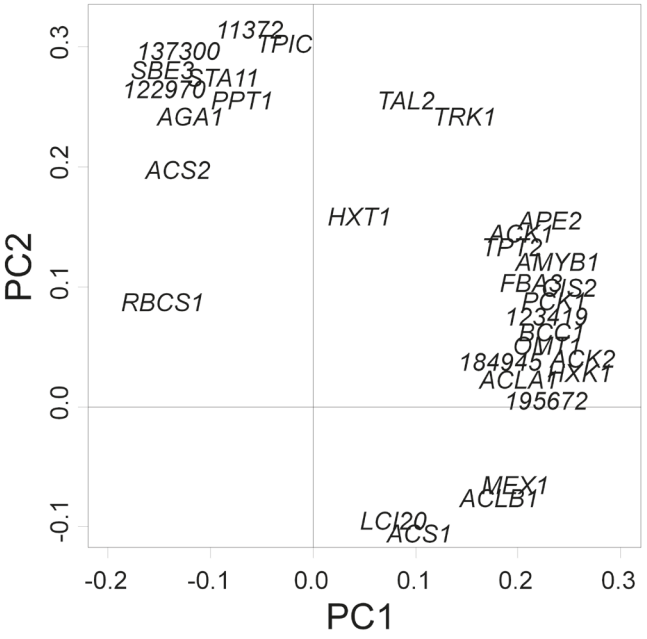

Fig. 4. PLS-DA loading plot obtained from the analysis of expression profiles of the autotrophic *Chlamydomonas reinhardtii* cc-124.

PLS-DA

The PLS-DA model of the transcriptional dynamics of the cultures in the process of culture growth included three main components explaining 44%, 23% and 10% of the dispersion (R2Y = 0.923, Q2Y = 0.785). Table 2 presents VIP values that characterize the connection between the gene of interest expression level and the distinction of the classes. For 12 genes, VIPs>1. In the case of PLS-DA, the distance of expression profiles at the beginning of exponential growth and other points in EG was related to PC1 (44%). The culture profiles in the second half of growth and at the end differed only in the PC2 (23%) space. And the variation of the expression profiles in the beginning of growth, between the 3rd and 6th day, fell within the PC3 (10%) space. Thus, the most radical changes occurred apparently in the middle of the phase of exponential growth. To find measure and direction of features variation determining the class distinction, the factor loadings of the components were considered (Table 2, Fig. 4). Most of the genes demonstrated relatively high PC1 loadings, both positive (expression higher at the beginning of exponential growth), and negative (expression higher in the second half of growth), which indicated dramatic multidirectional changes in the metabolic activity of cells in the middle of exponential growth. Most of the genes showed high PC2 factor loadings, which indicated a decrease in their expression level at the end of the growth phase. About

one-fifth of the genes demonstrated relatively high positive and negative PC3 factor loadings.

MAPPING OF GENES AND METABOLITES

To reveal the role of gene expression in the formation of a metabolite profile, a correlation analysis of gene expression levels was carried out among themselves and with the relative metabolites content per cell. The results are shown in Figure 5, where the nodes correspond to metabolites (ovals) and genes (rectangles). The edges correspond to correlations with a Pearson coefficient higher than 0.7. Since the most dramatic changes occur between the middle of the exponential phase and its completion, time points were considered in the interval of 6–20 days. Two clusters were visible. The first, of a smaller size, concentrates around the genes encoding plastid exporters, starch synthesis and enzymes of carbohydrate metabolism. The expression level of these genes has a peak in the second half of exponential growth (Fig. 2). These genes are mainly associated with carbohydrates, including glucose, fructose and sucrose. A certain amount of acylglycerols and fatty acids link to this cluster (mainly from the side of the second cluster). The second cluster is larger and more diverse, and it is made up of genes with a drop in the expression level in the second part of exponential growth (Fig. 2). It includes many of the genes encoding enzymes of fatty acids synthesis, energy and plastic pathways and plastid transporters. This cluster contains most of the amino acids, carboxylic acids and many fatty acids.

Discussion

With the elevation of cell density during batch culturing, algae needs to constantly adjust its metabolism to maintain proliferation. This adaptation occurs at the levels of transcriptome, proteome, metabolome, etc. Changes in the metabolite profile, photosynthetic intensity, respiration, and photosynthetic pigment content occur during the development of batch culture (Puzanskiy et al., 2011; 2015a; b; 2017). As it turned out, two-thirds of the genes (Table 2) demonstrated significant changes in expression during culture growth under autotrophic conditions. This allows the conclusion that large-scale metabolic rearrangements are regulated at the level of expression of genes encoding enzymes of primary metabolism and plastid transporters. That is, large-scale metabolic rearrangements are regulated at the gene expression level. Earlier, similar mechanisms were described for mixotrophic cultures (Puzanskiy et al., 2018).

The analysis showed that the dynamics of expression is non-monotonous and uneven. Application of the Tukey test (Table 2) and PCA (Fig. 3) revealed intensive variation in the middle of exponential growth. This might reflect adaptive metabolic adjustment triggered in response to changing growth conditions and aimed to maintain population growth. These rearrangements coincide with dramatic shifts in physiological status. The intensity of respiration and photosynthesis reaches its maximum and begins to decrease, and there are great changes in the metabolome (Puzanskiy et al., 2015a). During this period, the level of gene expression showing high positive PC1 factor loadings (Fig. 4) and belonging to clusters III and IV decreases (Fig. 2). Among it, significant differences (Tukey's test) in the expression level, high values of T^2 (Table 2) and high positive PC1 loads were shown in the following genes: *TPT2, MEX1, CHLREDRAFT_123419, BCC1, AMYB1, CIS2, HXK1, ACK2, FBA3, APE2, ACLB1* and *CHLREDRAFT_195672*. Three of them are genes that encode plastid transporters *TPT, APE* and *MEX* (Table 1). Triose transporters such as TPT and APE are involved in the function of triosophosphate shunt that maintains an energy balance and reduces power exchange between plastid and cytosol (Johnson and Alric, 2013). MEX exports sugar to the cytosol. High values of expression levels of these genes in the first half of the growth phase are consistent with the intensification of photosynthesis (Puzanskiy et al., 2015a) and, consequently, with the elevation of the export of its products from plastids. On the other hand, during this period, the expression of the *OMT* gene, encoding the 2-oxoglutarate/malate transporter, continued to decline, and the expression of the *LCI20* gene, encoding another 2-oxoglutarate/malate transporter, slightly decreased.

Moreover, in the second half of the exponential growth phase, the expression of several genes encoding carbohydrate metabolism enzymes dropped, including the β-amylase gene (*AMYB1)*, which encodes an enzyme that can participate in the mobilization of deposited starch. Interestingly, in *C. reinhardtii* cells under anaerobic and dark conditions, its expression notably increased (Mus et al., 2007). Expression of the gene encoding α,α-trehalase was also reduced. This enzyme cleaves glucose dimers, playing an important role in the degradation of polysaccharides. Also in the second half of exponential growth, a decrease in the expression of the gene encoding hexose kinase HXK1 was observed. This enzyme plays a central role in carbohydrate signaling and metabolism, including glycolysis. A similar dynamic was shown for the gene encoding fructose-1,6-bisphosphate aldolase 3 (FBA3), which has a chloroplast localization and is involved in the Calvin cycle and OPPP of *C. reinhardtii* (Yang et al., 2014). Thus, in the middle of the growth phase, changes in the metabolism of carbohydrates are associated with a decreasing level of carbohydrates' catabolism.

In addition to starch, TAGs are important stored substances of *C. reinhardtii*, and acetyl-CoA-carboxylase is a key enzyme that introduces acetyl groups into the

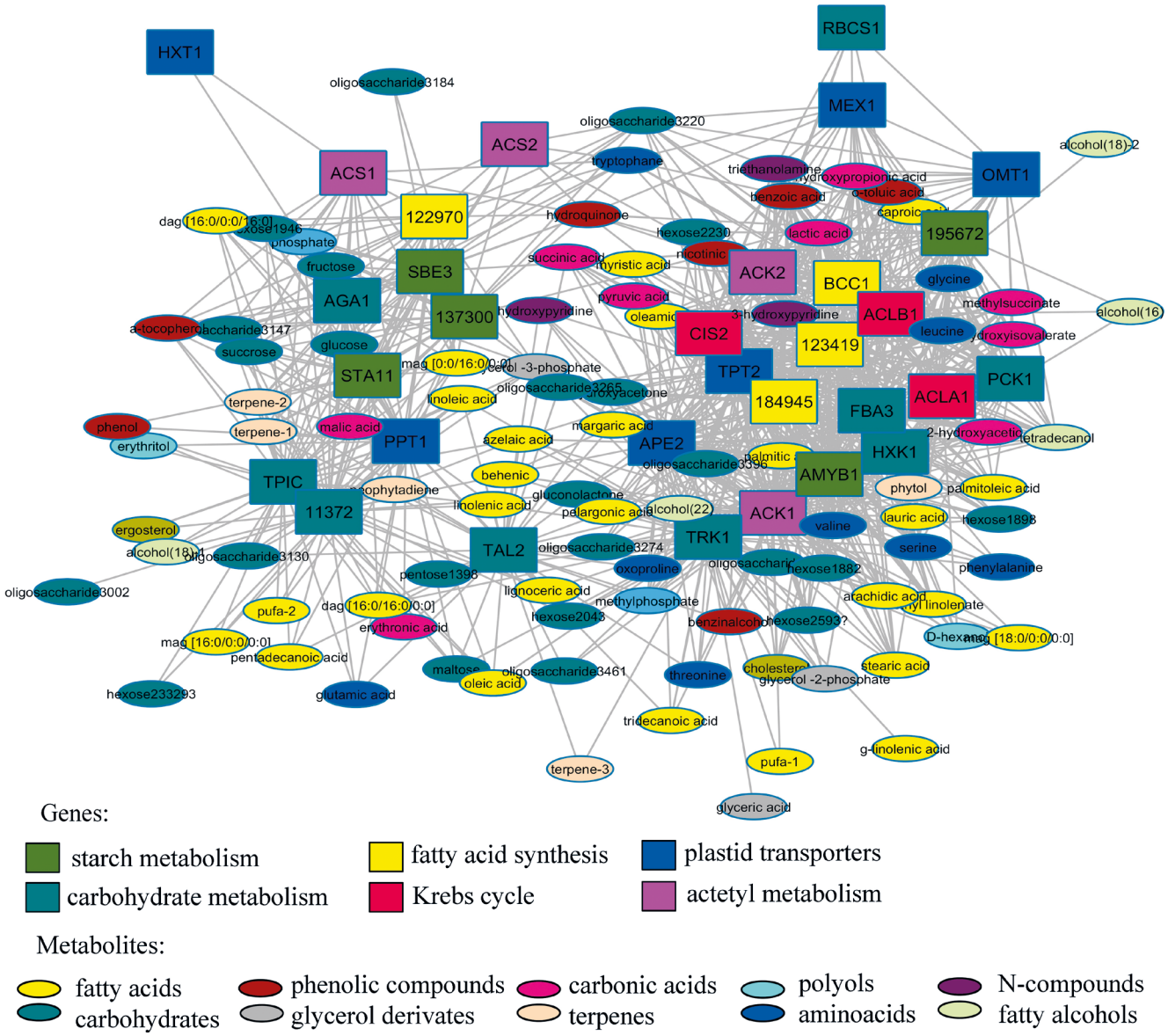

Fig. 5. Mapping of genes and metabolites by the correlation of their expression intensity and content (per cell) respectively during the middle and the end of the exponential growth phase. The figure shows a graph where the nodes are metabolites (ovals) and genes (rectangles), color denotes the class of metabolites or involvement of genes in certain biochemical process. The edges correspond to correlations with the Pearson coefficient greater than 0.7. Organic layout was used to build network.

synthesis of fatty acid chains (Cronan et al., 2002). Genes encoding the subunits of acetyl CoA carboxylase (*BCC1, CHLREDRAFT_184945*), as well as the KASIII enzyme (*CHLREDRAFT_123419*), which is also involved in the synthesis of fatty acids, showed maximum expression at the beginning of the exponential phase (Fig. 2, 4, Table 2). Acetyl-CoA is a point of carbon distribution. Acetyl-CoA carboxylase directs carbon to lipid biosynthesis. On the other hand, in the case of *C. reinhardtii* autotrophic cells, *ACC* gene activity is not enhanced during the accumulation of lipids, but expression is very high throughout growth. In comparison with the *PDH* gene, the number of ACCase transcripts is 10–20 times higher, indicating that the activity of this particular enzyme can be a "bottleneck" of the lipid biosynthesis (Lv et al., 2013).

In the second half of exponential growth, the level of expression of genes encoding enzymes regulating carbon entry and exit into the Krebs cycle, such as citrate synthase (CIS2) and isocitrate lyase (ACLB1, ACLA1), also decreases. However, during the growth of *C. reinhardtii* autotrophic culture, the level of expression of the gene encoding citrate synthase (CrCIS) first falls and then begins to grow gradually (Deng et al., 2013). In the case of myxotrophic cultures, the accumulation of CIS2 gene transcripts was reduced during culture growth. (Puzanskiy et al., 2017). On the other hand, citrate lyase (ACLA1 and ACLB1) can catalyze the regen-

eration of oxaloacetate and acetyl-CoA, which can be utilized in fatty acids synthesis (Plancke et al., 2014; Park et al., 2015). This corresponds with a decreased level of expression of the genes encoding fatty acid synthesis enzymes. Thus, a change in the activity of genes connecting the Krebs cycle with glycolysis and the synthesis of fatty acids could be associated with a decrease in both the level of respiration and the synthesis of lipids. The latter could be explained by a decrement in the membrane synthesis and the deposition of lipids.

After the culture passed the middle of the growth phase, the expression of a number of genes increased. Significant changes (Tukey's test) of the expression level, high values of T^2 (Table 2) and high negative factor loadings were shown in the following genes: *SBE3, CHLREDRAFT_122970, CHLREDRAFT_137300, PPT1* and *CHLREDRAFT_111372* (Fig. 2, 4). Among it, genes encoding enzymes (Table 1) of starch synthesis and degradation: starch phosphorylase (PHOB) and starch branching enzyme (SBE3). The *SBE3* gene was one of the few characterized by an increase in the accumulation of transcripts with an increase in the age of the mixotrophic cultures (Puzanskiy et al., 2017). Thus, it could be assumed that along with culture ageing, the value of the deposition and mobilization of starch increases. Moreover, expression of the gene (*CHLREDRAFT_111372)* encoding PfkB-type carbohydrate kinase, associated with OPPP, grew. Surprisingly, the expression of the gene encoding acetyl-CoA carboxylase, biotin carboxylase subunit, increased, whereas the level of transcripts of other subunits of this enzyme decreased. The multidirectional regulation of acetyl-CoA carboxylase subunits is also known in other cases. Thus, in the development of *C. pyrenoidosa* autotrophic culture, the level of expression of *accA* is higher during the stationary phase, and *bccp* is lower (Fan et al., 2014).

Significant alterations in the expression profile occur at the end of growth, reflecting changes in metabolism under stressful conditions when reproduction is impossible. Most of the genes show a decrease in the level of expression during transition to the stationary phase. Similar is observed under mixotrophic conditions (Puzanskiy et al., 2017).

None of the genes showed statistically significant acceleration at the end of culture growth (12–20 days). This result is in general agreement with the previously published data about the decrease of the expression level of genes involved in photosynthesis and energy metabolism (Lv et al., 2013). Nevertheless, a majority of tested genes demonstrated an increase in expression during the transition to a stationary phase, which seems rather unexpected and is at variance with our data. This can be explained by the differences in the experimental conditions: higher intensity of illumination, the presence of the circadian rhythm and differences in the medium composition.

Analysis of the correlation between the levels of gene expression and the content of metabolites revealed two gathered clusters (Fig. 5). The first, smaller cluster is centered around the genes of plastid exporters, enzymes involved in synthesis and degradation of starch and carbohydrate metabolism. The expression level of these genes has a peak in the second half of exponential growth. These genes are mainly linked to carbohydrates, including disaccharides and acylglycerols. Glycerol-3-phosphate is adjacent to this cluster. It could be suggested that this cluster is associated with carbon deposition, which is usually intensified in the later period of culture development.

The second cluster is larger and more diverse. It combines genes with a higher level of expression in the first half of exponential growth. Among them are the genes of enzymes of fatty acids synthesis, energy and plastic pathways, and plastid transporters. A high level of expression of these genes is associated with a high content of amino acids, carboxylic acids and a lot of fatty acids. Perhaps this metabolic cluster underlies the high growth of culture during the exponential phase. Different genes have a different number and set of correlation links. Some of the genes are associated with a small number of metabolites, while other genes are placed in the clusters' cores and linked to much bigger group of metabolites. This indicates different involvement in global metabolism rearrangements during culture growth.

Most of the genes of central metabolism changed their level of expression during the EG phase of *C. reinhardtii* autotrophic culture. The strongest alterations were observed in the middle of exponential growth. Most of the genes form two large clusters, showing maxima of expression at the beginning and second half of exponential growth.

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Abbreviations: ACK-PAT acetate kinase — phosphate acetyltransferase; ACS — acetyl-CoA synthetase; ANOVA — analysis of variance; EG — exponential growth; FDR — false discovery rate; C_i — C inorganic; DAG- diacylglycerol; LAP — lipid accumulation phase; MAG — monoacylglycerol; MEBA — Multivariate Empirical Bayesian Analysis; OPPP — oxidative pentose phosphate pathway; PAR — Photosynthetically active radiation; PCA — principal component analysis; PLS-DA — partial least squares (projections on latent structures) discriminant analysis; TAG — triacylglycerol; VIP — variable importance in projection.

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