DOI: 10.1007/s11099-015-0128-z PHOTOSYNTHETICA 53 (4): 625-629, 2015

BRIEF COMMUNICATION

Proteomic study reveals photosynthesis as downstream of both MAP kinase and cAMP signaling pathways in *Chlamydomonas reinhardtii*

C. LEE[*,†], J.-K. RHEE[**,†], D.G. KIM[*], and Y.-E. CHOI[***,+]

Department of Bioprocess Engineering, Chonbuk National University, Jeonju 561-756, Korea[*]
Department of Food Science and Engineering, Ewha Womans University, Seoul 03760, Korea[**]
Division of Environmental Science and Ecological Engineering, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, Korea[***]

Abstract

Previously, our data indicated that both cAMP and MAP kinase signaling play important roles in microalgal physiology as well as in lipid or carotenoid biosynthesis. In order to understand downstream genes of these signaling pathways, we employed proteomics approach. Both signal pathways were first altered with specific signaling inhibitors or modulators. Treatment of specific inhibitors changed microalgal size and increased lipid contents. With the microalgal cells after treatments of specific signaling inhibitor or modulators, we performed the proteomics analysis to identify downstream genes responsible for these phenotypes. Interestingly, multiple photosynthesis genes were identified, particularly proteins associated with PSII. Our data suggested that MAP kinase and cAMP signaling affect the photosynthesis, thereby leading to microalgal lipid or carotenoid biosynthesis.

Additional key words: gene regulation; mass spectrometry; two-dimensional gel electrophoresis.

Bioenergy derived from biomass has attracted public attention as a carbon neutral, renewable, and economical energy resource. In particular, biodiesels derived from microalgae have enormous potential due to their capacity to accumulate high lipid contents. Importantly, economic feasibility to utilize microalgal biomass can be further improved by the coproduction of value-added secondary metabolites such as carotenoids (Car). However, little is known about the mechanisms of microalgal lipid or Car biosynthesis.

Previously, we identified that microalgal mitogen-activated protein kinase (MAP kinase pathway) as well as cAMP signaling pathways regulate overall microalgal Car and lipid production (unpublished data). However, there are still large gaps and missing links to MAP kinase or cAMP signaling and microalgal Car and lipid biosynthesis in detail.

Proteins are final products of gene regulation so that both MAP kinase and cAMP signaling must significantly impact the expressions of downstream genes at the level of proteins, thereby rendering phenotypic characteristics of microalgal Car and lipid production. Since there have been found significant differences in the expression between protein and mRNA, it is always better to decipher the protein expressions as the final product for phenotypes. Therefore, in order to understand and identify these missing links, we employed proteomics to discover the possible downstream pathways of either MAP kinase or cAMP signaling. First, MAP kinase and cAMP signaling were altered with specific signaling inhibitors [MAP kinase kinase (MEK or MAP 2K) inhibitor] and modulators [IBMX (3-isobutyl-1-methylxanthine) inhibiting the activity of phosphodiesterase (PDE)], respectively. MAP kinase signaling cascade commonly consists of three

Received 31 July 2014, *accepted* 6 January 2015.
Corresponding author; phone: +82-2-3290-3042, fax: +82-2-3290-3040, e-mail: yechoi@korea.ac.kr
Abbreviations: cAMP – cyclic adenosine monophosphate; Car – carotenoid; DE – decrease; IBMX – 3-isobutyl-1-methylxanthine; IN – increase; MAP kinase – mitogen-activated protein kinase; MEK – MAP kinase kinase or MAP 2K; MEKK – MAP kinase kinase kinase or MAP 3K; OT – only in treated; OU – only in untreated; 2-DE – two-dimensional gel electrophoresis.
Acknowledgements: This research was also financially supported by the Ministry of Trade, Industry & Energy (MOTIE), Korea Institute for Advancement of Technology (KIAT), and Honam Institute for Regional Program Evaluation through the Leading Industry Development for Economic Region. This work was supported by the Advanced Biomass R&D Center (ABC) of Korea Grant funded by the Ministry of Education, Science and Technology (ABC-2012M3A6A2055032). This research was also financially supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2014R1A1A2055300). †These authors contributed equally to this work.

consecutive enzymes: MAP kinase kinase kinase (MEKK or MAP 3K), MAP kinase kinase (MEK or MAP 2K), and MAP kinase (MAPK). The MEK inhibitor is an organic compound that specifically inhibits activation of MAP kinase kinase by suppressing the kinase activity of MEK. On the other hand, cAMP is the key secondary messenger switching on/off multiple downstream cellular signals across many eukaryotic organisms. Therefore, it is crucial that the extent of cAMP in cells must be tightly regulated, which is mainly mediated by the activities of the adenylate cyclase and cAMP phosphodiesterase. Adenylate cyclase and cAMP phosphodiesterase are the enzymes that synthesize and degrade cAMP, respectively. IBMX (3-isobutyl-1-methylxanthine) is a well-established chemical modulator of cAMP signaling by inhibiting cAMP phosphodiesterase, thereby leading to the increase of cellular cAMP concentration. We hypothesized that proteins differentially expressed upon the treatment of either MEK inhibitor or IBMX are associated with phenotypic characteristics of microalgal lipid or Car biosynthesis. Protein expressional changes upon either cAMP signaling modulator (IBMX) or MEK inhibitor were thoroughly investigated using *Chlamydomonas reinhardtii*, since it is amenable to study the expression of various proteins with the complete genome sequence recently available.

Chlamydomonas reinhardtii was cultivated in Tris-acetate-phosphate (TAP) medium at 25°C under continuous illumination (average light intensity of 150 μmol m^{-1} s^{-1} PAR). Air was bubbled into the culture that was shaken at 200 rpm to support aeration. In the early exponential stage, we transferred *C. reinhardtii* suspensions into the appropriate inductive conditions. MEK inhibitor was purchased from *Promega* (MEK Inhibitor, U0126), and IBMX (IBMX, I5879) was supplied from *Sigma-Aldrich*. Microalgal biomass obtained from the treatment by either MEK inhibitor or IBMX was subjected to two-dimensional gel electrophoresis (2-DE) analysis. The same amount of proteins were loaded onto gels after the protein quantitative analysis by Bradford method (*Bio-Rad*, Hercules, CA, USA). Following fixation of the gels for 1 h in a solution of 40% (v/v) methanol containing 5% (v/v) phosphoric acid, the gels were stained with Colloidal Coomassie Blue G-250 solution (*ProteomeTech*, South Korea) for 5 h. The gels were destained in 1% (v/v) acetic acid for 4 h and then images processed using a *GS-710* imaging calibrated densitometer (*Bio-Rad*, Hercules, CA, USA). In addition, we also included *C. reinhardtii* without any treatment as a control.

Protein spots of interest were excised and digested in-gel with sequencing grade, modified trypsin (*Promega*, Madison, WI, USA) as previously described (Bahk *et al.* 2004). The gel slices were dehydrated by 100% acetonitrile (ACN), and then dried in a SpeedVac concentrator. After reduction [incubation at 56°C for 45 min with 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate (ABC) and alkylation (incubation at room temperature for 30 min at dark place with 55 mM iodoacetamide in 100 mM ABC)], the gel slices were dried by 100% ACN and *SpeedVac*. Then the gel slices were rehydrated in 30 μl of 25 mM ABC, containing 20 ng of trypsin. After incubation at 37°C for 20 h, the liquid was transferred to a new tube. Tryptic peptides remaining in the gel matrix were extracted for 40 min at 30°C with 20 μl of 50% (v/v) aqueous acetonitrile containing 0.1% (v/v) formic acid. The combined supernatants were evaporated in *SpeedVac* and dissolved in 8 μl of 5% (v/v) aqueous acetonitrile solution containing 0.1% (v/v) formic acid for mass spectrometric analysis.

The resulting tryptic peptides were separated and analyzed using reversed phase capillary HPLC directly coupled to a *Finnigan* LCQ ion-trap mass spectrometer (*ThermoQuest,* San José, USA) (LC-MS/MS) according to Zuo *et al.* (2001) with a slight modification. Both of a 0.1 × 20 mm trapping and a 0.075 × 130 mm resolving column were packed with *Vydac 218MS* low trifluoroacetic acid C18 beads (5 μm in size, 3 μm in pore size; *Vydac*, Hesperia, CA, USA) and placed in-line. The peptides were bound to the trapping column for 10 min with 5% (v/v) aqueous acetonitrile containing 0.1% (v/v) formic acid, then the bound peptides were eluted with a 50-min gradient of 5–80% (v/v) acetonitrile containing 0.1% (v/v) formic acid at a flow rate of 0.2 μl min^{-1}. For tandem mass spectrometry, a full mass scan range mode was m/z = 450–2,000 Da. After determination of the charge states of an ion on zoom scans, product ion spectra were acquired in MS/MS mode with relative collision energy of 55%.

The individual spectra from MS/MS were processed using the *TurboSEQUEST* software (*Thermo Quest*, San Jose, USA). The generated peak list files were used to query either *MSDB* database or *NCBI* using the *MASCOT* program (http://www.matrixscience.com). Modifications of methionine and cysteine [carbamidomethyl (C), deamidated (NQ), oxidation (M)], peptide mass tolerance at 2 Da, MS/MS ion mass tolerance at 1 Da, allowance of missed cleavage at 1, and charge states (+1, +2, and +3) were taken into account. Only significant hits as defined by *MASCOT* probability analysis were considered as matching proteins.

Before the proteomic analysis, we confirmed the phenotypic characteristics of increased lipid production by the treatment of either MAP kinase inhibitor or IBMX treatments. Both MAP kinase inhibitor and IBMX treatments could indeed alter lipid contents significantly, compared with the control, without any treatment (Fig. 1S, *supplementary data available online*). Increased lipid bodies (LBs) were observed upon the treatment of either MAP kinase inhibitor or IBMX treatment yielding a bright yellow fluorescence (Fig. 1S). We also confirmed that the treatment of MAP kinase inhibitor led to enhanced Car biosynthesis (Fig. 1S). After the confirmed consistency with the previous results concerning the effect of both MAP kinase inhibitor and IBMX treatments, we performed quantitative comparison of the proteins that were uniquely and differentially regulated in either MAP kinase inhibitor

or IBMX treatments. As a result of 2-DE analysis, 13 protein spots were confirmed as differentially expressed proteins by the treatment of MAP kinase inhibitor, whereas 14 differential expressions were identified *via* the IBMX treatment (Table 1, Fig. 1). Thus, totally, 27 protein spots were selected for subsequent LC-MS/MS.

After collection and analysis of MS/MS data, we identified multiple proteins associated with microalgal photosynthesis. Of 14 protein spots identified in IBMX treatment, 11 protein spots exhibited a significant difference in expression between the IBMX treatment and untreated samples, whereas three proteins were only identified from nontreatment. Among proteins identified as differential expression between the IBMX treatment and nontreatment, the expressions of five and six protein spots increased and decreased by the IBMX treatment, respectively. Particularly, the proteins in a group with the increased expression upon the IBMX treatment were identified as proteins closely associated with the photosynthesis (Table 1). For example, proteins categorized as the light-dependent proteins, including plastid-specific ribosomal protein (PSRP) (Yamaguchi *et al*. 2003), ATP synthase (Lemaire and Wollman 1989), major LHCII protein m1 (Ferrante *et al*. 2012), PSII, and Rubisco (Goldschmidt-Clermont and Rahire1986) could be discovered after increased expression upon IBMX. Our data suggested that microalgal cAMP signaling pathway must be tightly linked with the photosynthesis.

On the other hand, proteins, whose expressions were lost or downregulated upon IBMX, were suggested not to play any direct role in photosynthesis. For example, hydroxyproline-rich glycoprotein (Adair *et al*. 1990), isocitrate lyase (Godavari *et al*. 1973), G-strand telomere binding protein 1 (Petracek *et al*. 1994, Rhodes *et al*. 1995), and peroxiredoxin (Naumann *et al*. 2007) were identified as downregulated proteins by the IBMX treatment. Since the majority of proteins downregulated upon IBMX was not directly connected with photosynthesis, we speculated that IBMX triggered the degradation or suppressed expression of proteins, which were not associated with the

Table 1. Identification of differentially expressed proteins in *Chlamydomonas reinhardtii* by the manipulation of MAP kinase or cAMP signaling pathways. DE – decrease; IN – increase; OU – only in untreated; OT – only in treated.

Inhibitor	Spot No.	Acc. No.	Putative protein homolog	Mass	No. of peptides identified	Up/down regulation ratio
IBMX	DE01	gi|896259	hydroxyproline-rich glycoprotein, partial	29,382	2	0.436
	DE02	gi|159466892	beta subunit of mitochondrial ATP synthase	61,783	19	0.342
	DE03	gi|159474436	isocitrate lyase	45,720	6	0.132
	DE04	gi|159463672	G-strand telomere binding protein 1	24,046	24	0.425
	DE05	gi|11995220	peroxiredoxin	22,237	22	0.309
	IN01	gi|41179050	ATP synthase CF1 alpha subunit	54,718	13	3.319
	IN02	gi|41179057	ATP synthase CF1 beta subunit	53,129	21	2.920
	IN03	gi|159487801	plastid-specific ribosomal protein 3	33,346	3	2.922
	IN04	gi|20269804	major light-harvesting complex II protein m1	27,548	7	2.410
	IN05	gi|159489872	predicted protein (repair PSII)	28,296	4	2.380
	IN06	gi|16975084	chain I, Rubisco from *Chlamydomonas reinhardtii*	16,224	7	2.843
	OU01	gi|159467709	peptidyl-prolyl cis-trans isomerase, cyclophilin-type	44,667	7	0.016
	OU02	gi|159491492	light-harvesting complex II chlorophyll *a-b* binding protein M3	27,363	6	0.005
	OU03	-	no significant match	-	-	0.004
MEK	DE01	gi|159466892	beta subunit of mitochondrial ATP synthase	61,783	19	0.459
	DE02	gi|41179050	ATP synthase CF1 alpha subunit	54,718	2	0.434
	DE03	gi|41179050	ATP synthase CF1 alpha subunit	54,718	16	0.265
	DE04	-	no significant match	-	8	0.421
	DE05	-	no significant match	-	28	0.439
	DE06	gi|11995220	peroxiredoxin	22,237	13	0.491
	DE07	-	no significant match	-	14	0.381
	DE08	-	no significant match	-	-	0.428
	IN01	gi|159483223	2-cys peroxiredoxin	21,628	3	2.341
	IN02	gi|159489872	predicted protein (repair PSII)	28,296	4	2.371
	IN03	gi|16975084	chain I, Rubisco from *Chlamydomonas reinhardtii*	16,224	7	2.200
	OT01	gi|16975080	chain A, Rubisco from *Chlamydomonas reinhardtii*	52,520	15	254
	OT02	-	no significant match	-	-	357

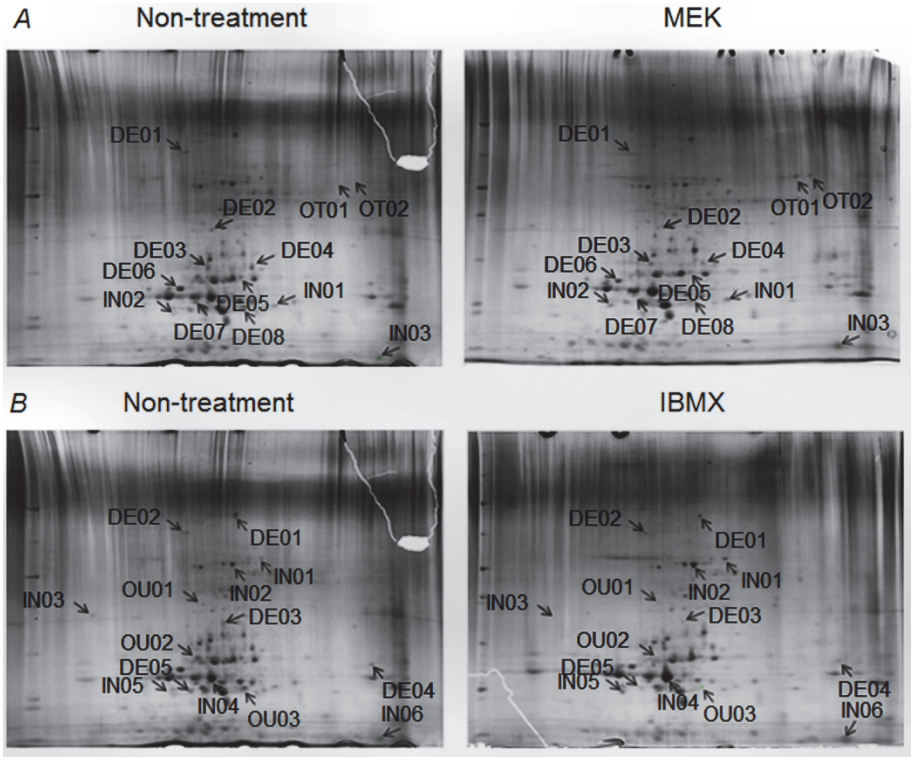

Fig. 1. Different protein expression profiles between nontreatment and the treatment of either IBMX or MEK inhibitor to alter or disrupt signaling in *Chlamydomonas reinhardtii*.

photosynthesis, to further concentrate the metabolic flux mostly on the photosynthesis. It in turn led to increased biosynthesis of microalgal lipids. For example, in this regard, the IBMX treatment led to the decrease of isocitrate lyase expression, which was not tightly related with the photosynthesis, thereby enhancing the photosynthesis. Only exception was LHCII chlorophyll *a-b* binding protein M3 that showed the decreased expression upon the IBMX treatment (Table 1). In contrast, along with multiple proteins linked with the photosynthesis, LHCII protein m1 increased upon the IBMX treatment (Table 1). Therefore, it is likely that different expression patterns of the major LHCII protein m1 and chlorophyll *a-b* binding protein M3 might be explained by the fact that the major LHCII protein m1 is directly bound to PSII contrary to chlorophyll *a-b* binding protein M3 (Ferrante *et al*. 2012). Taken together, although exception has been noted in LHCII chlorophyll *a-b* binding protein M3, it is likely that IBMX treatment could increase microalgal lipid production by maximizing the efficiency of photosynthesis. To this end, the expression of proteins directly related to PSII were increased upon IBMX treatment, whereas indirectly linked proteins with the photosynthesis were downregulated to achieve more efficient photosynthesis by preventing unnecessary energy consumption. In fact, consistent with our findings, it has been reported that the expression of PSII related gene was increased under nitrogen deprivation, which could enhance microalgal lipid contents (Ermilova *et al*. 2013).

We also examined the differential expression of proteins upon the treatment by the MEK inhibitor. Likewise the influence of IBMX on *C. reinhardtii* protein expression, we observed the increases in proteins related to PSII, which might in turn lead to the improvement of lipid synthesis. Of 13 protein spots identified by the MEK inhibitor treatment, three and seven protein spots increased and decreased, respectively (Table 1). In addition, only two proteins were identified by the treatment of the MEK inhibitor. Proteins downregulated upon the MEK inhibitor were beta subunit of mitochondrial ATP synthase, ATP synthase, LHCII protein m1, and peroxiredoxin. By contrast, protein upregulated by the MEK inhibitor were 2-cys peroxiredoxin, protein involved in repairing PSII, and chain I or A of Rubisco, indicating that *C. reinhardtii* MAP kinase signaling must be negatively associated with the photosynthesis. However, unlike the consistent positive influence of IBMX over proteins linked with photosynthesis, the MEK inhibitor treatment led to a different effect in that proteins such as β-subunit of mitochondrial ATP synthase and ATP synthase CF1 α-subunit, peroxiredoxin, related with photosynthesis were instead decreased in the expression. The protein, which exhibited the opposite patterns of regulation upon the MEK inhibitor, was ATP synthase protein. Particularly, protein spots corresponding to the family of peroxiredoxin displayed either increase or decrease by the treatment of the MEK inhibitor. We

speculated that there must be a different response of peroxiredoxin depending on the kind of peroxiredoxin, since the expression pattern varied with individually different peroxiredoxin (protein spot of DE06 *vs.* IN01 in the group of MEK inhibitor treatment).

Interestingly, multiple proteins identified by the IBMX and MEK inhibitor overlapped suggesting the similar effects of both signaling on the photosynthesis. In general, is has been well-accepted that carbohydrates generated from the photosynthesis must become a pool for subsequent microalgal metabolites, such as lipid or Car production. Since our previous study showed that microalgal MAP kinase and/or cAMP signaling pathways regulate overall microalgal Car and/or lipid production, it is likely that the missing link between microalgal major signaling (MAP kinase and cAMP signaling) and the Car and/or lipid biosynthesis must be the photosynthesis. Clearly, with the employment of proteomics, our data successfully revealed that MAP kinase and cAMP signaling affect the photosynthesis, thereby leading to microalgal lipid or Car biosynthesis. Further in-depth study on the photosynthesis could shed light on the impact of the photosynthesis on microalgal lipid or Car biosynthesis in detail.

References

Adair W.S., Apt K.E.: Cell wall regeneration in *Chlamydomonas*: Accumulation of mRNAs encoding cell wall HRGPs. – P. Natl. Acad. Sci. USA **87**: 7355-7359, 1990.

Bahk Y.Y., Kim S.A., Kim J.S. *et al*.: Antigens secreted from *Mycobacterium tuberculosis*: Identification by proteomics approach and test for diagnostic marker. – Proteomics **4**: 3299-3307, 2004.

Ermilova E., Lapina T., Zalutskaya Z. *et al*.: PII signal transduction protein in *Chlamydomonas reinhardtii*: localization and expression pattern. – Protist **164**: 49-59, 2013.

Ferrante P., Ballottari M., Bonente G. *et al*.: LHCBM1 and LHCBM2/7 polypeptides, components of major LHCII complex, have distinct functional roles in photosynthetic antenna system of *Chlamydomonas reinhardtii*. – J. Biol. Chem. **287**: 16276-16288, 2012.

Godavari H.R., Badour S.S., Waygood E.R.: Isocitrate lyase in green leaves. – Plant Physiol. **51**: 863-867, 1973.

Goldschmidt-Clermont M., Rahire M.: Sequence, evolution and differential expression of the two genes encoding variant small subunits of ribulose biphosphate carboxylaso/oxygenase in *Chlamydomonas reinhardtii*. – J. Mol. Biol. **191**: 421-432, 1986.

Lemaire C. and Wollman F. A.: The chloroplast ATP synthase in *Chlamydomonas reinhardtii*. II. Biochemical studies on its biogenesis using mutants defective in photophosphorylation. - – J. Biol. Chem. **264**: 10235-10242, 1989.

Naumann B., Busch A., Allmer J. *et al*.: Comparative quantitative proteomics to investigate the remodeling of bioenergetic pathways under iron deficiency in *Chlamydomonas reinhardtii*. – Proteomics **7**: 3964-3979, 2007.

Petracek M.E., Konkel L.M.C., Kable M.L., Berman J.: A *Chlamydomonas* protein that binds single stranded G-strand telomere DNA. – EMBO J. **13**: 3648-3658, 1994.

Rhodes D., Giraldo, R.: Telomere structure and function. – Curr. Opin. Struc. Biol. **5**: 311-322, 1995.

Yamaguchi K., Prieto S., Beligni M.V. *et al*.: Proteomic characterization of the *Chlamydomonas reinhardtii* chloroplast ribosome: Identification of proteins unique to the 70S ribosome. – J. Biol. Chem. **278**: 33774-33785, 2003.

Zuo X., Echan L., Hembach P. *et al*.: Towards global analysis of mammalian proteomes using sample prefractionation prior to narrow pH range two dimensional gels and using one-dimensional gels for insoluble and large proteins. – Electrophoresis **22**: 1603-1615, 2001.