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RESEARCH PAPER

Resistance and Proteomic Response of Microalgae to Ionizing Irradiation

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Abstract Microalgae have been drawing much attention as a platform for food supplements and biofuel production. Advance molecular tools are not available for manipulating microalgae, and therefore, methods for strain improvement mostly depend on random mutation. Radiation is frequently used mutagen in plant as well as microalgae breeding methods. In this study, the resistance of 7 microalgae species to ionizing irradiation was measured. To monitor the growth of microalgae, optical density and staining methods were used. Based on the D_{10} values, the dose required to reduce one log cycle of the cell population, *Chlorella protothecoides, Zygnema circumcarinatum*, and *Spirogyra varians* were shown to be highly resistant to ionizing radiation. The changes in protein expression levels in *S. varians* were further investigated. Using 2-dimensional electrophoresis and protein identification, it was shown that some proteins involved in energy and glyceride metabolisms were up-regulated. These results provide fundamental insights into metabolic changes that occur in a microalga species upon exposure to ionizing irradiation.

Keywords: ionizing irradiation, microalgae, proteome, resistance

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1. Introduction

Microalgae are incredibly valuable to the biotechnology industry for the production of food supplements and functional nutrients. Recently, microalgae biotechnology has drawing much attention as a platform for biofuel production [1]. However, methods used for improving microalgae strains have almost entirely depended on random mutation because of a lack of advance molecular tools [2]. Radiation is one of the traditional mutation methods for strain improvement and has been used to develop several microalgae species [3]. However, there have been limited studies on resistance and response of microalgal species to radiation.

Radiation is generally classified into ionizing and non-ionizing radiation, based on whether it has enough energy to knock electrons off atoms. Non-ionizing radiation, such as near- and medium-ultraviolet (UV) radiation, does not carry enough energy to break molecular bonds and ionize atoms. There are many reports on the deleterious effects of UVA and UVB on cyanobacteria, phytoplankton, and microalgae [4,5]. Ionizing radiation, such as cosmic rays, X-rays, and gamma rays emitted by radioactive materials, has enough energy to break molecular bonds and ionize atoms. Despite its more severe effects on cells, there are few studies on the effects of ionizing radiation on microalgae.

At low doses of ionizing radiation, the reactive radicals generated by radiation indirectly ionize DNA molecules, enzymes, or other parts of cells, resulting in metabolic changes. However, beyond a certain dose, along with indirect effects, direct collision action results in significant changes that cause cell death. The response of cells to radiation is generally expressed as D_{10} (kGy), the dose required to reduce one log cycle of the population. Knowing the D_{10} value is very important to decide the proper mutation

conditions for strain development [6].

Therefore, the D_{10} values of different microalgae species were investigated in this study. To monitor the growth of microalgae, optical density was measured and staining was conducted for comparison. Also, changes in protein expression caused by irradiation were investigated using two-dimensional electrophoresis. These results will be useful not only for understanding molecular responses of microalgae to radiation, but also for developing microalgae strains.

2. Materials and Methods

2.1. Microalgae

The microalgae *Botryococcus braunii* UTEX LB572, *Chlamydomonas reinhardtii* UTEX 90, *Chlorella protothecoides* (recently renamed as *Auxenochlorella protothecoides*) UTEX 25, *Neochloris oleoabundans* UTEX 1185, *Scenedesmus dimorphus* UTEX 746, and *Zygnema circumcarinatum* UTEX 1560 were purchased from the UTEX Culture Collection of Algae at the University of Texas, TX, USA. *Spirogyra varians* was received from Kongju National University, South Korea. The algae were cultivated in an incubator at 20°C, 12:12 h LD cycle, and under fluorescent light (>20 μmol photons m^{-2} s^{-1}). Each species was maintained in the medium suggested by UTEX.

2.2. Growth monitoring

Optical density or turbidity can be measured to determine the relative growth rate of microalgae. The optical density of each microalga in this study was measured at the optimum wavelength of 750 nm. In the case of *C. reinhardtii* and *N. oleoabundans*, optical density was measured at 540 nm following previously reported methods [7]. The optical density of the cells was measured with a UV-Vis spectrophotometer (Uvikon XL, BioTek Instruments, Winooski, VT).

Cell viability of microalgae was also measured with fluorescein diacetate (3′, 6′-diacetylfluorescein, FDA) [8]. FDA solution (100 mg FDA/20 mL dimethysulfoxide) was diluted 1:4000 with medium and mixed with cell broth. After incubation for 15 min, the viable cells were illuminated by blue light in a fluorescence microscope (Scope A1, Zeiss, Oberkochen, Germany). Cells emitting green fluorescence were considered viable.

2.3. Ionizing irradiation

To determine critical dose of D_{10} for microalgae, the microalgal cells were cultivated until the optical density reached to 0.5. Then, the cells were transferred to a 50 mL tube and irradiated using a ^{60}cobalt gamma irradiator (Point source, AECL; IR-79, Nordion, Canada) at various doses (0, 1, 3, 5, 8, and 10 kGy). The source strength was approximately 11.1 PBq with a dose rate at the location of the sample of approximately 10 kGy/h. Irradiation was carried out at 20 ± 2ºC. After irradiation, the cells were allowed to recover for 2 h before experiments. This experiment was performed at the Advanced Radiation Technology Institute, Korean Atomic Energy Research Institute (Jeongeup, South Korea).

2.4. Proteomic analysis

Two-dimensional electrophoresis (2-DE) was performed according to previously reported methods [9]. After 2-DE, the gel was stained with Coomassie Brilliant blue for 1 h and was treated with a de-staining solution containing 10% (v/v) methanol and 10% (v/v) acetic acid. Stained gels were scanned using a photo scanner (Perfection V700 Photo; Epson Corp., Nagano, Japan). To analyze the gel images, PDQuest software (Ver. 8.1.0; Bio-Rad, Hercules, CA, USA) was used. Differentially expressed protein spots were selected to compare the non-irradiated and irradiated samples.

Protein identification was performed using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (Voyager-DE STR MALDI-TOF Mass Spectrometer, Applied Biosystems, Foster City, CA). The obtained mass peptide profiles were internally calibrated using MoverZ (http://www.genomicsolutionscanada.com), using known autolysis peaks from porcine trypsin [9]. After subtraction of known background peaks derived from the matrix, trypsin and traces of keratin, the lists of peptide masses were compared to databases using Mascot (http://www.matrixscience.com) [9]. In Mascot, NCBInr and Swiss-Prot were used as the protein sequence databases. Additionally, the Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF optics was employed in this study for MALDI-TOF MS/MS analysis.

3. Results and Discussion

3.1. Determination of D_{10} values

In this study, several microalgae strains were tested to investigate the response to ionizing irradiation. *Spirogyra* and *Zygnema* were reported to accumulate a high concentration of carbohydrate up to 60% [9,10]. Therefore, if these microalgae can be cultivated in large quantities, they can be used to produce bioethanol from polysaccharides. *C. protothecoides* is a facultative heterotrophic green alga known for its potential application in biofuel production [11]. *S. dimorphus*, with 14-40% lipid content, has been used recently for developing biodiesel [12]. *C. reinhardtii*

has been used extensively in molecular and genetic studies and is a suitable species for overexpressing useful genes [13]. *B. braunii* and *N. oleoabundans* are the most actively used species in biodiesel research because they produce large amounts of lipids under stress [14]. These microalgae strains are drawing much attention for producing nutrients and bioenergy. Therefore, their response to abiotic stresses like irradiation needs to be investigated for strain development and cultivation optimization.

Measuring growth rate is paramount as a basis for measuring resistance and analyzing response to irradiation in these studies. The cell concentration of microalgae can be measured with optical density. Optical density is generally used for measuring cell concentration, but it is usually restricted to nonaggregative unicellular microorganisms [15]. To verify whether optical density is applicable for monitoring microalgae growth, a linear correlation was calculated between optical density and other assays. An alternative assay to measure microbial growth indirectly involves the use of a dye-based method. The viability of cells was evaluated by the FDA staining method. Hydrolytic esterase in living cells converts non-fluorescent FDA into the green fluorescent metabolite fluorescein, and living cells emit green fluorescence when illuminated with UV light under a fluorescence microscope [16]. Cells with and without green fluorescence were counted to obtain the ratio of live to dead cells.

Fig. 1 shows the cell viability of *S. varians* and *Z. circumcarinatum* after ionizing irradiation. As shown in Fig. 1, *S. varians* started dying at a radiation dose of 5 kGy; most filaments died at a dose of 8 kGy. On the other hand, *Z. circumcarinatum* largely survived up to a dose of 8 kGy, but at 10 kGy, all filaments died.

C. reinhardtii was cultivated and irradiated at different doses. After irradiation, its viability was compared by both optical density and FDA staining. As shown in Fig. 2, the growth rate measured by both methods was similar. This result suggested that optical density measurements are applicable to microalgae, regardless of their phylogeny. Therefore, we used only the optical density method for evaluating the sensitivity of microalgae to irradiation.

Seven microalgae strains used in this study were irradiated at different doses and their viability was measured. Using the linear regression of viability after irradiation, the D_{10} values of microalgae strains were calculated and are presented in Table 1. *Z. circumcarinatum* and *C. protothecoides* were shown to have high resistance to ionizing irradiation. On the other hand, *S. dimorphus* showed a very low radiation

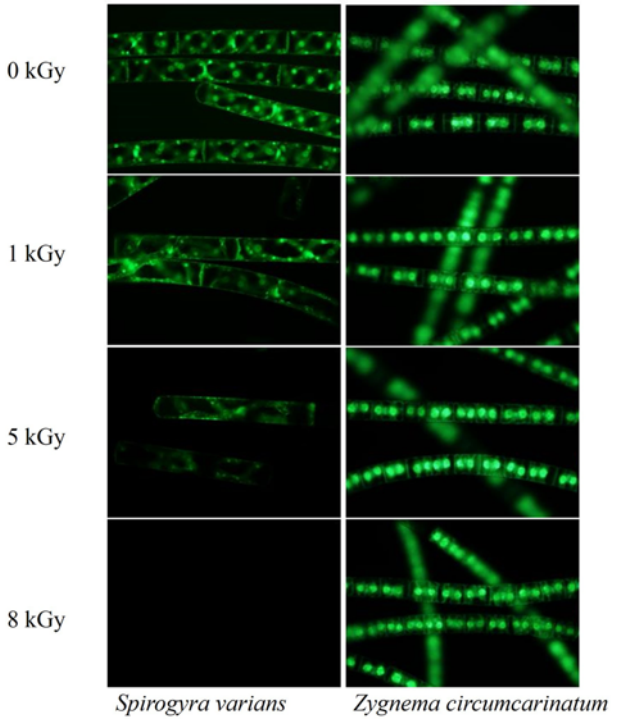

Fig. 1. Representative fluorescent images of FDA-stained microalgae cells of *Spirogyra varians* and *Zygnema circumcarinatum* under different doses of ionizing irradiation. Living cells emit green fluorescence.

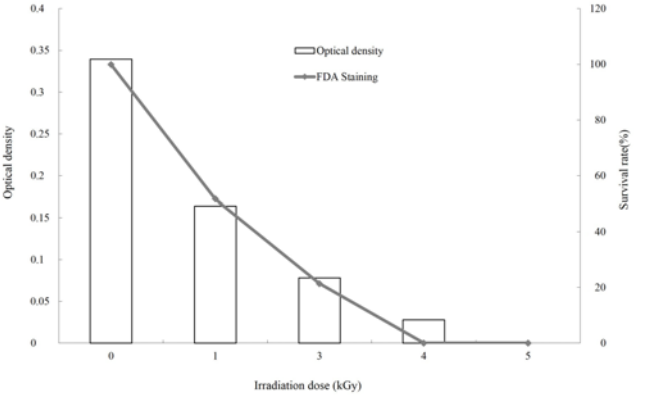

Fig. 2. Comparison of FDA staining and optical density for measuring growth of *Chlamydomonas reinhardtii* under different doses of ionizing irradiation.

Table 1. The D_{10} value of microalgae to gamma irradiation. D_{10} means the dose required to kill one log cycle of the population

Species	Mean D_{10} ± SD (kGy)
Botryococcus braunii	3.48 ± 0.15
Chlamydomonas reinhardtii	5.04 ± 0.14
Chlorella protothecoides	9.17 ± 0.31
Neochloris oleoabundans	4.21 ± 0.16
Scenedesmus dimorphus	0.81 ± 0.22
Spirogyra varians	6.75 ± 0.79
Zygnema circumcarinatum	9.52 ± 0.52

sensitivity compared to other species.

Zygnema is often found in shallow puddles and streamlets, and on the surface of water-saturated soil and stone particles exposed to ambient solar radiation [17]. The high resistance of this species to irradiation is likely based on its ecological environment. Several studies have investigated the tolerance of *Zygnema* sp. to UV radiation [18]. *C. protothecoides* inhibits low-pH environments [19]. The high acid-tolerance of *C. protothecoides* could protect the cells against free-radical ions generated by irradiation. *Deinococcus radioduranse*, one of the most irradiation-resistant strains, is well known for its high tolerance to desiccation and acidic conditions, alongside its radiation resistance [20]. *S. varians* is a cold-adapted species that blooms in winter and early spring in Korea, and can endure low temperatures and desiccation [10]. Therefore, all three species adapted to various stress environments are thought to express stress tolerance genes that increase the cell survival rate upon exposure to ionizing radiation [21]. Among the microalgae examined, several mutant strains of *Spirogyra* were isolated and the function of thioredoxin was investigated [10, 22]. Therefore, to investigate the response to ionizing irradiation, protein expression in *S. varians* was analyzed after irradiation.

3.2. Proteomic analysis

Proteomic analysis using 2-DE was performed to determine the effect of ionizing irradiation on *S. varians* to investigate the response mechanism for high radiation resistance. Fig. 3 shows the protein expression after irradiation at different doses. Approximately 300 protein spots were identified to be expressed. Results for the non-irradiated control and the irradiated samples were compared. It was confirmed that approximately 10 proteins were differentially overexpressed upon exposure to radiation.

MALDI-TOF MS analysis of proteins highly expressed under irradiation showed that the mascot scores of the proteins ranged from 56 to 73 and the sequence coverage was about 20 - 40% (Table 1). The highly expressed proteins were mostly enzymes such as glucose-1-phosphate adenylyl

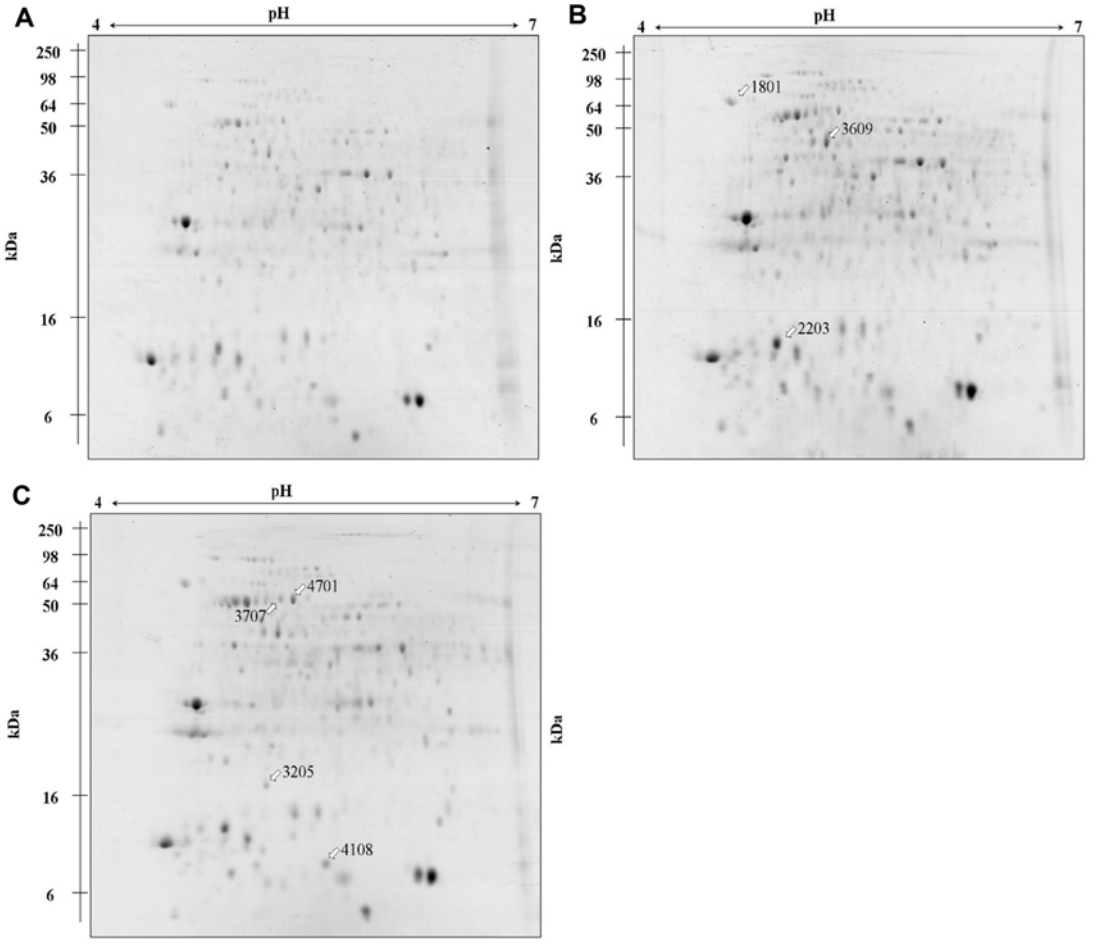

Fig. 3. Gel images showing protein expression by 2-dimensional electrophoresis of *Spirogyra varians*. (A) Non-irradiated; (B) Ionizing irradiated at doses of 1 kGy, and (C) 5 kGy.

Table 2. Identified proteins in *Spirogyra varians* highly expressed under ionizing irradiation

Spot No.	Protein name	Con→IR	Mascot score	Expected		Function
				MW (Da)	pI	
1801	Glucose-1-phosphate adenylyltransferase small subunit	UP	56	53762	5.59	Energy metabolism
3707	Pentatricopeptide repeat-containing protein	UP	62	97994	7.36	RNA editing
4701	ATP synthase subunit alpha	UP	62	55183	5.20	Electron transport / membraneassociated energy conservation
3609	ATP synthase subunit alpha	UP	73	54758	5.35	Electron transport / membraneassociated energy conservation
3202	ATP synthase subunit beta	UP	62	52872	4.98	Electron transport / membraneassociated energy conservation
2203	Nucleoside diphosphate kinase	UP	65	15431	5.36	Phosphate group transfer
4108	Glycerol-3-phosphate dehydrogenase	UP	70	71828	6.88	Glyceride Metabolism

transferase small subunit, ATP synthase, nucleoside diphosphate kinase involved in energy metabolism, and glycerol-3-phosphate dehydrogenase involved in glyceride metabolism. A pentatricopeptide repeat-containing protein involved in RNA editing was also highly expressed upon exposure to ionizing radiation.

Glucose-1-phosphate adenylyl transferase (EC:2.7.7.27) is known to play a role in starch synthesis. This enzyme catalyzes the synthesis of the activated glycosyl donor, ADP-glucose, from Glc-1-P and ATP. In a previous study, an inorganic phosphate deficiency stimulated the ADP-glucose pyrophosphorylase, and caused starch accumulation [23]. Therefore, the up-regulation of glucose-1-phosphate adenylyl transferase by radiation could be a defense mechanism to promote cell survival.

The role of ATP synthase (EC:3.6.3.14) is to convert the transthylakoid proton gradient into ATP and is related to cell growth. These proteins are involved in isoprene biosynthesis, and were previously reported to be down-regulated in *Haematococcus pluvialis* by oxidative stress [24]. The up-regulation of this protein by irradiation could be related to the high radiation tolerance of *Spirogyra* to radiation.

Nucleoside diphosphate kinases (EC:2.7.4.6) (NDK) are enzymes required for the synthesis of nucleoside triphosphates (NTPs) other than ATP. They provide NTPs for nucleic acid synthesis; CTP for lipid synthesis; UTP for polysaccharide synthesis; and GTP for protein elongation, signal transduction, and microtubule polymerization [25]. This protein was reported to increase after light irradiation [26,27].

The specific activity of glycerol-3-phosphate dehydrogenase (GPDH) (EC1.1.1.8), one of the two enzymes constituting the glycerol production pathway, increased markedly upon exposure to salt. Cells in the transition between fermentation and respiration or entering the stationary phase have been shown to contain elevated levels of GPDH. Glycerol accumulation and elevated levels of GPDH were factors that may have contributed to the capacity of the cells to withstand and recover from severe abiotic stress [28].

Pentatricopeptide repeat (PPR) proteins are characterized by tandem repeats of a degenerate 35 amino acid motif. PPR proteins are sequence-specific RNA-binding proteins that are involved in multiple aspects of RNA metabolism. Most have roles in mitochondria or plastids. Some of these proteins have been shown to play a role in post-transcriptional processes within organelles. In rice, PPR is important for chloroplast development, growth, and the maintenance of photosynthetic electron transport under cold stress [29]. The up-regulation of this protein also contributed to the high resistance of *Spirogyra* to radiation.

4. Conclusion

In this study, we demonstrated that radiation resistance of microalgae to ionizing irradiation and ionizing irradiation induced the expression of some proteins for survival. Proteomic analysis confirmed that energy and glyceride metabolism of *Spirogyra* sp. were significantly affected by ionizing irradiation. Overall, this study provides fundamental insights into ionizing irradiation-induced proteomic and metabolic changes in a microalgal species.

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