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Genome-Wide Gene Expression Profiling in γ-Irradiated Green Alga, *Pseudokirchneriella subcapitata*, by HiCEP

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Abstract Transcriptome was analyzed in γ-irradiated green alga *Pseudokirchneriella subcapitata* by high coverage gene expression profiling (HiCEP). Approximately 7,800 expressed genes were detected. Expression levels of 623–707 genes were affected at 100–300 Gy. Nucleotide sequences of 41 up-regulated genes were determined. The quantitative reverse transcription polymerase chain reaction validated the up-regulation. Two genes had homology to genes related to ionizing radiation. These results indicate usefulness of HiCEP for screening of stress-responsive genes in species that are ecotoxicologically important but for which genomic sequence information is lacking.

Keywords *Pseudokirchneriella subcapitata* · Transcriptome · HiCEP · Ionizing radiation

In ecotoxicological studies, it is important to identify genes involved in stress responses, because they provide a key for elucidation of toxicity mechanisms and can be used as biomarkers for ecotoxicity evaluation. Gene expression profiling is a powerful means for identifying such genes comprehensively. Currently, the standard approach to gene expression profiling is to use full genome microarrays, which require fully sequenced genomes (Snell et al. 2003). However, genomic sequence information is lacking for most species used in ecotoxicological studies. For example, a limited number of genes have been identified and sequenced in *Pseudokirchneriella subcapitata*, though this green alga is most commonly used alga in the algal growth inhibition test (OECD 2006). It is impossible to perform gene expression profiling in such species using full genome microarrays.

Recently, an amplified fragment length polymorphism (AFLP)-based gene expression method called "high coverage gene expression profiling" (HiCEP) analysis was developed for transcriptome analysis (Fukumura et al. 2003). This method requires no sequence information, and thus is applicable to eukaryotes for which genome information is lacking. Prokaryotes also can be analyzed with some modifications (Mitani et al. 2006). The HiCEP method is highly reproducible and sensitive enough to detect even a 1.2-fold difference in gene expression. This method has been able to detect 70–80% of all transcripts in mice, humans and yeasts (Fukumura et al. 2003). It has been used for gene expression profiling in mouse embryonic stem cells (Araki et al. 2006), and for identification of stress-responsive genes in human fibroblasts exposed to ionizing radiation (Fujimori et al. 2005, 2008) and arsenite (Suetomi et al. 2008), springtails irradiated with γ-rays (Nakamori et al. 2008) and the bacterium *Rhodococcus* exposed to isoniazid and ethambutol (Mitani et al. 2006).

In this study, HiCEP analysis was carried out in *P. subcapitata* exposed to ionizing radiation to demonstrate usefulness of HiCEP for screening of stress-responsive genes in species that are ecotoxicologically important but for which genomic sequence information is lacking.

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Materials and Methods

Four hundred milliliters of C medium (Ichimura 1971) were prepared in a 1 L Erlenmeyer flask stoppered with a silicone plug. Pre-cultured *P. subcapitata* ATCC22662 was inoculated to this medium at 1×10^4 cells mL^{-1}. Culturing was carried out at 25°C on a rotary shaker (120 rpm) with continuous illumination by fluorescent lamps (photosynthesis photon flux density: 170 μmol m^{-2} s^{-1}). After 64 h, when the culture was in a late log-phase, 95 mL of the culture was transferred to four 125-mL polypropylene bottles. Three bottles of the culture were irradiated with 100, 150 and 300 Gy of ^{60}Co γ-rays at a dose rate of 29 Gy min^{-1}, and the other one bottle of the culture served as a non-irradiated control. Each culture was transferred to a 300-mL Erlenmeyer flask stoppered with a silicone plug, and was incubated under the same conditions as mentioned above. Cells were counted under a microscope to monitor the population growth.

Two hours after the irradiation, total RNA was extracted using RNeasy Plant Mini Kit (QIAGEN, Düsseldorf, Germany). After each culture was centrifuged at 1,155*g* for 3 min at 4°C, the medium was removed completely. The cell pellet was suspended in 1.2 mL of Buffer RLT (lysis buffer of the extraction kit). After approximately 0.8 mL of glass beads (ϕ0.5 mm) were added to the cell suspension, bead milling was carried out using the Mini-Beadbeater (BioSpec Products, Bartlesville, OK, USA) at 4,800 rpm for 30 s three times. Thereafter total RNA was extracted in accordance with the manufacture's instructions. The series of culturing, irradiation and RNA extraction steps was done once for HiCEP and three times for the quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Gene expression profiling by HiCEP was carried out by the HiCEP unit team of the National Institute of Radiological Sciences (NIRS; Japan), and the details have been reported elsewhere (Fukumura et al. 2003). Briefly, 1 μg of total RNA was converted to cDNA by reverse transcriptase with 5′-biotynated oligo (dT) primers. Double-strand cDNA was prepared, digested by restriction enzyme MspI, and trapped by avidin bound to magnetic beads. After the fragments digested by MspI except for most of the 3′-region bearing oligo(dT)-biotin were washed off, a synthetic adaptor was ligated, and the trapped templates were digested by another restriction enzyme MseI. After ligation to MseI adaptor, 256 runs of selective PCR were performed based on AFLP. The PCR products labeled with fluorescent dyes were denatured and loaded onto an ABI PRISM 3100 electrophoresis system (Applied Biosystems, Foster City, CA, USA) for separation, and the fluorescent intensity of each peak, which represents an expression level of the corresponding gene, was recorded. Electrophoretic pattern data were normalized using a global normalization program developed by Maze Inc. (Tokyo, Japan). The HiCEP analysis was carried out twice for each RNA sample, and the mean of two fluorescent intensity data was used for analysis of each peak.

To characterize up-regulated transcripts, the PCR products were separated by electrophoresis in 4–10% polyacrylamide gels and the DNA bands corresponding to HiCEP peaks concerned were isolated. After reamplification by PCR, nucleotide sequences were determined directly. When direct sequencing was impossible, sequences were determined after target DNA was cloned in the pGEM-T Easy Vector (Promega, Madison, WI, USA). In this experiment, the technical services of MessengerScape Co., Ltd. (Tokyo, Japan) were used for some peaks. The basic local alignment search tool (BLAST) search was performed using the blastx against the non-redundant protein database of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) to find known genes that code similar amino acid sequences.

To validate the up-regulation of expression, qRT-PCR was performed. Nine hundred nanograms of total RNA were reverse-transcribed to the first-strand cDNA using the Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The reaction was done according to the manufacturer's protocol using a random hexamer primer. Quantitative PCR was done using the PRISM7500 Real Time PCR System with Power SYBR Green PCR Master Mix (Applied Biosystems) and peak-specific primers. The reaction conditions were 95°C for 10 min for denaturation, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min for amplification and quantification, and 95°C for 15 s, 60°C for 1 min and 95°C for 15 s for dissociation curve analyses. The data were normalized in relation to the expression level of the 18S rRNA gene (GeneBank accession number: AF169628).

Differences between irradiated and control data on the cell densities and gene expression levels determined by qRT-PCR were statistically tested by an analysis of variance (ANOVA), followed by Dunnett's test at the significance level of 5%. This test was carried out using the StatView software, version 5.0 (SAS Institute Inc., Cary, NC, USA).

Results and Discussion

This research represents the first survey and analysis of the *P. subcapitata* transcriptome. Approximately 7,800 HiCEP peaks, each of which theoretically represents a separate gene, were detected in this study. Since green alga *Chlamydomonas reinhardtii*, which is the most relevant species to *P. subcapitata* among genomic model organisms, has 15,143 genes (Merchant et al. 2007), genome coverage of

the HiCEP may have been approximately 50% in *P. subcapitata*. This limited genome coverage would be due to the combination of restriction enzymes used. That is, a considerable number of cDNA might lack restriction sites of MspI or MseI in *P. subcapitata*. A high GC content of *C. reinhardtii*, i.e., 68% in the coding sequence (Merchant et al. 2007), suggests that MseI sites (TTAA) might be rare in *P. subcapitata*. Several combinations of restriction enzymes would be necessary to improve the genome coverage of the HiCEP in this alga, as suggested from the estimation that more than 99% coverage is achieved using four combinations of restriction enzymes in humans and mice while the coverage is 75–80% in the MspI/MseI combination only (Fukumura et al. 2003).

In this study, gene expression profiling was performed in *P. subcapitata* γ-irradiated at high doses, i.e., 100–300 Gy, because, as the first step, sufficient gene expression responses were required to examine whether HiCEP was useful for screening of stress-responsive genes. Population growth of *P. subcapitata* was not affected at each dose 2 h after irradiation, when RNA was extracted for gene expression profiling (Fig. 1). The growth was not affected at 100 Gy thereafter, while, 25 h after irradiation, it was inhibited at 150 and 300 Gy in a dose-dependent manner. Of the approximately 7,800 peak profiles examined, 268, 280 and 310 peaks were up-regulated at 100, 150 and 300 Gy at more than twofold levels relative to control, respectively. At less than 1/2-fold levels, 355, 405 and 397 peaks were down-regulated at 100, 150 and 300 Gy, respectively. Almost the same number of genes was affected in this dose range. These affected genes are likely

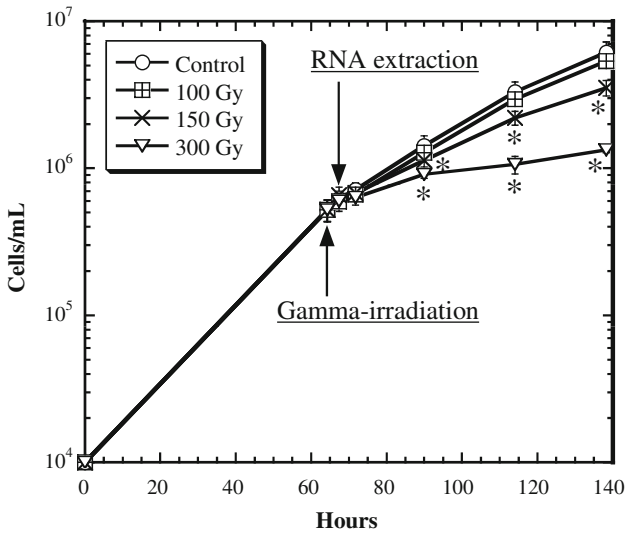

Fig. 1 Population growth of *Pseudokirchneriella subcapitata* in the γ-irradiated and control cultures. *Error bars* are standard deviations ($n = 3$–5). *Asterisks* indicate statistically significant differences from controls ($p < 0.05$)

biomarker candidates for high doses of ionizing radiation, which would arise from severe nuclear accidents or inadequate disposal of highly radioactive wastes.

Forty-one peaks selected from the up-regulated peaks were isolated, and the nucleotide sequences were determined to characterize these peaks. For the 21 peaks selected from these 41 peaks, primer sets were designed on the basis of the determined sequences, and qRT-PCR was carried out. Quantitative RT-PCR detected more than twofold up-regulation for all the peaks except peak 5 (Table 1). The result for peak 5 can be attributed to the possibility that an adjacent peak was cloned when the sequence was determined. The very high agreement between HiCEP and qRT-PCR in this study as well as some other studies (Fujimori et al. 2005, 2008; Mitani et al. 2006; Nakamori et al. 2008; Suetomi et al. 2008) indicates accuracy of the HiCEP.

The BLAST search against the protein database revealed that two peaks resembled some known genes in sequences of amino acids. Peak 14 was similar to putative DEAD/DEAH box helicases of *Arabidopsis thaliana* (NCBI ref: NP_196428.1; *E*-value: 1×10^{-4}), rice *Oryza sativa* (NCBI ref: NP_001045657.1; *E*-value: 6×10^{-4}) and *C. reinhardtii* (NCBI ref: XP_001691852.1; *E*-value: 1×10^{-3}). Peak 31 was similar to SNF2/RAD54 family protein of *C. reinhardtii* (NCBI ref: XP_00169622.1; *E*-value: 3×10^{-10}) and putative RAD26 of *O. sativa* (NCBI ref: BAD45511.1; *E*-value: 1×10^{-5}) and moss *Physcomitrella patens* (NCBI ref: XP_001764202.1; *E*-value: 2×10^{-4}). These results indicate that HiCEP is useful for screening of some genes responding to ionizing radiation, because ionizing radiation damages DNA and the known genes resembling the up-regulated peaks are implicated in the DNA damage response. Some DEAD box helicases, which unwind RNA–RNA or RNA–DNA duplexes, play a role in G1/S DNA-damage checkpoint recovery, function in a branch of homologous recombination involved in error-free bypass of DNA lesions, and facilitate repair of DNA double strand breaks by rapid clearance of RNA from the breaks (Li et al. 2008). The RAD54 has critical roles in DNA double-strand break repair and homologous recombination, and is involved in resistance to ionizing radiation in yeasts, chicken, mouse, *Drosophila* and *Arabidopsis* (Klutstein et al. 2008). The RAD26 is involved in transcription-coupled nucleotide excision repair (Aboussekhra and Al-Sharif 2005).

On the other hand, all the sequenced peaks except for the two peaks mentioned above had no similarity in the database. A possible explanation for this is that the sequences obtained might have been in the untranslated region, which is of little use in finding homologies to known genes. Another explanation is that they might have been too short (41–716 bp; Table 1) for performing a

Table 1 Expression profiling of HiCEP peaks up-regulated in γ-irradiated *Pseudokirchneriella subcapitata*

Peak no.	Fluorescent intensity in HiCEP analysis				Fold change to control						Peak length (bp)	GeneBank accession no.
	0 Gy	100 Gy	150 Gy	300 Gy	HiCEP			qRT-PCR[a]				
					100 Gy	150 Gy	300 Gy	100 Gy	150 Gy	300 Gy		
1	172	5,954	5,899	5,112	34.7	34.4	29.8	31.1 ± 4.9*	36.7 ± 5.6*	49.3 ± 11.5*	80	AB458260
2	192	5,751	5,860	4,536	30.0	30.5	23.6	6.4 ± 3.4*	6.7 ± 3.7*	7.5 ± 3.2*	84	AB458261
3	574	12,653	14,888	15,712	22.0	25.9	27.4	13.6 ± 1.8*	15.4 ± 2.9*	15.6 ± 1.4*	148	AB458262
4	211	2,783	2,759	2,793	13.2	13.1	13.2	12.4 ± 1.2*	11.9 ± 1.2*	17.8 ± 4.0*	112	AB458263
5	222	2,802	1,920	487	12.6	8.6	2.2	0.99 ± 0.46	0.98 ± 0.36	1.1 ± 0.3	139	AB458264
6	416	4,997	5,302	5,471	12.0	12.7	13.2	18.4 ± 2.9*	20.7 ± 2.6*	23.7 ± 5.4*	254	AB458265
7	430	4,213	4,737	6,195	9.8	11.0	14.4	3.1 ± 0.4*	3.3 ± 0.2*	4.4 ± 0.7*	98	AB458266
8	303	2,634	2,759	2,995	8.7	9.1	9.9	8.7 ± 0.9*	11.6 ± 0.5*	15.0 ± 1.6*	148	AB458267
9	105	883	573	169	8.4	5.5	1.6	3.3 ± 1.4*	2.6 ± 0.7*	0.75 ± 0.56	116	AB458268
10	528	4,388	6,109	5,884	8.3	11.6	11.2	11.8 ± 7.3*	10.6 ± 6.5*	16.4 ± 7.9*	100	AB458269
11	287	2,342	2,219	2,729	8.2	7.7	9.5	10.4 ± 1.7*	11.3 ± 2.0*	14.9 ± 3.6*	242	AB458270
12	62	485	539	520	7.8	8.7	8.4	–	–	–	102	AB458271
13	835	6,529	8,581	7,727	7.8	10.3	9.3	7.8 ± 1.6*	8.7 ± 1.2*	8.9 ± 0.4*	130	AB458272
14	394	3,028	3,410	3,846	7.7	8.7	9.8	8.2 ± 1.1*	9.5 ± 0.6*	11.7 ± 1.3*	128	AB458273
15	638	4,244	4,530	5,038	6.7	7.1	7.9	5.9 ± 0.7*	7.5 ± 0.8*	8.4 ± 1.2*	130	AB458274
16	560	3,640	3,887	4,345	6.5	6.9	7.8	8.8 ± 1.3*	10.6 ± 2.1*	11.8 ± 2.2*	179	AB458275
17	257	1,567	1,646	1,909	6.1	6.4	7.4	5.7 ± 0.6*	6.2 ± 1.0*	6.8 ± 1.1*	238	AB458276
18	111	638	279	266	5.8	2.5	2.4	–	–	–	45	AB458277
19	255	1,427	1,113	310	5.6	4.4	1.2	3.8 ± 0.8*	2.3 ± 0.8*	1.1 ± 0.4	100	AB458278
20	85	478	188	0	5.6	2.2	0	–	–	–	143	AB458279
21	208	1,022	670	390	4.9	3.2	1.9	–	–	–	117	AB458280
22	226	904	896	1,158	4.0	4.0	5.1	5.0 ± 1.5*	6.5 ± 1.3*	7.6 ± 2.5*	121	AB458281
23	478	1,731	1,918	1,918	3.6	4.0	4.0	4.1 ± 0.3*	4.2 ± 0.3*	4.7 ± 0.5*	117	AB458282
24	375	1,319	2,412	1,898	3.5	6.4	5.1	9.8 ± 3.3*	10.0 ± 2.6*	13.3 ± 4.0*	90	AB458283
25	55	186	139	190	3.4	2.5	3.5	–	–	–	91	AB458284
26	69	216	203	210	3.1	3.0	3.1	–	–	–	166	AB458285
27	121	379	466	421	3.1	3.8	3.5	–	–	–	65	AB458286
28	141	410	447	496	2.9	3.2	3.5	–	–	–	99	AB458287
29	1,408	3,936	4,293	3,290	2.8	3.0	2.3	–	–	–	118	AB458288
30	389	1,046	987	1,140	2.7	2.5	2.9	–	–	–	89	AB458289
31	78	213	237	246	2.7	3.0	3.1	3.7 ± 0.8*	4.3 ± 0.6*	4.7 ± 0.5*	443	AB458290
32	2,434	6,338	6,793	5,536	2.6	2.8	2.3	–	–	–	65	AB458291
33	355	890	841	699	2.5	2.4	2.0	–	–	–	237	AB458292
34	789	1,907	1,980	1,825	2.4	2.5	2.3	–	–	–	329	AB458293
35	626	1,519	1,664	1,902	2.4	2.7	3.0	–	–	–	154	AB458294
36	169	400	305	365	2.4	1.8	2.2	–	–	–	133	AB458295
37	242	551	443	470	2.3	1.8	1.9	–	–	–	110	AB458296
38	2,983	6,179	6,334	7,896	2.1	2.1	2.6	–	–	–	41	AB458297
39	8,250	16,597	15,973	17,288	2.0	1.9	2.1	–	–	–	140	AB458298
40	1,883	2,869	3,131	4,442	1.5	1.7	2.4	–	–	–	716	AB458299
41	1,154	1,329	1,814	2,418	1.2	1.6	2.1	–	–	–	237	AB458300

"–" Not determined

* Statistically significant differences in expression levels from controls ($p < 0.05$)

[a] Quantitative reverse transcription polymerase chain reaction

homology search. It therefore seems likely that rapid amplification of cDNA ends (RACE) is required to obtain sufficient sequence data. Although this is admittedly a time-consuming task, the advantage of HiCEP is that interesting peaks can be identified first, and thus RACE is required only on the handful of identified genes. This is in direct contrast to microarray-based approaches that, for non-model species, would require the construction and sequencing of cDNA or expressed sequence tag libraries to generate the sequence knowledge required for spotting on a chip.

There are some methods such as differential display (DD), serial analysis of gene expression (SAGE) and suppressive subtraction hybridization (SSH) that have been used to investigate genome-wide gene expression in organisms for which genomic sequence information is lacking. These methods, however, have some known limitations. For example, a critical limitation to DD analysis is that more than one cDNA can be obtained from a single DD band, which leads to the isolation of false positives. This can be attributed to nearly identically sized cDNA fragments that comigrate on display gels (Akhtar et al. 2005). Only short fragments, i.e., 10–20 bp, are obtained by SAGE (Griffitt et al. 2007), while a longer template would be informative in the case of an analysis of non-model species. Griffitt et al. (2006) have shown the limited utility of SSH as a tool for biomarker identification. In their study, only two of the eleven genes surveyed with semi-quantitative RT-PCR confirmed the response predicted by SSH. The HiCEP overcomes these limitations. For example, optimizing the selective PCR procedure in HiCEP reduces the number of peaks, including overlapping peaks, and the rate of false positive peaks is lowered to 4%. The HiCEP obtains longer cDNA fragments, most of which range from 40–700 bp (Fukumura et al. 2003), than SAGE. Accuracy of HiCEP was validated by qRT-PCR in this study and other studies (Fujimori et al. 2005, 2008; Mitani et al. 2006; Nakamori et al. 2008) as described above.

This study demonstrated the potential of HiCEP as an ecotoxicogenomic tool for rapid and accurate screening of genes affected by ionizing radiation in non-model species such as *P. subcapitata* which are important in ecotoxicology but for which there is insufficient sequence information to construct satisfactory DNA arrays. This conclusion is likely the case for chemicals as suggested from a study on application of HiCEP to bacterium *Rhodococcus*, which is important in pharmaceutical, environmental and industrial fields, but for which genomic sequence information is lacking, exposed to isoniazid and ethambutol (Mitani et al. 2006).

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