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Analysis of DNA methylation alterations in rice seeds induced by different doses of carbon-ion radiation

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ABSTRACT

To investigate the mechanism underlying differences in biological effects induced by low- versus high-dose heavy-ion radiation (HIR) in rice plants, two-dimensional gel electrophoresis (2-DE) coupled with methylation-sensitive amplification polymorphism (MSAP) analysis were used to check the expression changes in rice leaf proteome profiles and the changes in DNA methylation after exposure of seeds to ground-based carbon-ion radiation at various cumulative doses (0, 0.01, 0.02, 0.1, 0.2, 1, 2, 5 or 20 Gy; $^{12}C^{6+}$; energy, 165 MeV/u; mean linear energy transfer, 30 KeV/μm). In this study, principal component analysis (PCA) and gene ontology (GO) functional analysis of differentially expressed proteins of rice at tillering stage showed that proteins expressed in rice samples exposed to 0.01, 0.02, 0.1, 0.2 or 1 Gy differed from those exposed to 2, 5 or 20 Gy. Correspondingly, the proportion of hypermethylation was higher than that of hypomethylation at CG sites following low-dose HIR (LDR; 0.01, 0.2 or 1 Gy), whereas this was reversed at high-dose HIR (HDR; 2, 5 or 20 Gy). The hypomethylation changes tended to occur at CHG sites with both low- and high-dose HIR. Furthermore, sequencing of MSAP variant bands indicated that the plants might activate more metabolic processes and biosynthetic pathways on exposure to LDR, but activate stress resistance on exposure to HDR. This study showed that radiation induced different biological effects with low- and high-dose HIR, and that this may have been caused by different patterns of hyper- and hypomethylation at the CG sites.

Keywords: heavy-ion radiation; rice; DNA methylation; proteins

INTRODUCTION

In organisms, heavy-ion radiation (HIR) results in various biological alterations, including phenotype mutations and molecular level changes [1–3]. In addition, low-dose HIR (LDR) differs from high-dose HIR (HDR) in that it causes different biological responses and presents different risks. For example, dose-dependent mutations are not linear because of hormesis effects and adaptive responses observed at LDR [4, 5]. Current evidence indicates that <0.1 Gy can be considered to be a low-dose radiation range for both cell and mammalian [6]. A review indicates that approximately two-thirds of 64 publications reported that low-dose X-rays stimulated plant growth, seed germination or other phenotype changes [7]. Plants exhibit better radiation resistance than animals [8]; however, it has been demonstrated that the exposure of *Arabidopsis* seedlings to low-dose gamma-rays at 1 or 2 Gy stimulated plant growth [9] and accelerated photosynthesis, respiration and electron transport rates [10]. One report has revealed that the exposure of *Perilla frutescens* (L.) seeds to low-dose carbon-ion radiation (energy, 80 MeV/u) clearly promoted germination and survival rates [11]. Another report also found a stimulatory effect on germination and plant height of *Allium fistulosum* (L.) seedlings after exposure to carbon ions when the radiation dose increased [12]. It is known that heavy-ion particles have more lethal effects than X-rays and gamma-rays [13]. A series of reports about various doses of heavy-ion particle radiation inducing biological effects in rice have shown a stimulatory effect on plant height being induced by carbon-ion radiation (energy, 100 MeV/u; mean linear energy transfer (LET), 27.3 KeV/μm) at 0.02, 0.1 0.2, 1 or 2 Gy, but a suppressive effect being caused by

HDR (at 5, 10, 15 or 20 Gy) [14]. Furthermore, exposure to carbon-ion radiation (energy, 100 MeV/u, mean LET, 27.3 KeV/μm) induced a stimulatory response in mitotic activity of rice seedlings at 0.02 or 0.2 Gy, but an inhibitory response at 2 or 20 Gy [15]. Previous results showed that the superoxide dismutase (SOD) and hydrogen peroxidase (CAT) activities were significantly increased in rice seedlings as a result of carbon-ion radiation (energy, 100 MeV/u; mean LET, 27.3 KeV/μm) at 0.02 or 0.2 Gy, but decreased by such radiation at 2 or 20 Gy [16]. These findings showed that the biological effects of changing the radiation dose were similar, although the threshold of the change in biological effects when increasing the dose differed according to type of radiation, growth stage and water content. This suggests that different mechanisms may play key roles in regulating the different biological effects caused by low- and high-dose HIR. Accumulating evidence indicates that DNA methylation is an adaptation or a response to environmental factors [17, 18]. Recently, there have been several reports showing that DNA methylation profiles were altered by radiation [19]. Our previous results found that space flight (2 mGy) and HIR (2 Gy) induced significant alterations in the rice genome and DNA methylation [20]. In addition, hypermethylation of the rice cytidine deaminase gene was found to be associated with a decrease in gene expression [21].

These studies suggest that DNA methylation was changed by radiation, regardless of the dose. DNA methylation plays a central role in plant responses to environment stresses [22, 23]. Hypermethylation might protect genomic stability, while hypomethylation might participate in regulating the processes of the stress response. It is not clear whether the process of regulation is related to the radiation dose. The purpose of the current study was to investigate the pattern of DNA methylation remodeling caused by different doses of HIR. We also sought to understand the regulatory mechanisms of the epigenetic response at low- and high-dose HIR.

In this study, we focused on plant growth and the proteome in plants after seeds were irradiated with $^{12}C^{6}$ at 0, 0.01, 0.02, 0.1, 0.2, 1, 2, 5 or 20 Gy. Depending on the protein expression change patterns, the doses were divided into relatively low- and high-dose HIR. Qualitative analysis of differentially expressed proteins was used to indicate differences in protein function induced by low- versus high-dose HIR. Moreover, alteration in DNA methylation patterns and the proportion of hyper- and hypomethylation at CG and CNG sites [determined by methylation-sensitive amplification polymorphism (MSAP)] was used to reveal the epigenetic response caused by different doses of HIR. In addition, 18 DNA fragments showing polymorphisms related to differences in methylation were sequenced to find the differences produced in functional genes after exposure to low- versus high-dose HIR.

MATERIALS AND METHODS

Plant preparation and radiation

Approximately 50 dry rice seeds (*Oryza sativa* L. spp. *japonica*, var. Nipponbare, AA genome) were horizontally positioned in a 5-cm-diameter polystyrene chamber during irradiation (Supplementary Fig. 1A). The water content of the dry rice seeds was ~10–12%. The irradiation experiment was performed using equipment at the Heavy Ion Research Facility in Lanzhou, Gansu, China. The heavy ion type used was $^{12}C^{6+}$, with an energy of 165 MeV/u, and the mean LET within the rice seeds was 30 KeV/μm. The dose rate was 0.5 Gy/min. The particle count was used to measure the radiation dose. The dry seeds were irradiated with doses of 0, 0.01, 0.02, 0.1, 0.2, 1, 2, 5 or 20 Gy. The controls (0 Gy) were treated in the same way but without radiation.

After irradiation with the various doses, 30 dry rice seeds for each dose were soaked in distilled water at 25°C in darkness for 4 days. Seeds germinated on the third or fourth day. Then, the rice seedlings were cultured on moistened filter paper at 25°C for 28 days with a 16-h light/8-h dark cycle in an artificial climate room (270 × 420 × 200 cm) (Supplementary Fig. 1B). The light intensity was 300 μmol m^{-2}·s^{-1}. The Yoshida medium was changed at 9 a.m. and 9 p.m. every day. Ten individuals from each dose group were harvested at 28 days after the soaking (Supplementary Fig. 1C). Rice seedlings were transferred to the incubator with Yoshida medium and cultured to tillering stage (60 days after the soaking) at 25°C with a 16-h light/8-h dark cycle. The air humidity was 55%. The concentration of carbon dioxide in the room was 450 ppm. There were 30 rice seedlings in each incubator (60 × 50 × 30 cm). The Yoshida medium was changed every 3 days. To compare the growth of plants exposed to the various ion radiation doses, plant height was measured at tillering stage. Ten plants per replicate were used. The third leaf from the top was chosen from each rice plant for both the MSAP and two-dimensional gel electrophoresis (2-DE) analysis (Supplementary Fig. 1D). The remainder of the plants from both the exposed and control groups were cultured under the same conditions as described above and harvested at maturation stage (160 days after the soaking) (Supplementary Fig. 1E).

Protein preparation and 2-DE

The third leaf from the top was chosen from one rice plant at tillering stage as one sample. Three samples from each radiation treatment were prepared as biological duplicate sets. The rice leaf from each sample was powdered in liquid nitrogen and suspended in 10 ml cold acetone containing 10% (w/v) trichloroacetic acid solution and 0.07% (v/v) β-mercaptoethanol. Proteins were left to precipitate for 1 h at −20°C and centrifuged at 12 000 *g* for 10 min at 4°C. Pellets were washed twice with 10 ml cold acetone containing 0.07% (v/v) β-mercaptoethanol and once with 10 ml cold acetone containing 20% (v/v) H_2O and 0.07% (v/v) β-mercaptoethanol, and dried to powder under vacuum to remove any remaining acetone. Protein powder was resuspended in lysis buffer (8 M urea, 4% CHAPS, 30 mM Tris-HCl, pH 8.5), and insoluble material was removed by centrifugation at 12 000 *g* for 10 min at 4°C. The proteins were prepared using a 2-D Clean-Up Kit (GE Healthcare, Fairfield, CT, USA), and protein concentrations were determined using a 2-D Quant Kit (GE Healthcare, Fairfield, CT, USA).

Approximately 200 μg of protein sample was mixed with rehydration buffer {9.5 M urea, 2% 3-[(3-cholamidopropyl) dimethylamino]-1-propanesulfonate, 2% Triton X-100, 15 mM DTT (DL-Dithiothreitol) and 0.5% IPG buffer} to a total volume of 200 μl. The samples were then applied to 17 cm Immobiline Dry Strips, pH 4–7 (Bio-Rad, Hercules, California, USA). Isoelectric focusing (IEF) was

performed on a Protean IEF Cell (Bio-Rad) with the following settings: 250 V for 0.5 h, 1000 V for 1 h, 10 000 V for 4 h, 10 000 V for 60 000 Vh and 500 V for 1 h. After IEF, the strips were equilibrated in an equilibration buffer (6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), a trace of bromophenol blue and 50 mM Tris-HCl; pH 8.8) first with 2% DTT and then without DTT, each for 15 min, and transferred to 12% polyacrylamide gel electrophoresis (SDS-PAGE) gels for 2-DE using a Protean II xi Multi-Cell (Bio-Rad). SDS-PAGE was performed under a constant current of 10 mA per gel for 30 min followed by 60 mA per gel for 5 h. Proteins were visualized by silver staining. All electrophoretic profiles were confirmed by repeating the same procedure at least thrice before automatic analysis with ImageMaster 2D Eliteversion 3.10 (GE Healthcare, Fairfield, CT, USA).

Data analysis of 2-DE gel results

After protein acquisition, we identified the differentially expressed proteins by comparing the data with that for the standard gel [24]. UPLC/MS/MS analyses (SYNAPT G2, waters, Massachusetts, USA) were used to verify whether the proteins separated by 2-DE in this study were similar those in the standard gel. Five altered proteins randomly selected in this study were found to be the same as those identified in the standard gel. The chromatograph charts and mass spectrogram of the five proteins are shown in Supplementary Fig. 2. The identified proteins (matched with proteins in the Swiss-Prot protein database) are shown in Supplementary Table 1. A threshold was applied to select proteins with a statistically significant 1.5-fold (average ratio) differential expression in the normalized spot volume (Student's *t*-test, $P \leq 0.05$). The formula for calculating the ratio of differentially expressed proteins in each group was [(the number of altered proteins)/(the total number of detected proteins)] × 100%.

Principal component analysis (PCA) was employed to visualize any statistically significant difference between the groups. Furthermore, gene ontology (GO) annotation analysis was performed using the blast2GO software.

MSAP analysis

The third leaf from the top was chosen from one rice plant at tillering stage as one sample. Five samples from each radiation treatment were prepared as biological duplicate sets. Genomic DNA was extracted using the standard cetyltrimethyl ammoniumbromide (CTAB) method [25].

Aliquots of DNA were digested for 2 h at 37°C and for 15 min at 70°C with 5 U each of *Eco*RI and *Hpa*II/*Msp*I (New England Biolabs, Ipswich, Suffolk, England) in 50 µl buffer solution. DNA fragments from the two reactions were separately added to equal volumes of the adapter/ligation solution, and the ligation reaction was allowed to proceed overnight at 20°C. The ligation mixture was then diluted 1:10 with Tris-EDTA buffer solution (TE) and used as a template for the pre-selective amplification. The reaction was performed for 25 cycles of 30 s denaturation at 94°C, 30 s annealing at 56°C and 1 min extension at 72°C. The product was diluted 20-fold (v/v) with TE buffer and used as a template for the selective amplification reaction. In this step, *Eco*RI and *Hpa*II/*Msp*I primers with three additional selective nucleotides were used. One pair of pre-selective and five pairs of selective primers were used (Supplementary Table 2). Selective polymerase chain reaction (PCR) was performed in a final volume of 25 µl following the protocol of Vos [26]. MSAP amplification products were resolved by electrophoresis on a 6% denaturing polyacrylamide gel and visualized by silver staining [27].

The MSAP bands were scored '1' or '0' to indicate the presence or absence, respectively, of a band at a particular position. The MSAP patterns of the DNA fragments resulting from digestion with the isoschizomers were divided into the following four types. Type I: the sites that were free from methylation were recognized by both isoschizomers (1, 1). Type II: the full methylation sites were only recognized by *Msp*I (1, 0). Type III: the full methylation sites were only recognized by *Hpa*II (0, 1). Type IV: the absence of bands for both enzyme combinations, indicating that full methylation occurred at both cytosines (0, 0) [28, 29]. The frequency of DNA methylation polymorphism was calculated by the following formula: $(II \times 2 + III + IV \times 2)/[(I + II + III + IV) \times 2] \times 100\%$. The original electrophoresis photographs from the 0.1 and 0.2 Gy groups are shown in Supplementary Fig. 3.

Statistical analysis

Analysis of the raw data from 2-DE and MSAP was carried out with MS Excel 2010 software. SPSS was used to perform the ANOVA test. Changes were considered statistically significant (*) if $P < 0.05$ and (**) if $P < 0.01$.

RESULTS

Rice growth after exposure to various doses of carbon-ion radiation

Plant height is a key parameter used to determine plant response to stress conditions. To study the effect of different doses of HIR on plant growth, rice seeds were exposed to 0, 0.01, 0.02, 0.1, 0.2, 1, 2, 5 or 20 Gy of $^{12}C^{6+}$ and plant heights were recorded on Day 60 (tillering stage). Significant changes in the plant height of rice were observed for 0.01 and 0.2 Gy (Table 1). In this study, no significant

Table 1. Plant height of rice at tillering stage

Dose (Gy)	The plant height (cm)
0	60.65 ± 9.06
0.01	53.75 ± 10.74*
0.02	59.13 ± 10.19
0.1	54.82 ± 12.30
0.2	68.07 ± 6.14*
1	62.14 ± 12.02
2	64.24 ± 10.16
5	63.31 ± 7.92
20	65.33 ± 8.12

Asterisks (*) indicate differences significant at $P < 0.05$ between radiation groups and non-radiation groups. ± indicate the standard error of the mean plant height for $N = 10$ independent plants.

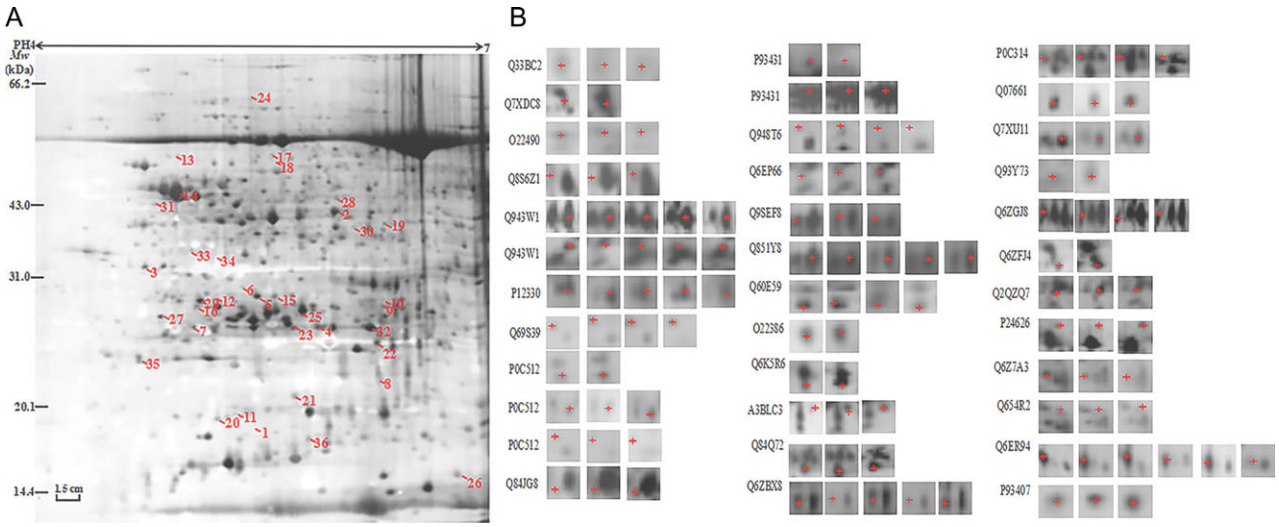

Fig. 1. Two-dimensional analysis of rice seeds after radiation. The 36 proteins that were significantly altered after heavy ion radiation treatments were marked and numbered. (A) The spots of changed proteins in the control sample (0 Gy). (B) The details of changed proteins. The first picture in each group was the spot of changed protein in the non-radiation groups (0 Gy).

differences in plant heights were observed when comparing plants grown from low- and high-dose HIR exposed seeds.

PCA of proteome expression profiles

In further analysis of the molecules in the rice that were affected by exposure to different doses of HIR [using protein separation by 2-DE (Fig. 1)], approximately 706–932 spots were reproducibly detected on 2-DE gels (Supplementary Table 3). PCA showed that proteins expressed after exposure to 0.01, 0.02, 0.1, 0.2 or 1 Gy were similar to those expressed after 0 Gy treatment. However, the proteins expressed at 2, 5 or 20 Gy differed from those expressed after 0 Gy or after irradiation of <2 Gy (Fig. 2). These results indicated that protein expression profiles differed between the relatively low- (<2 Gy) and high- (≥2 Gy) dose groups.

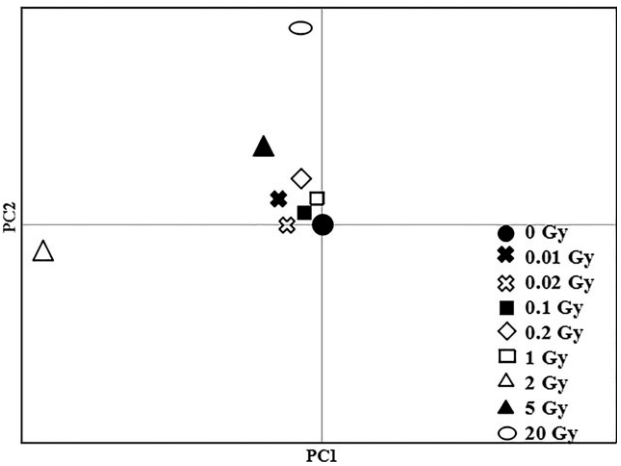

Fig. 2. PCA of proteomic expression profiles of rice seeds irradiated at all doses. The cumulative contribution rate was 80%.

Identification and GO functional analysis of differentially expressed proteins

Differentially expressed proteins in the radiation groups were compared with those in the control group (0 Gy), and the ratios of the differentially expressed proteins are shown in Supplementary Fig. 4A. To identify the proteins involved in the radiation response, we matched the significantly altered proteins in each radiation group with those found in our previous research, in which 204 expressed protein spots of rice at tillering stage were successfully identified by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) [23]. A total of 36 proteins were matched (Fig. 1, Table 2).

Based on the functional features of rice proteins, the altered proteins were classified into 12 biological process categories by GO analysis (Fig. 3). Among the upregulated proteins, most of the enriched biological processes were involved in photosynthesis, photorespiration or protein metabolic processes. Amino acid metabolism, cell death, response to biotic stimulus, and regulation of cellular processes were enriched by the upregulated proteins responding to LDR, but not by those responding to HDR exposure. On the other hand, energy metabolic processes were enriched by the upregulated proteins responding to HDR but not to LDR exposure. As a result of downregulation of proteins, seven categories of processes were enriched after both LDR and HDR exposures: energy metabolic processes, photosynthesis, photorespiration,

Table 2. Proteins showing altered expression in rice after radiation exposure

Protein AC	Name	Theoretical		Experimental		Function	Change fold at different doses (Gy)							
		Mw (kDa)	pI	Mw (kDa)	pI		0.01	0.02	0.1	0.2	1	2	5	20
Q7XDC8	Cytoplasmic malate dehydrogenase	42 942	6.08	35 568	5.75	Tricarboxylic acid cycle								2.21
O22490	Cytochrome c oxidase subunit 6b-1	33 881	4.50	19 266	4.46	Electron transport (respiration)	0.47		0.49					
Q8S6Z1	ATPase α subunit, 3′-partial	28 873	5.93	29 317	5.27	Oxidative phosphorylation						0.30	0.14	
Q943W1	Putative 33 kDa oxygen evolving protein of photosystem II	34 861	5.43	34 861	6.10	Light-harvesting reaction	0.29			0.46		0.48	0.48	
Q943W1	Putative 33 kDa oxygen evolving protein of photosystem II	34 817	5.30	34 861	6.10	Light-harvesting reaction				0.54	0.41	0.58		0.58
P12330	Chlorophyll a/b-binding preprotein	28 761	5.08	28 014	5.14	Light-harvesting reaction	0.46	0.58	0.52	0.45				
Q69S39	Rieske Fe-S precursor protein	21 431	6.37	23 884	8.55	Electron transport (photosynthesis)			2.97	3.12		3.03		
P0C512	Rubisco large chain	29 883	6.38	52 881	6.22	Calvin cycle, carbon fixation			2.10					
P0C512	Rubisco large subunit	32 299	6.42	52 881	6.22	Calvin cycle, carbon fixation	0.57						1.64	
P0C512	Rubisco large chain precursor	21 378	5.12	52 881	6.22	Calvin cycle, carbon fixation	0.38						0.38	
Q84JG8	Sedoheptulose-1,7-bisphosphatase precursor	34 175	5.23	42 245	5.83	Calvin cycle, regeneration of RuBP	2.80					2.50		
P93431	Rubisco activase	48 533	5.20	51 454	5.43	Regulation of photosynthesis							0.43	
P93431	Rubisco small isoform precursor	45 767	5.17	51 454	5.43	Regulation of photosynthesis		3.14			3.26			
Q948T6	Glyoxalase I	37 217	5.66	32 553	5.51	Photorespiration		0.40				0.40	0.67	
Q6EP66	Putative phosphoglycolate phosphatase precursor	32 937	5.11	33 516	4.99	Photorespiration			2.30					2.01
Q9SEF8	Translation elongation factor EF-Tu precursor, chloroplast	48 172	5.67	50 355	6.05	Translation		0.45					0.46	
Q851Y8	Chloroplast translation EF-Tu	48 172	5.75	48 424	6.04	Translation	0.48					0.29	0.40	0.66
Q60E59	Putative chloroplast ribosomal protein L1	40 542	6.29	38 780	6.87	Translation			0.59	0.40				0.25
O22386	50S ribosomal protein L12	21 310	4.85	18 590	5.36	Translation						1.89		
Q6K5R6	Putative ribosomal protein S15	23 042	5.59	14 818	9.94	Translation	1.77							

Continued

Table 2. Continued

Protein AC	Name	Theoretical		Experimental		Function	Change fold at different doses (Gy)							
		Mw (kDa)	pI	Mw (kDa)	pI		0.01	0.02	0.1	0.2	1	2	5	20
A3BLC3	Putative ribosome recycling factor	25 787	6.28	29 652	9.35	Translation		1.46				1.69		
Q84Q72	18.1 kDa class I heat shock protein	27 490	5.69	18 082	6.77	Protein folding	10.53		6.57					
Q6ZBX8	Putative aminopeptidase M	60 487	5.59	98 032	5.42	Proteolysis	0.22			2.62			1.61	1.55
P0C314	Endopeptidase CLPP2	28 686	5.71	24 728	4.64	Proteolysis					4.46	3.75	4.25	
Q07661	Nucleoside diphosphate kinase 1	20 196	6.61	16 861	6.30	Nucleoside metabolic process	0.40					0.65		
Q7XU11	Reverse transcriptases	29 471	4.81	204 692	8.96	RNA-dependent DNA replication	0.66			0.25				
Q93Y73	Aspartate-semialdehyde dehydrogenase family protein, expressed	45 906	6.08	40 178	6.72	Amino acid and derivative metabolism				3.58				
Q6ZGJ8	Putative inorganic pyrophosphatase	34 090	5.13	31 781	5.8	Phosphorus metabolic process	0.36		0.40			0.45		
Q6ZFJ4	Sulfite reductase, alpha subunit (Putative ferredoxin-NADP(H) oxidoreductase)	39 225	6.13	38 748	7.98	Sulfite assimilation				2.14				
Q2QZQ7	NB-ARC domain-containing protein	44 442	5.04	117 424	6.26	Programmed cell death	0.53	1.46						
P24626	Putative chitinase	32 259	6.17	33 681	4.84	Defence response to fungus		1.47		3.05				
Q6Z7A3	Putative C2 domain-containing protein	37 500	4.88	123 147	8.18	Signal transduction				0.50				0.52
Q654R2	Putative peroxidase	35 656	4.97	36 000	5.77	Cell redox homeostasis							0.41	0.40
Q6ER94	2-Cys peroxiredoxin	26 032	4.76	28 097	5.67	Cell redox homeostasis	0.49	0.83				0.56	0.61	0.74
P93407	Putative SOD[Cu–Zn], chloroplast precursor	20 255	5.76	21 301	5.79	Cell redox homeostasis		1.96			1.45			
Q33BC2	Hypothetical protein LOC-Os10g03230	20 752	5.28	24 528	7.12	Unknown protein			0.58			0.58		

Mw (kDa) indicates molecular weight of protein; PI indicates isoelectric point of protein.

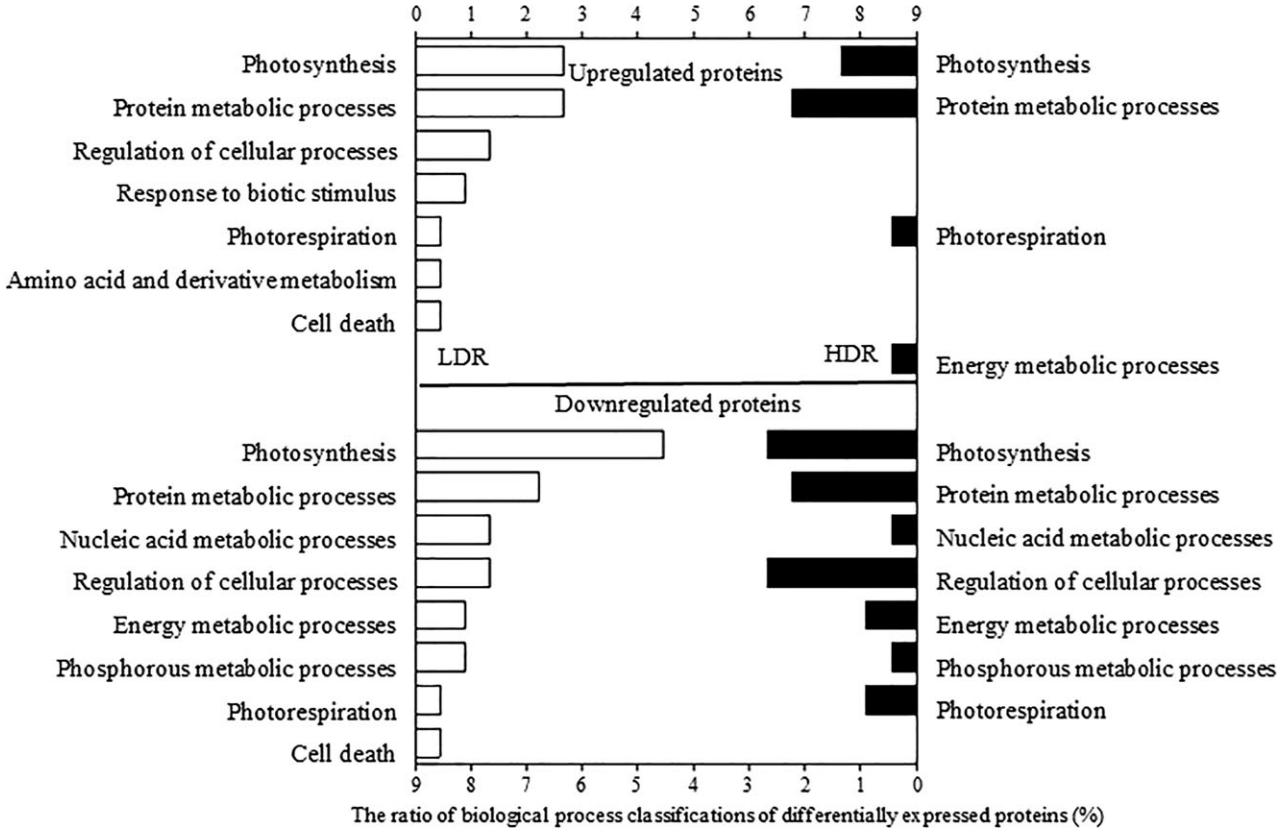

Fig. 3. Biological process classifications of the identified differentially expressed proteins in rice seeds irradiated with low- and high-dose radiation.

protein metabolic processes, nucleic acid metabolic processes, phosphorous metabolic processes, and regulation of cellular processes. Cell death was only enriched as a result of downregulation of proteins after LDR exposure.

Alteration of DNA methylation patterns after exposure to different doses of carbon-ion radiation

Genome methylation profiles were determined from DNA pools of each radiation group by MSAP analysis. The data showed that the five primer combinations assayed in MSAP generated 814 bands, with an average of 54 bands per primer pair (Fig. 4). The polymorphic rates in the nine groups were 1.11%, 4.25%, 4.30%, 3.15%, 7.03%, 9.20%, 9.87%, 7.61% and 7.22% (Supplementary Fig. 4B). Significant differences in overall relative cytosine methylation levels were found between the irradiation treatment and control groups ($P \leq 0.05$).

According to the digestion patterns of *Msp*I and *Hpa*II, mutation sites were divided into four types: hypermethylation at CG sites, hypomethylation at CG sites, hypermethylation at CNG sites, and hypomethylation at CNG sites. The number and percentage of mutation sites in each type are shown in Fig. 5. The data showed that there was a higher proportion of hypomethylation at 0.02, 0.1 and 2 Gy, but hypermethylation at 1 Gy (Fig. 5A). A greater number of altered CG sites than CNG sites were observed at 0.1, 5 and 20 Gy (Fig. 5B). The proportion of hypermethylation was more significant than that of hypomethylation at CG sites at 0.01, 0.2 and 1 Gy ($P \leq 0.05$), whereas the reverse was true at 2, 5 and 20 Gy ($P \leq 0.05$) (Fig. 5C). In addition, the hypomethylation level at CNG sites was more significant at 0.02, 0.1, 0.2 and 1, 5 Gy than hypermethylation at CNG sites ($P \leq 0.05$) (Fig. 5D). The multiple range ANOVA test analysing DNA methylation among the eight radiation groups is presented in Supplementary Table 4.

Sequencing of MSAP variant bands

Eighteen DNA fragments showing polymorphisms related to differences in methylation were sequenced (Table 3). Five variant bands showed homology to conserved hypothetical proteins with unspecified function, while the others were located in gene regulatory regions. In particular, the methylation patterns of genes coding oxophytodienoic acid reductase and cinnamoyl-coA reductase were altered at LDR, whereas the methylation patterns of genes coding DUF23 family proteins, heavy metal transport/detoxification protein domain-containing protein and RSH2 were altered at HDR. The methylation pattern of genes coding FAR1 domain-containing protein, cytochrome P450-like protein and the disease-resistance protein family proteins were altered in both LDR and HDR groups.

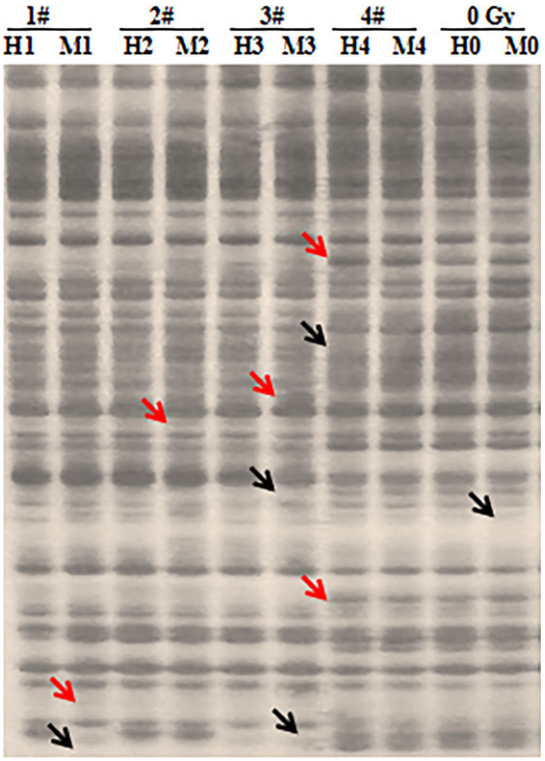

Fig. 4. Polymorphism of DNA methylation patterns of rice seeds following irradiation as determined by MSAP. Four rice plants (1#–4#) were randomly selected at 0.2 Gy. H: *Eco*RI and *Hpa*II; M: *Eco*RI and *Msp*I. 0 Gy, non-irradiated plants. Red and black arrows indicate the presence and absence of DNA methylation polymorphic bands, respectively.

DISCUSSION

In this study, no significant difference in plant height at the tillering stage was observed between LDR- and HDR-exposed seeds. This differs from our previous results, which found a stimulatory effect on the plant height of wet seeds induced by LDR, but a suppressive effect being induced by HDR [14, 15, 30–32]. Previous studies also found that rice plant height was inhibited by HDR, but increased by LDR at the three-leaf stage[14]. However, this phenomenon was found in irradiated wet seeds, but not in irradiated dry seeds. Those results indicate that the radiosensitivity level of plants depends on the water content of rice seeds. In our present results, there was no significant difference in rice plant height at the tillering stage after exposure of dry seeds to different doses of carbon-ion radiation.

Dose-dependent biological effects of radiation are not linear because of observed hormesis effects and adaptive responses to low-dose radiation [4, 5]. A series of reports showed that a dosage of 1 or 2 Gy is the boundary between low- and high-dose radiation in plants because of the biological effects were disorder caused by dosage below this threshold of radiation [14–16]. Although there was no dose effect in plant height after dry seeds were exposed to radiation, PCA and GO functional analysis of differentially expressed proteins showed that the proteins expressed after exposure dry seeds to 0.01, 0.02, 0.1, 0.2 or 1 Gy differed from those expressed after exposure to 2, 5 or 20 Gy, indicating that the different biological impacts observed in low- and high-dose–exposed dry seeds were reflected at the molecular level. The characteristics of the protein expression further suggested that 2 Gy might be the threshold between low- and high-dose HIR in this study.

The MSAP results showed that hyper- and hypomethylation both changed at CG and CNG sites. It is known that the average methylation level in CG contexts is 44.46% in the *Japonica* rice genome [33]. The CG sites are enriched in the promoter of genes. In our study, the proportion of hypermethylation was more significant than that of hypomethylation at CG sites after exposure to 0.01, 0.2 or 1 Gy, whereas the reverse was true at 2, 5 and 20 Gy. We speculate that hypermethylation at CG sites might play important roles in genomic stability in LDR groups, but the hypo-methylation at CG sites might upregulate the metabolic processes affected by HDR to protect the plant from radiation stress [34]. In addition, we found that the hypomethylation changes were more prone to occur at CNG sites after either low- or high-dose HIR. This remarkable change is in agreement with the idea that rice plant CNG methylation is more prone to perturbation by radiation stress than is CG methylation [35]. Investigations have indicated that DNA methylation at CNG sites is important not only for normal growth and development but also for the initiation of stress-defence mechanisms in plants [36, 37]. CNG sites have been found to be enriched in transposon and repetitive sequences in rice. The DNA methylation of these sites may be closely related to the formation of heterochromatin and the inactivation of transposons. Therefore, the hypomethylation of these regions may result in instability of the genome.

Our previous study used mature rice leaves irradiated by spaceflight and heavy HIR to investigate genomic/epigenomic mutations by using the amplified fragment length polymorphism (AFLP) and MSAP methods, respectively. Our results revealed correlations between the polymorphic rates of DNA methylation and the genomic sequence alterations: a higher level of DNA methylation changes and also of genomic sequence changes often appeared in the same individuals [38]. Another study investigated the genetic and DNA methylation stabilities of 11 randomly selected rice plants germinated from the space-flight seeds by AFLP and MSAP, and that analysis suggested that both the genetic and methylation changes manifested apparent mutational bias towards specific genomic regions [39]. However, further AFLP and MSAP mutation fragment sequencing indicated that the genetic and DNA methylation mutation sites showed different region preferences: the DNA methylation alterations were located at coding sequences and unknown function sequences, whereas the genomic mutations were located at repetitive sequences, introns and unknown function sequences [38]. Combined with the results of this and other current studies, this suggested that the DNA methylation might be the result of epigenetic mechanisms involved in radiation response and regulation.

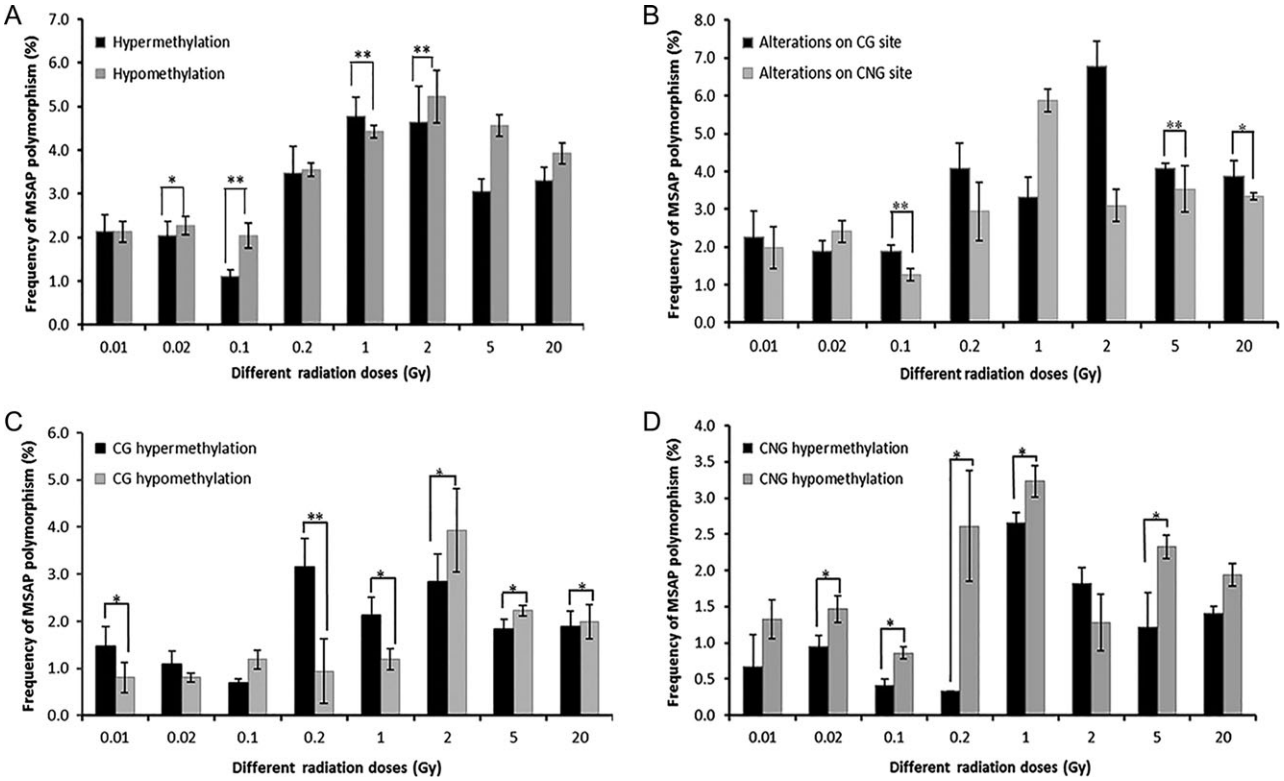

Fig. 5. Alterations of DNA methylation patterns of rice exposed to heavy ion radiation detected by MSAP. (A) Methylation changes of both types of cytosine methylation alterations: hyper- and hypomethylation. (B) Methylation changes in both types of cytosine methylation alterations: CG and CNG sites. (C) Methylation changes in both types of CG methylation alterations: CG hyper- and CG hypomethylation. (D) Methylation changes in both types of CNG methylation alterations: CNG hyper- and CNG hypomethylation. Data represents mean ± standard deviation of four replicates. A single asterisk indicates a difference significant at $P < 0.05$; two asterisks indicate a difference significant at $P < 0.01$.

Sequencing of MSAP variant bands showed that the changed DNA methylation genes differed between low- and high-dose HIR–exposed seeds. Oxo-phytodienoic acid reductase and cinnamoyl-coA reductase, which altered following LDR exposure, are associated with biosynthetic pathways [40], whereas DUF23 family protein, heavy metal transport/detoxification protein domain-containing protein and RSH2, which altered following HDR exposure, are involved in responses to various environmental stresses [41, 42]. This result implies that LDR enhances growth and increases the immune response of plants to stress by activating the biosynthetic pathways, whereas HDR is predominantly harmful for plants [43].

It is noteworthy that these results are based on a relatively small sample size. Because of the low resolution of our experimental methods, the relationship between the changes in DNA methylation, gene expression and genomic stability needs further study by whole-genome sequencing. This study showed that the proportion of hypermethylation was higher than that of hypomethylation at CG sites following LDR, and that this was reversed following HDR. The hypomethylation changes tended to occur at CNG sites both at low- and high-dose HIR. Furthermore, sequencing of MSAP variant bands suggested that, in this variety of rice plants, LDR might activate some metabolic processes and biosynthetic pathways, while HDR induce stress resistance. The results indicate that the different biological effects might be induced by different DNA methylation responses to low- and high-dose HIR. This may be helpful for further understanding of the mechanism of the biological effects caused by radiation.

CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Table 3. Sequence identification of DNA methylation change sites

No.	Clone primers	Dose (Gy)	Fragment length	Cytosine change type	Accession no.	Chromosome no.	Predicted homology
1	E3H3	0.01/0.1/0.2/5	55	CGde	ref|NC_008394.4|	Os01g0370000	Similar to oxo-phytodienoic acid reductase
2	E4H2	5/2/20	104	CGde	ref|NC_008401.2|	Os08g0121900	Protein of unknown function DUF23 family protein
3	E3H3	5/1/20	111	CGde	ref|NC_008396.2|	Os03g0152000	Heavy metal transport/detoxification protein domain-containing protein
4	E3H3	0.2/0.01/5	82	CGde	ref|NC_008395.2|	Os02g0608300	FAR1 domain-containing protein
5	E2H3	5/1/20	88	CGme&CNGde	ref|NC_008404.2|	Os11g0699100	Disease resistance protein family protein
6	E2H3	0.01/0.2	89	CNGme	ref|NC_008395.2|	Os02g0811600	Similar to cinnamoyl-CoA reductase
7	E1H1	0.2/0.01/2	141	CGme	ref|NC_008395.2|	Os02g0323600	Similar to cytochrome P450-like protein
8	E1H1	2/1/20	139	CGde	ref|NC_008402.2|	Os09g0442600	Similar to RSH2
9	E1H1	0.02/0.1/0.2/2	87	CGme&CNGde	ref|NC_008404.2|	Os11g0699100	Hypothetical protein; disease resistance protein family protein
10	E3H3	0.02/0.1/5	46	CGme	ref|NC_008403.2|	Os10g0516600	Conserved hypothetical protein
11	E3H1	2/1/5	96	CGme&CNGde	ref|NC_008394.4|	Os01g0622000	Conserved hypothetical protein
12	E4H2	0.01/0.02/0.1	96	CGme	ref|NC_008394.4|	Os01g0622000	Conserved hypothetical protein
13	E4H2	0.2	82	CGme	ref|NC_008404.2|	Os11g0180300	Conserved hypothetical protein
14	E3H3	0.1	266	CGme	ref|NC_008398.2|		Conserved hypothetical protein
15	E3H1	0.2/20	28	CGme	ref|NC_008404.2|		Between two genes
16	E3H3	5/2/20	49	CGme	ref|NC_008403.2|	Os10g0423000	At 1683 bp downstream of a conserved hypothetical protein
17	E3H3	0.01/0.1/0.2	81	CGde	ref|NC_008394.4|		At 2535 bp upstream of similar to GATA transcription factor 3 (AtGATA-3)
18	E1H1	2/1/5	120	CGde	ref|NC_008405.2|	Os12g0634500	At 3464 bp upstream of basic leucine zipper (bZIP) transcription factor domain-containing protein

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Radiation Research* online.

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