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 12C6 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 12C6+, 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−2s−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 maturiation 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) trichloroacid 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) H2O 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-propanesulfonylate, 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 Proteam 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 Proteam II xi Multi-Cell (Bio-Rad). SDS-PAGE was performed under a constant current of 20 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 Elite version 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 (SYNAP'T G2, waters, Massachusetts, USA) were used to verify whether the proteins separated by 2-DE in this study were similar to 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 ≤ 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 EcoRI and HpaII/MspI (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, EcoRI and HpaII/MspI 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 MspI (1, 0). Type III: the full methylation sites were only recognized by HpaII (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 × 2 + III + IV × 2)/[(I + II + III + IV) × 2] × 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 HR on plant growth, rice seeds were exposed to 0, 0.01, 0.02, 0.1, 0.2, 1, 2, 5 or 20 Gy of 13C6+ 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.
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.

**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 Mw (kDa) | Experimental Mw (kDa) | Function | Change fold at different doses (Gy) |
|------------|------|----------------------|----------------------|----------|-----------------------------------|
|            |      | 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 |
| Q5SEF8     | 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 |

C-ion radiation and DNA methylation alterations in rice seeds

Continued
| AC   | Name                                           | Mw (kDa) | pI | Mw (kDa) | pI | Function                        | Change fold at different doses (Gy) |
|------|------------------------------------------------|---------|----|----------|----|---------------------------------|-------------------------------------|
| 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 transcriptase                          | 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 |
| Q65R2  | 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.*
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 $MspI$ and $HpaII$, 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.
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.
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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| 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                | refNC_008394.4l       | Os01g0370000           | Similar to oxo-phytodienoic acid reductase                                          |
| 2   | E4H2          | 5/2/20    | 104            | CGde                | refNC_008401.2l       | Os08g0121900           | Protein of unknown function DUF23 family protein                                    |
| 3   | E3H3          | 5/1/20    | 111            | CGde                | refNC_008396.2l       | Os03g0152000           | Heavy metal transport/detoxification protein domain-containing protein              |
| 4   | E3H3          | 0.2/0.01/5 | 82             | CGde                | refNC_008395.2l       | Os02g0608300           | FAR1 domain-containing protein                                                     |
| 5   | E2H3          | 5/1/20    | 88             | CGme&CNGde         | refNC_008404.2l       | Os11g0699100           | Disease resistance protein family protein                                           |
| 6   | E2H3          | 0.01/0.2  | 89             | CNGme               | refNC_008395.2l       | Os02g0811600           | Similar to cinnamyl-CoA reductase                                                  |
| 7   | E1H1          | 0.2/0.01/2 | 141            | CGme                | refNC_008395.2l       | Os02g0323600           | Similar to cytochrome P450-like protein                                             |
| 8   | E1H1          | 2/1/20    | 139            | CGde                | refNC_008402.2l       | Os09g0442600           | Similar to RSH2                                                                    |
| 9   | E1H1          | 0.02/0.1/0.2/2 | 87          | CGme&CNGde         | refNC_008404.2l       | Os11g0699100           | Hypothetical protein; disease resistance protein family protein                    |
| 10  | E3H3          | 0.02/0.1/5 | 46             | CGme                | refNC_008403.2l       | Os10g0516600           | Conserved hypothetical protein                                                     |
| 11  | E3H1          | 2/1/5     | 96             | CGme&CNGde         | refNC_008394.4l       | Os01g0622000           | Conserved hypothetical protein                                                     |
| 12  | E4H2          | 0.01/0.02/0.1 | 96     | CGme                | refNC_008394.4l       | Os01g0622000           | Conserved hypothetical protein                                                     |
| 13  | E4H2          | 0.2       | 82             | CGme                | refNC_008404.2l       | Os11g0180300           | Conserved hypothetical protein                                                     |
| 14  | E3H3          | 0.1       | 266            | CGme                | refNC_008398.2l       |                         | Conserved hypothetical protein                                                     |
| 15  | E3H1          | 0.2/20    | 28             | CGme                | refNC_008404.2l       |                         | Between two genes                                                                  |
| 16  | E3H3          | 5/2/20    | 49             | CGme                | refNC_008403.2l       | Os10g0423000           | At 1683 bp downstream of a conserved hypothetical protein                           |
| 17  | E3H3          | 0.01/0.1/0.2 | 81     | CGde                | refNC_008394.4l       |                         | At 2535 bp upstream of similar to GATA transcription factor 3 (AtGATA-3)           |
| 18  | E1H1          | 2/1/5     | 120            | CGde                | refNC_008405.2l       | 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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