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Brief Communication
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Brief Communication

Progress Toward Rice Seed OMICS in Low-Level Gamma Radiation Environment in Iitate Village, Fukushima

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Abstract

Here, we present an update on the next level of experiments studying the impact of the gamma radiation environment, created post-March, 2011 nuclear accident at Fukushima Daiichi nuclear power plant, on rice plant and its next generation—the seed. *Japonica*-type rice (*Oryza sativa* L. cv. Koshihikari) plant was exposed to low-level gamma radiation (~4 μSv/h) in the contaminated Iitate Farm field in Iitate village (Fukushima). Seeds were harvested from these plants at maturity, and serve as the treated group. For control group, seeds (cv. Koshihikari) were harvested from rice grown in clean soil in Soma city, adjacent to Iitate village, in Fukushima. Focusing on the multi-omics approach, we have investigated the dry mature rice seed transcriptome, proteome, and metabolome following cultivation of rice in the radionuclide contaminated soil and compared it with the control group seed (non-radioactive field-soil environment). This update article presents an overview of both the multi-omics approach/technologies and the first findings on how rice seed has changed or adapted its biology to the low-level radioactive environment.

Subject area: Genomics and gene mapping, Bioinformatics and computational genetics
Key words: continuous low-level gamma radiation, DNA microarray, mass spectrometry, omics, rice plant, seed

Post-March 2011, following the "Great East Japan Earthquake" and the resulting nuclear accident at the Fukushima Daiichi Nuclear Power Plant led us to investigate the effects of low-level gamma radiation contamination in contaminated areas of Fukushima prefecture on the rice plant. Rice is a staple food crop in Japan and most of South-East Asia and a genome (read transcriptome in context of current research based on the DNA microarray technology utilized) model (Agrawal and Rakwal 2006, 2011) and has been our research material at the highly contaminated site (Iitate Farm, ITF) in Iitate village (Imanaka et al. 2012) of Fukushima prefecture 31 km from the damaged reactor site (Figure 1). Previously we had reported that low-level gamma radiation influences the rice seedling leaf transcriptome and proteome identifying numerous differentially regulated defense/stress responsive genes and proteins (Hayashi et al. 2014, 2015a). It was proposed therein that the next target should be to examine the effects of low-level gamma radiation in the whole plant to the level of seed, which is the next generation organ crucial to plant dissemination and survival. We have been conducting research on the rice seed in the radio-contaminated environment at ITF, Iitate village, since 2013 and are now able to present the an overview of the obtained data of our investigation using a multi-omics approach of transcriptomics, proteomics, and metabolomics. To note, we have also published a small part of the research as an "application note" with Agilent Technologies to showcase the use of high-through omics equipment's and tools used in DNA microarray analysis (transcriptome) and metabolomics with seed as a model case (Hayashi et al. 2015b). This present update discusses the multi-omics approach (transcriptome-proteome-metabolome), technologies used and findings on how the rice seed has changed or adapted its biology to the low-level (gamma) radioactive environment.

Materials and Methods

Methodologically, the Figure 2 presents an overview of the experiments designed and techniques used for obtaining the "seed omics" inventory of genes, proteins, and metabolites following a low-level gamma radiation environment for the whole growing season from seedling to maturity. Briefly, *Japonica*-type rice (*Oryza sativa* L. cv. Koshihikari) plant was grown till seedling stage in a greenhouse facility at the National Institute for Environmental Studies (NIES, Tsukuba, Japan) during May–June, 2013. Fourteen days after start of the germination protocol, healthy rice seedlings were transported to the designated experimental site (ITF rice field in Iitate village, Fukushima prefecture) for initiating the experiment. Rice plants, as they grew were exposed to low-level gamma radiation (ambient radiation level of ~4 μSv/h) in the contaminated field, which was 80 times higher than the natural background radiation for Japan (~0.05 μSv/h). Seeds were harvested from these plants at maturity, stored at ambient room temperature and served as the treated group. For control group, seeds (cv. Koshihikari) were harvested from rice grown in clean soil (rice field) in the Soma city (Fukushima). Radiation levels at Soma city (for control rice) were ~0.15 μSv/h. The choice or selection of the control field was made by requesting the rice farmer living next to the ITF farm in Iitate village, and who also had an evacuation (temporary) home in the Soma city. This was the nearest village where rice farming was being done, and the farmer agreed to let us use the rice he was cultivating, and thus providing us with the sample of control rice, as per our requirements. This was the only experimental design possible under the detailed experimental conditions we had set out to investigate. Therefore the data presented here will be a reflection of this experimental condition and conditions therein.

Prior to the analyses, a fine powder was prepared by grinding the seeds in liquid nitrogen using a mortar and pestle. The fine powder was stored in aliquots at −80 °C deep freezer till further analysis. Focusing on the multi-omics approach, we have investigated the dry mature rice seed transcriptome, proteome and metabolome following cultivation of rice in the radionuclide contaminated soil (i.e., exposed to low-level internal and external gamma radiation) and compared it with the control group seed (non-radioactive field-soil environment). This integrated omics approach provides us with a powerful platform to create an inventory of the major events occurrent at molecular level, and which might provide us with radiomarkers for rice plant (seed) under radiation stress.

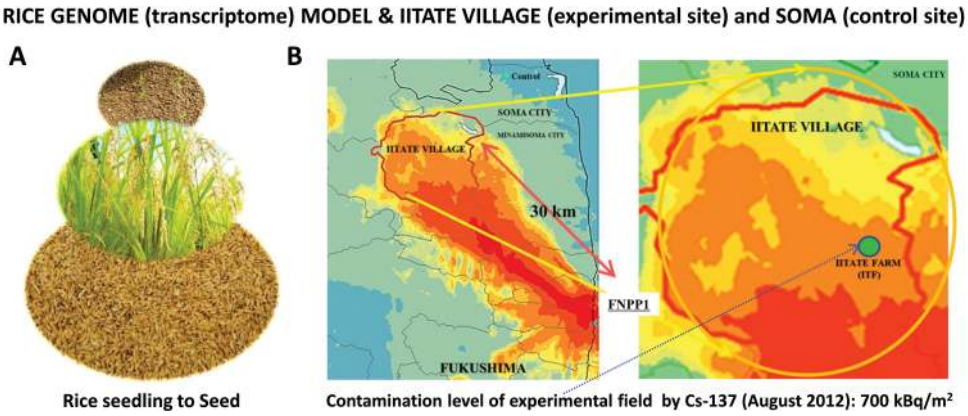

Figure 1. Rice as a genome (transcriptome) model and food for billions (**A**), and the litate village in Fukushima prefecture and location of the litate farm (ITF) and the contamination levels therein of the experimental field at ITF where the rice was grown in a radioactively contaminated environment (**B**).The adjoining area is the sample control site in Soma city, from where the control rice was obtained. For details see (Agrawal and Rakwal 2011; Imanaka et al. 2012; Hayashi et al. 2014).

First, the seed transcriptome and metabolome was examined using an Agilent-based multi-omics workflow (Figure 3). Total RNA was extracted from 250 mg of rice seed fine powder (in liquid nitrogen) by using the combination of CTAB, phenol-chloroform and Qiagen RNeasy kit methods and following quality control checks and labeling processes, the final cRNA was hybridized on to Agilent rice 4 × 44 k rice microarrays (AMADID: 015241). Agilent SureScan (p/n G4900DA) and Feature Extraction 12.0 software were used to generate data as per Agilent protocols, and data analysis was performed by using GeneSpring 13.1, and the differentially regulated

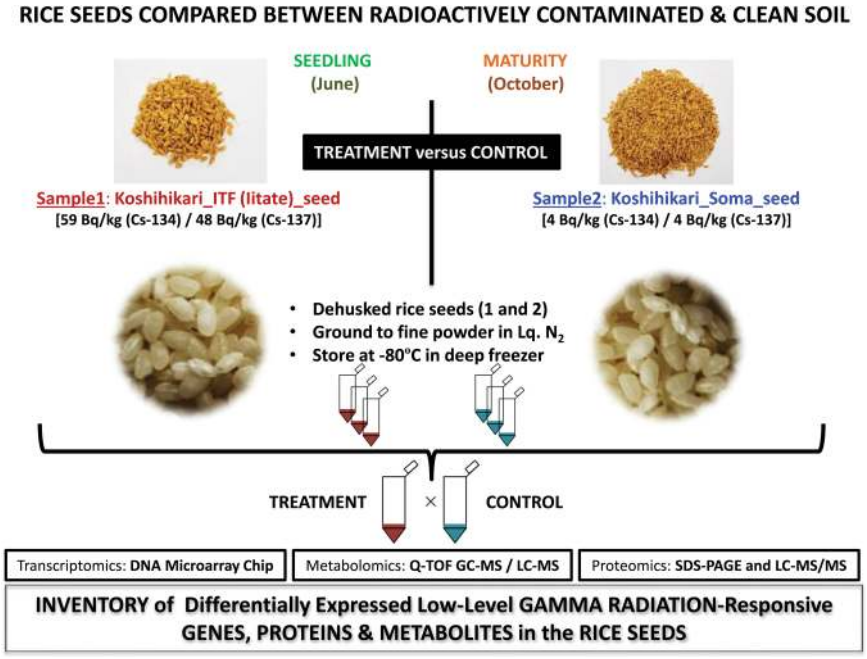

Figure 2. Experimental strategy for measuring effects of low-level dose of gamma radiation on molecular-level changes in dry mature seeds of rice (cv. Koshihikari) plant grown under low-level gamma radiation in litate village (ITF), Fukushima. Control rice (cv. Koshihikari) was grown during the same period in Soma city, Fukushima. Dry mature seeds were harvested and used for the omics analysis (genomics, proteomics and metabolomics) as detailed in the figure.

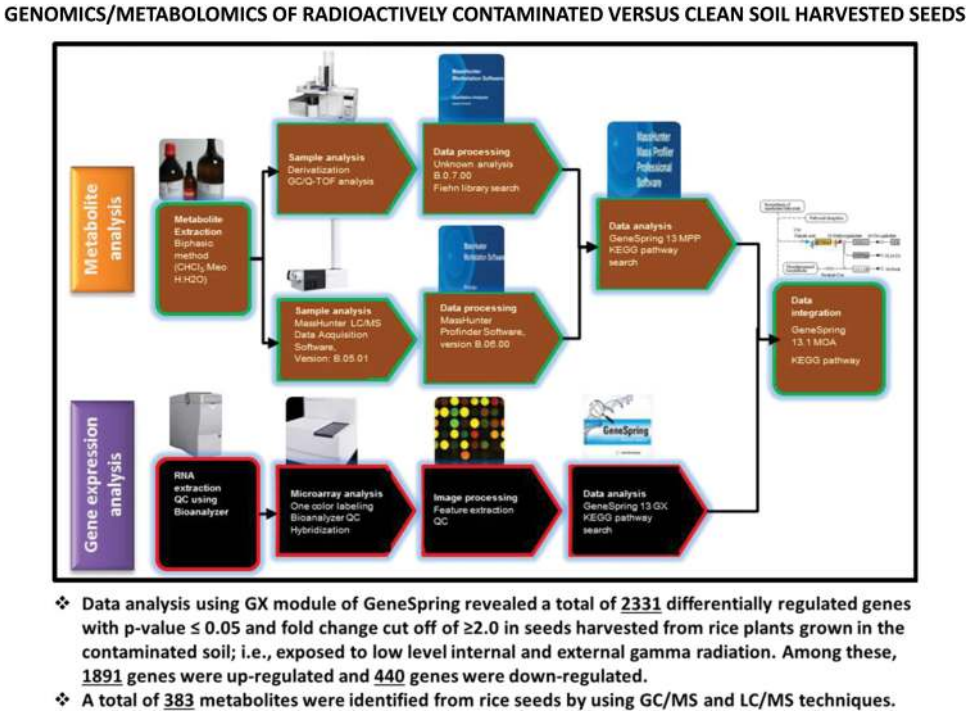

Figure 3. Transcriptomics and metabolomics analyses workflow for multi-omics analyses of rice seeds harvested from the rice plants cultivated in the radioactive environment (ITF) over the control seeds from clean soil (Soma city). Total RNA and metabolites were extracted and analyzed using Agilent analytical systems (DNA microarray and Q-TOF-GC/LC-MS) as described in the text. The number of identified genes and metabolites are indicated at the bottom of the image.

genes were mapped to KEGG pathways. For metabolite analysis, seed sample powder (50 mg) was extracted with mixture of chloroform: methanol:water = 1:2.5:1 (v/v/v) and both aqueous and organic phases (upper and lower layers, respectively) were dried after separation. Two approaches were followed; 1) using derivatization of metabolites for GC/Q-TOF analysis (Agilent 7200) and 2) the dried aqueous and organic fractions were taken for LC-MS analysis (Agilent 6550).

Second, the proteome of the seeds was examined to complement our data on the transcriptome and metabolome. Seed sample powder was mixed with Tris-Mg-NP-40 buffer containing 2% beta-mercaptoethanol followed by TCA-acetone precipitation to obtain total seed proteins. Total proteins were resolved on SDS-PAGE and which confirmed a good extraction and separation of proteins by CBB staining; some visible differences could also be discerned. Next, we utilized a shotgun-approach where the extracted total proteins were subjected to in-solution trypsin digestion followed by label-free quantitative proteome profiling using Q-exactive Orbitrap mass spectrometer (LC-MS/MS) (Figure 4A, B).

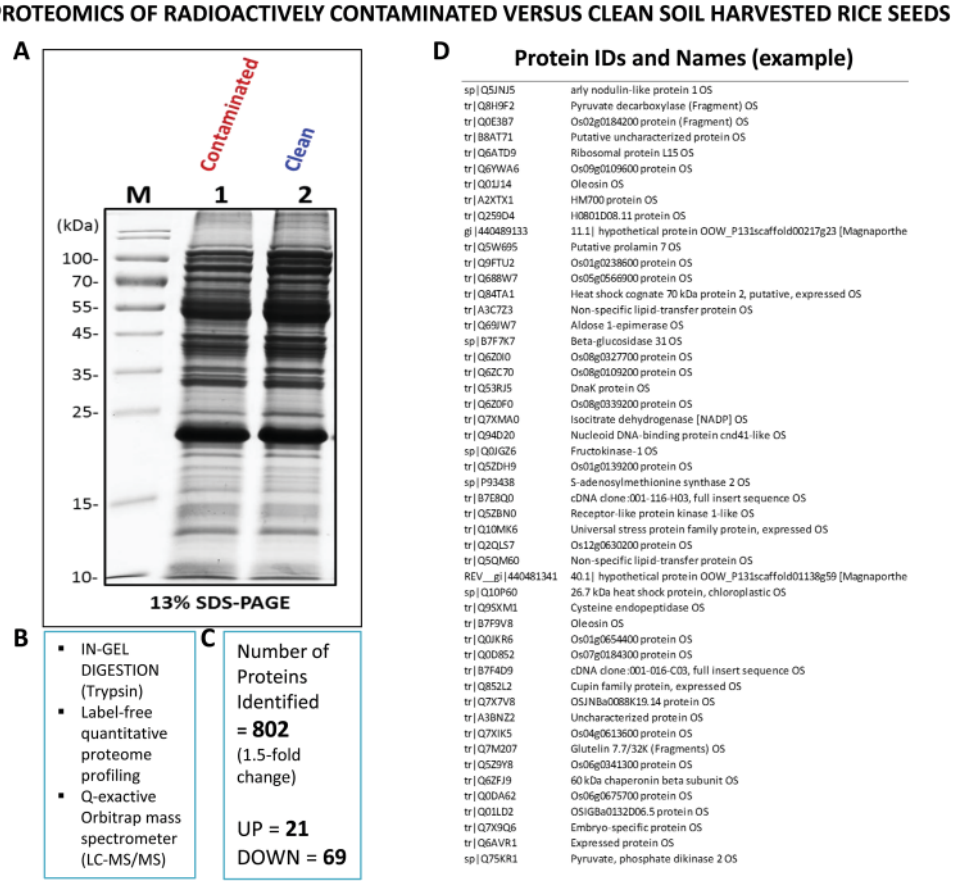

Figure 4. Proteome analysis of rice seed proteins harvested from radioactive environment (ITF) over clean soil (Soma city) control seeds. Following total protein extraction SDS-PAGE (**A**) was carried out to establish good quality isolation by CBB staining and then taken for in-gel digestion and label-free quantitative proteome profiling using LC-MS/MS approach (**B**) to identify the differentially changed proteins (**C** and **D**).

Table 1. Cultivar specific radionuclides in harvested mature rice seeds and characteristics of harvested dried culms and seeds

Sample name	Sample number	Sample site	Cultivar	Sample	Weight (g)	Cs-134 (Bq/kg)	Cs-137 (Bq/kg)
Koshihikari-γ-irradiated	1_2	Iitate Farm, Iitate Village	*Oryza sativa* L. cv. Koshihikari	Seed	10.5	59	48
Koshihikari-clean	4_	Soma City	*Oryza sativa* L. cv. Koshihikari	Seed	51.79	4	4

O. sativa L. cv. Koshihikari: γ-irradiated

O. sativa L. cv. Koshihikari: clean

Results and Discussion

Table 1 shows the data on the cultivar specific radionuclides in harvested mature rice seeds and characteristics of harvested dried culms and seeds, indicating a clear reduction in the rice seed harvested from the γ-irradiated sample site (ITF) compared to the control site (Soma). A recent paper by Matsunami and co-workers is worth mentioning here, as it deals with the suspected radioactive contamination in brown rice, in Minamisoma city, adjoining the Iitate village to the west and the Soma city to the north, in 2013, and where it was suggested based on bio-physical methods of autoradiograpy and gamma ray spectrometry that although the area was "clean" (refer to Figure 1 of Matsunami et al. 2016). It was indicated that the contamination might have occurred via external adherence of the radionuclides to the rice panicles (Matsunami et al. 2016; and data therein obtained from the Ministry of Agriculture, Forestry and Fisheries, Government of Japan: http://www.maff.go.jp/j/kanbo/joho/saigai/fukusima/pdf/genmai_h26_0214.pdf, http://www.maff.go.jp/j/kanbo/joho/saigai/fukusima/pdf/genmai_kousatu_141201.pdf). This indirectly supports our clean rice control field site outside the highly contaminated ground in Iitate village regions as shown in the map in Figure 1 and by other research (Imanaka et al. 2012; Matsunami et al. 2016).

At the molecular level, and the main focus of this study, the obtained large data-sets were analyzed using the bioinformatics tools subsequent to each transcriptome, proteome and metabolome experiment to reveal differential expressions of gene transcripts, proteins and metabolites (Figures 2–5). The GX module of GeneSpring to identify 2331 differentially regulated genes (up-regulated: 1891 genes, down-regulated: 440) with *P*-value ≤ 0.05 and fold change cut-off of ≥2.0 in seeds harvested from rice plants grown in the contaminated soil (Figure 3). On the other hand, metabolomics using GC/MS and LC/MS techniques revealed 383 metabolites. Pathway analysis using Pathway Architect module of GeneSpring characterized those genes to plant defense, cell wall synthesis, secondary metabolites production, fatty acid metabolism, antioxidant, energy cycling, etc., in KEGG pathways. The identified metabolites

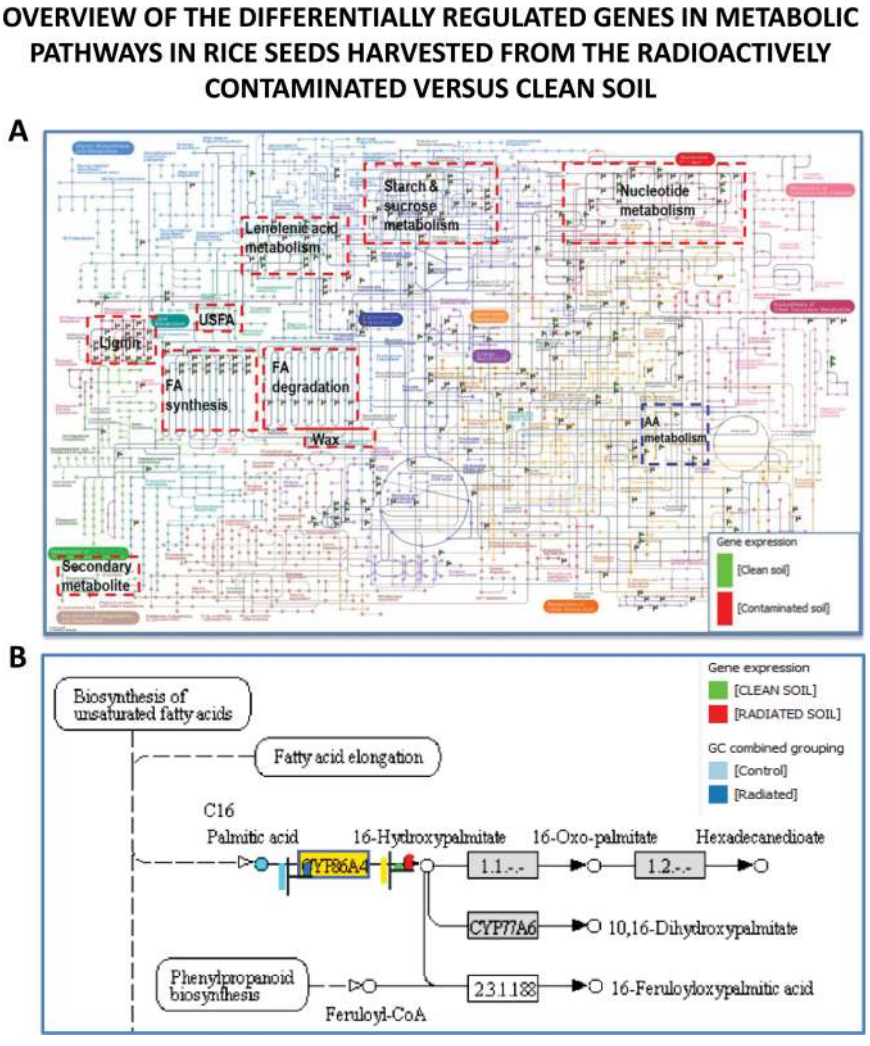

Figure 5. Overview of the differentially regulated genes in metabolic pathways inn radioactively contaminated following exposure to low-level gamma radiation. Heat strips shows the average differential abundance values for the control and radiated samples. The rice seeds exposed to radiation revealed major dysregulation of fatty acid metabolism, starch and sucrose metabolism, nucleotide metabolism, secondary metabolism pathways, etc. The changes in the subsequent linked pathways can also be traced out in the metabolic overview (**A**). Up-regulation of genes and metabolites, for example, as shown for the biosynthesis of unsaturated fatty acids, where HeatStrips shows average differential abundance values for the control and radiated samples (**B**).The vertical bars behind and after the boxed CYP86A4 indicates a result for metabolites (blue bar in online version) and genes (yellow bar in online version) , respectively.

mainly belonged to energy, fatty acid, amino acid, nucleotide, secondary metabolic pathways, etc., in rice KEGG pathways. For the proteome component, a total of 802 seed proteins were identified of which 90 showed differential expressions (more than 1.5-fold change) in either of the 2 experiments; 21 and 69 proteins showed increased and decreased abundances, respectively (Figure 4C, D). Using Multi-Omic Analysis (MOA) module of GeneSpring, we could map the gene and metabolites onto pathways as shown in Figure 5. This study provides the first inventory of a large number of gamma radiation-responsive proteins in dry mature rice seeds, suggesting the ultimate changes of surviving in a radiation environment.

Taken together, the experimental strategy/design and integrated-omics/MOA approach employed in the present research has provided new ways to test at the level of whole transcriptome, proteome and metabolome how a particular tissue (in this case, seed) of the rice plant responds to ionizing radiation under field conditions. The integrated-omic analyses by Agilent (transcriptome and metabolome) and non-Agilent (proteome; Thermo Fischer Scientific) platform has provided us with first knowledge on the seed response to low-level gamma radiation, which most probably occurs through a well-coordinated defense response. The obtained large volume molecular data will be further analyzed in greater detail for in-depth understanding of the molecular-level changes in the rice seed following cultivation in a low-level γ-irradiated contaminated environment.

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