A Multi-Omic Approach to Reveal the Effect of Low-Level Gamma Radiation on Rice Seeds

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ASMS 2016
Poster MP267

Agilent Technologies

Introduction

The exposure of plants to ionizing radiation (IR) is known to trigger a wide range of responses between initial absorption of energy and manifestation of final biological injury[1]. In this study, we examine the effects of low-level gamma radiation on seeds from rice plants grown in contaminated soil (rice fields) at litate farm (ITF) in Fukushima Prefecture, Japan. Here, we report the results of multi-omic analysis by studying both transcriptome and metabolome in rice seeds using Agilent multi-omics solutions.

Experimental

The workflow followed in the present study is presented in **Figure 1**.

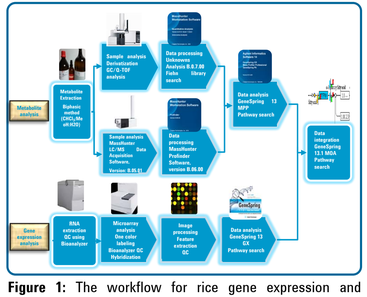

Figure 1: The workflow for rice gene expression and metabolite analysis

Collection of plant material

Rice plants (*Oryza sativa* L. cultivar Koshihikari) cultivated in radionuclide contaminated soil of a rice field in ITF (litate village in Fukushima prefecture) consequent to the nuclear (FDNPP: Fukushima Daiichi Nuclear Power Plant) disaster were collected and stored at ambient room temperature. The seeds for subsequent analysis were dehusked and flash frozen in liquid nitrogen and stored at -80 °C. Radiation levels from soil were measured using a Germanium semiconductor detector. Seeds from the same variety of rice plants grown in Minamisoma (Fukushima prefecture) were considered clean and served as a control.

RNA extraction and microarray analysis

RNA was extracted from 250 mg of rice seeds by using the combination of CTAB, phenol-chloroform and column based RNA isolation kit protocols.

Experimental

The RNA integrity was assessed using an Agilent 2100 Bioanalyzer system using 6000 Nano chip (p/n 5067-1511). 25 ng of total RNA was labelled by using one color Agilent Low Input Quick Amp (LIQA) Labeling Kit (p/n 5190-2305) and the cRNA quality was assessed by using NanoDrop and Bioanalyzer on a RNA 6000 Nano chip. 1.65 µg of cRNA was hybridized on to Agilent rice 4×44 k rice microarrays (AMADID: 015241). Scanning and feature extraction was performed on Agilent SureScan (p/n G4900DA) and Feature Extraction 12.0 software, respectively. Data analysis was performed using GeneSpring 13.1. The Moderated T test was used with Westfall Young Permutative multiple testing correction (*p* value cutoff of 0.05 and fold change > 2). The differentially regulated genes were mapped to pathways using Pathway Architect.

Quantative RT-PCR validation

One-step qRT-PCR was performed using Agilent QRT-PCR master mix (p/n 600886) in a 20 µl reaction in 96 well plates using an Agilent Real time PCR System (p/n G8830A). The Cq values obtained from targets/reference genes for control and radiated seeds were analyzed using GeneSpring 13.1. The amplicon sizes from the resulting reactions were confirmed by analyzing the products on a 2100 Bioanalyzer system using a DNA 1000 assay (p/n 5067-1504) according to the manufacturer's protocol.

Metabolite extraction

Rice seeds were powdered in liquid nitrogen using a mortar and pestle. Samples (50 mg) were extracted with a mixture of chloroform: methanol: water=1:2.5:1 (v/v/v).

Derivatization and GC/Q-TOF analysis

An Agilent 7200 Series GC/Q-TOF accurate mass high resolution mass spectrometer was used for analysis of derivatized metabolites using d27 myristic acid retention time locking standard from the Agilent Fiehn GC/MS Metabolomics Standards Kit (p/n 400505). The GC/Q-TOF conditions used are shown in **Table 1**. Data from the GC/Q-TOF was processed using MassHunter Unknown Analysis Software (version B.07.00). The spectral information was matched with the Agilent-Fiehn library (p/n G1676AA) with retention time indices (with respect to FAME mix).

LC/MS analysis

5 µl of the re-suspended fractions were injected on an Agilent 1290 Infinity LC System coupled to an Agilent 6550 LC/Q-TOF. Data of metabolites from the organic phase were acquired on both positive and negative ion modes. Data from the aqueous phase was acquired on positive mode. The LC/Q-TOF conditions are shown in **Table 2**.

Experimental

Table 1: GC/Q-TOF conditions

Column	DB-5ms: 30m x 0.25mmID x 0.25um, Guard Length: 10 m (Part No. 122-5532G)
Injection volume	1 µL
Split mode and ratio	Splitless
Split/Splitless inlet temperature	250 °C
Oven temperature program	60 °C for 1 min 10 °C/min to 325 °C, 10 min hold
Carrier gas	Helium at ~1.9 mL/min, constant flow
Transfer line temperature	290 °C
Q-TOF conditions	
Ionization mode	EI
Source temperature	230°C
Quadrupole temperature	150°C
Mass Range	50 to 600 m/z
Spectrum acquisition	5 spectra/s, 2679 transients/spectrum, centroid mode

Table 2: LC/Q-TOF parameters and conditions

Acquisition Mode	MS
Ion Polarity	Positive/Negative
Drying gas flow	15 L/min @ 250 °C
Nebulizer	40 psig
Sheath gas flow	10 L/min @ 350 °C
VCap	3,500 V
Nozzle voltage	1,000 V
Fragmentor	100 V
Mass Range	50-1700 m/z
LC column	Poroshell 120 EC-C18 3.0X50mm, 2.7 µm
Injection volume	5 µl
Flow rate	0.3 ml/min
Column temp.	30 °C
Ionization Mode (organic fractions)	Positive Ion Mode, Negative Ion Mode
Mobile Phase (organic fractions)	A) 10 mM ammonium acetate in acetonitrile : Water (2:3 v/v) B) 10 mM ammonium acetate in acetonitrile : Isopropanol (1:9 v/v)
Ionization Mode (aqueous fractions)	Positive Ion Mode
Mobile Phase (aqueous fractions)	A) Water with 0.2% Acetic acid B) Methanol with 0.2% Acetic acid

Results and Discussion

Table 2: LC/Q-TOF parameters and conditions

LC gradient (organic fractions)	Time	A	B
	0	100	0
	2	100	0
	40	0	100
	50	0	100
	50.1	100	0
	60	100	0

Table 2: LC/Q-TOF parameters and conditions

LC gradient (aqueous fractions)	Time	A	B
	0	95	5
	5	95	5
	30	0	100
	40	0	100
	40.1	95	5
	50	95	5

Results and Discussion

A total of 2,331 genes and 383 metabolites were differentially-expressed in rice seeds exposed to low level gamma radiation. Pathway analysis using Pathway Architect revealed that the differential genes/metabolites belonged to plant defense, cell wall synthesis, secondary metabolite production, fatty acid metabolism, antioxidant and energy cycling pathways demonstrated the elevated defense capability against stress in seeds.

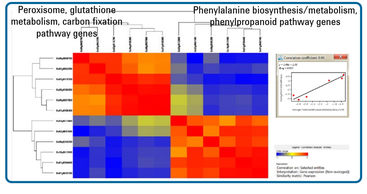

Figure 3: Similarity of differential entities from few pathways namely phenylalanine, tyrosine and tryptophan biosynthesis, phenylpropanoid biosynthesis, phenylalanine metabolism, peroxisome, carbon fixation in photosynthetic organisms and glutathione metabolism. Scatter plot showing positive relationship between chorismate mutase and peroxidase 72 (Insert).

Results and Discussion

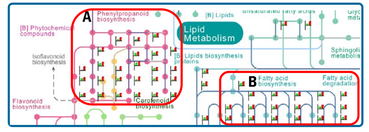

Figure 4: Differentially expressed metabolic pathways in rice due to exposure to low level gamma radiation in rice seeds. **A**. Phenylpropanoid biosynthesis pathway. **B**. Fatty acid metabolism pathways. HeatStrips show the average differential abundance values for the control and radiated samples.

Metabolite analysis

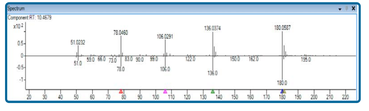

Figure 5: Fiehn library matched analysis using Agilent MassHunter Unknown Analysis Software (version: B.07.00/Build 7.0.457.0). Nicotinic acid spectral match with the library spectra.

Compound	Log FC	Regulation
	Amino acids	
L-Arginine	-18.2	down
L-Methionine	-17.9	down
D-Proline	17.5	up
L-alanine	22.7	up
Carbohydrates		
Glucoheptonic acid	-13.2	down
Trehalose	26.0	up
Raffinose	24.5	up
Organic acids		
Pimelic acid	-6.6	down
Phosphoric acid	2.9	up
Oxalic acid	15.2	up
Fatty acids		
Linolenic Acid	21.3	up
Stearic acid	21.8	up
Linoleic acid	-2.3	down
Secondary metabolites		
Ferulic acid	-3.4	down
12-OPDA	16.0	up

Table 3: Selected radiation induced differential metabolites in rice seeds with fold change and regulation (GeneSpring 13.1)

Combined analysis

The combined multi-omics analysis of transcriptome and metabolome data using Agilent GeneSpring/MPP 13.1 revealed significant overlaps in differential genes and metabolites in metabolic pathways that participate in the stress responses of plants to harmful environmental factors. These signatures can be used as radio markers for rice seeds under gamma radiation exposure.

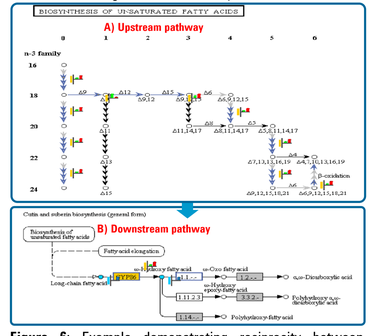

Figure 6: Example demonstrating reciprocity between entities in upstream and downstream pathways. (A) Up-regulation of genes and metabolites are evident in biosynthesis of unsaturated fatty acid pathway and (B) the subsequent cutin & suberin biosynthesis pathway. A yellow bar along the HeatStrip indicates genes; a blue bar indicates a result for metabolites.

Conclusions

- In this study, applying Agilent's multi-omic solutions, we identified that irradiated seeds respond to low-level gamma radiation through what appears to be a well-coordinated defense mechanism.
- Integrated-omic analysis using a combination of Agilent platforms and software tools is a powerful approach to identify the major events occurring in plants undergoing stress.

References:

1. Hayashi, G.; *et al.* Unraveling low-level gamma radiation-responsive changes in expression of early and late genes in leaves of rice seedlings at litate village, Fukushima. *J. Hered.* 2014, *105*, pp 723-738.