A Multi-omic Approach to Reveal the Effect of Low-level Gamma Radiation on Rice Seeds

Application Note

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Abstract

This Application Note describes the workflow for identifying the stress-related transcriptomics and metabolomics biomarkers in rice using Agilent multi-omics solutions. We studied the effects of low-level gamma radiation on seeds of rice plants grown in litate farm (ITF) of litate village in Fukushima prefecture, using Agilent sample preparation consumables, instrumentation, and software tools. We generated high quality transcriptomics/metabolomics data, and integrated them using Agilent GeneSpring/Mass Profiler Professional (MPP) 13.1 Software. The combined multi-omics analysis revealed modulation of several metabolic and defense pathways related to the stress response of plants. Our results suggest that the rice plants grown in radionuclide-contaminated soil form seeds with an elevated defense capability against stress. This study demonstrates the Agilent multi-omics workflow for performing gene expression and metabolite analysis on samples derived from plant sources.

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Introduction

The exposure of plants to ionizing radiation (IR) is known to trigger a wide range of responses between the 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 the radionuclide contaminated soil (paddy field) at litate farm (ITF). This report presents the results of multi-omic analysis by studying both the transcriptome and metabolome in rice seeds using Agilent multi-omics solutions.

Materials and Methods

Figure 1 shows the workflow followed.

Collection of plant material

Rice plants (*Oryza sativa* L. cultivar Koshihikari) cultivated in radionuclide-contaminated soil from a paddy field in ITF (litate village in Fukushima prefecture) were collected and stored at ambient temperature. The soil was contaminated as a result of the Fukushima Daiichi Nuclear Power Plant (FDNPP) disaster. The seeds for subsequent analysis were dehusked and flash frozen in liquid nitrogen and stored at –80 °C. Radiation levels from the soil were measured using a germanium semiconductor detector. The 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 using a combination of CTAB, phenol-chloroform, and a column-based RNA isolation kit. RNA quantity and quality were estimated using NanoDrop. The total RNA yield was >7 µg. The RNA integrity was assessed using an Agilent 2100 Bioanalyzer system with a 6000 Nano chip (p/n 5067-1511) (Figure 2). Twenty-five nanograms of total RNA was labeled using one color Agilent Low Input Quick Amp (LIQA) Labeling Kit (p/n 5190-2305), and the cRNA quality was assessed using NanoDrop and a Bioanalyzer with a RNA 6000 Nano chip. A 1.65 µg amount of cRNA was hybridized onto Agilent rice 4×44 k rice microarrays (AMADID: 015241). Scanning and feature extraction were performed on Agilent SureScan (p/n G4900DA) and Feature Extraction 12.0 software, respectively.

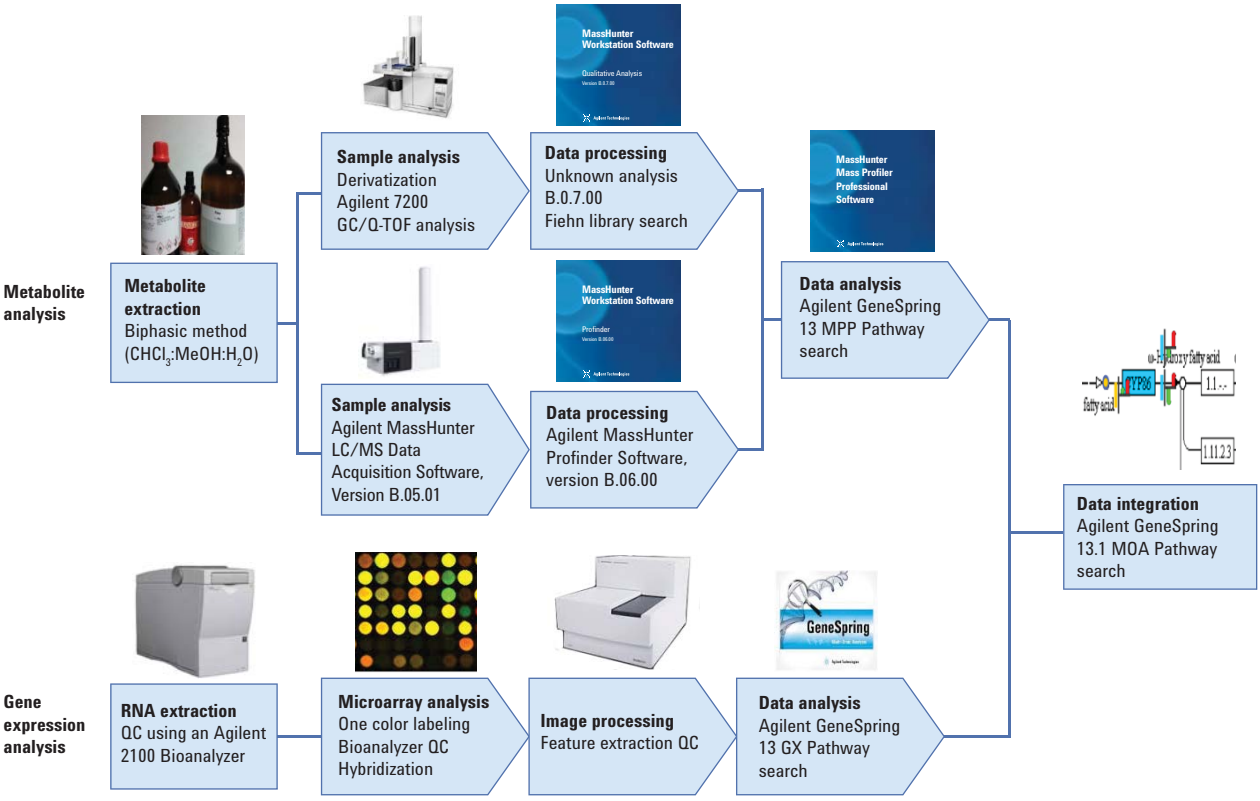

Figure 1. The gene expression and metabolite analysis workflow used for identifying radio markers in rice.

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 expressed genes were mapped to pathways using Pathway Architect.

Quantitative RT-PCR validation

One-step qRT-PCR was performed using the Agilent Brilliant III ultrafast SYBR Green QRT-PCR master mix (p/n 600886) in a 20 µL reaction in 96-well plates with an Agilent AriaMx Real time PCR System (p/n G8830A). After the reverse transcription step at 50 °C for 5 minutes, denaturation was performed at 95 °C for 3 minutes, followed by 40 cycles of 95 °C for 10 seconds, and annealing/extension at 60 °C for 12 seconds. No template controls (NTC) were maintained to ensure the absence of contamination. The Cq values obtained from targets/reference genes for control and radiated seeds were analyzed using the RT-PCR module in GeneSpring 13.1. The amplicon sizes from the resulting reactions were confirmed by analyzing the products on an Agilent 2100 Bioanalyzer system using a DNA 1000 assay (p/n 5067-1504) according to the manufacturer's protocol.

Metabolite extraction

Rice seeds, either from plants grown in radionuclide-contaminated soil or those grown in control clean soil, were powdered in liquid nitrogen using a mortar and pestle. The powder was stored at –80 °C. Samples (50 mg) were extracted with a mixture of chloroform:methanol:water = 1:2.5:1 (v/v/v) and vortexed for 5 minutes at 4 °C. The sample tubes were centrifuged at 20,800 rpm for 2 minutes, and the supernatant was transferred to fresh tubes. A 400 µL volume of ultrapure water was added to each mL of the supernatant, and the tubes were vortexed again for 10 seconds, followed by centrifugation at 20,800 rpm for 2 minutes. Both aqueous and organic phases (upper and lower layers, respectively) were dried after separation.

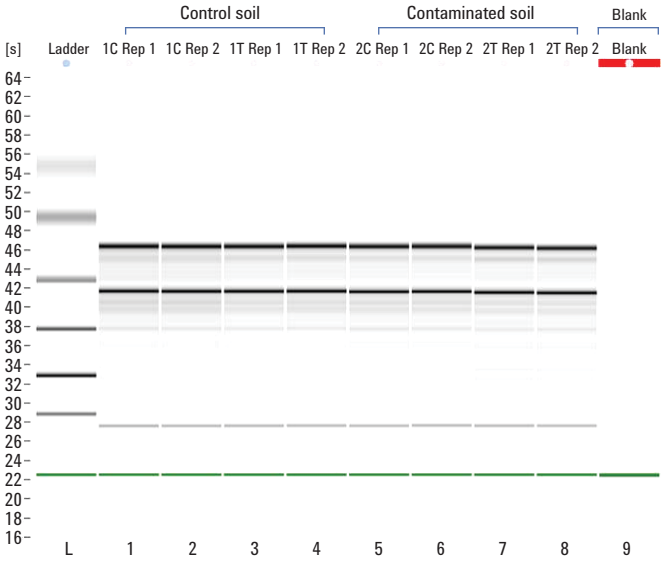

Figure 2. Bioanalyzer profiles of RNA extracted from rice seeds grown in contaminated and control soil.

Derivatization and GC/Q-TOF analysis

The derivatization of metabolites in the samples was performed following the protocol of Palazoglu and Fiehn[2]. An Agilent 7200 GC Q-TOF was used for analysis of metabolites using d_{27} myristic acid as a retention time locking standard from the Agilent Fiehn GC/MS Metabolomics Standards Kit (p/n 400505). Table 1 shows the GC Q-TOF conditions used. The 7200 GC/Q-TOF data were processed using Agilent MassHunter Unknown Analysis Software (version B.07.00). This software automatically deconvolutes spectra from coeluting compounds using model ion traces. The spectral information was matched with the Agilent-Fiehn library through retention time index with respect to FAME mix.

Table 1. Conditions used for GC/Q-TOF analysis.

GC conditions	
Column	Agilent DB-5ms, 30 m × 0.25 mm, 0.25 µm, guard length 10 m (p/n 122-5532G)
Injection volume	1 µL
Split mode and ratio	Splitless
Split/Splitless inlet temperature	250 °C
Oven temperature program	60 °C for 1 minute 10 °C/min to 325 °C, 10 minutes 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
m/z scan	50 to 600 *m/z*
Spectral acquisition rate	5 spectra/s, 2679 transients/spectrum, centroid mode

LC/MS analysis

The dried aqueous and organic fractions were resuspended in 100 µL of methanol:water (1:1 v/v) followed by centrifugation at 3,000 rpm for 10 minutes. Five microliters of the resuspended fractions were injected into an Agilent 1290 Infinity LC System interfaced to an Agilent 6550 Accurate mass Q-TOF LC/MS system. Reference solution was prepared by dissolving 10 µL of HP921, and 5 µL of purine in 1 L of methanol:acetonitrile:water (750:200:50) containing 0.1 % acetic acid, and was sprayed using an isocratic pump at a flow rate of 0.3 mL/min. MS data of metabolites from organic phase were acquired in both positive and negative modes. Metabolite data from aqueous phase were acquired in positive mode (Tables 2 and 3). Raw data were aligned and integrated using Agilent Profinder (version B.06.00). The resulting output CEF files were processed using MPP to determine the differential features between rice seeds exposed to gamma radiation, and the control. The differential features were then identified using an Agilent METLIN database.

The statistical analysis, fold change, and mapping onto rice pathways for compounds identified from LC/MS and GC/Q-TOF were performed using MPP/Pathway Architect 13.1.

Integration of gene expression and metabolite data

The gene expression and metabolomics datasets were combined by creating a multi-omics experiment in GeneSpring/MPP/Pathway Architect 13.1, which enables correlation and pathway analysis for different types of omics data.

Table 2. LC conditions used for organic and aqueous fractions.

LC conditions for organic phase	
LC column	Agilent Poroshell 120 EC-C18, 3.0 × 50 mm, 2.7 µm
Injection volume	5 µL
Flow rate	0.3 mL/min
Thermostatted column temperature	30 °C
Ionization mode	Positive mode MS, negative mode MS
Mobile phase	A) 10 mM ammonium acetate in aceonitrile:water (2:3 v/v) B) 10 mM ammonium acetate in acetonitrile: isopropanol (1:9 v/v)
LC gradient	Time A B 0 100 0 2 100 0 40 0 100 50 0 100 50.1 100 0 60 100 0
LC conditions for aqueous layer	
Ionization mode	Positive mode MS
Mobile phase	A) Water with 0.2 % acetic acid B) Methanol with 0.2 % acetic acid
LC gradient	Time A B 0 95 5 5 95 5 30 0 100 40 0 100 40.1 95 5 50 95 5

Table 3. MS source parameters used in LC/MS acquisition.

Agilent 6550 Q-TOF Acquisition parameters	
Acquisition mode	MS
Ion polarity	Positive/Negative
Drying gas flow	15 L/min at 250 °C
Nebulizer	40 psig
Sheath gas flow	10 L/min at 350 °C
VCap	3,500 V
Nozzle voltage	1,000 V
Fragmentor	100 V
MS, Range	50–1,700 *m/z*

Results and Discussion

Gene expression

Data analysis using a GX module of GeneSpring revealed a total of 2,331 differentially-expressed genes with *p*-value ≤ 0.05, and fold change cut off of ≥ 2.0 in seeds harvested from rice plants grown in the contaminated soil; that is, exposed to low level internal and external gamma radiation. Among these, 1,891 genes were up-regulated, while 440 genes were down-regulated. GeneSpring 13.1 offers support for curated pathways (WikiPathways/BioCyc/KEGG), which provide an interactive computing environment that promotes investigation and enables understanding of data within a biological context. Pathway analysis using Pathway Architect revealed that the differential genes belonged to plant defense, cell wall synthesis, secondary metabolite production, fatty acid metabolism, antioxidant and energy cycling pathways. Figure 3 shows the overview of overall gene expression alterations in rice metabolic pathways, which enables the user to visualize and understand the events occurring in the samples.

A few differentially-expressed genes belonging to starch/sucrose metabolism, plant hormone signal transduction, along with a couple of defense genes were validated by Agilent AriaMx qRT-PCR. The expression values obtained from gene expression microarrays were on par to the values of qRT-PCR. The relevance of some pathways to radiation stress is discussed below.

The expression of pathogenesis-related gene *PR10* was increased by 8.2 fold in rice seeds. This gene was identified as a potential marker for radiation stress in rice leaves exposed to radiation in radionuclide-contaminated soil from the exclusion zone around the Chernobyl reactor site[3]. *PR10* is known to play important roles against both abiotic and biotic environmental stress in plants.

The rice seeds exposed to radiation revealed major dysregulation of fatty acid metabolism (Figure 3A), phenylpropanoid biosynthesis (Figure 3B), carbohydrate metabolism (Figure 3C) pathways, and so forth. Changes in the subsequent linked pathways can also be traced in the metabolic overview. The genes belonging to the phenylpropanoid pathway, which produce monolignols, showed enhanced expression, indicating active reinforcement of the cell wall (Figure 3B). Lignin is known to resist gamma radiation due to its partial aromatic nature[4].

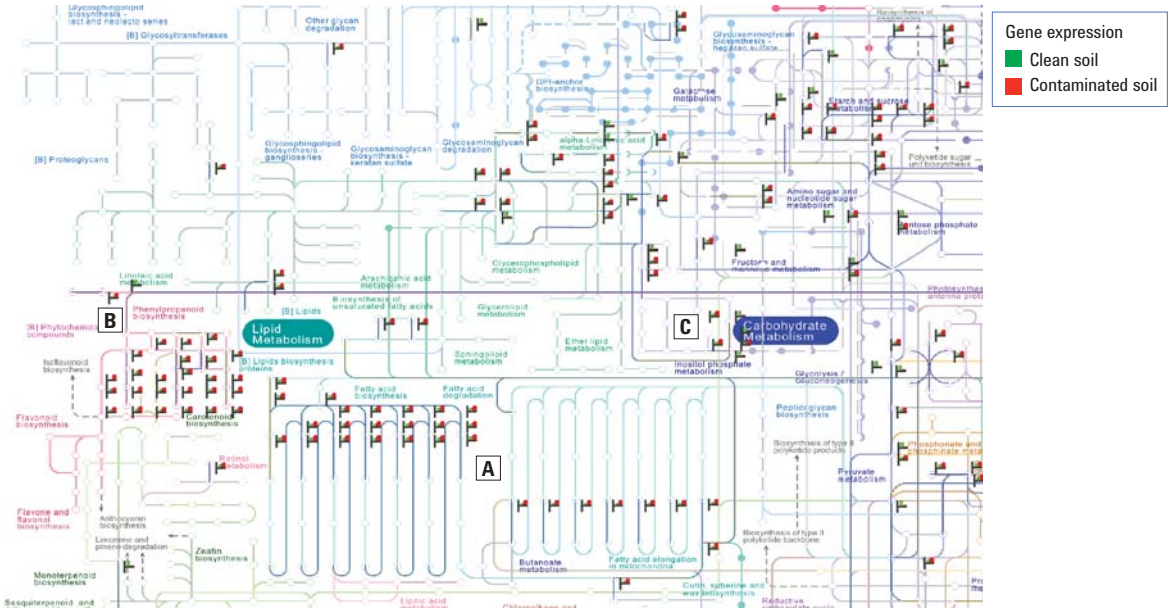

Figure 3. Overview of differentially regulated genes in rice KEGG metabolic pathways consequent to exposure to low-level gamma radiation. HeatStrips show the average differential abundance values for the control and radiated samples.

The correlation tool in GeneSpring GX can be used to look at associations between entities from a single technology, or between entities from two different technologies. Figure 4 shows the correlation analysis between differential entities from a few pathways in gene expression studies. The genes belonging to phenylalanine biosynthesis/metabolism and phenylpropanoid biosynthesis show positive correlation. The products of phenylalanine, tyrosine, and tryptophan pathways are used as a precursor for synthesis of defense-related secondary metabolites in the phenylpropanoid pathway. The activation of phenylpropanoid pathway genes leads to deposition of lignin, which is known to be a defense against biotic and abiotic stress in plants. A positive correlation between the genes in upstream and downstream pathways was evident, revealing a well-coordinated defense against radiation in rice seeds.

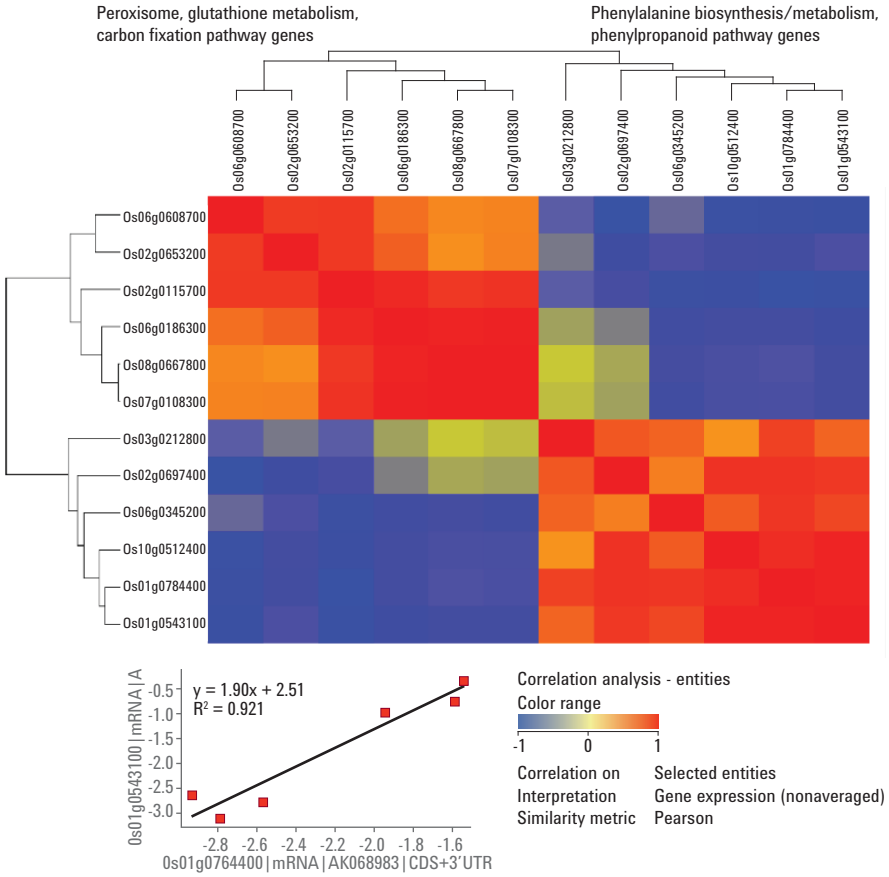

Figure 4. Similarity of differential entities from a few pathways: phenylalanine, tyrosine, and tryptophan biosynthesis, phenylpropanoid biosynthesis, phenylalanine metabolism, peroxisome, carbon fixation in photosynthetic organisms, and glutathione metabolism. The scatter plot shows a positive relationship between chorismate mutase and peroxidase 72.

Metabolite analysis

A total of 383 metabolites were identified from rice seeds using GC/MS and LC/MS techniques. Fifty differential metabolites were identified using the MPP module of GeneSpring. These metabolites mainly belonged to energy, fatty acid, amino acid, nucleotide, and secondary metabolic pathways in rice pathways (Table 4). The amino acid proline, an important component of cell wall proteins, was highly up-regulated, with with a +17.5 fold change in radiated seeds. With an important role in the protection of the cell against free radicals induced by harmful environmental effects, its accumulation is a good indicator of stress tolerance in plants[5].

Combined MOA analysis of gene expression and metabolite data

Multi-omic pathway analysis

The Multi-Omic Analysis (MOA) module of GeneSpring/MPP 13.1 allows researchers to perform integrated analysis of heterogeneous data, enabling them to identify linkages and data concordance that contribute to a more comprehensive understanding of the underlying biological mechanism. In this study, we used MOA to combine the gene expression and metabolome data. The results showed overlapping entities in several pathways between the two omics analyzed (Table 5). Two metabolites, linolenic acid and 12-OPDA, along with the ACX gene in *alpha* linolenic acid metabolism, were up-regulated. This pathway is involved in the production of the hormone jasmonic acid, which is involved in stress responses of plants.

Table 4. A few gamma radiation-induced differential metabolites in rice seeds identified by LC/MS and GC/MS with fold change and regulation (MPP 13.1).

Compound name	CAS Number	Mass	Log FC	Regulation
Amino acids				
L-Arginine	74-79-3	174.1121	-18.2	down
DL-Serine	302-84-1	105.0429	-16.5	down
L-Methionine	63-68-3	149.0516	-17.9	down
D-Proline	344-25-2	115.0632	17.5	up
L-alanine	56-41-7	116.0885	22.7	up
Carbohydrates				
Glucoheptonic acid	10094-62-9	259.1203	-13.2	down
Ribose	24259-59-4	307.1575	-2.7	down
Trehalose	6138-23-4	363.1725	26.0	up
Raffinose	17629-30-0	365.1696	24.5	up
Organic acids				
Pimelic acid	111-16-0	154.9934	-6.6	down
Phosphoric acid	7664-38-2	299.0720	2.9	up
Oxalic acid	144-62-7	147.0659	15.2	up
Fatty acids				
Methyl palmitoleate	1120-25-8	96.00481	-6.7	down
Linolenic acid	463-40-1	278.2248	21.3	up
Stearic acid	57-11-4	284.2717	21.8	up
Linoleic acid	60-33-3	337.1711	-2.3	down
Secondary metabolites				
Ferulic acid	1135-24-6	323.1081	-3.4	down
12-OPDA	85551-10-6	292.1996	16.0	up

Table 5. Few overlapping entities in rice pathways between gene expression and LC/MS metabolite data.

	Gene expression		Metabolites	
Pathway	Matched entities	Pathway entities	Matched entities	Pathway entities
Purine metabolism	3	132	2	92
Pentose phosphate pathway	2	48	1	35
Sulfur metabolism	1	35	1	29
Cysteine and methionine metabolism	4	85	3	57
alpha-Linolenic acid metabolism	3	33	1	40
Pyrimidine metabolism	2	112	1	66
Glycine, serine, and threonine metabolism	3	55	2	51
Sphingolipid metabolism	2	22	1	25
Aminoacyl-tRNA biosynthesis	2	114	4	52
Arginine and proline metabolism	2	63	3	90

In addition, the multi-omic pathway analysis enables direct visualization of components at various omic levels, and provides an overview of various linked events. For instance, an increase in unsaturated fatty acid pathway genes and metabolites resulted in a subsequent increase in downstream cutin and suberin biosynthesis genes and metabolites (Figure 5).

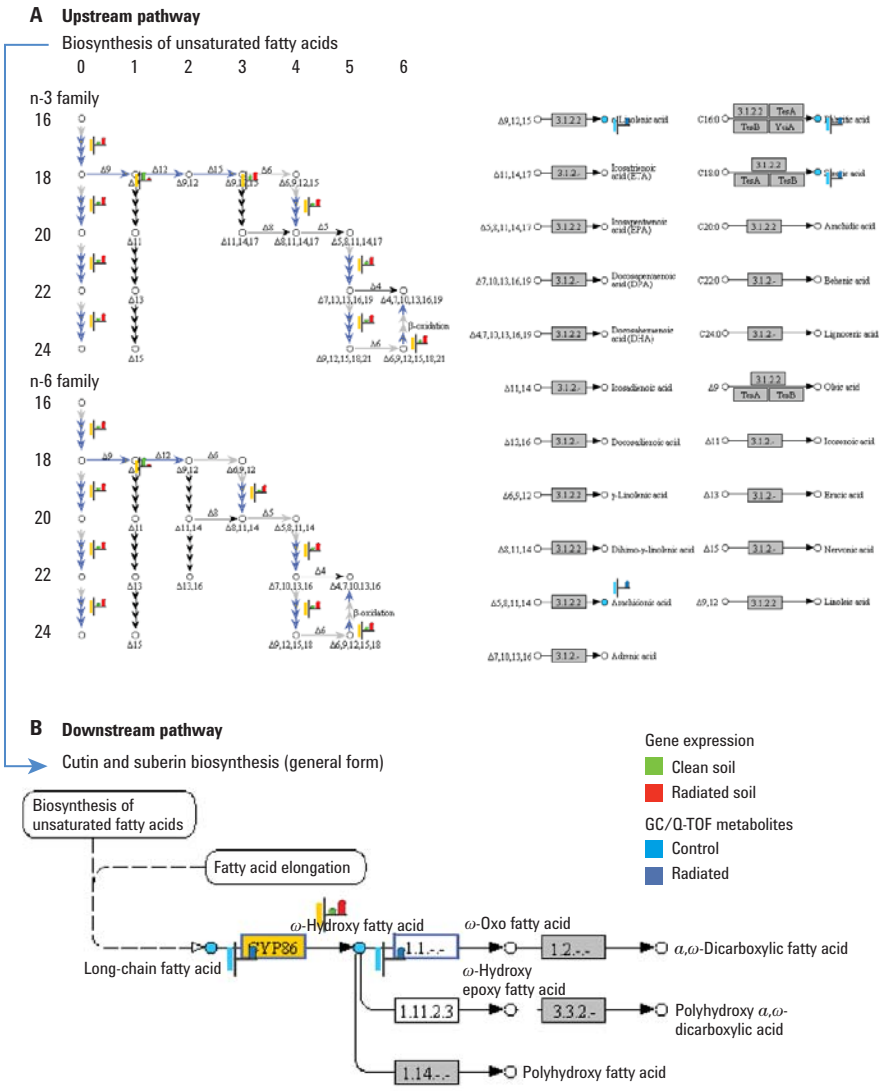

Figure 5. Selected examples demonstrating reciprocity between entities in up-stream and down-stream pathways. A) Up-regulation of genes and metabolites are evident in the biosynthesis of the unsaturated fatty acid pathway, and (B) the subsequent cutin and suberin biosynthesis pathway. Heat strips show the average differential abundance values for the control and radiated samples. A yellow bar along the heat strip indicates genes, and a blue bar indicates a result for metabolites.

Multi-omic correlation analysis

Correlation analysis between technologies allows the identification of coregulated entities such as genes and metabolites. Here, we performed a correlation analysis on a few differential genes and metabolites from carbohydrate metabolism and fatty acid metabolism pathways. We observed a positive correlation between the metabolites raffinose and trehalose with the genes in carbohydrate metabolism pathways (Figure 6).

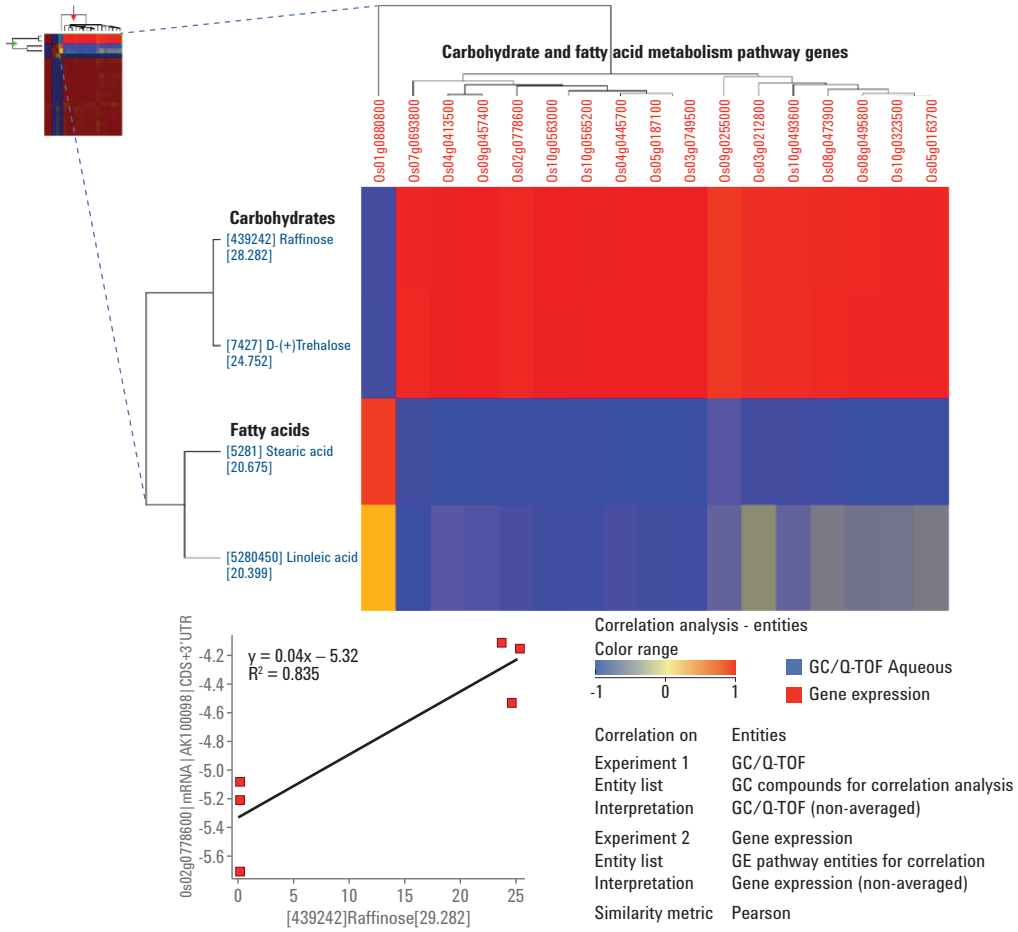

Figure 6. Correlation analysis (Pearson) heatmap between gene expression and metabolic differentials in carbohydrate metabolism/fatty acid metabolism in rice seeds exposed to gamma radiation. The scatter plot shows a positive relationship between the cellulose 5 (Os02g0778600) gene and the metabolite, raffinose.

Conclusions

In this study, applying Agilent multi-omic solutions, we confirmed that rice seeds respond to low-level gamma radiation through what appears to be a well-coordinated defense mechanism.

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.

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.

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