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Identification of rice genes associated with cosmic-ray response via co-expression gene network analysis

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ABSTRACT

In order to better understand the biological systems that are affected in response to cosmic ray (CR), we conducted weighted gene co-expression network analysis using the module detection method. By using the Pearson's correlation coefficient (PCC) value, we evaluated complex gene–gene functional interactions between 680 CR-responsive probes from integrated microarray data sets, which included large-scale transcriptional profiling of 1000 microarray samples. These probes were divided into 6 distinct modules that contained 20 enriched gene ontology (GO) functions, such as oxidoreductase activity, hydrolase activity, and response to stimulus and stress. In particular, modules 1 and 2 commonly showed enriched annotation categories such as oxidoreductase activity, including enriched *cis*-regulatory elements known as ROS-specific regulators. These results suggest that the ROS-mediated irradiation response pathway is affected by CR in modules 1 and 2. We found 243 ionizing radiation (IR)-responsive probes that exhibited similarities in expression patterns in various irradiation microarray data sets. The expression patterns of 6 randomly selected IR-responsive genes were evaluated by quantitative reverse transcription polymerase chain reaction following treatment with CR, gamma rays (GR), and ion beam (IB); similar patterns were observed among these genes under these 3 treatments. Moreover, we constructed subnetworks of IR-responsive genes and evaluated the expression levels of their neighboring genes following GR treatment; similar patterns were observed among them. These results of network-based analyses might provide a clue to understanding the complex biological system related to the CR response in plants.

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1. Introduction

Cosmic ray (CR) is generated by the solar system and is comprised of high-energy particle radiation (Sharma, 2008). CR causes the ionization of nitrogen and oxygen molecules in the atmosphere, and this reaction maintains the level of carbon-14 (Noller et al., 2000). Because plant biology is certainly affected by CR, the CR-responsive pathway involves complex biological processes such as changes in plant growth, morphology, and overall biology. The biological effects of irradiations such as CR, gamma rays (GR), and ion beam (IB) might involve direct and indirect actions. These actions might cause molecular changes such as enzymatic repair and oxidative mutilation in key biological molecules. However, the biological impact of ionizing radiation (IR), especially that of CR, in plant biology remains unknown because biological experiments based on irradiation treatment are very limited.

An integrated study of functional genomics and computational biology provides a powerful approach for understanding complex biological systems. Established high-throughput resources with large microarrays facilitate a systematic approach such as network-based analysis on a genome-wide scale. Gene co-expression networks are being increasingly used to explore the functional relationships of specific responsive genes under various conditions. Furthermore, the clustering of co-expressed genes into distinct modules might reflect functional associations in a complex biological system and provide the predicted functions of unknown genes through the principle of guilt-by-association (Dandekar et al., 1998). Various methods for generating a co-expression gene network have been developed, e.g. weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008), rank-based methods (Stuart et al., 2003), and permutation testing (Carter et al., 2004). Several online resources, including ATTED-II (Obayashi and Kinoshita, 2010) and RiceArrayNet (Lee et al., 2009), are also available for searching for functional interactions between co-expressed gene pairs in plants. Recently, a weighted gene co-expression network was developed to identify a

Abbreviations: ABA, abscisic acid; ARACNE, algorithm for the reconstruction of accurate cellular networks; BP, biological process; CC, cellular component; CR, cosmic ray; FC, fold change; FBX322, 322 F-box proteins; GR, gamma ray; GID1, ga insensitive dwarf1; GO, gene ontology; IB, ion beam; IR, ionizing radiation; MF, molecular function; NADPH, nicotinamide adenine dinucleotide phosphate; NADH, nicotinamide adenine dinucleotide; NAM (or NAC), no apical meristem; PCC, Pearson's correlation coefficient; RMA, robust multi-array average; ROS, reactive oxygen species; rpoS, RNA polymerase, sigma S; SOG1, suppressor of gamma response 1; UDP-GT, UDP-glucuronyl transferase; WGCNA, weighted correlation network analysis.

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module of putative genes for a specific phenotype in rice, and has been further described as a method for identifying genotype–phenotype associations between maize and rice (Ficklin and Feltus, 2011; Ficklin et al., 2010). In addition, this method has been used for the identification of gene modules associated with the drought response in rice (Zhang et al., 2012).

Rice is one of the most important food crops in the world, and has been used as a model monocot for molecular and genetic studies because of its commercial value, relatively small genome size of approximately 430 Mb, complete genome sequences, diploid origin ($2x = 24$), and ease of transformation. Physical mutagenesis techniques such as GRs, X-rays, and fast neutrons are widely used methods for crop improvement. Recently, space-induced mutation techniques, such as CR, have proven highly efficient in inducing mutations, such as those affecting seed germination (Dutcher et al., 1994), plant growth (Liu and Zheng, 1997), and the generation of new germplasms and materials (Liu et al., 2000). Halsted and Dutcher (1987) have reported space-induced mutations in plants in space flight experiments. The greatest difference observed in the first generation of space-induced seeds was the reduced damage effect, compared with other treatments such as gamma ray (Liu et al., 2008). In particular, some high-quality mutations in rice can be easily stabilized in later generations (Chen et al., 1994). It has therefore been shown that space breeding might be useful in breeding new varieties and characteristic genetic resources.

A better understanding of the CR-responsive molecular pathway is of great importance to improve our knowledge regarding CR biology. In this study, we conducted network-based analysis with a module detection method to understand CR-responsive genes on the basis of microarray transcriptional profiling in rice plants that were grown for 3 weeks after their seeds were irradiated with CR in space. Enriched *cis*-regulatory elements of CR-responsive genes were identified by using motif enrichment analysis. Subsequently, we surveyed the irradiation-responsive genes that were commonly upregulated or downregulated under various irradiation treatments such as GR and IB radiations, using microarray data sets. The expression patterns of co-expressed genes, predicted based on functional interactions in the cosmic ray-response network, were confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Thus, the discovery of CR-responsive genes on the basis of network-based analyses may provide insight into significant biological relationships regarding the complex CR-response pathway in plants.

2. Materials and methods

2.1. Plant materials for CR and non-gravity treatments

In order to examine the plant response to CR irradiation, seeds of *Oryza sativa* L. (cv. "Ilpum-byeo") were exposed to CR for 15 days of space flight by a Shijian-8 spacecraft at the China Atomic Energy Authority (CAEA). For GR stress treatment, mature seeds were irradiated with two different doses, 100 and 300 Gy, for both acute (24-h) and chronic (5-week) exposure, as generated by a gamma irradiator (60 Co, ca. 150 TBq capacities, Atomic Energy of Canada Limited, Ottawa, Canada) at the Korea Atomic Energy Research Institute. IB radiation was generated by an AVF-cyclotron (Japan Atomic Energy Agency, Takasaki, Japan) with 220 MeV carbon ions (LET 107 keV/lm) at a dose of 40 Gy. Non-gravity stress treatments were conducted following the proper procedures described in Volkmann and Tewinkel (1996). Rice seeds treated with various stresses were sterilized using 5% (v/v) sodium hypochlorite solution for 20 min at room temperature and then washed with distilled water four or five times. The sterilized seeds were cultured on 1/2 MS (Murashige and Skoog, 1962) medium, and grown in climate controlled rooms at 24 °C for 3 weeks with a photoperiod of 16 h light and 70% humidity.

2.2. Microarray hybridization

All rice leaf samples for microarray analysis were frozen in liquid nitrogen and ground to powder using a chilled mortar and pestle. Total RNA was isolated using the TRIzol reagent (Gibco BRL, Cleveland, OH) according to the manufacturer's instructions. cDNAs of CR-treated and untreated plants were synthesized using the Affymetrix One-Cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, CA), and then biotin-cRNAs were labeled using the GeneChip IVT Labeling Kit (Affymetrix). GeneChip Rice Genome Array was performed after purification of labeled cRNAs with the GeneChip Sample Cleanup Module according to the manufacturer's instructions and then scanned using a Hewlett-Packard GeneArray Scanner (Zhou et al., 2011).

2.3. Genome–transcriptome analysis of CR-responsive genes

A total of 1000 cell intensity files (.CEL) from Affymetrix GeneChip genome arrays of *O. sativa* (GPL2025) were manually downloaded from the Gene Expression Omnibus (GEO) data sets of the NCBI database (http://www.ncbi.nlm.nih.gov/geo/); the list of arrays is provided in Supplementary Table 2. The CEL files were normalized by R statistical software with the RMA method (http://www.r-project.org/). In order to isolate differentially expressed genes (DEGs) with 2-fold higher or lower expression in treated than in untreated cells, the Student's t-test (two-tailed) was performed with a p-value < 0.05. We constructed a coexpression network of CR-responsive genes using the R package WGCNA, following the procedure described in Zhang and Horvath (2005). Module detection analysis was performed by the dynamicTreeCut R package (Langfelder et al., 2008). The CR-responsive network was visualized using Cytoscape software (Shannon et al., 2003). The GR- and IB-related microarray data sets were those used by Kim et al. (2012), elsewhere. Functional enrichment analysis of CR-responsive genes was conducted by agriGO with significance levels (p-values) < 0.05 (Du et al., 2010).

2.4. cis-Regulatory motif analysis

One-kilobase upstream sequences of rice genes were downloaded from the TIGR database (http://rice.plantbiology.msu.edu/). The *cis*-motifs of 1-kb upstream sequences of members in co-expressed modules were retrieved by the AlignACE program with default parameters (Hughes et al., 2000). For redundancy removal in the retrieved motifs, the similarities between each motif were evaluated by CompareACE (Hughes et al., 2000) with Pearson's correlation coefficient (PCC) with a cut-off of 0.65, and then each similar motif was merged to yield a non-redundant group. In order to study enriched *cis*-regulatory motifs, we retrieved the top 5 non-redundant motifs based on the maximum a posteriori probability (MAP) score between each module in the CR-responsive network, and these higher-ranking motifs were compared by CompareACE (Hughes et al., 2000). The *cis*-regulatory motif groups that were enriched in both co-expressed modules were annotated by the PLACE database (Higo et al., 1999).

2.5. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from rice leaves at the three-leaf stage following various stress treatments such as non-gravity, CR, GR, and IB, using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two micrograms of total RNA (2 μg) from each sample was used to synthesize cDNA using the Power cDNA synthesis kit (Intron Biotech Inc., Sungnam, Korea). The synthesized cDNAs (1 μl) were used as templates for the qRT-PCR reaction. Specific gene primers were designed using Primer-BLAST (NCBI, www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Supplementary Table 1. The *Os18S-rRNA* gene was used as the internal control (Os09g00999) (Kim et al., 2003). qRT-PCR was performed with a Rotor-Gene Q (Qiagen, Hilden, Germany) utilizing the green fluorescence signal of SYBR, and

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