Genetics and Molecular Research

Characterization of microRNAs and their target genes associated with transcriptomic changes in gamma-irradiated *Arabidopsis*

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Genet. Mol. Res. 15 (3): gmr.15038386
Received January 6, 2016
Accepted February 19, 2016
Published July 29, 2016
DOI http://dx.doi.org/10.4238/gmr.15038386

ABSTRACT. MicroRNAs (miRNAs) regulate gene expression in response to biotic and abiotic stress in plants. We investigated gamma-ray-responsive miRNAs in *Arabidopsis* wild-type and *cmt3-11t* mutant plants using miRNA microarray analysis. miRNA expression was differentiated between the wild-type and *cmt3-11t* mutants. miR164a, miR169d, miR169h, miR172b*, and miR403 were identified as repressible in the wild-type and/or *cmt3-11t* mutant in response to gamma irradiation, while miR827, miR840, and miR850 were strongly inducible. These eight miRNA genes contain UV-B-responsive *cis*-elements, including G-box, I-box core, ARE, and/or MBS in the putative promoter regions. Moreover, Box 4, MBS, TCA-element,

and Unnamed_4, as well as CAAT- and TATA-box, were identified in these eight miRNA genes. However, a positive correlation between the transcriptions of miRNAs and their putative target genes was only observed between miR169d and At1g30560 in the wild-type, and between miR827 and At1g70700 in the *cmt3-11t* mutant. Quantitative RT-PCR analysis confirmed that the transcription of miR164a, miR169d, miR169h, miR172b*, miR403, and miR827 differed after gamma irradiation depending on the genotype (wild-type, *cmt3-11t*, *drm2*, *drd1-6*, and *ddm1-2*) and developmental stage (14 or 28 days after sowing). In contrast, high transcriptional induction of miR840 and miR850 was observed in these six genotypes regardless of the developmental stage. Although the actual target genes and functions of miR840 and miR850 remain to be determined, our results indicate that these two miRNAs may be strongly induced and reproducible genetic markers in *Arabidopsis* plants exposed to gamma rays.

Key words: *Arabidopsis thaliana*; Gamma rays; Genetic marker; MicroRNA; Microarray; Transcriptome

INTRODUCTION

Small non-coding RNAs, which can be categorized into microRNAs (miRNAs) and small interfering RNAs (siRNAs), are important regulators of gene expression in biotic and abiotic stress responses in plants (Khraiwesh et al., 2012). Both miRNAs and siRNAs control gene expression at transcriptional and post-transcriptional levels, and are distinguished based on their biogenesis and function. Recently, the involvement of miRNAs in plant stress responses has been demonstrated in model plants under environmental stress conditions, including nutrient deficiency, drought, cold, salinity, bacterial infection, UV-B radiation, and mechanical stress (Khraiwesh et al., 2012; Kruszka et al., 2012). miR168, miR171, and miR396 commonly respond to high salinity, drought, and cold stress (Liu et al., 2008).

Ionizing radiation has been used commercially for mutation breeding of plants, for the prolonged storage of harvested commercial plants, and for plant quarantine services (Kim et al., 2013b). Ionizing radiation is a potent genotoxic agent and inducer of oxidative stress, which generates a wide range of genetic and epigenetic changes in the plant genome both directly, by energetic disruption of DNA integrity, and indirectly, through the formation of reactive oxygen species via water radiolysis (Esnault et al., 2010). Many of the miRNAs that are altered by ionizing radiation respond to other DNA-damaging agents, and are associated with the DNA damage response (Simone et al., 2009). Several miRNAs and their putative targets, such as miR-24/miR-138 (H2AX), miR-182 (BRCA1), miR-421/miR-101 (ATM), and miR-504/miR-125b (p53), are known to be key regulators in the DNA damage response pathway (Joly-Tonetti and Lamartine, 2012). In contrast, many of the miRNA changes observed in response to ionizing radiation are also induced by H_2O_2 and are associated with reactive oxygen species or oxidative stress (Simone et al., 2009). Therefore, it is logical that many of the radiation-responsive miRNAs may play a role in the generalized cellular response to various oxidative stresses.

Since the study of miRNAs in response to ionizing radiation only began recently, the number of articles published in this research field is increasing exponentially. Strategies

used in these studies included the identification of putative targets of miRNAs involved in the DNA damage response, and the development of miRNA biomarkers of radiation exposure or radiosensitivity (Joly-Tonetti and Lamartine, 2012). The interactome of putative target genes of radiation-responsive miRNAs has facilitated the comprehensive modeling of the cellular stress response to ionizing radiation (Lhakhang and Chaudhry, 2012). However, since the response of miRNAs to ionizing radiation is limited in a cell type-, dose- and time-dependent manner, their use as indicators of radiation sensitivity rather than as useful biomarkers of radiation exposure, has been suggested (Joly-Tonetti and Lamartine, 2012).

Until recently, very few studies have been published on the identification of radiation-responsive miRNAs in plants. In relation to UV-B radiation, various miRNA gene families, including miR156, miR159, miR160, miR164, miR167, miR169, miR395, and miR171, were identified as UV-B radiation-responsive genes in *Arabidopsis thaliana*, *Populus tremula*, and/or *Triticum aestivum* (Zhou et al., 2007; Jia et al., 2009; Wang et al., 2013). UV-B-responsive miRNA genes were found to possess various light-responsive and stress-related *cis*-elements in their upstream regions. In contrast, the only article to describe ionizing radiation and miRNAs in plants reported that miR164a, miR164c, miR164d, and miR156a-j were heavy ion-induced miRNAs in rice seedlings produced from germinated rice seeds exposed to heavy ion (Zhang et al., 2011a). Although highly conserved 5'-*cis*-element sequences have been investigated in gamma-ray-responsive *Arabidopsis* genes (Nagata et al., 2005), little is known about 5'-*cis*-elements in the putative promoter regions of plant miRNA genes responding to ionizing radiation. In addition, only a few miRNAs have been identified as regulators of DNA double-strand break repair during the DNA damage response (Joly-Tonetti and Lamartine, 2012); therefore, further study is needed to explore plant-specific miRNAs and their putative targets that respond to ionizing radiation-induced damage and discriminate between ionizing radiation and other environmental stresses. Therefore, our study aimed to identify gamma-ray-responsive miRNAs in *Arabidopsis* wild-type and *cmt3-11t* mutant plants after acute irradiation with gamma rays, in order to compare *cis*-elements in their putative promoter regions, and to identify their putative target genes via a plant small RNA target analysis server in association with genome-wide transcriptome data. The use of radiation-responsive miRNAs as widely applicable radiation markers or radiosensitivity indicators will also be discussed.

MATERIAL AND METHODS

Plant materials, gamma-ray treatment, and RNA extraction

Arabidopsis thaliana (ecotype Columbia wild-type and *cmt3-11t* mutant) plants were grown in soil or half-length MS medium under a 16-h photoperiod at 22/18°C (day/night) for 28 days or at 23/20°C for 14 days, respectively. The *cmt3-11t* allele carries a T-DNA insertion in the predicted DNA methyltransferase domain of CMT3 predicted to create a null mutation (SALK_148381c). The wild-type and *cmt3-11t* T-DNA insertion mutant can be discriminated by DNA methylation and genome-wide transcriptome changes after exposure to gamma rays (Kim et al., 2013a).

Plants were irradiated with gamma rays at a dose of 50 Gy/h for 4 h using a ^{60}Co gamma irradiator (IR-222, MDS Nordion Inc., Kanata, Canada) at the Advanced Radiation Technology Institute (ARTI). Next, plants were placed under growth conditions until 1 or 5 days after irradiation. For miRNA chip analysis, rosette leaves from at least 10 soil-grown plants

were pooled for use as individual biological replicates. In contrast, at least 10 medium-grown whole plants, including roots, were harvested for miRNA quantitative PCR (qPCR) analysis. All biological replicates were independently prepared from different batches of plants.

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for miRNA microarray and qRT-PCR analyses, while RNeasy® Plant Mini Kit (Qiagen, Chatsworth, CA, USA) was used for gene chip analysis. RNA integrity was assessed by analyzing the ribosomal RNA bands after gel electrophoresis or by using an Agilent 2100 Bioanalyser with an RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA, USA).

Microarray analysis

MicroRNA microarray experiments

A total of 259 different miRNAs were identified from mature miRNA sequences for *A. thaliana* in the miRBase database (http://microrna.sanger.ac.uk/). Probes for each miRNA were designed using the Agilent eArray platform (http://earray.chem.agilent.com/earray/). Eight control probes were also designed as recommended by the eArray and used as negative controls. A total of 267 probes were synthesized and printed onto glass slides in an 8 x 15-K format by Agilent Technologies. For miRNA microarray analysis, biological triplicates of total RNA were isolated from three different batches of samples. Target RNA labeling and hybridization were performed using the Agilent miRNA Labeling Reagent and Hybridization Kit (Agilent Technologies) according to the manufacturer instructions. In brief, total RNA was subjected to dephosphorylation, denaturation, and labeling. Labeled RNA was hybridized with the Agilent miRNA microarray after desalting and denaturation. The hybridized microarrays were scanned using a DNA microarray scanner (Agilent Technologies) after washing. Scanned images were quantified by the Agilent Feature Extraction Software (Agilent Technologies).

Gene chip experiments

Genome-wide transcriptome analysis was performed with biological duplicates of total RNA from two different batches of samples using GeneChip® *Arabidopsis* ATH1 Genome Array (Affymetrix, Santa Clara, CA, USA). Experimental procedures have been previously described (Kim et al., 2013a). Raw data were registered as accession No. GSE43947 and GSM1074824-GSM1074839 in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/).

Microarray data processing

Raw data obtained from miRNA microarray or gene chip analyses were further processed by the GeneSpring GX 7.3 software (Agilent Technologies) as follows; i) probe signal values less than 0.01 were set to 0.01, ii) per-chip normalization was set to the 50th percentile, and iii) each gene signal was normalized to the median value obtained in the experiment. Lists of differentially expressed genes (DEG) were obtained from probes with a >2-fold difference in the normalized signals between control and test samples. Gene ontology analysis of DEGs was performed via the web-based tool DAVID (Database for Annotation, Visualization, and Integrated Discovery, http://david.abcc.ncifcrf.gov/).

miRNA qRT-PCR analysis

Several gamma-ray-responsive miRNAs were selected from the miRNA microarray results and confirmed by quantitative RT-PCR analysis. Using Custom TaqMan® Small RNA Assay Kit (Applied Biosystems, Foster City, CA, USA), which includes ath-miR164a, ath-miR169d, ath-miR403, ath-miR840, or ath-miR850-specific primers, 5 g of each RNA sample was reverse-transcribed using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer instructions. The resulting cDNA was amplified in 40 cycles on a quantitative thermal cycler, ABI 7300 (Applied Biosystems), using TaqMan® Small RNA Assays and TaqMan® Universal PCR Master Mix II, No. UNG (Applied Biosystems) according to the manufacturer instructions.

Cis-element analysis of miRNA genes and prediction of miRNA target genes

To analyze putative promoter regions of gamma-ray-responsive miRNA genes, each pre-miRNA sequence obtained from miRBase was aligned with the *A. thaliana* genome sequence in the NCBI database. Approximately 1500 bp of the upstream sequence of each miRNA gene was retrieved and used to identify *cis*-acting motifs in the putative promoter regions via PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/) (Lescot et al., 2002).

Target genes of the gamma-ray-responsive miRNAs were predicted in psRNATarget, a plant small RNA target analysis server, using a preloaded *A. thaliana* transcript library (TAIR, version 10, cDNA, removed miRNA gene) released on 12/14/2010 for target search (Dai and Zhao, 2011). The default cut-off threshold of the maximum expectation was increased to 3.5 for higher prediction coverage.

RESULTS AND DISCUSSION

microRNA microarray and gene chip experiments in gamma-irradiated *Arabidopsis* plants

Biological effects of ionizing radiation, which acts as a genotoxic agent and oxidative stress inducer, are often compared with those of H_2O_2 or UV-B radiation (Nagata et al., 2005; Moon et al., 2008; Simone et al., 2009; Gill et al., 2015). For comparison, we performed a literature search to investigate what miRNAs respond to H_2O_2 or UV-B radiation as well as ionizing radiation (Table 1); however, only a few articles could be identified. Expression of the miR156 and miR164 families was found to respond to UV-B radiation and carbon ions (Jia et al., 2009; Zhang et al., 2011a; Casati, 2013; Wang et al., 2013), while expression of the miR169 family was affected by UV-B radiation and H_2O_2 (Zhou et al., 2007; Jia et al., 2009; Li et al., 2011). Previously, we demonstrated that genome-wide transcriptional changes in *Arabidopsis* seedlings subjected to gamma irradiation were most clear at 6 h and then decreased until 48 h (Kim et al., 2013b). The majority of transcriptomes responding to gamma rays differed from those responding to H_2O_2, especially at 24 h. Therefore, in the present study, the putative association of gamma-ray-responsive miRNAs with gamma-ray-responsive transcriptomes was investigated using control and irradiated samples 24 h after gamma irradiation using miRNA microarray and gene chip experiments. In addition, since *Arabidopsis* wild-type and *cmt3-11t* mutants, which have a defective non-CG DNA methyltransferase, exhibited different

genome-wide transcriptomes after gamma irradiation (Kim et al., 2013a), the gamma-ray-responsive miRNAs were compared between the two different genotypes of *Arabidopsis*.

Table 1. H_2O_2-, UV-B-, and carbon ion-responsive miRNAs in plants.

Stress type	Species	miRNA family	Reference
UV-B	*Arabidopsis thaliana*	miR156, miR157, miR159, miR160, miR165, miR166, miR167, miR169, miR170, miR171, miR172, miR393, miR398, miR401	Zhou et al., 2007
UV-B	*Populus tremula*	miR156, miR160, miR164, miR165, miR166, miR167, miR168, miR398, miR408, miR159, miR169, miR390, miR393, miR395, miR399, miR472	Jia et al., 2009
H_2O_2	*Oryza sativa*	miR169, miR397, miR528, miR827, miR1425, miR319a.2, miR408-5p	Li et al., 2011
Carbon ion	*Oryza sativa*	miR156, miR164, miR2097-5p	Zhang et al., 2011a
UV-B	*Zea mays*	miR156, miR164, miR165, miR166, miR171, miR172, miR395, miR396, miR398, miR399, miR444, miR529, miR533a*, miR896, miR903, miR1427, miR1858a	Casati, 2013
UV-B	*Triticum aestivum*	miR156, miR159, miR164, miR167, miR171, miR395	Wang et al., 2013

Identification of gamma-ray-responsive miRNAs in *Arabidopsis* wild-type and *cmt3-11t* mutant plants

The overall expression pattern of the gamma-ray-responsive miRNAs differed by time after gamma irradiation and by the genotype of genome-wide DNA methylation (Table 2). The levels of miR164a, miR172b*, and miR403 expression 1 day after gamma irradiation significantly decreased in the *cmt3-11t* mutant, but not in the wild-type, while the level of miR827 increased only in the wild-type. In particular, transcriptional changes of miR164a, miR169d, and miR403 followed an opposite trend in the *cmt3-11t* mutant compared to the wild-type in response to gamma rays. This difference can be explained in part by the pronounced differences in genome-wide gamma-ray-responsive transcriptome profiles between the wild-type and *cmt3-11t* mutant (Kim et al., 2013a). The gamma-ray-responsive miRNAs seem to be significantly affected by the genome-wide DNA methylation level. Expression of the miR164 family has also been reported to be induced by UV-B radiation and carbon ion (Jia et al., 2009; Zhang et al., 2011a) and expression of miR169 and miR827 was substantially increased after H_2O_2 treatment (Li et al., 2011). In the present study, these miRNAs may be associated with general light- or stress-related signals induced by gamma irradiation. In contrast, the expression levels of miR840 and miR850 were remarkably increased in both the wild-type and *cmt3-11t* mutant 1 day after gamma irradiation. There are no reports to date on such transcriptional induction of miR840 and miR850 in response to environmental stress factors. This indicates that these two miRNAs are highly specific to gamma rays and may be used as radiation markers for the early radiation

Table 2. Gamma-ray-responsive miRNAs in *Arabidopsis* wild-type and *cmt3-11t* mutant identified by microarray analysis.

miRNA	miRBase accession	miRBase sequence	Wild-type		*cmt3-11t*	
			1 day	5 days	1 day	5 days
miR164a	MIMAT0000185	UGGAGAAGCAGGGCACGUGCA	>2.0, 1 of 3†		0.011 ± 0.000	
miR169d	MIMAT0000908	UGAGCCAAGGAUGACUUGCCG	>2.0, 1 of 3†	0.196 ± 0.264	<0.5, 1 of 3†	0.160 ± 0.259
miR169h	MIMAT0000913	UAGCCAAGGAUGACUUGCCUG		0.297 ± 0.107		0.380 ± 0.150
miR172b*	MIMAT0000204	GCAGCACCAUUAAGAUUCAC			0.362 ± 0.024	
miR403	MIMAT0001004	UUAGAUUCACGCACAAACUCG	>2.0, 1 of 3†		0.011 ± 0.001	
miR827	MIMAT0004243	UUAGAUGACCAUCAACAAACU	138.184 ± 21.127			
miR840	MIMAT0004262	ACACUGAAGGACCUAAACUAAC	181.913 ± 11.394	194.855 ± 6.120	181.396 ± 28.143	
miR850	MIMAT0004272	UAAGAUCCGGACUACAACAAAG	166.951 ± 33.511		150.423 ± 27.080	

Eight miRNAs in the list showing a >2-fold change in expression at 1 and/or 5 days after gamma irradiation in 2-3 independent experiments. Values represent the mean fold-change and standard deviation of 2-3 independent experiments. †2-fold change in one of three independent experiments.

response 1 day after gamma irradiation. In addition, since the levels of miR169d and miR169h expression were substantially reduced in both plants 5 days after gamma irradiation, the miR169 family may also be a radiation marker for the late radiation response in *Arabidopsis* plants.

Prediction of *cis*-elements in the upstream regions of gamma-ray-responsive miRNA genes

Distinctive *cis*-elements in the 5'-upstream sequences or putative promoter regions of UV-B-responsive miRNA genes have been identified in *A. thaliana*, *Populus tremula*, and *Triticum aestivum* (Zhou et al., 2007; Jia et al., 2009; Wang et al., 2013). Many of the UV-B-responsive *cis*-elements including G-box, I-box core, ARE, and MBS were frequently found in the putative promoter regions of gamma-ray-responsive miRNA genes (Table 3). However, GT-1 sites were relatively rare in the gamma-ray-responsive miRNA genes, while Box 4 and TCA-elements were found preferentially in these genes. Light relevant G-box and I-box core, and stress-related ARE and MBS may be important *cis*-elements for the transcriptional regulation of miRNAs responding to both UV-B radiation and gamma rays. Instead, stress-related TCACG-motif and TCA-element seem to be associated with the expression of gamma-ray-responsive miRNA genes. Additionally, all the miRNA genes listed included a large number of CAAT- and TATA-boxes, which are generally found in the 5'-upstream regions of most eukaryotic genes (Table 4). As well as Box 4, G box, GA motif, MBS, and TCA-elements, CGTCA-motif, Skn-1 motif, and Unnamed 4 were identified as common *cis*-elements in the highly induced miRNAs, (e.g., miR827, miR840, and miR850), responding to gamma rays. Although many of the miRNA genes responding to gamma rays and UV-B radiation showed similar levels of *cis*-elements in their putative promoter regions, our results suggest that the possession of specific *cis*-elements, novel gamma-ray-specific cis-elements, or other

Table 3. Major predicted light-related and stress-responsive *cis*-elements in the putative promoter regions of gamma-ray-responsive miRNA genes.

	miR164a	miR169d	miR169h	miR172b*	miR403	miR827	miR840	miR850	Total
3-AF1 binding site (AAGAGA)	1			1				1	3
AE-box (AGAAACTT)			1	1		1			3
Box 4 (ATTAAT)	8	2	1	4	5	1	1	1	23
Box I (TTTCAAA)	1	1	1				2	5	10
Box II (TGGTAATAA)	1				1		1		3
CATT-motif			1	1			1		3
G-box (CACGTG)	6			1	4	1	2	8	22
GA-motif		1	1			1	1	1	5
GAG-motif	2		3			4		2	11
GT-1 site (GGTTAA)		1			2		1	2	6
I-box core (GATAA)	1	3	3	2	3	1	1		14
TCT-motif (TCTTAC)	2		2					1	5
ARE		2	1	1	3	2		2	11
ABRE	4		1			1		3	9
LTR		1	1		2	1	1		6
MBS	2	2	2	2	1	2	3	1	15
TCACG-motif	1	1	2	3		1		2	10
TCA-element	1	2	3	1	1	3	1	2	14
TC-rich repeats (ATTTTCTTCA)	1	2	2	2	1	1			9

3-AF1 binding site to TCT-motif: light-related *cis*-elements, ARE to TC-rich repeats: stress-responsive *cis*-elements, ARE: anaerobic response element, ABRE: ABA-responsive element, LTR: low temperature-responsive element, MBS: MYB binding site, TCACG-motif: MeJA-responsive element, TCA-element: SA-responsive element. The listed *cis*-elements exist in more than three of the eight gamma-ray-responsive miRNA genes. Numbers represent the number of *cis*-elements.

regulatory mechanisms, is necessary for the expression of gamma-ray-specific miRNAs, such as miR840 and miR850. In fact, it was previously reported that miRNA gene promoters are frequently affected by aberrant DNA methylation in human breast cancer, thereby resulting in the decreased expression of miRNAs (Vrba et al., 2013). Therefore, DNA methylation of promoters, or their interaction with active chromatin markers on histones, may also be important for the transcriptional induction of gamma-ray-specific miRNAs.

Table 4. Major predicted *cis*-elements frequently found in the putative promoter regions of gamma-ray-responsive miRNA genes.

	miR164a	miR169d	miR169h	miR172b*	miR403	miR827	miR840	miR850	Total
AAGAA-motif	2	2	2	1	1	1			9
Box-W1 (TTGACC)	1	1	3	1		1			7
CAAT-box	18	43	28	19	35	25	32	23	223
CGTCA-motif	1	1	2	3		1		2	10
Skn-1_motif (GTCAT)	2	1	3	2		2	2	2	14
TATA-box	76	42	75	94	132	25	15	204	663
TATCCAT/C-motif	1	2		2		1	1		7
Unnamed_4 (CTCC)	5	2	6	7	3	9	13	9	54
Unnamed_6 (TAAATAT)	1	1	1	1				2	6

Listed *cis*-elements exist in more than five of the eight gamma-ray-responsive miRNA genes. Numbers represent the number of the *cis*-elements.

Prediction and expression of putative target genes of gamma-ray-responsive miRNAs

Putative target genes of the gamma-ray-responsive miRNAs were predicted by psRNATarget, a plant small RNA target analysis server (Dai and Zhao, 2011). Target accessions of each miRNA were subjected to post-transcriptional cleavage rather than translational inhibition (Table 5). Twenty-three target accessions were associated with miR164a, while eight accessions were predicted as putative targets for miR840. All target accessions of miR840 are subjected to post-transcriptional cleavage only. However, only limited information is available on the interaction of each miRNA with its target genes. For example, the translation of AGO1 mRNA is inhibited by miR168, which is induced by p19 RNA-silencing suppressor (Várallyay et al., 2010), while miR403 induces the post-transcriptional silencing (cleavage) of the AGO2 gene via interaction with AGO1 (Allen et al., 2005; Harvey et al., 2011). MEMB12 mRNA is targeted by miR393b* in association with AGO2, which is induced by DC3000 (Zhang et al., 2011b). Therefore, we analyzed genome-wide gamma-ray-responsive transcriptomes in order to correlate gamma-ray-responsive miRNAs with the expression of their putative target genes. Only 8 of 121 target accessions (Table 1) were identified in the gamma-ray-responsive transcriptomes with at least a 2-fold change (Table 6). Transcription of At1g70700 or At1g30560 was closely correlated with miR169d in the *cmt3-11t* mutant at 5 days or with miR827 in the wild-type plants 1 day after gamma irradiation, respectively, suggesting that these genes are subjected to miRNA-mediated post-transcriptional control. In contrast, there was only a weak correlation between miR164a and At2g38340, and between miR403 and At3g32110, which can be explained by miRNA-mediated translational inhibition as predicted in Table 5. Moreover, miR403, which showed significantly decreased transcription in the *cmt3-11t* mutant 1 day after gamma irradiation, did not directly affect the expression of its target gene, At1g31280 (AGO2), in the wild-type and *cmt3-11t* mutant plants. Increased transcription of the AGO2 gene in the wild-type may be due to its enhanced promoter activity rather than

the release of miR403-mediated post-transcriptional silencing, which is induced by pathogen infection in plants. Taken together, the data shown in Tables 1, 5, and 6 suggest that gamma-ray-responsive miRNAs are rarely associated with their putative target genes in response to gamma rays. Nevertheless, the high gamma-ray-specific inductions of miR840 and miR850 indicate that these miRNAs may affect other target genes unpredictable by psRNATarget.

Table 5. Putative target genes of gamma-ray-responsive miRNAs in *Arabidopsis*.

miRNA	Inhibition	Target accession
miR164a	Cleavage	AT1G05890, AT1G31100, AT1G56010, AT1G78240, AT2G17115, AT3G12977, AT3G15170, AT3G26360, AT3G31935, AT3G33142, AT4G01210, AT4G39400, AT5G07680, AT5G39610, AT5G50770, AT5G53950, AT5G61430, AT5G65910
	Translation	AT1G10530, AT2G37960, AT2G38340, AT4G37705, AT5G19097
miR169d	Cleavage	AT1G17590, AT1G48500, AT1G54160, AT1G68560, AT1G70700, AT1G72830, AT2G39210, AT3G05690, AT3G09915, AT3G20910, AT3G45940, AT4G35080, AT5G06510, AT5G12840, AT5G42120
	Translation	AT1G03060, AT1G19810, AT1G80770, AT3G48770, AT4G03795
miR169h	Cleavage	AT1G17590, AT1G54160, AT1G68560, AT1G72830, AT3G05690, AT3G20910, AT4G35080, AT5G06510, AT5G12840, AT5G42120
	Translation	AT2G03590, AT2G03600, AT2G10100, AT3G15730, AT3G19540
miR172b*	Cleavage	AT1G79730, AT2G03820, AT2G22950, AT2G43465, AT2G47830, AT3G07050, AT3G57330, AT4G10970, AT4G32420, AT5G48410, AT5G48830, AT5G65720
	Translation	AT1G77840, AT2G04110, AT2G16500, AT3G49601, AT3G61820, AT4G12990, AT5G13690
miR403	Cleavage	AT1G03060, AT1G31280, AT1G53210, AT2G01720, AT2G27880, AT3G17310, AT3G17450, AT4G01030, AT4G08930, AT4G30825, AT5G37110
	Translation	AT1G13910, AT1G75820, AT1G77550, AT3G26190, AT3G32110, AT3G49040, AT4G09060
miR827	Cleavage	AT1G02860, AT1G30560, AT3G04750, AT4G00610, AT4G08263, AT4G17550, AT4G37590
	Translation	AT1G31760, AT2G10910, AT2G16365, AT2G21450, AT4G01975, AT5G33428, AT5G60615
miR840	Cleavage	AT1G10580, AT1G50770, AT1G58848, AT1G59218, AT1G64740, AT1G69570, AT2G03150, AT2G26790
miR850	Cleavage	AT1G09340, AT1G41746, AT2G04842, AT2G43240, AT2G44500, AT3G11930, AT3G28007, AT3G42206, AT3G46630, AT3G50390, AT3G52250
	Translation	AT3G59750, AT4G23100, AT4G23470, AT5G16610

Target accessions of eight miRNAs listed in Table 1 were predicted by psRNATarget, a plant small RNA target analysis server, as described by Dai and Zhao (2011).

Table 6. Transcriptional changes of putative target genes of gamma-ray-responsive miRNAs.

miRNA	Target accession	Target description	Wild-type		*cmt3-11t*	
			Transcription	Correlation	Transcription	Correlation
miR164a	AT2G38340	DREB-like AP2 domain transcription factor			Increase†	Weak
	AT5G07680	NAM (no apical meristem)-like protein; ANAC080			Decrease†	Weak
	AT5G39610	NAM/CUC2-like protein CUC2; ATNAC6	Decrease†	Weak		
miR169d	AT1G70700	hypothetical protein; TIFY7			Decrease*; increase†	Weak; strong
miR169d, h	AT5G06510	transcription factor-like protein; NF-YA10			Increase*	Weak
miR403	AT1G31280	unknown protein; AGO2	Increase*	Weak		
	AT3G32110	non-LTR reverse transcriptase, putative	Increase†	Weak		
miR827	AT1G30560	unknown protein; glycerol-3-phosphate permease homolog	Decrease*	Strong		

*,†Transcriptional change of the putative target genes 1 or 5 days after gamma irradiation, respectively. The strength of the correlation was determined by the association between transcriptional changes of the putative target genes and the expression of miRNAs as shown in Table 2. miRNAs and putative target genes with the same change in transcription after gamma irradiation were excluded.

Transcriptional variation of gamma-ray-responsive miRNAs in *Arabidopsis* wild-type and RNA-directed DNA methylation (RdDM) mutant plants

Arabidopsis wild-type and *cmt3-11t* mutant plants undergo significantly different DNA methylation and genome-wide transcriptome changes following exposure to gamma rays (Kim et al., 2013a). Similarly, the profile of gamma-ray-responsive miRNAs differed between the wild-type and *cmt3-11t* mutant plants (Table 2 and **Table S1**) and putative target genes of these miRNAs were differentially expressed (Table 6). These results indicate that

transcriptional changes of miRNA genes in response to gamma rays may be affected by different genotypes such as genome-wide DNA hypomethylation. Moreover, results of our previous study showed that the developmental stage is the main determinant for the profile of gamma-ray-responsive transcriptomes in *Arabidopsis* (Kim et al., 2013b).

Therefore, we compared the transcription of the eight gamma-ray-responsive miRNAs listed in Table 2 in the wild-type and several RdDM mutants, including such as *cmt3-11t*, *drm2*, *drd1-6*, and *ddm1-2* using MS medium-grown 14-day-old whole seedlings. In all mutants, transcription of the four gamma-ray-responsive marker genes, At2g30360, At4g19130, At4g22960, and At5g24280, was induced (Kim et al., 2013b; **Figure S1**). However, most of the miRNAs tested had distinct expression patterns among the wild-type and RdDM mutants, which differed from those observed in the miRNA microarray analysis using soil-grown 28-day-old rosette leaves (Figure 1 and Table 2). Overall expressions of miR164a, miR169d, miR169h, miR172b*, and miR403 were differentially decreased among the wild-

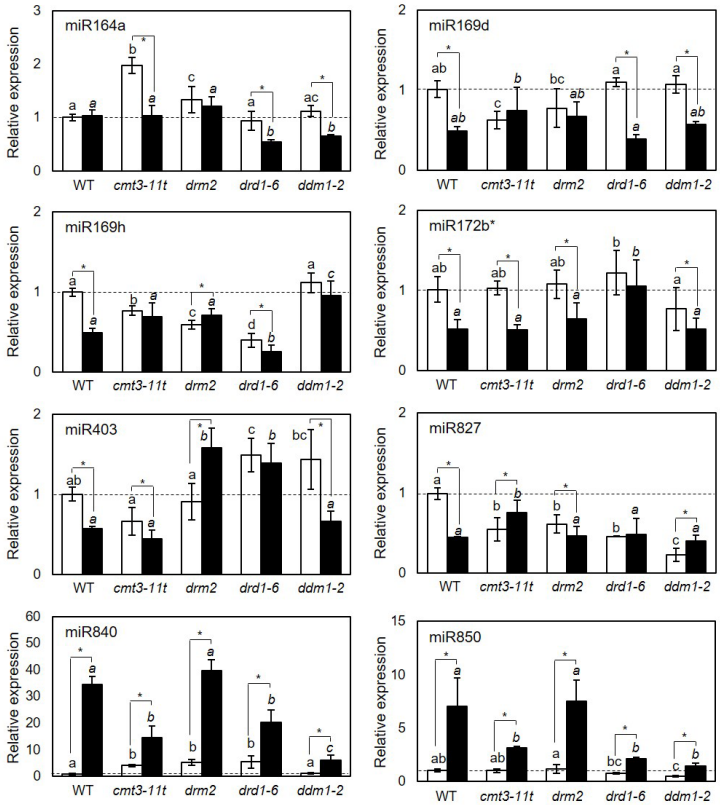

Figure 1. Expression of gamma-ray-responsive miRNAs in *Arabidopsis* wild-type, *cmt3-11t*, *drm2*, *drd1-6*, and *ddm1-2* mutant plants. Total RNA was isolated from MS medium-grown 14-day-old whole seedlings and used for quantitative RT-PCR analysis of each miRNA. All transcript levels are expressed relative to the control samples of the wild-type (WT) plants, as indicated by dashed lines. White and black bars represent means ± SD (N = 6 from two independent experiments) for the control and irradiated samples, respectively. Bars with the same letters are not significantly different at P = 0.05 by the Tukey HSD test. Asterisks indicate statistically significant differences between the control and irradiated samples, as determined by the Student *t*-test (P < 0.05).

type and mutant plants 1 day after gamma irradiation, while those of miR169h and miR403 were increased specifically in the *drm2* mutant (Figure 1). In contrast to the data presented in Table 2, transcription of miR164a, miR172b*, and miR403 was significantly repressed in the wild-type and in the *cmt3-11t* mutant. Transcription of miR827, which was strongly induced in the wild-type plants (Table 2), was also found to be repressed by miRNA qRT-PCR analysis using MS medium-grown younger whole seedlings. In contrast, the transcription of miR840 and miR850 was highly induced in the *drm2*, *drd1-6*, and *ddm1-2* mutants as well as the wild-type and *cmt3-11t* mutant plants following gamma irradiation. These results indicate that the high transcriptional induction of miR840 and miR850 in response to gamma rays is not significantly affected by the genotype, growth condition, and developmental stage of *Arabidopsis* plants.

In conclusion, in the present study, investigation of common gamma-ray-responsive miRNAs in *Arabidopsis* wild-type and various RdDM mutants revealed high transcriptional induction of miR840 and miR850 genes after gamma irradiation. Although the universal radiomiR has yet to be identified in response to ionizing radiation (Joly-Tonetti and Lamartine, 2012), our results suggest that the miRNAs miR840 and miR850, which are induced by gamma rays, may be reproducible and reliable radiation markers in *Arabidopsis*. However, further research is needed to elucidate the target genes and functions of these miRNAs in response to gamma rays.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Nuclear R&D Program funded by the Ministry of Science, ICT and Future Planning (MSIP), Republic of Korea.

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Supplementary material

Table S1. Gamma-ray-responsive miRNAs in Arabidopsis wild-type (WT) or cmt3-11t mutant plants identified by microarray analysis. Ten miRNAs exhibited a >2-fold-change in expression 1 day after gamma irradiation in one of three independent experiments. †: 2-fold-change observed in two of three independent experiments.

Figure S1. Expression of gamma-ray-responsive marker genes in Arabidopsis wild-type (WT), cmt3-11t, drm2, drd1-6, and ddm1-2 mutant plants. C and R represent the control and irradiated samples, respectively. ACTIN2 was used as an endogenous control gene. Sense/antisense primer sequences (product size) are as follows: 5'-AGATGGAGGGGCAAAATGGG-3' / 5'-TGACGACGTCTGATCGCAAA-3' (173 bp) for At2g30360, 5'-TGGAGAAGTGACGACTGAAGC-3' / 5'-ACCTCCAGTTGCGGAACAAT-3' (267 bp) for At4g19130, 5'-AGGGTACAAAAGGGCTCACG-3' / 5'-CTGCAGCATGGTAGCGAAGTGATTGT-3' (167 bp) for At4g22960, 5'-GAGACCGCAGAGCATCTTGA-3' / 5'-GCAAGAGAAGGCATTGGTGC-3' (169 bp) for At5g24280, and 5'-GCCCAGAAGTCTTGTTCCA-3' / 5'-CTTGGTGCAAGTGCTGTGAT-3' (199 bp) for ACTIN2.