Comparative genomic hybridization analysis of rice dwarf mutants induced by gamma irradiation

J.E. Hwang[1,2], S.-H. Kim[1], I.J. Jung[1], S.M. Han[1], J.-W. Ahn[1], S.-J. Kwon[1], S.H. Kim[1], S.-Y. Kang[1], D.S. Kim[3] and J.-B. Kim[1]

[1]Advanced Radiation Technology Institute,
Korea Atomic Energy Research Institute, Jeongeup, Jeonbuk,
Republic of Korea
[2]Division of Ecological Conservation, Bureau of Ecological Research,
National Institute of Ecology, Seocheon, Republic of Korea
[3]NJ Biopia Co. Ltd., Gwangju, Republic of Korea

Corresponding author: J.-B. Kim
E-mail: jbkim74@kaeri.re.kr

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ABSTRACT. Comparative genomic hybridization (CGH) is a powerful tool used to analyze changes in copy number, polymorphisms, and structural variations in the genome. Gene copy number variation (CNV) is a common form of natural diversity in the genome, which can create new genes and alter gene structure. Thus, CNVs may influence phenotypic variation and gene expression. In this study, to detect CNVs, we irradiated rice seeds with gamma rays (300 Gy) and selected two dwarf mutagenized plants, GA-III-189 and -1052, in the M_3 generation. These plants were subjected to CGH analysis using Agilent's RICE CGH array. Most of the CNVs identified were less than

10 kb in length. We detected 90 amplified and 18 deleted regions in GA-III-189, and 99 amplified and 11 deleted regions in GA-III-1052. Of note, CNVs were located on chromosome 12 in both GA-III-189 and -1052, which contained 39 commonly amplified regions in 29 genes. The commonly amplified genes included six genes encoding F-box domain-containing proteins. Alterations in these F-box domain-containing genes were confirmed by quantitative RT-PCR. Integration of CGH and gene expression data identified copy number aberrations and novel genes potentially involved in the dwarf phenotype. These CGH and gene expression data may be useful for uncovering the mechanisms underlying the dwarf phenotype.

Key words: Comparative genomic hybridization; Gamma ray; F-box domain-containing protein; Copy number variation

INTRODUCTION

Individuals of the same species are generally thought to possess very similar genomes (Swanson-Wagner et al., 2010). However, there is growing evidence that structural variation in the form of copy number variation (CNV) can lead to variations in the genome contents of individuals of a single species. CNVs can create new genes, alter gene dosage, reshape gene structure, and modify elements regulating gene expression (Zhang et al., 2009). Indeed, structural variation can have major phenotypic consequences (Beckmann et al., 2007; Sebat, 2007). Structural shifts in the entire genome as the result of gene CNVs represent major genetic variables in the human genome, which may account for human disease etiology and phenotypic variability between individuals (Estivill and Armengol, 2007; McCarroll and Altshuler, 2007). CNVs comprise segments of DNA ranging from 1 kb to 3 Mb in size, which have been deleted, inserted, or duplicated in the genomes of some individuals. CNVs can be detected using cytogenetic techniques such as fluorescent *in-situ* hybridization, array-based comparative genomic hybridization (CGH), and single nucleotide polymorphism genotyping arrays (Scherer et al., 2007; Sudmant et al., 2010).

CGH is a powerful tool used to analyze changes in copy number, as well as polymorphisms and structural variation, in the genome. This method, which relies on techniques originally developed for use in tumor tissues, employs differential DNA hybridization on chromosome spreads to visualize deleted or amplified genomic regions (Kallioniemi et al., 1992). Array CGH was developed to analyze the number of genome-wide DNA sequences in a single experiment (Pinkel and Albertson, 2005) and has been widely used to detect DNA copy number polymorphisms between closely related genomes (Mockler et al., 2005; Pinkel and Albertson, 2005; Vianna et al., 2016). A modified method that offers the advantages of high-resolution and high-throughput genome-wide screening of genomic imbalances has been successfully used to detect single-feature polymorphisms and structural variations created by mutagenesis in rice (Kumar et al., 2007; Bruce et al., 2009).

Ionizing radiation is typically classified into low-liner energy transfer (LET) radiation, such as gamma rays and X rays, and high-LET radiation, such as α-particles and heavy ion particles (Yang and Tobias, 1979). Low-LET radiation provides relatively low energy per unit length of the particle's path, and transfers energy evenly to the irradiated area (Sachs et

al., 2000). Among the types of low-LET radiation used to induce mutagenesis, gamma rays are most widely utilized in plant breeding. Exposure to gamma rays tends to induce random deletions in large genomic segments, causing serious phenotypic damage to exposed plants (Xu et al. 2006). The resulting morphological and functional changes depend on the strength and duration of the gamma irradiation (Stoeva et al., 2001).

Plant height and branching behavior are important agronomic traits affecting crop yields. Although a variety of factors determine plant height, recent molecular genetic studies in dwarf mutants of *Arabidopsis* and other dicot species have indicated that gibberellins (GA) and brassinosteroids (BR) are the most important (Fujioka and Yokota, 2003). GA-deficient or -insensitive rice (*Oryza sativa* L. *japonica*) mutants display the dwarf phenotype characteristics of GA-deficient dicot species (Itoh et al., 2002). Rice plants normally exhibit a regular, well-described growth pattern. During vegetative development, leaves are generated in alternate phyllotaxy, and axillary meristems are produced in the axils of leaves. Some axillary buds grow to form tillers, while the remaining buds are maintained as dormant until the environmental and developmental conditions become suitable for their growth (Ishikawa et al., 2005). A group of dwarf mutants known as tillering dwarf mutants exhibits an increase in tiller number as well as reduced plant height (Kinoshita and Takahashi, 1991).

In the present study, we conducted an array-based CGH analysis to detect CNVs in two dwarf rice mutants (GA-III-189 and -1052) produced from seeds irradiated with 300 Gy gamma rays. We detected high levels of CNVs in the mutants, some representing large amplified/deleted regions. The same CNV locations were detected on chromosome 12 in both GA-III-189 and GA-III-1052, which contained commonly amplified genes.

MATERIAL AND METHODS

Plant materials and dwarf mutant isolation following gamma irradiation

Dry seeds (5000) of the cultivar Dongan (*O. sativa* L. *japonica*) were irradiated with 300 Gy generated by a gamma irradiator ([^{60}Co], with approximately 150 TBq capacity, Atomic Energy of Canada, Limited) for 24 h at the Korea Atomic Energy Research Institute. After irradiation, the M_1 seeds were germinated and sown in soil. After flowering, the plants were self-pollinated and the M_2 seeds were harvested. A total of 2961 M_2 seeds were sown, and five M_3 seeds per M_2 plant were obtained and grown to maturity. Among these M_3 plants, two rice mutants with a noticeable dwarf phenotype were isolated at the vegetative stage and designated GA-III-189 and GA-III-1052. Plants from these lines and the wild-type control, cultivar Dongan, were used in all of the experiments.

Genomic DNA labeling and hybridization for array-based CGH analysis

All array hybridizations were performed according to the recommended manufacturer protocols. Genomic DNA was isolated from aboveground seedling tissue using the hexadecyltrimethylammonium bromide mini prep method as described by Fang et al. (1992). The quantity and quality of the prepared genomic DNA were checked using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., MA, USA). For each CGH hybridization, 1-mg samples of genomic DNA from the wild-type control and the GA-III-189 and -1052 mutant lines were digested with 50 units each of the restriction enzymes Alu1 and

Rsa1 (Roche, Mannheim, Germany) in a final volume of 100 µL, to which 10 µL 10X Promega Buffer C had been added. The digestions were performed for 2 h at 37°C. Digested DNA from GA-III-189 and -1052 was purified using QIAprep Spin Miniprep columns (Qiagen, Hilden, Germany) and eluted according to the manufacturer instructions.

*Alu*1/*Rsa*1 digested DNA from GA-III-189 and -1052 was labeled using a Bioprime Array CGH Labeling Kit (Invitrogen, USA) according to the manufacturer instructions in a volume of 50 µL with a modified dNTP pool containing 120 µM each of dATP, dGTP, and dCTP, as well as 60 µM dTTP and 60 µM Cy5-dUTP (GA-III-189 and -1052) or Cy3-dUTP (for the control sample). The labeled targets were subsequently purified using a QIAquick PCR clean-up kit (Qiagen). The purified targets for each hybridization were pooled and mixed with 50 mg human Cot-1 DNA (Applied Genetics Laboratories Inc., USA)/52 µL 10X Blocking reagent (Agilent Technology, USA), followed by 250 µL 2X aCGH hybridization buffer (Agilent Technology).

Before hybridization to the array, 520-µL aliquots of the hybridization mixtures were denatured at 95°C for 3 min and incubated at 37°C for 30 min. The hybridization mixtures were then centrifuged at 16,000 *g* for 1 min at room temperature and immediately applied to the assembled Agilent RICE aCGH 4X44K microarray. The arrays were hybridized at 65°C for 24 h with rotation at 20 rpm using an Agilent Hybridization oven (Agilent Technology). The hybridized microarrays were washed according to the manufacturer protocol (Agilent Technology).

Data acquisition and analysis

The hybridization images were analyzed with a DNA microarray scanner (Agilent Technology), and data were quantified using the Agilent Feature Extraction V9.3.1 software (Agilent Technology). The average fluorescence intensity for each spot was calculated, and the local background was subtracted. Pre-processing of raw data and normalization steps were performed using the Agilent Genomic Workbench software according to the manufacturer instructions (Agilent Technology). All regions exhibiting statistically significant changes in copy number were determined using ADM algorithms (1 and 2), which identify genomic regions with copy number differences between the sample and the reference based on $\log_2$ ratios of fluorescent signals from probes in the interval. To detect aberrations in each sample, the genome-wide scanning array data were analyzed using the stringent ADM-2 algorithm at a threshold of 6.2. Centralization and fuzzy zero corrections were applied to remove putative variant intervals with small average $\log_2$ ratios. Alterations in gene copy number were defined based on the following criteria: 1) minimal number of probes for amplification ≥2; and 2) minimal average absolute log ratio for amplification ≥0.05.

Quantitative RT-PCR analysis

To analyze the expression of candidate genes in the dwarf mutants, total RNA was isolated from aboveground seedling tissue of GA-III-189 and GA-III-1052 plants using Trizol reagent according to the manufacturer protocol (Gibco BRL, USA). Total RNA samples (1 mg) were reverse transcribed using a Power cDNA Synthesis Kit (Intron Biotech Inc., Sungnam, Korea) for 60 min at 42°C using 1 mg oligo(dT)15 primers. The synthesized cDNAs were

used as templates for quantitative RT-PCR. Quantitative RT-PCR was performed on an Eco Real-Time PCR system (Illumina, USA) using SYBR Premix Ex Taq™ (TaKaRa). The PCR thermal cycle conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, and 60°C for 30 s. The primer sequences are listed in Table 1. The relative expression levels of the selected genes were calculated using the relative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 1. Primer sequences used for quantitative RT-PCR.

Name	GenBank accession No.	Forward and reverse primers	Expected size (bp)
OsFBX450	LOC_Os12g34200	5'-AGTTACCATCGGAGGACACC-3'	352
		5'-AGCATCCGAACATACTTCCC-3'	
OsFBX451	LOC_Os12g34240	5'-TAATTGCAGTCAGCCAGAGG-3'	451
		5'-AGCAATAGTCGCAAGAGGCT-3'	
OsFBX452	LOC_Os12g34270	5'-TGGTTTCTTCAGCACTACGC-3'	551
		5'-TCAACTCGGTAACATTGGGA-3'	
OsFBX453	LOC_Os12g34290	5'-AGCCATGTCCACAGATACCA-3'	335
		5'-CAATGAGCTTGGTTGTGCTT-3'	
OsFBX454	LOC_Os12g34300	5'-CCTCGACGTCGTACTGAAGA-3'	462
		5'-ATTGCATGTGTATCCACGGT-3'	
OsFBX455	LOC_Os12g34310	5'-CCAGGGACGTAATCAGTGTG-3'	507
		5'-CAATTCGAAGGCACTCAAGA-3'	
OsFBL61	LOC_Os12g34220	5'-TGATGACACTGGTCTCGGTT-3'	383
		5'-TTGCAATTGATGAGGTGGAT-3'	
Actin1	LOC_Os03g50885	5'-TGAAGTGCGACGTGGATATTAG-3'	411
		5'-CAGTGATCTCCTTGCTCATCC-3'	

RESULTS

Isolation of rice dwarf mutants induced by gamma irradiation

To identify structural variations induced by gamma irradiation, we observed the phenotypes of M_3 rice plants. In total, 5000 dry rice seeds were irradiated with 300 Gy gamma rays and then germinated. After flowering, the plants were self-pollinated, and the M_2 seeds were harvested. In total, 2961 M_2 seeds were sown, and five M_3 seed lines per M_2 plant were obtained. Among the M_3 plants, we isolated two rice mutants with noticeable dwarf phenotypes, GA-III-189 and -1052, which we subjected to array-based CGH analysis.

Figure 1A and B shows the growth morphology of the GA-III-189 and -1052 mutants at the tillering (Figure 1A) and grain-filling stages (Figure 1B). Compared to the control, the GA-III-189 and -1052 mutants showed higher tiller numbers (Table 2), late and asynchronous heading, and partial male sterility (Figure 1B). The GA-III-189 and -1052 mutants also had smaller panicles and shorter rachis-branches than the control (Table 2 and Figure 1B and C). The grains of GA-III-189 and -1052 plants were shorter than those of the control (Figure 1D and E). Plant heights for GA-III-189 and -1052 were less than 50% of the control cv. 'Dongan'. The number of tillers in GA-III-189 and -1052 was approximately 3-fold higher that in control plants (Table 2). These observations indicate that the GA-III-189 and -1052 mutants exhibited multiple morphological defects.

Figure 1. Morphological characterization of the control and dwarf mutants (GA-III-189 and GA-III-1052). Morphology at the tillering stage (**A**); grain-filling stage (**B**); panicles (**C**); grains (**D**); and brown rice (**E**). Bars: 5 cm (**C**), 0.5 cm (**D** and **E**).

Table 2. Plant height, tiller number, and panicle length of the dwarf mutants.

Type	Number of tillers	Plant height (cm)	Length of panicles (cm)
WT	10 ± 0.47	66.40	19.33 ± 1.31
GA-III-189	37 ± 4.96	40.43	9.23 ± 0.20
GA-III-1052	31 ± 5.55	38.36	8.56 ± 0.80

CNV detection using array-based CGH

To detect CNVs in these dwarf rice mutants, we performed array-based CGH analysis of GA-III-189 (Figure 2A) and -1052 (Figure 2B). Multiple CNVs were detected on the rice chromosomes (Figure 2A, B).

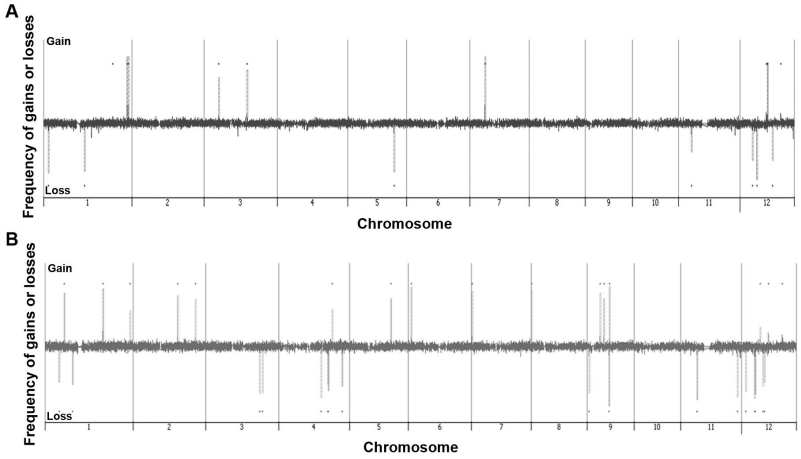

Figure 2. CNV (copy number variation) events detected by array-based CGH (comparative genomic hybridization) analysis of dwarf rice mutants. Full chromosome views of CNV events are depicted for all 12 rice chromosomes. **A.** GA-III-189 mutant. **B.** GA-III-1052 mutant.

However, in the GA-III-189 mutant, CNVs were not detected on chromosome 10, whereas, in the GA-III-1052 mutant, CNVs were not detected on chromosomes 2, 4, 6, 8, 9, or 10 (Table 3). The genomes of the two dwarf mutants (GA-III-189 and -1052) contained 108 and 110 CNVs, respectively (Figure 3). The GA-III-189 mutant contained 90 gains and 18 losses, whereas the GA-III-1052 mutant contained 99 gains and 11 losses (Figure 3).

Table 3. Number of CNVs (copy number variations) on individual chromosomes of the dwarf mutants revealed by CGH analysis.

Chr	GA-III-189	GA-III-1052	No. of CNVs
1	5	24	29
2	2	0	2
3	2	2	4
4	5	0	5
5	1	1	2
6	1	0	1
7	1	1	2
8	1	0	1
9	5	0	5
10	0	0	0
11	2	5	7
12	83	77	160

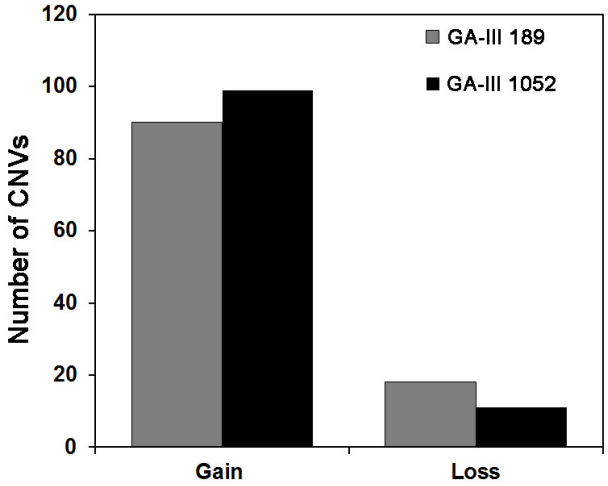

Figure 3. Number of CNVs detected by CGH analysis of dwarf rice mutants.

Table 3 shows the number of CNVs detected on individual chromosomes of the dwarf mutants. Most of the CNVs in both mutants were located on chromosome 12, with chromosome 1 having the second highest number of CNVs (Table 3). The CNVs in line GA-III-189 ranged from 54 bp to 164.7 kb in length, averaging 2.3 kb. The CNVs in line GA-III-1052 ranged from 47 bp to 164.7 kb in length, averaging 2.9 kb (data not shown). Most CNVs (88%) were small variants of less than 5 kb (Table 4).

Table 4. Size range distribution of CNVs in the dwarf mutants as revealed by CGH (comparative genomic hybridization) analysis.

ID	<5 kb	5-10 kb	10-500 kb	≥500 kb
GA-III-189	34	0	2	0
GA-III-1052	12	1	3	0
CNV	48	1	5	0
CNVR	1	1	5	0

Expression of genes affected by copy number alterations

We detected CNVs on chromosome 12 that were present in both mutants (Table 5); this region contains genes of the FBX (F-box domain containing protein) family. To confirm the altered expression of FBX family genes in the mutants, we performed quantitative reverse-transcription PCR and the results are shown in Figure 4. All of the genes were upregulated in line GA-III-189, with the exception of *OsFBX450*. These differences in expression were striking for *OsFBX452*, *OsFBX452*, *OsFBX454*, and *OsFBX455*. It is likely that the enhanced expression of these genes in GA-III-189 is due to the presence of multiple copies of FBX genes resulting from gene duplications. In GA-III-1052, reverse-transcription PCR analysis showed that four of the seven genes were differentially expressed, namely, *OsFBX450*, *OsFBX451*, *OsFBX452*, and *OsFBL61* (Figure 4). The CNVs in the three genes without altered expression might affect factors including gene structure or protein expression.

Table 5. Description of commonly amplified genes on chromosome 12 of the dwarf mutants as revealed by CGH analysis.

Gain/Loss	Location	LOC_No.	Size (bp)	Description
Gain	Chr. 12	Os12g34140	1632	Retrotransposon protein, putative, unclassified
		Os12g34144	378	Mitochondrial ribosomal protein S12, putative
		Os12g34148	1763	Expressed protein
		Os12g34154	1472	Ribosomal protein S2, putative, expressed
		Os12g34160	6312	Transposon protein, putative, CACTA, En/Spm subclass
		Os12g34170	585	Retrotransposon protein, putative, unclassified
		Os12g34180	5418	Retrotransposon protein, putative, Ty3-Gypsy subclass
		Os12g34190	399	Retrotransposon protein, putative, unclassified
		Os12g34200	1881	Osfbx450 - F-box domain-containing protein
		Os12g34210	5544	Transposon protein, putative, unclassified
		Os12g34220	2084	Osfbl61 - F-box domain- and LRR-containing protein, expressed
		Os12g34230	576	Hypothetical protein
		Os12g34240	1605	Osfbx451 - F-box domain-containing protein
		Os12g34250	2766	Transposon protein, putative, CACTA, En/Spm subclass
		Os12g34260	2619	Transposon protein, putative, CACTA, En/Spm subclass
		Os12g34270	1596	Osfbx452 - F-box domain-containing protein
		Os12g34280	519	Conserved hypothetical protein
		Os12g34290	1665	Osfbx453 - F-box domain-containing protein
		Os12g34300	1954	Osfbx454 - F-box domain-containing protein, expressed
		Os12g34310	1581	Osfbx455 - F-box domain-containing protein
		Os12g34320	3115	S-locus-like receptor protein kinase, putative, expressed
		Os12g34330	2782	Protein-binding protein, putative, expressed
		Os12g34340	1960	Pentatricopeptide, putative, expressed
		Os12g34350	1934	Expressed protein
		Os12g34360	2686	Expressed protein
		Os12g34370	2690	Adaptor complexes medium subunit family protein, expressed
		Os12g34380	1467	Glutathione synthetase, chloroplast precursor, putative, expressed
		Os12g34390	1269	Conserved hypothetical protein
		Os12g34400	876	Hypothetical protein

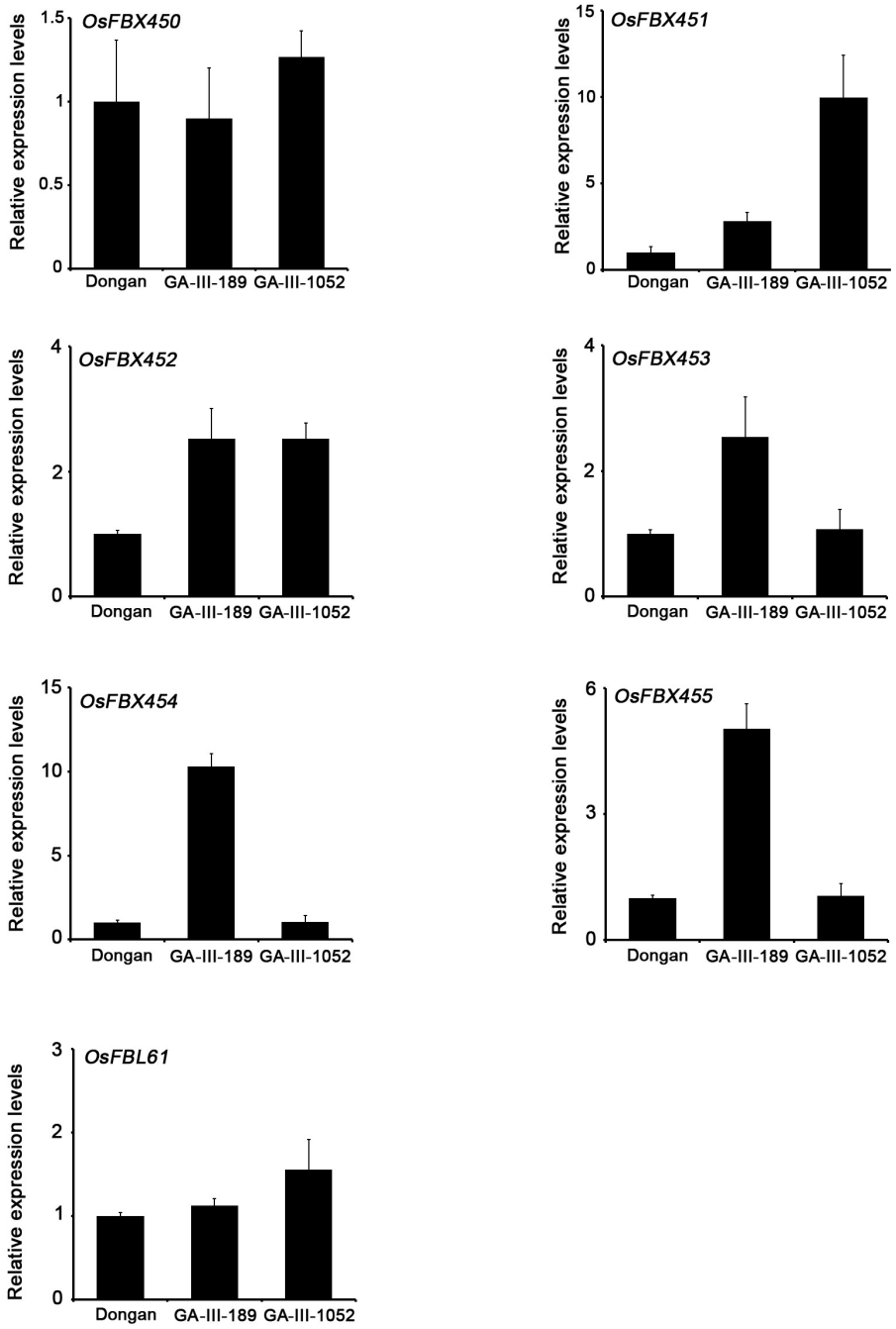

Figure 4. Validation of FBX family gene expression in the dwarf mutants by RT-PCR. RT-PCR confirmation of the expression of genes of interest containing CNVs located on chromosome 12: *OsFBX450*, *OsFBL61*, *OsFBX451*, *OsFBX452*, *OsFBX453*, *OsFBX454*, and *OsFBX455*. FBX family genes were differentially expressed between the control and two dwarf mutants.

DISCUSSION

Dwarf mutants are excellent tools for elucidating regulatory mechanisms underlying plant growth and development, and have been isolated in many species. Various reasons account for the dwarf phenotypes in these mutants, involving factors such as altered GA or BR levels, abnormal cell walls, or abnormal cell elongation. Ionizing radiation can generate chromosome breakages that lead to mutations upon repair, and a large proportion of these probably involve deletions (Vizir et al., 1996). In particular, exposure to gamma rays tends to induce random deletions in large genomic segments, causing serious phenotypic damage to exposed plants (Xu et al., 2006). In the present study, we characterized the rice mutants GA-III-189 and -1052, which were induced by gamma irradiation. Under normal growth conditions, GA-III-189 and -1052 plants exhibited many abnormal phenotypes throughout development, including dwarfism, increased tiller number, and smaller panicles (Figure 1 and Table 2). Thus, these specific phenotypes represent suitable criteria that can be used to determine whether a novel dwarf mutant is related to CNV.

CNV is a genomic polymorphism of major importance in human biology (Sebat, 2007). In plants, exploration of the extent and role of CNV has recently begun (Żmieńko et al., 2014). Despite the prevalence of CNVs in plant genomes and their frequent overlap with protein-coding regions, only a few CNVs have been associated with particular phenotypes on morphological, physiological, or developmental levels. CNVs play an important role in phenotypic variation. A good example of a CNV affecting phenotype is the diversity of flowering times and plant heights in wheat (Díaz et al., 2012; Li et al., 2012). For instance, a CNV determines the extreme dwarf phenotype in the Aibian 1 line (Li et al., 2012). However, little is known about CNVs in the rice genome. CNV in an ultraviolet-B tolerance related gene, *UTR319*, has been shown to play significant roles in exposure to carbon-ion beams in rice (Takano et al., 2013). This report also supports the role of CNVs in phenotypic variations of rice. In the current study, 108 and 110 genomic regions were gained or lost in GA-III-189 and -1052, respectively (Table 3). These CNVs may influence the dwarf phenotypes of these mutants. Additionally, our data suggest that smaller CNVs (<10 kb) are much more frequent than larger CNVs (Table 4); this finding is supported by those from other studies (McCarroll and Altshuler, 2007; Beló et al., 2010).

CNVs that overlap a gene may alter its expression level by changing the number of functional copies. Additionally, CNVs that partially overlap a gene sequence may disrupt the structure of the gene and impair its function (Żmieńko et al., 2014). We analyzed the gene expression patterns in a region on chromosome 12 with CNVs detected in both mutants (Figure 4). Almost all genes of the FBX family on chromosome 12 were upregulated in both dwarf mutants (Figure 4). F-box proteins, which constitute a large family of proteins in eukaryotes, play pivotal roles in regulating various developmental processes in plants (Lechner et al., 2006). Duan et al. (2012) found that, in rice, *DDF1* encodes an F-box protein that is likely to control both cell division and cell expansion, thereby controlling organ size by affecting these processes concurrently. The reduced plant height observed in the current study probably resulted from CNV of FBX genes, which might function via a similar mechanism.

In this study, we demonstrated that CNVs could be detected in dwarf rice mutants using array-based comparative genome hybridization. These CNVs, which are likely to be linked to specific phenotypes, should provide an important resource for understanding the mechanisms underlying the dwarf phenotype. Our results suggest that changes in gene copy

number are an important source of genetic diversity. Indeed, some of these changes could be directly associated with important traits in crops.

Conflicts of interest

The authors declare no conflict of interest.

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