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Molecular characterization and concerted evolution of two genes encoding RING-C2 type proteins in rice

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ABSTRACT

RING (Really Interesting New Gene) finger proteins are believed to play a critical role in mediating the transfer of ubiquitin to heterogeneous substrate(s). While the two canonical types, RING-H2 and RING-HC, have been well-characterized, the molecular functions of the modified types, particularly the RING-C2 types, remain elusive. We isolated two rice genes harboring the RING-C2 domain on the distal parts of rice chromosomes 11 and 12, termed *OsRINGC2-1* and *OsRINGC2-2*, respectively. A comparison of sequence divergences between 10 duplicate pairs on the distal parts of rice chromosomes 11 and 12 and randomly selected duplicate pairs suggested that *OsRINGC2-1* and *OsRINGC2-2* have evolved in concert via gene conversion. An *in vitro* ubiquitination assay revealed that both proteins possess E3 ligase activity, suggesting that the innate functions of these RING domains have not been affected by their modifications during evolution. Subcellular localizations were strikingly different; OsRINGC2-1 was found only in the cytoplasm with many punctate complexes, whereas OsRINGC2-2 was observed in both the nucleus and cytoplasm. The expression patterns of both genes showed striking differences in response to salt stress, whereas plants heterogeneous for both genes mediated salt tolerance in *Arabidopsis*, supporting the notion of concerted evolution. These results shed light on the molecular functions of *OsRINGC2-1* and *OsRINGC2-2* and provide insight into their molecular evolution.

1. Introduction

RING (Really Interesting New Gene) finger proteins are believed to play a critical role in mediating the transfer of ubiquitin to heterogeneous substrate(s), contributing to various post-translational regulation processes such as degradation, functional and structural modification, and alternation of localization (Deshaies and Joazeiro, 2009). Conjugation of ubiquitin to a target protein typically requires the sequential activity of three classes of enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin-ligase). In eukaryotes, RING finger proteins, most of which possess E3 ligase activity, are a large gene family. Particularly in plants, botanical model genomes, such as rice and *Arabidopsis* are known to harbor at least 425 and 469 RING domain-containing proteins, respectively (Lim et al., 2010; Stone et al., 2005). Completed sequencing and assembly of the apple genome demonstrated about 663 predicted RING domain-harboring proteins (Li et al., 2011).

The canonical RING motifs have been defined by the presence of consensus sequence cysteine (Cys) and histidine (His) residues (Cys-*X*2-Cys-X9-39-Cys-X1-3-His-*X*2-3-Cys/His-*X*2-Cys-X4-48-Cys-*X*2-Cys, where X can be any amino acid), which can mediate binding of two zinc atoms (Freemont et al., 1991). Two canonical RING finger protein types, RING-H2 and RING-HC, are subdivided on the basis of the fifth coordination Cys or His sites, respectively, and exist with high frequency in the genome as compared to other modified RING finger proteins. For example, the frequencies of RING-H2 and RING-HC are about 50.5% and 39.0% in *Arabidopsis*, 55.5% and 25.9% in rice and 53.3% and 29.3% in apple, respectively (Li et al., 2011; Lim et al., 2010; Stone et al., 2005), indicating similar expansion patterns of the gene family over a broad array of plants. Additionally, some modified types of RINGs including RING-v, RING-D, RINGS/T, RING-G, and RING-C2 exist in various plant genomes. Interestingly, only two RING-C2 types exist in the rice genome, whereas 10 occur in *Arabidopsis* and 10 in apple, respectively.

RING finger proteins with canonical domains have been well characterized in their diverse biological roles in light-inactivatable repression (McNellis et al., 1994), cell cycle progression (Liu et al., 2008), abscisic acid (ABA) signaling (Bu et al., 2009), chromatin remodeling (Liu et al., 2007), and maintenance of cell viability (Koiwai et al., 2007). In contrast, functions of the modified RING domains remain largely unidentified.

Abbreviations: RING, really interesting new gene; DDC, duplication-degeneration-complementation; ABA, abscisic acid; SA, salicylic acid; JA, jasmonic acid; MBP, maltose binding protein; dsm, shared-motif divergences; Mya, million years ago; EYFP, enhanced yellow fluorescent protein.

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Complete genome sequences and bioinformatics provide new insights into the evolutionary history of plant genomes. In particular, the *Poaceae* genome, which includes major food crops such as rice, wheat, corn, and sorghum, is believed to have undergone a pregrass whole-genome duplication event with a common ancestor 70–90 million years ago (Mya) (Paterson et al., 2009; Salse et al., 2008, 2009; Wang et al., 2005) followed by independent gene loss during speciation. In contrast, the distal parts (about 3 Mbp) of rice chromosomes 11 and 12 were previously suggested to have been affected by a recent segmental duplication approximately 7.7 Mya (Rice chromosomes 11 and 12 sequencing consortia, 2005; Yu et al., 2005). However, the comparative analysis of rice and sorghum genomes and physical and genetic maps have suggested that segmental duplication originally resulted from the pan-cereal whole-genome duplication (Paterson et al., 2009; Wang et al., 2009).

Gene duplication occurring via whole/segmental duplication and/or tandem duplication is generally believed to contribute to establishing novel gene functions (Moore and Purugganan, 2005; Sémon and Wolfe, 2007). Several hypotheses exist regarding the evolutionary mechanisms. The pioneer, Ohno (1970), proposed that either one of the duplicates loses (pseudogenization, the most common fate) or gains a new function (neofunctionalization) after gene duplication; however, all evolutionary mechanisms of duplicate genes cannot be adequately explained by this model. Additionally, duplication–degeneration–complementation (DDC) has been proposed as another attractive model that accounts for duplicate gene preservation (Force et al., 1999). The DDC model is generally described as a process by which daughter genes accumulate degenerative mutations in both the regulatory regions and protein coding regions, resulting in a separation of ancestral gene functions. In contrast, an alternative hypothesis has advanced the buffering of crucial functions via gene conversion (Chapman et al., 2006).

Our previous investigation on the dynamic expansion and genomic localization of rice RING finger protein genes over evolution revealed only two RING-C2 type protein genes located on the distal part on each of rice chromosomes 11 and 12 (Lim et al., 2010). We questioned whether the rice RING-C2 domains harbor E3 ligase activity and what their evolutionary fate is after duplication. We isolated two RING-C2 type genes from a Korean rice variety and examined their expression patterns, subcellular localization, E3 ligase activities, and functions via induced heterogeneous overexpression. This study presents an example of the evolutionary fate of duplicate genes, which appear to be homogenized frequently via gene conversion and illegitimate recombination.

2. Materials and methods

2.1. Plant materials and stress treatments

Rice (*O. sativa* spp. *japonica* cv. 'Donganbyeo') seeds were germinated in half-strength Murashige and Skoog (MS) nutrient solution and incubated in a photoperiod-controlled growth chamber (16 hr/8 hr day/night) at 25/23 °C (day/night) with 70% relative humidity. Fourteen day-old seedlings were transferred to a fresh nutrient solution containing 200 mM NaCl for the salinity treatment, and then sampled at 1, 6, 12, and 24 hr post-treatment. For the drought treatment, 14 day-old seedlings were positioned without water and then sampled at 1, 6, 12, and 24 hr. Plants were incubated in a refrigerator at 4 °C in the dark for cold stress, whereas another container with plants covered with foil was maintained in a growth chamber (25/23 °C, day/night) as a control, and leaves were harvested at 1, 6, 12, and 24 hr. Heat stress treatment was conducted by incubating the plants at 45 °C and then sampling them at 1, 6, 12, and 24 hr. Fourteen day-old seedlings were treated with 0.1 mM ABA, 1 mM salicylic acid (SA), or 0.1 mM jasmonic acid (JA) for hormone treatments, and then sampled at 1, 6, 12, and 24 hr. Panicle samples were harvested from plants cultivated in an experimental field of Kangwon National University, Chuncheon, Korea.

2.2. Isolation and cloning of OsRINGC2-1 and OsRINGC2-2

The coding sequences for *OsRINGC2-1* (Os11g01190) and *OsRINGC2-2* (Os12g01190) were obtained from the Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu/). Primer pairs harboring restriction enzyme sites were designed and listed in Supplementary Table 1. Total RNA was extracted from 4-week-old rice leaves using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and first-strand cDNA was synthesized using MMLV reverse transcriptase (Takara Bio, Kyoto, Japan). The full-length cDNAs of *OsRINGC2-1* and *OsRINGC2-2* were amplified using high-fidelity *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA, USA). The amplified polymerase chain reaction (PCR) products were digested by *Bam*HI/*Kpn*I enzymes and introduced into pBIN35S plasmids. Then, the inserted pBIN35S plasmids were isolated and treated with diacritical enzymes *Hind*III and *Kpn*I to identify *OsRINGC2-1* or *OsRINGC2-2*. Two constructs were verified at a DNA sequencing facility (Macrogen, Seoul, Korea).

2.3. Phylogenetic analyses

Viridiplantae peptide sequences were downloaded from the Phytozome database V. 6.0 (http://www.phytozome.net). Proteins harboring the RING-C2 domain were retrieved depending on the presence and distance between each of the zinc-coordinating cysteine residues using a specially designed Perl script and then manually inspected. Sequences were aligned using the ClustalW2 program (ftp://ftp.ebi.ac.uk/pub/software/clustalw2/2.1/) with default parameters (Larkin et al., 2007). A phylogenetic tree was generated using the MEGA software (version 5.05) with the maximum likelihood method based on the JTT matrix-based model with 1000 bootstrap replications (Jones et al., 1992).

2.4. RT-PCR

Total RNA was extracted using TRIzol (Invitrogen). First, cDNA was synthesized using the PrimeScript™ RT-PCR kit (Takara Bio) in accordance with the manufacturer's protocol. To normalize each sample for cDNA quality and quantity, the rice *Os18S rRNA* gene (Os09g00999) was employed as an internal control with specific primers (for sequences see Supplementary Table 2). The reliable reference genes, such as *OssalT* (Os01g24710), *OsLIP19* (Os05g03860), *OsHsp90-1* (Os04g01740), *OsPR1b* (Os01g28450), and *OsPBZ1* (Os12g36830), were also employed as positive controls to validate each stress treatment. Gene-specific primers were designed via the NCBI Primer BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The PCR program was run as follows: denaturing for 5 min at 95 °C, followed by 30–33 cycles of 30s of denaturation at 95 °C, 30 s of annealing, and 60 s of extension at 72 °C, with a final extension step at 72 °C.

2.5. OsRINGC2-1 and OsRINGC2-2 ubiquitination assay

Each of the full-length *OsRINGC2-1* and *OsRINGC2-2* clones was inserted into the pMAL-c5X vector for protein expression in the BL21(DES) pLysS *E. coli* strain. Recombinant proteins were tagged with maltose binding protein (MBP) and then purified by amylose resin column chromatography according to the manufacturer's recommendation (New England BioLabs, Ipswich, MA, USA). *AtUBC10* (At5g53300) cDNA was cloned into the pET-28a vector for protein expression in the BL21(DES) pLysS *E. coli* strain. Recombinant protein tagged with 6x His-tagged protein was purified by Ni-NTA column chromatography in accordance with the standard commercial protocol (Invitrogen). *In vitro* ubiquitination assay was performed as described previously by Hardtke et al. (2002) with some modifications. Ubiquitination reactions were performed in a total volume of 20 μl with approximately 250 ng of each purified full-length MBP-*OsRINGC2* fusion protein mixed with 50 ng of yeast E1 (Boston