MORF-RELATED GENE702, a Reader Protein of Trimethylated Histone H3 Lysine 4 and Histone H3 Lysine 36, Is Involved in Brassinosteroid-Regulated Growth and Flowering Time Control in Rice1[OPEN]

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The methylation of histone H3 lysine 36 (H3K36) plays critical roles in brassinosteroid (BR)-related processes and is involved in controlling flowering time in rice (Oryza sativa). Although enzymes that catalyze this methylation reaction have been described, little is known about the recognition mechanisms to decipher H3K36 methylation information in rice. In this study, biochemical characterizations showed that MORF-RELATED GENE702 (MRG702) binds to trimethylated H3K4 and H3K36 (H3K4me3 and H3K36me3) in vitro. Similar to the loss-of-function mutants of the rice H3K36 methyltransferase gene SET DOMAIN GROUP725 (SDG725), the MRG702 knockout mutants displayed typical BR-deficient mutant and late-flowering phenotypes. Gene transcription analyses showed that MRG702 knockdown resulted in the down-regulation of BR-related genes, including DWARFT1, BRASSINOSTEROD INSensitive1, and BRASSINOSTEROID UPREGULATED1, and several flowering genes, including Early heading date1 (Ehd1), Ehd2, Ehd3, OsMADS50, Heading date 3a, and RICE FLOWERING LOCUS T1. A binding analysis showed that MRG702 directly binds to the chromatin at target gene loci. This binding is dependent on the level of trimethylated H3K36, which is mediated by SDG725. Together, our results demonstrate that MRG702 acts as a reader protein of H3K4me3 and H3K36me3 and deciphers the H3K36 methylation information set by SDG725. Therefore, the role of MRG702 in the BR pathway and in controlling flowering time in rice is to function as a reader protein to decipher methylation information.

Histone Lys methylation is an evolutionarily conserved epigenetic modification that plays important roles in the dynamic regulation of chromatin structure and gene expression (Liu et al., 2010; Berr et al., 2011). Generally, methylations at histone H3 lysine 9 (H3K9), H3K27, and H4K20 residues are considered to be repressive chromatin marks, whereas methylations of H3K4 and H3K36 are strongly correlated with transcriptional activation. Therefore, methylation to activate or repress transcription can greatly affect the variability of gene expression, ultimately affecting many aspects of plant development (Liu et al., 2010; Berr et al., 2011).

Enzymes responsible for the deposition of methyl groups on histone Lys residues contain a conserved SET domain, named after the three proteins first identified in fruit fly (Drosophila melanogaster): SUPPRESSOR OF VARIEGATION3-9, ENHANCER OF ZESTE, and Trithorax (Tschiersch et al., 1994). Functional analyses of plant Trithorax and ABSENT, SMALL, OR HOMEOtIC DISCS1 (ASH1) family SET DOMAIN GROUP (SDG) proteins, which mediate H3K4 and/or H3K36 methylation, have demonstrated their crucial roles in plant growth and development. For instance, Arabidopsis (Arabidopsis thaliana) SDG2/ARABIDOPSIS TRITHORAX-RELATED3 is the major H3K4 methyltransferase, and sdg2 mutants display pleiotropic phenotypes associated with the perturbation of many genes (Berr et al., 2010b; Guo et al., 2010; Yao et al., 2013). Arabidopsis SDG8, also named ASH1 HOMOLOG2, EARLY FLOWERING IN SHORT DAYS, or CAROTENOID CHLOROPLAST REGULATORY1, is the major H3K36 methyltransferase,
and its loss-of-function mutants also show pleiotropic defects, including early flowering, reduced organ size, increased shoot branching, altered carotenoid composition, misformed reproductive organs, reduced fertility, and impaired defenses against pathogens (Kim et al., 2005; Zhao et al., 2005; Dong et al., 2008; Xu et al., 2008; Cazzonelli et al., 2009; Grini et al., 2009; Berr et al., 2010a). Interestingly, SDG725, an H3K36 methyltransferase and the closest homolog of SDG8 in rice (Oryza sativa), is involved in promoting brassinosteroid (BR)-related gene expression and rice flowering (Sui et al., 2012, 2013). SDG724, another H3K36 methyltransferase in rice, also promotes flowering by affecting H3K36 methylation levels at OsMADS50 and RICE FLOWERING LOCUS T1 (RFT1) chromatin (Sun et al., 2012).

Although several enzymes that methylate histone Lys residues have been identified in plants, the mechanisms to decipher histone methylation information are not well understood. The different modifications of histone residues (also known as histone codes) are thought to serve as signals that are recognized by effector modules (reader proteins), which might ultimately implement the downstream functions of histone codes (Liu et al., 2010; Berr et al., 2011; Yun et al., 2011). Recent studies on such effector modules have shown that a variety of reader domains, including chromodomain (chromatin organization modifier domain), plant homeodomain (PHD), Tudor, malignant brain tumor, PWWP (named after a conserved Pro-Trp-Trp-Pro motif), Zinc finger-CW (named after conserved Cys and Trp residues), WD40, and ankyrin repeats, bind to different methylated Lys residues of histones (Yun et al., 2011). However, only a few histone methylation reader proteins have been identified in plants. In Arabidopsis, the PHD-containing proteins Origin Recognition Complex1, Inhibitor of growth, and ALF1N1-like and the WD40 repeat domain-containing protein WDR5a were shown to bind methylated H3K4 in vitro or in vivo (de la Paz Sanchez and Gutierrez, 2009; Jiang et al., 2009; Lee et al., 2009; Molitor et al., 2014). The CW domain in SDG8 was shown to associate with methylated H3K4, subsequently causing the enzyme to methylate H3K36 (He et al., 2010; Hopfmann et al., 2011). LIKE HETEROCHROMATIN PROTEIN1/TERMINAL FLOWER2, which contains a chromodomain, was shown to bind and colocalize with the Polycomb Repressive Complex2-mediated trimethylated H3K27 modification (H3K27me3) in Arabidopsis (Turck et al., 2007; Zhang et al., 2007). The Arabidopsis CHD3 (for Chromodomains, Helicase/ATPase, and DNA-binding) domain protein PICKLE was shown to directly associate with genes enriched with H3K27me3 (Zhang et al., 2012). The rice CHD3 protein CHR729 was shown to interact with methylated H3K4 and H3K27 via its chromodomain and PHD finger, respectively. Thus, CHR729 affects many aspects of plant development as a bifunctional chromatin regulator (Hu et al., 2012).

Very recently, our laboratory identified the Arabidopsis MORF-RELATED GENE (MRG) group proteins MRG1/2 as readers of H3K4me3/H3K36me3 via their chromodomains. Our results showed that MRG1/2 physically interacted with the transcription factor CONSTANS (CO) to activate FLOWERING LOCUS T (FT) expression. Different from the pleiotropic defects of sgd8 mutants, the mrgl mrgr double mutant exhibits normal growth phenotypes, except late flowering, under long-day (LD) conditions (Zhao et al., 2005; Bu et al., 2014). MRG1/2 also interact with the histone H4-specific acetyltransferases HAM1 and HAM2 and thus bridge H3K4/H3K36 methylation and histone H4 acetylation in modulating the expression of flowering genes (Xu et al., 2014). The rice genome contains two closely related homologs of Arabidopsis MRG1/2: MRG701 and MRG702. Because of the different functions of H3K36 methylation between Arabidopsis (a dicot) and rice (a monocot), we questioned the function of rice MRG proteins. We speculated that further information on MRG proteins may increase our understanding of the mechanisms to decipher histone methylation information in plants.

Here, we report that MRG702, rather than MRG701, plays a primary function in rice growth and development. In vitro binding assays showed that MRG702 preferentially associates with trimethylated H3K4 and H3K36. Similar to SDG725 knockdown mutants, MRG702 knockdown mutants displayed pleiotropic defects, including typical BR-deficient morphologies and late flowering associated with the down-regulation of the BR pathway and flowering regulatory genes. Our results further showed that MRG702 directly binds to the chromatin of target genes in an SDG725-dependent manner, thus providing a direct link between H3K36 methylation and its functional outcomes in rice.

RESULTS

MRG702 Plays a Primary Function in Rice Growth and Development

MRG group proteins are widely distributed among eukaryotes, from fungi to plants and animals. The members of this group share similar domain architecture, with a chromodomain near the N terminus and an MRG domain close to the C terminus (Bu et al., 2014). We generated a phylogenetic tree from a sequence-based alignment of the full-length sequences of plant MRG proteins. Proteins from dicots, monocots, and a moss, Physcomitrella patens, were included in this analysis, as were some previously identified MRG proteins from yeast (Sacharomyces cerevisiae; Essential Something about silencing2-related acetyltransferase1-associated factor3; Sun et al., 2008), fruitfly (DmMRG15; Zhang et al., 2010), and human (HsMRG15; Zhang et al., 2006). In the phylogenetic tree, the plant MRG proteins formed one group with three distinct clades (monocot, dicot, and moss; Fig. 1A). Interestingly, most dicots contain two homologs, which were grouped into two separate subclades (Fig. 1A), suggesting that the amplification of these proteins occurred through duplication events before the divergence of dicots. Some monocots, such as maize (Zea mays),
sorghum (Sorghum bicolor), and wheat (Triticum aestivum), have only one MRG protein, while rice has two, MRG701 and MRG702 (Fig. 1A). These two MRG proteins appear to have duplicated after the divergence of monocots during evolution. The analysis showed that MRG701 and MRG702 share 93% amino acid sequence identity, suggesting that they have similar functions in rice.

Quantitative reverse transcription (RT)-PCR with RNA extracted from different rice tissues showed that both MRG701 and MRG702 were transcribed ubiquitously throughout the plant. There were much higher transcript levels of MRG702 than those of MRG701 in all of the tested tissues (Fig. 1B). Thus, we speculated that MRG702, rather than MRG701, may function primarily in rice.

To explore the roles of MRG proteins in planta, we used an RNA interference (RNAi) approach to knock down MRG701/702. As shown in Supplemental Figure S1, MRG701 shares extremely high identity with MRG702, except at the N terminus. Thus, we selected nucleotides 206 to 39 and 670 to 896 of MRG702 to create the inverted repeats of the hairpin structure; the two RNAi constructs were p35S::MRG-RNAi-1 and p35S::MRG-RNAi-2, respectively. The construct p35S::MRG-RNAi-1 was designed to knock down MRG702 only, because the nucleotides 206 to 39 exist only in MRG702. In contrast, p35S::MRG-RNAi-2 was designed to knock down both MRG genes, because the nucleotides 670 to 896 are conserved in MRG701 and MRG702. Control plants transformed with the empty vector were also generated. We obtained 11 independent transgenic lines containing the RNAi construct p35S::MRG-RNAi-1 (hereafter, 702Ri-1) and 15 lines containing p35S::MRG-RNAi-2 (702Ri-2). Four 702Ri-1 lines and five 702Ri-2 lines showed stable phenotypes after three to five generations. Plants of 702Ri-1 and 702Ri-2 displayed a dwarf phenotype, and their overall morphologies were similar (Fig. 1C).
As expected, p35S:MRG-RNAi-1 eliminated MRG702 transcription, while p35S:MRG-RNAi-2 greatly decreased the transcript levels of both MRG701 and MRG702 (Fig. 1D). The similar phenotypes of 702Ri-1 and 702Ri-2 plants indicated that MRG702 knock down and knock down of both MRG701 and MRG702 had similar effects on growth and development. This result suggested that MRG702 acts dominantly in rice. Interestingly, the overall phenotypes of 702Ri-1 and 702Ri-2 plants were similar to those of 725Ri-1 plants but less severe (Fig. 1C), indicating that MRG701/702 and SDG725 may function in the same genetic pathway.

MRG702 Affects BR Biosynthesis and Signaling Pathways

Given its functional dominance, MRG702 became the focus of our research. To confirm the phenotypes of MRG702 knockdown plants, we analyzed the 702Ri-1 transgenic lines in more detail. Consistent with their MRG702 transcript levels, 702Ri-1-2 plants showed less severe phenotypes than 702Ri-1-1 plants (Supplemental Fig. S2). This result suggested that the dwarf phenotype indeed resulted from the knockdown of MRG702. Although the phenotype of 702Ri-1-1 plants was less severe than that of 725Ri-1 plants, their phenotypes were similar in that both lines had erect leaves, short internodes, fewer spikelets and rachis branches, and small grains (Fig. 2, A–D; Table I), which are common features of rice BR-deficient mutants.

To assess BR-related defects in MRG702 knockout mutants in more detail, we conducted a lamina joint-bending assay after treating wild-type, 725Ri-1, and 702Ri-1-1 plants with the bioactive BR compound brassinolide (BL). When increasing concentrations of BL were supplied, as described previously (Sui et al., 2012), the angle of the lamina joint increased in 725Ri-1, but to a much smaller extent than that in the wild type under the same conditions (Fig. 2, E and F). In the 702Ri-1-1 mutant plants, the angle also increased upon treatment with increasing concentrations of BL; the extent of bending was greater than that of 725Ri-1 but markedly less than that of the wild type (Fig. 2, E and F). This result showed that the 702Ri-1-1 plants could also respond to exogenous BL treatment, but the response was less sensitive than that of wild-type plants. Taken together, these data suggested that, similar to SDG725, MRG702 also affects BR biosynthesis and signaling pathways.

Next, we analyzed the transcript levels of several rice BR-related genes (Sui et al., 2012), including three genes involved in BR signaling pathways (Brassinazole
resistant1, BRI1, and BU1) and three genes related to BR biosynthesis (D11, D2, and BRASSINOSTEROID-deficient Dwarf1), by quantitative RT-PCR. The transcript levels of most of the tested genes were down-regulated to varying extents, with larger decreases for D11, BRI1, and BU1 than for the other genes (Fig. 2G). This finding further demonstrated the role of MRG702 in BR biosynthesis and in BR signaling pathways by modulating the transcription of BR-related genes.

MRG702 Promotes Rice Flowering

The mrg702 mutants showed not only a typically BR-deficient morphology but also a late-flowering phenotype. When 702Ri-1-1 and wild-type plants were grown side by side at two locations with different latitudes, Shanghai, with LD conditions, and Sanya, with short-day (SD) conditions, the 702Ri-1-1 plants showed a late-flowering phenotype. At both locations, the average heading dates were much later for 702Ri-1-1 than for the wild type (Fig. 3A). The heading dates for 702Ri-1-1 were at 95 to 102 d in Shanghai and at 81 to 86 d in Sanya. These findings suggested that, like SDG725, MRG702 may be involved in promoting flowering in rice.

Rice has two flowering genes, Heading date 3a (Hd3a) and its close paralog RFT1 (Sun et al., 2014). Similar to their Arabidopsis homolog FT, both Hd3a and RFT1 are expressed in leaf phloem tissues, and their products are transported from leaves to the shoot apex to promote flowering (Tamaki et al., 2007; Komiya et al., 2009).

Many genes, such as OsGI, Hd1, Ehd3, Ehd2/OsID1/RID1, OsMADS50, OsMADS51, Ghd7, and Ehd1, have

Table I. Measurements of mature wild-type and mutant plants

| Parameter                  | Wild Type | 702Ri-1-1 | 725Ri-1 |
|----------------------------|-----------|-----------|---------|
| Plant height (cm)          | 77.94 ± 2.50 | 55.65 ± 2.1 | 46.8 ± 1.99 |
| Flag leaf length (cm)      | 30.95 ± 3.73 | 22.48 ± 2.24 | 21.43 ± 1.41 |
| Panicle length (cm)        | 20.02 ± 1.17 | 15.57 ± 0.87 | 15.7 ± 0.85 |
| Length of first internode (cm) | 32.49 ± 1.87 | 22.6 ± 0.98 | 20.53 ± 1.64 |
| Length of second internode (cm) | 15.17 ± 1.43 | 9.73 ± 0.98 | 6.76 ± 0.72 |
| Length of third internode (cm) | 8.56 ± 0.96  | 4.27 ± 1.2  | 3.13 ± 0.43  |
| Lamina joint angle (˚)     | 16.59 ± 1.49 | 11.38 ± 1.05 | 10.01 ± 1.49 |
| 1,000 unhulled grain weight (g) | 27.72 ± 1.52 | 21.27 ± 1.26 | 16.22 ± 0.92 |

Figure 3. MRG702 promotes flowering in rice. A, Heading time of wild-type (WT), 702Ri-1-1, and 725Ri-1 plants grown under LD (Shanghai) or SD (Sanya) conditions. Values shown are means ± SD (n = 30). Asterisks indicate statistically significant differences between the indicated genotypes and the wild type (P < 0.01). B, Schematic of two core flowering regulatory pathways in rice. C, Relative transcript levels of flowering regulatory genes in the indicated plants. Quantitative RT-PCR analyses were performed using leaves collected at 2 h after dawn from 28-d-old rice seedlings grown in a growth chamber under an LD photoperiod (14 h of light/10 h of dark). OsACTIN1 served as the internal control, and fold change relative to the wild-type level is shown. Values shown are means ± SD from three independent replicates. Asterisks indicate statistically significant differences between the indicated genotypes and the wild type (P < 0.01).
been identified as regulators of Hd3a/RFT1 flowering via their involvement in two core flowering regulatory pathways (Fig. 3B). We examined the transcript levels of these flowering regulatory genes and the two flowering genes Hd3a and RFT1 under SD and LD conditions in the wild type and 702Ri-1-1 mutants. For comparison, 725Ri-1 mutants were included in these analyses. Leaves from 21-d-old (for SD analysis) and 28-d-old (for LD analysis) seedlings were collected 2 h after dawn for RNA extraction and quantitative RT-PCR. Because the transcription patterns were almost the same under SD and LD conditions, only the transcript profile under LD conditions is shown (Fig. 3C). Consistent with the late-flowering phenotype of 702Ri-1-1, most of the tested genes (except for OsGI, Hd1, and Ghd7) were down-regulated compared with their transcript levels in the wild type. Similar results were obtained for 725Ri-1. Based on these findings, we concluded that MRG702 is involved in promoting rice flowering by regulating the expression of flowering regulatory genes.

The Chromodomain of MRG702 Binds Trimethylated H3K4 and H3K36 in Vitro

Members of the MRG family, such as ScEa3, HsMRG15, and AtMRG1/2, can bind to methylated H3K4/H3K36 via their N-terminal chromodomains (Zhang et al., 2006; Sun et al., 2008; Bu et al., 2014). To determine whether the chromodomain of MRG702 could bind to specific histones, we produced an MRG702 protein with glutathione S-transferase (GST) fused to its N-terminal chromodomain (amino acids 1–94) to use in a pull-down assay. In this assay, we used H3 peptides with different methylations at Lys-4, Lys-9, Lys-27, or Lys-36. Dot-blot analyses of the elution products using specific anti-histone antibodies showed that the chromodomain of MRG702 could bind to dimethylated and trimethylated H3K4 (H3K4me2/3) and trimethylated H3K36 (H3K36me3) peptides (Fig. 4A) but not to H3K9me2 and H3K27me2 (Fig. 4A). This analysis showed that binding of the MRG702 chromodomain occurs in a substrate-specific manner.

To further verify the binding specificity of MRG702, we performed an isothermal titration calorimetry (ITC) assay using free-labeled histone peptides as substrates. The binding isotherms showed that the MRG702 chromodomain bound to H3K4me3 (residues 1–9; dissociation constant [Kd] = 0.63 mM) and H3K36me3 (residues 31–41; Kd = 0.71 mM; Fig. 4B). By contrast, the MRG702 chromodomain bound to H3K4me2 (residues 1–9) at a relatively low affinity (Kd = 3.51 mM; Supplemental Fig. S3). The MRG702 chromodomain did not bind to H3K4me1 (residues 1–9), H3K36me1/2 (residues 31–41), H3K9me2 (residues 4–13), or H3K27me2 (residues 23–31). This analysis clarified that the MRG702 chromodomain binds specifically to trimethylated H3K4 and H3K36 in vitro.

Yellow Fluorescent Protein-Fused MRG702 Colocalizes with Trimethylated H3K4 and H3K36 in the Nucleus

To gain insights into the function of MRG702 in vivo, we made a Yellow Fluorescent Protein (YFP) fusion construct (MRG702-YFP) driven by the maize Ubiquitin promoter (pUBI::MRG702-YFP) and transformed it into wild-type rice plants. Using the root cells of the
transgenic plants overexpressing MRG702-YFP, which showed a wild-type phenotype and normal flowering time, we performed dual immunofluorescence staining with an antibody against GFP and specific residue-methylated H3 antibodies against H3K4me3, H3K36me3, H3K9me3, and H3K27me2. As expected, the euchromatic histone modifications H3K4me3 and H3K36me3 were ubiquitously localized in the euchromatic regions of the nucleus. The heterochromatic histone modifications H3K9me2 and H3K27me2 were more concentrated in the 4',6-diamidino-2-phenylindole-dense heterochromatin (Fig. 5). Consistent with a previous report that Arabidopsis MRG proteins function as chromatin effectors (Bu et al., 2014), MRG702-YFP was distributed exclusively in the nucleus in a similar pattern to those of H3K4me3 and H3K36me3 (Fig. 5). The fluorescence of MRG702-YFP merged with the signals of H3K4me3 and H3K36me3 but not with those of H3K9me2 or H3K27me2 (Fig. 5). The overlapping localization of MRG702 and H3K4me3/H3K36me3 in vivo suggested that the function of MRG702 may be closely associated with the histone modifications of H3K4me3 and/or H3K36me3.

MRG702 Function Is Dependent on SDG725-Mediated H3K36me3

To uncover the molecular mechanisms by which MRG702 regulates the BR pathway and controls flowering time, we performed a chromatin immunoprecipitation (ChIP) assay. This allowed us to analyze the association between MRG702 and histone methylations at the chromatin of target genes in vivo. First, the antibodies against H3K4me3 and H3K36me3 were used in ChIP assays to analyze histone methylation patterns in wild-type and 702Ri-1-1 plants. Probably due to the down-regulation of the genes, slightly reduced H3K4me3 levels were observed in 702Ri-1-1 at D11 and RFT1/Hd3a loci, while the H3K36me3 levels in most regions of D11 and RFT1/Hd3a were not obviously affected in 702Ri-1-1 compared with the wild type (Supplemental Fig. S4). We obtained the MRG702-YFP/725Ri-1 plants by genetic crossing, which overexpressed MRG702-YFP in 725Ri-1 mutant background. Both MRG702-YFP (wild-type) and MRG702-YFP/725Ri-1 plants showed similar levels of the transgene product (Supplemental Fig. S5). As described previously (Sui et al., 2012), 725Ri-1 mutants showed reduced levels of H3K36me3 but not H3K4me3 in most D11 chromatin regions. In contrast, there was no obvious change in the levels of H3K4me3 and H3K36me3 in transgenic pUBI::MRG702-YFP (wild-type) plants as compared with control plants overexpressing YFP (wild-type). This finding indicated that MRG702 does not affect the deposition of H3K4me3 and H3K36me3 at D11 chromatin regions (Fig. 6A). In the ChIP experiments using the antibody against GFP, the YFP-tagged MRG702 was clearly enriched in the D11 chromatin, especially in the promoter and 3' coding regions (Fig. 6A). We conducted further ChIP experiments to test the enrichment of MRG702-YFP in 3' coding regions of other BR-related genes. The results showed that MRG702 directly targets the chromatin of BR-related genes (Supplemental Fig. S6). However, with the reduced H3K36me3 levels in 725Ri-1 plants, MRG702-YFP enrichment at these target gene loci was clearly decreased (Supplemental Fig. S6), suggesting that MRG702 may directly bind to the BR-related genes by recognizing H3K36 methylation in planta.

We also investigated the histone methylation status and MRG702-binding capacity at the chromatin of flowering genes. We selected the closely located florigen genes Hd3a and RFT1 for detailed analyses. As shown in Figure 6B, MRG702-YFP was more enriched at the chromatin regions of both genes than was the YFP control. With the decreased H3K36me3 levels in 725Ri-1 plants, there was markedly decreased MRG702-YFP binding to the chromatin of Hd3a and RFT1 (Fig. 6B). There were similar patterns of MRG702 binding at other flowering genes in wild-type and 725Ri-1 plants (Supplemental Fig. S7). These findings further confirmed that, in vivo, MRG702 can directly target specific flowering genes in an SDG725-mediated, H3K36me3-dependent manner.

DISCUSSION AND CONCLUSION

H3K36 methylation plays critical functions in the BR pathway and in controlling flowering time in rice (Sui et al., 2012, 2013; Sun et al., 2012). Here, to our knowledge for the first time, we show that MRG702 serves as a reader protein for H3K36 trimethylation in rice. The first evidence came from the in vitro binding assay, which showed that the chromodomain of MRG702 preferentially binds to H3K4me3 and H3K36me3 peptides. The immunolocalization analysis showed that MRG702-YFP

Figure 5. MRG702-YFP proteins colocalize with H3K4me3 and H3K36me3 in vivo. Dual immunolocalization of MRG702-YFP (green) and methylated histones (red) in nuclei of root cells is shown. 4’,6-Diamidino-2-phenylindole (DAPI)-stained DNA is shown in blue. Merged green and red signals are shown at right. The heterochromatin regions are indicated by arrows. Bars = 10 μm.
colocalizes with trimethylated H3K4/H3K36. More importantly, MRG702 binding to chromatin depends on the level of H3K36me3 in vivo, because the binding of MRG702 was markedly decreased in the sdg725 mutant, in which H3K36me3 deposition is impaired. Similar to MRG702, its homologs such as Eaf3 from yeast, MRG15 from animals, and MRG1/2 from Arabidopsis also bind to H3K36me3 (Zhang et al., 2006; Sun et al., 2008; Bu et al., 2014), demonstrating the evolutionary conservation of the ability of MRG family proteins to bind to H3K36me3.

Although MRG proteins share the same domain organization and show similar binding ability in rice and Arabidopsis, they have different roles in controlling flowering time. In Arabidopsis, the loss-of-function mutant of the H3K36 methyltransferase SDG8 displays FLOWERING LOCUS C (FLC) repression and an early-flowering phenotype (Zhao et al., 2005), whereas the loss-of-function mutants of the reader protein genes MRG1/2 show an opposite late-flowering phenotype (Bu et al., 2014; Xu et al., 2014). Although the expression of the floral repressor gene FLC, the target of SDG8, is indeed reduced in the mrg1 mrg2 double mutant (Xu et al., 2014), its effect on flowering appears to be overridden by the down-regulation of the floral gene FT in the mrg1 mrg2 double mutant under LD conditions in a CO-dependent pathway. Studies on the H3K36 methyltransferases SDG725 and SDG724 in rice have demonstrated that SDG725-mediated H3K36 methylation plays a crucial function in BR regulation (Sui et al., 2012)

Figure 6. ChIP assays at D11 and RFT1/Hd3a loci. Leaves were collected at 2 h after dawn from 14-d-old (for BR-related gene analysis) or 28-d-old (for flowering gene analysis) rice seedlings grown in a growth chamber under an LD photoperiod (14 h of light/10 h of dark). ChIP analyses were performed at the D11 (A) and RFT1-Hd3a (B) chromatin regions using antibodies against H3K4me3, H3K36me3, and GFP. Data shown are percentage input values (ratio obtained by taking a fixed aliquot of the DNA extracted from the immunoprecipitated samples and the input). Values shown are means ± sd from three parallel biological replicates. Asterisks indicate statistically significant differences between the MRG702-YFP/725Ri-1 and the MRG702-YFP (wild-type) plants (P < 0.01).
and that SDG724 and SDG725 are both involved in controlling flowering time, with overlapping and specific targets (Sun et al., 2012; Sui et al., 2013). Similarly, MRG702 is also required to promote BR-related gene expression and rice flowering by directly binding to H3K36 methylation at target gene loci. Considering the similar dwarf and late-flowering phenotypes of mutants and the shared target genes of MRG702 and SDG725, we believe that MRG702 is more likely to function through SDG725-mediated than through SDG724-mediated H3K36 methylation.

There are several possible explanations for the functional divergence of MRG proteins in controlling flowering time between rice and Arabidopsis. One explanation is related to the key floral repressor FLC. Many studies have revealed that the FLC gene can be modulated by various active and repressive chromatin modifications, such as histone methylation, acetylation, and ubiquitination (He, 2012; Sun et al., 2014). However, this key floral repressor that is present in Arabidopsis is absent from rice. In addition to FLC, another key flowering integrator, florigen FT, is promoted by the transcription factor CO in a day-length-dependent pathway to trigger flowering transition in Arabidopsis (Turck et al., 2008). The CO-FT flowering pathway appears to be conserved between rice and Arabidopsis, because the respective homologs Hd1 and Hda3a have been identified in rice (Yano et al., 2000; Kojima et al., 2002). In Arabidopsis, loss of MRG1/2 functions resulted in the down-regulation of FT but not of CO (Bu et al., 2014). The mrg702 mutant of rice showed reduced mRNA levels of Hda3a but not of Hd1. In Arabidopsis, CO accelerates FT expression and flowering under LD conditions (Turck et al., 2008). In rice, however, Hd1 promotes Hda3a expression and flowering under SD conditions and represses them under LD conditions (Sun et al., 2014), suggesting that Hd1 functions as either an activator or a suppressor of Hda3a. Therefore, although members of the Hd1/CO-Hda3a/FT pathway are conserved between rice and Arabidopsis, their functions in flowering responses may have diverged during the evolution of the two plant species. More importantly, Arabidopsis MRG1/2 activate FT not only by H3K4me3/H3K36me3 recognition but also by interacting with the transcription factor CO. The binding between rice MRG702 and the CO homolog Hd1 cannot be detected in our in vitro binding assay (Supplemental Fig. S8), indicating that, in addition to the different flowering regulatory mechanisms between the two species, the distinct functional performance of MRG702 in rice is also probably due to the divergence of the MRGs themselves.

In rice, a distinct flowering regulatory Ehd1-RFT1 pathway has been identified that is absent from Arabidopsis. This pathway in rice involves Ehd1, RFT1, and several other flowering regulators, such as Ehd2, Ehd3, Ghd7, OsMADS550, and OsMADS551 (Doi et al., 2004; Sun et al., 2014). This unique floral pathway may provide direct evidence that MRG702 functions in an SDG725-dependent manner, because in both mrg702 and sdg725 mutants, the regulatory genes involved in the Ehd1-RFT1 pathway (except for the repressor Ghd7) were all down-regulated. SDG724 is only responsible for the specific deposition of H3K36 methylation at RFT1 and OsMADS50 (Sun et al., 2012). Our results show that MRG702 directly binds to the chromatin of target genes, while the loss of SDG725 impairs this binding. These results further demonstrate that the function of MRG702 is dependent on SDG725-mediated H3K36 methylation. The MRG702 and SDG725 double knockdown line 725Ri-1 702Ri-1-1 was obtained by genetic crossing. The morphologies of 702Ri-1-1 725Ri-1 plants resembled those of 725Ri-1, with more severe phenotypes than 702Ri-1-1 plants. The genetic analysis, together with the H3K36me3-dependent MRG702 recruitment to the target genes, suggest that, unlike Arabidopsis MRG1/2, which acts as a cofactor of the transcription factor CO to activate FT, MRG702 looks more like a relatively broader spectrum reader protein to decipher the H3K36 methylation set by SDG725. In this way, MRG702 regulates the expression of BR pathway and flowering genes in rice.

It is worth noting that the chromodomain of MRG702 showed a high affinity for H3K36me3 and H3K4me3 in our dot-blot binding assays and ITC experiments. The ChIP assays revealed that MRG702-YFP proteins are enriched at the H3K4me3-enriched D11 promoter, where H3K36me3 is not abundant. Thus, although mrg702 and sdg725 mutants share a similar overall morphology and the same targets, we cannot exclude the function of H3K4me3 in recruiting MRG702 to target genes in planta. Very recently, a Trithorax group protein, OsTrx1, was shown to control flowering time in rice via its interaction with Ehd3 (Choi et al., 2014). Although whether OsTrx1 possesses H3K4 methyltransferase activity needs to be determined, additional molecular and genetic studies are required to determine how MRG702 recognizes the histone methylation marks and activates gene expression in rice.

**MATERIALS AND METHODS**

**Phylogenetic Analysis**

According to the predictions of the SMART program and the ChromDB database (www.chromdb.org), the full-length sequences of plant MRG proteins and some previously identified members from yeast and animals were aligned using ClustalW. The phylogenetic tree was constructed using MEGA4.0 software with bootstrapping set at 500 replicates.

**Plant Material and Growth Conditions**

Rice (Oryza sativa 'Nipponbare') plants were used in this study. For the phenotype study, plants were grown in two locations with different latitudes: Shanghai, under a natural LD photoperiod, and Sanya, under a natural SD photoperiod. The seedlings used for molecular experiments were grown in artificial growth chambers under an LD photoperiod (14 h of light at 30°C and 10 h of dark at 28°C) or an SD photoperiod (10 h of light at 30°C and 14 h of dark at 28°C).

**RNA Extraction and Quantitative RT-PCR**

We extracted RNA from 14-d-old (for BR-related gene analysis), 21-d-old (for flowering gene analysis under SD conditions), and 28-d-old (for flowering gene analysis under LD conditions) seedlings. The RNA was extracted using Trizol Reagent according to the manufacturer’s instructions (Invitrogen;
http://www.invitrogen.com. RT was performed using standard procedures with Invitrogen-TM reverse transcriptase (Promega; http://www.promega.com). Quantitative PCR was performed using gene-specific primers (Supplemental Table S1). We used OsACTIN1 as the reference gene to normalize data.

Transgene Constructs and Plant Transformation

DNA fragments containing nucleotides 1–206 to 39 and 670 to 896 of the MRG702 transcript were used as the inverted repeats to create the hairpin structure. These fragments were amplified using the primers listed in Supplemental Table S1. Each of the constructs was inserted into the plant expression vector pHB containing a double cauliflower mosaic virus 35S promoter, yielding p35S::MRG-RNAi-1 and p35S::MRG-RNAi-2, which were used for plant transformation. The full-length open reading frame of MRG702 was amplified by RT-PCR using the primers listed in Supplemental Table S1 and then fused with the sequence encoding Enhanced Yellow Fluorescent Protein (EYFP) and inserted into the plant expression vector pU1301 containing the maize Ubiquitin promoter, yielding pU1B::MRG702-EYFP. Agrobacterium tumefaciens (strain EHA105)-mediated plant transformation was performed as described previously (Sun and Zhou, 2008).

In Vitro Binding Assay

The DNA fragment encoding the chromodomain of MRG702 (residues 1–94) was cloned into the pGEX-4T1 expression vector (GE Healthcare; http://www.gelifesciences.com). The GST-fused proteins were expressed in bacteria and purified as described previously (Bu et al., 2011). The dot-blot binding assay was performed as described previously (Bu et al., 2014). Methylation H3K4 (H3K4me1/2/3; residues 1–21) and H3K36 (H3K36me1/2/3; residues 21–44) peptides were synthesized by Scilight Biotechnology (http://www.scilight-peptide.com). Anti-monomethyl-H3K4 (07-436), anti-dimethyl-H3K4 (07-030), anti-trimethyl-H3K4 (07-473), and anti-dimethyl-H3K27 (07-452) antibodies were purchased from Millipore (http://www.millipore.com). Anti-dimethyl-H3K9 (ab1220), anti-trimethyl-H3K36 (ab9050) and anti-dimethyl-H3K9 (ab1220) antibodies from Abcam, anti-trimethyl-H3K4 (07-473) and anti-dimethyl-H3K36 (ab9050; Abcam), and anti-GFP (A-11122; Invitrogen) antibodies. To determine the enrichment of immunoprecipitated DNA, quantitative PCR was performed using a kit from Takara (http://www.takara-bio.com). Gene-specific primers are listed in Supplemental Table S1. The efficiency values are the ratios determined by taking a fixed aliquot of the DNA extracted from the immunoprecipitated samples and the input. Values shown are means and so from three parallel biological replicates.

Sequence data from this article can be found in the Rice Annotation Project Database (http://rice.plantbiology.msu.edu) under accession numbers Os04g01130 (MRG701) and Os11g34300 (MRG702).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Nucleotide sequence alignment of MRG701 and MRG702.

Supplemental Figure S2. Correlation between phenotype and MRG702 expression.

Supplemental Figure S3. ITC measurements of binding between MRG702 chromodomain and various histone peptides.

Supplemental Figure S4. ChIP assays at D11 and RFT1/Hd3a loci in wild-type and 702Ri-1-1 plants.

Supplemental Figure S5. Protein levels of YFP or YFP-tagged MRG702 in the indicated genotypes.

Supplemental Figure S6. ChIP assays at BR-related genes.

Supplemental Figure S7. ChIP assays at flowering regulatory genes.

Supplemental Figure S8. Interaction of purified His-tagged MRG702 and GST-fused Hd1 from Escherichia coli by pull-down assays.

Supplemental Table S1. Primers used in the study.

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