Arabidopsis MRG domain proteins bridge two histone modifications to elevate expression of flowering genes

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Received April 14, 2014; Revised July 30, 2014; Accepted August 18, 2014

ABSTRACT

Trimethylation of lysine 36 of histone H3 (H3K36me3) is found to be associated with various transcription events. In Arabidopsis, the H3K36me3 level peaks in the first half of coding regions, which is in contrast to the 3′-end enrichment in animals. The MRG15 family proteins function as ‘reader’ proteins by binding to H3K36me3 to control alternative splicing or prevent spurious intragenic transcription in animals. Here, we demonstrate that two closely related Arabidopsis homologues (MRG1 and MRG2) are localised to the euchromatin and redundantly ensure the increased transcriptional levels of two flowering time genes with opposing functions, FLOWERING LOCUS C and FLOWERING LOCUS T (FT). MRG2 directly binds to the FT locus and elevates the expression in an H3K36me3-dependent manner. MRG1/2 binds to H3K36me3 with their chromodomain and interact with the histone H4-specific acetyltransferases (HAM1 and HAM2) to achieve a high expression level through active histone acetylation at the promoter and 5′ regions of target loci. Together, this study presents a mechanistic link between H3K36me3 and histone H4 acetylation. Our data also indicate that the biological functions of MRG1/2 have diversified from their animal homologues during evolution, yet they still maintain their conserved H3K36me3-binding molecular function.

INTRODUCTION

In eukaryotes, including plants, post-translational covalent modifications on histones play a pivotal role in controlling gene expression at the chromatin level. Different histone modifications act sequentially or in combination to confer distinct transcriptional outcomes. Although histone modifications are conserved to a large extent amongst eukaryotes, there is some divergence in terms of the distribution of these histone modifications in the genome and their biological functions between plants and animals. For example, in yeast, worms and mammals, trimethylation of lysine 36 of histone H3 (H3K36me3) preferentially marks the exons of transcribed genes and peaks at the 3′-end of the coding region, and it has been shown to be involved in various activities, including the control of alternative splicing and the prevention of spurious intragenic transcription (1–5). In contrast, the H3K36me3 level in Arabidopsis peaks at the 5′-end of the coding region, which resembles the distribution patterns of active transcription-linked histone modifications, H3K4me2/3 and acetylated H3 (6). This preferential enrichment at the first half of the coding region in plants suggests that the mechanism governing H3K36me3 deposition, and possibly its effect on transcriptional events, may differ between plants and other eukaryotes.

Whilst some histone modifications such as acetylation can directly modulate chromatin structures, an increasing body of evidence suggests that individual histone modifications, or a combination of them, may serve as a platform to recruit specific ‘reader’ proteins, which then determine the transcriptional outcome of the target genes. The yeast homologue of the human MORF4-related gene on chromosome 15 (MRG15), Esal-associated factor 3 (Eaf3), was the first identified ‘reader’ for H3K36me3 (2–4). MRG15 proteins are highly conserved across multiple species, including fruit flies (Drosophila melanogaster), worms (Caenorhabditis elegans) and yeast (Schizosaccharomyces pombe and Saccharomyces cerevisiae) (7,8). The MRG15 family proteins contain the conserved chromodomain at its amino terminus, which binds to H3K36me3. Chromodomain and chromo-like domain (such as Tudor and PWWP domains) are protein modules that are found in many chromatin-related proteins in nucleoprotein complexes (9). They have been shown to recognise and bind to methylated-Lys at the histone tails and hence recruit the protein complexes to play an important role in histone modifications and chromatin-remodelling, which control the transcription status of a large number of genes. For example, the chromodomain of chromatin-binding proteins heterochromatin-binding protein 1 (HP1) and Polycomb (Pc) bind to methylated Lys9
and Lys27 of histone H3, respectively, directing heterochromatin formation and/or gene silencing (10–13). The chromodomain of human MRG15 and its homologue in yeast, Eaf3, which is unlike the typical chromodomain found in HP1 and Pc proteins, is assumed to be an auto-inhibited chromo barrel domain and binds to trimethylated H3K36 and H3K4 in vitro with a relatively weak affinity (2–4,14–16).

In addition to the chromodomain, all members of the MRG family proteins contain the MRG domain, which shares sequence similarity with the Mortality factor on chromosome 4 (MORF4), a cell-senescence protein in humans, and that may be involved in protein–protein interactions (2–4,8,17,18). Biochemical assays have shown that the animal MRG15 proteins and the yeast homologues associate with at least two independent and antagonising nucleo-protein complexes that contain either histone acetyltransferases (HAT) or histone deacetylases (HDAC) (2–4,18–20). In mouse and Drosophila, deletion or knockdown of MRG15 genes cause embryonic-lethality phenotypes, whilst loss-of-function of S. cerevisiae Eaf3 and S. pombe altered polarity mutant-13 (Alp13) are viable (21,22). The loss of Alp13 in fission yeast causes growth arrest, sterility, defects in cell polarity and is associated with global hyperacetylation of histones and chromosome instability (22). Alp13 represses the expression of repeated regions and maintains the heterochromatin through the recruitment of histone deacetylation complexes to the repeat regions (23). Eaf3 in S. cerevisiae was suggested to suppress intragenic transcriptional initiation by recruiting the histone deacetylase complex to H3K36me3-containing nucleosomes (2–4). Eaf3 also specifically targets promoter regions of heat-shock and ribosomal protein genes for transcriptional activation through the recruitment of NuA4-dependent histone H4 acetylation complexes (24–26). Due to these dual functions, the deletion of Eaf3 greatly alters the global genomic profile of histone modification, with increased acetylation levels at coding sequences and decreased acetylation levels at the promoter regions (21). MRG-1, an MRG15 homologue in C. elegans, was recently reported to be involved in homologous chromosome pairing, which is independent of both the pairing centre and meiotic homologous recombination (27). These data suggest that the function of MRG15 family proteins have diversified in different species and participate in varied biological processes. The Arabidopsis genome contains two MRG15 homologues with high similarities in their protein sequences (8). However, whether they maintain their functions as H3K36me3 readers and effectors and which biological process they are involved in are largely unknown.

Histone acetylation is one of the histone modifications that is well known to be linked with active transcription (28). Histone acetylation may neutralise a positive charge that is well known to be linked with active transcription (28). Histone acetylation may neutralise a positive charge that is well known to be linked with active transcription.

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of sdg7 (SALK_131218) in which T-DNA is inserted in the exon 4 were isolated from SALK collections. Plants were grown in LDs (16-h light/8-h dark) at 23°C. Genotyping primer sequences are shown in Supplementary Table S1.

Plasmid construction and plant transformation

To construct 35S::MRG2-HA and 35S::GFP-MRG2 plasmids, the full length MRG2 coding sequence was amplified by primer set MRG2- MluI_F and MRG2-XmaI_R, or MRG2-ApaI_F and MRG2-XmaI_R2, cloned into vectors pGREEN-35S-HA by using MluI and XmaI or pGREEN-35S-GFP by using ApaI and XmaI.

For pMRG2::MRG2-GUS, pMRG2::HA-MRG2 and pHAM1::HA-HAM1 plasmids, a ∼7.2-kb MRG2 genomic fragment (from −3406 to +3845; A of the start codon was set as +1) and a ∼4.0-kb HAM1 genomic fragment (from −1371 to +2579) amplified using primers gDMRG2-cacc_F and gDMRG2-XmaI_R or gDHAM1-cacc_F and gDHAM1_R were cloned into pENTR/D-TOPO vector (Invitrogen). SfoI restriction site was introduced after ATG or in front of the MRG2 stop codon, or after ATG of HAM1 through mutagenesis using primers mMRG2-ATG-SfoI_F and mMRG2-ATG-SfoI_R, mMRG2-Stop-SfoI_F and mMRG2-Stop-SfoI_R, or mHAM1-ATG-SfoI_F and mHAM1-ATG-SfoI_R, respectively. H4 or GUS fragments were then inserted into the SfoI site of the pENTR/D-TOPO-gDMRG2 or pENTR/D-TOPO-gDHAM1 plasmid. Finally, gDMRG2-GUS, HA-gDMRG2 or HA-gDHAM1 in pENTR/D-TOPO plasmids were recombined into pBGW or pHGW using LR Clonase II (Invitrogen), respectively.

For pMRG1::MRG1-GUS plasmid, a ∼3.2-kb MRG1 genomic fragment (from −321 to +2878) spanning to the neighbouring genes located to the upstream and downstream of MRG1 was cloned into Topo-PCRII (Invitrogen) first using the primer set gDMRG1-NotI_F and gDMRG1_R. The genomic fragment was then released with NotI digestion and cloned into the modified pENTR vector. Mutagenesis was performed using the primers mMRG1-Stop-Scal_F and mMRG1-Stop-Scal_R. The GUS fragment was then inserted into the Scal site of the pENTR-gDMRG1 plasmid. Finally, MRG1 in pENTR was recombined into pBGW using LR Clonase II (Invitrogen). All the vectors were verified by sequencing. Primer sequences are listed in Supplementary Table S1.

Transgenic plants were generated by floral dipping with Agrobacterium tumefaciens with the corresponding constructs. The additional pSOUP helper plasmid was co-transfected for the transformation of pGREEN-35S::MRG2-HA and pGREEN-35S::GFP-MRG2.

RNA extraction and expression analysis

For the tissue expression analysis of MRG1 and MRG2, total RNAs were isolated from the root, juvenile rosette leaf, mature rosette leaf, cauline leaf, stem, flower bud, open flower and silique. For the flowering time gene expression analysis, the aerial parts of 6-day-after-germination (DAG) and 9 DAG seedlings were harvested at dusk (ZT16). For a time course assay over a 24-h-long day cycle, the aerial parts of 9 DAG seedlings were harvested every 2 or 4 h. The total RNAs were extracted using an RNasy plant mini kit (Qiagen) according to the manufacturer’s instructions. Approximately 500 ng total RNAs were used for reverse transcription with the Superscript III RT-PCR system (Invitrogen). Semi-quantitative PCR (semi-qPCR) with gene-specific primers (Supplementary Table S1) were performed using HotStarTag DNA Polymerase (Qiagen) on a Thermocycler (Bio-Rad) at 25–35 cycles. Real-time qPCR was performed on ABI PRISM 7900HT sequence detection system (Applied Biosystems) using KAPA SYBR FAST ABI Prism qPCR Master Mix (KAPA Biosystems). The ubiquitously expressed Tip41-like (AT4G34270) (39) was used as an internal reference gene. Primer sequences were shown in Supplementary Table S1.

GUS staining

GUS staining was performed as previously described (40). Briefly, plant tissues were fixed with ice-cold 90% acetone for 30 min and rinsed with rinsing solution. The tissues were then incubated in staining solution at 37°C for approximately 8 h. After staining, the tissues were incubated in fixation solution overnight and washed with a serial of ethanol solution. For histological analysis, the tissues were mounted on slides with clearing solution, and images were taken with an AxioCam Icc 3 (Zeiss) under a SteREO Discovery.V12 Stereomicroscope (Zeiss).

Chromatin immunoprecipitation (ChIP) assays

ChIP experiments were performed as previously described with minor modifications (41). Briefly, total chromatin was extracted from 10 DAG seedlings and immuno-precipitated using anti-HA (Santa Cruz Biotechnology, #sc-7392) and normal mouse IgG as a control (Santa Cruz Biotechnology, #sc-2025), or anti-H3Ace3 (Millipore, #06-599), anti-H4K5Ace (Active Motif #39699), anti-H3K4me3 (Active Motif #61379) and anti-H3K36me3 (Active Motif #61021), with anti-H3 (Abcam, #ab1791) as a control. DNA fragments were recovered by phenol-chloroform extraction and ethanol precipitation. Quantitative PCR with locus-specific primers (Supplementary Table S1) was performed to measure the amounts of FT relative to that of the constitutively expressed ACTIN2 (AT3G18780) on ABI PRISM 7900HT sequence detection system (Applied Biosystems) using KAPA SYBR FAST ABI Prism qPCR Master Mix (KAPA Biosystems).

Bimolecular fluorescence complementation (BiFC) assay

The full-length coding sequences for MRG1, MRG2, HAM1 and HAM2 were fused in frame with either the coding sequence for an N-terminal yellow fluorescent protein (YFP) fragment or for a C-terminal YFP fragment in the primary pSAT1 vectors (42). To detect the interaction in tobacco, leaves of 2- to 4-week-old tobacco plants were infiltrated with Agrobacterium containing the respective plasmid pairs (43). Epidermal cell layers were examined 2–4 days after infiltration and imaged with a Zeiss LSM 5 EXCITER upright laser scanning confocal microscope (Zeiss) (41).
Co-immunoprecipitation (Co-IP) assays

Co-IP experiments were performed as described previously with minor modifications (41). Briefly, 10-day-old seedlings (~2 g) expressing pHAM1::HA-HAM1 355::GFP-MRG2 or pHAM1::HA-HAM1 were harvested and total proteins were extracted and immunoprecipitated with anti-GFP antibody (Invitrogen, #A-11122). The HA-HAM1 protein in the immunoprecipitates was detected by western blotting with anti-HA-HRP (Santa Cruz Biotechnology, #sc-7392).

Immunostaining and in situ proximity ligation assay

Isolation of nuclei and in situ immunolocalisation of chromatin proteins were performed as described with minor modifications (44). Young leaves from sdg8, sdg7 sdg8, wild-type or pMRG2::HA-MRG2 transgenic plants were used for nuclei extraction. Sample suspension was air-dried on glass slides. Each slide was blocked with 1% BSA in 1x PBS before incubating with mouse anti-H3K36me3 (Active Motif #61021) and rabbit anti-H3K36me2 (Abcam, #ab9049), or with rabbit anti-HAM1/2 (Aviva, #ARP33345_P050) and mouse anti-HA (Santa Cruz Biotechnology, #sc-7392), rabbit anti-H3K36me3 (Abcam, #ab9050) and mouse anti-H3K4me3 (Active Motif #61379), and rabbit anti-H3K9me2 (Abcam, #ab1220). Later the slides were incubated with CF555 goat anti-mouse IgG (Biotium, #20030) and/or CF 488A goat anti-rabbit IgG (Biotium, #20010). Slides were stained with DAPI and imaged with Leica TCS SP5 confocal microscope (Leica).

The in situ proximity ligation assay (PLA) assay was carried out with Duolink II kits (Genome holdings) according to the manufacturer’s instructions, with nuclei prepared with pMRG2::HA-MRG2 transgenic plants, wild-type plants and pMRG2::MRG2-HA in a sdg8 background, in a way similar to the in situ immunolocalisation assay. Mouse anti-HA (Santa Cruz Biotechnology, #sc-7392) and rabbit anti-H3K4me3 (Millipore, #07-473), rabbit anti-H3K36me3 (Abcam, #ab9050), rabbit anti-H3 (Abcam, #ab1791), rabbit anti-HAM1/2 (Aviva, #ARP33345_P050), rabbit anti-H3K9me2 (Abcam, #ab1220) or rabbit IgG (Santa Cruz, #SC-2027) were used as the primary antibodies. After washing, Duolink II PLA Probe anti–Mouse Plus (Genome holdings, #92001–0030) and Duolink II PLA Probe anti–Rabbit Minus (Genome holdings, #92005–0030) were applied as the probes. Duolink II Detection Reagents (Genome holdings, #92008–0030) were used to amplify the interaction signal, and the fluorescing signal was imaged with a Leica TCS SP5 confocal microscope (Leica).

Peptide pull-down assays

The chromodomain of MRG1 (1-131 aa) or MRG2 (1-130 aa) was cloned into pGST-4T-1 (GE Healthcare) with the primer set GST-MRG1-CD-BamHI_F and GST-MRG1-CD-XmaI_R, or GST-MRG2-CD-BamHI_F and GST-MRG2-CD-XmaI_R. GST alone, GST-MRG1 CD or GST-MRG2 CD (1 μg) expressed in E. coli Rosetta was incubated in binding buffer [20 mM Tris-HCl pH 8.0, 250 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 1x protease inhibitor cocktail (Roche)] with 10 μg of BSA and 1 μg biotinylated trimethylated histone H3K36 peptide (Epigentek, #R–1050–100), trimethyl histone H3K4 (Epigentek, #R–1023–100), trimethyl histone H3K9 (Epigentek, #R–1029–100), trimethyl histone H3K27 (Epigentek, #R–1035–100) or biotinylated unmodified histone H3 aa 22–44 (Epigentek, #R–1060–100) and biotinylated unmodified histone H3 aa 1–21 (Epigentek, #R–1004–100) immobilised on streptavidin-agarose. The beads were then washed with binding buffer and analysed by western blotting with antibodies against GST (Santa Cruz, #sc-138).

RESULTS

The mrg1 mrg2 double mutant shows a late-flowering phenotype

In Arabidopsis, there are two closely related MRG15 homologues MRG1 (AT4G37280) and MRG2 (AT1G02740) that share high sequence similarities (Supplementary Figure S1; (8)). We first identified the mrg2-1 (SALK_035089) mutant, in which the T-DNA insertion in the fourth exon (Figure 1A) abolished the expression of the full-length transcript of MRG2 (Figure 1B). Whilst the homozygous plants showed male and female sterility due to a meiosis defect, fertility was normal in other mrg2 loss-of-function alleles, indicating that it is not linked with the mrg2 mutation. We also identified the mrg1-1 (SALK_057762) mutant, in which T-DNA is inserted in the first intron with no full-length transcript produced (Figure 1A and B). Although the single mrg1-1 did not show any mutant phenotypes, mrg1-1 mrg2-1 showed a late-flowering phenotype under long-day (LD) growth conditions (Figure 1C, Supplementary Figure S2A, B). We isolated two more T-DNA mutant alleles of mrg2, mrg2-2 (SK28487) and mrg2-3 (GK-255G06) (Figure 1A). T-DNA insertion in the first and ninth intron, respectively, abolished the production of the MRG2 full-length transcript (Figure 1A and B, Supplementary Figure S2A, E), but they did not show any sterility phenotype. Consistent with mrg1-1 mrg2-1, mrg1-1 mrg2-2 and mrg1-1 mrg2-3 showed similar late-flowering phenotypes (Figure 1C and D, Supplementary Figure S2A–D). Moreover, introduction of the pMRG2::HA-MRG2 transgene into mrg1-1 mrg2-2 completely rescue the late-flowering phenotype in the mrg1 mrg2 double mutant (Figure 1C and D). These results show that MRG1 and MRG2 are redundantly involved in flowering control. Due to the similarity in their phenotype, we used mrg1-1 mrg2-2 (herein mrg1 mrg2) for all subsequent experiments.

MRG1 and MRG2 are required for higher expression levels of FLC and FT

To investigate which flowering genes are responsible for the late-flowering phenotype in mrg1 mrg2, we harvested the aerial parts of the seedlings at 9 DAG (day after germination, at the beginning of the dark photoperiod under LD conditions) just before the floral induction. We then compared the expression of a series of flowering time genes between WT and mrg1 mrg2. Whilst AGL24, MAF1-5 and SVP were un-affected in mrg1 mrg2 (Supplementary Figure
**Figure 1.** MRG1 and MRG2 are required for high transcriptional levels of FT and FLC. (A) Schematic drawings of the genomic structures of MRG1 and MRG2. The smaller gray boxes indicate the 5′ and 3′ UTRs. Black lines indicate the introns. The larger boxes indicate exons with gray boxes and white boxes further showing the chromodomain and MRG domain coding regions, respectively. T-DNA insertion sites are marked by downward arrows above the schematic diagrams. The pairs of arrowheads on MRG1 and MRG2 genomic structures indicate the position for the semi-quantitative RT-PCR primers designed to detect MRG1 and MRG2 transcripts. Bar, 500 bp. (B) Semi-quantitative RT-PCR of MRG1 and MRG2 in Col wild-type (WT), mrg1-1, mrg2-1 and mrg2-2, respectively. Primers are shown in A. Ubiquitously expressed Tip41-like gene (AT4G34270) was used as a control. (C) Total number of rosette leaves before bolting in Col WT, mrg1-1 mrg2-1, mrg1-1 mrg2-2 and mrg1-1 mrg2-2 pMRG2::HA-MRG2 plants grown in 16 h light/8 h dark long-day condition (LD) at 23°C were counted (two independent experiments with 15–20 plants each). *P < 0.05 with Student’s t-test to indicate the difference of flowering time between Col WT and mrg1-1 mrg2-1, mrg1-1 mrg2-2 and mrg1-1 mrg2-2 pMRG2::HA-MRG2. No significant difference between mrg1-1 mrg2-2 and mrg1-1 mrg2-3 (P = 0.50), Col WT and mrg1-1 mrg2-2 pMRG2::HA-MRG2 (P = 0.49). (D) mrg1-1 mrg2-2 bolted later than Col WT when grown in LD 23°C (2 plants each, left panel), and pMRG2::HA-MRG2 rescued the late-flowering phenotype of mrg1-1 mrg2-2 (right panel) grown at the same condition. (E) Quantitative RT-PCR-based expression levels of FLC and FT in 6 and 9 DAG seedlings of Col WT and mrg1-1 mrg2-2 grown in LD 23°C. Bars indicate SD of three biological replicates. *P < 0.05 with Student’s t-test.

S3A–G), FLC, FT and SOC1 transcription levels were reduced approximately 2-fold in mrg1 mrg2 (Figure 1E, Supplementary Figure S3H). As SOC1 is one of the downstream targets of FT, the reduction of SOC1 could be due to the indirect effect of loss-of-function of mrg1 and mrg2. Because FT functions downstream of FLC, the FT reduction at 9 DAG may lead to late-flowering. Down-regulation of FT in spite of the reduced FLC expression in the mutant indicates that FT may be directly controlled by MRG1 and MRG2. To further characterise the transcriptional change of FLC and FT in mrg1 mrg2, we next compared their expression between WT and mrg1 mrg2 plants at an earlier stage at 6 DAG, and found that FLC expression is reduced to half in mrg1 mrg2 at both 6 DAG and 9 DAG (Figure 1E). Although FT showed a 2-fold decrease in expression at 9 DAG, the FT expression at 6 DAG was not affected (Figure 1E). FT is expressed at a relatively low level in the WT plant at 6 DAG compared with that at 9 DAG (45). Similar expression patterns of FT and FLC were also observed in the different allele mrg1-1 mrg2-3 (Supplementary Figure S2F). These data suggest that two redundant MRG1 and MRG2 are essential in ensuring the maximum endogenous expression level of their putative targets FT and FLC but may not act in the initial transcriptional activation of FT. Transcriptional up-regulation by MRG1 and MRG2 on FT may require a threshold level of FT expression or the accumulation of the transcription-associated epigenetic marks, such as H3K36me3.
MRG1 and MRG2 are ubiquitously expressed and localised in euchromatin. Quantitative RT-PCR with RNA extracted from different tissues showed that MRG1 and MRG2 are ubiquitously expressed in the whole plants (Figure 2A). A GUS staining assay of plants harbouring the GUS fusion transgene pMRG1::MRG1-GUS and pMRG2::MRG2-GUS confirmed that MRG1 and MRG2 expression is detectable in all tissue types, especially in the vasculatures of true leaves and young leaf primordia (Figure 2B), which match the expression patterns of FLC and FT. The subcellular localisation of the MRG proteins was examined using the root of 35S::GFP-MRG2 transgenic plants. GFP-MRG2 was found to be localised in the euchromatic regions of the nucleus but not in the DAPI-dense heterochromatin (Figure 2C), consistent with the previous reports that MRG proteins function as chromatin remodelling factors (8).

MRG2 binds to H3K36me3 in vitro and in vivo

It has been shown that the chromodomain at the N-terminus of MRG15 homologues can bind to H3K36me3 in both yeast and humans and to H3K4me3 in yeast with low affinity (2–4,14–16). To check whether the chromodomain of Arabidopsis MRG proteins interacts directly with these modified histones, we cloned the N-terminal chromodomain (1–131 aa) of MRG1 and chromodomain (1–130 aa) of MRG2 to produce the GST-MRG1/2 chromodomain fusion proteins (MRG1-CD and MRG2-CD in Figure 3A) and performed a pull-down assay using different histone H3 peptides trimethylated at K4, K9, K27, K36, dimethylated at K36 or two unmodified histone H3 peptides (H3 1–21 and H3 21–44), all of which were biotin-labelled and immobilised on streptavidin agarose beads. Both of the chromodomains of MRG1 and MRG2 were found to bind to H3K36me2, H3K36me3 and H3K4me3 with different affinities, but not to the other histone modifications (H3K9me3 and H3K27me3) nor the unmodified histone H3 (Figure 3A). Thus, the histone-binding function of MRG1/2 to H3K36me2/3 is rather conserved across different species, but like the yeast homologue, they also bind to H3K4me3.

An in situ PLA provides another piece of evidence to support the model that MRG2 binding to histone H3 is mediated through H3K36me3 and/or H3K4me3 in vivo. An in situ PLA is able to detect the direct protein interaction with high sensitivity and specificity by integrating traditional immunolocalisation techniques with DNA-based signal amplification (46). We first carried out the immunofluorescence staining with antibody against HA-MRG2, MYST family HATs HAM1/2, H3K36me3 and H3K9me2, and confirmed that HA-MRG2 and HAM1/2 are co-localised at the euchromatin (Supplementary Figure S4C) in a similar pattern with the euchromatic histone modifications H3K4me3 and H3K36me3 (Supplementary Figure S4B), whilst H3K9me2 is specifically detectable at the heterochromatin region (Supplementary Figure S4A). Next, we performed in situ PLA assay with the tested antibodies. Using antibodies against HA and H3K4me3 and H3K36me3, the fluorescence signals of PLA were detected in the nuclei harvested from pMRG2::HA-MRG2 plants but not in WT plants without the transgene (Figure 3B and C). The negative control, using an H3K9me2 antibody, showed there is no interaction between H3K9me2 and HA-MRG2 in transgenic plants. These results indicate the close localisation between MRG2 with H3K4me3 and H3K36me3 in vivo (Figure 3C).

MRG proteins function through SDG8-mediated H3K36me3

Immunostaining using the nuclei of wild-type and sdg8 mutant leaf cells showed that mutation of the Arabidopsis H3K36 methyltransferase SDG8 leads to a near complete abolishment of H3K36me3 and a slight reduction in H3K36me2 (Figure 4A) (37,38). The double mutant with another close homologue of SDG8, sdg7 sdg8 fully abol-
Figure 3. MRG2 directly binds to H3K36me3 in vitro and in vivo. (A) In vitro binding assay. Bacteria-expressed GST alone or GST-MRG1/2 chromodomains (MRG1-CD and MRG2-CD) were tested for binding to biotinylated unmodified histone H3 (H3 1–21 amino acid and H3 21–44 amino acid) or modified histone H3K4me3, H3K9me2, H3K27me3, H3K36me2 and H3K36me3 peptides immobilised on streptavidin-agarose. MRG1/2-CD were pulled down only by H3K4me3 and H3K36me2/3. GST was used as the negative control. (B) Schematic diagram showing in situ PLA assay between MRG2 and H3K4/36me3. (C) In situ PLA assay performed using anti-HA and anti-H3K4me3/H3K36me3 antibodies to show the interaction between HA-MRG2 and H3K4me3/H3K36me3. Blue, DAPI. Red, in situ PLA signals showing the interaction between HA-MRG2 and H3K4me3/H3K36me3. Purple, merge. Top two rows, in situ PLA assay using anti-HA and anti-H3K4me3/H3K36me3 antibodies in pMRG2::HA-MRG2; third row, in situ PLA assay between pMRG2::HA-MRG2 and H3K9me2 as a negative control; bottom row, in situ PLA assay using anti-HA and anti-H3K36me3 antibodies in WT as a negative control. Scale bar, 5 μm.

Figure 4. MRG1/2 function is dependent on H3K36me3. (A) Dual immunolocalisation of H3K36me2 and H3K36me3 in Col wild-type (WT, top panels), sdg8 (middle panels) and sdg7 sdg8 (bottom panels) nuclei. H3K36me2 (green) and H3K36me3 (red) were detectable using the respective antibodies in WT but not in sdg7 sdg8, whilst sdg8 could still retain a reduced level of H3K36me2. Blue, DAPI. Scale bar, 5 μm. (B-C) RT-qPCR of FLC (B) and FT (C) expression in Col WT, sdg8, sdg7 sdg8, sdg8 mrg1 mrg2, 35S::MRG2-HA (OX-MRG2) and sdg8 OX-MRG2. Error bars indicate SD of three biological replicates. *P < 0.05 between FLC expression in Col WT and other genotypes including sdg8, sdg7 sdg8, OX-MRG2 and sdg8 OX-MRG2, Col WT and OX-MRG2 at both 6 DAG and 9 DAG with Student’s t-test. *P < 0.05 between FT expression in Col WT and other genotypes including sdg8, sdg7 sdg8, sdg8 mrg1 mrg2, OX-MRG2 and sdg8 OX-MRG2 at 9 DAG only. (D, E) The interaction between MRG2 and histone H3 is dependent on H3K36me3. Schematic diagram for in situ PLA assay between MRG2 and histone H3 (D). In situ PLA assay using anti-HA and anti-H3 antibodies in pMRG2::HA-MRG2 WT showed positive signals (upper panels in E), whilst in situ PLA assay with pMRG2::HA-MRG2 sdg8 showed no signals (lower panels in E). Scale bar, 5 μm. Blue, DAPI. Red, in situ PLA signals showing the interaction between HA-MRG2 and histone H3. Purple, merge.

ishes both the di- and tri-methylation marks at H3K36 (Figure 4A). In sdg8 and sdg7 sdg8, FLC expression is almost fully abolished, suggesting that the H3K36me3 is essential for FLC expression (Figure 4B) (37). We found that H3K36me2, as in Drosophila, may function differently from H3K36me3 in Arabidopsis, because detectable, albeit dras-
tically reduced FLC expression was observed in sdg7 sdg8 (Figure 4B) (47). Whilst FLC expression is reduced dramatically in sdg8 mutant seedlings as well as in sdg7 sdg8 at both DAG 6 and 9 (Figure 4B), the FT transcript is reduced to half at 9 DAG but not affected at 6 DAG in either sdg8 or sdg7 sdg8 (Figure 4C). These expression profiles of FT are similar to those observed in the mrg1 mrg2 mutant (Figure 1E), suggesting that the SDG H3K36 methyltransferases and MRG proteins may function in the same pathway to regulate FT.

To test if the function of MRG1 and MRG2 requires H3K36 methylation, we performed genetic analyses. As the effects on FLC and FT in sdg7 are as severe as those in sdg7 sdg8, we used the sdg8 single mutant. In the sdg8 mrg1 mrg2 triple mutant background, FT and FLC expression levels are similar to those in the sdg7 single mutant (Figure 4B and C). This indicates that the function of MRG1 and MRG2 in flowering time control are dependent on SDG8-mediated H3K36me3. Overexpression of MRG2 under the constitutive Cauliflower Mosaic Virus p35S promoter causes increased expression of FLC (∼2 and 1.5-folds in 6 and 9 DAG, respectively) and decreased expression of FT at 9 DAG (∼1.7-folds) (Figure 4B and C), leading to a late-flowering phenotype (Supplementary Figure S5). However, 35S::MRG2-HA failed to affect the FLC transcript or flowering time in the sdg8 mutant background (Figure 4B and Supplementary Figure S5), further confirming that the function of MRG2 is dependent on SDG8. FLC reduction is more pronounced in sdg8 than in mrg1-1 mrg2-2 (Figures 1E and 4B), suggesting that H3K36me3 may have different effect in FT and FLC expression. It is possible that additional readers other than the MRG proteins, e.g. the PHD finger proteins, may prevent further down-regulation of FLC expression in mrg1 mrg2 (48,49), and/or other H3K36me3-independent factors may maintain certain FT transcription level in sdg8 and mrg1 mrg2 (37,50–52).

Next, we tested whether the interaction of MRG proteins and histone H3 is dependent on H3K36me3 in vivo. We carried out the in situ PLA assay with antibodies for HA and histone H3 using pMRG2::HA-MRG2 transgenic plants in the wild-type and sdg8 mutant backgrounds. We observed positive signals that showed histone H3-MRG2 interaction in the wild-type background (Figure 4D and E). But the signals were abolished by the mutation of sdg8 (Figure 4E), in which H3K36me3 is greatly decreased, whilst H3K4me3 and H3K36me2 are not changed or only moderately decreased (Figure 4A) (53,54), suggesting that MRG2 binding to histone H3 requires the H3K36me3 mark, whilst H3K4me3 and H3K36me2 are not sufficient to recruit MRG2 to histone H3 in vivo.

MRG2 directly binds to FT genomic regions

FT expression is controlled by CONSTANS (CO) and several other genes, including some histone modifiers involved in the activation and repression along the light-dark phase change (55). We next examined how MRG1 or MRG2 regulates FT expression during LD conditions. Taking samples from the aerial part of seedlings at 9 DAG, we showed that FT expression in mrg1 mrg2 is decreased to approximately half at ZT 16 (the highest FT expression) and at most tested time points (Figure 5A). Interestingly, even at ZT 0 and ZT 24, at which the FT expression is at the lowest level during the entire light-dark cycle, the FT expression level in mrg1 mrg2 is further reduced compared to WT (Figure 5A). In contrast, the FT expression level at 6 DAG did not show any obvious change between mrg1 mrg2 and WT (Figure 1E). These results suggest that MRG1/2-mediated up-regulation may require pre-existing active transcription and the accumulation of the transcription-linked H3K36me3 mark to a certain level and that MRG1 and MRG2 may function continuously during the entire light-dark cycle once they have started.

To check if MRG2 directly binds to the FT locus throughout the day and night.

MRG2 interacts with HATs to induce H4 acetylation

We next compared the histone acetylation levels of histone H3 (H3Ace) and histone H4K5 (H4K5Ace), and the activation marks of H3K4me3 and H3K36me3 between WT and mrg1 mrg2 seedlings (Figure 6A–C, Supplementary Figure S6A–C). For ease of sampling, we harvested the seedlings at 10 DAG and tested the histone modification at the FT locus. Interestingly, whilst the levels of H3K36me3 were not obviously changed in mrg1 mrg2 (Figure 6B), the H3K4me3 levels were clearly reduced in all tested primers at the promoter, exon 1 and intron 1 (Supplementary Figure S6B). Both the levels of H3Ace and H4K5Ace were decreased in mrg1 mrg2 compared to WT at the regions around the promoter and exon 1, but not at the intron 1 of the FT locus (Figure 6C, Supplementary Figure S6C), suggesting that the function of MRG1 and MRG2 in Arabidopsis is mediated through histone acetylation, and possibly H3K4me3. When we compared the distribution patterns of these histone modifications, H3K36me3 and H4K5Ace levels were peaked at the exon 1, whilst H3Ace and H3K4me3 showed higher levels at the promoter (Figure 6D). Thus, we checked the possible interaction between MRG1/2 and MYST HATs, HAM1/2 proteins, that catalyse H4K5Ace (33,34). First, we carried out bimolecular fluorescence complementation (BiFC) assay by transiently co-expressing a translational fusion of MRG1/2 to the N-terminal portion of YFP (MRG1/2-nYFP) and fusion of HAM1/2 to the C-terminal portion of YFP (HAM1/2-cYFP) in tobacco. We were able to detect fluorescence signal in the nuclei of tobacco epidermal cells whilst the control (MRG1/2-nYFP and vector cYFP, vector nYFP and HAM1/2-cYFP) did not show any signals, suggesting that the observed BiFC between MRG1/2-nYFP and HAM1/2-cYFP are specific (Supplementary Figure S7). In addition, using a rabbit antibody against both HAM1 and HAM2, together with the
Figure 5. MRG2 binds directly to the FT locus. (A) FT mRNA levels in Col WT and mrg1-1 mrg2-2 seedlings over a 24-h LD cycle. FT transcript levels were normalised to Tip41-like; bars indicate SD of triplicate measurements. White and dark bars below the x-axis indicate light and dark periods, respectively. One of two biological repeats with similar trends was shown. (B) Schematic drawing of the FT genome structure showing regions amplified by primers used for ChIP analysis. (C–D) ChIP analysis of HA-MRG2 enrichment at the FT locus at (C) ZT16 and (D) ZT24 with the pMRG2::HA-MRG2 transgenic line. Amounts of the immunoprecipitated genomic fragments were measured by qPCR and normalised first to the endogenous control ACTIN2 (ACT2). The fold enrichment of HA-MRG2 in each examined region was calculated by dividing the ACT2-normalised amount of the examined region from the sample with the anti-HA antibody by that with mouse IgG. Error bars indicate SD of six quantifications (three technical measurements of two biological replicates). * P < 0.05 with Student’s t-test.

mouse HA antibody against HA-MRG2, we performed an in situ PLA assay. We detected positive signals, further indicating the direct interaction between MRG2 and HAM1/2 (Figure 7A and B). Furthermore, we prepared and transformed the genomic DNA fusion construct of HAM1 with HA inserted just after its ATG into 35S::GFP-MRG2 or wild-type plants. We performed Co-IP using the seedlings of pHAM1::HA-HAM1 35S::GFP-MRG2, with the seedling of pHAM1::HA-HAM1 as the control. Indeed, anti-GFP antibody immunoprecipitated HA-HAM1 from the seedlings but not in the negative control with HA-HAM1 alone (Figure 7C). These results suggest that MRG proteins interact with HAM1/2 to induce histone H4 acetylation at the target loci that contained H3K36me3 (Figure 7D).

**DISCUSSION**

In this study, we have shown that Arabidopsis MRG15 homologues MRG1 and MRG2 function redundantly to stimulate the amplitude of expression of the flowering time genes FT and FLC in an H3K36me3-dependent manner. Due to the epistatic relationship between FLC and FT, the mutant shows late-flowering phenotype, caused by FT reduction.
MRG1/2 bridges H3K36me3 and histone acetylation to achieve higher levels of flowering gene expression

Two flowering time genes, FT and FLC, with the opposing functions in floral transition, are down-regulated in mutant mrg1 mrg2 (Figure 1E, Supplementary Figure S2F). It is not uncommon that one epigenetic regulation controls these two genes with opposing functions. For example, PRC2-like complex, including H3K27 methyltransferases CURLY LEAF (CLF) and SWINGER (SWN), deposits H3K27me3 at both FLC and FT to repress its expression (56,57). FT expression levels are similarly reduced in sdg8, mrg1 mrg2 and sdg8 mrg1 mrg2 at 9 DAG (Figures 1E and 4C), which support the idea that MRG proteins function in the same pathway as H3K36 methyltransferase SDG8. One paradox was why the sdg8 mutant showed an early flowering phenotype (37) despite reduced expression of FT at 9 DAG. It could be caused by a significant reduction of FLC or increased FT expression at later time points. We found that FT was indeed increased at 12 DAG for an unknown reason (Supplementary Figure S8). Such up-regulation of the FT transcript appears to be independent of MRG1/2 because the early flowering phenotype of sdg8 is epistatic to the overexpression of MRG2 (Supplementary Figure S5). Our other genetic data of sdg8 mrg1 mrg2 also suggested that sdg8 is epistatic to mrg1 mrg2 in flowering control. These data do not contradict the model that MRG1 and MRG2 bind to SDG8-mediated-H3K36me3 and function through the mark to control downstream activities.

The sdg8 mutation has different locus-specific effects on gene expression (54,58,59). Whilst the transcription of FT in 9 DAG is similarly impaired in sdg8 and mrg1 mrg2 mutant plants (Figures 1E and 4C), the sdg8 mutation has a drastic effect on FLC expression compared with mrg1 mrg2 mutation (Figures 1E and 4B). It is of note that H3K27me3 co-exists with H3K4me3 at the 5′-end nucleosome of FLC (50). As H3K36me2/3 were reported to inhibit PcG-mediated repressive mark H3K27me3 (51,52), it is possible that the loss of H3K36me3 (Figure 4A), and also the dramatic reduction of H3K36me2 level at the FLC locus (37), lead to the spreading or increased level of H3K27me3 at the FLC region, thus greatly reducing FLC expression. However, it is interesting to note that FT chromatin also has a bivalent structure, simultaneously carrying the active H3K4me3 and repressive H3K27me3 marks (56,57). Thus, it is likely that the different levels of H3K4me3 on FLC and FT regions, which is also responsible to prevent H3K27me3 (51), determine the different effects of sdg8 mutation on FT and FLC expression. This speculation requires further detailed study in the dynamic change of epigenetic mark in inducible rescue lines.

MRG proteins directly bind to HAM1/2 acetyltransferase and bridge the two histone modifications, H3K36me3 and H4 acetylation (Figures 6 and 7, Supplementary Figure S7). The ham1 ham2 double mutant is not viable, and the single mutation of either ham1 or ham2 did not show any defects in the vegetative and reproductive stages (32). Interestingly, ham1/+ ham2/+ and RNAi knockdown plants showed the reduction in FLC expression levels, as observed in mrg1 mrg2, and both overexpression of HAM1 and MRG2 caused late flowering phenotype with the increased
Figure 7. MRG1/2 recruits HAM1/2 to their targeted loci. (A) In situ PLA assay to show the interaction between MRG2 and HAM1/2. Blue, DAPI. Red, in situ PLA signal showing the interaction between HA-MRG2 and HAM1/2 (upper panels); the assay between HA-MRG2 and rabbit IgG was carried out as the negative control (lower panels). Purple, merge. Scale bar, 5 μm. (B) Schematic diagram for in situ PLA assay between MRG2 and HAM1/2. (C) Co-IP assay to show the interaction between MRG2 and HAM1. Total protein extracted from 10 DAG seedlings co-expressing HA-HAM1 and GFP-MRG2 or solely expressing HA-HAM1 (as the negative control), were subjected to immunoprecipitation with anti-GFP antibody. HA-HAM1 protein in the immunoprecipitates was detected by western blotting using anti-HA-HRP antibody. (D) A working model for the control of the target genes by MRG1/2 through the recruitment of HAM1/2-containing complex to increase the histone acetylation level at the target loci.

FLC transcription (Figures 1E and 4B and Supplementary Figure S5) (32,34). These results suggest that the MRG proteins and the HAM acetyltransferases function together to regulate FLC.

We showed that the approximately 2-fold reduction of FT expression is correlated with a reduced level of H4K5Ace at the 5′ region of the FT locus in mrg1 mrg2 seedlings (Figure 6C). In addition to H4K5Ace, we showed that the level of H3Ace is also decreased at the FT locus in the mrg1 mrg2 mutant (Supplementary Figure S6C). As MYST family proteins specifically catalyse histone H4 acetylation, H4K5Ace may help to recruit other histone acetyltransferases to amplify the effect of histone acetylation in different histones. The similar distribution pattern between H3K36me3 and H4K5Ace but not with H3Ace or H3K4me3 on the FT locus also indicates that the change of H4K5Ace level could
be the direct effect of mrg1 mrg2 mutation (Figure 6A–D, Supplementary Figure S6A–C). Further experiments using the inducible line of MRG1/2 in the mrg1 mrg2 mutant could provide a suitable system to check the sequential steps of histone modifications.

**FT** expression is controlled by CO in a rhythmic pattern in LD conditions (55). Whilst several genes, including some histone modifiers, are required for the activation and repression of FT along the light-dark phase change. Newly identified SAP30 FUNCTION-RELATED 1/2 (AFR1/2) only represses FT transcription at ZT 16, at the end of the light photoperiod (60). FT transcription is first detectable in seedlings at 4 DAG and dramatically increases at approximately 9–10 DAG during the floral transition (45). It is interesting to note that at 6 DAG, we were unable to detect a difference in FT transcription levels between the seedlings of WT and mrg1 mrg2 plants suggesting MRG1/2 function is dispensable for FT expression at the early developmental stage (Figure 1E). Such a difference is only detectable at later stages such as 9 DAG (Figure 1E). This suggests that, upon reaching a certain developmental stage (between 6 and 9 DAG), MRG1 and MRG2 begin acting on the FT locus, which then affect FT expression throughout the day and night (Figure 5), unlike AFR1/2 which only regulates FT expression at ZT 16. MRG2 binding to H3K36me3 (Figure 3A–C) and the unaffected FT expression at 6 DAG suggest that the activating H3K36me3 epigenetic mark may need time to accumulate to recruit MRG1/2-containing complex to fine-tune transcription. The continuous binding of MRG2 to the FT locus during the light-dark cycle (Figure 5B–D) suggests that H3K36me3 is relatively stable throughout the day and night.

**Conservation and divergence of plant MRG protein functions**

Similar to its homologous MRG15 proteins, in vivo and in vitro assays confirmed that MRG1 and MRG2 can bind to H3K36me3. The MRG15 family proteins are evolutionarily conserved in yeast and animals, and the chromodomains of MRG15 and Eaf3 bind to trimethylated H3K36 and H3K4 in vitro with a relatively weak affinity (2–4,14–16). Consistently, we found that the binding specificity of plant MRG15 homologues to H3K36me3 is also relatively conserved during evolution. An in vitro pull-down assay showed that the chromodomains of MRG1/2 directly binds to trimethylated H3K36 and H3K4, but the binding affinity is relatively weak (Figure 3A). Yeast Eaf3 has been reported to interact with other protein partners (e.g. PHD proteins) and shows a wide range of binding strength (from unstable to robust) to H3K36-methylated nucleosomes, which suggests that the combinatorial action of Eaf3 and PHD proteins strengthens the binding affinity (61). It is likely that other chromatin- or DNA-binding proteins also help MRG proteins in binding to their target loci with higher affinity in vivo. Alternatively, such weak affinity may allow MRG-containing complexes to spread histone acetylation across the target loci, as implicated in the dosage compensation complex (62).

Although the in vitro data showed that the chromodomain of MRG2 can bind to both H3K4me3 and H3K36me3 (Figure 3A), the in situ PLA assay suggested that MRG2 binds to H3K36me3 specifically in vivo because the binding of MRG2 is completely abolished in the sdg8 mutant, in which H3K36me3 is dramatically reduced, but H3K36me2 is still maintained albeit at a reduced level and H3K4me3 is intact (Figure 4A, D and E)(53,54). These data indicate that H3K36me3 is essential for MRG protein binding in vivo.

Whilst the human MRG15 and yeast Eaf3 are involved in both HATs and HDACs, most studies focus on the defects of its HDAC function because this effect is more obvious and the mutation effect is more severe (2–4,18–20). For example, in yeast, the deletion of Eaf3 greatly alters the global genomic profile of histone acetylation, with increased acetylation levels at coding sequences and decreased acetylation levels at the promoter regions (21). However, these studies were rather focused on the function of Eaf3 binding to coding regions, recruiting an HDAC complex to H3K36me3-containing nucleosomes and thus prevents spurious intragenic transcription (2–4). Moreover, the loss of Alp13, the MRG15 homologue in fission yeast, causes global hyperacetylation of histones and chromosome instability (22). Thus, the function of MRG15 and its homologues associated with HATs was overlooked, partly because the loss of the MRG15 mutants causes phenotypes that are more linked with elevated histone acetylation on a gene-specific level or globally.

The interaction between MRG1/2 and MYST family proteins HAM1/2, characterised by an in situ PLA assay, BiFC assay and Co-IP analysis (Figure 7A–C, Supplementary Figure S7), confirmed the protein interaction between MRG1/2 and HATs. Whilst we could detect the interaction between MRG2 and HDA6 when transiently co-overexpressed in tobacco (Supplementary Figure S9), the resulting effects with the locus-specific decreased acetylation suggested that MRG1 and MRG2 could have diversified from its homologues in animal and yeast and primarily function in the fine-tuning of transcription regulation associated with HATs. It is possible that MRG1/2 proteins recruit both HAT and HDAC dynamically, and loss-of-function of mrg1 mrg2 leads to the disruption of a balance between these two counteracting activities, resulting in the reduction of histone acetylation levels and transcription. In human T cells, genome-wide mapping of HATs and HDACs binding showed that regions enriched with both HATs and HDACs are linked with active genes (63). They showed that in both active genes and primed genes (genes poised for expression later), inhibition of HDAC activity causes increased acetylation level at the promoter regions (63), rather than the reduced acetylation level we observed in mrg1 mrg2 (Figure 6C, Supplementary Figure S6C). In addition, at active gene loci, HDAC physically interacts with the elongating form of RNA Pol II and is directly recruited to actively transcribed regions by RNA Pol II, which may bypass the need for H3K36me3 (63). Moreover, the histone deacetylase HDA6 in Arabidopsis is recruited to remove acetylated histone marks at the FT locus specifically at dusk under long-day conditions, thereby dampening FT mRNA expression after its transcriptional activation by CO. Mutation of the HDAC recruiter increases the histone acetylation level at the FT locus (60). As perturbation of HDAC generally leads to increased histone acetylation levels, the opposite effect of
the reduction of histone acetylation level at the FT locus in \textit{mrg1 mrg2} mutant suggests that the function of MRG1/2 on FT is majorly dependent on its interaction with HATs.

The loss-of-function of \textit{MRG1} and \textit{MRG2} causes an approximately 2-fold decrease in the expression levels, together with the reduction of the histone acetylation levels at their target genes. It can be argued that the decreased FT expression is due to the decreased H3K4me3 and H3Ace.

Although we have shown that H3K36me3 is essential for \textit{MRG2} binding to chromatin (Figure 4E), it is still possible that H3K4me3 is also necessary for \textit{MRG2}-dependent expression of FT, as transcriptional regulation is mediated by complex cross-talk of multiple histone modifications. However, based on our results that \textit{MRG1} can form complex with the HATs specific for H4, HAM1/2, \textit{in vitro} and \textit{in vivo} (Figure 7 and Supplementary Figure S7) and co-localisation between H3K36me3 and H4 acetylation at the promoter region (Figure 6D), we favour the hypothesis that the binding of MRG proteins to H3K36me3 leads to the H4 acetylation for transcriptional induction and that the reduction of H3K4me3 and H3Ace levels in \textit{mrg1 mrg2} could be the secondary effects of transcription reduction. This suggests that the MRG1/2 functions resemble the dosage compensation complex in \textit{Drosophila} that contains MSL3 (MRG protein not belonging to the MRG15 subfamily) and MOF (MYST family HAT) (64,65). The binding of MRG2 to H3K36me3 at the target loci could facilitate the spread of histone acetylation through the recruitment of the MRG2-containing HAT complex, in a similar manner as the MSL3-containing dosage compensation complex (64,65).

In \textit{Arabidopsis}, H3K36me3 co-localises with other activation marks, including H3K4me3 and H3K56Ace, at the active chromatin (6). This simultaneous occurrence of multiple epigenetic marks has no equivalent in \textit{Drosophila}, \textit{C. elegans} and humans (6). Moreover, in \textit{Arabidopsis}, H3K36me3 peaks in the 5′-end of the coding region, which is in contrast to the 3′-end enrichment in other organisms (6). MRG1 and MRG2 may be involved in the association of H3K36me3 and histone acetylation marks. Indeed, at the FT locus, H4K5Ace distribution pattern is similar to that of H3K36me3 peaking at the 5′-end of the coding region (Figure 6D). Such a difference of the distribution pattern of H3K36me3 between plants and other organisms may be a result of co-evolution with the functional diversification of MRG15 proteins.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENT

We thank Aiwu Dong for communication on the \textit{mrg2-1} mutant and for research discussion. 

Author Contributions. T.I. conceived of the study, supervised and coordinated the study. Y.X., E.-S.G. J.Z. and T.I. designed the experiments. Y.X., E.-S.G. and W.-Y.W. performed the ChiP experiments, immunolocalisation analysis, \textit{in situ} PLA assays and the microarray studies. Y.X., J.Z. and W.-Y.W. performed the genetic analysis. Y.X. and T.I. wrote the manuscript. All authors discussed the results and approved the final manuscript.

FUNDING

Temasek Life Sciences Laboratory (TLL) [to T.I.]; National Research Foundation Singapore under its Competitive Research Program [CRP Award No. NRF-CRP001-108]. Conflict of interest statement. None declared.

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