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Knockdown of SAMS genes encoding *S*-adenosyl-L-methionine synthetases causes methylation alterations of DNAs and histones and leads to late flowering in rice

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ABSTRACT

S-Adenosyl-L-methionine synthetase (SAMS) [EC 2.5.1.6] catalyzes to produce SAM (*S*-adenosyl-L-methionine), a universal methyl group donor in biochemical reactions in cells. However, less is known how SAMS controls plant development. Here, we demonstrate that *OsSAMS1*, *2* and *3* are essential for histone H3K4me3 and DNA methylation to regulate gene expression related to flowering in *Oryza sativa*. RNA interference (RNAi) transgenic rice with downregulated transcripts of *OsSAMS1*, *2* and *3* showed pleiotropic phenotypes, including dwarfism, reduced fertility, delayed germination, as well as late flowering. Delayed germination was largely rescued by application of SAM in the knockdown lines. Knockdown of *OsSAMS1*, *2* and *3* led to distinguished late flowering and greatly reduced the expression of the flowering key genes, *Early heading date 1* (*Ehd1*), *Hd3a* and *RFT1* (rice *FT-like* genes). Moreover, the histone H3K4me3 and symmetric DNA methylation at these genes were greatly reduced. Thus, SAM deficiency suppressing DNA and H3K4me3 transmethylations at flowering key genes led to a late-flowering phenotype in rice. This information could help elucidate the mechanism of epigenetic control flowering transition.

Introduction

Flowering, the transition from the vegetative to the reproductive growth phase, is controlled by both endogenous signals and environmental factors (Baurle and Dean, 2006). Molecular genetic studies have revealed that major genes regulating flowering time are highly conserved between *Arabidopsis* and rice. For example, *OsGI*, *OsMADS50*, and *Hd3a* in rice function in flowering time control similar to their *Arabidopsis* orthologs *GI (GIGANTEA)*, *SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CO 1)*, and *FT (FLOWERING LOCUS T)* (Higgins et al., 2010). Furthermore, some genes have evolved a new unique flowering pathway in rice; examples are *Ghd7* and *Ehd1*. *Ghd7* has a key role in flowering by regulating the *Ehd1–Hd3a* pathway. Also, *Ehd1*, encoding a B-type response regulator, promoted flowering by upregulating its downstream *Hd3a* and *RFT1* (Doi et al., 2004). Therefore, a preliminary network of genes controlling flowering has been established.

Epigenetic control is determinative in plants for coordinating the switch to flowering in *Arabidopsis* and rice. Histone acetylation, H3K4 di-and tri-methylation and H3K36 di- and tri-methylation, are associated with actively transcribed *FLC* chromatin to repress flowering. In contrast, histone H3K9 and H3K27 di- and tri-methylation are tightly coupled to *FLC* repression to promote flowering (He, 2009). Recently, PRMT5 (protein arginine methyltransferase 5) was found to participate in the regulation of pre-messenger-RNA splicing to affect flowering (Deng et al., 2010; Sanchez et al., 2010). In addition, changed DNA methylation also resulted in altered flowering time (Finnegan et al., 1996; Kakutani et al., 1996). However, little is known about the effect of the methyl supply on epigenetic modification.

SAM, a universal methyl group donor involved in numerous transmethylation reactions, participates in the regulation of various cellular functions. Also, it functions as a precursor of polyamines

Abbreviations: DAG, days after germination; *Ehd1*, *early heading date 1*; *Hd3a*, *heading date 3a*; ^{m}CG, CG methylation; ^{m}CNG, CNG methylation; Met, methionine; OsSAMS, *S*-adenosyl-L-methionine synthetase; *RFT1*, rice *FLOWERING LOCUS T*; RNAi, RNA interference; SAM, *S*-adenosyl-L-methionine; WT, wild-type; Zhonghua 10, ZH 10.

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and ethylene in plants (Tabor et al., 1984; Yang and Hoffman, 1984). In biosynthesis, SAM is synthesized from L-methionine and ATP by SAMS. The silencing of *SAMS* in tobacco resulted in a stunted phenotype (Boerjan et al., 1994). Moreover, the *mto3* (Met over accumulating mutant 3) mutation in *SAMS3* in *Arabidopsis (AtMTO3)*, accumulated remarkably high levels of free Met (Shen et al., 2002). However, the response of flowering to SAMS expression changes is still unknown.

In this paper, *OsSAMS1*-RNAi transgenic plants with repressed transcripts of all 3 *OsSAMS* genes exhibited a severe late-flowering phenotype. Our data suggested that H3K4me3 and symmetric DNA methylation (^{m}CG and ^{m}CNG) at specific genes were more sensitive to methyl supply. The alterations in these modifications suppressed genes expression and subsequently led to late flowering. We concluded that SAM, as a major methyl donor, plays a critical role in the key genes involved in epigenetic control of flowering.

Materials and methods

Gene-specific primers in this study are listed in Table S2.

Plant materials and treatments

The rice plants used in this study were the variety Zhonghua 10 (*Oryza sativa* L. spp *japonica* cv Zhonghua 10, ZH 10). The *sams3* mutant (PFG_3A-02149.R) and Dongjin variety were ordered from the Rice Insertion Sequence Database (RISD). Rice plants were grown in the field under a natural photoperiod (14 h light/10 h dark) between May and August. The T_1 generation of transgenic rice plants was used in the phenotype analysis and following experiments.

For SAM treatment, ZH 10 and *OsSAMS1*-RNAi weak transgenic seeds were surface-sterilized and immersed in water supplemented with various concentrations of SAM for germination. After the seeds germinated for 10–120 h under dark conditions at 30, the germination rate was analyzed (providing 30 seeds per sample).

Plasmid construction and rice transformation

Full-length cDNAs of *OsSAMS1*, *2* and *3* were used for overexpression construction and inserted into the binary plasmid pUN1301 (Chen et al., 2011). Constructed pTCK303-*OsSAMS1* was used for creating an RNAi knockdown transgenic line. The detailed protocols were as described previously (Wang et al., 2004). The gene transformation followed a previous method (Wang et al., 2004).

Genetic complementation in yeast

The *Saccharomyces cerevisiae SAMS*-deficient double-mutant strain W744-1A (*MATa*, *ade2-1*, *canl-100*, *his3-11,15*, *leu2-3, 112*, *trpl-1*, *ura3*, *saml*::*LEU2*, *sam2*::*HlS3*) was used. W744-1A yeast strain used in this investigation was derived from W303-1A (*MATa*, *ade2-1*, *canl-100*, *his3-11,15*, *leu2-3,112*, *trpl-1*, *ura3*) (Thomas and Rothstein, 1989). The W744-1A strain required supplemented SAM for growth (Robertson et al., 1995). The coding region of *SAMS1* in *Arabidopsis* (*AtSAM1*) or *SAMS1*, *2* or *3* in rice (*OsSAMS1*, *2* or *3*) was cloned into the vector pJN92 (2 μ, URA3), behind the galactose-inducible GAL1 promoter. AtSAM1 was used as a positive control (Lindermayr et al., 2006). Transformants were selected on uracil-less synthetic glucose or galactose media.

RT-PCR and real-time RT-PCR

RNA samples were prepared from leaves at the beginning of the light phase at 6:00 am (for flowering key genes expression analysis). The cDNA samples were synthesized by use of M-MLV Reverse Transcriptase (Promega, USA). LA Taq polymerase (Takara Bio Inc., Japan) and SYBR Green Real-time PCR Master Mix (TOYOBO, Osaka, Japan) were used for semi-quantitative and Real-time RT-PCR.

Expression of OsSAMS and polyclonal antibody production

The full-length *OsSAMS1* was cloned into the expression vector pGEX4T-1 and then transformed into *Escherichia coli DH5α*. Cells were grown at 37 °C and then induced by use of 1 mM isopropyl β-D-1-thiogalactoside (IPTG) for 4 h. Bacteria were harvested and resuspended in denaturing buffer. Purified recombinant protein was separated by 12% SDS-PAGE. Polyclonal antisera were raised by subcutaneously inoculating New Zealand white rabbits with about 2.5 mg of recombinant proteins extracted from gels emulsified in an equal volume of Freund's adjuvant.

Protein extraction and protein gel blot analysis

Protein was extracted from the aerial part of seedlings at 14 DAG and boiled for 10 min. Proteinase inhibitors were then added and cells were sonicated. Cells underwent 12% SDS-PAGE and proteins were transferred to polyvinylidene fluoride (BioTrace™, USA) membrane. The transferred membrane was blocked with 3% non-fat milk powder in TBS-T and then probed with anti-OsSAMS (1:1,000). The blots were developed by use of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Zhongshan Golden Bridge Biotechnology, China) and an ECL protein gel blot detection system (Amersham, Sweden), mouse anti-tubulin (Beyotime, China) (1:10,000) was used as a loading control.

For protein gel blot analysis, histone-enriched fractions were extracted from young rice leaves as described previously (Bowler et al., 2004).

Chromatin immunoprecipitation (ChIP) assays

Leaves of 4-week-old rice seedlings were used for ChIP assays according to the methods as described (Bowler et al., 2004). ChIP primers of *RFT1* were used as described (Komiya et al., 2008).

Antibodies used were anti-H3K4me2 (Upstate of Millipore 04-790), anti-H3K4me3 (17-614), anti-H3K9me3 (07-523), anti-H3K27me3 (17-622), anti-H3K9ace (06-599), and anti-H3 (07-690; www.millipore.com).

Bisulphite genomic sequencing

DNA methylation was performed according to the method described by Mathieu et al. (2007). Bisulphite conversion was performed with use of the EpiTect Bisulphite kit (Qiagen) following recommended methods. Nested PCRs were used. PCR products were purified by use of the AxyPrep™ PCR Cleanup Kit (Axygen, USA) and cloned into pGEM-T Easy vector (Promega, USA). Fifteen clonal replicates for each of the bisulphite-treated samples were sequenced on the ABI377 automatic DNA sequencer.

Results

Characterization of OsSAMS and phylogenetic analysis

There are 3 members of the predicted *SAMS* family in rice, named *OsSAMS1*, *2* and *3*, respectively (GenBank accession number: Z26867; U82833; AK241882). They were highly identical in DNA and deduced amino acid sequences (Fig. S1). *OsSAMS1* was expressed abundantly in almost every tissue. *OsSAMS2* was preferentially expressed in young roots and panicles. By contrast, the

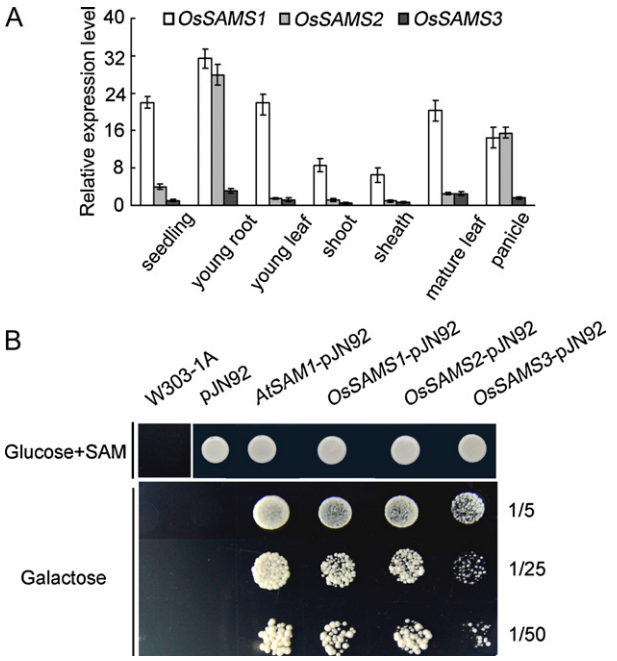

Fig. 1. Biochemical characterization of *Oryza sativa S*-adenosylmethionine synthetase (OsSAMS1, 2, 3) and expression patterns. (A) Expression profile of *OsSAMS1*, *2*, *3* in different ZH 10 organs detected by real-time RT-PCR. Error bars represent ±SD. (B) *OsSAMS1*, *2*, *3* complemented yeast *sam1 sam2* double mutant W744-1A. Yeast transformants were grown as patches on uracil-less synthetic glucose media (top panel), and then were diluted and replicated to uracil-less media containing galactose (bottom panel). 1/5, 1/25 and 1/50 meant dilution ratio. W303-1A was used as WT yeast, *AtSAM1* was used as a positive control.

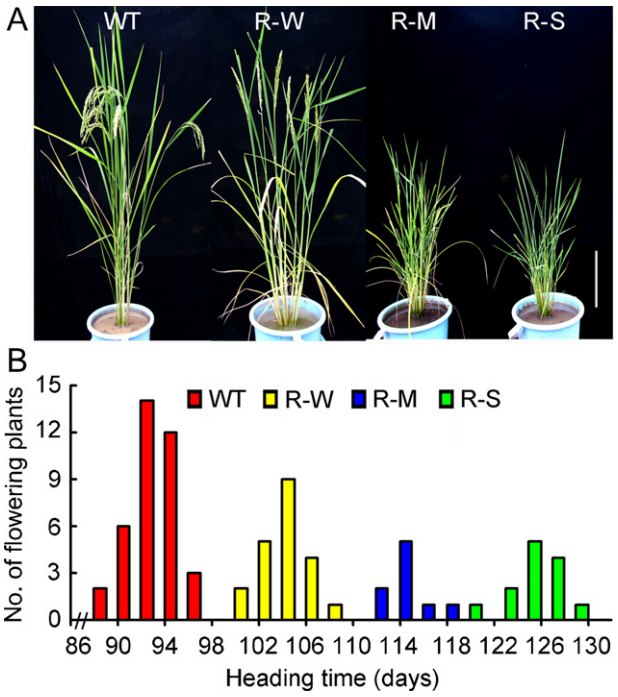

Fig. 2. Phenotypic comparison of the *OsSAMS*1-RNAi transgenic plants was shown during the heading period. (A) Transgenic plants showed late-flowering phenotype in the field. Rice plants were photographed at 114 DAG. Bar = 20 cm (B) Heading time distributions of the transgenic rice plants grown in the field. *OsSAMS1*-RNAi-weak, medium-strength and strong transgenic plants were abbreviated to R-W, M, S, respectively.

expression of *OsSAMS3* was weak compared with *OsSAMS1* (Fig. 1A). Thus, *OsSAMS* genes were constitutively expressed but the various homologues may have different functions during development.

To confirm their function in rice, we performed a genetic complementation experiment in the yeast W744-1A *sam1 sam2* double mutant. The W744-1A mutant strain is disrupted for two SAMS-encoding genes, *SAM1* and *SAM2*, which exist in yeast, and therefore requires SAM for growth. Transformants were first selected on uracil-less synthetic glucose media. Following growth, these transformants with appropriate dilutions were then plated on uracil-less synthetic galactose media to check the SAMS function and determine the rate of formation of SAM. The double mutant containing *OsSAMS1* or *2*, or *3* alone no longer depended on externally added SAM when their expression was induced in the presence of galactose (Fig. 1B). Thus, *OsSAMS1*, *2* and *3* were all genetically complemented for the functional SAMS enzyme in the yeast double mutant *in vivo*. Moreover, as shown in Fig. 1B, the OsSAMS1 activity was comparable to OsSAMS2, and OsSAMS3 activity was weak.

Knockdown of OsSAMS resulted in pleiotropic phenotypes including flowering

OsSAMS1-RNAi transgenic plants displayed various degrees of developmental defects, such as flowering time alteration and reduced fertility. The weak lines (W) in the phenotype showed only late-flowering and delayed germination phenotypes. The flowering time was delayed 2 weeks in W lines (Fig. 2). By contrast, flowering in the medium-strength (M) and the strong (S) lines was delayed for 3-5 weeks as compared with the WT (Fig. 2). The M and S lines also showed other developmental defects, including dwarfism (Fig. 2A) and reduced fertility.

Real-time RT-PCR analysis of *OsSAMS1* revealed reduced expression in the transgenic lines W, M and S, by 60.4%, 4.8%, 2.6% that of the WT, respectively, and *OsSAMS2* and *OsSAMS3* also showed a significant decrease in expression levels (Fig. 3A). The expression of *OsSAMS2* was decreased to 76.1%, 31.5% and 15.9% in the W, M and S lines. Similarly, the expression of *OsSAMS3* was decreased to 69.7%, 25.9% and 20.2% in the W, M and S lines. Thus, transcripts levels in *OsSAMS1*, *2* and *3* corresponded with their phenotypes.

The protein gel blot analysis showed OsSAMS expression was greatly decreased in the transgenic lines as compared with the WT. The expression level in the blot was also comparable to the phenotype of the S, M and W lines (Fig. 3B).

Inhibition of SAMS by trifluoromethionine prevents germination and outgrowth of spores in *Saccharomyces cerevisiae* ascospores (Choih et al., 1977). Moreover, changed *SAMS* gene expression could influence SAM production (He et al., 2006; Liu et al., 2011). Our transgenic lines showed delayed germination as compared with the WT. Thus, this germination block in yeast spores and plant seeds could result from SAM deficiency. With 1 mM SAM supplementation, this blocked germination was largely alleviated in W transgenic line (Fig. 3C). In contrast, ZH 10 seeds showed no obvious response to this treatment. These data suggest that SAM production was reduced in the knockdown lines.

Alteration of the key gene patterns for flowering in the knockdown transgenic rice lines

To further investigate the molecular basis for flowering in transgenic plants, we checked the expression patterns of key genes, such as *Ehd1*, *Hd3a* and *RFT1*. The mRNA expression of all these key genes was decreased in the knockdown rice lines (Fig. 4A). The reduced transcript levels were greater in *Hd3a* and *RFT1* than in *Ehd1*. Furthermore, these trends were related to the phenotypical strength of the knockdown lines, S, M and W.

A series of the upstream genes of *Ehd1* and/or *Hd3a* and *RFT1* have been identified; examples are *OsMADS50*, *OsGI* and *Ghd7*. The W transgenic line showed no significant changes in mRNA

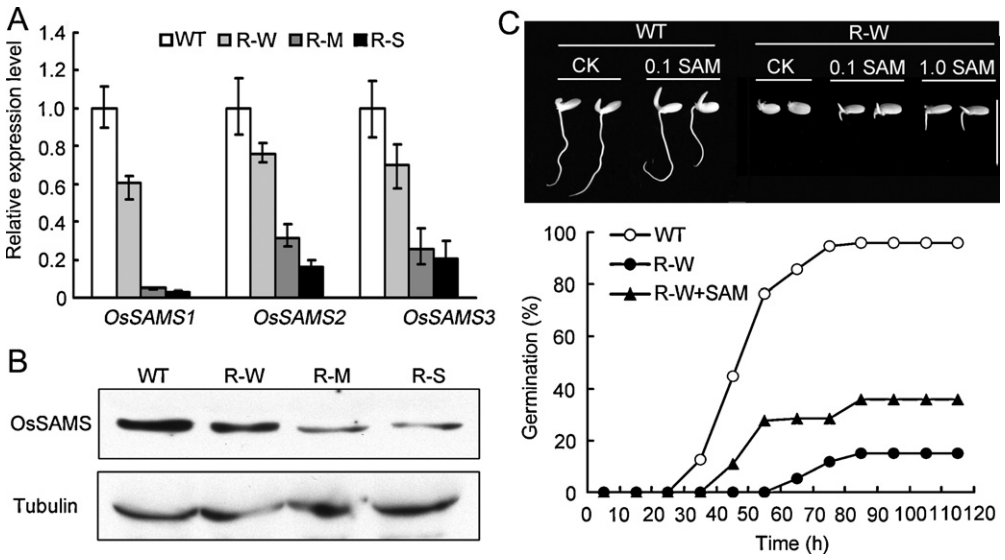

Fig. 3. Identification of *OsSAMS1*-RNAi transgenic plants. (A) Real-time RT-PCR analysis of the expression of *OsSAMS1*, 2, 3 genes in *OsSAMS1*-RNAi transgenic seedlings. Error bars represent ±SD. (B) Protein gel blot analysis of the expression of OsSAMS protein in transgenic seedlings. (C) Effect of SAM on seed germination and seedling growth in ZH 10 and *OsSAMS1*-RNAi transgenic weak (W) line. Top panel: morphology of seedlings after 40 h germination. SAM concentration was 0.1 mM or 1 mM. Bar = 2 cm; bottom panel: germination. SAM (1 mM).

expression of these genes. The S line showed a slight reduction in transcript level of these genes (Fig. 4A). Thus, knockdown of *OsSAMS1* led to suppressed expression of the key genes *Ehd1*, *Hd3a* and *RFT* for late flowering.

Histone modifications in the knockdown transgenic rice lines

As SAM is a universal methyl group donor, it is possible the patterns of histone modification and/or DNA methylation may be altered in rice transgenic plants. Our analysis of the global change in histone modification showed that a significant decrease in H3K4me3 appeared in the transgenic lines, for a dosage response in the various transgenic lines S, and W (Fig. 4B). In contrast, H3K4me2 and H3 acetylation, H3K9me3 as well as H3K27me3, were not affected under the same conditions (Fig. 4B).

To confirm whether SAM deficiency affected histone methylation on *Ehd1*, *Hd3a* and *RFT1*, ChIP assays were performed. The H3K4me3 level was substantially lower in regions II, III and IV of *Ehd1* and *Hd3a* in the S transgenic line than in the WT. In contrast, the W transgenic line showed little decrease in H3K4me3 modifications of these 2 genes (Fig. 4C and D). In contrast, the H3K4me3 level was not changed in *RFT1* promoter or the body region (Fig. S2). These results suggested that knockdown of *SAMS1* mainly caused a decrease in H3K4me3 to mediate gene suppression of *Ehd1* and *Hd3a* at the promoter, especially in the initial translation regions.

DNA modification response in the transgenic plants

DNA methylation patterning was checked by an approach of genomic bisulphite sequencing (Fig. 5). *Ehd1* promoter methylation was substantially lower at CNG sites (from 76.5% to 18.5%) in the S transgenic line than in WT plants. In contrast, the S transgenic line showed a slight decrease at CG sites (from 95.2% to 90.3%) and asymmetric methylation CNN sites (from 13.3% to 8.3%) (Fig. 5A). The DNA methylation patterns at floral key genes were more severe in the S line than in the W line (Table S1). Similarly, at the *RFT1* body region, methylation was substantially reduced at CG sites (from 60.5% to 26.1%) and slightly decreased at CNG sites (from 7.5% to 2.5%) and asymmetric methylation sites (from 4.4% to 4.0%) in the S transgenic line (Fig. 5C). In contrast, DNA methylation was unchanged at *Hd3a* or *OsMADS50* (Fig. 5B and Table S1). Therefore, the supply of SAM also functions in ^{m}CG and ^{m}CNG of the specific key genes for flowering with various sensitivities in rice.

To date, three types of cytosine DNA methyltransferases have been identified in rice (Tariq and Paszkowski, 2004), named METHYLTRANSFERASE 1(MET1), CHROMOMETHYLASE 3 (CMT3) and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2). In our study, the expression of genes encoding for 3 putative types of DNA methyltransferases (MET1-1, 2; CMT3-1, 2; DRM2-1, 2) was greatly reduced and showed spatio-temporal specificity in the S transgenic line (Fig. S3). These suppressed transcripts also showed a dosage response in the various transgenic lines. Thus, it is possible that SAM deficiency may directly disrupt the homeostatic expression state of these DNA methyltransferases in specific stages, leading to hypomethylation of specific genes. The suppressed expression of *DEMETER1*, *2* (*DME1* and *2*), encoding 5-methylcytosine glycosylases, probably represented a corresponding requirement for insufficient DNA methylation patterns in the knockdown lines (Fig. S3).

Discussion

Knockdown SAMS leads to late flowering due to reduced transcription of Ehd1, Hd3a and RFT1

In *Arabidopsis*, mutants of *AtSAMS3 (AtMTO3)* did not, for the most part, influence plant development due to redundant functions of 4 SAMSs (Goto et al., 2002; Shen et al., 2002). In rice, no alterations in growth or development were observed in the *sams3* homozygote and *OsSAMS1* overexpressing plants as compared with the WT (Figs. S4E and S5A). In contrast, our evidence suggests that knockdown of rice *SAMS* leads to aberrant morphological phenotypes such as late flowering, dwarfism, and reduced fertility. In this study, as a 522 bp fragment of *OsSAMS1* cDNA, 281 bp in ORF (open reading frame) and 241 bp in 3′ untranslated region (UTR) was used to construct *OsSAMS1*-RNAi vector, we presumed that this highly conserved 281 bp sequence inhibited the transcripts of *OsSAMS2* and *OsSAMS3* except for *OsSAMS1* (Fig. 3A). This finding is consistent with At*SAM1* transgenic tobacco plants being stunted, since the

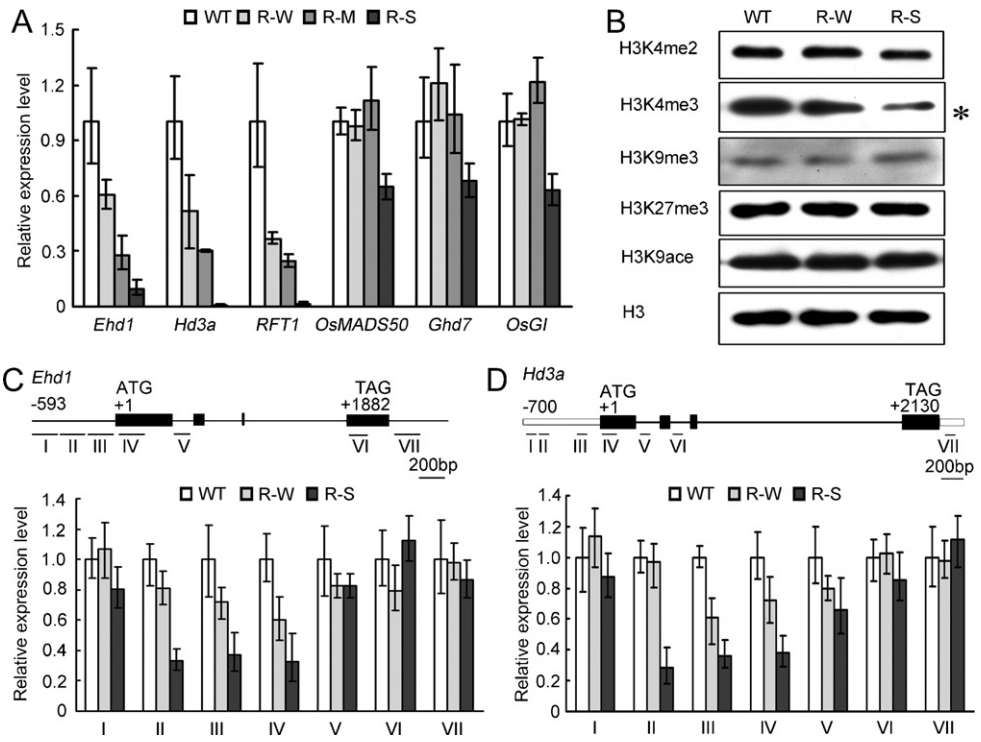

Fig. 4. Transcripts and H3K4me3 level of flowering control genes in *OsSAMS1*-RNAi transgenic seedlings compared with WT ZH 10. (A) Transcripts of flowering control genes in *OsSAMS1*-RNAi lines compared with WT ZH 10 plants. Leaves were collected from WT and transgenic plants at 70 DAG. Bars represent mean values ±SD. (B) Protein gel blot analysis of *in vivo* histone methylation status. Asterisks denoted noticeable changes in H3K4me3. (C and D) Histone modification analysis in *OsSAMS1*-RNAi transgenic and WT seedlings. Top panel: schematic diagram of *Ehd1* and *Hd3a*. I through VII represent the regions in which H3K4 trimethylation was examined by ChIP. The translation initiation site is +1. Filled boxes represent exons. Regions analyzed by ChIP are indicated by short straight lines. Bottom panel: ChIP analysis of H3K4me3 levels of *Ehd1* and *Hd3a*. Bars represent mean values ±SD.

transgenic *AtSAM1* and the homologous endogenous genes in tobacco were all silenced due to cosuppression (Boerjan et al., 1994). Unfortunately, knockdown of *SAMS* with the conserved fragments could not produce any positive regenerated plants. Importantly, *OsSAMS1* was predominantly expressed in almost every tissue, with *OsSAMS2* preferentially in young roots (Fig. 1A). Therefore, although *SAMS1*, *2* and *3* probably have redundant functions, *OsSAMS1* is mainly involved in development, especially in flowering.

Later flowering was clearly displayed in the *OsSAMS1*-RNAi S transgenic line (Fig. 2). The transcripts of *Ehd1*, *Hd3a* and *RFT1* were reduced in all knockdown lines, but the transcripts of *OsMADS50*, *OsGI* and *Ghd7* were reduced only slightly (Fig. 4A). *Ehd1* promoted flowering by upregulating *Hd3a* and *RFT1,* which were rice orthologues of *Arabidopsis FT* (Doi et al., 2004; Higgins et al., 2010). Double *RFT1-Hd3a* RNAi plants with distinct late flowering suggested that *RFT1* and *Hd3a* are the only genes encoding rice florigens (Komiya et al., 2008, 2009). Therefore, the reduced transcripts of *Ehd1*, *Hd3a* and *RFT1* were involved in the knockdown transgenic lines with delayed flowering.

H3K4me3 at Ehd1 and Hd3a was critical in SAMS-mediated flowering

SAM-dependent methylation of histone and DNA play a crucial role in the organization of chromatin structure and transcriptional regulation in cells. In addition, H3K4me3 is proposed to mark an active state allowing stronger transcriptional activity, which is required for normal plant development (Santos-Rosa et al., 2002; Li et al., 2007). For instance, the loss of SDG2, a specific H3K4 methyltransferase in *Arabidopsis*, led to a severe reduction in H3K4me3 and to early flowering and dwarfed and abnormal inflorescence development. H3K4me3 is also involved in the regulation of *FT* expression to modulate floral transition in *Arabidopsis* (Jeong et al., 2009; Yang et al., 2010).

Our data suggest that decreased SAM supply in *OsSAMS1*-RNAi transgenic plants led to a marked reduction in global histone H3K4me3 level (Fig. 4B). Meanwhile, other types of histone modification remained stable situation in knockdown lines. Further ChIP analysis indicated that H3K4me3 on *Ehd1* and *Hd3a* was lower in level in the knockdown lines (Fig. 4C and D). There-

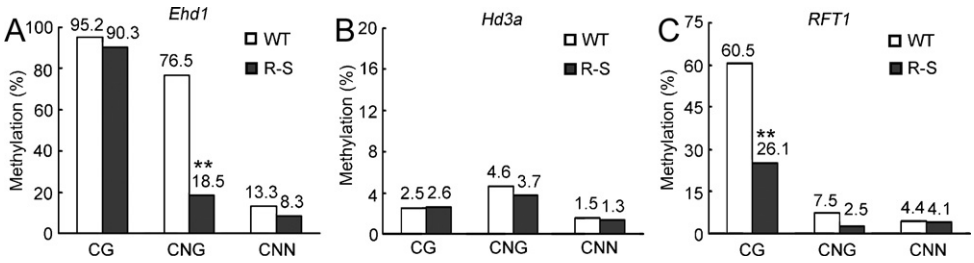

Fig. 5. Histogram about DNA methylation at flowering control genes in *OsSAMS1*-RNAi S transgenic seedlings: *Ehd1* (A), *Hd3a* (B) and *RFT1* (C). Asterisks denoted noticeable changes.

fore, H3K4me3 is sensitive to SAM supply to regulate flowering in rice.

Recent studies have suggested that *de novo* methylation in the embryo may be excluded at CG islands because of the presence of H3K4me (Cedar and Bergman, 2009). This finding is consistent with the observation that H3K4me2/3 and DNA methylation appear to be mutually exclusive in *Arabidopsis* (Zhang et al., 2009). By contrast, DNA methylation may dictate a closed chromatin structure that prevents H3K4me2 and H3K4me3 in the region (Okitsu and Hsieh, 2007).

In our knockdown lines, decreased SAM supply led to marked reduction in both H3K4me3 and symmetric DNA methylation (Figs. 4B–D and 5A,C), but the H3K4me3 modification may play a determinant role in flowering time control. There were apparently decreased transcriptions of *Ehd1*, *Hd3a* and *RFT1* and late-flowering phenotype in W transgenic line, with no obvious changes on DNA methylation at *Ehd1* in this line (Figs. 2 and 4 and Table S1). By contrast, different severities of reduction in H3K4me3 resulted in repressed transcripts of flowering key genes and the late-flowering phenotype both in W and S lines (Figs. 2 and 4). Further analysis of the respective function of DNA methylation and histone modifications with their specific mutations or inhibitors is necessary.

Decreased DNA methylation induced flowering key genes inactive by loss of function of OsSAMS

DNA methylation in transcriptional regulatory regions leads to decreased gene expression or gene silencing in cells. However, sometimes it can induce transcriptional activation. For instance, ^{m}CG in a negative regulatory sequence of *Igf2* de-repressed this gene by interfering with the binding of a transcriptional repressor GCF2 (Eden et al., 2001; Murrell et al., 2001). In another case, the reduced DNA methylation suppressed the transcripts of *ROS1* and *DME* (both encoding DNA demethylases) in *met1-3* mutant (Mathieu et al., 2007). In our study, decreased ^{m}CG on *RFT1* and ^{m}CNG on *Ehd1* (Fig. 5A and C) and suppressed gene transcription (Fig. 4A) were all observed in *OsSAMS1*-RNAi transgenic plants. Therefore, we propose that the repressed expression of the key genes may be due to reduced DNA methylation in the negative regulatory sequence or hypomethylation of specific genes directly inhibiting gene transcription or the coaction of reduced H3K4me3 and DNA methylation.

OsSAMSs mediated methyl supply were essential for epigenetic modifications

Our studies proposed that SAM deficiency led to decreased H3K4me3 and DNA methylation, which subsequently triggered pleiotropic defects. Consistent with this notion, the *homology-dependent gene silencing of HDG silencing* mutations in the *Arabidopsis* S-*adenosylhomocysteine* (AdoHcy) *hydrolase* gene caused genome-wide hypomethylation (Rocha et al., 2005) and induced many developmental defects. The mechanism is that AdoHcy competed with SAM to bind SAM-dependent methyltransferases to reduce their activities. Moreover, decreased SAM flux into plastids also induced growth-retarded phenotype in *Arabidopsis*, probably due to hypomethylation and decreased histone methylation (Bouvier et al., 2006). This evidence strongly supports the idea that the inhibition of SAM supply or transport reduces SAM-dependent methyltransferase activities, leading to hypomethylation and decreased histone methylation in plants.

Moreover, the transcripts of genes encoding putative DNA methyltransferases showed a significantly suppressed alteration in spatio-temporal and gene specificity in the knockdown lines (Fig. S3). Consistent with our data, others have found that various DNA methyltransferase genes have different expression patterns and differed quantitatively and spatially in their mode of expression in plants. In addition, analysis of rice SET-domain proteins in the plant chromatin databases (www.chromdb.org) identified 33 putative histone methyltransferase genes in rice. By analyzing the expression patterns from Rice eFP Browser databases (http://www.bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi), we found that some genes showed specific expression patterns in certain tissues at certain stages, examples are SDG714 and SDG717. On the other hand, scientists demonstrated that allele-specific differences in G protein α expression correlated in a tissue-specific manner with allele-specific differences in the extent of H3K4 methylation, and the chronic transcriptional activation in mammals is correlated with H3K4me3 (Sakamoto et al., 2004). Thus, we proposed that the tissue-specific epigenetic modification and specific expression pattern of methyltransferase genes led to changed H3K4me3 in *OsSAMS1*-RNAi transgenic seedlings (Fig. 4B).

Therefore, we propose that SAM deficiency inhibits DNA methyltransferases and transmethylation, which subsequently decreases DNA methylation and H3K4me3 in specific sites. The reduced H3K4me3 mainly repressed the *Ehd1*, *Hd3a* and *RFT1* transcripts to result in a late-flowering phenotype in the knockdown lines. Meanwhile, the decreased symmetric DNA methylation probably also participated in this transcription regulation, which also contributed to late flowering in our knockdown lines. This finding has potential for use in molecular breeding.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2011.05.020.

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