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Regulation of flowering time by the protein arginine methyltransferase AtPRMT10

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In plants, histone H3 lysine methyltransferases are important in gene silencing and developmental regulation; however, the roles of histone H4 methylation in plant development remain unclear. Recent studies found a type II histone arginine methyltransferase, AtPRTM5, which is involved in promoting growth and flowering. Here, we purified a dimerized plant-specific histone H4 methyltransferase, plant histone arginine methyltransferase 10 (PHRMT10), from cauliflower. *Arabidopsis thaliana* protein arginine methyltransferase 10 (AtPRMT10)—the *Arabidopsis* homologue of PHRMT10—was shown to be a type I PRMT, which preferentially asymmetrically methylated histone H4R3 *in vitro*. Genetic disruption of *AtPRMT10* resulted in late flowering by upregulating *FLOWERING LOCUS C* (*FLC*) transcript levels. In addition, we show that *AtPRMT10* functions genetically separate from *AtPRMT5*, but that each acts to fine-tune expression of *FLC*. This work adds an extra layer of complexity to flowering-time regulation and also sheds light on the importance of asymmetric arginine methylation in plant development.

Keywords: AtPRMT10; asymmetric arginine methylation; FLC; flowering time

INTRODUCTION

Histone methylation at lysine and arginine residues is one of the most complex and important covalent modifications in eukaryotic organisms (Bannister & Kouzarides, 2005). In animals, crucial roles of arginine methylation in transcriptional regulation, RNA processing, nuclear transport, DNA-damage response and signal transduction are just emerging (Bedford & Richard, 2005; Wysocka *et al*, 2006).

Protein arginine methyltransferases (PRMTs) catalyse the transfer of methyl groups from *S*-adenosyl-L-methionine (SAM) to the guanidino nitrogen atoms of arginine residues; all active PRMTs in mammals catalyse the formation of ω-monomethyl-arginine (MMA). Type I and type II enzymes further catalyse the formation of asymmetric (ADMA) or symmetric (SDMA) dimethyl arginine, respectively. In mammals, PRMT1 and PRMT5 represent the main type I and type II methyltransferase activities to both mono- and dimethylate histone H4R3 asymmetrically or symmetrically, respectively (Bedford & Richard, 2005). Embryos of *prmt1*^{−/−} knockout mice died soon after implantation, indicating its necessity for development (Pawlak *et al*, 2000).

In *Arabidopsis thaliana*, several histone H3 lysine methyltransferases have been identified to be important in gene silencing and developmental regulation (Jackson *et al*, 2002; Kim *et al*, 2005; Naumann *et al*, 2005; Zhao *et al*, 2005; Ebbs & Bender, 2006). Recently, *A. thaliana* protein arginine methyltransferase 5 (AtPRMT5), a type II enzyme and the *Arabidopsis* homologue of human PRMT5, was shown to symmetrically dimethylate histone H4R3. Mutations in *AtPRMT5* resulted in several developmental defects, such as growth retardation, dark green leaves and late flowering (Pei *et al*, 2007; Wang *et al*, 2007); however, the roles of asymmetrical dimethylation of histone H4 in plant development remain unknown.

RESULTS AND DISCUSSION

Purification of a histone H4 methyltransferase

To identify histone methyltransferases in plants, we used a conventional biochemical approach to directly purify a histone H4-specific methyltransferase from cauliflower by following methyltransferase activity towards calf thymus histones (Fig 1). The H4-specific methyltransferase activity peak was separated into two peaks on a phenyl Sepharose column (Fig 1B), and the latter was separated into another two peaks on a DEAE 5PW column (Fig 1C). After final fractionation over a gel filtration column, two closely migrating bands of around 42 kDa were resolved by using SDS–polyacrylamide gel electrophoresis (SDS–PAGE), which were eluted with a predicted mass of 85 kDa from

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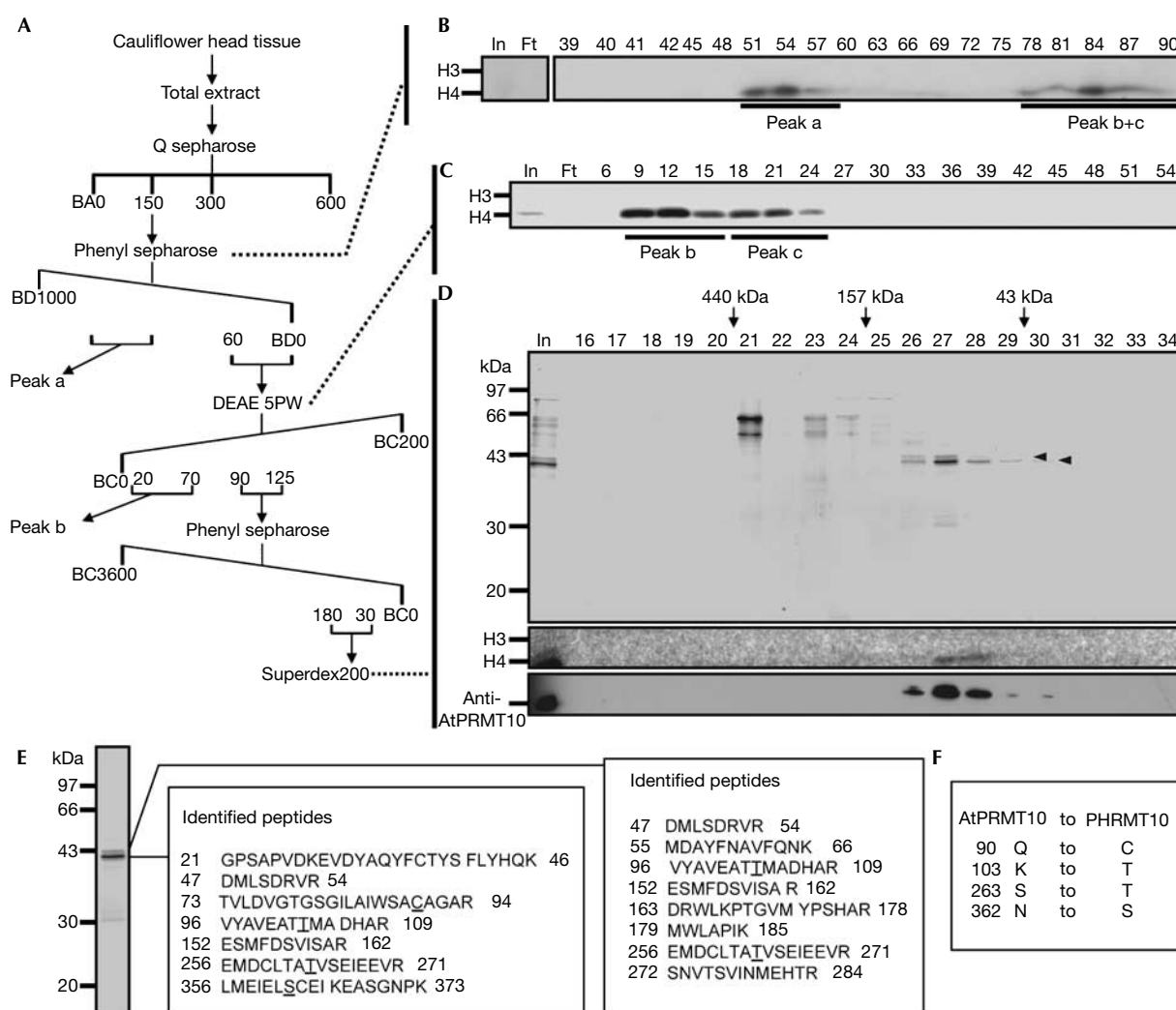E-mail: xfcao@genetics.ac.cn

Fig 1 | Purification and identification of a plant histone H4 methyltransferase from cauliflower. (A) Purification scheme of the histone H4 methyltransferase. BA, BD and BC represent different buffers and detailed information is listed in the Supplementary information online. Numbers represent salt concentration (mM). (B,C) Histone methylation assay of fractions derived from (B) the phenyl Sepharose column and (C) the DEAE-5PW column. In and Ft represent input and flow through, respectively. Numbers above the gel lanes indicate fraction numbers. (D) Silver staining (top), methyltransferase activity (middle) and immunoblot analysis using anti-AtPRMT10 (bottom) of fractions derived from the Superdex200 column. Proteins co-fractionated with H4 methyltransferase activity are indicated by arrowheads. Elution profile of molecular weight standard is indicated at the top. Numbers on the left indicate molecular weight standard of SDS–PAGE. (E) Both bands with similarity to AtPRMT10 were analysed by mass spectrometry. Peptides obtained from each band by tandem mass spectrometry analysis are listed. Numbers represent the amino acid number of AtPRMT10. Amino acids that are underlined are different between PHRMT10 and AtPRMT10, and are summarized in (F). AtPRMT10, *Arabidopsis thaliana* protein arginine methyltransferase 10; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

the gel filtration column (Fig 1D, top panel). These proteins correlated with the enzymatic activity, indicating that the putative purified enzyme exists either as a dimer or in a protein complex (Fig 1D, middle panel). These bands were excised and subjected to mass spectrometry analysis and both showed great similarity to a putative AtPRMT at locus *At1g04870* except for a few amino-acid substitutions (Fig 1E,F).

Previously, nine PRMTs (PRMT1–PRMT9) have been reported in mammals (Bedford & Richard, 2005; Cook *et al*, 2006). We searched the *Arabidopsis* genome for AtPRMTs, and a gene family containing nine members was identified and named based on their animal counterparts (supplementary Fig S1 online). Phylogenetic analysis showed that the polypeptide encoded by *At1g04870* had low similarity to other *Arabidopsis* and mammal PRMTs, but had high similarity to a rice putative methyltransferase encoded by rice *Os06g05090* (supplementary Figs S1,S2 online). Thus, we named this protein AtPRMT10 and the *Brassica* counterpart PHRMT10 for plant histone arginine methyltransferase 10.

The *AtPRMT10* complementary DNA encodes a 383-amino-acids polypeptide (NP_563720) with a calculated molecular mass of 43,130 Da. The final gel filtration fractions probed with polyclonal antibody against full-length AtPRMT10 showed two closely migrating
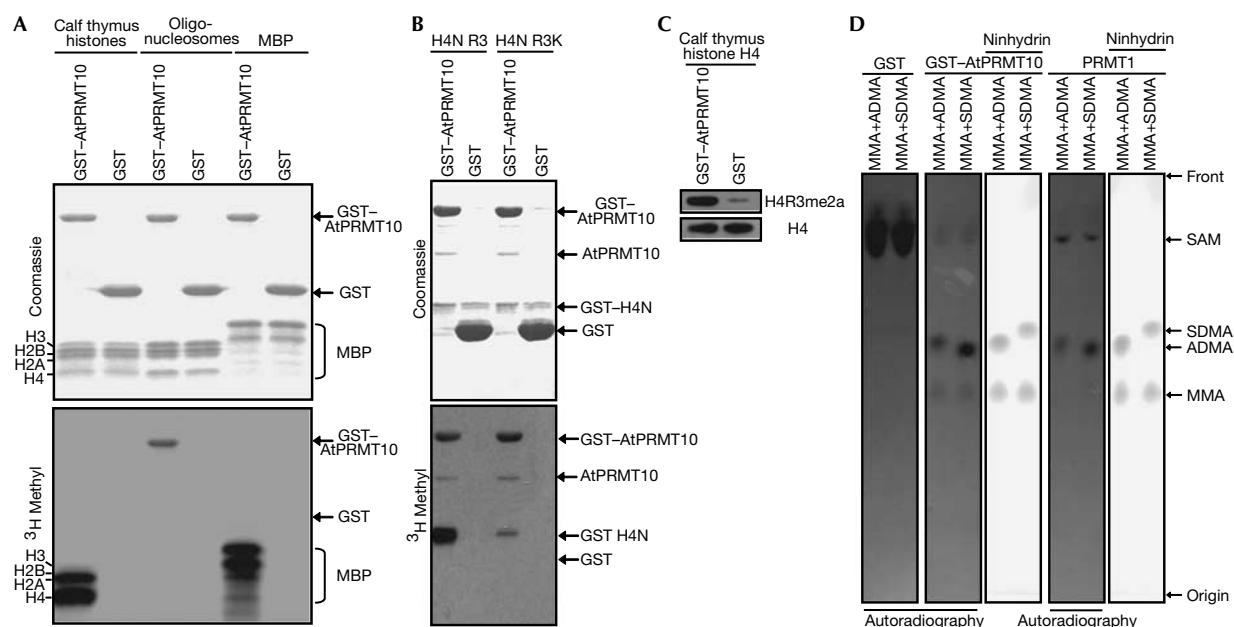
Fig 2 | AtPRMT10 is a type I arginine methyltransferase that catalyses H4R3 methylation *in vitro*. The GST–AtPRMT10 fusion protein was used to methylate (**A**) calf thymus histones, oligonucleosomes from HeLa cell and MBP, and (**B**) GST–H4N R3 and GST–H4N R3K. Top panels are Coomassie-stained gels and the bottom panels are autoradiographs of the top gels. Note: exposure time of (**B**) is five times more than that of (**A**). (**C**) Histone H4 methylated by GST–AtPRMT10 was probed with an antibody against asymmetrically dimethylated H4R3 (H4R3me2a). The membrane was re-probed with antibodies against histone H4 to act as a loading control. (**D**) Acidic hydrolysed histone methylation mixture was mixed with either MMA/ADMA or MMA/SDMA standards (30 nmol each) and separated by TLC. The positions of MMA, ADMA and SDMA were visualized by ninhydrin staining (right panel). Autoradiography of this plate showed that the radioactivities co-migrated with ninhydrin-stained MMA and ADMA but not SDMA, which was identical to that of a well-characterized type I enzyme, PRMT1. The radioactive signal migrated slower in MMA + SDMA than in MMA + ADMA because of the excess amount of standard used, which affected the migration of amino acids close to the standards. GST alone was used as a negative control and its autoradiography is shown in the first panel. Free [^{3}H]SAM is indicated. The separation starting site is indicated as 'Origin' and the solvent front is indicated as 'Front'. ADMA, asymmetric dimethyl arginine; AtPRMT10, *Arabidopsis thaliana* protein arginine methyltransferase 10; GST, glutathione-*S*-transferase; MBP, myelin basic protein; MMA, ω-monomethyl-arginine; PRMT, protein arginine methyltransferase; SDMA, symmetric dimethyl arginine; TLC, thin-layer chromatography.

bands around 42 kDa that matched the activity and the silver-stained gel (Fig 1D, bottom panel). In combination with the gel filtration and mass spectrometry data, it is confirmed that PHRMT10 is responsible for the methyltransferase activity we identified.

Asymmetrical dimethylation of H4R3 by AtPRMT10

We also used *Arabidopsis* as a model system to explore the biological roles of PHRMT10. AtPRMT10 has relatively conserved *S*-adenosyl methionine (AdoMet)-dependent methyltransferase motifs I, post I, II, III and THW loop domains that are common to all PRMTs indicating that AtPRMT10 might have enzymatic activity (Bedford & Richard, 2005; supplementary Fig S2 online). *In vitro* protein methylation assays indicated that AtPRMT10 predominantly methylated calf thymus histones H4 and H2A, and a non-histone protein myelin basic protein, which is a common PRMT substrate, but not oligonucleosomes (Fig 2A). The ability of AtPRMT10 to methylate a glutathione-*S*-transferase (GST) fusion protein containing the amino-terminal amino acids (1–54) of histone H4 (H4N R3), but only weakly methylate a mutant form with arginine 3 changed to lysine (H4N R3K), indicated that AtPRMT10 was mainly an H4R3-specific methyltransferase (Fig 2B). We also observed automethylation of AtPRMT10 when

the substrate was not optimal or when no substrate was present (Fig 2A,B). Furthermore, calf thymus histone H4 methylated by AtPRMT10 could be recognized by an antibody against asymmetrically dimethylated H4R3 (H4R3me2a), indicating that AtPRMT10 might be a type I PRMT (Fig 2C). In addition, a thin-layer chromatography (TLC) assay confirmed that arginine residues methylated by AtPRMT10 and PRMT1—a well-known type I PRMT—co-migrated with MMA and ADMA, but not with SDMA (Fig 2D). These results indicate that AtPRMT10 is a type I PRMT and preferentially methylates R3 of histone H4 *in vitro*.

Characterization of *atprmt10* mutants

To study further the *in vivo* functions of *AtPRMT10*, we identified *atprmt10-1*, *atprmt10-2* and *atprmt10-3* mutants, which contain transferred DNA insertions in exon 5, intron 3 or exon 1, respectively (Fig 3A). Full-length *AtPRMT10* messenger RNA was not detected in any of the three mutant alleles (Fig 3B); the AtPRMT10 antibody detected a single band of 42 kDa in wild type, which was absent in all three *atprmt10* mutants (Fig 3C). Therefore, *atprmt10-1*, *atprmt10-2* and *atprmt10-3* seem to be null alleles.

The *atprmt10-1*, *atprmt10-2* and *atprmt10-3* homozygous mutants showed delayed flowering time with increased total leaf
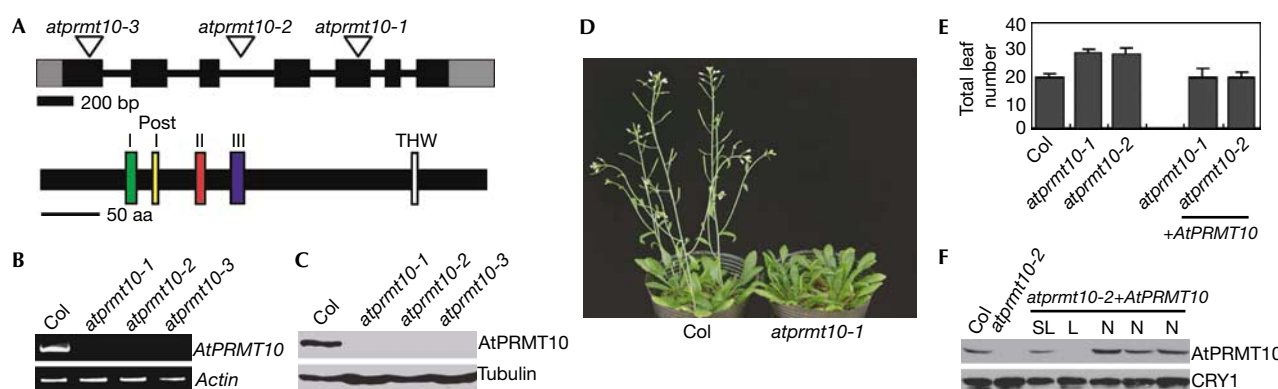
Fig 3 | *atprmt10* mutants are late flowering. (**A**) The gene structure of *AtPRMT10* (top). The triangles indicate the three identified T-DNA insertions into the *Arabidopsis* genome. The structure of conserved domains of AtPRMT10 is shown below. (**B**) RT–PCR analysis of full-length *AtPRMT10* transcript in wild-type Col and three *atprmt10* mutants (top panel). Actin was used as an internal control (bottom panel). (**C**) Western blot analysis of AtPRMT10 protein in wild-type Col and three *atprmt10* mutants (top panel). Tubulin was used as an internal control (bottom panel). (**D**) *atprmt10* plants are late flowering. The plants shown here were grown under long-day conditions. (**E**) Total leaf number of wild-type Col, *atprmt10* mutants and *atprmt10* mutants transformed with a genomic DNA fragment of *AtPRMT10* (restored flowering time lines are indicated by $+ AtPRMT10$) was calculated under long-day conditions. Error bars represent $2 \times$ s.e. (**F**) Western blot analysis of AtPRMT10 in complementary lines. Five representative complementary lines of *atprmt10-2* transformed with *AtPRMT10* genomic fragment were probed with AtPRMT10 antibody. SL, L or N indicates slightly late flowering, late flowering or normal flowering complementary lines, respectively, as compared with wild-type Col plants. Wild-type Col was used as a positive control, whereas *atprmt10-2* was used as a negative control. The membrane was re-probed with antibodies against CRY1 to act as a loading control (bottom). AtPRMT10, *Arabidopsis thaliana* protein arginine methyltransferase 10; RT–PCR, reverse transcription–PCR.

number when grown under long-day conditions (Fig 3D; data not shown). The late-flowering phenotype of both *atprmt10-1* and *atprmt10-2* mutants was complemented by the transformation of *AtPRMT10* genomic DNA (Fig 3E). The flowering time of these complemented transgenic plants tended to correlate reversely with the level of AtPRMT10 (Fig 3F). Hence, we conclude that asymmetrical dimethylation of arginine residues mediated by AtPRMT10 is required for the promotion of flowering in *Arabidopsis* under long-day conditions.

Molecular genetic analysis has indicated that the photoperiod, gibberellin, vernalization and autonomous pathways are four main pathways that regulate flowering time in *Arabidopsis* (Blazquez *et al*, 1998; Henderson & Dean, 2004; Searle & Coupland, 2004; Amasino, 2005; Dennis *et al*, 2006). Here, we show that the late-flowering phenotype of *atprmt10* mutants phenocopies mutants in the autonomous pathway (Fig 4A), as *atprmt10* mutants flowered later than wild-type plants under both long- and short-day conditions. Vernalization and gibberellin treatments rescued the late-flowering phenotype of *atprmt10* mutants, and the flowering time of *flc-3* FLOWERING LOCUS C *atprmt10* double mutants was similar to that of *flc-3* plants (Fig 4B). Consistent with our genetic observations, we detected elevated levels of *FLC* transcripts and decreased expression of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) transcripts (a floral pathway integrator, repressed by *FLC*) in *atprmt10* mutants (Fig 4C). Therefore, we conclude that AtPRMT10 is a new component in the autonomous pathway, which controls the floral transition in an *FLC*-dependent manner.

FLC chromatin has been shown to be regulated by histone acetylation and lysine methylation (He & Amasino, 2005). To investigate whether arginine methylation might also be involved in modifying chromatin of the *FLC* locus, chromatin immunoprecipitations (ChIPs) were carried out using antibodies against H4R3me2a, trimethylated H3K4 (H3K4me3), dimethylated H3K4 (H3K4me2) and acetylated H3 (H3ac). However, no significant differences were found between *atprmt10* mutants and wild-type Col (supplementary Fig S3A,B online). In addition, four control assays worked well, indicating that the ChIP assays were carried out correctly (supplementary Fig S3C–E online).

Histone modifications are believed to be a conserved mechanism in regulating chromatin dynamics in eukaryotic organisms. In mammals, methylation of histone H4R3 by PRMT1 has been shown to be important in transcriptional activation by facilitating histone H4 acetylation (Wang *et al*, 2001). Therefore, asymmetrical H4R3 dimethylation in plants might also promote transcription. If AtPRMT10 modifies *FLC* chromatin directly, it might be expected to reduce *FLC* expression in *atprmt10* mutants. However, loss-of-function mutations in *AtPRMT10* showed elevated levels of *FLC* mRNA. Together with ChIP results we conclude that AtPRMT10 might be an indirect modulator of *FLC* by activating other repressors of *FLC*.

To test this, we evaluated the transcript levels of genes in the autonomous pathway, including those involved in RNA processing (*FCA, FY, FLOWERING LOCUS K* (*FLK*), *FPA*) (Simpson *et al*, 2004) and transcription regulation (*FVE, FLD, RELATIVE OF EARLY FLOWERING 6* (*REF6*) and *LUMINIDEPENDENS* (*LD*)) (Lee *et al*, 1994; He *et al*, 2003; Ausin *et al*, 2004; Noh *et al*, 2004), by using real-time PCR. However, all tested genes showed no marked differences in mRNA levels between *atprmt10* mutants and wild-type Col (supplementary Fig S4 online). This raised the possibility that AtPRMT10 might regulate an as yet unidentified *FLC* repressor, or that AtPRMT10 might function at post-transcriptional or post-translational levels in controlling flowering time.
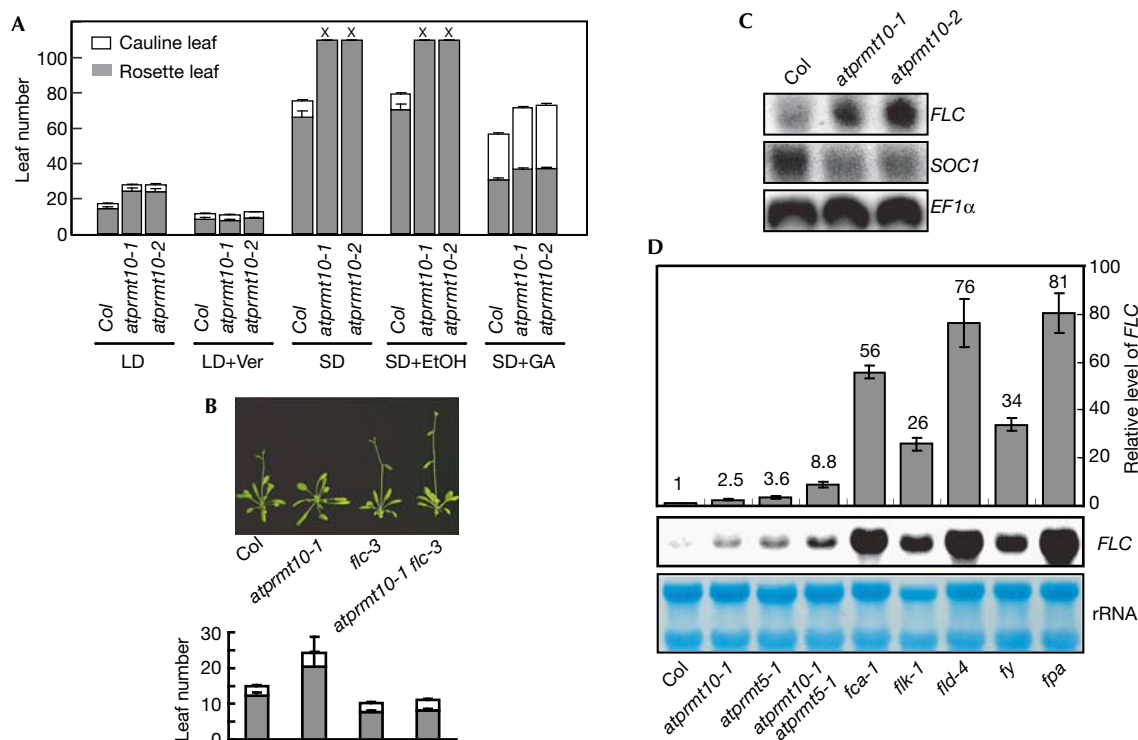
Fig 4 | Regulation of flowering time by AtPRMT10 is FLC-dependent. (A) Flowering time was assessed by rosette leaf and cauline leaf number of *atprmt10* mutants under long-day conditions (LD), vernalization (LD + Ver), short day (SD) and gibberellin (GA) treatment (SD + GA). Short day with ethanol (SD + EtOH) was used as a control for GA treatment. White boxes indicate cauline leaf number and grey boxes indicate rosette leaf number. 'X' indicates that we stopped counting after they had produced 110 rosette leaves. Error bars represent 2 × s.e. **(B)** The late-flowing phenotype of *atprmt10* mutants was rescued by *flc-3*. The upper panel shows the phenotype of *atprmt10-1 flc-3* double mutant and single mutants under LD. The lower panel shows leaf number of the corresponding plants above. **(C)** RNA gel blot analysis of *FLC* and *SOC1* transcripts. *EF1α* (*ELONGATION FACTOR 1α*) (AT5G60390) was used as a loading control. **(D)** Real-time PCR (top) and RNA gel blot analysis (medium) of *FLC* transcripts in 11-day old seedlings of wild-type Col, *atprmt10-1*, *atprmt5-1*, *atprmt10-1atprmt5-1* with some autonomous pathway mutants indicated. The real-time PCR result was normalized to *actin*, and the membrane stained by methylene blue (bottom) was used as a loading control of RNA gel blot. Error bars represent 2 × s.e. Numbers represent the transcript amount of *FLC* in mutants relative to that in wild-type Col. AtPRMT10, *Arabidopsis thaliana* protein arginine methyltransferase 10; FLC, FLOWERING LOCUS C; SOC, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS.

As AtPRMT5 was shown to promote flowering time in an *FLC*-dependent manner (Pei *et al*, 2007; Wang *et al*, 2007), we wanted to know whether there is some genetic interaction between *AtPRMT10* and *AtPRMT5* genes in repressing *FLC*. Under long-day conditions, the *atprmt5 atprmt10* double mutant showed additive effects on flowering time and *FLC* mRNA levels as compared with either single mutant, indicating that *AtPRMT5* and *AtPRMT10* might act in a genetically parallel manner (Fig 4D; supplementary Fig S5 online). In addition, real-time PCR and northern blot analysis showed that the double mutant expressed lower levels of *FLC* mRNA than the autonomous pathway mutants tested (Fig 4D). These indicate that the two genes are weaker suppressors compared with the classic autonomous pathway mutants and the minor effects also indicate further redundancies in the repression of *FLC*.

In mammals, proteomic analysis identified more than 200 putative arginine-methylated proteins, many of which are heterogeneous nuclear ribonucleoproteins acting in RNA processing (Boisvert *et al*, 2003). For example, sam68, a K homology domain RNA-binding protein, was an *in vivo* substrate of PRMT1 and relocalized to the cytoplasm when methylation was blocked (Cote *et al*, 2003). Therefore, besides histones, AtPRMT10 might also act on many other cellular proteins to modulate their functions. It will be of interest to dissect deeper molecular mechanisms of AtPRMT10 in flowering time regulation.

METHODS

Purification of the histone H4 methyltransferase. Fresh cauliflower head was purchased from the local market. The 3–7 mm surface layer mainly composed of inflorescence and floral meristem was collected. The purification procedure is detailed in the supplementary information online.

Histone methyltransferase assay. Column fractions or recombinant enzyme was incubated with appropriate substrates and [³H]SAM as described previously (Wang *et al*, 2001). The reaction mixture was separated by SDS–PAGE and stained with Coomassie blue. The gel was then treated with Amplifier (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), dried and exposed to Kodak Biomax MS film at −80 °C for the appropriate time.

Antibodies. Antibodies used in this study are as follows: tubulin (Sigma-Aldrich, St Louis, MO, USA); horseradish peroxidase-conjugated secondary antibodies (Pierce Biotechnology Inc, Rockford, IL, USA); CRY1 (a gift from C. Lin). The mouse polyclonal antibody against AtPRMT10 was raised using recombinant GST-AtPRMT10 fusion protein. The antiserum was affinity purified before use.

Thin-layer chromatography analysis. *In vitro* histone methylation reactions were carried out with [³H]SAM (GE Healthcare Bio-Sciences AB), histone H4 calf thymus (Roche Diagnostics GmbH, Mannheim, Germany) and the appropriate enzymes. Sample preparation and TLC analysis were carried out as reported previously (Pei *et al*, 2007).

Plant materials. *atprmt10-1* (SALK_047046), *atprmt10-2* (SALK_024289), *atprmt10-3* (SALK_049430), *fy-5* (SALK_053604) and *fpa-9* (SALK_011615) were isolated from the Salzburger Landeskliniken Collection (http://signal.salk.edu/). The *flk-1* seeds were a gift from C. Lin. The primers used for genotyping are listed in the supplementary information online.

Complementation assay. The complement fragment of *AtPRMT10*, including 1,157 bp before ATG, the coding region, and 530 bp after TGA, the stop codon, was ligated into pCAMBIA1300 vector. This construct was then transformed into *Agrobacterium* strain *AGLO* and transformed into *Arabidopsis* through floral dipping.

Flowering time assessment. Plant growth conditions are listed in the supplementary information online. Mutants and control plants were directly grown in soil side by side in Versatile Environmental Test Chamber (MLR-350H, *SANYO*). Flowering time was assessed by counting the number of rosette and cauline leaves after they had flowered. At least 15 plants were counted for each line.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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