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The catalytic subunit of *Arabidopsis* DNA polymerase α ensures stable maintenance of histone modification

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SUMMARY

Mitotic inheritance of identical cellular memory is crucial for development in multicellular organisms. The cell type-specific epigenetic state should be correctly duplicated upon DNA replication to maintain cellular memory during tissue and organ development. Although a role of DNA replication machinery in maintenance of epigenetic memory has been proposed, technical limitations have prevented characterization of the process in detail. Here, we show that INCURVATA2 (ICU2), the catalytic subunit of DNA polymerase α in *Arabidopsis*, ensures the stable maintenance of repressive histone modifications. The missense mutant allele *icu2-1* caused a defect in the mitotic maintenance of vernalization memory. Although neither the recruitment of CURLY LEAF (CLF), a SET-domain component of Polycomb Repressive Complex 2 (PRC2), nor the resultant deposition of the histone mark H3K27me3 required for vernalization-induced *FLOWERING LOCUS C* (*FLC*) repression were affected, *icu2-1* mutants exhibited unstable maintenance of the H3K27me3 level at the *FLC* region, which resulted in mosaic *FLC* de-repression after vernalization. ICU2 maintains the repressive chromatin state at additional PRC2 targets as well as at heterochromatic retroelements. In *icu2-1* mutants, the subsequent binding of LIKE-HETEROCHROMATIN PROTEIN 1 (LHP1), a functional homolog of PRC1, at PRC2 targets was also reduced. We demonstrated that ICU2 facilitates histone assembly in dividing cells, suggesting a possible mechanism for ICU2-mediated epigenetic maintenance.

KEY WORDS: DNA polymerase α, Epigenetic maintenance, Chromatin assembly

INTRODUCTION

During eukaryotic chromosome replication, both genetic and epigenetic information are accurately duplicated, which maintains identical cellular memory through mitoses (Margueron and Reinberg, 2010). DNA replication accompanies chromatin assembly, and the cooperation between these two processes has long been proposed to be important for the mitotic maintenance of epigenetic information (Alabert and Groth, 2012; Ransom et al., 2010; Smith and Whitehouse, 2012).

DNA polymerase α is responsible for initiating replication at both the origins and the lagging strand (Kunkel and Burgers, 2008). In *Arabidopsis*, the single copy gene *INCURVATA2* (*ICU2*) encodes the catalytic subunit of DNA polymerase α, and transfer DNA (T-DNA)-inserted knockout mutants of *ICU2* showed a lethal phenotype during gametogenesis and embryogenesis (Barrero et al., 2007). A missense mutation in *ICU2*, *icu2-1*, was isolated by the resulting upward curling phenotype of the rosette leaves (Serrano-Cartagena et al., 2000). This mutant exhibits early flowering and homeotic transition of the floral organs, as observed in mutants of Polycomb Repressive Complex 2 (PRC2) (Barrero et al., 2007). Accordingly, certain PRC2 target genes are de-regulated in *icu2-1* (Barrero et al., 2007). Another missense allele of *ICU2*, *polα*, was identified by suppressor mutant screening of the *repressor of transcriptional silencing 1* (*ros1*) (Liu et al., 2010a), which has a defect in the DNA demethylase ROS1 (Gong et al., 2002). The *polα* mutant exhibited a similar phenotype to *icu2-1*, and showed de-repression of some heterochromatic elements in the *Arabidopsis* genome, although the DNA methylation levels in the centromeric repeats and transposons were not affected. ICU2 has a genetic relationship with CURLY LEAF (CLF), a SET-domain component of PRC2; LIKE-HETEROCHROMATIN PROTEIN 1 (LHP1), a potential PRC1 component; and FASCIATA1 (FAS1), a chromatin assembly factor (Barrero et al., 2007).

The vernalization response in *Arabidopsis thaliana* is an example of the mitotic inheritance of histone marks in plants (Kim et al., 2009). Vernalization is the acquisition of floral competence through exposure to prolonged cold in winter. In *Arabidopsis*, the two strong floral repressors *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) synergistically interact to cause late flowering (Shindo et al., 2005). *FRI* induces the expression of *FLC*, and the resulting high level of *FLC* inhibits floral induction. However, vernalization induces the downregulation of *FLC*, and this repression is mitotically remembered until reproduction, even after a return to warm conditions (Choi et al., 2009; Kim et al., 2009; Sheldon et al., 2008). As a result, plants exposed to long-term cold treatment undergo the transition to flowering.

Covalent histone modifications at the *FLC* genomic region is a key process for maintaining *FLC* repression (Kim et al., 2009). During vernalization, *FLC* repression is initiated by VERNALIZATION INSENSITIVE 3 (VIN3) through the deacetylation of histone H3. Simultaneously, VERNALIZATION2 (VRN2)-PRC2 deposits H3K27me3 at the *FLC* region (De Lucia et al., 2008; Finnegan and Dennis, 2007; Sung and Amasino, 2004; Wood et al., 2006). The accumulation of H3K27me3 results in the stable repression of *FLC*. Accordingly, a loss-of-function *vrn2* mutant normally shows a downregulation of *FLC* during vernalization. However, the mutant fails to accumulate H3K27me3, so *FLC* is de-repressed when the *vrn2* plants return to warm conditions (Gendall et al., 2001). Remarkably, the H3K27me3 mark is inherited and propagated to nearby histones in the *FLC*

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region in subsequent warm growing conditions by an unknown mechanism (De Lucia et al., 2008; Finnegan and Dennis, 2007). Therefore, stable inheritance of H3K27me3 and the concomitant *FLC* silencing are crucial for the acquisition of floral competence after vernalization.

In this study, we presented new evidence that ICU2 is specifically involved in the maintenance of repressive histone marks during mitoses, but not in the mark deposition on histones, by analyzing the mitotic maintenance of vernalization memory in *icu2-1* mutants. In addition, the role of ICU2 in silencing diverse chromatin loci and the functional relationship of ICU2 with PRC2 and LHP1 were also examined. Lastly, we identified a possible mechanism for ICU2-mediated epigenetic inheritance by analyzing DNA replication-dependent chromatin assembly in *icu2-1* mutant plants.

MATERIALS AND METHODS

Plant materials, growing conditions, histochemical GUS staining and microscopy

All plants used in this study originated from the Col-0 background except for the *icu2-1* (En-2), *polα* (C24) and *clf-2* (L*er*) mutants. To generate *icu2-1 FRI* and *clf-2 FRI*, each mutant allele was introduced into *FRI*-Col through five backcrosses. The plants were grown in either long-day (16 hour light/8 hour dark) or short-day (8 hour light/16 hour dark) photoperiodic conditions under cool white fluorescent light (100 μmole/m^2/s) at 22°C with 60% relative humidity. For the vernalization treatment, seeds or plants grown in soil were incubated at 4°C under short-day conditions. To prepare samples in equivalent developmental stages, seeds of non-vernalized (NV) samples were germinated at 3 days before the end of vernalization treatment of vernalization +21 days warm growth (V+21d) samples. Under our experimental conditions, this condition provided the most equivalent developmental stages between NV and V+21d samples.

β-Glucuronidase (GUS) activity in vernalized plants was analyzed by incubation in 50 mM $NaPO_4$ (pH 7.0), 1 mM X-Gluc, 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, 10 mM EDTA and 0.2% Triton X-100 at 37°C for 8-10 hours. The stained tissues were photographed using an Axio Imager A1 microscope with an AxioCam HRc camera (Carl Zeiss).

Gene expression analysis

Total RNA was extracted from whole seedlings using an RNeasy Plant Mini Kit (Qiagen). After RNase-free DNase (TaKaRa Bio) treatment, 2 μg of total RNA was converted to cDNA using an oligo-dT primer and M-MLV reverse transcriptase (Ambion). Real-time qPCR was performed using a CFX96 Real-Time System (Bio-Rad), and the relative transcript level of each gene was determined by normalization of the resulting expression levels to that of *TUB2*. The primer sequences for real-time qPCR are listed in the supplementary material Table S1.

Generation of *ICU2::GUS* construct

To generate *ICU2::GUS* recombinant DNA, ~1.7 kb of the promoter of *ICU2* was cloned by PCR (supplementary material Table S1). The amplified DNA was inserted into a pBI101.1 binary vector (Clontech).

Chromatin immunoprecipitation (ChIP) analysis

ChIP analysis was performed as described previously (Ko et al., 2010) with minor modifications. After chromatin isolation, immunoprecipitation was performed using H3K27me3 (Upstate), H3K4me3 (Upstate), H3K9me2 (Upstate) and GFP (Invitrogen) antibodies. Real-time qPCR was performed, and the relative enrichment of the IP/Input at each genomic region was normalized to that of the *ACT7* reference genomic locus as described previously (De Lucia et al., 2008).

Generation of anti-CLF antibody

A polyclonal anti-CLF antibody was obtained using a synthesized peptide antigen (390-TNSDKVSSSPKVKG-405; Genscript). Synthesized peptide was conjugated to keyhole limpet hemocyanin (KLH) and immunized in rabbit by active immunization. The resultant antisera were further purified by affinity purification (Genscript).

Generation of partial ICU2 protein series

To construct a *35S::partial ICU2* series, the partial cDNA fragments of *ICU2* were amplified by PCR as presented in Fig. 5A. The amplified partial *ICU2* cDNAs were cloned into a binary vector myc-pBA. To examine the phenotypes of the partial *ICU2* transgenic plants, we analyzed at least 20 independent transgenic lines for each construct.

Yeast two-hybrid analysis

Yeast two-hybrid analysis using the Matchmaker Two-Hybrid system (Clontech) was performed according to the manufacturer's protocol with minor modifications. Full- and partial-length cDNAs from *ICU2* and *LHP1* were reciprocally cloned into the pGBKT7 bait vector and pGADT7 prey vector in the Matchmaker Two-Hybrid system (Clontech). The constructed recombinant clones were introduced into AH109 yeast cells following the manufacturer's protocol. Transformed yeast colonies were examined their growth on –Ade/–His/–Leu/–Trp quadruple dropout media to determine interactions.

Bimolecular fluorescence complementation (BiFC) analysis

BiFC analysis was performed as described previously (Jeong et al., 2011). Full-length cDNAs from *ICU2* and *LHP1* were reciprocally cloned into *pSAT4-nEYFP-C1* and *pSAT4-cEYFP-C1-B* vectors containing the *CaMV 35S* promoter. The constructed recombinant clones were introduced into *Arabidopsis* mesophyll protoplasts by polyethylene glycol transfection, as described previously (Yoo et al., 2007). The fluorescence images were photographed and processed using a DV ELITE DeltaVision microscope (Applied Precision).

In vivo co-immunoprecipitation (co-IP) analysis

To generate *ICU2::H3.1:GFP*, the *ICU2* promoter used in *ICU2::GUS* construction was cloned into a pBI101.1 binary vector (Clontech) containing the sGFP coding sequences. Next, the *H3.1* cDNA was sequentially inserted in frame with the *GFP* open reading frame to generate an *ICU2::H3.1:GFP* binary vector. To perform *in vivo* co-IP analysis with intact chromatin, the nuclear fraction was purified and immunoprecipitated following the same protocol that was used in the ChIP experiment. The input and immunoprecipitated samples were separated on 10% acrylamide SDS-PAGE gel and hybridized with H2B (Abcam), GFP (Invitrogen) and H3 (Abcam) antibodies. After signal detection, the relative band intensity was quantified using ImageJ software (NIH).

RESULTS

Spatiotemporal expression pattern of *ICU2*

To understand the role of *ICU2*, we first quantified the spatiotemporal expression pattern of *ICU2* in *Arabidopsis*. Our reverse transcription-real-time qPCR (RT-qPCR) results revealed that *ICU2* is highly expressed in seedlings, inflorescence meristems with floral buds, open flowers and pistils, which all have highly proliferating cells (Fig. 1A,B). We also generated *ICU2::GUS* transgenic plants and analyzed the *GUS* expression pattern (Fig. 1C-K). The transgene contains the 1.7 kb promoter region of *ICU2* before the start codon, and the promoter includes the E2F-binding motif TTTCCCGC, which is characteristic of cell cycle-regulated genes (Chabouté et al., 2000). *ICU2::GUS* expression was detected in the shoot apex, young emerging leaves, meristematic zone in root tip, lateral root primordia, young floral buds and ovules in open flowers (Fig. 1C-K). These expression patterns of *ICU2* and *ICU2::GUS* suggest that *ICU2* might function in the proliferating tissues of *Arabidopsis*.

Vernalization memory in *icu2-1 FRI* population

As *icu2-1* mutants show phenotypes similar to that of the PRC2 mutants, we hypothesized that *ICU2* might play a role in

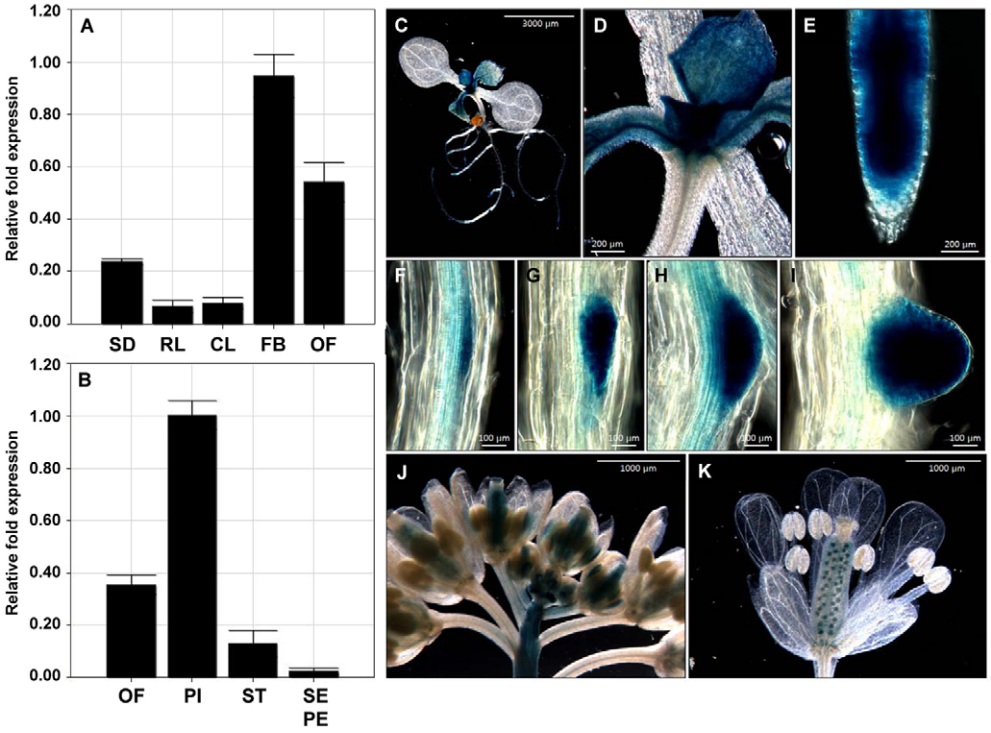

Fig. 1. Spatiotemporal expression pattern of *ICU2*. (**A**) RT-qPCR analysis of *ICU2* mRNA level in wild-type 10-day-old *Arabidopsis* seedlings (SD), rosette leaves (RL), cauline leaves (CL), floral bud clusters including inflorescence meristems (FB) and open flowers (OF). (**B**) RT-qPCR analysis of *ICU2* mRNA level in wild-type open flowers (OF), pistils (PI), stamens (ST) and sepals plus petals (SE PE). (**C-K**) GUS activity in wild-type *ICU2::GUS* 10-day-old seedlings (C), shoot apical meristem regions (D), root tips (E), lateral root meristems (F-I), floral bud clusters (J) and open flowers (K). Error bars indicate s.e.m.

vernalization memory. To check this, *icu2-1* mutation was introduced into a *FRI*-Col ecotype that requires vernalization for flowering using backcrosses repeated five times. The resultant *icu2-1 FRI* plants showed an early flowering phenotype under both long-day (LD) and short-day (SD) photoperiod conditions (Fig. 2A; Fig. 4B). In RT-qPCR analysis, we observed a slight downregulation of *FLC* and the ectopic expression of the floral activator *FLOWERING LOCUS T* (*FT*) in *icu2-1 FRI* (Fig. 2B). These results are consistent with the previous study of *icu2-1* plants lacking an active *FRI* gene (Barrero et al., 2007).

To investigate the *ICU2* regulation on *FLC* in vernalization memory, we crossed *icu2-1 FRI* to the *FLC::GUS* transgenic plants in *FRI*-Col background (Choi et al., 2009; Michaels et al., 2005), and the *GUS* expression pattern was examined in the *FLC::GUS icu2-1 FRI* plants. In *FRI*-Col, vernalization induced *FLC::GUS* repression, and the repression was maintained until 32 days after vernalization (V+32d) (Fig. 2C,E,F,M,O) as we reported previously (Choi et al., 2009). In non-vernalized (NV) conditions, *FLC::GUS* was weaker in the *icu2-1 FRI* plants than in the *FRI*-Col plants (Fig. 2C,D,M,N). *FLC::GUS* expression in the *icu2-1 FRI* was effectively repressed in response to vernalization (Fig. 2Q). However, the repression of *FLC::GUS* was not maintained in the *icu2-1 FRI* after vernalization treatment (Fig. 2G-L,P). Surprisingly, the degree of de-repression was variable among individual *icu2-1 FRI* plants. In addition, the differential defects were also observed even in cell populations or organs (Fig. 2G-L), thereby resulting in a mosaic *GUS* staining pattern in the *icu2-1 FRI* plants. This is similar to a previous result showing the mosaic de-repression of *AGAMOUS (AG)::GUS* in *icu2-1* (Barrero et al., 2007).

To examine the maintenance of endogenous *FLC* repression, the *FLC* expression was analyzed by RT-qPCR. Efficient repression of *FLC* was observed in both *FRI*-Col and *icu2-1 FRI* at V+0d and at V+5d, but was partially released at V+10d and V+21d in the *icu2-1 FRI* plants (Fig. 2Q). Interestingly, a similar partial mosaic de-repression of the silenced loci was also observed in the mutants of the components of CHROMATIN ASSEMBLY FACTOR-1 (CAF-1), *fasciata 1* (*fas1*) and *fas2*, as well as in *brushy 1* (*bru1*), the cooperation factor for CAF-1 (Ono et al., 2006; Ransom et al., 2010; Takeda et al., 2004). Recently, two studies reported that the mosaic *FLC::GUS* pattern can also be observed in plants that were only vernalized for two weeks, showing that the length of the cold treatment is crucial for a stable *FLC* repression (Angel et al., 2011; Satake and Iwasa, 2012). To ensure that our results were not biased by the relatively short vernalization period of 4 weeks, we analyzed *FLC* expression in plants vernalized for 8 weeks. In this condition also, *icu2-1 FRI* showed a defect in the maintenance of *FLC* repression (Fig. 2Q).

Taken together, we suggest that *ICU2* is required for the stable maintenance of the mitotic memory of vernalization, and that its defect induces a mosaic de-repression of *FLC* after vernalization.

Role of ICU2 in the maintenance of H3K27me3 during mitoses

To characterize the epigenetic function of ICU2 in vernalization memory, the chromatin state at the *FLC* genomic region was analyzed by performing a chromatin immunoprecipitation (ChIP) experiment. During cold treatment, H3K27me3 initially accumulates around the transcriptional start region of *FLC*, and the histone mark is stably inherited and propagated to the gene body region of *FLC* in subsequent warm conditions (De Lucia et al., 2008; Finnegan and Dennis, 2007). Consistently, we observed H3K27me3 enrichment in the specific start region at V+0d and the subsequent inheritance and propagation of H3K27me3 to the *FLC* gene body region at V+21d in *FRI*-Col plants (Fig. 3B). The *icu2-1 FRI* plants showed specific H3K27me3 enrichment similar to that of *FRI*-Col plants in response to vernalization (Fig. 3B, V+0d). However, at V+21d, the inheritance and propagation of H3K27me3 failed in *icu2-1 FRI* plants (Fig. 3B). Consistent with *FLC* expression (Fig. 2Q), the *icu2-1 FRI* mutants exhibited reduced H3K27me3 level at V+10d, but not at V+5d (supplementary material Fig. S1A,B). Taken together, these results suggest that

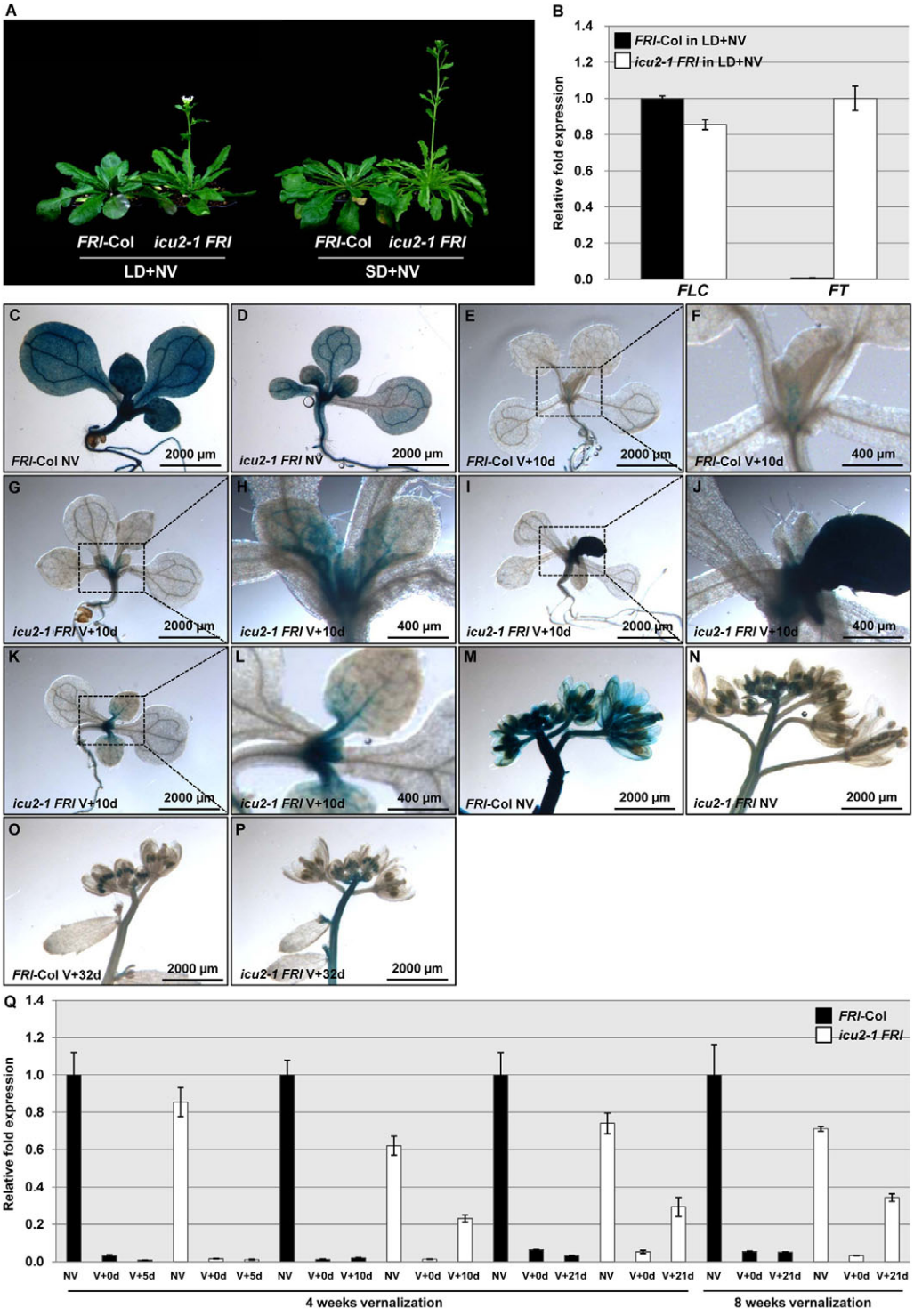

Fig. 2. Vernalization memory in *icu2-1 FRI* population. (**A**) Early flowering phenotype of *icu2-1 FRI*. (**B**) RT-qPCR analysis of *FLC* and *FT* mRNA levels in 3-week-old *FRI*-Col and *icu2-1 FRI Arabidopsis* seedlings. Error bars indicate s.e.m. (**C-P**) *FLC::GUS* expression in *FRI*-Col and *icu2-1 FRI* plants before and after vernalization. (C) *FLC::GUS FRI*-Col in non-vernalized (NV) condition. (D) *icu2-1 FLC::GUS FRI* in NV condition. (E,F) *FLC::GUS FRI*-Col in 4 weeks vernalization followed by 10 days further growth at 22°C under LD (V+10d); the shoot apical meristem region in E is enlarged in F. (G-L) Three independent *icu2-1 FLC::GUS FRI* siblings at V+10d; their enlarged images are shown in H, J and L. (M) *FLC::GUS FRI*-Col in NV. (N) *icu2-1 FLC::GUS FRI* in NV. (O) *FLC::GUS FRI*-Col at V+32d. (P) *icu2-1 FLC::GUS FRI* at V+32d. (**Q**) RT-qPCR analysis of *FLC* mRNA expression at NV, V+0d and V+21d time points after a 4- or 8-week vernalization. Three biological replicates were performed with similar results. Error bars indicate ±s.e.m. of PCR replicates (*n*=3).

ICU2 is involved not in the deposition of the histone mark but in the mitotic inheritance of H3K27me3 after vernalization.

To delineate further the chromatin state at *FLC* region, we analyzed the enrichment of H3K4me3, an active chromatin mark. H3K4me3 was specifically enriched at the transcriptional start region of *FLC* under NV conditions (Fig. 3C) (Ko et al., 2010; Pien et al., 2008; Tamada et al., 2009). In response to vernalization, both *FRI*-Col and *icu2-1 FRI* plants showed a loss of H3K4me3 enrichment (Fig. 3C, V+0d), consistent with *FLC* downregulation (Fig. 2Q). At V+21d, *icu2-1 FRI* plants showed a greater accumulation of the H3K4me3 whereas the wild-type plants showed a stable reduction of the mark (Fig. 3C).

To understand the molecular mechanism of ICU2 on H3K27me3 inheritance, we examined whether ICU2 mediates the recruitment and/or retention of PRC2. Using an antibody against CLF, a SET-domain component in PRC2 (supplementary material Fig. S1C), we performed ChIP analysis. During cold treatment, CLF was specifically recruited to the region where H3K27me3 initially accumulated in both *FRI*-Col and *icu2-1 FRI* plants (Fig. 3B,D, V+0d). The level of CLF was reduced in the *FRI*-Col plants at

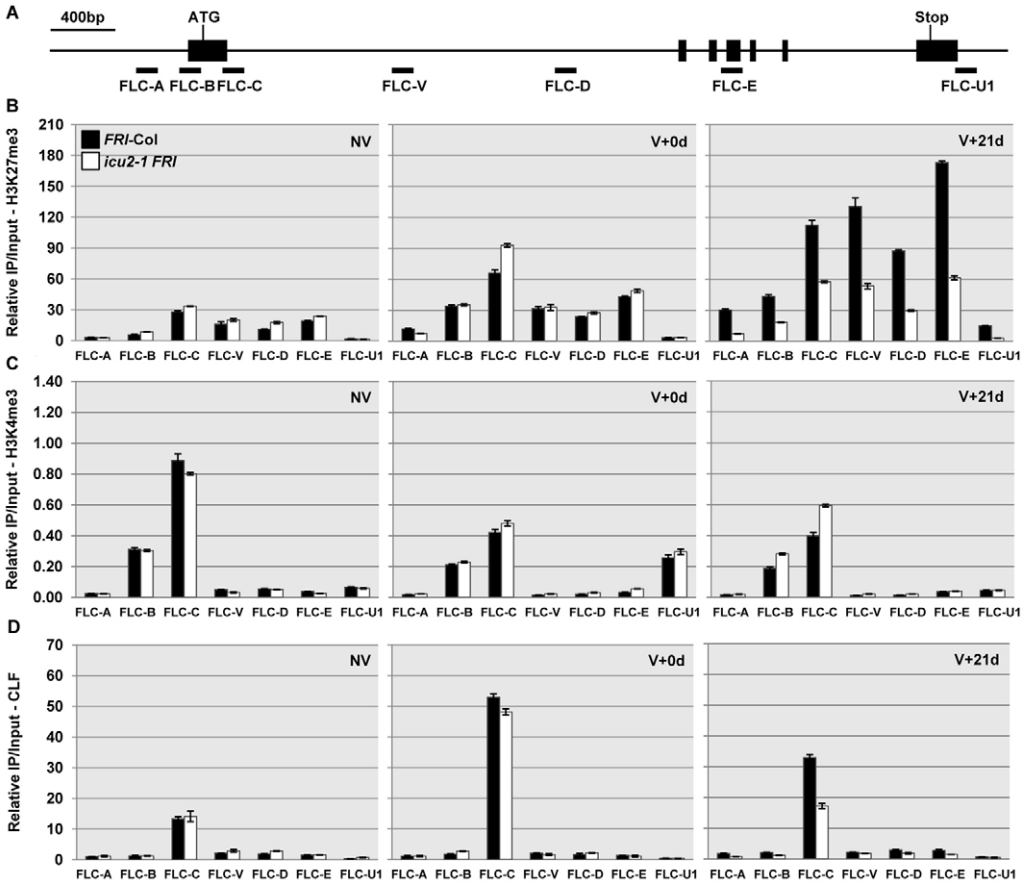

Fig. 3. Role of ICU2 in mitotic H3K27me3 maintenance and CLF retention. (**A**) Schematic of the *Arabidopsis FLC* locus. The exons are represented by black boxes. The location of each PCR amplicon is represented by a black bar. (**B-D**) ChIP-qPCR analysis at *FLC* in *FRI*-Col and *icu2-1 FRI* plants at NV, V+0d and V+21d time points; the levels of H3K27me3 (B), H3K4me3 (C) and CLF (D) binding are shown. Three biological replicates were performed with similar results. Error bars indicate ±s.e.m. of PCR replicates (*n*=3).

V+21d, which is consistent with the previous result that the protein abundance in VRN2-PRC2 components is reduced after vernalization (Wood et al., 2006). However, the enrichment level was still higher than that observed in NV conditions (Fig. 3D), supporting the retention of CLF on *FLC* after vernalization (De Lucia et al., 2008; Heo and Sung, 2011). In *icu2-1 FRI*, the enrichment of CLF was lower than that in *FRI*-Col only at V+21d (Fig. 3D). This demonstrates that the *icu2-1* mutation resulted in the failure of CLF retention during cell proliferation after vernalization.

Taking these data together, we suggest that ICU2 is involved not in the establishment of the epigenetic states for the vernalization memory, but in the mitotic maintenance of these states.

Chromatin states at various genomic loci in *icu2-1 FRI* plants

To determine whether ICU2 is required for general epigenetic maintenance in *Arabidopsis*, the chromatin states at additional genomic loci were analyzed. We examined two PRC2 target genes, *FT* and *AG*, and two heterochromatic retroelements, *Transcriptionally Silent Information* (*TSI*) and *Ta3*. We also explored the chromatin states in the *clf-2* knockout mutants to compare biochemical functions of ICU2 and CLF. We introduced an active *FRI* allele into *clf-2* knockout mutants by genetic crosses and compared the epigenetic states in *icu2-1 FRI* plants with those in *clf-2 FRI* plants.

In RT-qPCR analysis, all of the mutants exhibited ectopic expression of *FT* and *AG* (Fig. 4A). In LD conditions, all mutants showed an early flowering phenotype compared with *FRI*-Col (Fig. 4B). In SD conditions, the delay of flowering is slight in *icu2-1 FRI*, whereas the *clf-2 FRI* plants were still sensitive to the photoperiod conditions (Fig. 4B), implying an additional defect in *icu2-1* compared with *clf-2*. The early flowering in *icu2-1 FRI* was still observed after vernalization although the mutant showed *FLC* de-repression (Fig. 2). It is possible that the ectopic expression of *FT* resulted in the early flowering because the *FT* is downstream and epistatic to *FLC* in the floral pathway (Searle et al., 2006). Consistently, the same flowering phenotype was observed in the *lhp1-4* mutants showing ectopic *FT* expression and *FLC* de-repression after vernalization (Mylne et al., 2006; Sung et al., 2006).

To check the H3K27me3 states at *FT* and *AG* loci, we performed ChIP analysis. Consistent with the previous study (Turck et al., 2007), our ChIP-qPCR analysis revealed that H3K27me3 is abundant at the 3′ region of *FT* and the entire genomic region of *AG* in *FRI*-Col (supplementary material Fig. S2). The *icu2-1 FRI* plants showed a loss of H3K27me3 at both the *FT* and *AG* regions (supplementary material Fig. S2), but the reduction in the *icu2-1 FRI* plants was weaker than that in the *clf-2 FRI* plants (Fig. 4C), consistent with a defect in H3K27me3 inheritance but not in deposition. Although the H3K27me3 reduction in the *clf-2 FRI* plants was higher than in the *icu2-1 FRI*, the *FT* expression in the *clf-2 FRI* was less elevated (Fig. 4A). This can probably be explained by the fact that the *clf* mutants exhibit increased *FLC* mRNA levels (Jiang et al., 2008), and that the *FT* expression is repressed by *FLC* in a PRC2-independent manner (Helliwell et al., 2006; Searle et al., 2006). To extend our analysis, we investigated ten additional loci known to be targeted by PRC2 (Lu et al., 2011). In the *icu2-1 FRI* mutant, all the loci showed decreases in the H3K27me3 level (supplementary material Fig. S3).

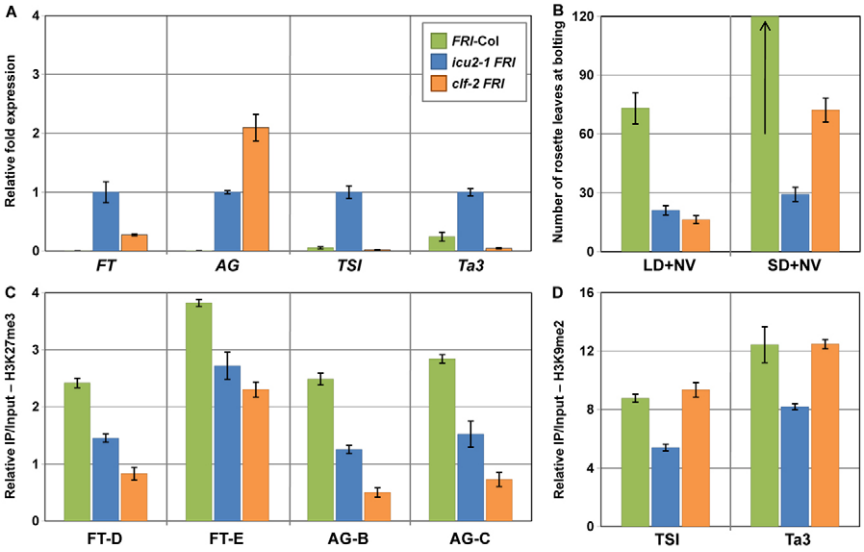

Fig. 4. Loss of epigenetic states at various genomic loci in *icu2-1 FRI*. (**A**) RT-qPCR analysis of *FT*, *AG*, *TSI* and *Ta3* mRNA levels. (**B**) Photoperiod sensitivity in flowering in *Arabidopsis*. *FRI*-Col did not flower under short day photoperiod conditions in our study. Arrow indicates no flowering of *FRI*-Col in SD conditions. (**C**) Levels of H3K27me3 at *FT* and *AG* chromatin. (**D**) Levels of H3K9me2 at *TSI* and *Ta3* chromatin. The location of each PCR amplicon is represented in supplementary material Fig. S2A. All of the samples were obtained at the 21-day-old seedlings grown under NV+LD. Three biological replicates were performed with similar results. Error bars indicate ±s.e.m. of PCR replicates (n=3).

In contrast to the repression of PRC2 targets, the silencing of the heterochromatic elements *TSI* and *Ta3* was released only in the *icu2-1 FRI* plants, not in the *clf-2 FRI* plants (Fig. 4A). Accordingly, H3K9me2, the heterochromatic mark enriched at *TSI* and *Ta3* loci, was reduced only in the *icu2-1 FRI* mutants (Fig. 4D).

Conclusively, our results suggest that ICU2 plays a role not only in the repression of PRC2 targets but also in the silencing of retroelements. Moreover, *icu2-1 FRI* mutant shows epigenetic defects that are not observed in the *clf-2 FRI* mutants.

Relationship between ICU2 and LHP1

In a previous report, an *in vitro* GST pull-down assay suggested that the C-terminal portion of ICU2 interacts with LHP1, possibly through the putative MIR domain in the protein. In addition, the LHP1:GFP fusion protein was mislocalized in the rosette leaves of *icu2-1* mutants (Barrero et al., 2007). Therefore, we explored the *in vivo* biochemical relationship between ICU2 and LHP1 to elucidate the mechanism by which *ICU2* is involved in epigenetic maintenance.

We first confirmed that *icu2-1* and *polα* mutants, both of which have a missense mutation near the putative MIR domain, showed similar epigenetic defects (supplementary material Fig. S4). However, we did not observe the reduction of *FLC* and the de-repression of *Ta3* in NV conditions in the *polα* mutants. Similarly, the *polα* mutation caused milder defects in flowering time and *FLC* expression in the *FRI*-Col background under NV conditions (our unpublished data). Although these results imply that *polα* is a weaker mutant allele than *icu2-1*, the same defects in both mutants still suggest the importance of MIR domain for ICU2 function. To investigate the function of the MIR domain, we generated transgenic plants that overexpress partial ICU2 proteins under the *35S* promoter. We observed that only the overexpression of transgenes with a C-terminus that includes the MIR domain phenocopied the *icu2-1 FRI* mutants, displaying, for example, early flowering and the de-repression of *FT* and *AG* (Fig. 5A-C). This raises the possibility that overexpression of the MIR-containing partial domain of ICU2 sequesters the ICU2-interacting partners for epigenetic maintenance, thereby preventing endogenous ICU2 protein from functioning in this process.

To test whether ICU2 interacts with LHP1 through the putative MIR domain, we performed a yeast two-hybrid analysis using both full- and partial-length ICU2 proteins. In our results, the interaction between ICU2 and LHP1 was not observed in any of the combinations (Fig. 5D). To analyze further the interaction between ICU2 and LHP1 *in planta*, a bimolecular fluorescence complementation (BiFC) assay was also performed using protoplasts from *Arabidopsis* mesophyll cells. Although the interaction between the F-box protein UCL1 and CLF was observed as previously reported (Jeong et al., 2011), we failed to detect any reconstituted YFP fluorescence from ICU2 and LHP1 reciprocal combinations in our experimental conditions (Fig. 5E). Therefore, our yeast two-hybrid assay and BiFC results do not support a direct interaction between ICU2 and LHP1.

LHP1 is known to be colocalized with H3K27me3 in the *Arabidopsis* genome (Exner et al., 2009; Turck et al., 2007; Zhang et al., 2007). Our study also revealed that *icu2-1FRI* resulted in the reduction of H3K27me3 at the PRC2 target loci (Fig. 4; supplementary material Fig. S3). These results raise the possibility that the retention of LHP1 on PRC2 targets is also reduced by the *icu2-1* mutation, which is consistent with previous cytological results (Barrero et al., 2007). To examine the retention of the LHP1:GFP fusion protein at its target loci, we generated *icu2-1 LHP1:GFP lhp1-4* plants from a genetic cross between *icu2-1* and *LHP1:GFP lhp1-4* plants, which fully rescued the *lhp1-4* knockout mutant phenotypes (Sung et al., 2006). The ChIP-qPCR results demonstrated that *icu2-1* caused a reduced accumulation of LHP1:GFP at its binding loci, *FT* and *AG* (Fig. 5F,G). Taken together, our data suggest that ICU2 and LHP1 do not interact directly *in vivo*, but that *icu2-1* causes loss of LHP1 binding at its target loci in *Arabidopsis*.

Role of ICU2 in H3.1 inheritance and incorporation in proliferating tissues

We suggested that *icu2-1* mutants share distinctive epigenetic defects, such as the mosaic de-repression of silenced loci and the de-repression of both PRC2 targets and retroelements, with the knockout mutants of chromatin assembly factors *FAS1*, *FAS2* and *BRU1* (Figs 2, 4) (Ono et al., 2006; Takeda et al., 2004). Abnormal phyllotaxy and fasciation of stems, a typical phenotype of *fas1*, *fas2* and *bru1* mutants, were also observed in both *icu2-1 FRI* and *polα* mutants (Fig. 6A; supplementary material Fig. S4C). As

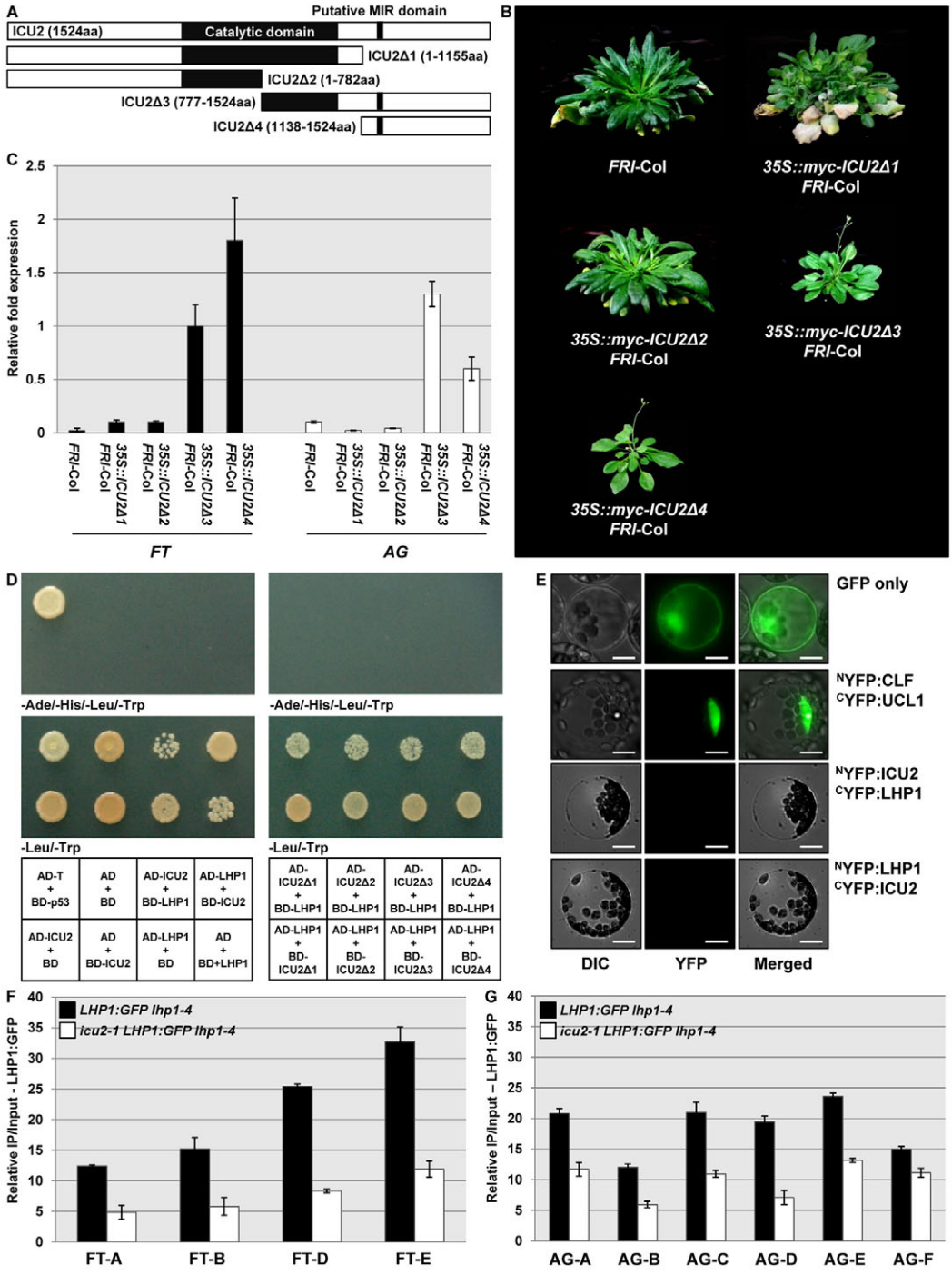

Fig. 5. Biochemical interaction between ICU2 and LHP1. (**A**) Schematic of full- and partial-length ICU2 proteins. (**B**) Recapitulation of *icu2-1* phenotype by overexpression of partial-length ICU2 possessing the MIR domain. (**C**) RT-qPCR analysis of *FT* and *AG* mRNA levels in partial-length *ICU2* overexpressors. (**D**) Interaction test between full- and partial-length ICU2 and LHP1 in a yeast two-hybrid assay. The combination for the interaction assay is presented in the bottom panel. AD-T + BD-p53 and AD + BD were used as positive and negative interaction controls, respectively. –Ade/–His/–Leu/–Trp, quadruple (adenine, histidine, leucine and tryptophan) dropout media; –Leu/–Trp, double (leucine and tryptophan) dropout media; AD, GAL4 activation domain; BD, GAL4-binding domain. (**E**) BiFC assay of protein-protein interaction of ICU2 and LHP1 in *Arabidopsis* protoplasts. Deconvoluted images of protoplasts are shown. The *35S::GFP* construct was used as a transfection control, and the binding of CLF and UCL1 was used as a positive control for BiFC. Scale bars: 10 μm. (**F**,**G**) Retention of LHP1:GFP on *FT* (F) and *AG* (G) chromatin in 21-day-old seedlings grown under NV+LD conditions. Two biological replicates were performed with similar results. Error bars indicate ±s.e.m. of PCR replicates (*n*=3).

reported in *bru1* mutants, the degree of abnormal phyllotaxy and fasciation varies between individual plants and even between neighboring branches of the same plant (Fig. 6B). Therefore, these aspects led us to examine whether chromatin assembly upon DNA replication is affected by *icu2-1*, which could explain various epigenetic defects in the mutant.

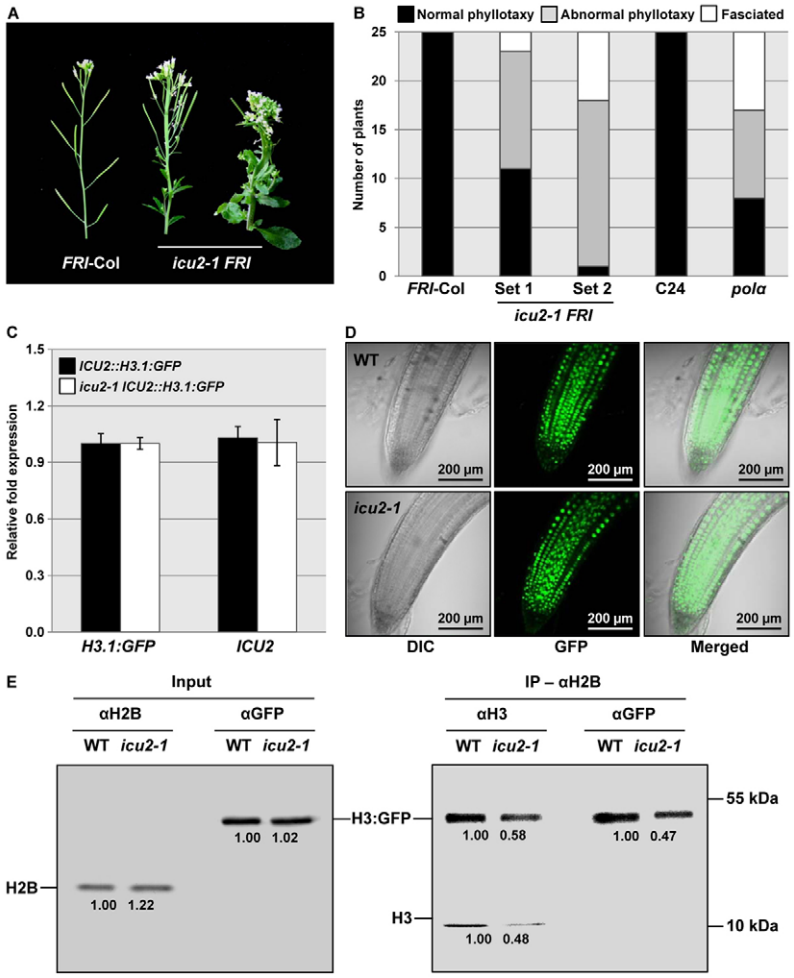

Fig. 6. Role of ICU2 in chromatin assembly in proliferating tissues. (**A**) Abnormal phyllotaxy and fasciation of stems in *icu2-1 FRI*. Primary shoots from two independent *icu2-1 FRI* plants showing the defects are presented. (**B**) Ratio of shoot defects in *icu2-1 FRI* and *polα* populations. The numbers of plants with normal shoot, abnormal phyllotaxy and fasciated shoot in two independent *icu2-1 FRI* populations and one *pol* population are presented. (**C**) RT-qPCR analysis of *H3.1:GFP* and *ICU2* in *ICU2::H3.1:GFP* wild-type and *icu2-1* siblings. Error bars indicate s.e.m. (**D**) Confocal microscopy GFP images of root tips from *ICU2::H3.1:GFP* wild-type and *icu2-1* siblings. (**E**) *In vivo* co-IP analysis of H3.1:GFP with H2B in *ICU2::H3.1:GFP* wild-type and *icu2-1* siblings. The relative band intensity was quantified using ImageJ software and is indicated under each immunodetected band. The band intensity of the wild-type sample was set to 1. Two biological replicates were performed with similar results in two independent transgenic lines.

To analyze this impact, an *ICU2::H3.1:GFP* construct was transformed into *icu2-1*/+ heterozygous plants, and wild-type and *icu2-1* siblings showing the same expression levels of the transgene were isolated (Fig. 6C). The canonical histone variant H3.1 is known to be incorporated into nucleosomes in a DNA replication-dependent manner (Ransom et al., 2010), and it is more enriched at genomic regions with a low transcription rate in *Arabidopsis* (Stroud et al., 2012). A previous study suggested that the H3.1:GFP fusion protein behaves similarly to the endogenous H3.1 in *Arabidopsis* (Ingouff et al., 2010). As seen in our *ICU2::GUS* analysis (Fig. 1), *ICU2::H3.1:GFP* showed strong GFP fluorescence in proliferating tissues (supplementary material Fig. S5A-C). This GFP pattern is similar to the fluorescence pattern of 5-ethynyl-2′-deoxyuridine (EdU), a chemical used for S-phase cell cycle-specific detection (Kotogány et al., 2010). We could also observe H3.1:GFP fluorescence at the segregating chromosomes in dividing root cells (supplementary material Fig. S5D-G), suggesting efficient incorporation of the fusion protein during DNA replication. No significant difference was observed in the subcellular localization of H3.1:GFP between wild-type and *icu2-1* siblings (Fig. 6D), and H3.1:GFP was also observed at the segregating chromosomes of *icu2-1* root cells (supplementary material Fig. S5H), suggesting that the intrinsic biochemical feature of H3.1:GFP is not likely to be altered in *icu2-1* mutants. Therefore, *ICU2::H3.1:GFP* can provide a tool to examine the DNA replication-dependent chromatin assembly in *Arabidopsis* proliferating tissues.

Next, we compared the association of H3.1:GFP in assembled chromatin in wild-type and *icu2-1* plants with an *in vivo* co-immunoprecipitation (co-IP) analysis using an antibody against histone H2B (αH2B). Because the histone octamer is formed after the binding of two molecules of an H2A-H2B dimer to an H3.1-H4 tetramer at the DNA replication fork, an IP with αH2B should purify the assembled histone octamer as well as any free H2A-H2B dimers not incorporated in nucleosomes. The co-IP experiment demonstrated that H3.1:GFP was efficiently associated with H2B in the nuclear fractions from wild-type siblings (Fig. 6E). By contrast, we found that the association of H3.1:GFP and endogenous H3 was reduced in the *icu2-1* nuclear fraction (Fig. 6E), suggesting that the *icu2-1*mutation resulted in inefficient chromatin assembly in proliferating tissues. We also examined nucleosome density in the ChIP experiment using an H3 antibody. In the *icu2-1* mutant, less DNA was immunoprecipitated at *ACT7*, *FT*, *AG*, *FLC* and *TSI* loci with a similar reduction ratio (supplementary material Fig. S5I). However, the ratio between the histones with or without trimethylation at K27 was specifically reduced at *FT* and *AG* loci in NV conditions (supplementary material Fig. S5J), suggesting that low nucleosome density is not the technical reason for the reduced H3K27me3 levels observed at those loci. Combining these findings with the observed epigenetic defects in chromatin assembly factor mutants, we suggest that ICU2 facilitates chromatin assembly and ensures stable maintenance of repressive histone marks in dividing cells.

DISCUSSION

Epigenetic inheritance during DNA replication is crucial for the maintenance of identical cellular memory (Margueron and Reinberg, 2010). In this study, we elucidated a role of ICU2, the catalytic subunit of DNA polymerase α, in the maintenance of repressive chromatin states in *Arabidopsis*.

Loss of epigenetic memory in *icu2-1* mutants during mitoses

During vernalization, the PHD-PRC2 complex deposits H3K27me3 on the *FLC* locus, which is crucial for establishment of *FLC* silencing (Kim et al., 2009). For stable *FLC* repression after vernalization, which is known to be impaired in mitotically quiescent cells (Finnegan and Dennis, 2007), the H3K27me3 has to be correctly inherited upon DNA replication in subsequent mitoses. Recently, a transiently expressed long noncoding RNA, known as COLDAIR, was identified; this RNA recruits CLF to the *FLC* region during cold treatment (Heo and Sung, 2011). Interestingly, CLF remains in association with the *FLC* region in the absence of COLDAIR even after vernalization (Fig. 3D) (De Lucia et al., 2008; Heo and Sung, 2011). In animals, PRC2 associates with its target DNA loci through replication (Hansen et al., 2008; Margueron et al., 2009; Margueron and Reinberg, 2010; Petruk et al., 2012). These findings indicate that the retention of PRC2 at its targets could be the mechanism for the mitotic maintenance of H3K27me3 enrichment. Although we could not examine the retention of SWINGER (SWN), a major SET-domain component in PHD-PRC2 for vernalization (De Lucia et al., 2008; Heo and Sung, 2011), owing to a lack of antibody, our study suggested that ICU2 is involved in retention of the PRC2 complex. However, in *Arabidopsis*, GFP:CLF fusion proteins were not detected in the segregating chromosomes in the dividing root cells (Schubert et al., 2006), suggesting that the PRC2 complex could act differently upon DNA replication in animals and plants. It is possible that ICU2 mediates the retention of PRC2 through unknown factors that re-recruit the complex after cell division at the PRC2 target loci. Interestingly, the C-terminal domain of ICU2, which includes the putative MIR motif, is important for the epigenetic function of the protein (Fig. 5A-C). Protein complexes interacting with the C-terminal portion of ICU2 could be the factors for the retention of PRC2 in *Arabidopsis*. In addition to the H3K27me3 maintenance, propagation of H3K27me3 to the gene body region of *FLC* was also impaired in the *icu2-1 FRI* mutants (Fig. 3B). As the *icu2-1 FRI* mutants showed defects in the retention of CLF, the failure of the histone mark propagation could be caused by a reduced level of PRC2 complexes at the *FLC* locus. However, it is also possible that ICU2 could mediate the activation or the recruitment of additional factors for the histone mark propagation during mitoses.

Roles of ICU2 that are independent to functions of PRC2 and LHP1

A previous study identified that knockout mutants of the PRC2 complex show slight upregulation of *FLC* in NV conditions (Jiang et al., 2008). However, in the *icu2-1 FRI* plants, the expression of *FLC* and *FLC::GUS* were reduced (Fig. 2). If the *icu2-1* mutation induces the epigenetic defect only in PRC2- and H3K27me3-mediated gene regulation, the mutant should show increased expression of *FLC* in NV conditions. In this study, we characterized the defects in *icu2-1 FRI* plants, such as the low sensitivity in photoperiodic flowering and the release of heterochromatic loci, that were not observed in *clf-2 FRI* plants. These findings suggest that there should be additional epigenetic defects in the *icu2-1* mutants that are not related with the functions of PRC2. As diverse epigenetic mechanisms for *FLC* regulation have been characterized so far (Kim et al., 2009), those defects could result in the downregulation of *FLC* in NV conditions.

In the fission yeast *Saccharomyces pombe*, the DNA polymerase α catalytic subunit Swi7 binds to the HP1 homolog Swi6, and a missense mutation in Swi7 inducing the failure of the interaction with Swi6 resulted in the de-repression of heterochromatin loci (Ahmed et al., 2001; Nakayama et al., 2001), further supporting additional roles of ICU2 that are not related with PRC2 functions. Although LHP1 has been isolated as a homolog of HP1 in *Arabidopsis*, we could not detect a direct interaction between ICU2 and LHP1 (Fig. 5D,E). Diverse studies have suggested that the epigenetic function of LHP1 is limited to H3K27me3-mediated epigenetic gene regulation (Lindroth et al., 2004). LHP1 is not likely to be the interaction partner of ICU2 for heterochromatin maintenance. However, in *icu2-1* mutants, the retention of the LHP1:GFP fusion protein at the *FT* and *AG* regions was impaired (Fig. 5F,G). As LHP1 is known to be colocalized with the H3K27me3 mark (Exner et al., 2009; Turck et al., 2007; Zhang et al., 2007), the loss of LHP1:GFP retention in the *icu2-1* mutants could be caused by a reduction of H3K27me3 levels at those loci.

Roles of DNA replication machinery in epigenetic maintenance

The DNA polymerase α complex, including the primase subunit, initiates DNA replication by the synthesis of a primer at the replication fork. Replication factor C (RFC) binds to the DNA at the template-primer junctions and displaces DNA polymerase α. The binding of RFC to DNA creates a loading site for recruitment of proliferating cell nuclear antigen (PCNA) complexes. PCNA then acts as a scaffold for recruiting histone modification enzymes and chromatin assembly factors (Majka and Burgers, 2004; Petruk et al., 2012; Ransom et al., 2010). In this study, we suggested that the *icu2-1* and *polα* mutants share distinctive epigenetic defects with the knockout mutants of chromatin assembly factors in *Arabidopsis* (Figs 2, 4; supplementary material Fig. S4) (Kaya et al., 2001; Kirik et al., 2006; Ono et al., 2006; Takeda et al., 2004). In addition, inefficient chromatin assembly in proliferation tissues was observed in the *icu2-1* mutants (Fig. 6). Although the direct relationship between ICU2 and the chromatin assembly factors has yet to be identified, our results suggest that ICU2 could facilitate chromatin assembly at the replication fork through a direct or indirect biochemical relationship with those factors. Consistent with this possibility, the catalytic subunit of DNA polymerase α in the budding yeast *S. cerevisiae* has been reported to form a complex with SPT16, a subunit of the FACT chromatin assembly factor (Wittmeyer and Formosa, 1997). Fortunately, many recent reports have characterized diverse epigenetic defects in *Arabidopsis* and mammals with mutations in various components of the DNA replication machinery (del Olmo et al., 2010; Liu et al., 2010b; O'Donnell et al., 2010; Xia et al., 2006; Yin et al., 2009). Additional studies that investigate the functional and biochemical relationship between ICU2 and these factors will provide further insights into DNA replication-coupled epigenetic inheritance.

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Competing interests statement
The authors declare no competing financial interests.

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