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RESEARCH ARTICLE

Identification of Elements Responsible for Maternally-Silenced Imprinted Gene Expression of *Upward Curly Leaf1*, an F-box Protein Gene that Regulates Curly Leaf in *Arabidopsis*

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Abstract

Upward Curly Leaf 1 (UCL1) is an *Arabidopsis thaliana* E3 ligase that targets the Curly Leaf (CLF) SET-domain polycomb-group (PcG) protein for degradation via the ubiquitin-26S proteasome system. *UCL1* is a paternally-expressed imprinted gene in the endosperm. To precisely locate the promoter elements required for *UCL1* imprinting pattern, various gene constructs were created in which the imprinting control region (ICR), endosperm-specific expression (ENSE) element, and/or the linker sequence were altered. By fusing these constructs with a GUS reporter gene, GUS expression patterns were monitored after reciprocal crosses with wild-type Columbia-0 allowing the determination of parent-of-origin expression. Analysis of publicly-available data on the *UCL1* promoter region facilitated the search for allele-specific DNA and H3K27 methylation patterns. Overall, three promoter elements are required for maternal repression of *UCL1*; the ICR sequence located from − 2.5 to − 2.4 kb upstream of the translation start site, a differentially methylated region 2 (DMR2) that overlaps the short *ATLINE1-1* transposable element in the linker region, and a minimal 271 bp ENSE element. In addition, DNA methylation patterns in the DMR2 contribute to the repression of the maternal *UCL1* allele. Our findings would help to understand how parent-of-origin epigenetic patterns are created and maintained in the endosperm.

Keywords Imprinting · DNA methylation · Polycomb group · Endosperm · Promoter elements

Introduction

A distinctive feature of the plant life cycle is the alternation of generations in which multicellular diploid (sporophytic) and haploid (gametophytic) generations alternately produce each other. In flowering plants, the sporophyte is the dominant generation, with the gametophyte being shorter-lived, contained within sporophyte tissues and consisting of only a few cells. In addition to the transition from the sporophytic generation to the gametophytic generation seen in the reproductive cycle, other significant developmental transitions occur at distinct points during the plant life cycle, such as the transition from embryonic development to vegetative growth and from vegetative to reproductive growth (Xiao and Wagner 2015). In plants, these phase transitions are controlled genetically by polycomb-group (PcG) protein complexes.

PcG proteins mediate chromatin structure remodeling, causing the epigenetic silencing of genes. As a result, PcG protein complexes play an important role in transitioning from one developmental stage to the next by modulating suitable gene repression (Mozgova et al. 2015). Alteration of PcG protein composition will shift the gene expression/repression patterns associated with chromatin structure. Epigenetic control of gene expression patterns throughout the plant life cycle allows radical shifts in cell fate and organ morphology in plants undergoing developmental

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transitions as well as morphological responses to environmental change (Bracken et al. 2006; Hennig and Derkacheva 2009).

Due to their modulation of chromatin structure, PcG proteins play important roles in cell proliferation, cell differentiation, stem cell identity, and genomic imprinting (Schwartz and Pirrotta 2008; Margueron and Reinberg 2010). The three main PcG complexes are Polycomb-repressive complex I (PRC1), PRC2, and PcG-like PRC2. Although these complexes work together to carry out epigenetic remodeling of chromatin, the specific function of PRC2 is to methylate lysine 27 residues in the H3 histone proteins of nucleosomes (H3K27me2/3). H3K27me3 leads to increased chromatin condensation, which reduces the transcription of genes located in the area of condensation. The PRC2 complex is highly conserved in *Drosophila*, mammals and flowering plants and is composed of four protein subunits: Extra sex combs (Esc), Supressor of Zeste 12 (Su(z)12), Enhancer of Zeste (E(z)) and p55. Both Esc and p55 contain WD40 domains that interact each other, Su(z)12 encodes the C2H2-zinc finger domain, and E(z) encodes the SET-domain protein that exerts H3K27 methylation activity (Czermin, Melfi et al. 2002).

In *Arabidopsis thaliana* there are three distinctive PRC2 complexes, named after their Su(z)12 homologs: EMBRYNIC FLOWER2 (EMF2)-PRC2, VERNALIZATION2 (VRN2)-PRC2, and FERTILIZATION INDEPENDENT SEED2 (FIS2)-PRC2. There are also *Arabidopsis* homologs to other complex components. A homolog of Esc is FERTILIZATION INDEPENDENT ENDOSPERM (FIE); homologs of E(z) include CURLY LEAF (CLF), SWINGER (SWN), and MEDEA (MEA); and a homolog of p55 is MULTICOPY SUPRESSOR OF IRA1 (MSI1) (Hennig and Derkacheva 2009).

PRC2 activity itself is under cellular control, with increasing evidence for its regulation through posttranslational events such as selective protein degradation. The length of time that PcG proteins remain in the cell is regulated by the ubiquitin-26S proteasome system. The enzyme E3 ubiquitin ligase transfers the ubiquitin to a target protein, and this protein is subsequently degraded by proteases (Hershko and Ciechanover 1998). *UPWARD CURLY LEAF1* (*UCL1*) is a gene encoding an E3 ligase that degrades the CURLY LEAF (CLF) PcG protein. This F-box protein is known to bind to the CLF PcG protein in yeast and *in planta* and targets CLF proteins for degradation through the 26S proteasome pathway (Jeong et al. 2011). Overexpression of *UCL1* during vegetative growth phenocopies *clf* mutant phenotypes: upward leaf curling, early flowering, decreased CLF protein levels, and altered H3K27me3 chromatin marks of the CLF-target genes (Jeong et al. 2011). *UCL1* is primarily expressed in the endosperm; interestingly, the endosperm is a tissue in which CLF does not function.

In terms of *UCL1* inheritance, the sex of the parent contributing the allele determines whether the allele is expressed or not—*UCL1* is an imprinted gene. Genomic imprinting refers to parent-of-origin-dependent differential allelic expression, thereby showing predominantly as either maternally expressed or paternally expressed. A few theories have been proposed to explain the evolution of imprinting: (1) parental conflict over resource allocation to the embryo (Haig and Westoby 1989; Haig 1997), (2) limitation of the gene dosage of key genes during early embryo development (Iwasa 1998; Garnier et al. 2008), and (3) a byproduct of the silencing of invading foreign DNA that inserted near key genes, such as transposable elements (TE) (Barlow 1993; Hsieh et al. 2009). In this sense, imprinted gene expression might be one consequence of large-scale epigenome remodeling, primarily directed at TEs, that occurs in gametes and seeds. This remodeling could be important for maintaining the epigenome in the embryo as well as for establishing gene imprinting (Gehring 2013).

UCL1 is a paternally-expressed gene (PEG), meaning that the maternal allele is silenced during female gametophyte development as well as during seed development (Jeong et al. 2015). In *Arabidopsis*, genomic imprinting occurs primarily in the endosperm, the triploid tissue created during double fertilization of the female gametophyte, by the fusion of a sperm nucleus with the two polar nuclei of the central cell. The placenta-like endosperm tissue surrounds and nourishes the embryo during its development (Gehring 2013). The maternal *UCL1* allele is silenced by the FIS2-PRC2 complex in the central cell before fertilization and in the endosperm after fertilization (Jeong et al. 2015).

For many imprinted genes, the parental allele-specific expression is determined by imprinting control regions (ICRs) that are marked by DNA or histone methylation on the maternal or paternal allele. The ICR of *UCL1* was found to be adjacent to a transposable element (TE) in the *UCL1* 5′-upstream region, between − 2.7 and − 2.0 kb from the translation start site, whereas the cis-element(s) responsible for the default bi-allelic endosperm-specific expression (ENSE) was within the − 1.0 kb upstream sequence (Jeong et al. 2015) (Supplementary Fig. 1). The maternal *UCL1* allele is repressed by the FIS2-PRC2 complex. In addition, DEMETER (DME) is required for silencing the maternal *UCL1* allele (Jeong et al. 2015).

In this study, we systematically deleted the ICR region to narrow down the region required to reproduce the *UCL1* imprinting pattern. Next, we added this region to a basal bi-allelic *UCL1* promoter to see whether this element is sufficient for imprinted expression. In addition, we analyzed the allele-specific methylation pattern of the short TE region in the *UCL1* 5′-upstream region using publicly-available data, thereby providing evidence of the necessity of DME-mediated hypomethylation for maternal *UCL1* silencing. Lastly,

we identify the *UCL1* minimal promoter that required for gene expression specifically in the endosperm, but not in the embryo or maternal tissue, of the seeds during reproduction.

Results

The Region Between − 2.5 kb and − 2.4 kb 5′-Upstream of the *UCL1* Translation Start Site is Essential for *UCL1* Imprinting

To more precisely delineate the ICR, we systematically deleted the ICR region in approximately 100 bp intervals, and then *β-glucuronidase* (*GUS*) fusion constructs were generated (Fig. 1a). GUS staining before fertilization revealed that inclusion of the − 2.7 kb to − 2.5 kb upstream region stably inhibited maternal GUS expression in the ovules, and inclusion of only the − 2.4 kb to − 2.1 kb upstream region resulted in maternal GUS expression in the central cell, which is the precursor of post-fertilization endosperm (Fig. 1b, upper row). Thus, the cis-element necessary for *UCL1* maternal repression is located within the − 2.5 kb to − 2.4 kb upstream region. After fertilization, all constructs exhibited endospermal GUS expression (Fig. 1b, bottom row).

To further narrow down the location of the essential cis-element for maternal repression within that − 2.5 kb to − 2.4 kb upstream region and to determine whether this potential element was able to change the bi-allelic expression of *UCL1_1.0k::GUS* to mono-allelic expression when fused to the *UCL1_1.0k::GUS* ENSE upstream region, we sequentially deleted within this portion of the *UCL1* promoter region by 30 bp into 200 bp sizes and then fused that fragment to the 5′ end of the *UCL1_1.0 k::GUS* construct (Fig. 2a). To check for parent-of-origin effect, *UCL1_− 2.56 ~ − 2.36/− 2.53 ~ − 2.33/− 2.50 ~ − 2.30/− 2.47 ~ − 2.27/− 2.44 ~ − 2.2*

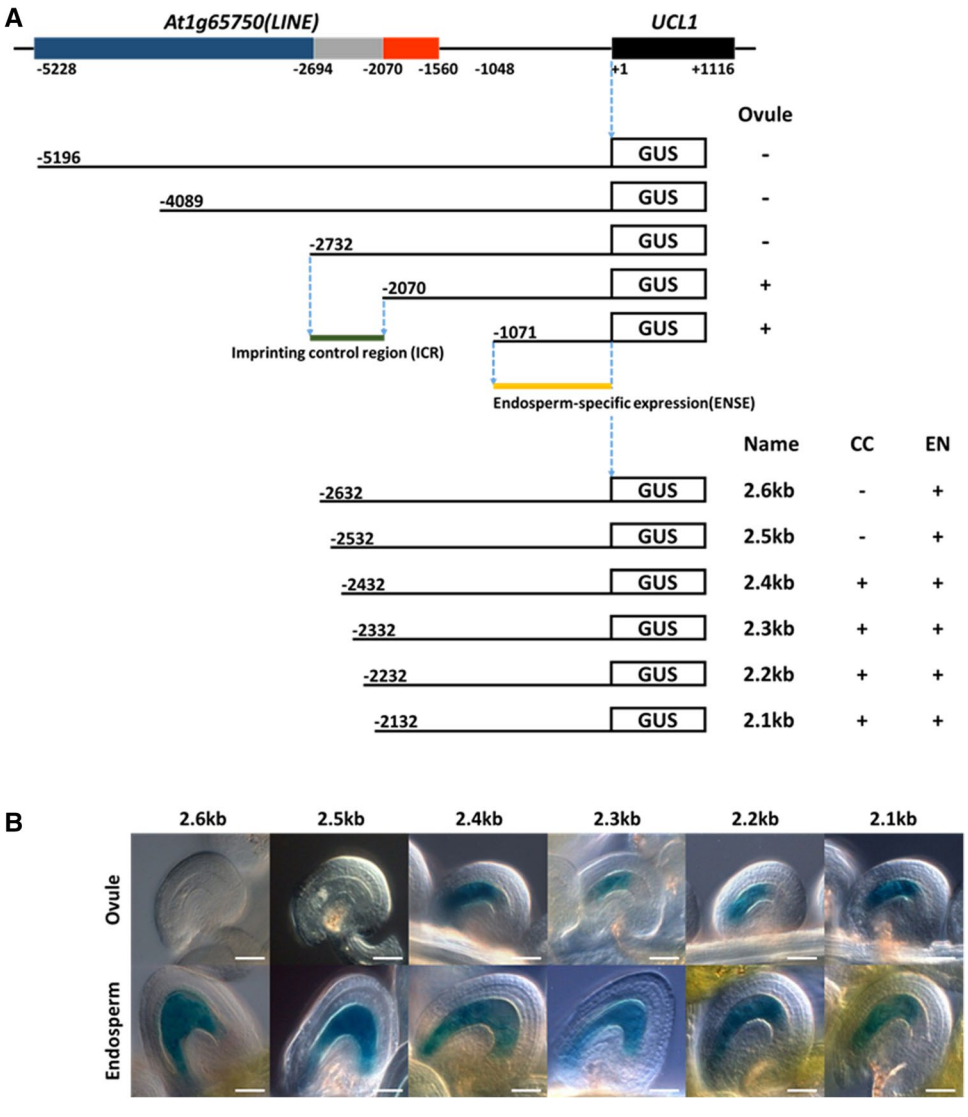

Fig. 1 Diagram of the *UCL1::GUS* reporter constructs for the imprinting control region (ICR) mapping and the resulting GUS expression pattern in transgenic plants. **a** The relative locations of the *At1g65750* transposable element (TE), the imprinting control region (ICR), *ATLINE1_1* short TE (red box), and the cis-element responsible for bi-allelic endosperm-specific expression (ENSE) are shown. The name and GUS expressions of each deletion construct are displayed. *CC* central cells, *EN* endosperm. **b** Micrographs of GUS staining, before (ovule) and after (endosperm) fertilization, for each construct. Scale bars: 200 µm

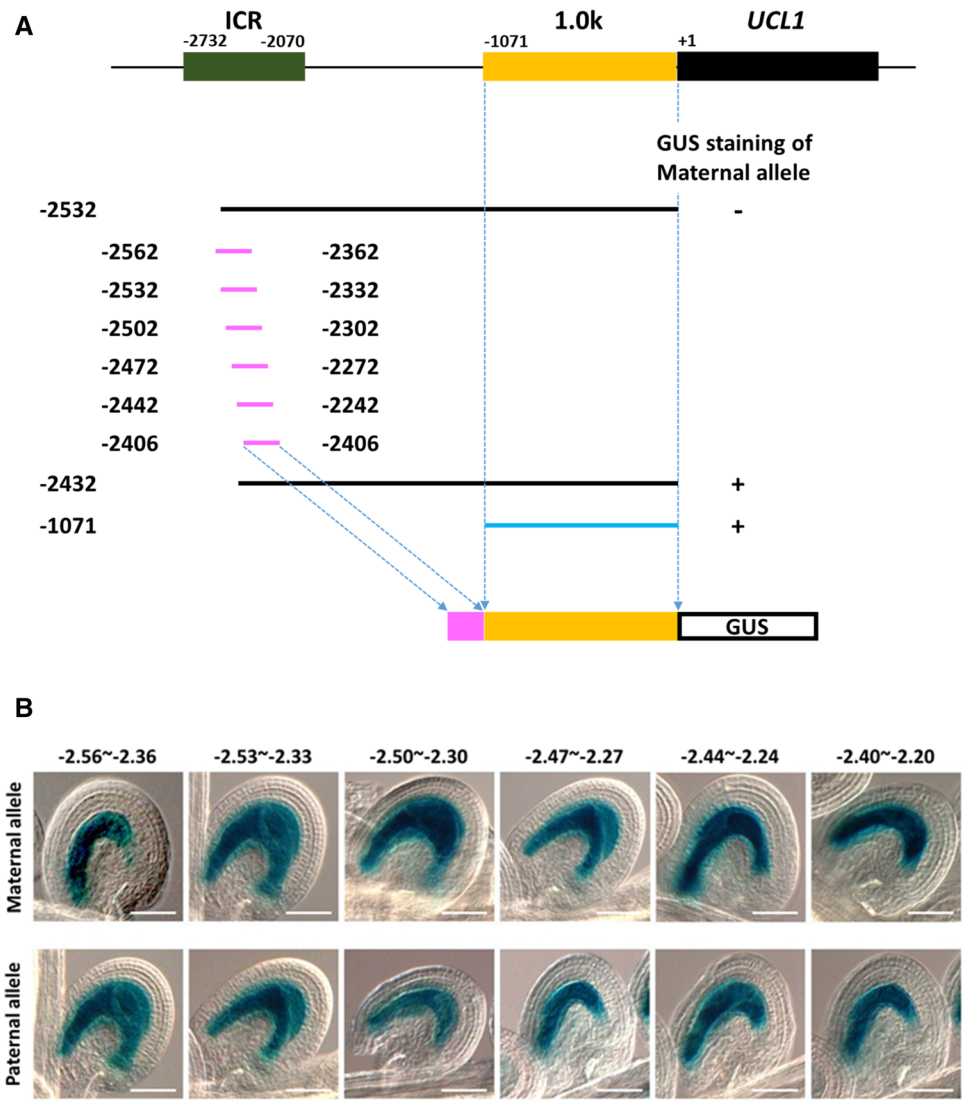

Fig. 2 Diagram of the *UCL1::GUS* reporter constructs for fine mapping of the imprinting control region (ICR) and the resulting expression patterns in transgenic plants. **a** Each 200 bp fragment (pink) within the ICR was fused to 5′ end of the *UCL1_1.0k::GUS* (blue). **b** GUS activity was analyzed at 1 DAP in the developing endosperm after reciprocal crosses with wild-type plants. Scale bars: 200 µm

4/− 2.40 ~ − 2.20 kb + UCL1_1.0k::GUS ENSE transgenic plants were reciprocally crossed with *Col-0* plants, and GUS activity was checked in the endosperm at 1DAP. Examination of the endosperm in all of the transgenic plants revealed bi-allelic *GUS* expression (Fig. 2b). So, the addition of shorter 200 bp segments from within the − 2.5 to − 2.4 kb ICR region was not sufficient to repress the *UCL1* maternal allele in the endosperm. Therefore, while the sequence between − 2.5 and − 2.4 kb is critical for *UCL1* maternal repression, the shorter 200 bp segments were not sufficient to drive *UCL1_1.0k::GUS* to an imprinted pattern, indicating that additional element(s) between − 2.4 and − 1.0 kb are required for maternal repression.

The Role of ICR, ENSE and the Linker Region in *UCL1* Imprinting

To examine the possible role of the linker sequence (− 2070 to − 1071 bp) between the ICR and ENSE region [including the *ATLINE1_1* short TE (red box, Fig. 1a)], a *UCL1_ICR + 1.0k::GUS* construct was generated in which the linker region was deleted (Fig. 3a), and transgenic plants were obtained. First, GUS staining of ovules was performed prior to fertilization to see whether the maternally-derived *UCL1_ICR + 1.0k::GUS* allele was stably repressed. In the homozygous transgenic plants (Fig. 3b, left), 36.2% ($n = 1880$) of the *UCL1_ICR + 1.0k::GUS* ovules exhibited

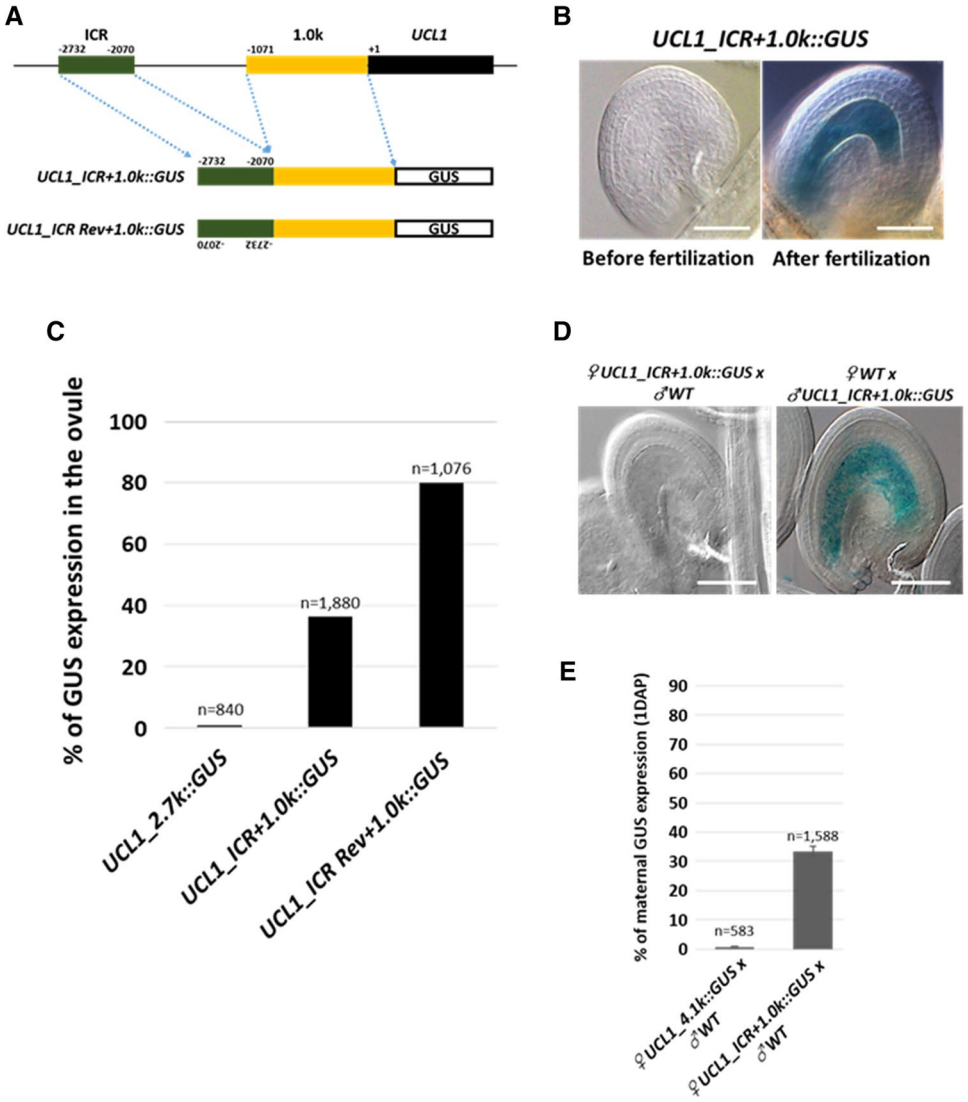

Fig. 3 Linker region and ICR orientation are important for repression of maternal *UCL1* allele. **a** Diagram of *UCL1_ICR+1.0k::GUS* and *UCL1_ICR Rev+1.0 k::GUS*. **b** Micrographs of GUS staining of *UCL1_ICR+1.0k::GUS* ovule and endosperm (before and after fertilization). **c** De-repression of maternal allele in the ovules before fertilization at 2 DAE. **d** GUS activity of maternal and paternal *UCL1_ICR+1.0k::GUS* allele. Images are taken at 1DAP in the developing endosperm. **e** % of maternal *UCL1_ICR+1.0k::GUS* expression in the endosperm at 1 DAP. Scale bars: 200 µm

GUS expression in the central cell at 2 days after emasculation (DAE) (Fig. 3c). This result is in contrast to the *UCL1_2.7k::GUS* reference plants showing 0.7% ($n = 840$) ovule expression at 2 DAE (Fig. 3c). Without the 1 kb linker sequence, the maternal *UCL1_ICR + 1.0 k::GUS* allele was not completely repressed. So, having the ICR and ENSE without the linker region was not sufficient for repression of the maternal *UCL1* allele in the ovules before fertilization.

Second, we examined the *UCL1_ICR + 1.0k::GUS* expression pattern in the fertilized seeds. GUS activity was only detected in the endosperm of the self-pollinated plants (Fig. 3b, right). Third, to verify the parental origin of endosperm GUS expression, *UCL1_ICR + 1.0k::GUS* plants and wild-type Col-0 plants were reciprocally crossed, and then GUS staining was performed in the developing seeds. GUS expression was observed in seeds with paternally-inherited *UCL1_ICR + 1.0k::GUS* (Fig. 3d). Whereas 33.4% ($n = 1588$) of seeds with the maternally inherited *UCL1_ICR + 1.0k::GUS* showed GUS expression in the developing endosperm, only 0.8% ($n = 583$) of maternal GUS expression was detected in maternally inherited *UCL1_4.1k::GUS* plants (Fig. 4e). This result is consistent with the incomplete maternal repression observed in the pre-fertilization ovules of *UCL1_ICR + 1.0k::GUS* plants.

To check whether ICR orientation affects the *UCL1* imprinting pattern, a *UCL1_ICR Rev + 1.0 k::GUS* construct was generated that contains the 700 bp ICR in reverse orientation (Fig. 3a). GUS staining revealed that 80% ($n = 1076$) of the unfertilized ovules in reverse-orientated ICR construct plants showed maternal GUS expression in the central cell compared to 36.2% ($n = 1880$) expression observed in the ovules of forward ICR plants, *UCL1_ICR + 1.0k::GUS* (Fig. 3c). Unlike other conventional cis-enhancers, the reverse orientation of the ICR was far less effective in maternal *UCL1* allele repression.

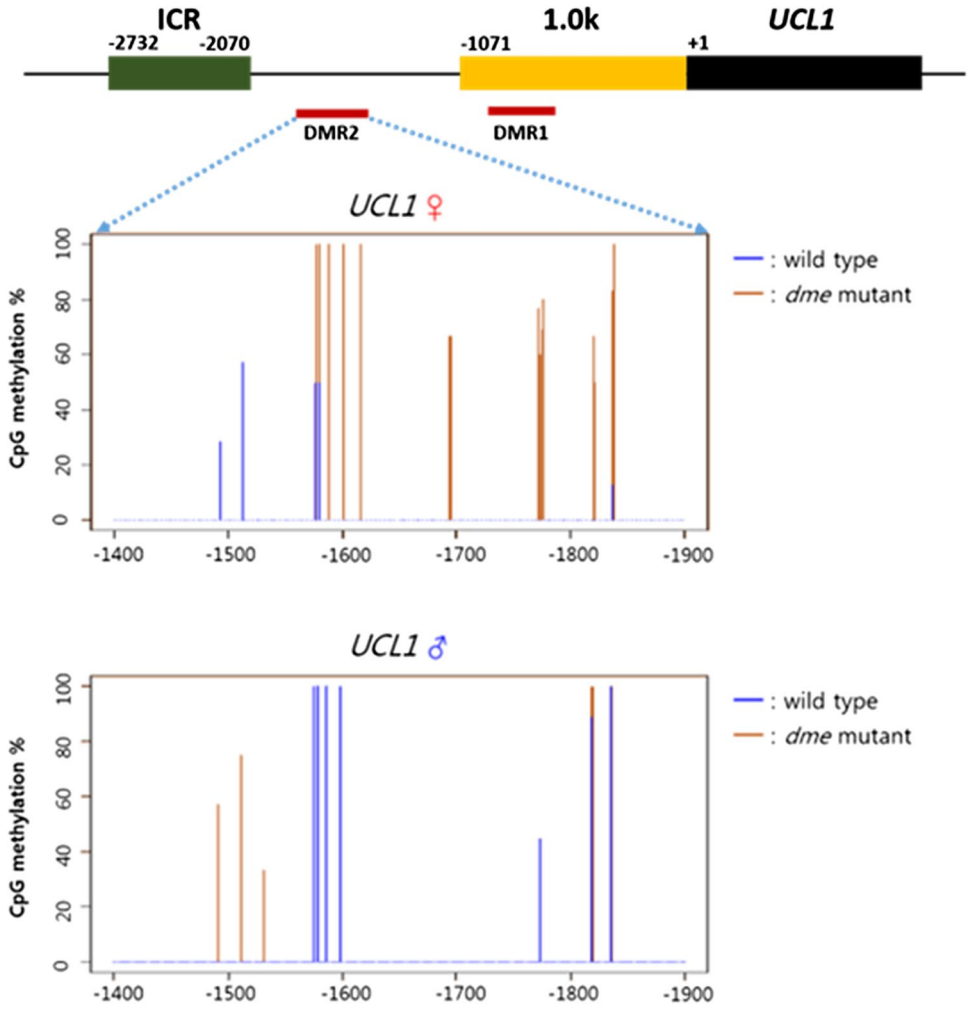

Fig. 4 Allele-specific differentially methylated regions (DMR) in the 5′ upstream region of *UCL1* in wild type and dme mutant endosperm. Percentage of CpG methylation in the *UCL1* DMR2 region of the female (Col-0) and male (Ler) in the endosperm. The data were extracted from the published whole genome-wide DNA methylome in the endosperm (Ibarra et al. 2012)

DMR2, Which Overlaps the Short TE, Plays an Important Role for *UCL1* Imprinting

To compare allele-specific methylation differences in maternally- and paternally-inherited *UCL1* alleles, we examined the endospermal maternal and paternal *UCL1* methylation pattern using publicly-available data (Ibarra et al. 2012). Two differentially methylated regions (DMRs) were found in which the silenced maternal *UCL1* allele is hypomethylated, but the expressed paternal allele is hypermethylated: the region around − 700 bp (hereafter DMR1) and the short TE region (hereafter DMR2) (Fig. 4 and Supplementary Fig. S1). These maternally hypomethylated DMR regions exhibited significantly increased methylation in the *dme* mutant (Fig. 4 and Supplementary Fig. S1), suggesting that DME is required for hypomethylation of *UCL1* maternal allele, which is a prerequisite for silencing of the maternal allele. Consistent with this, silenced *UCL1* maternal allele is activated in *dme* mutants (Jeong et al. 2015). Expressed paternal *UCL1* allele shows hypermethylation in DMR regions (Fig. 4 and Supplementary Fig. S1). Methylation changes in the *UCL1* paternal allele are not as dramatic as that of the maternal allele in *dme* mutants.

We also analyzed publicly-available data on the H3K27me3 pattern in the *UCL1* promoter region using the integrated genome browser (IGB) 8.3.1 program (https://wiki.transvar.org/display/igbman/Quick+start). In this analysis, H3K27me3 was not found in the region − 1814 to − 1478 bp upstream of the *UCL1* translation start site that overlaps with the DMR2 region; however, H3K27me3 was found on both sides of this region (Supplementary Fig. S2). This alternation of histone and DNA methylation pattern is reminiscent of the mutually-exclusive pattern of DNA methylation and H3K27me3 observed in some other genes (Bogdanovic et al. 2011).

To confirm whether the DMR2-overlapping − 1478 to − 1814 bp region (hereafter DMR2′) is important for *UCL1* imprinting, *UCL1_ICR+DMR2′+1.0k::GUS* transgenic plants were generated (Fig. 5a), and GUS activity was examined in ovules. Almost no maternal GUS expression ($n = 1855$) was detected in the pre-fertilization ovules compared to 38% maternal GUS expression ($n = 1880$) in

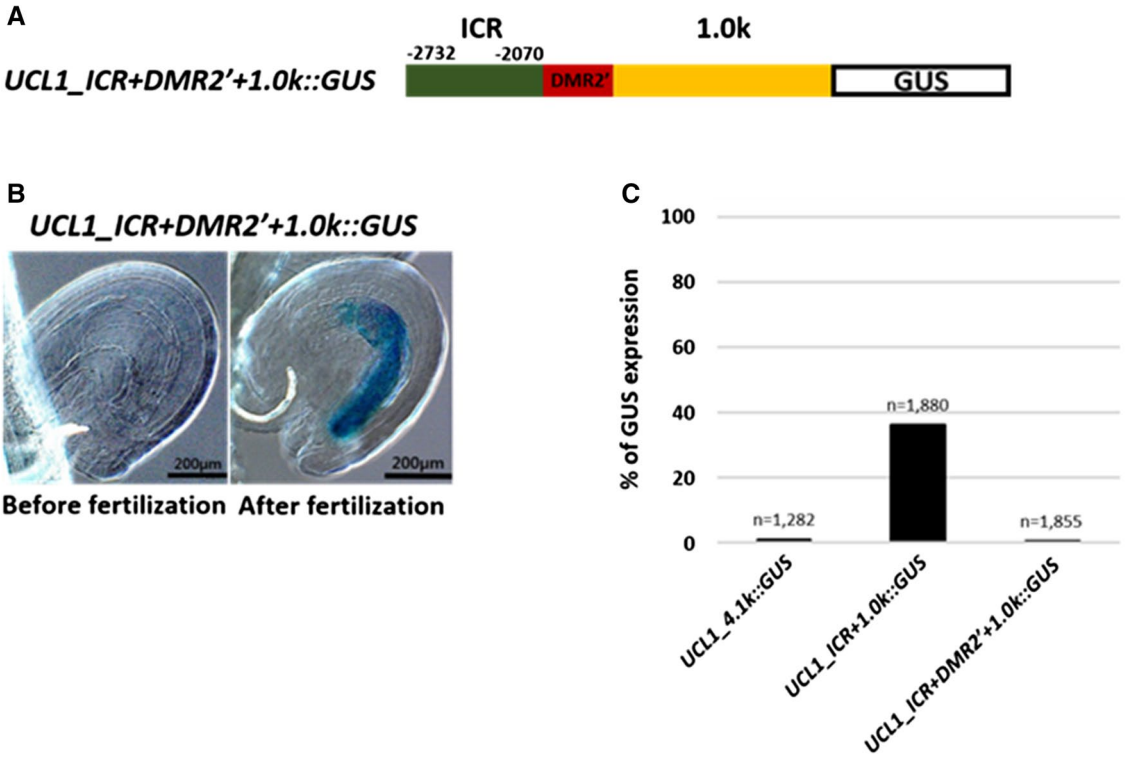

Fig. 5 DMR2′ is important for *UCL1* imprinting. **a** Diagram of *UCL1_ICR+DMR2′+1.0k::GUS*. **b** GUS staining of *UCL1_ICR+DMR2′+1.0 k::GUS* before (2DAE) and after fertilization (1DAP). **c** Maternal GUS activity of each construct in 2 DAE ovules prior to fertilization. Scale bars: 200 μm

UCL1_ICR+1.0k::GUS ovules (Fig. 5b, c). Evidently, adding the DMR2′ region into the *UCL1_ICR + 1.0k::GUS* construct that previously showed partial derepression in the ovules enabled complete silencing of the maternal allele prior to fertilization. This result is comparable to our reference construct, *UCL1_4.1k::GUS* (Fig. 5c) (Jeong et al. 2015). After fertilization of the self-pollinated seeds, GUS was strongly detected in the endosperm (Fig. 5b). Therefore, the DMR2′ region that is overlapping with the short TE is regulated by DME, and this hypomethylated DMR region is critical to the stable repression of maternal *UCL1* allele, thereby controlling *UCL1* imprinting.

The 271 bp Segment Immediately Upstream of the *UCL1* Translation Start Site is Sufficient for *UCL1* Endosperm-Specific Expression

To investigate the minimal promoter sequence in the 5′ upstream region responsible for the endosperm-specific expression of *UCL1*, we sequentially deleted the − 1071 bp ENSE upstream sequence by 100 bp intervals and fused those segments with GUS (Fig. 6). When GUS activity was examined, constructs with − 1071 to − 271 bp upstream sequence fused to GUS exhibited endosperm expression. However, further deletion, − 171 and − 71 bp upstream sequences, did not direct GUS expression. Based on this result, the cis-element responsible for *UCL1* ENSE resides between − 271 and − 171 bp 5′ upstream region of *UCL1*. Since only this shortened − 271 bp upstream sequence is necessary for endosperm-specific expression, that segment could provide a useful promoter when any genes need to be expressed exclusively in the endosperm, and not in the embryo or in the maternal seed coat.

Discussion

UCL1 encodes a E3 ligase that degrades the CLF polycomb protein and is expressed in the endosperm of the developing seed. Previous study of *UCL1* expression demonstrated that *UCL1* is an imprinted, paternally-expressed gene. For repression of the maternally-inherited allele, the general region between − 2.7 kb and − 2.0 kb 5′-upstream from the translation start site was identified as the ICR involved in the recruitment of the FIS-PRC2 complex (Jeong et al. 2015). In this present study, a more precise location of the cis-element necessary for maternal repression was narrowed down to the region between − 2.5 kb to − 2.4 kb region upstream of the gene (Fig. 1). We demonstrated that the DMR2′ region was critical for the stable repression of the maternal UCL1

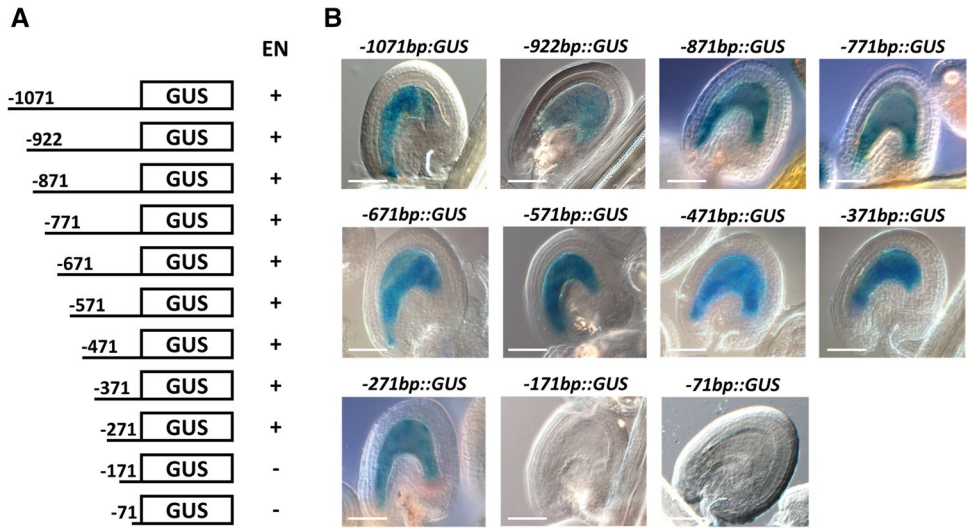

Fig. 6 Diagram of the GUS reporter constructs for fine mapping of endosperm-specific expression (ENSE). The activity of GUS and their constructs are coordinated. ENSE is sequentially deleted from − 1071 bp ENSE upstream sequence with 100 bp interval. Scale bars: 200 µm

allele (Fig. 5). In addition, we elucidated that the sequence within − 271 bp 5′ upstream of the start site contains the minimal promoter region necessary for endosperm-specific gene expression (Fig. 6).

In an attempt to more specifically locate the ICR cis-element within the − 2.5 kb to − 2.4 kb region, we discovered that smaller fragments from within this region were not sufficient to repress *UCL1* maternal allele expression when fused directly to the *UCL1_1.0k::GUS* construct (Fig. 2b). This result suggested that additional element(s) in the intervening linker region between the ICR and ENSE could be required for maternal repression. Creation of transgenic plants with the *UCL1_ICR+1.0k::GUS* construct, which contains the ICR and ENSE without the linker sequence, allowed us to explore this hypothesis. Without the 1 kb linker sequence, maternal expression of *UCL1* was not completely repressed in either ovule or in the endosperm (Fig. 3). In total, these results demonstrate that although ICR and ENSE play considerable roles in *UCL1* imprinting in the endosperm, the linker sequence is additionally required for the stable repression of maternal *UCL1*.

Within the linker sequence of the *UCL1* promoter is the ATLINE1_1 short TE. Jeong et al. (2015) reported that significant hypomethylation near the TE was detected in the endosperm compared to hypermethylation of that same area in the embryo. In this study, we investigated endospermal *UCL1* maternal and paternal DNA methylation as well as histone methylation patterns. In the *UCL1* promoter, two differentially methylated sections were identified: DMR1 (at about − 700 bp) and DMR2 (which overlaps the short *ATLINE1_1* TE sequence). In both regions, the silenced maternal *UCL1* allele is hypomethylated (Fig. 4 and Supplementary Fig. S1). In addition, H3K27me3 is found both upstream and downstream, but not within, the DMR2 region (Supplementary Fig. S2). Transgenic plants that contain a *UCL1* construct that included the ICR, DMR2, and ENSE showed almost total repression of maternal expression in ovules (Fig. 5).

Maternal dme mutants exhibit derepression of silenced maternal *UCL1* allele (Jeong et al. 2015). Thus, maternal hypomethylation by DME in the DMR2 is important for maternal allele silencing. Although DME is an active demethylase that catalyzes efficiently at CG, CHG, and CHH (Gehring et al. 2006), DME functions more in a targeted manner and tends to demethylate relatively euchromatic TE that are short, AT rich, or nucleosome poor, and generally interspersed or link with genes (Ibarra et al. 2012). For *UCL1*, the short *ATLINE1_1* TE is located in the − 2070 to − 1560 bp upstream of the *UCL1*translation start site (DMR2), and this DMR2 region plays an important role in silencing of the maternal allele, presumably recruiting FIS2-PRC2 complex and binding to it.

The *UCL1* methylation pattern is similar to that of the *PHERES* (*PHE*) gene in which the silenced maternal *PHE* allele is hypomethylated and the expressed paternal *PHE* allele is hypermethylated, except that the *PHE* DMR is located in the 3′ region (Makarevich et al. 2008). It is known that FIS2-PRC2 complex is recruited to the hypomehtylated maternal *PHE* allele, thereby induce silencing of the maternal allele. In *dme* mutant endosperm, the maternal *UCL1* allele showed significant hypermethylation in the two DMR regions, whereas paternal *UCL1* allele showed methylation similar to wild type (Fig. 4 and Supplementary Fig. S1). Since the silenced maternal *UCL1* allele is activated in *dme* mutant ovules and endosperm (Jeong et al. 2015), this result provides evidence that derepression of the silenced maternal *UCL1* allele in the *dme* mutant is due to the hypermethylation of the maternal allele and that this hypermethylation in maternal *UCL1* allele presumably results in failure of FIS2-PRC2 complex binding.

In *Arabidopsis*, active DNA demethylation occurs through the base excision repair (BER) pathway by replacement of methylcytosine with cytosine, and DME DNA glycosylase directly removes methylcytosine (Gehring et al. 2006). Since DME acts as an active demethylase and is expressed in the central cell of the female gametophyte (Choi et al. 2002), which is a precursor cell of the endosperm, active DNA demethylation is initiated in the central cell prior to fertilization by DME (Park et al. 2016). Maternal alleles of the DME targets, e.g. *MEDEA* and *FWA*, are hypomethylated compared to the methylation state of the corresponding paternal alleles in the endosperm after fertilization (Kinoshita et al. 2004; Gehring et al. 2006; Hsieh et al. 2009; Ibarra et al. 2012). Based on this information, it is tempting to speculate that the maternal *UCL1* allele is demethylated by DME in the central cell of the female gametophyte and that this hypomethylated region provides a FIS2-PcG binding site to silence the maternal UCL1 allele.

In terms of histone modification, the H3K27me3 marker extends both upstream and downstream of the DMR2. In *dme* mutant plants, the DMR2 region is hypermethylated, and perhaps FIS2-PRC2 fails to bind. As a result, the H3K27me3 pattern would not be established and maintained. Alternatively, since MEA and FIS2 PcG genes are MEGs in the endosperm and these two genes are known to be the targets of DME (Choi et al. 2002; Jullien et al. 2006), FIS1-PRC2 cannot be formed in *dme* mutant and cannot then repress the maternal *UCL1* allele. Further study will tell us which hypothesis is more plausible if the functional FIS2-PRC2 can be generated in the endosperm of *dme* mutants by exogenous transgenes, which is challenging.

Materials and Methods

Plant Materials and Growth Condition

Arabidopsis thaliana Columbia (Col-0) ecotype was used as the wild type. Plants were grown in an environmentally-controlled growth room at 22 °C under long-day conditions (16 h light/ 8 h dark) with 60% relative humidity.

Recombinant Plasmid Construction

UCL1_ICR+1.0k::GUS construct was generated using the PCR-amplified fragments of − 2.7 kb to − 2.0 kb and − 1.0 kb to + 1 sequences 5′-upstream of the *UCL1* translation start site fused with GUS reporter gene. Those regulatory regions of *UCL1* were obtained by PCR amplification with primer sets of JYHong1/2 and JYHong8/7 using the *pJET-40PRO_2.7 K* construct as a template. For the binary vector, *pBI101* was used. To analyze the role of differentially methylated regions of *UCL1* in the stable suppression of the maternal allele, *UCL1_ICR+DMR2'+1.0k::GUS* was generated using primer sets of JYHong1/2, JYHong4/5, and JYHong6/7 for the ICR, DMR2′ and ENSE 1 kb region, respectively. The fragments were all amplified using the *pJET-40PRO_2.7K* construct as a template and subcloned into the SalI/BamHI sites of the *pBI101* vector. To generate a *UCL1_ICR Rev+1.0k::GUS* construct in which the ICR orientation is reversed, PCR primer sets of JYHong82/83 were used to amplify reverse orientation of the ICR, and then the fragment was subcloned into the SalI/HindIII sites of the pBI-*UCL1_1.0 k::GUS* vector. All of the primers used are listed in Supplementary Table 1.

Histochemical GUS Staining Analysis and Microscopy

For analysis of GUS expression in the female gametophyte, mature floral buds were emasculated and left for 2 days to obtain the ovules synchronized and fully matured, if not otherwise specified. To analyze GUS gene expression in the endosperm, floral buds were emasculated and left for 1 day, pollinated, and then grown for 1 day. The tissues were dissected under the microscope, and the samples were put into X-Gluc staining solution containing 100 mM sodium phosphate buffer (pH 7.0), 2 mM each of potassium ferricyanide and ferrocyanide, 2 mM X-Gluc (GOLDBIO) and 0.1% Triton X-100 (SIGMA) overnight at 37 °C under dark conditions. After staining, tissue clearing with 70% EtOH was done as previously described (Choi et al. 2009). GUS-stained samples mounted on a slide glass were photographed using an Axio Imager A1 microscope (Carl Zeiss) with an AxioCam HRc camera.

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Author contributions JH, YC, and JSL conceived and designed the study. JH, JL and CWJ performed the experiments. JH, JL, CWJ, JSB, YC and JSL analyzed the data. JSB and YC wrote the manuscript with the contribution of JH, JL, CWJ and JSL.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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