이학석사학위논문

Study on the Imprinting Mechanism of the *UPWARD CURLY LEAF1* (*UCL1*) Gene in Arabidopsis

애기장대에서 *UPWARD CURLY LEAF1* (*UCL1*) 유전자의 각인 조절 메커니즘에 관한 연구

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ABSTRACT

Study on the Imprinting Mechanism of the *UPWARD CURLY LEAF1* (*UCL1*) Gene in Arabidopsis

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Genomic imprinting, an epigenetic process in mammals and flowering plants, refers to the differential expression of alleles of the same genes in a parent-of-origin-specific manner. In flowering plants like *Arabidopsis*, genomic imprinting has been detected only in the endosperm and it is regulated by Polycomb Repressive Complex 2 (PRC2) through trimethylation of lysine

27 on histone H3 (H3K27me3). Recent high-throughput sequencing analyses revealed that more than 200 loci are imprinted in *Arabidopsis*; however, only a few of these imprinted genes and their imprinting mechanisms have been examined in detail. In a previous study, it was reported that *UPWARD CURLY LEAF1* (*UCL1*), a gene encoding an E3 ligase that degrades the CURLY LEAF (CLF) polycomb protein, is a paternally expressed imprinted gene (PEG). After fertilization, paternally inherited *UCL1* is expressed in the endosperm, but not in the embryo. FERTILIZATION INDEPENDENT SEED2-PRC2 (FIS2-PRC2) silences the maternal *UCL1* allele in the central cell before fertilization and in the endosperm after fertilization.

In this study, the expression pattern of the *UCL1::GUS* genes suggests that the polycomb response element (PRE) of *UCL1* is located between -2.5 and -2.4 kb upstream of the *UCL1* translation start codon. To investigate exact PRE sequences of *UCL1*, I generated *UCL1_2.7k::GUS* constructs with 10 bp-scanning transversion mutagenesis between -2.5 and -2.4 kb. Their GUS expressions need to be checked in the Col-0 background transformants after floral dip. The PRE cooperated with the endosperm-specific factor binding element (ESFE), -1.0 kb upstream of *UCL1*, to drive the paternal imprinting and endosperm-specific expression of the *UCL1::GUS* gene. However, the imprinting pattern of the *UCL1_PRE+ESFE::GUS*

was relatively unstable. To identify additional element which is essential for repression of maternal *UCL1* allele with PRE, new construct containing −1814 to -1478 bp upstream of *UCL1*, the putative differentially methylated region (DMR), was generated. *UCL1_PRE+DMR+ESFE::GUS* showed complete suppression of maternal *UCL1* allele, that indicates DMR is essential for the paternal imprinting of the *UCL1* gene. On the other hand, the expression pattern of the *UCL1::GUS* genes those contain sequential deleted ESFE suggests that ESFE of *UCL1* is located between -271 bp and -171 bp. Specific transcription factor may bind to this ESFE sequence for endosperm−specific expression of *UCL1.*

keywords : *UPWARD CURLY LEAF1* (*UCL1*), genomic imprinting, paternally expressed imprinted gene (PEG), FIS−PRC2, endosperm, *Arabidopsis*

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ABBREVIATIONS

PcG	Polycomb Group
PRC2	Polycomb Repressive Complex 2
H3	Histone 3
K27	Lysine 27
PEG	Paternally Expressed imprinted Gene
PCR	Polymerase Chain Reaction
MS	Murashige-Skoog
GUS	β-glucuronidase
ICR	Imprinting Coding Region
PRE	Polycomb Response Element
ESFE	Endosperm-Specific Factor binding Element
DMR	Differentially Methylated Region

Ⅰ. Introduction

1. Composition of eukaryotic polycomb group complexes

One feature of the plant's life cycle is the alternations of diploid sporophytic generation and haploid gametophytic generation. During the life cycle of a plant, significant developmental changes occur, including the transition from gametophytic generation to sporophytic generation, as well as the transition from embryonic development to vegetative growth and from vegetative to reproductive growth (Xiao et al., 2015). The transition of the developmental stage of the plant leads to the suitable regulation of gene expression. Polycomb group complexes (PcG) play an important role in the transition of these developmental stages through modulation of chromatin structure (Mozgova et al., 2015).

The PcG complexes formed by the eukaryotic PcG proteins inhibit the expression of target genes at the transcriptional stage and lead to cell differentiation, stem cell formation, genomic imprinting, and X chromosome inactivation in eukaryotes and so on (Bemer et al., 2012; Derkacheva et al., 2013). Three types of PcG complexes are known in animals: Polycomb-repressive

complex 1 (PRC1), PRC2 and Pcl-PRC2 and they cooperate with each other to suppress the expression of target genes (Nekrasov et al., 2007; Hennig et al., 2009; Morey et al., 2010).

In eukaryotes, the PRC2 complexes are known to exist in a highly conserved form. The PRC2 complex of Drosophila consists of Extra sex combs (Esc), p55, Suppressor of Zeste [Su(z)12] and Enhancer of Zeste [E(z)]. Esc and p55 encode the WD40 domains, Su(z)12 encodes the C2H2 zinc finger domain and E(z) encodes the SET domain protein respectively (Czermin et al., 2002). The SET domain protein has methyltransferase activity, which is linked to the trimethylation of lysine 27 (H3K27) on histone H3 (H3K27me3), which inhibits the expression of the corresponding gene by altering the chromatin structure (Cao et al., 2002, Sims et al., 2003).

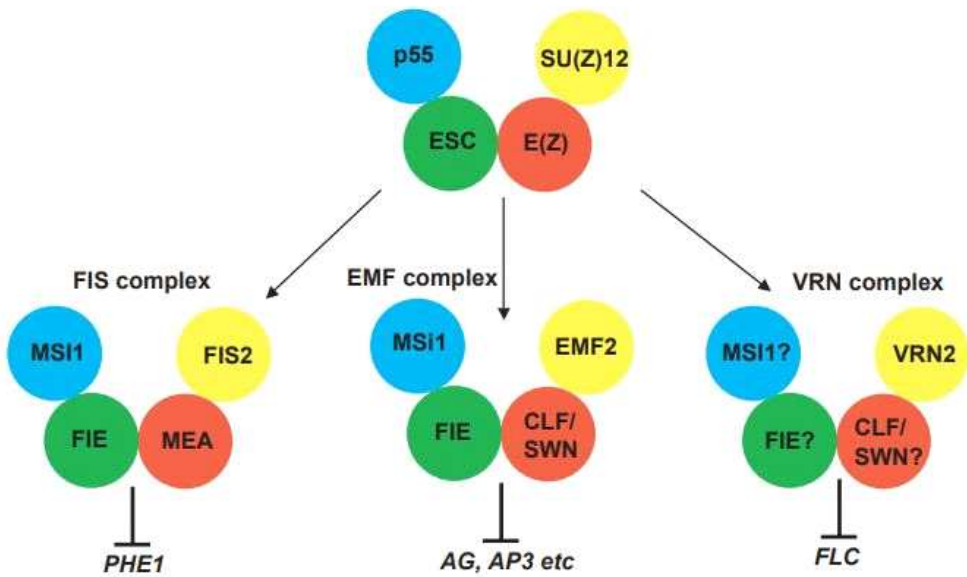
2. Polycomb group complexes in *Arabidopsis thaliana* and their functions

In *Arabidopsis thaliana*, a homologue of Esc is FERTILIZATION INDEPENDENT ENDOSPERM (FIE), homologues of Su(z) are EMBRYONIC FLOWER2 (EMF2), VERNALIZATION2 (VRN2) and FERTILAZATION INDEPENDENT SEED2 (FIS2), a homologue

of p55 is MULTICOPY SUPPRESSOR OF IRA1 (MSI1), and homologues of E(z) are CURLY LEAF (CLF), SWINGER (SWN) and MEDEA (MEA)(Goodrich et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999; Chanvivattana et al., 2004; Hennig et al., 2005). *Arabidopsis* PRC2 complexes are named after Su(z)12, EMF-PRC2, VRN-PRC2 and FIS-PRC2, respectively (Figure 1).

The epigenetic mark by PRC2 allows the cell to remember gene silencing. As PRC2 is involved not only in the transition from the vegetative growth stage to the reproductive growth stage in the plant development process, but also in the role of recognizing the response to the external environment, its role on the plant life cycle is expanding (He et al., 2013, Baulcombe et al., 2014). Recently, the existence of PRC2 in Arabidopsis and its target genes have been actively studied.

Figure 1



Chanvivattana et al., 2004

Figure 1. Arabidopsis Polycomb-group protein complexes

3. Arabidopsis genomic imprinting and Polycomb group complexes

The genes specifically expressed in Arabidopsis central cell and endosperm are differentially regulated (Gehring et al., 2006,

Hsieh et al., 2010). FIS-PRC2 is involved in the suppression of the genes. In some of the genes whose expression is inhibited by FIS-PRC2, the maternal gene is expressed while the paternal gene is inhibited. In other cases, the maternal gene is suppressed while the paternal gene is expressed. These are called maternally expressed imprinted genes (MEGs) and paternally expressed imprinted genes (PEGs), respectively (Chaudhury et al., 1997, Zhang et al,, 2013). MEGs are well known and *MEA*, *FIS2* and *FWA* belong to them (Kinoshita et al., 1999, Kinoshita et al., 2004, Jullien et al., 2006). The PEGs are *PHERES1* (*PHE1*), *ADMETOS* (*ADM*) and *UPWARD CURLY LEAF1* (*UCL1*) (Köhler et al., 2005, Kradolfer et al., 2013, Jeong et al., 2015, Wolff et al., 2015). Genomic imprinting is described in several theories, the most notorious theory being the parental conflict theory that the distribution of nutrients to the embryo evolved by the interests of the parents (Haig et al., 1989, Feil et al., 2007). In plants, genomic imprinting occurs mainly in endosperm.

MEGs are demethylated by DNA glycosylation by *DME* in the central cell, resulting in the expression of the maternal allele, while the paternal allele of the MEG is regulated by methylation and self-regulated by self-contained FIS-PRC2 (Choi et al., 2002, Gehring et al., 2006, Kinoshita et al., 2004, Jullien et al., 2006, Jullien et al., 2009). On the other hand, a maternal allele of *PHE1* belonging to PEGs is repressed by histone methylation

by FIS-PRC2. Meanwhile, it is known that DNA methylation of the repetitive sequences existing on the 3' end of the *PHE1* are necessary for the *PHE1* imprinting (Makarevich et al., 2008, Vilar et al., 2009). As in *PHE1*, the maternal allele of *UCL1* is inhibited by FIS-PRC2, but the expression of paternal *UCL1* allele seems unrelated to the repeat sequences in the *UCL1* promoter region (Jeong et al., 2015). This fact implies that genes expressed in endosperm are controlled by various mechanisms such as DNA methylation and histone methylation. However, the mechanism by which FIS-PRC2 recognizes the target gene (PRC2 recruitment) is not yet known. In particular, since the *UCL1* gene is expressed specifically in the endosperm and promotes the degradation of CLF, it not only maintains the function of FIS-PRC2 but also is a PEG (Jeong et al., 2011). Therefore, investigation of endosperm-specific expression mechanism and imprinting mechanism of *UCL1* is expected to contribute to food production by not only making an important contribution to the recognition of gene expression in endosperm, but also understanding the principle of endosperm development. In animals, imprinted genes are clustered on chromosomes, controlled by an imprinting control region (ICR), and ICRs have a differentially methylated region (DMR) (Bartolomei, 2009). In contrast, imprinted genes in plants are scattered throughout the genome and are known to be regulated by their respective ICRs (Feil et al., 2007, Makarevich et al., 2008).

Several target genes of PRC2 complexes have been identified so far. It is known that EMF-PRC2 containing CLF protein regulates *AG*, *AGL17*, *AP3*, *SEP3*, *KNAT2*, *FLC*, *FT*, *MEA*, *FUS3* and *PHE1* by methylation of histones and FIS-PRC2 containing MEA protein regulates *MEA*, *PHE1* and *FUS3* (Köhler et al., 2003, Katz et al., 2004, Guyomarc'h et al., 2005, Jiang et al., 2008, Raissig et al., 2013). Although EMF-PRC2 and FIS-PRC2 have been identified as having common target genes such as *PHE1*, it is thought that other genes are generally controlled by different PRC2. The reason for the differential regulation of different genes is unknown. Furthermore, CLF and MEA, belonging to EMF-PRC2 and FIS-PRC2 respectively, are functionally different and can not be interchanged. When *CLF* is overexpressed in endosperm, it has been observed that FIS-PRC2 fails to perform its function by replacing MEA, resulting in embryo abortion (Jeong et al., 2011). Therefore, it is crucial to identify how the different PRC2 complexes adapt to differential development of the endosperm that results from the replacement of CLF with MEA in the central cell and endosperm of Arabidopsis during the course of evolution.

4. Purpose of this study

UCL1 is a PEG in which a paternal allele is imprinted. It is specifically expressed in Arabidopsis endosperm, and degrades CLF protein, which forms EMF-PRC2, by ubiquitination to ensures the formation of FIS-PRC2 (Jeong et al., 2011, Jeong et al., 2015). Jeong (2015) revealed that the imprinting control region (ICR/PRE) of *UCL1*, that recognize FIS-PRC2 recruitment protein, is located between -2.7 and -2.0 kb upstream of the *UCL1* translation start codon. On the other hand, Jeong et al. (2015) also revealed that 922 bp upstream of the *UCL1* is sufficient for endosperm-specific expression of *UCL1*.

In this study, I generated 100 bp-sequential deleted *UCL1::GUS* genes to identify polycomb response element (PRE) and endosperm-specific factor binding element (ESFE) of *UCL1*. The expression pattern of *UCL1::GUS* genes suggested that the PRE of *UCL1* is located between -2.5 to -2.4 kb and the ESFE of *UCL1* is located between -271 and -171 bp upstream of *UCL1* translation start codon. To investigate PRE sequences between -2.5 and -2.4kb of *UCL1*, I generated 10 bp-scanning transversion mutagenesis and their expressions need to be checked in the Col-0 background transformants. Based on hypothesis that PRE may cooperate with ESFE to drive the paternal imprinting and endosperm-specific expression of *UCL1*, I also carried out experiments to check whether

UCL1_PRE+ESFE::GUS gene shows imprinting pattern. The expression pattern of *UCL1_PRE+ESFE::GUS* gene showed paternal imprinting and endosperm-specific expression, however unstably. It shows the possibility that additional element may be required for stable suppression of the PRE by FIS complexes. To check this possibility, I also generated new construct, which contains specific sequence predicted to be differentially methylated region (DMR) near short transposable element (TE) of *UCL1* promoter region. This *UCL1_PRE+DMR+ESFE::GUS* gene showed stable repression of maternal *UCL1* allele, indicating the essential role of DMR on paternal imprinting of the *UCL1.*

In addition, a transcription factor which is expressed specifically in the endosperm and functions in endosperm development, binding to ESFE, is expected to be involved in the endosperm-specific expression pattern of *UCL1.* *AGL62* is a gene that is expressed specifically in antipodal cells and endosperm and is known to be unable to develop normal endosperm development due to early cellularization when mutation occurs (Kang et al., 2008). Therefore, *AGL62* could be a candidate transcription factor which binds to ESFE of *UCL1*. In order to confirm this possibility, I examined the expression of *UCL1* in the *agl62*; *UCL1_4.1kb :: GUS* plants obtained by crossing the *agl62* mutant with *UCL1_4.1kb :: GUS*. As a result, the expression of *UCL1* was decreased in the *agl62*; *UCL1_4.1kb*

∷ *GUS* F1 plants, however not in the *UCL1_4.1kb* ∷ *GUS*$^{+}$ / $^{+}$;*agl62*$^{-}$ / $^{+}$ F2 and F3 generations. Therefore, other endosperm-specifc expressed transcription factors could be involved in the expression pattern of *UCL1* and further study is required.

Ⅱ. MATERIALS AND METHODS

1. Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild type. *agl62-1* (SALK_137707) and *agl62-2* (SALK_022148) plants were ordered from the ABRC, Ohio State University. Arabidopsis plants were grown in the growth room or growth chamber under long day conditions (16h of light at 24℃ /8h of darkness at 22℃). Seeds were sown on the surface of soil (Sunshine #5, Sungro), then put in 4℃ cold chamber with darkness for 2~5 days before moved to growth room. For transgenic plant screening, surface of seeds were sterilized by treating with 75% ethanol containing 0.08% Triton X-100 (SIGMA) for 10min thrice, followed by washing briefly with 100% ethanol (MERCK). The seeds were dried on Whattman filter paper and plated on solidified MS agar (0.5X Murashige and Skoog salts including Gamborg' s B5 vitamins [DUCHEFA], 0.025% MES monohydrate [DUCHEFA], 1% sucrose [JUNSEI] and 0.07% plant agar [DUCHEFA], pH between 5.7 and 5.8 into 1L of distilled water was autoclaved in 121℃ for 20min) plates containing appropriate antibiotics (25㎍/ml of kanamycin). Before transferring to the growth room, MS plates with seeds

were also put in 4℃ for cold treatment for 2-5 days. Per construct, around 20 Columbia-0 (Col-0) wild-type plants and 200 *fis2-11* plants were used for *Agrobacterium*-mediated transformation by the floral dipping method.

2. Characterization of the *agl62-1* and *62-2* allele

To confirm the T-DNA insertion, genotyping PCR was carried out by using genomic DNA as a template. According to Kang (2008), T-DNA in *agl62-1* is inserted into the intron, 474 nucleotides downstream of the start codon and T-DNA in *agl62-2* is inserted in the second exon, 620 nucleotides downstream of the start codon. The left border junction of *agl62-1* and *agl62-2* were determined using the T-DNA primer LBa1 (JYHong10) and genomic primer agl62 RP(JYHong50). For amplification of the *agl62-1* and *agl62-2* wild-type allele, gene specific primers JYHong49/JYHong50 and JYHong51/JYHong50 primers sets were used respectively. After genotyping, phenotype of the heterozygous *agl62-1* and *agl62-2* plants were observed, and they contained ~25% defective seeds in the siliques.

3. Recombinant plasmid construction

For analysis of interaction between PRE and ESFE of *UCL1*, *UCL1_PRE+ESFE::GUS* construct including -2.7~-2.0 kb and -1.0~0 kb sequences upstream of the *UCL1* translational start codon was fused with GUS reporter gene sequences. The regulatory regions of *UCL1* were obtained by PCR amplification with primer sets of JYHong1/2 and JYHong8/7 using *pJET-40PRO_2.7K*, construct which contains -2.7~0 kb upstream of UCL1 translation start codon, as a template. Amplified fragments were cloned into *Sal I/BamH I* sites of the *pBluescriptⅡ SK(+)* vector and then blue-white screening was performed. Cloned insert inside the *pBluescriptⅡ SK(+)* vector was then subcloned into *Sal I/BamH I* sites of the *pBI101* vector. Additionally, *UCL1_PRE+DMR+ESFE::GUS* construct was generated for investigating role of differentially methylated region (DMR) on stable suppression of maternal *UCL1* allele by FIS complex. For these, PRE, DMR and ESFE regions of *UCL1* were obtained by PCR with primer sets of JYHong1/2, JYHong4/5 and JYHong6/7 using same template with previous construct. All the fragments were amplified using *pJET-40PRO_2.7K* as a template and subcloned into *Sal I/BamH I* sites of the *pBI101* vector.

For investigation of *UCL1* PRE, six different constructs were generated. The *UCL1_2.6/2.5/2.4/2.3/2.2/2.1K::GUS* constructs

include 2632/2532/2432/2332/2232/2132 bp sequences upstream of the translational start codon of *UCL1* that was fused with GUS reporter gene sequences. The regulatory regions of *UCL1* were obtained by PCR amplification with primer sets of JYHong35/7, JYHong36/7, JYHong37/7, JYHong38/7, JYHong39/7 and JYHong40/7 using *pJET-40PRO_2.7K* as a template. Fragments were then subcloned into *Sal I*/*BamH I* sites of the *pBI101* vector. Also, for identification of accurate PRE region, six more constructs were generated. The *UCL1_-2.56~-2.36/-2.53~-2.33/-2.50~-2.30/-2.47~-2.27/-2.44~-2.24/-2.40~-2.20 kb + ESFE :: GUS* constructs include 2562~2362/2532~2332/2502~2302/2472~2272/2442~2242/2406~2206 bp sequences upstream of the translational start codon of *UCL1*, amplified by PCR with primer sets of JYHong69/70, JYHong36/79, JYHong71/72, JYHong73/74, JYHong75/76 and JYHong77/78, that was fused with *UCL1_1.0k::GUS.*

To figure out whether PRE orientation is significant for its role on *UCL1* paternal imprinting, *UCL1_-2.7~-2.0kbRev+ESFE::GUS* construct including reversed sequences of PRE and ESFE was generated. Reversed PRE sequences harboring *HindIII* and *Sal I* sites were obtained by PCR with primer sets JYHong82/83 and subcloned into *Sal I*/*HindIII* sites of *pBI101-UCL1_ESFE::GUS* cloned vector.

For investigation of *UCL1* ESFE, nine different constructs were generated. The *UCL1_0.8/0.7/0.6/0.5/0.4/0.3/0.2/0.1/0.07k::GUS*

constructs include 871/771/671/571/471/371/271/171/71-bp sequences upstream of the translational start codon of *UCL1* that was fused with GUS reporter gene sequences. The regulatory regions of *UCL1* were obtained by PCR amplification with primer sets of JYHong26/JYHong7, JYHong27/JYHong7, JYHong28/JYHong7, JYHong29/JYHong7, JYHong30/JYHong7, JYHong31/JYHong7, JYHong32/JYHong7, JYHong33/JYHong7 and JYHong34/JYHong7 using *pJET-40PRO_2.7K* as a template. Fragments were then subcloned into *Sal I*/*BamH I* sites of the *pBI101* vector.

4. *Agrobacterium tumefaciens* transformation and plant transformation by floral dipping

The cloned constructs were introduced into *Agrobacterium* GV3101 by electroporation for transforming Arabidopsis genome. For transformation, 50㎕ *Agrobacterium tumefaciens* (GV3101) cell stock in a 1.5ml tube was thawed on ice for 10min, and then 2㎕ of DNA of interest was added to the competent cell mixing by gentle pipetting. After transferring the mixture into glass cuvette, it was pursed by 1.8kV for 5.8mS with Micro-Pulser™ (BIORAD). After electroporation, 400㎕ of Luria Broth (LB) medium was added to the cuvette and mixture was transferred to a 1.5ml tube and incubated in shaking incubator at 28℃ for 1h before spreading on solidified LB plate with proper

antibiotics (50㎍/ml kanamycin, 50㎍/ml gentamicin). The plate was incubated at 28℃ for 2 days.

For Arabidopsis transformation, floral dipping method was used. Single colony of transformant *Agrobacterium* was selected and inoculated into 5 ml of LB medium with adequate antibiotics (50㎍/ml kanamycin, 50㎍/ml gentamicin) and grown for 24h at 28℃ shaking incubator. To amplify the bacterium cell, mini-prep cultures were then inoculated again into 300 ml of LB medium containing same antibiotics and grown in the same condition. Incubated *Agrobacterium* cell was harvested by centrifuge at 4000 rpm for 20min at 20℃ (SORVALL® RC 6 PLUS with SLC-3000 rotor). After discarding supernatant, the cell pellet was resuspended in infiltration media. 300ml of infiltration media contains 0.63 g of MS salt, 15 g of sucrose and 150 ㎕ of Silwet (Vac-In-Stuff, Silwet L-77, LEHLE SEEDS). For successful Arabidopsis transformation, Col-0 plants were grown on soil until adequate number of inflorescences are generated in a long day condition. Right before transformation, siliques and open flowers were removed. Young buds were then dipped into the infiltration media containing *Agrobacterium* for 10 sec. After floral dipping, plants were laid on a tray and covered with black plastic bag for blocking light for 24 h. Next day, the plants were uncovered, put vertically and grown until their seeds were mature enough to harvest.

5. Histochemical GUS staining analysis

The expression of GUS was analyzed in the *UCL1_4.0/2.7/2.6/2.5/2.4/2.3/2.2/2.1/1.0/0.8/0.7/0.6/0.5/0.4/0.3/0.2/0.1/0.07k::GUS*, *UCL1_PRE+ESFE::GUS*, *UCL1_PRE+DMR+ESFE::GUS*, *UCL1_-2.7~-2.0kbRev+ESFE::GUS*, *UCL1_-2.56~-2.36/-2.53~-2.33/-2.50~-2.30/-2.47~-2.27/-2.44~-2.24/-2.40~-2.20k+ESFE::GUS* plants. For analysis of gene expression in the female gametophyte, matured floral buds were emasculated and left for 2 days. Whereas, to investigate gene expression in the developing endosperm, flowers were emasculated, left for 1 day and pollinated, then grown for 1day. The tissues were dissected and sampled in the X-GLUC staining solution containing 100mM sodium phosphate buffer (pH 7.0), 2mM each of potassium ferricyanide and ferrocyanide, 2mM X-GLUC (GOLDBIO) and 0.1% Triton X-100 (SIGMA) for overnight at 37℃ in the dark condition.

6. Microscopy

The tissues were mounted by the clearing solution (1ml 70% glycerol, 2.5g chloral hydrate) on a slide glass. GUS expressing samples were observed on a Zeiss Axio Imager A1 light microscope under different interference contrast optics with 10x, 20x, 40x objectives and photographed by AxioCam HRc camera

(Carl Zeiss).

7. Confocal laser scanning microscopic analysis

For the analysis of developing endosperm in *UCL1_4.1k::GUS;agl62-1* mutant and *UCL1_4.1k::GUS* mutant, CLSM of the endosperms was performed as previously described with slight modification (Christensen et al., 1997, Maruyama et al., 2015). Developing seeds were harvested 36 hours after pollination (36 HAP) and stained with GUS staining solution as described above. For fixation, GUS-stained endosperms were dipped into 4% glutaraldehyde (in 12.5 mM cacodylate buffer, pH 6.9) under vacuum (~200) for 20min. For stable fixation, fixed samples were stored for 5 days at 4℃. Samples were then dehydrated with 20/35/50/65/80/95% ethanol series for 10min respectively. The tissues were subsequently cleared in 2:1 benzyl benzoate:benzyl alcohol and observed with a LSM700 (Carl Zeiss).

Table 1. List of primer sequences

Label	Oligo name	Sequences
Cloning		
JYHong1	Sal_40PRO_2.7K_F	ACG CGT CGA CCA ACC CTT ACT CCC TTT CTT TC
JYHong2	Hind_40PRO_2.0K_R	GCC GGC AAG CTT TCG TTA TTA AAT AAA ATG TAG GAG AAA AA
JYHong4	Hind_40PRO_1.8K_F	GGC CAA GCT TAA TAT CCC TAA TAT CTA ACT ATA TTA AAC C
JYHong5	Eco_40PRO_1.5K_R	GCC GAA TTC AAT TAA CCT CTA TCG TTT CAC CT
JYHong6	Eco_40PRO_1.0K_F	GCC GAA TTC TGA TTG ATT TTA TGA GTT TTC ACA
JYHong7	Bam_40PRO_R	CGC GGA TCC TTT GCT ACT TTG ATT GTT TGT GAT
JYHong8	Hind_40PRO_1.0K_F	CGC GCA AGC TTT GAT TGA TTT TAT GAG TTT TCA CA
JYHong26	Hind_40PRO_0.8K_F	GCG GCC AAG CTT ATA AAT CTC TTA AGA AAC AAG GAA
JYHong27	Hind_40PRO_0.7K_F	GCG GCC AAG CTT TAA GAC ATA TCT CTT AAC ATA AGA AT
JYHong28	Hind_40PRO_0.6K_F	GCG GCC AAG CTT TGT TCT CTT ATC GCA AAA ACA ATA C
JYHong29	Hind_40PRO_0.5K_F	GCG GCC AAG CTT CAA ATT GTC ATT CTA AAA GTC AT
JYHong30	Hind_40PRO_0.4K_F	GCG GCC AAG CTT TTG TTG GGC TTT TGT TTT TGT TTA
JYHong31	Hind_40PRO_0.3K_F	GCG GCC AAG CTT TGT TTT GGT GTA GTT TAG GGA AG
JYHong32	Hind_40PRO_0.2K_F	GCG GCC AAG CTT AAT GAT GAA CCA TGA AAA TCA TT
JYHong33	Hind_40PRO_0.1K_F	GCG GCC AAG CTT GTA TTC ATT CTT ATT TAT GGG AAT G
JYHong34	Hind_40PRO_0.07K_F	GCG GCC AAG CTT ACT TTT AGG TAA GTA TAC GTA GTA
JYHong35	Sal_40PRO_2.6K_F	ACG CGT CGA CTA TAC CAT AAA TGG TTT TCA TGG

JYHong36	Sal_40PRO_2.5K_F	ACG CGT CGA CTA GAT GAA AGA TTT TGG GTT GA
JYHong37	Sal_40PRO_2.4K_F	ACG CGT CGA CAT AAT TTA TTA TCA GAA TTA ACT TGA
JYHong38	Sal_40PRO_2.3K_F	ACG CGT CGA CAA AAA GAA AAG AAA AGA AAA GTA AAT C
JYHong39	Sal_40PRO_2.2K_F	ACG CGT CGA CTG AGA TTG AAG GGA TAA CTT TT
JYHong40	Sal_40PRO_2.1K_F	ACG CGT CGA CTA TCT TCT ATG TAC GCA CAT CG
JYHong69	Sal_40PRO_2.56K_F	ACG CGT CGA CTG TAG CAT TAC AAA ATA TTC TCT CC
JYHong70	Hind_40PRO_2.36K_R	CGG CAA GCT TAA ATC TCT GAA ATA TCA AAT CCC T
JYHong71	Sal_40PRO_2.50K_F	ACG CGT CGA CAA CAC CAA ATA TAG TGT TTA TTG TT
JYHong72	Hind_40PRO_2.30K_R	CGG CAA GCT TTG AGA TTT ACT TTT CTT TTC TTT TCT T
JYHong73	Sal_40PRO_2.47K_F	ACG CGT CGA CTT ACT TTT TTG TGG TAT GGA TAT CT
JYHong74	Hind_40PRO_2.27K_R	CGG CAA GCT TTG TAT TTC TTA AAA TTA AAA CTA AAA CTG A
JYHong75	Sal_40PRO_2.44K_F	ACG CGT CGA CGC ATT AAT ATA TAA TTT ATT ATC AGA ATT AAC T
JYHong76	Hind_40PRO_2.24K_R	CGG CAA GCT TTC TCT CCT TTC TTA TAT TTC CAT T
JYHong77	Sal_40PRO_2.40K_F	ACG CGT CGA CTT TAC AGA TTG TAT TAG ATT AGG GAT
JYHong78	Hind_40PRO_2.20K_R	CGG CAA GCT TAG TCA AAA GTT ATC CCT TCA AT
JYHong79	Hind_40PRO_2.33K_R	CGG CAA GCT TGT TTT TAA AAG AAT ACA TAA CAA CCT
JYHong82	Hind_40PRO_2.7K_F	CGG CAA GCT TCA ACC CTT ACT CCC TTT CTT TC
JYHong83	Sal_40PRO_2.0K_R	ACG CGT CGA CAA AAT CTC GTA CAA TCT ACC AA
Genotyping		
JYHong10	LBa1	TGG TTC ACG TAG TGG GCC ATC G
JYHong49	agl62-1 LP	AGT TGT GTT CTC ACC TGG

		TCG
JYHong50	agl62 RP	CAA GAA CAA GAA AAA CAA CAA CAA C
JYHong51	agl62-2 LP	TGG ATC TTT CTG GCA GAT TTG

Ⅲ. Results

1. Analysis of an interaction between the polycomb response element (PRE), involved in *UCL1* expression, and endosperm-specific factor binding element (ESFE)

In the previous study, *UCL1* was reported as a paternally expressed imprinted gene (PEG) which is not expressed in the ovule but expressed only in the endosperm after fertilization (Jeong et al., 2015).

Also, Jeong et al. (2015) reported that *UCL1_2.7k::GUS* transgenic plants show a paternally expressed imprinted pattern of *UCL1* while *UCL1_2.0k::GUS* transgenic plants show biallelic expression of *UCL1*, which means that ICR/PRE of the *UCL1* is located between -2.7 kb and -2.0 kb of the *UCL1* promoter region. On the other hand, the −1.0 kb promoter region was verified to be sufficient for endosperm-specific expression of *UCL1* (Jeong et al., 2015). To confirm whether the −2.7 to −2.0 kb region present in the *UCL1* promoter and the −1.0 kb promoter region expressing the endosperm-specific expression induce paternal imprinting and endosperm-specific expression, a basic construct containing these two regions was generated

(Figure 2A). I checked GUS activity of the *UCL1_PRE+ESFE::GUS* transgenic plant in the ovules before fertilization to see whether the maternal allele of *UCL1* is repressed. As a result, maternal *UCL1* expression was not detected in the ovules; however *UCL1* expressed strongly in the endosperm after fertilization (Figure 2B). To check parent-of-origin specificity of *UCL1*, I performed reciprocal crosses between *UCL1_PRE+ESFE::GUS* transgenic plants and Col-0 wild type plants. GUS activity was not detected in the developing endosperm of *UCL1_PRE_ESFE::GUS* transgenic plants pollinated by Col-0; however, Col-0 plants pollinated by the *UCL1_PRE+ESFE::GUS* transgenic plants showed high percentage of GUS expression in the endosperm (Figure 2C). However, *UCL1_PRE+ESFE::GUS* transgenic plants showed incomplete maternal gene suppression compare to *UCL1_4.1k::GUS* transgenic plants. When unfertilized floral ovules were emasculated and GUS was stained after 1 day and 4 days, 0.9% (The total number of ovules scored=424) and 24.63% (total n=1141) of *UCL1_PRE+ESFE::GUS* ovules showed GUS expression, otherwise 0% (total n=413) and 1.2% (total n=557) of *UCL1_4.1k::GUS* ovules showed GUS expression (Figure 3A). I also performed reciprocal crosses between *UCL1_PRE+ESFE::GUS* transgenic plants and Col-0 wild type plants. 33.43% (The total number of seeds scored=1588) of GUS activity was detected in the developing endosperm of

UCL1_PRE+ESFE::GUS transgenic plants pollinated by Col-0; however, *UCL1_4.1k::GUS* transgenic plants pollinated by Col-0 showed 0.8% (total n=583) of GUS expression and *UCL1_1.0k::GUS* transgenic plants pollinated by Col-0 showed 76.32% (total n=561) of GUS activity in the developing endosperms (Figure 3B). These results demonstrate considerable role of PRE and ESFE for the *UCL1* imprinting, but also show the possibility that a specific element between −1.0 kb and −2.0 kb may be additionally required for stable suppression of PRE by FIS complexes.

Figure 2

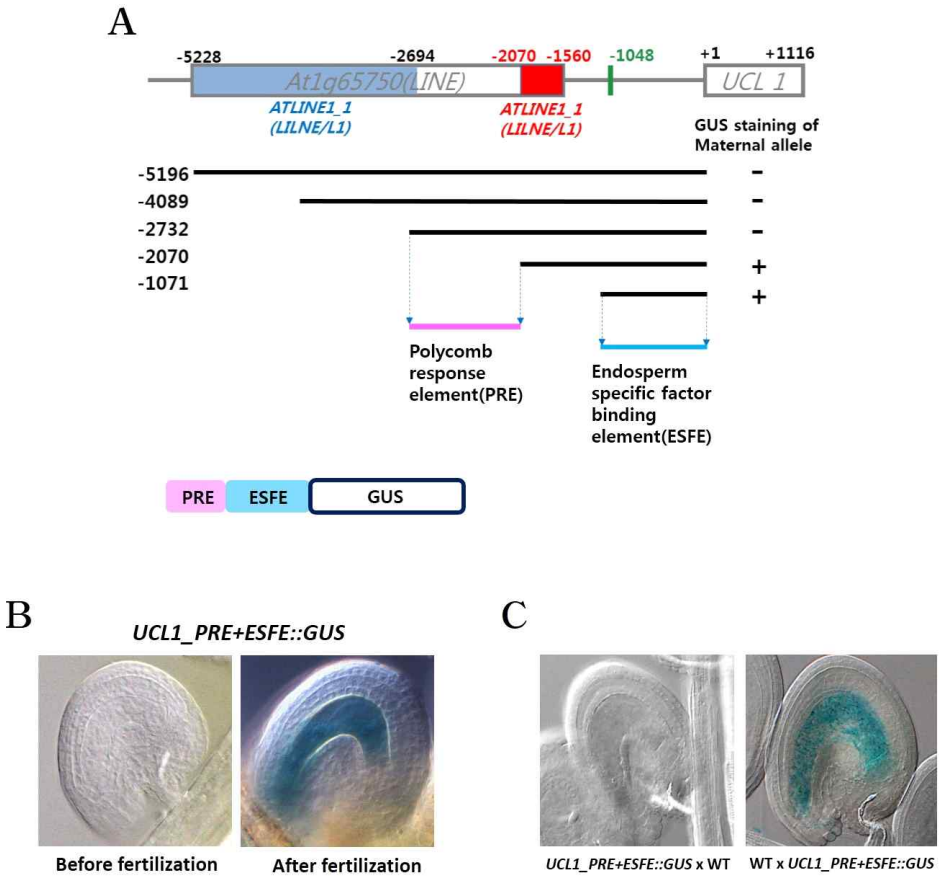


Figure 2. Expression pattern of *UCL1_PRE+ESFE::GUS*

(A) *UCL1_PRE+ESFE::GUS* construct containing PRE and ESFE of *UCL1* fused with GUS.

(B) Expression of *UCL1_PRE+ESFE::GUS* in the ovule before fertilization and in the endosperm after fertilization.

(C) Cytoplasmic GUS expression of endosperm resulting from reciprocal crosses between *UCL1_PRE+ESFE::GUS* transgenic plants and Col-0 wild-type plants.

Figure 3

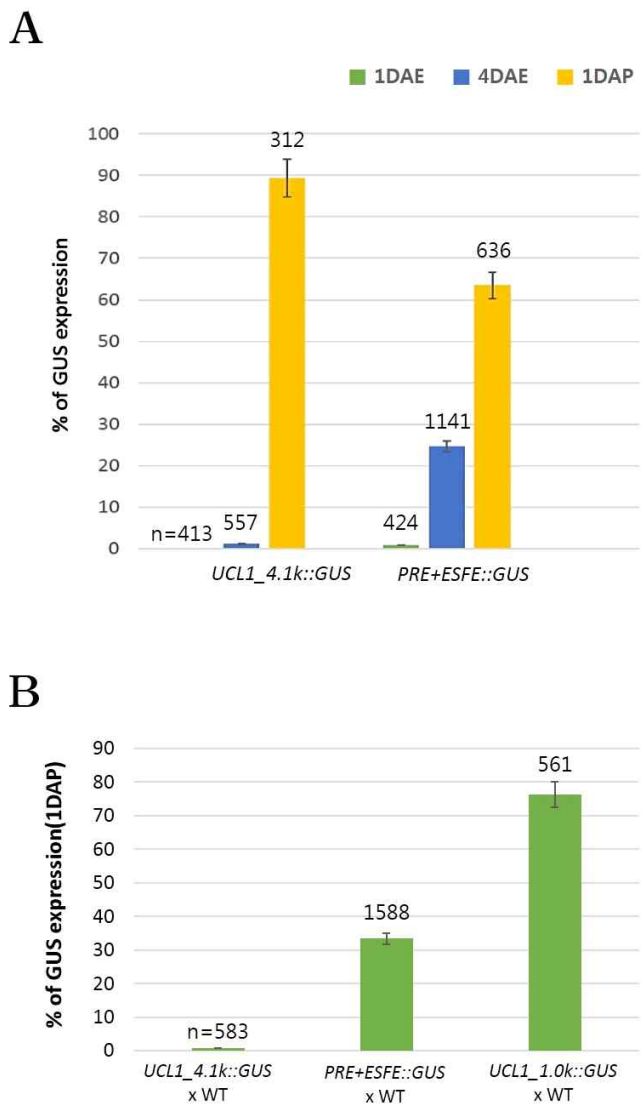

A
1DAE
4DAE
1DAP
% of GUS expression
100
90
80
70
60
50
40
30
20
10
0
312
636
1141
557
n=413
424
UCL1_4.1k::GUS
PRE+ESFE::GUS
B
% of GUS expression(1DAP)
90
80
70
60
50
40
30
20
10
0
561
1588
n=583
UCL1_4.1k::GUS
x WT
PRE+ESFE::GUS
x WT
UCL1_1.0k::GUS
x WT

Figure 3. Percentage of GUS expression in the *UCL1_PRE+ESFE::GUS*

(A) Expression percentage of GUS activity in *UCL1_4.1k::GUS* and *UCL1_PRE+ESFE::GUS* at 1 day after emasculation (DAE), 4 DAE and 1 day after pollination (DAP). The total number of ovules scored is denoted on top of the graph column.

(B) Expression percentage of GUS activity in developing endosperm after being pollinated with wild-type pollen. Maternal *UCL1* allele is de-repressed at 33.43%. The total number of seeds scored is denoted on top of the graph column.

2. Investigation of the polycomb response element (PRE) of *UCL1* by the FIS-PRC2 complex

A polycomb response element (PRE) −2.0 to −2.7 kb, which is present in the *UCL1* gene promoter region, was sequentially deleted by 100 bp, and then GUS fusion constructs were prepared (Figure 4A). The results showed that the expression of the maternal allele was repressed in the GUS reporter fused with -2.7/−2.6/−2.5 kb of the *UCL1* promoter region whereas the expression of the maternal allele was observed from −2.4kb (Figure 4B). These results suggest that the PRE of the *UCL1* gene is located between −2.5 and −2.4 kb of the *UCL1* promoter region.

Therefore, fine-deletion of the *UCL1* promoter −2.5 to −2.4 kb site was performed for more accurate PRE identification. For this, −2.5~-2.4 kb of the *UCL1* promoter region was deleted sequentially by 30 bp into 200 bp size and then combined with *UCL1_1.0kb::GUS* construct (Figure 5A). For checking the allele-specific expression, *UCL1_−2.56~-2.36 / −2.53~-2.33 / −2.50~-2.30 / −2.47~-2.27 / −2.44~-2.24 / −2.40~-2.20 kb + ESFE :: GUS* transgenic plants were reciprocally crossed with Col-0 wild-type plants, respectively and the endosperms were stained with GUS solution 1 day after pollination. As a result, all of the transgenes showed biallelic expression of GUS

activity(Figure 5B). These results suggest that the 200 bp sequence of the PRE is not sufficient for repression of the *UCL1* maternal allele by the FIS complex. Another possibility is the requirement of DMR to repress the maternal *UCL1* allele stably.

On the other hand, generation of 10 bp-scanning transversion mutagenesis on -2.5 to -2.4 kb of *UCL1* promoter region is on going (Figure 6A). These constructs contain -2.7 to 0 kb upstream of the *UCL1* translation start codon with 10 bp transversion (Figure 6B). Once the sequence is identified, Chromatin Immunoprecipitation (ChIP) will be performed around this location to check H3K27me3 accumulation on this PRE region.

Figure 4

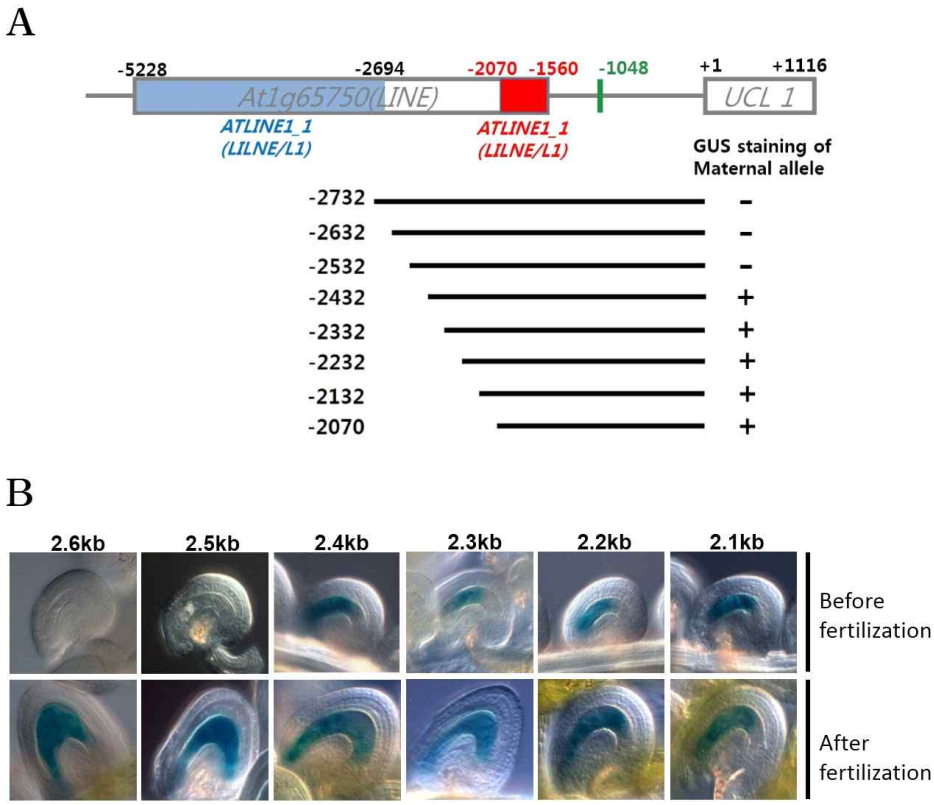


Figure 4. Identification of PRE in the *UCL1* promoter region

(A) Constructs of sequential deletion of PRE fused with GUS.

(B) Analysis of GUS activity in the ovules and developing endosperms, before and after fertilization. The results show PRE is located between -2.5 and -2.4kb of *UCL1* promoter region.

Figure 5

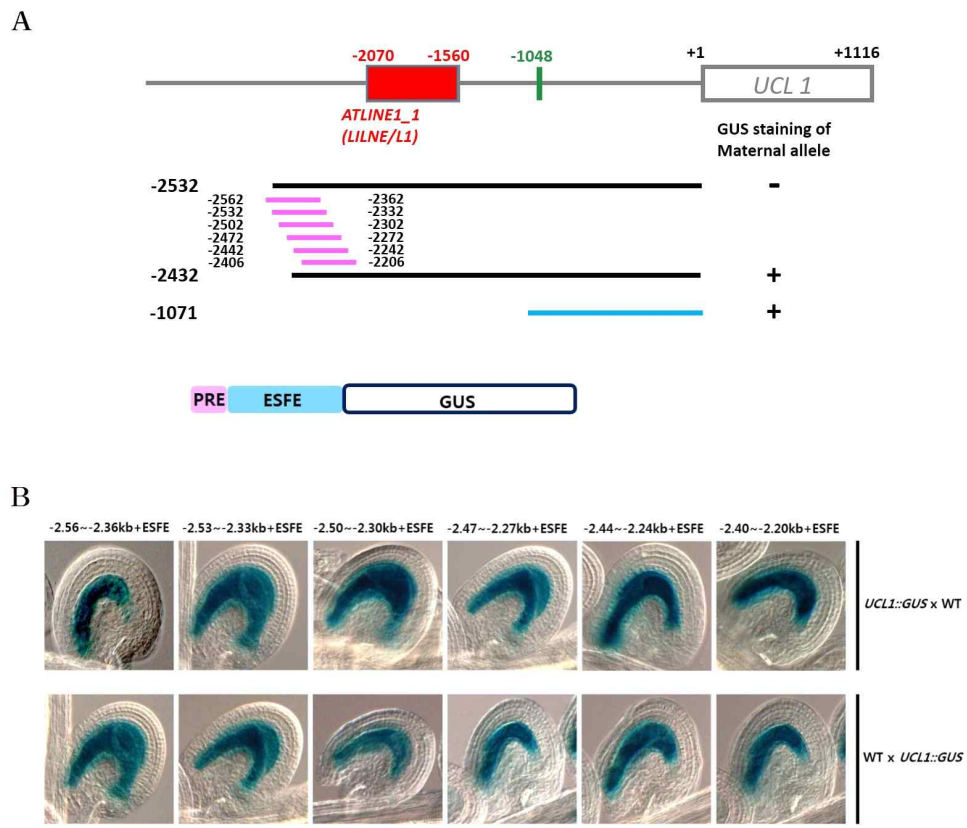


Figure 5. Identification of PRE

(A) Constructs of fine-deleted PRE fused with *UCL1_ESFE::GUS.*

(B) Analysis of GUS activity in the developing endosperm after reciprocal crosses. The results show biallelic expression of *UCL1.*

Figure 6

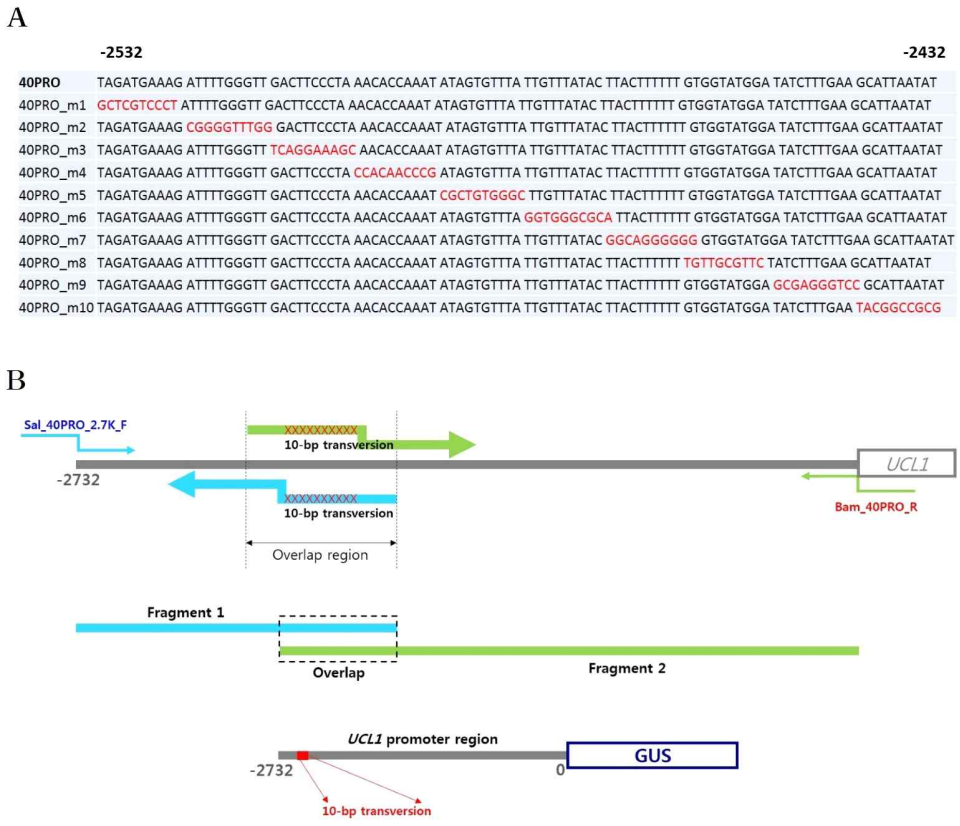


Figure 6. 10-bp scanning transversion mutagenesis of PRE

(A) Sequences showing 10-bp scanning transversion between -2532 and -2432 bp upstream of *UCL1*, that was confirmed as PRE. T and G were substituted while A and C were substituted each other.

(B) Scheme for generating 10-bp scanning transversion mutagenesis constructs.

3. Significance of PRE orientation, controlled by the FIS complex

In order to confirm whether the orientation of the *UCL1* PRE affects the repression of the maternal *UCL1* allele by the FIS complex, I generated *UCL1_−2.7~−2.0kbRev+ESFE::GUS* construct containing the *UCL1* promoter region -2.0~−2.7 kb, reversed PRE sequences of *UCL1*, and ESFE fused with GUS (Figure 7A). *UCL1_−2.7~−2.0kbRev+ESFE::GUS* transgenic plants were then reciprocally crossed with Col−0 wild−type plants and the expression of GUS activity was analyzed in the developing endosperms 1 day after pollination (1DAP). As a result, there were transgenic plants showing maternal allele repression, GUS activity is only expressed when wild−type plant was pollinated by *UCL1_−2.7~−2.0kbRev+ESFE::GUS* plant, which were the same as *UCL1_−2.7~−2.0kb+ESFE::GUS* (Figure 7B, C). To investigate this result clearly, I checked the GUS expression in the unfertilized ovules of 20 different *UCL1_−2.7~−2.0kbRev+ESFE::GUS* transgenic plants and analyzed statistically. As a result, 40% (total n=1076) of *UCL1_−2.7~−2.0kbRev+ESFE::GUS* transgenic plants showed none or weakly stained GUS expression, which means repression of the maternal *UCL1* allele. On the other hand, *UCL1_−2.7~−2.0k+ESFE::GUS* transgenic plants that contain

forward oriented PRE and ESFE fused with GUS, showed 76.59% (total n=1880) of none or weakly stained GUS expression and homozygous *UCL1_4.1k::GUS* transgenic plants showed 99.6% (total n=1282) of maternal *UCL1* repression (Figure 8A, B).

Partial repression of maternal *UCL1* allele at low probability in *UCL1_-2.7~-2.0kbRev+ESFE::GUS* transgenic plants suggests that orientation of the PRE is significant for recruitment of FIS-PRC2 and repression of maternal allele of *UCL1* by FIS-PRC2.

Figure 7

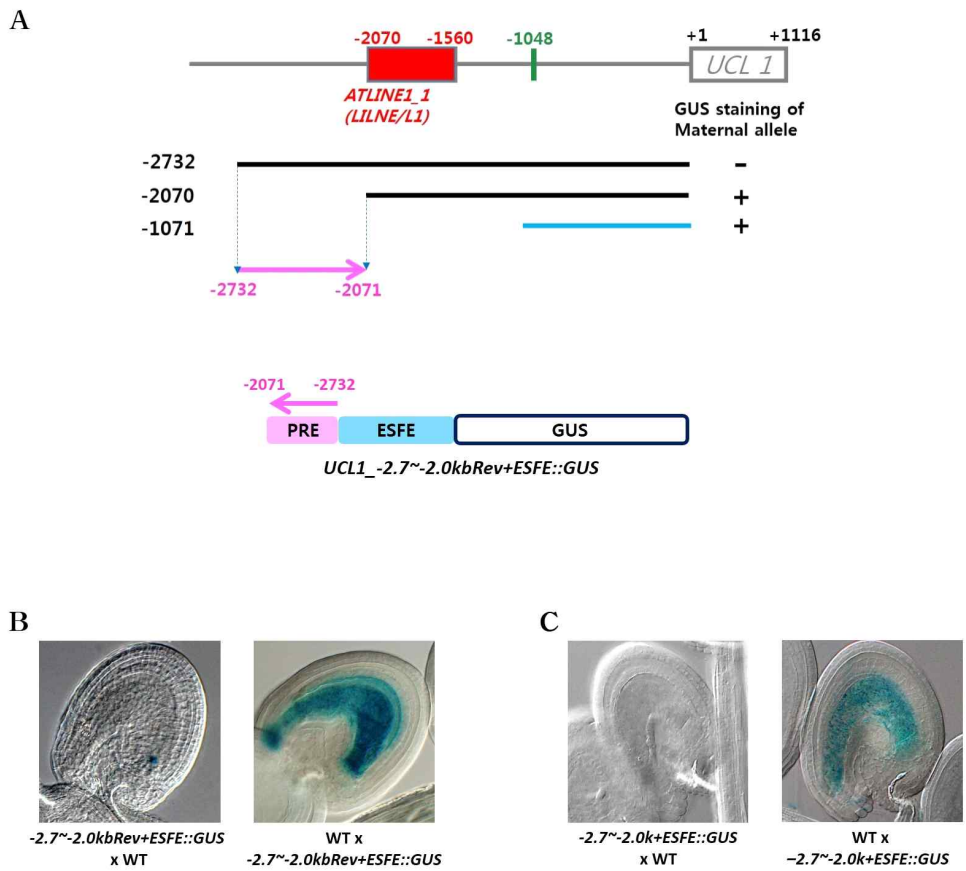


Figure 7. Investigation on significance of PRE orientation

(A) Construct of *UCL1_-2.7~-2.0kbRev+ESFE::GUS*.

(B) GUS expression in the endosperm after reciprocal cross between *UCL1_-2.7~-2.0kbRev+ESFE::GUS* transgenic

plant and Col-0 wild-type plant (1DAP). Paternal *UCL1_-2.7~-2.0kbRev+ESFE::GUS* is detected otherwise maternal is not.

(C) GUS expression in the endosperm after reciprocal cross between *UCL1_-2.7~-2.0k+ESFE::GUS* (*UCL1_PRE+ESFE::GUS*) transgenic plant and Col-0 wild-type plant (1DAP).

Figure 8

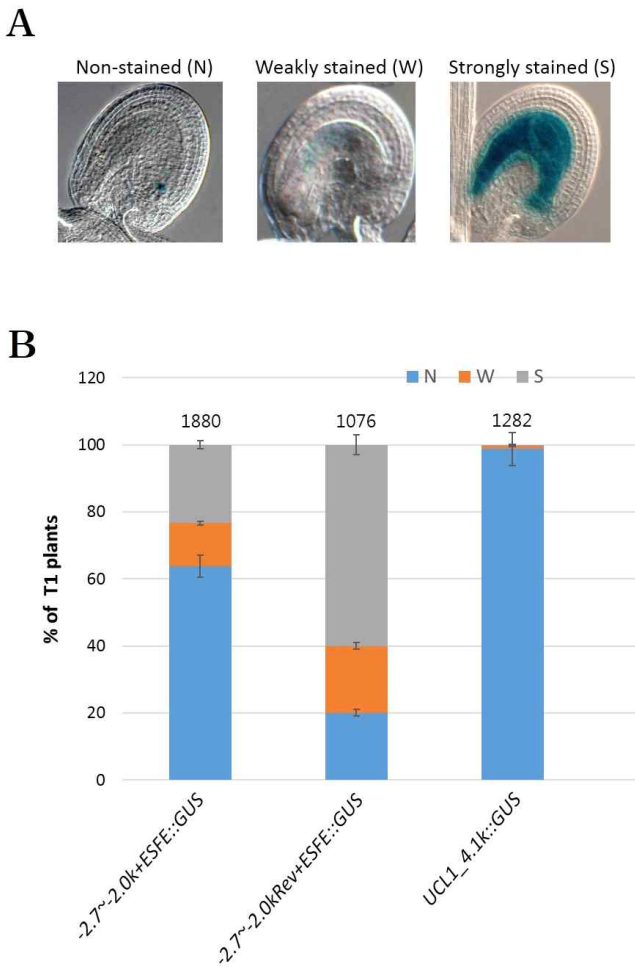

A
Non-stained (N)
Weakly stained (W)
Strongly stained (S)
B
N
W
S
120
100
80
60
40
20
0
% of T1 plants
1880
1076
1282
-2.7~-2.0k+ESFE::GUS
-2.7~-2.0kRev+ESFE::GUS
UCL1_4.1k::GUS

Figure 8. GUS Expression pattern in *UCL1_−2.7~−2.0kbRev+ESFE::GUS* compare to other transgenic plants

(A) Division of GUS expression pattern in the transgenic plants.

(B) Expression pattern of GUS in unfertilized ovules of *UCL1_−2.7~−2.0k+ESFE::GUS*, *UCL1_−2.7~−2.0kbRev+ESFE::GUS* and *UCL1_4.1k::GUS* transgenic plants (2DAE).

4. Additional element near short transposable element (TE) is essential for stable repression of *UCL1* maternal allele

The results of the *UCL1_PRE + ESFE :: GUS* suggested that additional element could be required for stable suppression of the maternal *UCL1* allele. Jeong et al. (2015) reported that repression of the maternal *UCL1* allele is related to CpG DNA methylation. Jeong et al. (2015) also demonstrated that there is a short transposable element (TE) in the −2070 to -1560 bp upstream of the *UCL1* translation start codon, and this region is significantly hypomethylated, suggesting this region may be required for maternal *UCL1* silencing. Based on these facts, I checked H3K27me3 pattern of the *UCL1* promoter using the integrated genome browser (IGB) 8.3.1 program (https://wiki.transvar.org/confluence/display/igbman/Quick+Start).

The result showed that H3K27me3 disappears at the -1814 to -1478 bp upstream of the *UCL1* translation start codon, nearby short TE, while H3K27me3 was found on both sides of this region(Figure 9). This alteration of H3K27me3 could be related to different methylation in this region, designated as differentially methylated region (DMR). To confirm whether this putative DMR is a specific element essential for repression of the maternal *UCL1* allele, I generated *UCL1_PRE+DMR+ESFE::GUS* construct

(Figure 10A). GUS activity of the *UCL1_PRE+DMR+ESFE::GUS* transgenic plant was analyzed in the ovules 2 days after emasculation to see whether the maternal allele of *UCL1* is repressed. As a result, maternal *UCL1* expression was not detected in the ovules; however *UCL1::GUS* was strongly expressed in the self fertilized seeds after fertilization (Figure 10B).

To compare the imprinting pattern of *UCL1_PRE+DMR+ESFE::GUS* with that of *UCL1_PRE+ESFE::GUS*, I checked the GUS activity in the unfertilized ovules of the *UCL1_PRE+DMR+ESFE::GUS* transgenic plants 2 days after emasculation (2 DAE) and analyzed statistically (Figure 11). As a result, 99.57% (total n=1855) of none or weakly stained GUS expression was detected in the *UCL1_PRE+DMR+ESFE::GUS* ovules, whereas 76.59% (total n=1880) of which was detected in the *UCL1_PRE+ESFE::GUS* ovules. Suppression percentage of the maternal *UCL1* allele in the *UCL1_PRE+DMR+ESFE::GUS* transgenic plants was similar with that of UCL1_4.1k::GUS, 99.6% (total n=1282). The results so far suggests that DMR region is essential for the stable repression of the maternal *UCL1* allele.

Figure 9

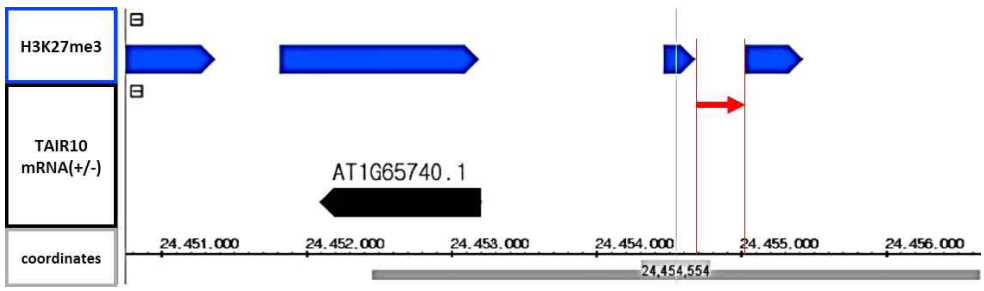


Figure 9. H3K27me3 pattern in *UCL1* promoter region

Red arrow indicates -1814 bp to -1478 bp upstream of *UCL1* translation start codon, that is predicted to be differentially methylated region (DMR).

Figure 10

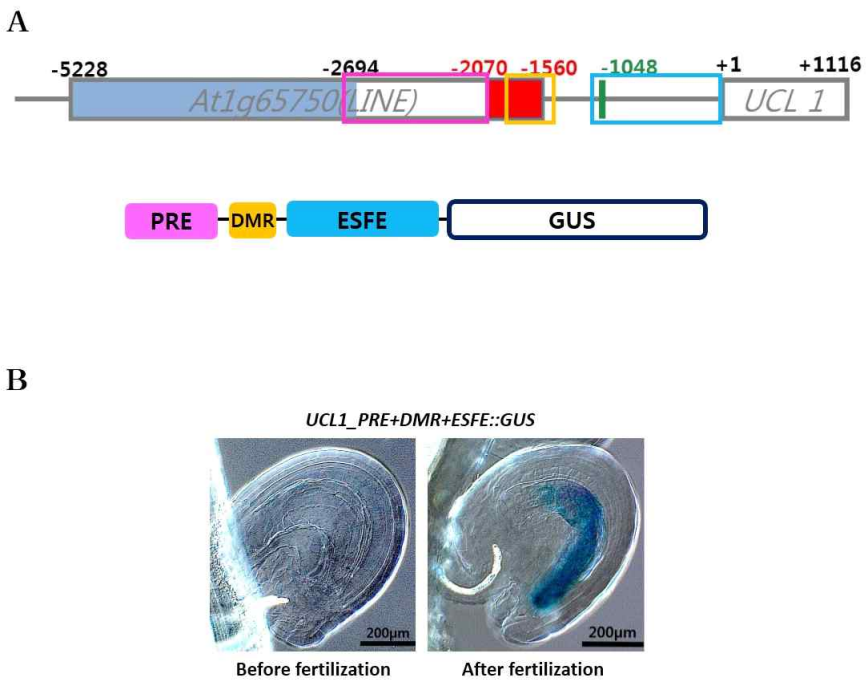


Figure 10. Expression pattern of *UCL1_PRE+DMR+ESFE::GUS*

(A) *UCL1_PRE+DMR+ESFE::GUS* construct containing PRE, DMR and ESFE of *UCL1* fused with GUS. The pink box, yellow box and blue box indicate PRE, DMR and ESFE respectively.

(B) Expression of *UCL1_PRE+DMR+ESFE::GUS* in the ovule 2 DAE and in the endosperm 1 DAP.

Figure 11

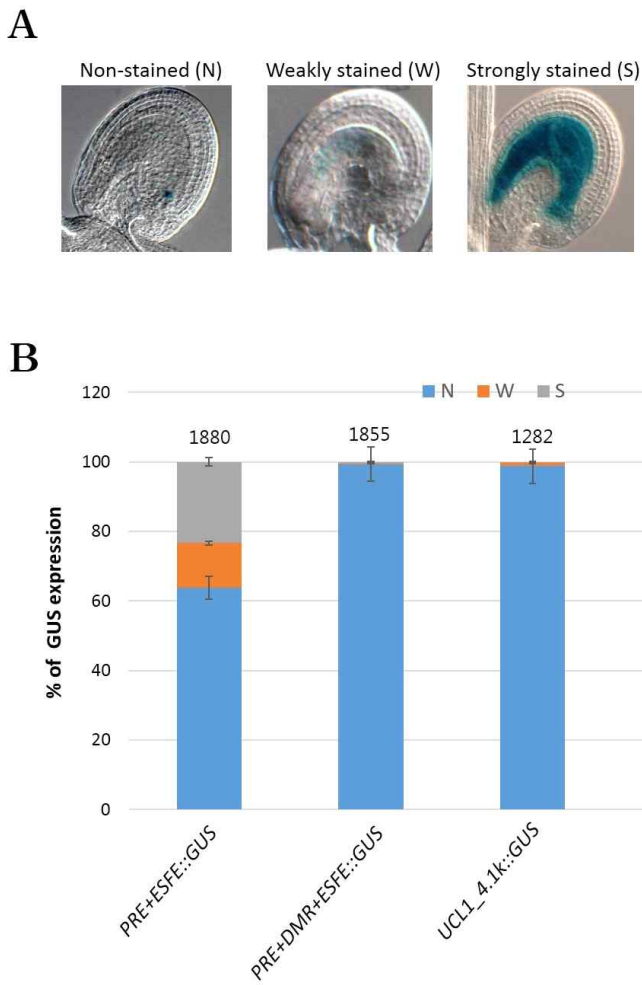

A
Non-stained (N)
Weakly stained (W)
Strongly stained (S)
B
N
W
S
120
100
80
60
40
20
0
% of GUS expression
1880
1855
1282
PRE+ESFE::GUS
PRE+DMR+ESFE::GUS
UCL1_4.1k::GUS

Figure 11. GUS Expression pattern in *UCL1_PRE+DMR+ESFE::GUS* compare to other transgenic plants

(A) Division of GUS expression pattern in the transgenic plants.

(B) Expression pattern of GUS in unfertilized ovules of *UCL1_PRE+ESFE::GUS*, *UCL1_PRE+DMR+ESFE::GUS* and *UCL1_4.1k::GUS* transgenic plants (2DAE).

5. Investigation of the endosperm-specific factor binding element (ESFE) of *UCL1*

In order to investigate whether the specific nucleotide sequence is involved in endosperm-specific expression of *UCL1*, 1 kb of the *UCL1* promoter region, which is presumed to be Endosperm-Specific Factor binding Element (ESFE), was sequentially deleted in 100 bp and fused with GUS gene (Figure 12A).

As a result, cytoplasmic GUS activity was detected in the endosperm of *UCL1_0.8k::GUS* to *UCL1_0.2k::GUS* transgenic plants; however, not in *UCL1_0.1k::GUS* and *UCL1_0.07k::GUS* (Figure 12B). Based on the results thus far, it is expected that there will be a region that regulates endosperm-specific expression of *UCL1*, between -271 and -171 bp of the *UCL1* promoter region.

Figure 12

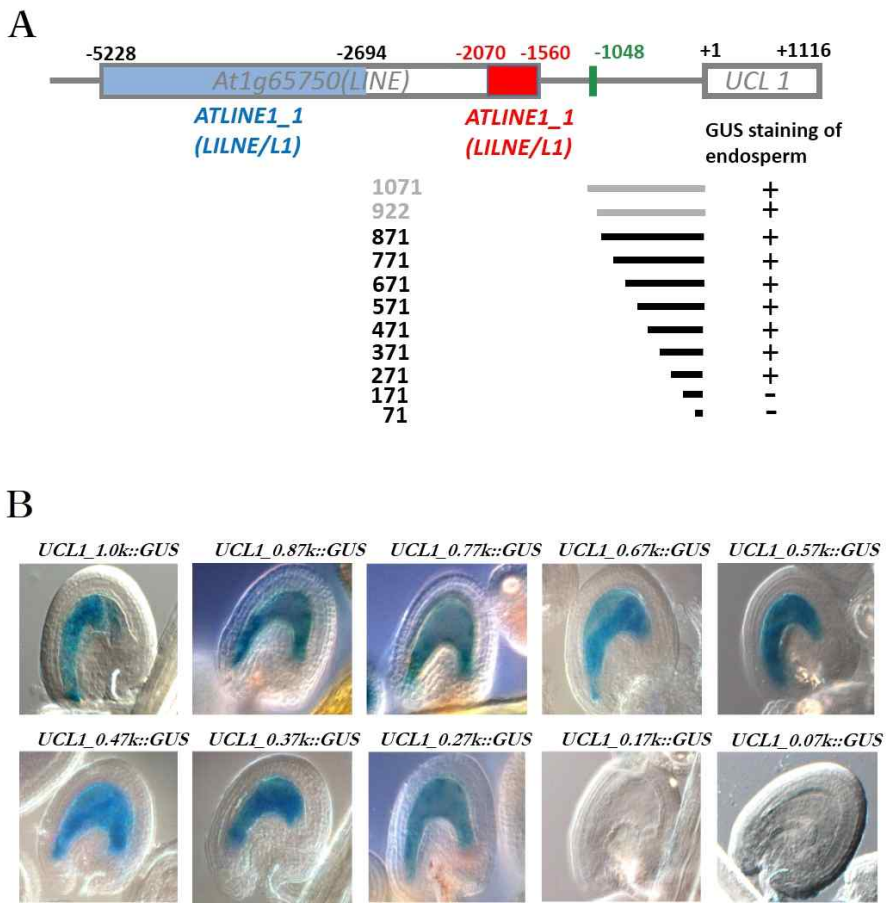


Figure 12. Identification of ESFE

(A) Constructs of 100bp-deleted ESFE.

(B) Analysis of GUS activity in the developing endosperm of transgenic plants. The result indicate ESFE is located between -271 and -171 bp upstream of *UCL1* translation start codon.

6. Analysis of the *UCL1* gene expression in the endosperm development-defective mutants

In Arabidopsis, transcription factors are likely to be involved in recognition of *UCL1* PRE by the FIS complex or endosperm-specific expression. One of factors that are expressed specifically in the central cell or endosperm could be the candidate. *AGL62* is a gene which is expressed specifically in the endosperm after fertilization, and repressed by FIS complex just before cellularization (Kang et al., 2008).

To confirm the possibility, I crossed *UCL1_4.1k::GUS* transgenic plants with *agl62* mutant plants and obtained *UCL1_4.1k::GUS*$^{+/-}$;*agl62*$^{-/+}$ F1 generation plants. As a result, the expression of GUS in the *agl62* mutant was reduced by an average of 34% (total n=1095) compared to wild-type Arabidopsis (Figure 13). This suggests that *AGL62* may directly or indirectly affect the endosperm-specific expression of *UCL1*.

Based on these possibilities, *UCL1_4.1k::GUS* homozygous plants among *UCL1_4.1k::GUS;agl62* F2 plants were selected and the GUS expression pattern of specimens 24~36 hours after fertilization was observed. As a result, most of the samples showed GUS activity while some of the samples did not show GUS activity (Figure 14A). To confirm whether absent GUS expression is related to effect of *AGL62*, I also observed cross

section with confocal laser scanning microscope of the endosperm which did not show GUS activity. Confocal laser microscopy of specimens without GUS expression revealed a phenotype different from that of normal GUS expression (Figure 14B, C). In the seed development stage of normal wild-type Arabidopsis, cellularization occurs after 5 days of fertilization, whereas in the case of *agl62* mutant, early cellularization occurs 3 days after fertilization (Kang et al., 2008). As a result, the specimen of abnormal GUS expression was similar to the "early cellularization" phenotype of the *agl62* mutant, suggesting that the expression of *UCL1* may be regulated by *AGL62*. To confirm these results further, I increased the number of *UCL1_4.1k::GUS*$^{+/+}$;*agl62*$^{-/+}$ F2 plants and *UCL1_4.1k::GUS*$^{+/+}$;*agl62*$^{-/+}$ F3 plants and observed the GUS expression patterns 39 hours after fertilization. As a result, I observed that GUS expression pattern of *UCL1_4.1k::GUS*$^{+/+}$;*agl62*$^{-/+}$ F2 plants and *UCL1_4.1k::GUS*$^{+/+}$;*agl62*$^{-/+}$ F3 plants were almost similar with that of *UCL1_4.1k::GUS* in wild-type plants (Figure 15). Therefore, there is no direct relation between the expression of *UCL1* and the function of *AGL62*. Other endosperm-defective mutants are under investigation, and it is expected that transcription factors that regulate the endosperm-specific expression of genes could be elucidated.

Figure 13

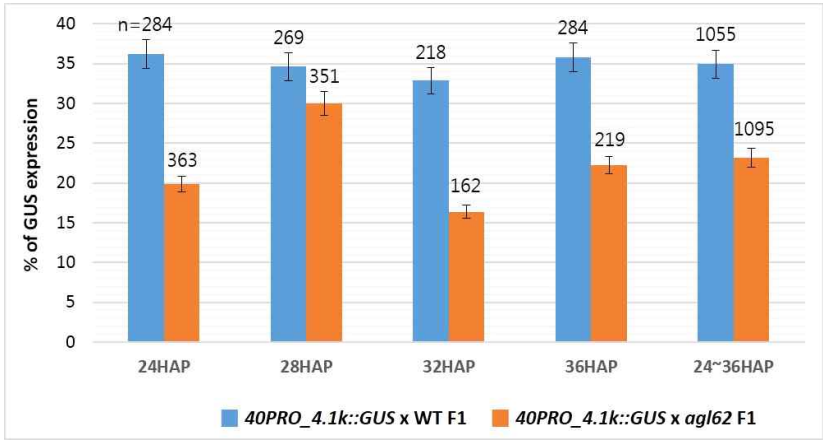


Figure 13. Expression pattern of *UCL1::GUS* in the developing endosperms of *UCL1_4.1k::GUS;agl62* F1 mutants and *UCL1_4.1k::GUS*$^{+/-}$ plants

GUS expression in the *agl62* mutants were decreased by 34% compare to that in the wild-type.

Figure 14

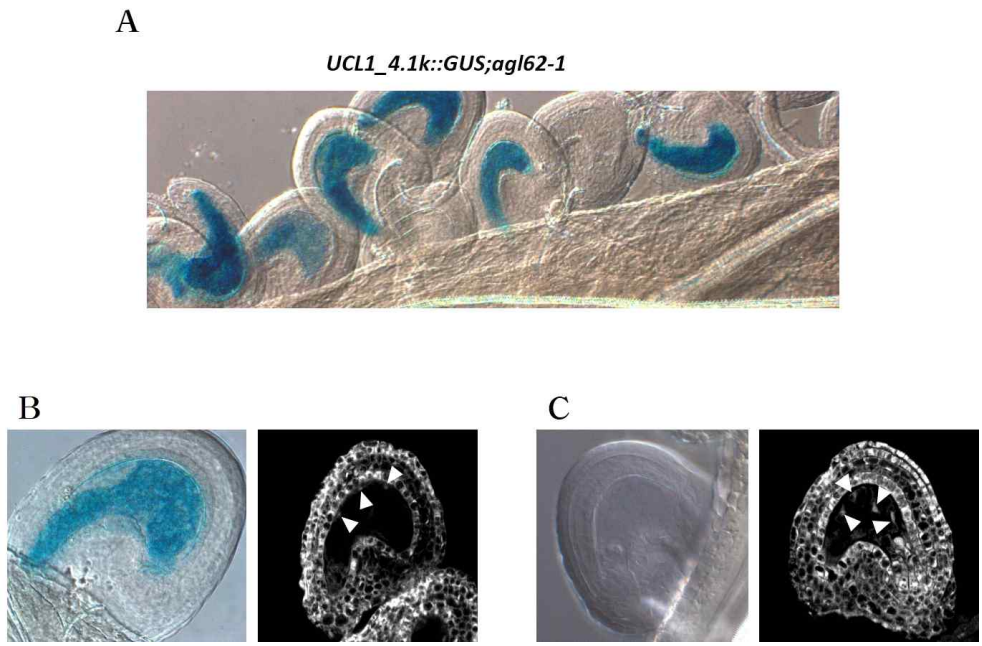


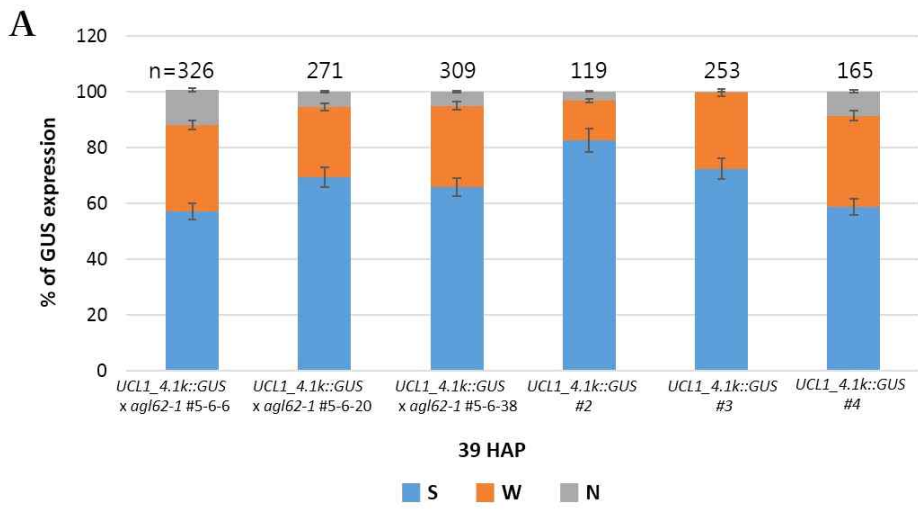
Figure 14. Analysis of *UCL1::GUS* activity in the *UCL1_4.1k::GUS;agl62-1* and phenotype of early cellularization

(A) Analysis of GUS expression in the developing endosperm of *UCL1_4.1k::GUS;agl62-1* mutant.

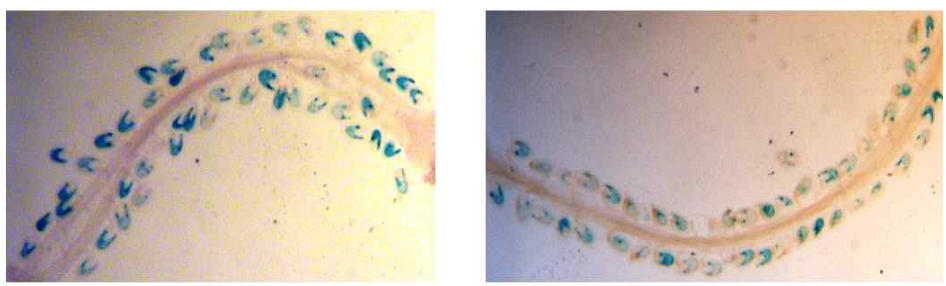
(B) Cross section of the specimen which showed strong GUS expression. Arrowheads indicate the phenotype of wild-type seed (24~36HAP).

(C) Cross section of the specimen which did not show GUS expression. Arrowheads indicate the early cellularization phenotype of *agl62* mutant seed (24~36HAP).

Figure 15



B



UCL1_4.1k::GUS $^{+/+}$, 39HAP

UCL1_4.1k::GUS $^{+/+}$*;agl62-1* $^{+/-}$, 39HAP

Figure 15. Expression pattern of *UCL1::GUS* in *UCL1_4.1k::GUS;agl62* F3 plants, 39HAP

(A) Percentage of GUS expression (39HAP) in *UCL1_4.1k::GUS*$^{+/+}$;*agl62*$^{-/+}$ F3 mutants compare to *UCL1_4.1k::GUS*$^{+/+}$. There was no significant difference in GUS expression. (S: Strongly stained, W: Weakly stained, N: Non-stained)

(B) GUS expression in *UCL1_4.1k::GUS*$^{+/+}$ and *UCL1_4.1k::GUS*$^{+/+}$;*agl62*$^{-/+}$ F3 mutant, 39HAP. Significant difference of GUS activity between them was not observed.

Ⅳ. Discussion

The previous study on the *UCL1* gene suggested that *UCL1* is a paternally expressed imprinted gene which is expressed specifically in the developing endosperm only after fertilization (Jeong et al., 2015). Also, Jeong et al. (2015) identified −2.0 to −2.7 kb region of *UCL1* promoter as ICR/PRE, that involves in recruitment of FIS2−PRC2 complex to repress maternal allele of *UCL1*. Otherwise, 1.0 kb of *UCL1* promoter region showed biallelic expression, specifically in the endosperm which shows repeat sequences in this region is not the imprinting control region (ICR) of *UCL1* (Jeong et al., 2015).

In this study, I analyzed the interaction between polycomb response element (PRE) and endosperm−specific factor binding element (ESFE) on the expression of *UCL1* paternal allele. The −2.7~−2.0 kb upstream region of *UCL1* translation start codon was used as PRE, while the -1.0~0 kb region was used as ESFE. In the analysis of GUS activity in the *UCL1_PRE+ESFE::GUS* transgenic plants, GUS activity was detected in the 24.63% of unfertilized ovules. Therefore, imprinting pattern of the *UCL1_PRE+ESFE::GUS* was unstable as compared to *UCL1_4.1k::GUS* which displayed only 1.2% of ovules showed de−repression. When *UCL1_PRE+ESFE::GUS*

plants were pollinated with Col-0 wild-type pollen, 33.43% (total n=1588) of the 1DAP developing endosperms showed de-repression of maternal *UCL1* allele. When PRE was inserted in the reversed orientation, only 40% (total n=1076) of ovules was repressed, compared to 76.59% (total n=1880) of ovules was repressed in *UCL1_PRE+ESFE::GUS*. This result indicate that the orentation of PRE might be significant for suppression of the maternal *UCL1* allele.

Many imprinted genes are orchestrated by histone and DNA methylation and their control mechanism is various. DNA methylation can regulate expression or repression of the imprinted genes, and TEs have been suggested as differentially methylated region (DMR) that is related to this regulation mechanism (Lippman et al., 2004, Martienssen et al., 2004, Kinoshita et al., 2006).

Jeong et al. (2015) reported that maternal allele of *UCL1* was de-repressed not only in the *mea* and *fie* mutants but also in the *met1* and *dme* mutants, demonstrating that the maternal allele of *UCL1* is suppressed by the FIS complex as well as DNA methylation multiply. Also, the promoter region of the *UCL1* includes a short transposable element (TE), from -2070 to -1560 bp, conserved in Col-0, En-2, Ler, RLD, C24 ecotypes (Jeong et al., 2015). Therefore, it is possible that specific element in or nearby this short TE of the *UCL1* promoter could be involved in *UCL1* imprinting. H3K27me3 pattern in *UCL1*

promoter using IGB program showed that H3K27me3 disappears at the -1814 to -1478 region of the *UCL1* promoter. This region was considerably overlapped with short TE of *UCL1* promoter, -2070 to -1560 bp upstream region. Normally H3K27me3 is related to hypomethylation of the target genes. Based on possibility that the disappearance of H3K27me3 could be related to hypermethylation of this region, I designated this region as differentially methylated region (DMR).

UCL1_PRE+DMR+ESFE::GUS including PRE, putative DMR and ESFE fused with GUS showed stable repression of the maternal *UCL1* allele with 99.57% (total n=1855) ratio in the ovules. This may suggests that putative DMR is essential for the complete repression of the maternal *UCL1* allele cooperating with PRE.

In case of *PHE1* gene belonging to PEGs, hypomethylation of maternal *PHE1* allele in tandom triple repeat of 3' region is required for repression of maternal *PHE1* allele by FIS-PRC2. By contrast, this tandom triple repeat of paternal *PHE1* allele is hypermethylated, so that FIS-PRC2 can not bind to this locus. Also, lack of DNA methylation caused de-repression of the maternal *PHE1* and reduced expression of the paternal *PHE1*, ensuring that different methylation level in the different parental allele is essential for *PHE1* imprinting. However, imprinting mechanism of the *UCL1* seems different from that of *PHE1*. DMR did not affect expression of the paternal *UCL1* allele, but

only affected repression of the maternal *UCL1*. Further sequence analysis of the PRE and DMR is needed to elucidate imprinting mechanism of the *UCL1.*

On the other hand, 100-bp sequential deletion from -2.7 to -2.1 kb upstream of the *UCL1* translation start codon showed that PRE of *UCL1* is located between -2.5 and -2.4 kb of *UCL1* promoter region. Therefore PRE fragments in 200 bp size were fused with *ESFE::GUS* to drive paternal imprinting of the UCL1, however showed biallelic expression. This may suggests that sequences longer than 200 bp may be necessary for the recruitment and maintenance of FIS-PRC2 by PRE, unlike other imprinted genes. Further studies based on 10-bp scanning transversion of this region may trace the critical sequences those involve in recruitment of FIS-PRC2 to repress maternal allele of *UCL1.*

Meanwhile, 100-bp sequential deletion from -971 to -71 bp of the *UCL1* promoter region fused with GUS showed that ESFE of *UCL1* is located between -271 and -171 bp sequences of the *UCL1* promoter region.

To elucidate a transcription factor that binds to ESFE of *UCL1*, inducing endosperm-specific expression, I set a candidate as *AGL62* which expresses specifically in the endosperm and checked the expression of *UCL1* in *agl62* mutants. F1, F2 and F3 generations of *UCL1_4.1k::GUS;agl62* double mutants were obscured statistically. As a result, *UCL1_4.1k::GUS*$^{+/+}$*;agl62*$^{-/+}$

F3 mutants showed no significant difference with *UCL1_4.1k::GUS*$^{+/+}$ transgenic plants, that suggests *AGL62* is not realted to endosperm-specific expression of the *UCL1* gene.

One possibility is that another transcription factor that is specifically expressed in the endosperm could be a candidate. Based on this possibility, I also conducted investigation on *AGL61*, *AGL80*, *MYB64* and *MYB119* genes, those are specifically expressed in the central cell and developing endosperm. In the *agl61* and *fem111*, loss-of-function mutants of *AGL61* and *AGL80,* and *myb64;myb119* double mutants show arrest on female gametophyte development, that causes seed abortion (Portereiko et al., 2006, Bemer et al., 2008, Steffen at al., 2008, Rabiger et al., 2013). However, when the expression of *UCL1_4.1k::GUS* was analyzed in those mutants background, there was no significant relation between those genes and endosperm-specific expression of *UCL1*. Therefore other genes, those are specifically expressed in the endosperm and related to endosperm development, are required to be investigated.

Another possibility is that there is unknown factor that represses paternal *UCL1* allele in the pollen grain that is absent in the endosperm (Jeong et al., 2015). In this case, specific factor that is expressed only in the mature pollen grain may involve in endosperm-specific expression of paternal *UCL1* allele. Further studies on these two points of view are needed.

V. References

Bartolomei, M. S. (2009). Genomic imprinting: employing and avoiding epigenetic processes. *Genes Dev* **23:** 2124-2133

Baulcombe, D. C., & Dean, C. (2014). Epigenetic regulation in plant responses to the environment. *Cold Spring Harb Perspect Biol* **6:** a019471

Bemer, M., Wolters-Arts, M., Grossniklaus, U., & Angenent, G. C. (2008). The MADS domain protein DIANA acts together with AGAMOUS-LIKE80 to specify the central cell in Arabidopsis ovules. *Plant Cell* **20:** 2088-2101

Bemer, M., & Grossniklaus, U. (2012). Dynamic regulation of Polycomb group activity during plant development. *Curr Opin Plant Biol* **15:** 523-529

Berger, F. (2003). Endosperm: the crossroad of seed development. *Curr Opin Plant Biol* **6:** 42-50

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., ... & Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* **298:** 1039-1043

Chanvivattana, Y., Bishopp, A., Schubert, D., Stock, C., Moon,

Y. H., Sung, Z. R., & Goodrich, J. (2004). Interaction of Polycomb-group proteins controlling flowering in Arabidopsis. *Development* 131: 5263-5276

Chaudhury, A. M., Ming, L., Miller, C., Craig, S., Dennis, E. S., & Peacock, W. J. (1997). Fertilization-independent seed development in Arabidopsis thaliana. *Proc Natl Acad Sci USA* 94: 4223-4228

Choi, Y., Gehring, M., Johnson, L., Hannon, M., Harada, J. J., Goldberg, R. B., ... & Fischer, R. L. (2002). DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in Arabidopsis. *Cell* 110: 33-42

Christensen, C. A., King, E. J., Jordan, J. R., & Drews, G. N. (1997). Megagametogenesis in Arabidopsis wild type and the Gf mutant. *Sex Plant Reprod* 10: 49-64

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., & Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell* 111: 185-196

Derkacheva, M., & Hennig, L. (2013). Variations on a theme: Polycomb group proteins in plants. *J Exp Bot* 65: 2769-2784

Feil, R., & Berger, F. (2007). Convergent evolution of genomic imprinting in plants and mammals. *Trends Genet* 23: 192-199

Gehring, M., Huh, J. H., Hsieh, T. F., Penterman, J., Choi, Y.,

Harada, J. J., ... & Fischer, R. L. (2006). DEMETER DNA glycosylase establishes MEDEA polycomb gene self-imprinting by allele-specific demethylation. *Cell* **124:** 495-506

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E. M., & Coupland, G. (1997). A Polycomb-group gene regulates homeotic gene expression in Arabidopsis. *Nature* **386:** 44-51

Grossniklaus, U., Vielle-Calzada, J. P., Hoeppner, M. A., & Gagliano, W. B. (1998). Maternal control of embryogenesis by *MEDEA*, a polycomb group gene in Arabidopsis. *Science* **280:** 446-450

Guyomarc'h, S., Bertrand, C., Delarue, M., & Zhou, D. X. (2005). Regulation of meristem activity by chromatin remodelling. *Trends Plant Sci* **10:** 332-338

Haig, D., & Westoby, M. (1989). Parent-specific gene expression and the triploid endosperm. *Am Nat* **134:** 147-155

He, C., Huang, H., & Xu, L. (2013). Mechanisms guiding Polycomb activities during gene silencing in Arabidopsis thaliana. *Front Plant Sci* **4**

Hennig, L., Bouveret, R., & Gruissem, W. (2005). MSI1-like proteins: an escort service for chromatin assembly and remodeling complexes. *Trends Cell Biol* **15:** 295-302

Hennig, L., & Derkacheva, M. (2009). Diversity of Polycomb

group complexes in plants: same rules, different players? *Trends Genet* **25:** 414-423

Hsieh, T. F., Shin, J., Uzawa, R., Silva, P., Cohen, S., Bauer, M. J., ... & Fischer, R. L. (2011). Regulation of imprinted gene expression in Arabidopsis endosperm. *Proc Natl Acad Sci USA* **108:** 1755-1762

Jeong, C. W., Roh, H., Dang, T. V., Do Choi, Y., Fischer, R. L., Lee, J. S., & Choi, Y. (2011). An E3 ligase complex regulates SET-domain polycomb group protein activity in Arabidopsis thaliana. *Proc Natl Acad Sci USA* **108:** 8036-8041

Jeong, C. W., Park, G. T., Yun, H., Hsieh, T. F., Do Choi, Y., Choi, Y., & Lee, J. S. (2015). Control of paternally expressed imprinted *UPWARD CURLY LEAF1*, a gene encoding an F-box protein that regulates CURLY LEAF polycomb protein, in the Arabidopsis endosperm. *PLoS One* **10:** e0117431

Jiang, D., Wang, Y., Wang, Y., & He, Y. (2008). Repression of FLOWERING LOCUS C and FLOWERING LOCUS T by the Arabidopsis Polycomb repressive complex 2 components. *PLoS One* **3:** e3404

Jullien, P. E., Kinoshita, T., Ohad, N., & Berger, F. (2006). Maintenance of DNA methylation during the Arabidopsis life cycle is essential for parental imprinting. *Plant Cell* **18:** 1360-1372

Jullien, P. E., & Berger, F. (2009). Gamete-specific epigenetic mechanisms shape genomic imprinting. *Curr Opin Plant Biol* **12:** 637-642

Kang, I. H., Steffen, J. G., Portereiko, M. F., Lloyd, A., & Drews, G. N. (2008). The AGL62 MADS domain protein regulates cellularization during endosperm development in Arabidopsis. *Plant Cell* **20:** 635-647

Katz, A., Oliva, M., Mosquna, A., Hakim, O., & Ohad, N. (2004). FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression during sporophyte development. *Plant J* **37:** 707-719

Kiyosue, T., Ohad, N., Yadegari, R., Hannon, M., Dinneny, J., Wells, D., ... & Fischer, R. L. (1999). Control of fertilization-independent endosperm development by the *MEDEA* polycomb gene in Arabidopsis. *Proc Natl Acad Sci USA* **96:** 4186-4191

Kinoshita, T., Yadegari, R., Harada, J. J., Goldberg, R. B., & Fischer, R. L. (1999). Imprinting of the MEDEA polycomb gene in the Arabidopsis endosperm. *Plant Cell* **11:** 1945-1952

Kinoshita, T., Miura, A., Choi, Y., Kinoshita, Y., Cao, X., Jacobsen, S. E., ... & Kakutani, T. (2004). One-way control of FWA imprinting in Arabidopsis endosperm by DNA methylation. *Science* **303:** 521-523

Kradolfer, D., Wolff, P., Jiang, H., Siretskiy, A., & Köhler, C. (2013). An imprinted gene underlies postzygotic reproductive isolation in Arabidopsis thaliana. *Dev Cell* **26**: 525-535

Köhler, C., Hennig, L., Spillane, C., Pien, S., Gruissem, W., & Grossniklaus, U. (2003). The Polycomb-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene PHERES1. *Genes Dev* **17**: 1540-1553

Köhler, C., Page, D. R., Gagliardini, V., & Grossniklaus, U. (2005). The Arabidopsis thaliana MEDEA Polycomb group protein controls expression of PHERES1 by parental imprinting. *Nat Genet* **37**: 28-30

Lippman, Z., Gendrel, A. V., Black, M., Vaughn, M. W., Dedhia, N., McCombie, W. R., ... & Carrington, J. C. (2004). Role of transposable elements in heterochromatin and epigenetic control. *Nature* **430**: 471-476

Makarevich, G., Villar, C. B., Erilova, A., & Köhler, C. (2008). Mechanism of PHERES1 imprinting in Arabidopsis. *J Cell Sci* **121**: 906-912

Martienssen, R., Lippman, Z., May, B., Ronemus, M., & Vaughn, M. (2004). Transposons, tandem repeats, and the silencing of imprinted genes. *Cold Spring Harb Symp Quant Biol* **69**: 371-379

Maruyama, D., Endo, T., & Nishikawa, S. I. (2015). BiP3

supports the early stages of female gametogenesis in the absence of BiP1 and BiP2 in Arabidopsis thaliana. *Plant signal Behav* **10**: e1035853

Morey, L., & Helin, K. (2010). Polycomb group protein-mediated repression of transcription. *Trends Biochem Sci* **35**: 323-332

Mozgova, I., Köhler, C., & Hennig, L. (2015). Keeping the gate closed: functions of the polycomb repressive complex PRC2 in development. *Plant J* **83**: 121-132

Nekrasov, M., Klymenko, T., Fraterman, S., Papp, B., Oktaba, K., Köcher, T., ... & Müller, J. (2007). Pcl-PRC2 is needed to generate high levels of H3-K27 trimethylation at Polycomb target genes. *EMBO J* **26**: 4078-4088

Portereiko, M. F., Lloyd, A., Steffen, J. G., Punwani, J. A., Otsuga, D., & Drews, G. N. (2006). AGL80 is required for central cell and endosperm development in Arabidopsis. *Plant Cell* **18**: 1862-1872

Rabiger, D. S., & Drews, G. N. (2013). MYB64 and MYB119 are required for cellularization and differentiation during female gametogenesis in Arabidopsis thaliana. *PLoS Genet* **9**: e1003783

Raissig, M. T., Bemer, M., Baroux, C., & Grossniklaus, U. (2013). Genomic imprinting in the Arabidopsis embryo is partly

regulated by PRC2. *PLoS Genet* **9:** e1003862

Sims, R. J., Nishioka, K., & Reinberg, D. (2003). Histone lysine methylation: a signature for chromatin function. *Trends Genet* **19:** 629-639

Steffen, J. G., Kang, I. H., Portereiko, M. F., Lloyd, A., & Drews, G. N. (2008). AGL61 interacts with AGL80 and is required for central cell development in Arabidopsis. *Plant Physiol* **148:** 259-268

Villar, C. B. R., Erilova, A., Makarevich, G., Trösch, R., & Köhler, C. (2009). Control of PHERES1 imprinting in Arabidopsis by direct tandem repeats. *Mol Plant* **2:** 654-660

Wolff, P., Jiang, H., Wang, G., Santos-Gonz□llez, J., & Köhler, C. (2015). Paternally expressed imprinted genes establish postzygotic hybridization barriers in Arabidopsis thaliana. *Elife* **4:** e10074

Xiao, J., & Wagner, D. (2015). Polycomb repression in the regulation of growth and development in Arabidopsis. *Curr Opin Plant Biol* **23:** 15-24

Zhang, H., Chaudhury, A., & Wu, X. (2013). Imprinting in plants and its underlying mechanisms. *J Genet Genomics* **40:** 239-247

국문 초록

애기장대에서 *UPWARD CURLY LEAF1* (*UCL1*) 유전자의 각인 조절 메커니즘에 관한 연구

포유동물과 현화식물에서 후생유전학적으로 양친의 기원에 따라 유전자의 발현이 차별적으로 나타나는데, 이를 유전체 각인 현상이라고 일컫는다. 애기장대와 같은 현화식물에서 유전체 각인 현상은 배유에서 주로 기술되었으며, Polycomb Repressive Complex 2 (PRC2)가 히스톤 H3 단백질 라이신 27에서 trimethylation (H3K27me3)을 통해 이러한 현상을 조절한다. 최근 고수율 시퀀싱 분석을 통해 애기장대에서 200 개 이상의 유전자좌가 각인되어있다는 사실이 밝혀졌지만, 각인 메커니즘이 자세히 규명된 각인 유전자는 소수에 제한되어 있다. 선행 연구에서는, E3 ligase를 암호화함으로써 CURLY LEAF (CLF) 폴리콤 단백질을 분해시키는 역할을 수행하는 유전자인 *UPWARD CURLY LEAF1* (*UCL1*)가 부계 발현 각인 유전자라는 사실이 보고되었다. 수정 이후에는 부계로부터 기원된 *UCL1*이 배유 특이적으로 발현되는 반

면 배에서는 발현되지 않으며, FERTILIZATION INDEPENDENT SEED2-PRC2 (FIS2-PRC2)가 수정 전 중심세포와 수정 후 배유에서 모계 *UCL1* 대립유전자를 침묵화한다.

본 연구에서는 *UCL1::GUS* 의 발현 양상을 통해 *UCL1*의 polycomb response element (PRE)가 *UCL1* 번역 개시 코돈 상류 -2.5 kb에서 -2.4 kb 사이에 존재한다는 사실을 확인하였다. 보다 정확한 *UCL1*의 PRE 염기서열을 규명하기 위하여, 본 연구자는 -2.5 kb에서 -2.4 kb 사이에 10 bp-scanning transversion mutagenesis를 포함하는 *UCL1_2.7k::GUS* 구조체를 제조하였다. 이러한 10개 구조체를 Col-0 야생형 애기장대에 도입하여 GUS 발현을 확인할 예정에 있다. 또한 PRE는 *UCL1*의 배유 특이적 발현을 보이는 프로모터 부위 (endosperm-specific factor binding element; ESFE)인 1.0 kb와 상호작용함에 따라 *UCL1::GUS* 유전자의 부계 각인 현상과 배유 특이적인 발현을 정상적으로 유도하였으나, *UCL1_PRE+ESFE::GUS*의 유전체 각인 현상은 비교적 불안정한 결과를 보였다. *UCL1*의 모계 유전자 발현 억제에 어떠한 추가적 요소가 필요한 지 규명하기 위하여, differentially methylated region (DMR)로 추정되는 *UCL1* 상류 -1814 bp에서 -1478 bp 부위를 포함한 새로운 구조체를 제조하였다.

UCL1_PRE+DMR+ESFE::GUS 는 완전한 모계 *UCL1*의 발현 억제를 보였으며, 이러한 결과는 DMR이 *UCL1* 유전자의 부계 각인 현상에 중요한 역할을 수행한다는 것을 의미한다. 다른 한편으로 ESFE를 순차적으로 절단하여 제조한 *UCL1::GUS* 유전자의 발현 양상을 통해 *UCL1*의 ESFE가 -271 bp 와 -171 bp 사이에 존재함을 확인하였다. 특정한 전사인자가 이 ESFE 염기서열에 결합함으로써 *UCL1*의 배유 특이적 발

현을 유도할 것이라 예상하는 바이다.

주요어 : *UPWARD CURLY LEAF1* (*UCL1*), 유전체 각인, 부계 발현 각인 유전자, FIS-PRC2, 배유, 애기장대

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