A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Biochemical characterization of rice histone methyltransferase OsiEZ1

BY

YEJIN KWON

FEBRUARY, 2016

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
COLLEGE OF AGRICULTURAL AND LIFE SCIENCES
THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

ABSTRACT

Flowering time is an important agronomic trait for grain production in crop. In Arabidopsis, *FLOWERING LOCUS C* (*FLC*) is known as a key controller of flowering time and its expression is epigenetically regulated by chromatin modification through a histone methyltransferase Enhancer of Zeste [E(z)] which is a core component of polycomb repressive complex 2 (PRC2). Long intronic non-coding RNA known as a COLD ASSISTED INTRONIC NONCODING RNA (*COLDAIR*) is also required for epigenetic repression of *FLC* expression through its interaction with PRC2. However, the epigenetic regulation mechanism of flowering time is not characterized well in rice. The functional loss of histone methyltransferase OsiEZ1 results in late flowering under short day (SD). Therefore, we investigated the biochemical characteristics of OsiEZ1. Result showed that OsiEZ1 directly interacted a intronic LncRNA expressed in the first intron of *OsMADS56*, a *FLC* homolog gene, named *OsCOLDAIR*. In addition, we found direct interaction between OsiEZ1 and rice E3 SUMO ligase OsSIZ1. These data indicate that OsiEZ1-mediated methylation of *OsMADS56* via *OsCOLDAIR* and that of other target genes can be controlled by sumoylation through E3 SUMO ligase activity of OsiEZ1.

Keywords : Histone methyltransferase, Enhancer of Zeste [E(z)], OsiEZ1, Long non-coding RNA, E3 SUMO ligase, OsSIZ1.

Student number : 2014-20024

CONTENTS

1. LIST OF FIGURES

2. INTRODUCTION

In these days, many studies have identified a close relation of epigenetic regulation to flowering in plants. For example, during and after the process of vernalization in Arabidopsis, polycomb repressive complex 2 (PRC2)-mediated tri-methylation of histone H3 lysine 27 (H3K27me3) represses the expression of a potent floral repressor, Flowering Locus C (FLC), to promote flowering (Sung and Amasino, 2004; De Lucia et al., 2008; Heo et al., 2009).

In rice, SDG724 mediates H3K36me2/3 deposition at *OsMADS50* and *RFT1* stimulating flowering and identifying a different function between paralogs RFT1 and Hd3a under LD or SD conditions (Sun et al., 2012). Also, SDG725 mediates H3K36me2/3 deposition at Ehd2, Ehd3, OsMADS50, Hd3a, and RFT1, stimulating flowering under LD or SD conditions (Sui et al., 2013).

The PRC2 complex was first established in Drosophila, which has four core components: ENHANCER OF ZESTE [E(z)], SUPPRESSOR OF ZESTE 12 [Su(z)12], EXTRA SEX COMBS (ESC) and P55 (Schuettengruber and Cavalli, 2009). The E(z) protein has a function as the H3K27 methyltransferase (Czermin et al., 2002; Cao et al., 2002).

In Arabidopsis, there are three homologs of E(z) genes [CURLY LEAF (CLF), SWINGER (SWN), and MEDEA (MEA)] and Su(z)12 genes [FERTILIZATION INDEPENDENT SEED 2 (FIS2), VERNALIZATION 2 (VRN2) and EMBRYONIC FLOWER 2 (EMF2)],

and one ESC gene [FERTILIZATION INDEPENDENT ENDOSPERM (FIE)] (Luo et al., 1999; Ohad et al., 1999; Gendall et al., 2001; Yoshida et al., 2001; Hennig et al., 2003; Hsieh et al., 2003).

These genes form three PRC2-like complexes. The FIS complex containing MEA/SWN, FIS2, FIE, and MSI1 (Multicopy Suppressor of IRA1) functions during gametogenesis and seed development. The EMF complex and the VRN complex are involved in control of flowering. The EMF complex is composed of CLF/SWN, EMF2, FIE, and MSI1 and suppresses the early flowering. The VRN complex plays critical roles in the vernalization pathway by retaining the high level of H3K27me3 on the FLC locus after vernalization (Hennig and Derkacheva, 2009; Heo et al., 2009). Also it is associated with VERNALIZATION INSENSITIVE 3 (VIN3, a PHD-domain containing protein) and VIN3-like proteins to form PHD-VRN PRC2 complexes (Wood et al., 2006; De Lucia et al., 2008). The VIN3 protein enhances H3K27me3 to the target loci for stable silencing.

In rice, there are two homolog genes of E(z) (OsiEZ1 and OsCLF), Su(Z)12 (OsEMF2a and OsEMF2b) and ESC (OsFIE1 and OsFIE2) respectively (Luo et al., 2009). While morphological changes are not observed in *osclf* and *osfie1* mutant plants, earlier flowering at LD and abnormal flower organs are observed in *osfie2* and *osemf2b* mutant plants (Luo et al., 2009). Recent results have shown that rice VIN3-like proteins OsVIL2, OsVIL3, or RICE LEAF INCLINATION 2 (LC2) promote flowering in rice through the photoperiod pathway (Wang et al., 2013; Yang et al., 2013). These results suggest that

PRC2 and PRC2-associated genes are involved in photoperiod regulation of flowering in rice. However, how PRC2-mediated gene repression is involved in accurate photoperiod control of rice flowering is not clear.

Several long non-coding RNAs (lncRNAs) have been shown to target repressive histone-modifying activities and epigenetically silence transcription through a molecular interaction with specific chromatin domains (Ponting et al., 2009, Lee et al., 2009). Notably, PRC2-mediated silencing includes the interaction with such non-coding RNAs (Rinn et al., 2007, Tsai et al., 2010). A group of related antisense ncRNAs (termed COOLAIR) from FLC have been proposed to be involved in vernalization-mediated FLC repression (Swiezewski et al., 2009). And a long intronic noncoding RNA [termed COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR)] is required for the vernalization-mediated epigenetic repression of FLC (Heo et al., 2009). But, it is not identified that non-coding RNA interacts with PRC2 in rice.

Despite some genes are shared between Arabidopsis and rice flowering regulatory pathways, there are considerable differences between the regulation of flowering of both species such as absence of the vernalization pathway in rice (Shrestha et al., 2014). In this work, we have characterized that the rice E(z) genes, OsiEZ1 (or SDG718, Os03g19480, here after referred to as OsiEZ1), and displayed function in photoperiod regulation of flowering in rice. OsiEZ1 is induced in SD and represses OsLF, a repressor of Hd1

(Liu et at., 2014, Zhao et al., 2011), leading to a higher expression of Hd1 (that activates Hd3a in SD) and thus promotes early flowering. The data suggested that the two E(z) genes are involved respectively, in LD and SD signals to differently control key flowering genes expression and contribute to the accurate photoperiod control of flowering time in rice.

The activities of DNA and histone methyltransferases in mammals are regulated by post-translational modifications such as sumoylation. however, it is not clear how the activities of these enzymes are regulated at the post-translational level in plants (kim et al., 2015). In plants, SUMO and AtSIZ1-mediated sumoylation regulates response to nutrient deficiency, hormones and environmental stress, and they also control vegetative growth and development(Park et al., 2012).

To date, numerous SUMO conjugates have been identified in plants (Conti et al., 2014, Son et al., 2014, Kim et al., 2015). Transgenic plants expressing OsSIZ1 under the control of the CaMV35S promoter in a SIZ1-knockout Arabidopsis line (atsiz1-2) complemented functions of SIZ1. And sumoylation by the OsSIZ1 as E3 SUMO ligase plays an important role in regulating growth and development in rice (HC Park et al.. 2012).

3. MATERIALS AND METHODS

Construction of recombinant plasmids

To produce GST-OsiEZ1, the cDNA encoding full-length OsiEZ1 was amplified by PCR and inserted into the pDEST15 vector. For the maltose-binding protein (MBP)–OsSIZ1 fusion, a cDNA encoding full-length OsSIZ1 was amplified by PCR and inserted into the pMALc2x vector (New England Biolabs). All constructs were transformed into strain R2 cells. The transformed cells were treated with IPTG (isopropyl-β-d-thiogalactoside) to induce fusion protein expression. All the constructs were verified by automatic DNA sequencing to ensure that no mutations were introduced.

Plant material and growth conditions

Oriza sativa var. japonica cultivar Dongjin was used for characterization in this study. T-DNA insertion line of OsiEZ1 (4A-01953) and OsSIZ1 (3A-02154) was obtained from the Postech rice mutant database (http://www.postech.ac.kr/life/pfg/risd). The genomic DNA sequence was acquired from the Rice Annotation Project Database (RAP-DB;http://rapdb.dna.affrc.go.jp; Tanaka, Antonio

et al., 2008) and the TIGR Rice Genome Annotation Project (http://rice.plantbiology.msu.edu; Ouyang, Zhu et al., 2007). Insertion was confirmed by PCR using the specific forward primers, reverse primer and a T-DNA left border primer. The rice plants were grown either in a paddy field in summer in Suwon or in controlled growth chambers for 6 week-old under continuous SDs (10h light at 30℃/14h dark at 25℃) of 8 week-old under continuous LDs (14h light at 30℃ /10h dark at 25℃).

Purification of recombinant proteins

All of the recombinant proteins were expressed in strain BL21 and were purified in accordance with the manufacturer's instructions. Briefly, for MBP-OsSIZ1, GST-OsiEZ1 purification, bacteria were lysed in 50mM NaH_2PO_4 (pH8.0), 300mM NaCl, 1% TritonX-100, 1mM imidazole, 5mM dithiothretiol (DTT), 2mM phenyl methyl sulphonyl fluoride(PMSF), and a proteinase inhibitor cocktail(Roche), and purified on maltose and glutathione resins (Qiagen). For GST, GST–OsiEZ1 purification, bacteria were lysed in PBS buffer (pH 7.5) containing 1% Triton X-100, 2mM PMSF, and a proteinase inhibitor cocktail (Roche), and purified on glutathione resins (Pharmacia). For MBP, MBP-SIZ1-HA purification, bacteria were lysed in bacteria were lysed in 20mM TRIS-HCl(pH 7.4), 200mM NaCl, 1mM EDTA, 1% Triton X-100, and 2mM PMSF containing a proteinase inhibitor cocktail

(Roche), and purified on amylose resins (New England Biolabs).

5'RACE PCR to identify long non-coding RNA around the *OsMADS56*

RT-PCR analysis was performed with or without 5'RACE reaction. 5'RACE PCR reactions were performed using random primer and subsequently amplified using *OsMADS56* region specific primer sets in a combination with Abridged Anchor Primer (AAP).

In vitro RNA pull down assay

Biotin-labeled RNAs were *in vitro* transcribed using the Biotin RNA Labeling Mix (Roche, Indianapolis, IN, USA) and T7 RNA polymerase (Roche, Indianapolis, IN, USA), treated with RNase inhibitor (Intron, Korea) and precipitated at -20°C.

In addition, recombinant OsiEZ1 proteins were purified using *E. coli* (BL21) expression system. 1μg biotin-labeled RNAs and recombinant OsiEZ1 protein were mixed in pull-down buffer (50mM Tris pH 7.5, 150mM NaCl, 2mM DTT, 0.05% NP-40 and protease inhibitor tablet (Roche, Indianapolis, IN, USA)) and incubated for 2 hour at 4℃ and then 30μL washed Streptavidin agarose beads (Promega, Medison, WI, USA) were added to each binding reaction and further incubated for 1

hours at 4℃. Beads were washed briefly 12 times using binding buffer and boiled in SDS buffer, and then Western blots against anti-GST antibody (young in frontier, Seoul, Korea).

In vitro protein pull down assay

To carried the *in vitro* binding of MBP–OsSIZ1 to GST-OsiEZ1, 5μg of full-length MBP–OsSIZ1 and 5 μg of full-length GST-OsiEZ1 were added to 1ml of binding buffer[50mM Tris-HClpH7.5), 100mM NaCl, 10mM $MgCl_2$, 1% TritonX-100, 0.5mM β-mercaptoethanol]. Pre-incubation at 4 °C for 1h and the reaction mixtures were incubated with a glutathione resin for 1h. before washing six times with buffer 50mM Tris-HCl (pH 7.5). Absorbed proteins were analysed by 8% SDS–PAGE and detected by western blotting using an anti-GST antibody (Santa Cruz Biotechnology).

RT-PCR

For real-time PCR, RT-PCR were carried out the after cDNA synthesis, primer designed using SYBR Green I. In brief, cDNA was synthesized using random hexamers using 1μg of each total RNA sample. RT-PCR amplification mixtures (20μl) contained 50ng template cDNA, 2x SYBR Green I Master Mix buffer (10μl) (Enzynomics) and

10pcM forward and reverse primer. The cycling conditions comprised 10 min polymerase activation at 95°C and 40 cycles at 95°C for 15 sec and 60°C for 60 sec. and RT-PCR was carried out using cDNA. mRNA was isolated from rosette leaves of 4-weeks -old. and cDNA synthesize using cDNA synthesis premix(Enzynomics) and carried out using target primer for amplified PCR products.

4. RESULTS

OsiEZ1 physically interacts with OsCOLDAIR

There are some evidences that PRC2-mediated silencing includes the interaction with non-coding RNA (Rinn et al., 2007, Tsai et al., 2010). In Arabidopsis, a candidate non-coding RNA from *FLC* that is distinct from COOLAIR is identified and it is designated COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR) (Heo et al., 2009). To predict the intronic long non-coding RNA from *FLC* homolog genes in rice, we first executed blast search in the database. Numerous *FLC* homolog genes were sorted from rice genome data. We selected four genes that have long first intron like a *FLC*.

To verify expression of RNA in intron, we investigated the total mRNA in four genes used primer set (Fig. 1A). RT-PCR analysis showed that mRNA level at 10P2 on Os10g39130 was highest (Fig. 1B). We identified expression region of intronic long non-coding RNA and designated the RNA *OsCOLDAIR*.

OsCOLDAIR was transcribed in the 5'→3' direction (Fig. 2A). Also, three transcript start points and a transcript termination point were identified (Fig. 2B). And RNA transcript length was 1131bp, 977bp, and 962bp, respectively.

To determine whether OsiEZ1 can interact with *OsCOLDAIR*, a recombinant plasmid expressing GST and GST-tagged OsiEZ1 were generated. The over-expression of these protein in E. coli was induced by IPTG treatment and the protein was purified with glutathione (fig. 3A). After purification, in vitro RNA pull-down assay was performed using GST or GST-OsiEZ1 and biotinylated *OsCOLDAIR*, and *OsCOLDAIR* was detected using an anti-GST antibody. As expected, this experiment showed a physical interaction between OsiEZ1 and *OsCOLDAIR* (fig. 3B).

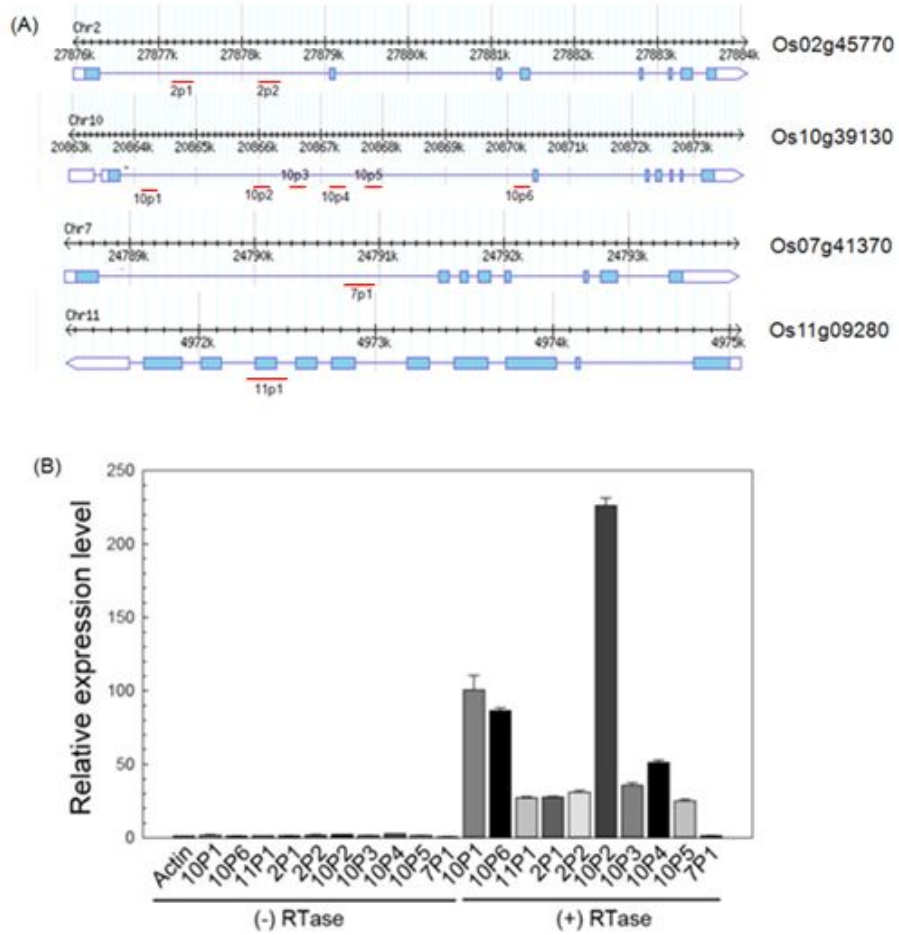

Figure 1. Prediction of long non-coding RNA expression region. (A) *FLC* homolog gene was sorted from rice genome data and four *FLC* homolog genes Os02g45770 Os10g39130, Os07g41370, Os11g09280 that have the long first intron was selected. (B) Transcript expression pattern of total RNA in rice leaf using primer set designed at the first intron.

Figure 2. Sequence of rice long non-coding RNA *OsCOLDAIR*. (A) Schematic representation of structure and location of *OsCOLDAIR* at the first intron of *OsMADS56*. Arrow represent location and direction of *OsCODLAIR*. (B) Genomic DNA sequence of *OsCOLDAIR* and transcript start points (G2059, T2213, A2228) and termination point (A3190). Transcript start points were indicated with red arrow and zoomed amino acid sequence. Transcript termination point was indicated with zoomed amino acid sequence. TATA box was indicated with yellow highlight.

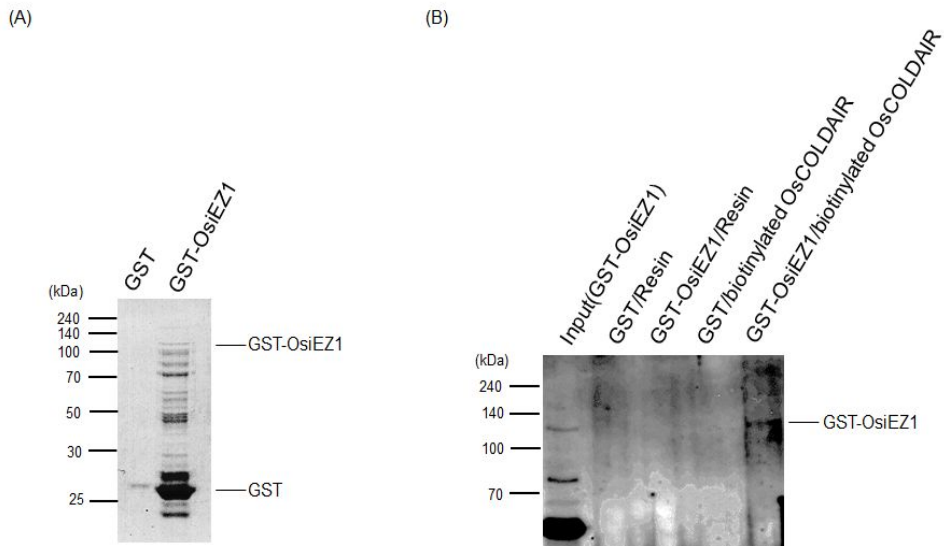

Figure 3. Rice histone methyltransferase OsiEZ1 physically interacts with *OsCOLDAIR*. (A) The purified GST, GST-OsiEZ1 were separated by 12% SDS-PAGE. (B) GST-OsiEZ1 was pulled down with biontinylated *OsCOLDAIR*. GST-OsiEZ1 absorbed with biontinylated *OsCODLAIR* was detected using western blotting with anti-GST antibody.

Expression analyses of *OsiEZ1*, *OsMADS56* and *OsCOLDAIR* under LD and SD

OsiEZ1 is induced by SD and transcript level of *OsMADS56* peaked at 4 weeks under LD (Liu et al., 2014, Ryu et al., 2009). But there is no evidence when these genes are expressed.

To study whether expression of *OsiEZ1*, *OsMADS56* and *OsCOLDAIR* were photoperiod-dependent, we analyzed transcript levels of these genes under LD and SD.

The analysis revealed that *OsiEZ1* mRNA levels were higher under SD than under LD, whereas those of *OsMADS56* were higher under LD than under SD. These mean that expression of *OsiEZ1* is increased under SD and expression of *OsMADS56* is decreased under SD. Interestingly, *OsCOLDAIR* were higher under SD than under LD. This result suggests that expression of *OsCOLDAIR* is short day-dependent.

Polycomb group (PcG) proteins are involved in stable repression of homeotic genes (Van et al., 1998). In Arabidopsis, FLOWERING LOCUS C (*FLC*) is repressed by histone methylation through histone methyltranferae *CLF* (Bastow et al., 2004). Therefore, we suggest that *OsMADS56* transcript levels are decreased under SD because *OsiEZ1* is induced under SD and *OsiEZ1* is critical for expression of *OsMADS56*, *OsCOLDAIR*.

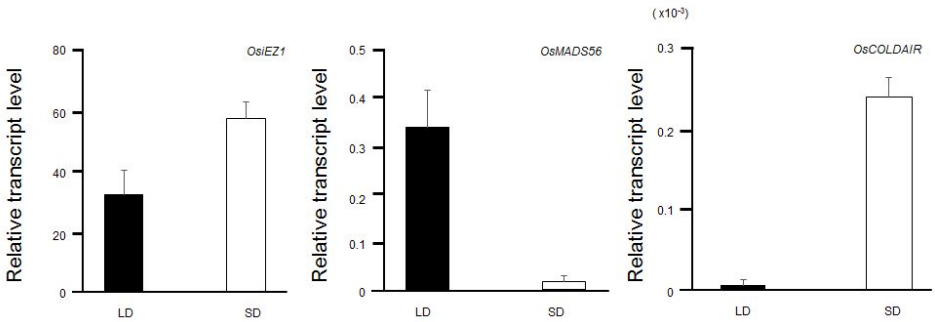

Figure 4. Relative transcript levels of *OsiEZ1*, *OsMADS56*, *OsCOLDAIR* under long days (LD) and short days (SD). There was no conspicuous difference at numerical value. We isolated mRNA from leaves of 6 week-old under SD (10h light/ 14h dark) and 8 week-old under LD (14 light/ 10 dark) grown plant leaves harvested at ZT 2h during a 24h period. Relative transcript levels of *OsiEZ1*, *OsMADS56*, *OsCOLDAIR* were measured by RT-PCR.

Relationship of OsiEZ1, OsMADS56 and OsCOLDAIR

To examine more direct relationship between them, we compared transcript levels of *OsMADS56* and *OsCOLDAIR* in wild-type (WT) and *osiez1* plants. Because *OsMADS56* was increased under LD, mRNA of OsMADS56 was isolated from leaves of 10 day-old grown under LD (14 light/ 10 dark) harvested at ZT 2h during a 24h period. Whereas, because *OsCOLDAIR* was increased under SD, we isolated mRNA from leaves of 10 day-old grown under SD (10h light/ 14h dark) harvested at ZT 2h during a 24h period.

Compared to wild-type, difference of transcript levels in *osiez1* was observed. Transcript levels of *OsMADS56* were increased in *osiez1* plants. In contrast, transcript levels of *OsCOLDAIR* were decreased in *osiez1* plants. It suggested that *OsMADS56* and *OsCOLDAIR* be affected by *OsiEZ1*.

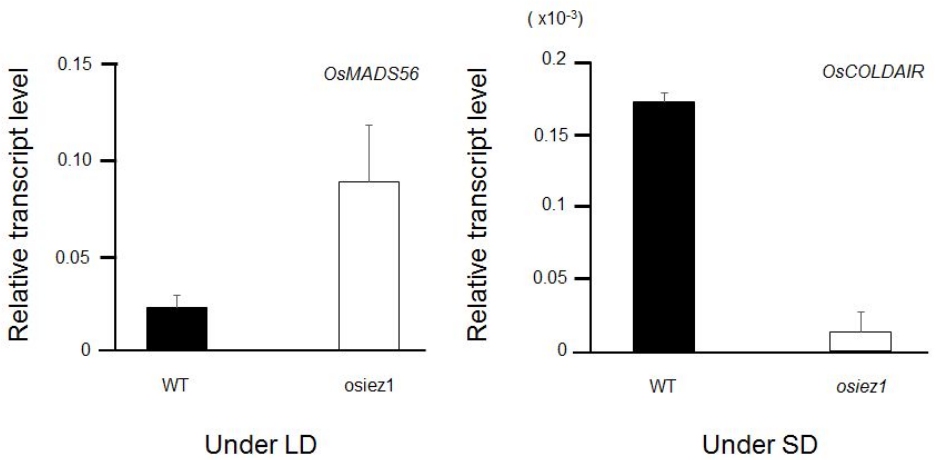

Figure 5. Relative transcript levels of *OsMADS56* and *OsCOLDAIR* in wild-type (WT) and *osiez1* plants. There was no conspicuous difference at numerical value. We isolated mRNA from leaves of 10 day-old under SD (10h light/ 14h dark) and 10 day-old under LD (14 light/ 10 dark) grown plant leaves harvested at ZT 2h during a 24h period. Relative transcript levels of *OsiEZ1*, *OsMADS56*, *OsCOLDAIR* were measured by RT-PCR.

OsiEZ1 interacts with rice E3 SUMO ligase OsSIZ1

In Arabidopsis, AtSIZ1 interacted with CHROMOMETHYLASE 3 (CMT3), which is DNA methyltransferase (Kim et al., 2015). It suggest that AtSIZ1 can interact with other methyltransferase. And *CLF* has several sites that have high sumoylation possibility like a *CMT3* (Fig. 6A). To identify whether AtSIZ1 can interact with CLF ,which is histone methyltransferase in Arabidopsis, in vitro pull-down were performed using MBP or MBP-AtSIZ1. And CLF was detected using an anti-His antibody. As a result, we observed that AtSIZ1 physically interacted with CLF (Fig. 6B).

OsSIZ1 which is SIZ/PIAS-type SUMO E3 ligase was suggested that be able to functionally complement *AtSIZ1* in the SUMO conjugation pathway (HC Park et al., 2010). To determine whether OsSIZ1 interacts with *CLF* homolog gene in rice, we chose to examine *OsiEZ1*. *OsiEZ1* contains four sumoylation sites. Especially there were high possible sumoylation site at amino acid positions K764, K149 (Fig. 7A). So *OsiEZ1* had a possibility that it interacts with OsSIZ1. In vitro pull-down assay showed that OsiEZ1 physically interacts with OsSIZ1 (Fig. 7B).

(A)

```
  1 MASEASPSSS ATRSEPPKDS PAEERGPASK EVSEVIESLK KKLAADRCIS
 51 IKKRIDENKK NLFAITQSFM RSSMERGGSC KDGSDLLVKR QRDSPGMKSG
101 IDESNNNRYV EDGPASSGMV QGSSVPVKIS LRPIKMPDIK RLSPYTTWVF
151 LDRNQRMTED QSVVGRRRIY YDQTGGEALI CSDSEEEAID DEEEKRDFLE
201 PEDYIIRMTL EQLGLSDSVL AELASFLSRS TSEIKARHGV LMKEKEVSES
251 GDNQAESSLL NKDMEGALDS FDNLFCRRCL VFDCRLHGCS QDLIFPAEKP
301 APWCPPVDEN LTCGANCYKT LLKSGRFPGY GTIEGKTGTS SDGAGTKTTP
351 TKFSSKLNGR KPKTFPSESA SSNEKCALET SDSENGLQQD TNSDKVSSSP
401 KVKGSGRRVG RKRNKNRVAE RVPRKTQKRQ KKTEASDSDS IASGSCSPSD
451 AKHKDNEDAT SSSQKHVKSG NSGKSRKNGT PAEVSNNSVK DDVPVCQSNE
501 VASELDAPGS DESLRKEEFM GETVSRGRLA TNKLWRPLEK SLFDKGVEIF
551 GMNSCLIARN LLSGFKSCWE VFQYMTCSEN KASFFGGDGL NPDGSSKFDI
601 NGNMVNNQVR RRSRFLRRRG KVRRLKYTWK SAAYHSIRKR ITEKKDQPCR
651 QFNPCNCKIA CGKECPCLLN GTCCEKYCGC PKSCKNRFRG CHCAKSQCRS
701 RQCPCFAADR ECDPDVCRNC WVIGGDGSLG VPSQRGDNYE CRNMKLLLKQ
751 QQRVLLGISD VSGWGAFLKN SVSKHEYLGE YTGELISHKE ADKRGKIYDR
801 ENCSFLFNLN DQFVLDAYRK GDKLKFANHS PEPNCYAKVI MVAGDHRVGI
851 FAKERILAGE ELFYDYRYEP DRAPAWAKKP EAPGSKKDEN VTPSVGRPKK
901 LA
```

(B)

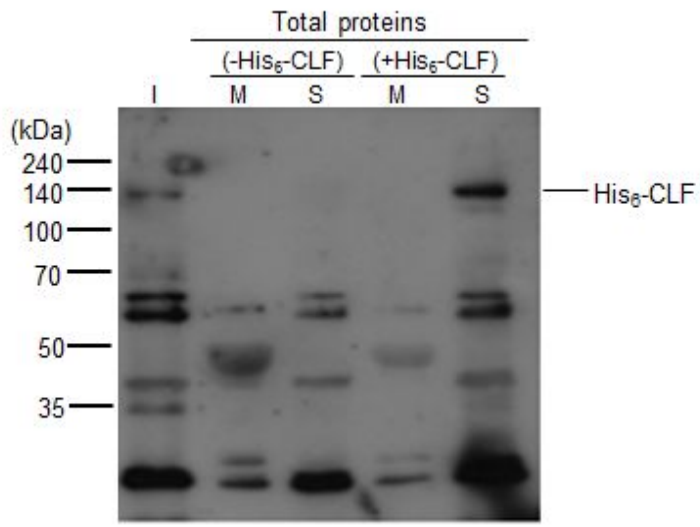

Figure 6. Arabidopsis histone methyltransferase AtCLF physically interact with E3 SUMO ligase AtSIZ1. (A) The deduced amino acid sequence of the AtCLF protein. Putative sumoylation sites identified using the SUMO-plot Analysis Program are shown in bold and underlined type. (B) AtCLF was pulled-down with full-length AtSIZ1. MBP, MBP-AtSIZ1 and His_6-CLF were over-expressed in E. coli and purified with amylose and nickel resins, respectively. His_6-CLF absorbed with MBP or MBP-AtSIZ1 was detected using western blotting with anti-His antibody. M: MBP, S: MBP-AtSIZ1, I: Input.

(A)

```
  1 MASSSSKASD SSSQRPKRPD QGPSGKDAAG LVALHGKLAQ LKRQVQSTRL
 51 AAIKERVEAN RKALQVHICA LFDVAAAAEV ASRGAEGGNA LSRGAAEGHR
101 RFVGWDSASG PGERELVHVQ EENLVAGTLV LSSSGGSGAS HRTVVQLVKL
151 PVVDKIPPYT TWIFLDKNQR MADDQSVGRR RIYYDPIVNE ALICSESDDD
201 VPEPEEEKHV FTEGEDQLIW KATQDHGLSR EVLNVLCQFV DATPSEIEER
251 SEVLFEKYEK QSQSSYKTDL QLFLDKTMDV ALDSFDNLFC RRCLVFDCRL
301 HGCSQNLVFP SEKQPYGHEL DENKRPCGDQ CYLRRREVYQ DTCNDDRNAC
351 TTYNMDSRSS SLKVSATILS ESEDSNRDED NIKSTSIVET SRSKITNSEY
401 ADKSVTPPPG DASETENVSP DMPLRTLGRR KISKHASKSN DHSPDKRQKI
451 YSSPFPFAMS VLNKQSVPEI GETCPDSIES AVDQLPSLDD PNKKISTKDM
501 CAGSTTNTTE NTLRDNNNNL FISNKEHSIS HWSALERDLY LKGIEIFGKN
551 SCLIARNLLS GLKTCMEVAS YMYNNGAAMA KRPLSGKSIL GDFAEAEQGY
601 MEQDLVARTR ICRRKGRARK LKYTWKSAGH PTVRKRIGDG KQWYTQYNPC
651 GCQQMCGKDC ACVENGTCCE KYCGCSKSCK NRFRGCHCAK SQCRSRQCPC
701 FAASRECDPD VCRNCWVSCG DGSLGEPLAR GDGYQCGNMK LLLKQQQRIL
751 LGKSDVAGWG AFIKNPVNRN DYLGEYTGEL ISHREADKRG KIYDRANSSF
801 LFDLNEQYVL DAYRKGDKLK FANHSSNPNC YAKVMLVAGD HRVGIYAKDR
851 IEASEELFYD YRYGPDQAPA WARRPEGSKK DEASVSHHRA HKVAR
```

No.	Pos.	Group	Score
1	K764	GWGAF **IKNP** VNRND	0.84
2	K149	TVVQL **VKLP** VVDKI	0.82
3	K581	NGAAM **AKRP** LSGKS	0.69
4	K753	QRILL **GKSD** VAGWG	0.67

(B)

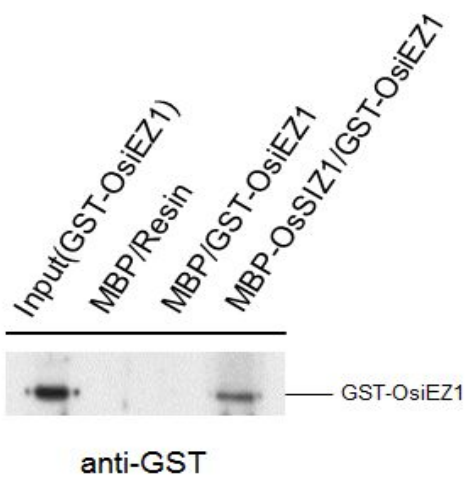

Figure 7. Rice histone methyltransferase OsiEZ1 physically interact with E3 SUMO ligase OsSIZ1. (A) The deduced amino acid sequence of the OsiEZ1 protein. Putative sumoylation sites identified using the SUMO-plot Analysis Program are shown in bold and underlined type. (*up*). The table showed the location of sites, amino acid sequence and score (*down*). (B) OsiEZ1 was pulled-down with full-length OsSIZ1. MBP, MBP-OsSIZ1 and GST-OsiEZ1 were over-expressed in E. coli and purified with amylose and glutathione, respectively. GST-OsiEZ1 absorbed with MBP or MBP-AtSIZ1 was detected using western blotting with anti-GST antibody.

OsiEZ1 is stimulated by rice E3 SUMO ligase OsSIZ1

SAP and MIZ 1 (SIZ1) proteins exhibit SUMO E3-ligase activity that facilitates the conjugation of SUMO proteins to target substrates (HC Park et al., 2010). And sumoylation modulates protein stability, subcellular localization and activity (Li et al., 2013).

To verify that OsSIZ1 post-transcriptionally affect OsiEZ1, we analyzed expression pattern of *OsiEZ1* and *OsSIZ1* in wild-type (WT), *osiez1* and *ossiz1* plants.

OsiEZ1 transcript levels were decreased in *OsSIZ1* mutant plants (Fig 8A), whereas *OsSIZ1* transcript levels were increased in *OsiEZ1* mutant plants (Fig 8B). This indicates that expression of *OsiEZ1* is affected by *OsSIZ1* post-transcriptionally. Although, transcript levels do not mean protein levels, we suggest that OsiEZ1 is stabilized by sumoylation through E3 SUMO ligase OsSIZ1.

Furthermore, we anticipate that OsiEZ1-mediated methylation of *OsMADS56* via *OsCOLDAIR* and that of other target genes can be controlled by sumoylation of OsiEZ1 through E3 SUMO ligase OsSIZ1.

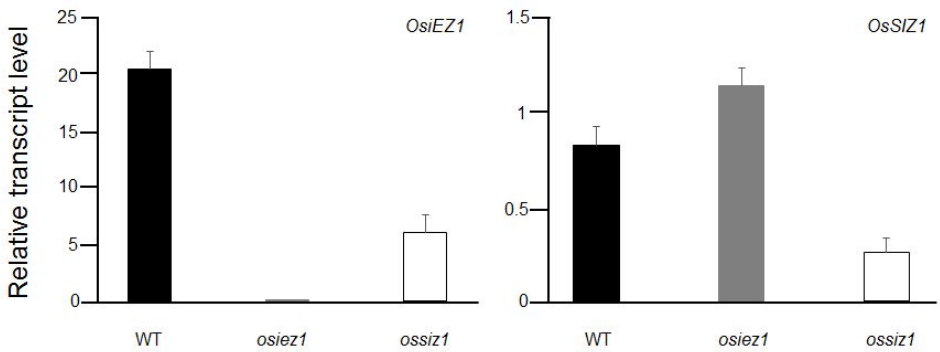

Figure 8. Change of expression pattern in wild-type (WT), *osiez1, ossiz1* plants. There was no conspicuous difference at numerical value. We isolated mRNA from leaves of 10-day-old under SD (10h light/ 14h dark). Relative transcript levels of *OsiEZ1*, *OsMADS56*, *OsCOLDAIR* were measured by RT-PCR.

6. DISCUSSIONS

In this study, we identified that histone methyltransferase *OsiEZ1* is closely connected with genes controlling flowering time. As we mentioned in result, long non-coding RNA, which guided polycomb repressive complex2, was identified from FLOWERING LOCUS C (*FLC*) chromatin in *arabidopsis thaliana*. So we thought that intronic long non-coding RNA would be founded at *FLC* homolog genes in rice. Same the prediction, long non-coding RNA candidate gene was identified at *FLC* homolog gene (Fig 1, 2).

Several long non-coding RNA have been known to target repressive histone-modifying activities and be required for epigenetic gene silencing through a molecular interaction with specific chromatin domains (Ponting, 2009; Lee, 2009). And PRC2-mediated silencing includes the interaction with such non-coding RNAs (Rinn et al., 2007, Tsai et al., 2010). Refers to these previous study, we checked the interaction OsiEZ1 and long noncoding RNA *OsCOLDAIR*. It was observed that OsiEZ1 physically interacted with *OsCOLDAIR* (Fig 3).

Previous study about histone methylation by *OsiEZ1* showed that transcript levels and H3 lysine 27 tri-methylation of key flowering genes were decreased in *OsiEZ1* knock-down plants (Liu et at., 2014). And *OsVIL3,* which is component of PRC2, could

promote flowering initiation via repressing floral repressors including *OsMADS56* (Wang et al., 2013). Understanding how the polycomb repressive complex 2 is recruited to the target gene is useful in explain the regulatory mechanism of PRC2 group on target genes. For identification of the correlation between *OsiEZ1*, *OsMADS56*, *OsCOLDAIR*, we checked expression pattern under LD and SD and compared relative transcript levels in wild-type and *osiez1* plants (Fig 4, 5). The SD-induced *OsiEZ1* expression is consistent with the results showing that *OsVIL3* that promote flowering under SD are also induced in SD (Wang et al., 2013). The heading date of *OsiEZ1* knock-down plants was similar as the wild-type under LD, whereas flowered later (>20days) than wild-type under SD (Liu et al., 2014). These results supported that *OsiEZ1* did not methylated *OsMADS56* under LD, because expression of *OsiEZ1* was almost none. We need to analysis OsMADS56 trnascript levels in lncRNA knockdown plant under SD and LD.

It is known that the activity of histone methyltransferases is regulated by post-translational modifications such as phosphorylation and sumoylation. But it is not clear how the enzyme was regulated by post-translational modification in plants. In Arabidopsis, activity of CHROMOMETHYLASE 3 (*CMT3*), DNA methyltransferase, was regulated by sumoylation through E3 SUMO ligase AtSIZ1. And CHROMOMETHYLASE 3 (*CMT3*)

containing three sumoylation sites interacted with AtSIZ1 (Kim et al., 2015). It suggest that CURLY LEAF(*CLF*) and *OsiEZ1*, which are histone methyltransferase having several sumoylation sites, will interact with E3 SUMO ligase OsSIZ1. Interaction of these histone methyltransferases and E3 SUMO ligase OsSIZ1 was found in result (Fig 6, 7). Also, we observed that transcript levels of *OsSIZ1* in *osiez1* plant was increased and transcript levels of *OsiEZ1* in *ossiz1* plant was decreased (Fig 8). Although sumoylation of OsiEZ1 through rice E3 SUMO ligase OsSIZ1 was not analyzed, it is suggested that the stability of OsiEZ1 was regulated by OsSIZ1-mediated sumoylation. Further, we anticipate that flowering pathway through *OsiEZ1* was regulated by *OsSIZ1*.

7. REFERENCES

Bel, S., et al. (1998). "Genetic interactions and dosage effects of Polycomb group genes in mice." Development 125(18): 3543-3551.

Cao, R., et al. (2002). "Role of histone H3 lysine 27 methylation in polycomb-group silencing." Science 298(5595): 1039-1043.

Conti, L., et al. (2014). "Small Ubiquitin-like Modifier protein SUMO enables plants to control growth independently of the phytohormone gibberellin." Dev Cell 28(1): 102-110.

Czermin, B., et al. (2002). "Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal polycomb sites." Cell 111(2): 185-196.

De Lucia, F., et al. (2008). "A PHD-Polycomb Repressive Complex 2 triggers the epigenetic silencing of FLC during vernalization." Proceedings of the National Academy of Sciences of the United States of America 105(44): 16831-16836.

Ding, B., et al. (2007). "Molecular characterization of three rice SET-domain proteins." Plant Science 172(6): 1072-1078.

Gendall, A. R., et al. (2001). "The VERNALIZATION 2 gene mediates the epigenetic regulation of vernalization in Arabidopsis." Cell 107(4): 525-535.

.

Hennig, L. and M. Derkacheva (2009). "Diversity of Polycomb group complexes in plants: same rules, different players?" Trends in Genetics 25(9): 414-423.

Heo, J. B. and S. Sung (2011). "Vernalization-Mediated Epigenetic Silencing by a Long Intronic Noncoding RNA." Science 331(6013): 76-79.

Hsieh, T. F., et al. (2003). "From flour to flower: how Polycomb group proteins influence multiple aspects of plant development." Trends in Plant Science 8(9): 439-445.

Kim, D. Y., et al. (2015). "Arabidopsis CMT3 activity is positively regulated by AtSIZ1-mediated sumoylation." Plant Science 239: 209-215.

Kim, S. I., et al. (2015). "E3 SUMO ligase AtSIZ1 positively regulates SLY1-mediated GA signalling and plant development." Biochemical Journal 469: 299-314.

Kohler, C., et al. (2003). "Arabidopsis MSI1 is a component of the MEA/FIE Polycomb group complex and required for seed development." Embo Journal 22(18): 4804-4814.

Lee, J. T. (2009). "Lessons from X-chromosome inactivation: long ncRNA as guides and tethers to the epigenome." Genes & Development 23(16): 1831-1842.

Liang, Y. K., et al. (2003). "OsSET1, a novel SET-domain-containing gene from rice." Journal of Experimental Botany 54(389): 1995-1996.

Liu, N., et al. (2014). "H3K27me3 and H3K4me3 Chromatin Environment at Super-Induced Dehydration Stress Memory Genes of Arabidopsis thaliana." Molecular Plant 7(3): 502-513.

Liu, X. Y., et al. (2014). "The rice enhancer of zeste [E(z)] genes SDG711 and SDG718 are respectively involved in long day and short day signaling to mediate the accurate photoperiod control of flowering time." Frontiers in Plant Science 5.

Luo, M., et al. (1999). "Genes controlling fertilization-independent seed development in Arabidopsis thaliana." Proceedings of the National Academy of Sciences of the United States of America 96(1): 296-301.

Luo, M., et al. (2009). "Expression, Imprinting, and Evolution of Rice Homologs of the Polycomb Group Genes." Molecular Plant 2(4): 711-723.

Nallamilli, B. R. R., et al. (2013). "Polycomb Group Gene OsFIE2 Regulates Rice (Oryza sativa) Seed Development and Grain Filling via a Mechanism Distinct from Arabidopsis." Plos Genetics 9(3).

Ohad, N., et al. (1999). "Mutations in FIE, a WD polycomb group gene, allow endosperm development without fertilization." Plant Cell 11(3): 407-415.

Park, B. S., et al. (2012). "Arabidopsis SIZ1 positively regulates alternative respiratory bypass pathways." Bmb Reports 45(6): 342-347.

Park, H. C., et al. (2010). "Functional characterization of the SIZ/PIAS-type SUMO E3 ligases, OsSIZ1 and OsSIZ2 in rice." Plant Cell and Environment

33(11): 1923-1934.

Ponting, C. P., et al. (2009). "Evolution and Functions of Long Noncoding RNAs." Cell 136(4): 629-641.

Rinn, J. L., et al. (2007). "Functional demarcation of active and silent chromatin domains in human HOX loci by Noncoding RNAs." Cell 129(7): 1311-1323.

Ryu, C. H., et al. (2009). "OsMADS50 and OsMADS56 function antagonistically in regulating long day (LD)-dependent flowering in rice." Plant Cell and Environment 32(10): 1412-1427.

Schuettengruber, B. and G. Cavalli (2009). "Recruitment of Polycomb group complexes and their role in the dynamic regulation of cell fate choice." Development 136(21): 3531-3542.

Shi, J., et al. (2015). "Epigenetic regulation of rice flowering and reproduction." Frontiers in Plant Science 5.

Shrestha, R., et al. (2014). "Molecular control of seasonal flowering in rice, arabidopsis and temperate cereals." Annals of Botany 114(7): 1445-1458.

Son, G. H., et al. (2014). "FLC-mediated flowering repression is positively regulated by sumoylation." Journal of Experimental Botany 65(1): 339-351.

Sui, P. F., et al. (2013). "H3K36 Methylation Is Involved in Promoting Rice Flowering." Molecular Plant 6(3): 975-977.

Sun, C. H., et al. (2012). "The Histone Methyltransferase SDG724 Mediates H3K36me2/3 Deposition at MADS50 and RFT1 and Promotes Flowering in Rice." Plant Cell 24(8): 3235-3247.

Sung, S. B. and R. M. Amasino (2004). "Vernalization and epigenetics: how plants remember winter." Current Opinion in Plant Biology 7(1): 4-10.

Swiezewski, S., et al. (2009). "Cold-induced silencing by long antisense transcripts of an Arabidopsis Polycomb target." Nature 462(7274): 799-U122.

Thakur, J. K., et al. (2003). "A POLYCOMB group gene of rice (Oryza sativa L. subspecies indica), OsiEZ1, codes for a nuclear-localized protein expressed preferentially in young seedlings and during reproductive development." Gene 314: 1-13.

Tsai, M. C., et al. (2010). "Long Noncoding RNA as Modular Scaffold of Histone Modification Complexes." Science 329(5992): 689-693.

Wang, J., et al. (2013). "LC2 and OsVIL2 Promote Rice Flowering by Photoperoid-Induced Epigenetic Silencing of OsLF." Molecular Plant 6(2): 514-527.

Wood, C. C., et al. (2006). "The Arabidopsis thaliana vernalization response requires a polycomb-like protein complex that also includes VERNALIZATION INSENSITIVE 3." Proceedings of the National Academy of Sciences of the United States of America 103(39): 14631-14636.

Yang, J., et al. (2013). "OsVIL2 functions with PRC2 to induce flowering by

repressing OsLFL1 in rice." Plant Journal 73(4): 566-578.

Yoshida, N., et al. (2001). "EMBRYONIC FLOWER2, a novel polycomb group protein homolog, mediates shoot development and flowering in Arabidopsis." Plant Cell 13(11): 2471-2481.

Zhao, X. L., et al. (2011). "An atypical HLH protein OsLF in rice regulates flowering time and interacts with OsPIL13 and OsPIL15." New Biotechnology 28(6): 788-797.

Bastow, R., et al. (2004). "Vernalization requires epigenetic silencing of FLC by histone methylation." Nature 427(6970): 164-167.

Li, Z. G., et al. (2013). "Heterologous expression of OsSIZ1, a rice SUMO E3 ligase, enhances broad abiotic stress tolerance in transgenic creeping bentgrass." PlantBiotechnologyJournal 11(4): 432-445.

초록

작물에서의 개화 시기는 곡식의 생산량을 결정하는데 있어 중요한 특성 중 하나이다. 애기 장대에서, 개화 시기의 주요 조절 유전자인 FLOWERING LOCUS C (*FLC*)의 발현이 크로마틴 변형에 의해 후성 유전학적으로 조절된다고 잘 알려져 있다. 이러한 크로마틴 변형은 POLYCOMB REPRESSIVE COMPLEX 2 (PRC2)라는 억제 복합체의 핵심 구성성분인 히스톤 메칠화 효소 Enhancer of Zeste [E(z)]에 의해 일어난다. 그리고 COLD ASSISTED INTRONIC NONCODING RNA (*COLDAIR)*라고 알려진 long intronic non-coding RNA는 PRC2와의 상호작용을 통해 *FLC* 크로마틴을 안정적으로 억제시키는데 있어서 필수적이라고 알려져 있다. 그러나 벼의 개화 억제 유전자의 조절과 관련된 크로마틴 메커니즘은 현재 잘 알려져 있지 않다. 그러므로 우리는 OsiEZ1의 생화학적 특성을 조사하였다. OsiEZ1과 벼의 *FLC* 상동 유전자인 *OsMADS56*에서 발현되는 intronic long non-coding RNA *OsCOLDAIR*이 상호한다는 작용하는 것을 확인하였다. 또한 OsiEZ1과 벼의 E3 SUMO ligase인 OsSIZ1이 상호 작용 한다는 사실을 보았다. 이러한 발견들은 *OsCOLDAIR*를 통한 OsiEZ1의 *OsMADS56* 메칠화와 다른 대상 유전자들의 메칠화가 E3 SUMO ligase인 OsSIZ1에 의한 OsiEZ1의 수모화를 통해 조절될 수 있다는 것을 보여 준다.

주요어: 히스톤 메칠화 효소, Enhancer of Zeste[E(z)],
OsiEZ1, Long non-coding RNA, E3 SUMO ligase, OsSIZ1.

학번 : 2014-20024