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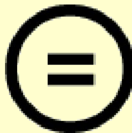
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THESIS FOR DEGREE OF MASTER OF SCIENCE

OsiEZ1 negatively regulates the expression of floral repressor *OsMADS56* by forming complex with long noncoding RNA *RIFLA*

OsiEZ1 와 *RIFLA* 복합체에 의한
개화 억제 유전자 OsMADS56 의 발현 조절

BY

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AUGUST, 2018

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ABSTRACT

There are many long noncoding RNAs(LncRNAs) in plant. They control various mechanisms. For instance, the lncRNA COLDAIR is one of regulator that is required for the repression of a floral repressor *FLOWERING LOCUS C* (*FLC*) in *Arabidopsis thaliana*. The repression of *FLC* is regulated by Polycomb Repressive Complex 2 (PRC2) and trimethylation of Histone H3 Lysine 27 (H3K27me3) on *FLC* chromatin and thereby regulates

methylation of target genes and their expression. But, the mechanism how a rice enhancer of zeste rice, OsiEZ1, a homologue of Arabidopsis CLF, methylates target genes and regulates gene silencing is still remained unclear. Here, we show that OsiEZ1 can control expression of rice MADS-box protein gene 56 (*OsMADS56*), an orthologue of the Arabidopsis floral repressor *FLC*. LncRNA *RIFLA* was expressed in the first intron of *OsMADS56*. lncRNA *RIFLA* was physically associated with OsiEZ1 and also interacted with histone proteins. Besides, OsiEZ1 interacted with histone proteins and their interaction was enhanced by *RIFLA*. Moreover, OsiEZ1 strongly interact with rice E3 SUMO ligases OsSIZ1 and OsSIZ2, suggesting that histone OsiEZ1- and lncRNA *RIFLA*-mediated target gene methylation and expression is controlled by E3 SUMO ligase OsSIZ1 and OsSIZ2. Taken together, our data indicate that OsiEZ1 can regulate expression of *OsMADS56* through direct interaction with lncRNA *RIFLA* and OsiEZ1 activity will be modulated by sumoylation through E3 ligase SUMO activity.

Keyword : *FLOWERING LOCUS C* (*FLC*); CURLY LEAF (CLF); Rice enhancer of zeste rice(OsiEZ1); Rice MADS-box protein gene 56 (*OsMADS56*); Long noncoding RNA (lncRNA) *RIFLA*

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ABBREVIATION

OsiEZ1	*ORYZA SATIVA INDICA* ENHANCER OF ZESTE1
OsMADS56	*ORYZA SATIVA* MADS-BOX PROTEIN GENE 56
LncRNA	ONG NON-CODING RNA
RIFLA	RICE FLOWERING-ASSOCIATED
FLC	*FLOWERING LOCUS C*
WT	Wild-Type
LP	Left Primer
RP	Right Primer
LB	Left Border.

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INTRODUCTION

Epigenetic regulation of gene expression is a series of molecular regulations, including DNA modification, chromatin modification and long noncoding RNAs (lncRNAs). PTM(Post Translational Modification) such as ubiquitination, sumoylation and methylation influence on gene expression to regulate chromatin status (Gan et al., 2013). Histone methylation is a modification which add methyl groups to lysine or arginine residues of histone proteins. The methylation of the lysine residues of histone proteins is catalyzed by the histone methyltransferase (HMTase) containing the Enhancer of zeste (Ez), SET (Suppressor of variegation 3-9 (Suv3-9) and Trithorax (Trx) domain.

The vernalization is a example in which epigenetic regulation of gene expression in *Arabidopsis*. Vernalization is the exposure to an extended period of cold. And the functions of that is the repression of the FLOWERING LOCUS C (FLC) gene. The repression of *FLC* in vernalization is regulated by the changes Histone H3 Lys 27 (H3K27me3) at *FLC* chromatin. In addition, lncRNA *COLDAIR* is transcribed from the *FLC* first intron locus. It suggests the functions of vernalization that mediated repression of *FLC*(Sung et al., 2011). Modifications at *FLC* chromatin are regulated by various chromatin remodeling complexes. In particular, studies revealed that POLYCOMB REPRESSION COMPLEX 2 (PRC2) is necessary for remodeling at *FLC* chromatin. The PRC2 complex which is composed of Sex comb extra (Sce), Ez, Suppressor of zeste 12 (Suz12), and Chromatin Assembly Factor 1 (CAF1) was first identified in Drosophila and it catalyzes the trimethylation of H3K27 through HMTase activity of Ez domain (Margueron and Reinberg, 2011). In Arabidopsis, there are four homologs of core

PRC2 components, FERTILIZATION INDEPENDENT ENDOSPERM (FIE), EMBRYONIC FLOWER 2 (EMF2), MULTICOPY SUPRESSOR OF IRA 1 (MSI1) and CURLY LEAF (CLF)/SWINGER (SWN), and they mediate gene repression via the deposition of H3K27me3 marks on the chromatin (Gan et al., 2013; Bemer and Grossniklaus, 2012). Recently, the working mechanism about how PRC2 complexes function has been reported. The PRC2 complex deposits the H3K27me3 mark onto specific location (Margueron and Reinberg, 2011) and thereby silences gene expression at the region.

There are three EZ homologs in Arabidopsis that is SWINGER (SWN), MEDEA (MEA) and CURLY LEAF (CLF) (Chanvivattana et al., 2004; Hennig and Derkacheva, 2009). The function of CLF is a major H3K27 methyltransferase and its functional roles have been reported. For instance, CLF was involved in embryonic development including post-fertilization development (Chanvivattana et al., 2004; Spillane et al., 2007). H3K27me3 modifications on FLC, a key flowering repressor at which vernalization and autonomous pathways and that indicate the role of CLF-mediated H3K27me3 in flowering pathway.

Rice genome contains a conservative set of PRC2 components (Luo et al., 2009) although little is known about their function in rice. The rice genome contains two Ez-like genes, *OsiEZ1* and *OsCLF*. *OsiEZ1* is induced in short day (SD) and represses *OsLF*, a repressor of Hd1 (Zhao et al., 2011), leading to a higher expression of *Hd1* that activates *Hd3a* and thus early flowering. *OsCLF* is induced in long day (LD) and represses *OsLF*, *Ehd1* and other flowering-promoting genes leading to late flowering (Liu et al, 2014) indicating that they display distinct function in photoperiod regulation of flowering in rice.

ORYZA SATIVA MADS-BOX PROTEIN GENE 56 (OsMADS56) is a rice orthologue of Arabidopsis Suppressor of Overexpression of Constans1 (SOC1) which functions as a flowering activator downstream of CONSTANS and integrates signals from multiple flowering pathways. (Lee et al. 2000; Onouchi et al. 2000; Moon et al. 2003). OsMADS56 contains a MIKC-type MADS-box protein that is composed of MADS-domain(Kaufmann et al., 2005), intervening, coiled-coil keratin-like and highly variable C-terminal domain (Riechmann & Meyerowitz 1997). The MADS and intervening domains are required for DNA-binding/dimerization (Schwarz-Sommer et al. 1990; Krizek & Meyerowitz 1996). Interactions between MIKC-type proteins are largely achieved via the keratin-like domain (Fan et al. 1997; Yang & Jack 2004). But, the flowering of OsMADS56-overexpressing rice was delayed in LD but not in SD (Ryu et al., 2009), indicating that OsMADS56 is an LD-specific floral repressor.

It is well known that lnc RNAs are involved in epigenetical transcription silencing of various genes. In plant, flowering is regulated by ncRNA. Arabidopsis COLDAIR associates with a component of PRC2 and target PRC2 to FLC gene, which represses FLC expression and thereby induces flowering (Heo and Sung, 201). Because of the significance of the *OsMADS56* gene in rice flowering, we examined the whether lncRNA can be produced from the introns of the *OSMADS56* gene or not and found that the lncRNAs were transcribed in the first intron of the *OSMADS56* gene. Recent results proved that OsiEZ1, a polycomb repressive complex2 (PRC2) key subunit required for trimethylation of H3K27, was involved in regulation of key flowering genes (Liu et al., 2014). These suggest that OsiEZ1 can regulate flowering by OsMADS56 expression control through its

HTMase activity with OsiEZ1-lncRNA. Thus, we identify the role of OsiEZ1 in flowering pathway.

Long non-coding RNAs (lncRNAs) are a RNA wasn't translated and is longer than 200 nucleotides(Kung et al., 2013), and they are new regulators of transcriptional and post-transcriptional regulation (Kornienko et al., 2013). Furthermore, few of them have been functionally characterized such as HOTAIR, ANRIL and COLDAIR (Heo and Sung, 2010; Rinn et al., 2010; Tsai et al., 2010; Burd et al., 2010) and they influence chromatin-remodeling complexes and interact with the PRC2 (Heo et al., 2013). For instance, Arabidopsis lncRNA COLDAIR interacts with CLF protein that is a homolog of E(z) component, the PRC2 complex and the enrichment of CLF and H3K27me3 at the FLC chromatin indicating that COLDAIR is necessary for recruiting PRC2 at FLC chromatin.

Here, we provide the first evidence that OsiEZ1 functions as an HTMase for *OsMADS56*, directly interacting with OsiEZ1-lncRNA *RIFLA*. These findings indicate that OsiEZ1 can negatively regulate floral repressor OsMADS56 by forming complex with *RIFLA*.

Materials and methods

Plant material and growth conditions

The *Oriza sativa var. japonica cultivar* Dongjin (WT) and the T-DNA insertion mutant plants *osmads56* (PFG-4A-00363), *ossiz2* (PFG-3A-3A-13223) were used for this study. T-DNA insertion mutant lines were obtained from the Gynheung An's lab (Kyung hee university). Seed sterilized in 5% sodium hypochlorite for 20 min, rinsed ten times in sterilized water and sterilized seeds were spreaded on plates. T-DNA insertion site was confirmed by PCR using the specific forward primers, reverse primers and T-DNA PFG 2715 vector Right or left border primer. The plants were grown in fully automated growth chambers. The plants in growth chambers were grown for 4 weeks under continuous short day condition (10h light at 30℃ /14h dark at 25℃). For phenotype study of *ossiz2*, The plants were grown on plates composed of Murashige and Skoog(MS) medium, 2% sucrose and 1% agar (pH 5.8). The plants in soil, seeds were directly planted in sterile soil in 3 weeks on continuous long day condition (14h light at 30℃ /10h dark at 25℃).

Production of rice transgenic plants

To produce plants over-expressing *RIFLA*, the full-length cDNA sequences were amplified by PCR using primer(Table 1) and inserted into pBA002 that is a plant over-expression vector. The recombinant plasmid in LBA4404 an *Agrobacterium* strain was infected to the callus embryos from the japonica rice cultivar 'Oryza sativa', and infected callus was selected on N6D medium containing 50 μg/ml of BASTA (glufosinate ammonium). Transformants were confirmed by PCR with RIFLA genomic fragment primers (Table 1).

RNA isolation and Quantitative real-time PCR analysis

Total RNA was isolated from rice leaves using Plant Total RNA Purification Mini kit(FAVORGEN), according to the manufacturer's instructions. The transcripts level of the putative histone methyltransferase OsiEZ1, flowering repressor OsMADS56, RIFLA and E3 SUMO ligase OsSIZ1 and OsSIZ2 were examined by real-time PCR in WT, OX;RIFLA, *osmads56, ossiz2* plants. 1 ug of total RNA isolated from the WT, OX;RIFLA, *osmads56, ossiz2* plants were reverse transcribed with TOPscript^{TM} RT DryMIX (dT16 plus) (Enzynomics). The resulting cDNA:RNA hybrids were treated with RNase H (Enzynomics) for 20 minutes at 37℃, and used as template for real-time PCR, which was conducted with the Light Cycler 480 (Roche) and KAPA SYBR FAST qPCR Kits (Kapa Biosystems) according to the manufacturer's instruction. Primers for UBQ10 were added as a control with gene specific primers. All reactions of this study were repeated three times. The primers used are listed in Table 1.

Construction of recombinant plasmids

To produce MBP-OsiEZ1 recombinant plasmid, a cDNA of *Oriza sativa* OsiEZ1 encoding full-length protein was amplified by PCR and inserted into the pENTRY 3C vector. To produce MBP–OsSIZ1 recombinant plasmid, a cDNA of *Oryza sativa* OsSIZ1 encoding full-length protein was amplified by PCR and inserted into the pMAL-c2x vector (New England Biolabs). To produce GST-SAP, GST-Histone H3 and GST-Gutelin, a cDNA of Os11g29340 encoding full-length protein was amplified by PCR and inserted into and pGEX4T-1 (GE Healthcare) vectors. All of constructs were transformed into *Escherichia coli* BL21 cell lines. Isopropyl-*β*-D-thiogalactoside (IPTG) to produce fusion protein expression in the transformed

cells. The primers used in this reseach are listed in Table 1.

Purification of recombinant proteins

The recombinant proteins for studies were expressed in *E. coli* strain BL21 and were purified with the manufacturer's instructions. Briefly, for GST–OsiEZ1, GST–OsSAP, and GST-Histone H3 purification, E. coli was lysed in PBS buffer (pH 7.5) added 0.5% Triton X-100 and 2mM PMSF and purified on glutathione resins (GE Healthcare Life Science). For MBP-OsiEZ1 and its derivatives, MBP-OsSAP, MBP-OsGlutelin and purification, *E. coli* were lysed in lysis buffer added 20mM TRIS-HCl(pH 7.4), 150mM NaCl, 1mM EDTA, 0.5% Triton X-100, and 2mM PMSF] and purified on amylose resins (New England Biolabs). Protein concentrations were determined by comparision with serial concentration of Bovine serum albumin(BSA) through SDS-PAGE and brilliant blue staining.

In vitro transcription

To produce lncRNAs, the full-length cDNA sequences were amplified by PCR using primers that containing T7 promoter and a reverse primer. lncRNA RIFLA and its derivatives is transcribed with the EZ™ High Yield In Vitro Transcription Kit (Enzynomics), treated with RNase-free DNase I (TAKARA), and purified with the Plant Total RNA Purification Mini kit(FAVORGEN). Biotin-labeled RNAs were transcribed using the Biotin RNA Labeling Mix (Roche, Indianapolis, IN, USA) and T7 RNA polymerase (Roche, Indianapolis, IN, USA), treated with RNase inhibitor (Intron biotechnology, Korea) and purified with Plant Total RNA Purification Mini kit(FAVORGEN).

In vitro RNA pull down assay

For research, 1 μg biotin-labeled RNAs and recombinant OsiEZ1 protein were mixed in pull-down buffer containing 50mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P40 and incubated for 2 hours at 4℃ and then 30μL washed Streptavidin agarose resin (Promega) were added to each binding reaction mixtures and further incubated for 1 hour at 4℃. Resin was washed briefly 10 times using binding buffer and boiled in SDS sample buffer containing 50mM Tris-Cl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 1% bromophenol blue for 10 minutes and then subjected to Western blots against anti-MBP antibody (Santa Cruz Biotechnology).

in vitro protein pull down assay

To determine the *in vitro* binding of OsEZ1 to OsSAP, OsHistone H3 and OsGlutelin with or without *in vitro* transcribed RIFLA through T7 RNA polymerase (Roche), 2 μg of full-length MBP-OsiEZ1 and 2 μg of full-length GST-OsSAP, GST-OsHistone H3 and GST-OsGlutelin were added to 1ml of binding buffer containing 50 mM Tris-HCl pH7.5, 100 mM NaCl, 1% Nonidet P40. After incubation at 4 ℃ for 2hour, the reaction mixtures were incubated with amylose resin for 1hour followed by washing six times with binding buffer. Resin was boiled in SDS sample buffer containing 50 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 1% bromophenol blue for 10 minutes and then subjected to Western blots against anti-GST antibody (YOUNG IN FRONTIER).

Yeast two hybrid assays

For yeast two hybrid library screening bait, a cDNA of *Oriza sativa* OsiEZ1 was amplified by PCR and inserted to pGBT (CLONTECH). As a bait, a cDNA of *Oriza sativa* OsSIZ1, OsSIZ2, OsSAP was amplified by PCR and inserted to pGAD (CLONTECH). All constructs were verified by DNA sequencing. All constructs were transformed into AH109 one of yeast strains using the lithium acetate method. Yeast cells on medium (–Leu/–Trp) were grown for 3 days. Transformants were spreaded on to medium (–Leu/–Trp/–His/+ 3-amino-1,2,4-triazole) to study the association between OsiEZ1 and OsSAP domain protein.

Table1. Primers used in this study

A. Primers for verification of OsRL3 T-DNA insertion		
Product	Forward primer(5'→3')	Reverse primer(5'→3')
PFG_4A-00363	TTACACTCAACCACCGTCCC	CTATGGGGGTACTATGCCCC
PFG_3A-13223	GCTTGGACTTCCAAAGCAAG	AAAGGGGCAAACAAATTGTG
PFG_2715_LB	ACGTCCGCAATGTGTTATTAAG	
PFG_2715_RB	TTGGGGTTTCTACAGGACGTAAC	
B. Gene-specific primers used for qRT-PCR		
Gene	Forward primer(5'→3')	Reverse primer(5'→3')
OsiEZ1	TTGCGCCTGTGTGGAAAATG	CTAGTGAGCCATCTCCGCAG
OsMADS56	GCTGAAAGAGAAGGAGCGGA	TCGTCGTCATGTGGTTAGCC
RIFLA	AGGTCATTTGCCTTTCCTCA	GGACGGTGGTTGAGTGTAAGT
OsSIZ1	GCTTTGGTTGGCGATGATCC	CTTCGTTACCACCTCCACCC
OsSIZ2	CCAACCCACCTGAAGCTTCA	GCAGCATCTTGAGAGCTCGA
OsUBQ10	CCTCCGTGGTGGTCAGTAAT	GCATACTGCTGTCCCACAGG
C. Gene-specific primers used for PCR		
Gene	Forward primer(5'→3')	Reverse primer(5'→3')
RIFLA	ACCTCAACTACCCTCCCACT	AGAGAGATGGAGGTGGGACG

D. List of primers used for construction		
Consturct	Forward primer(5'→3')	Reverse primer(5'→3')
pCR8_*COLDAIR*	CTTTGTTCCTATTCGTTAA	AACATATACGAGAAAACTTTTCGGA
pCR8_Anti-*RIFLA*	GCATTTAAAATTTATCCA	TGTAATTATGAGCAATTGTATTTCA
pCR8_*RIFLA*	TGTAATTATGAGCAATTGTAT	GCATTTAAAATTTATCCACA
pCR8_Δ69-210*RIFLA*	TGCCTTTCCTCATTATTTGGCTC	TATTTTGGGAAGAGGGATCAGT
pCR8_Δ267-639*RIFLA*	ATTACTTTGCCTTGAATCTTCCAAGC	ATAAATGAATAAGCTACTTTATCACT
pCR8_Δ646-823 *RIFLA*	AGTAATTTCAGAACAAACTTT	AGGCACCACACTTCTCATGG
pCR8_Δ825-1057 *RIFLA*	TACACGTATATATATACTTGCTTGGA	AGTATGATCGGTGTGCTGTCT
pGEX_4T-1-OsiEZ1	TCGTACCCCGGGATGGCTGGCGATTCCC	TCGTACGACGTCTCAGTGGGCGAGCTTC
pGEX_4T-1-OsSAP domain protein	AATTGAATTCATGGCGGCCGAGGGTTCC	AATTGTCGACTCAGTCCTGTGATTCTCTTAAGCG
pGEX_4T-1-Histone H3	AATTGTCGACTTACGACCTGCTCTTGAATGGA	AATTGAATTCATGGCCCGCACGAAGCAG

pGEX_4T-1- Glutelin	AATTGAATTCATGGCGAGTTCCGTTTTCTCT	AATTGTCGACTTACTCTGAGGTCTCGCTTTCG
pMAL_c2X-OsiEZ1	TCGTACCCCGGGATGGCTGGCGATTCCC	TCGTACGACGTCTCAGTGGGCGAGCTTC
pMAL_c2X-OsSAP domain protein	AATTGAATTCATGGCGGCCGAGGGTTCC	AATTGTCGACTCAGTCCTGTGATTCTCTTAAGCG
pMAL_c2X-D1	GCAGGAATTCATGGCGTCGTCCTCGTCC	ATAAAAGCTTCTAAATAGAGTGCTCCTTGTTGGA
pMAL_c2X-D2	GCAGGAATTCGGAAGCACAACTAACACTACTGAAAA	ATAAAAGCTTCTATCTAGCAACTTTGTGCGCT
pMAL_c2X-D3	GCAGGAATTCATGGCGTCGTCCTCGTCC	ATAAAAGCTTCTAAGTTGGATGCCCTGCAG
pMAL_c2X-D4	GCAGGAATTCATGGCGTCGTCCTCGTCC	ATAAAAGCTTCTATAGTGAGCCATCTCCGCAG
pMAL_c2X-D5	GCAGGAATTCACACCACCTCCTGGAGAT	ATAAAAGCTTCTATAGTGAGCCATCTCCGCAG
pMAL_c2X-D6	GCAGGAATTCACACCACCTCCTGGAGAT	ATAAAAGCTTCTAAGTTGGATGCCCTGCAG
pGAD424-OsiEZ1	GCAGGAATTCGGAAGCACAACTAACACTACTGAAAA	AAAGGATCCATGGCGTCGTCCTCGTCCA
pGAD424-OsSIZ1	ACAGGAATTCATGGCGGACCTGGTTTCC	GCAGCTCGAGGACTCAGAATCAGTATCTAT
pGAD424-OsSIZ2	CAATGAATTCATGGCGCTCGACCCCG	GCTCGATATCtATCAGAGTCTGACTCGA
pGBT9-OsiEZ1	GCAGGAATTCGGAAGCACAACTAACACTACTGAAAA	AAAGGATCCATGGCGTCGTCCTCGTCCA
pGBT9- OsSAP	AATTGAATTCATGGCGGCCGAGGGTTCC	AATTGTCGACTCAGTCCTGTGATTCTCTTAAGCG

Results

OsMADS56 expression is downregulated in *RIFLA* overexpression line

OsMADS56 may regulate *RIFLA* trascription level. To study the functional roles of *OsMADS56*, we identified a T-DNA insertional mutant line, 4A-00363, in which T-DNA was inserted at the first intron of *OsMADS56*(Fig.2A). PCR analyses showed that osmads56 mutant line indicating that these are knock-out alleles(Fig.2B). Although the transcript was not present in the mutant (Fig.2B), the insertion did not affect *RIFLA* trascription. Since T-DNA was inserted at the *RIFLA*(Fig.2A).To confirm our observation with *osmads56* plant, we generated transgenics overexpressed *RIFLA* constructs of the gene, and then checked OsMADS56 trascription level. PCR analyses of three normal plants demonstrated that RIFLA trascription level is high in #1 and #2 plants among 3 primary (T1) plants(Fig.1B). We then check the expression level of OsMADS56(Fig.1C). These results suggest that OsMADS56 and *RIFLA* role is in complementary relations. Opposite expression pattern between *OsMADS56* and *RIFLA* suggests that *OsMADS56* expression can be regulated by *RIFLA*. We thus examined whether *OsMADS56* expression is downregulated in *RIFLA* overexpression line. In a result, we found that *OsiEZ1, OsSIZ1* and *OsSIZ2* expression is lower in *RIFLA* overexpression line than wild-type. Together, *OsiEZ1* and *OsSIZ1* expression is lower in *osmads56* mutant line than wild-type. This suggests that OsiEZ1 negatively control *OsMADS56* expression and that *RIFLA* expression is regulated by OsiEZ1.

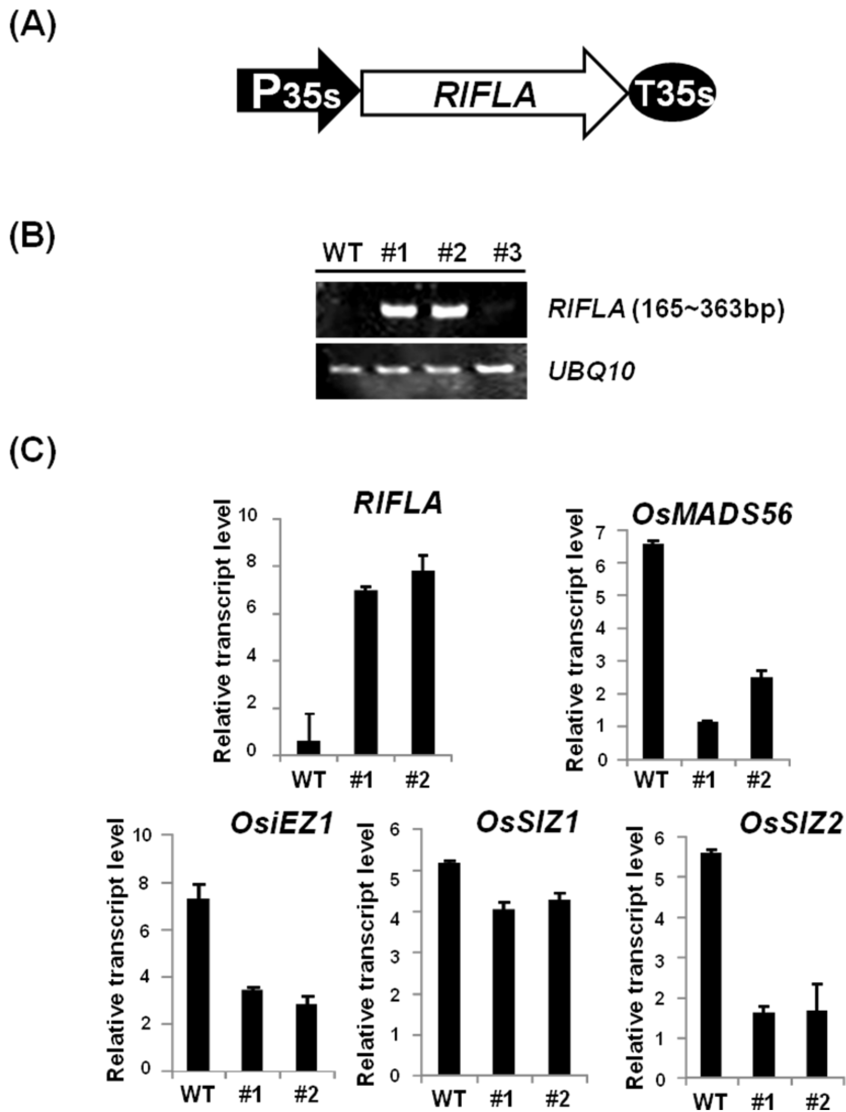

Figure 1. Gene structure and relative transcript levels of *P35S::RIFLA* plants

(A) Schematic diagram of overexpression *RIFLA construct. RIFLA* full-length cDNA clone was placed between maize *35s* promoter (P*35s*) and terminator (T*35s*). (B) PCR verification of the *RIFLA* overexpression transgenic line. (C) Relative transcript levels of *RIFLA, OsMADS56*, *OsiEZ1, OsSIZ1* and *OsSIZ2*. Plants were grown under continuous light during 10 days and transferred under short day conditions. After further incubation for 10 days, total RNAs were isolated from

leaves of each sample and then the transcript levels of *RIFLA, OsMADS56*, *OsiEZ1, OsSIZ1* and *OsSIZ2* genes were examined by qRT-PCR.

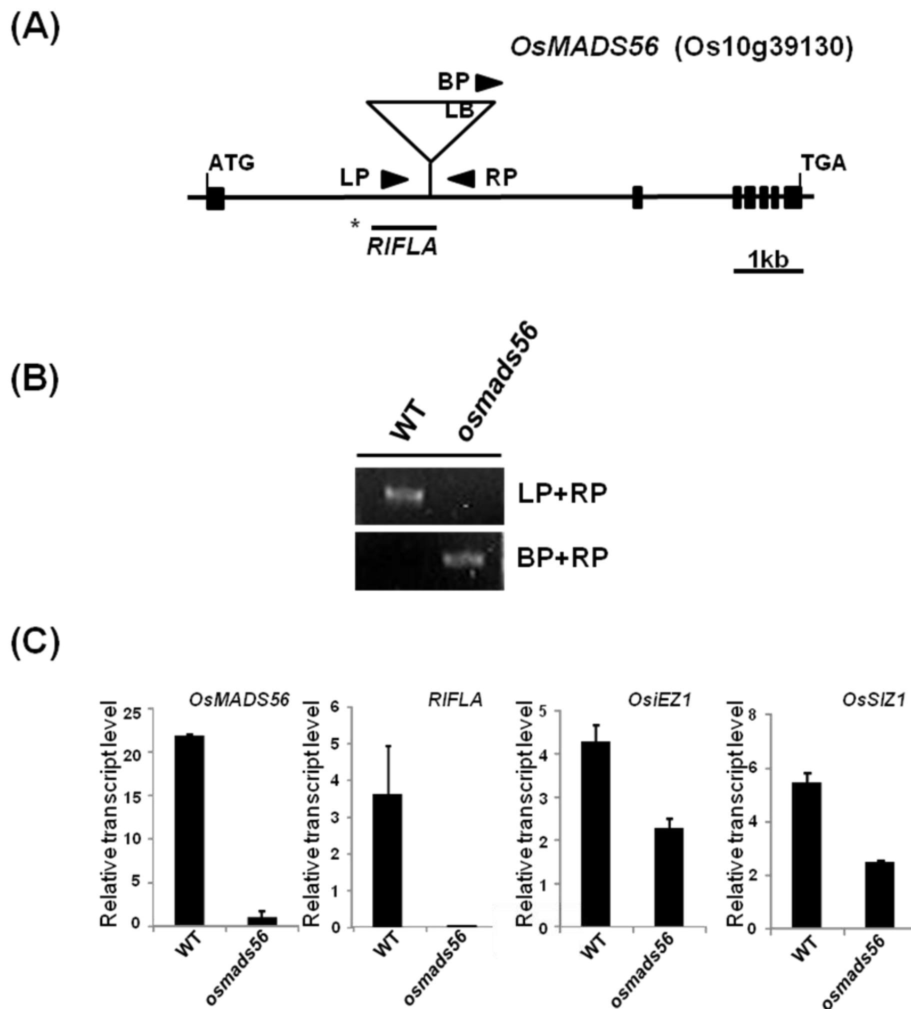

Figure 2. Schematic diagram of *OsMADS56* genomic structure and the regions of T-DNA insertional mutant alleles and relative transcript levels

(**A**)Schematic diagram of *OsMADS56* genomic structure, positions of T-DNA insertional mutant alleles and flowering time of mutants. *OsMADS56* (Os10g39130) consists of seven exons (boxes) and six introns (lines between boxes). T-DNAs were inserted into the first intron in *osmads56* (Line 4A-00363), respectively. Arrows indicate LP and RP primers used for amplification of gDNA. LB is left border of T-DNA. (B) PCR verification of the homozygous T-DNA

insertion alleles of *osmads56*. (C) Relative transcript levels of *OsMADS56*, *RIFLA*, *OsiEZ1* and *OsSIZ1*. Plants were grown under continuous light during 10 days and transferred under short day conditions. After further incubation for 10 days, total RNAs were isolated from leaves of each sample and then the transcript levels of *OsMADS56*, *RIFLA*, *OsiEZ1* and *OsSIZ1* genes were examined by qRT-PCR.

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RIFLA physically interacts with OsiEZ1

In Arabidopsis, *COLDAIR* guides CLF to FLC chromatin as a complex with CLF, leading to *COLDAIR*-mediated epigenetic silencing of *FLC* gene. We also presumed that *RIFLA* may interact with OsiEZ1 to recruit it on *OsMADS56* chromatin. To prove this, we first investigated a possible interaction between *RIFLA* and OsiEZ1. To do this, we predict secondary structures of *RIFLA* and its derivatives(Fig.3) and we prepared recombinant DNA *MBP-OsiEZ1* and then introduced it into *E.coli*. After overexpression by IPTG treatment, MBP-OsiEZ1 was purified with amylose column. Biotin-conjugated RILFA was also prepared as described in Materials and Methods. For examination their interaction, MBP-OsiEZ1 and Biotin-*RIFLA* or MBP and Biotin-*RIFLA* were mixed and pull-downed with avidin resin. After elution, MBP-OsiEZ1 was detected by western blot with anti-GST antibody. Result showed that OsiEZ1 was clearly detected in the lane pull-downed with RIFLA (Fig.4B), indicating that OsiEZ1 directly interacts with *RIFLA*.

Many lncRNAs specific motifs that are formed by modular structures in the RNA is necessary for interaction with protein components. To identify the region of *RIFLA* that mediates the interaction with OsiEZ1, we utilized an *in vitro* RNA binding assay. Genomic DNA from wild-type that express T7 promoter-tagged full-length and partial transcripts of *RIFLA* with PCR analysis. Then, products were transcribed, biotinylated full-length and partial transcripts of RIFLA(Fig.4A). Proteins interact with each RNA fragment were precipitated with streptavidin beads and MBP-tagged OsiEZ1 protein was detected by western blot. From these experiments, we determined that only RIFLA fragments containing nucleotides

from 646 to 823 precipitate OsiEZ1-containing PRC2. In addition, the fragment of *RIFLA* that only contains nucleotides 646 to 823 bases of *RIFLA* was able to bind to OsiEZ1 (Fig.4B and Fig.4C).

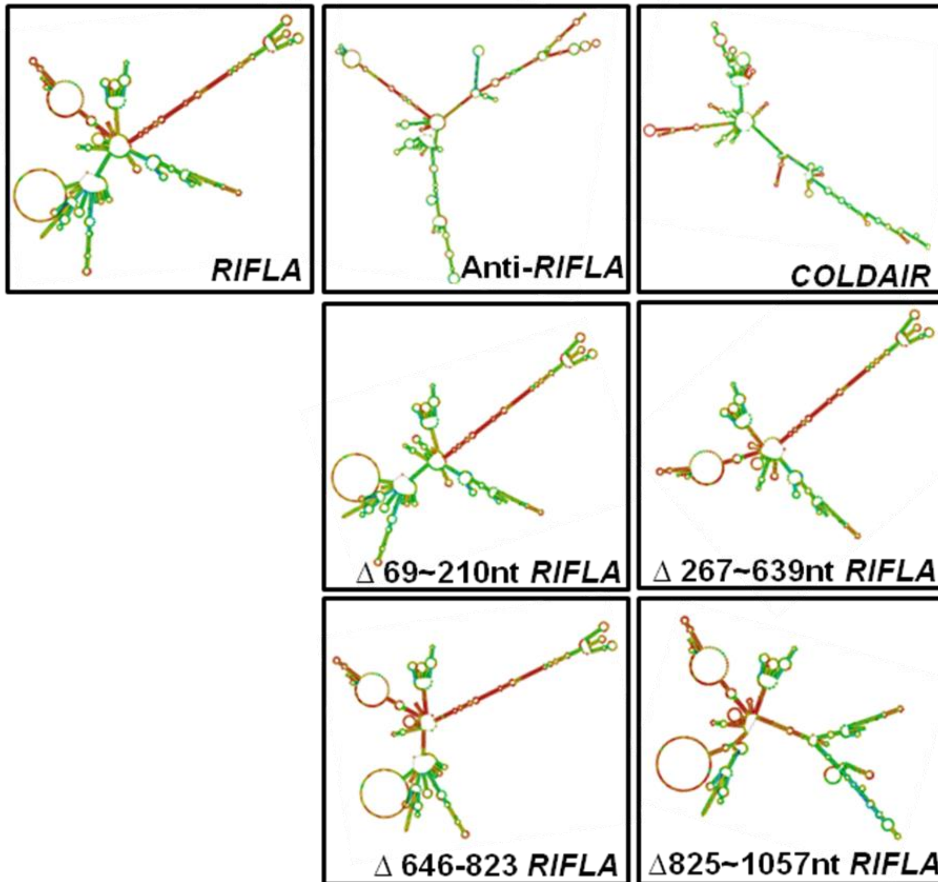

Figure 3. Secondary structure of *RIFLA* and its derivatives

An artificial stem-loop structure of *RIFLA* and its derivatives**.** Predicted RNA secondary structure of *RIFLA* and its derivatives. Structures were predicted via the Vienna RNAfold web server. The heat map indicates the probability of second structure formation, from low (green) to high (red).

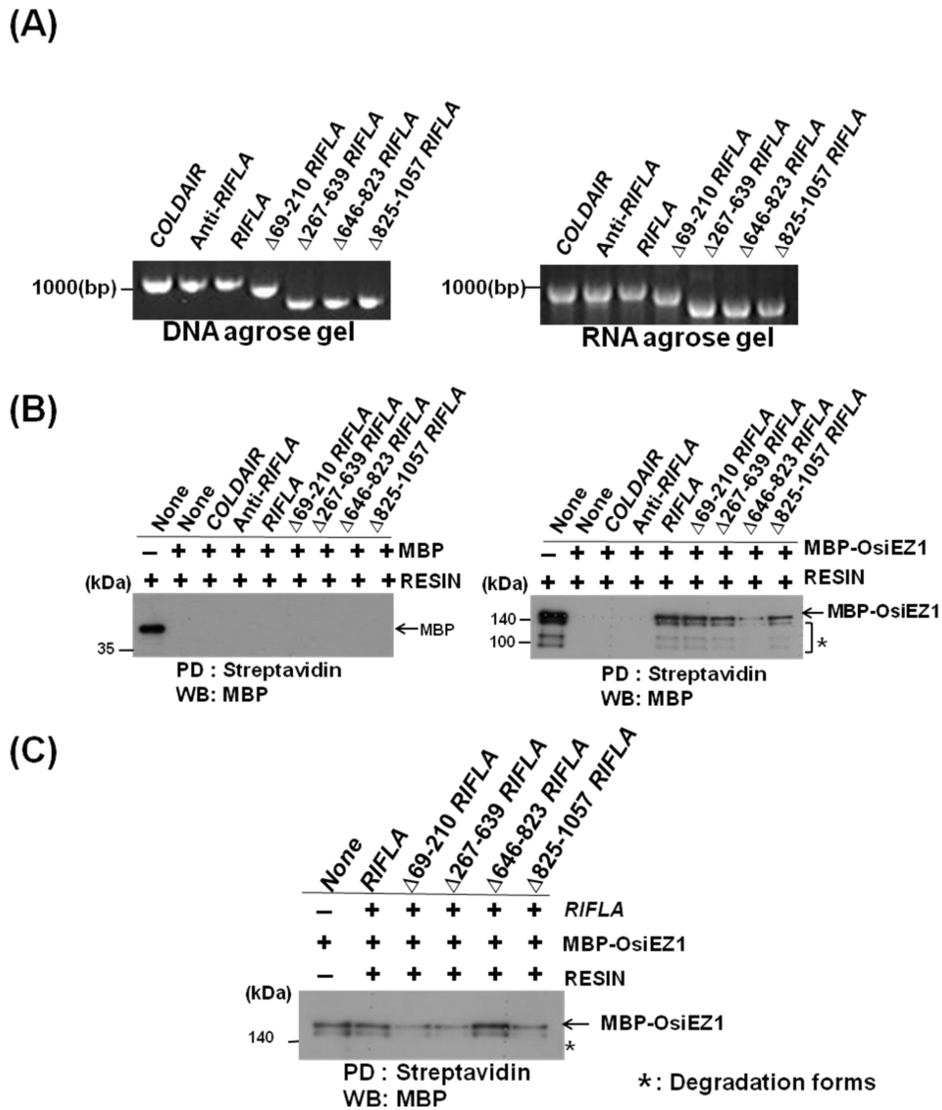

Figure 4. ***in vitro*** **pull-down assays** ***RIFLA*** **and its derivatives**

Rice histone methyltransferase OsiEZ1 physically interacts with *RIFLA*. (A) *RIFLA*, anti-*RIFLA*, arabidopsis *COLDAIR* and three of delete *RIFLA* form. *RIFLA* was deleted sequence. Each 68~210nt, 267~639nt, 646~823nt, 825~1057nt sequence was deleted from whole *RIFLA* sequence. (B)MBP-OsiEZ1 was pulled down with biontinylated *RIFLA*. MBP-OsiEZ1 absorbed with biontinylated *RIFLA* was detected using western blotting with anti-MBP antibody. (C) MBP-OsiEZ1 was pulled down with biontinylated *RIFLA* and included RIFLA for competition.

MBP-OsiEZ1 absorbed with biontinylated *RIFLA* was detected using western blotting with anti-MBP antibody.

OsiEZ1 physically interacted with OsSAP, Histone H3 and OsGlutelin mediated by *RIFLA*

It is known that ncRNAs should associate with PRC2 chromatin-modifying complex to exert their function. To identify the protein working with *RIFLA*, we performed yeast two hybrid assay. To do this, we check the OsSAP, Histone H3 and OsGlutelin that were isolated as binding protein of *RIFLA* by yeast three hybrid assay. One of them was SAP domain-containing protein which the function was not unknown yet. Since OsiEZ1 binds to *RILFA*, we examined whether SAP domain protein can interact with OsiEZ1. To do this, yeast two-hybrid assay was performed to identify this interaction. The full-length cDNAs of OsiEZ1 and OsSAP were cloned into yeast expression vectors. After introducing the constructs into yeast strain AH109, we examined the protein interactions. we found that OsiEZ1 and OsSAP doesn't interact with OsiEZ1 (Fig.5).

There are many lncRNAs that known as scaffolds to make a number of proteins into a complex or spatial proximity and may also act as recruiting proteins to guide, such as chromatin modification enzymes. To identify that *RIFLA* that mediates the association with OsiEZ1 and other proteins, we utilized an *in vitro* RNA binding assay. For this experiment, two recombinant plasmids expressing MBP-tagged OsiEZ1 and GST-SAP domain protein, Histone H3 and Glutelin were generated; overexpression of these tagged proteins in *E. coli* was induced by IPTG treatment, and the proteins were purified with glutathione and amylose resins, respectively. After purification, *in vitro* pull-down assays were performed using MBP-SAP domain protein and GST-OsiEZ1 was detected using an anti-MBP antibody. Result showed that SAP domain protein did not interact with OsiEZ1. But, we found that OsiEZ1 physically interacted with OsSAP, Histone H3 and OsGlutelin mediated by

RIFLA(Fig. 6). Thus, we hypothesized that if RIFLA is added, OsiEZ1 and SAP domain protein can form complex. In this case, RIFLA may function as a bridge.

We therefore examined specific motifs of RIFLA for interaction between OsiEZ1 and SAP domain. To end this, we also performed *in vitro* pull-down assays using MBP-SAP domain protein with RIFLA then detected MBP-SAP by western blot using an MBP antibody. Interestingly, we found that *RIFLA* without 267-639 base fragment specifically interacts with SAP domain protein (Fig. 7A) and GST-SAP is specific than MBP-OsiEZ1 for complex(Fig. 7B).

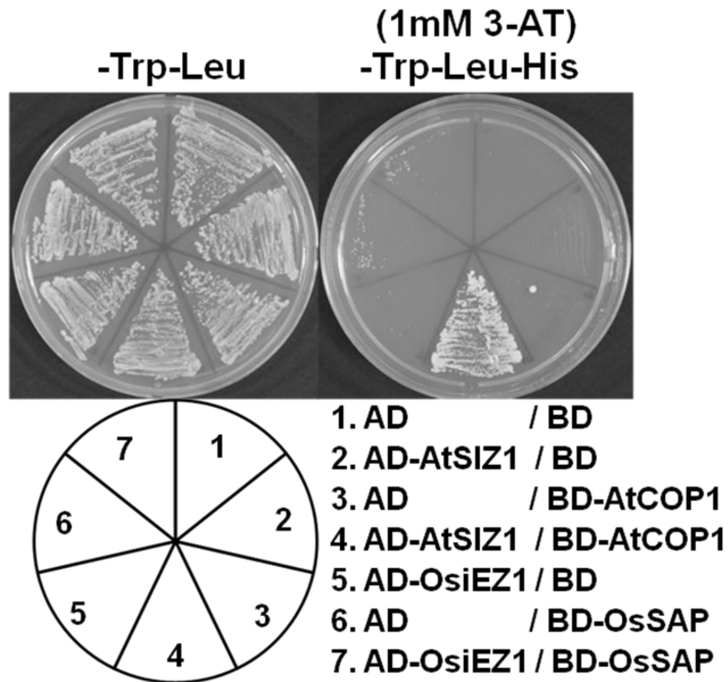

Figure 5. Interaction analyses between SAP domain protein and OsiEZ1

Full-length OsiEZ1 was inserted to region encoding the Gal4 AD in pGAD424. OsSAP cDNAs was inserted to region encoding the Gal4 DNA BD in pGBT9. Recombinant plasmids were transformed into AH109, the yeast strain. Numbers indicate the yeast cells transformed with pGAD424 and pGBT9 vectors or with recombinant plasmids. Transformants were spreaded on to media, –Leu/–Trp and –Leu/–Trp/–His/+ 1mM 3-AT, and incubated for 5 days. AD-COP1 and BD-SIZ1 were used as positive control.

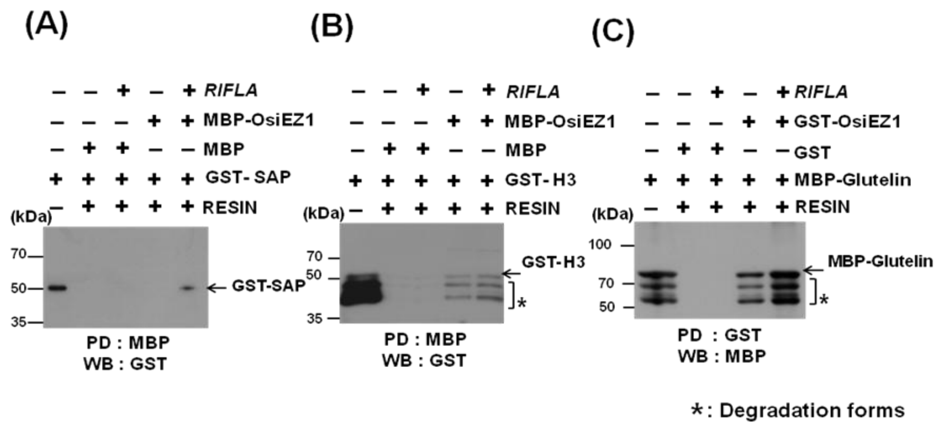

Figure 6. *in vitro* pull-down assays using SAP domain protein, Histone H3 protein, glutelin and MBP-OsiEZ1 with or without RIFLA

(A) *in vitro* pull-down assays using GST-OsSAP domain protein and MBP-OsiEZ1 with or without RIFLA and then detected GST-OsSAP by western blot using an anti-GST antibody. (B) *in vitro* pull-down assays using GST-Histone H3 protein and MBP-OsiEZ1 with or without RIFLA and then detected GST-Histone H3 by western blot using an anti-GST antibody. (C) *in vitro* pull-down assays using MBP-Glutelin protein and GST-OsiEZ1 with or without *RIFLA* and then detected MBP-Glutelin by western blot using an anti-MBP antibody.

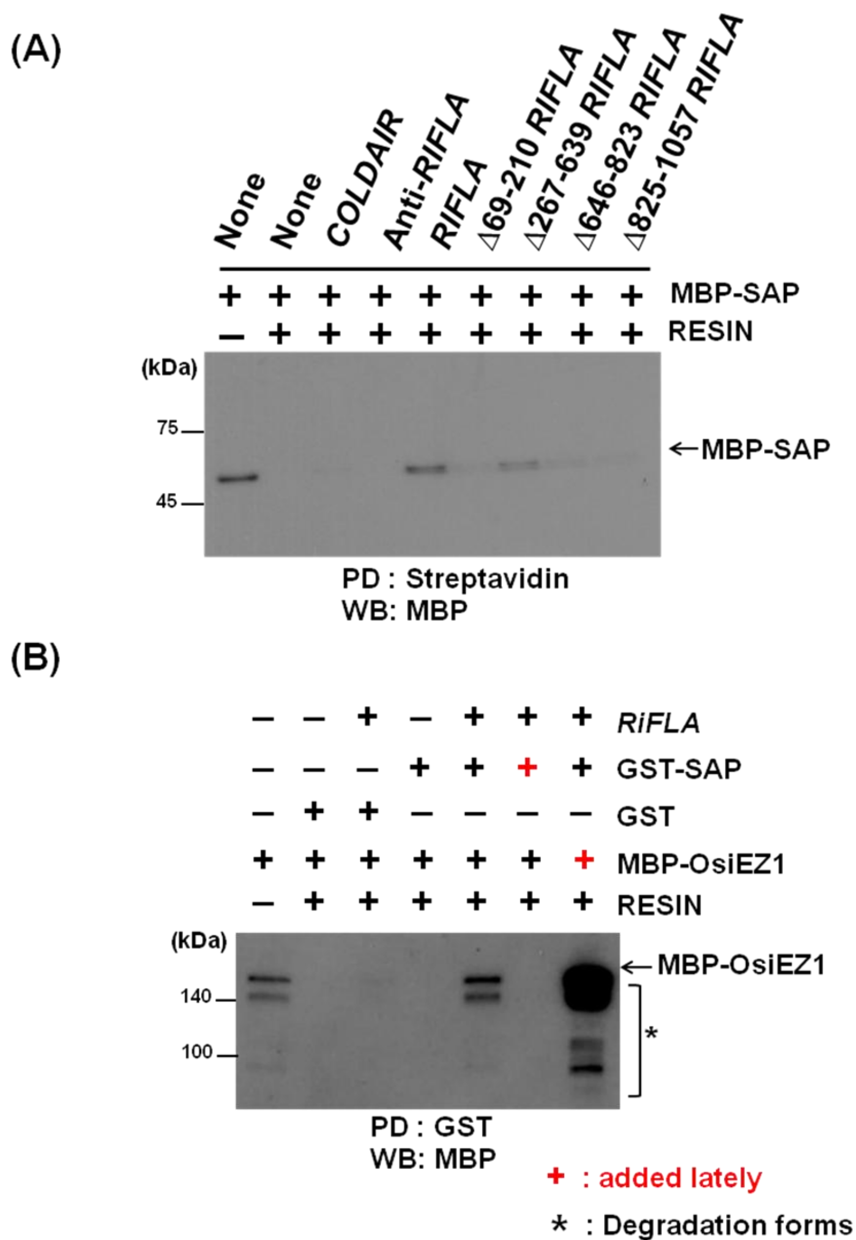

Figure 7. ***In vitro*** **binding assays using domains of** ***RIFLA*** **and time course**

(A)MBP-OsSAP domain protein was pulled down with biontinylated *RIFLA* and its derivatives. MPB-OsiEZ1 absorbed with biontinylated *RIFLA* was detected using western blot with MBP antibody. (B) *in vitro* pull-down assays using GST-

OsSAP domain protein and MBP-OsiEZ1 with or without *RIFLA* in time course and detected GST-OsSAP by western blotting using an MBP antibody.

RIFLA specifically interacts with SANT and CXC domain of OsiEZ1

Arabidopsis *SANT and CXC domain* interact with COLDAIR. N-terminal region of CLF is required for CLF-COLDAIR interaction. To confirm the domain of OsiEZ1 that associates the interaction with RIFLA, we studied an *in vitro* binding assay. To prove this, we prepared recombinant DNA *MBP-OsiEZ1* and *MBP-OsiEZ1 derivatives* and then introduced it into *E.coli*. After overexpression by IPTG treatment, MBP-OsiEZ1 was purified with amylose column. For examination their interaction, MBP-OsiEZ1 or MBP-OsiEZ1 derivatives with Biotin-RIFLA were mixed and pull-downed with streptavidin resin. After elution, MBP-OsiEZ1 and MBP-OsiEZ1 derivatives were detected by western blot with anti-MBP antibody. These results indicate that, *RIFLA* specifically interacts with SANT and CXC domain of OsiEZ1. To examine domain of OsiEZ1 for complex, MBP-OsiEZ1 or MBP-OsiEZ1 derivatives and GST-SAP with *RIFLA* were mixed and pull-downed with glutathione resin. After elution, MBP-OsiEZ1 and MBP-OsiEZ1 derivatives were detected by western blot with anti-MBP antibody. These results indicate that, *RIFLA* specifically interacts with SANT and CXC domain of OsiEZ1.

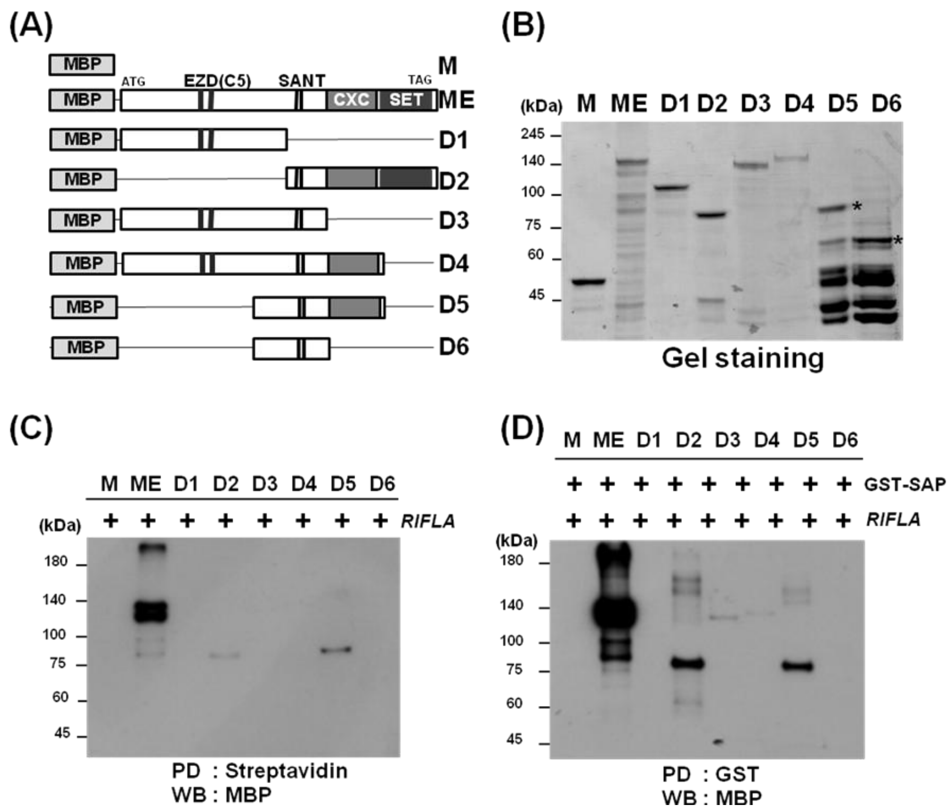

Figure 8. Schematic diagram of OsiEZ1 and its derivatives and *In vitro* binding assays using domains of OsiEZ1

(A) The schematic diagram of full-length or a series of deletion constructs of OsiEZ1 used for protein interaction assay. Numbers denote the deletions relative to the full-length OsiEZ1 amino acid sequences. EZD(C5); EZ domain, SANT; DNA binding domain, CXC; cysteine-rich domain, SET; SET-domain. **(B)** Each proteins were identified on SDS PAGE Gel and then stained using Coomassie blue. (C) MBP-OsiEZ1 and its derivatives protein was pulled down with biontinylated *RIFLA*. Each proteins absorbed with biontinylated *RIFLA* was detected using western blotting with anti-MBP antibody. **(D)** MBP-OsiEZ1 and its derivatives protein and GST-SAP was pulled down with *RIFLA*. Each proteins absorbed with GST-SAP was detected using western blot with anti-MBP antibody.

OsiEZ1 physically interacts with OsSIZ1 and OsSIZ2

A large number of proteins can be modified by SUMO and their activity can be modulated by sumoylation. Recently, we also reported that the activity of DNA methyltransferase CMT3 is stimulated by sumoylation through AtSIZ1 activity (Kim et al., 2015). From these data, we reasoned that the activity of OsiEZ1 can also be regulated by sumoylation. Small ubiquitin-related modifier (SUMO) is a small modulator that regulates abiotic and biotic responses and hormone signaling in plants. Dwarf plants of arabidopsis *siz1-2* flower early, show abnormal seed development. To identify the phenotype of ossiz, we identified a T-DNA insertional mutant line, 3A-13223, in which T-DNA was inserted at the second exon of *OsSIZ2*(Fig. 10A). PCR analyses showed that *ossiz2* mutant line indicating that these are knock-out alleles(Fig. 10B). Here we show the dwarf phenotype of ossiz2 that OsSIZ regulates development stages in Oryza sativa through its E3 SUMO ligase function(Fig. 10C). OsSIZ1 and OsSIZ2 is a rice homologs of Arabidopsis Siz1. Thus to determine whether OsSIZ1 and OsSIZ2 can interact with OsiEZ1. To do this, we confirm this interaction through yeast two-hybrid assay. The full-length cDNAs of OsiEZ1, *OsSIZ1* and OsSIZ2 were cloned into yeast expression vectors. After introducing the constructs into yeast strain AH109, we examined the protein interactions. we found that OsSIZ1 and OsSIZ2 strongly interacted with OsiEZ1 (Fig. 9A). The direct interaction between OsiEZ1 and Rice E3 SUMO ligase OsSIZ1 and OsSIZ2 and similar expression level(Fig 9B) suggested that OsSIZ1 and OsSIZ2 may act as an E3 SUMO ligase for OsiEZ1.

OsSIZ2 regulates OsiEZ1, RIFLA and OsMADS56

We predict that OsiEZ1 activity is stimulated by sumoylation through E3 ligase activity of OsSIZ1. Thus, we now want to know whether *OsiEZ1* expression can be controlled by OsSIZ1 or not. To end this, we examined *OsiEZ1* transcript level by qRT-PCR. As a result, we found that *OsiEZ1* transcript level was decreased in *Ossiz2* mutants (Fig. 10D), suggesting that OsSIZ2 positively controls OsiEZ1 expression as well as its activity. In addition, we also found that *RIFLA* expression level is similar with OsiEZ1 expression level but OsMADS56(Fig. 10D), suggesting that OsiEZ1 and RIFLA can control OsMads56 expression.

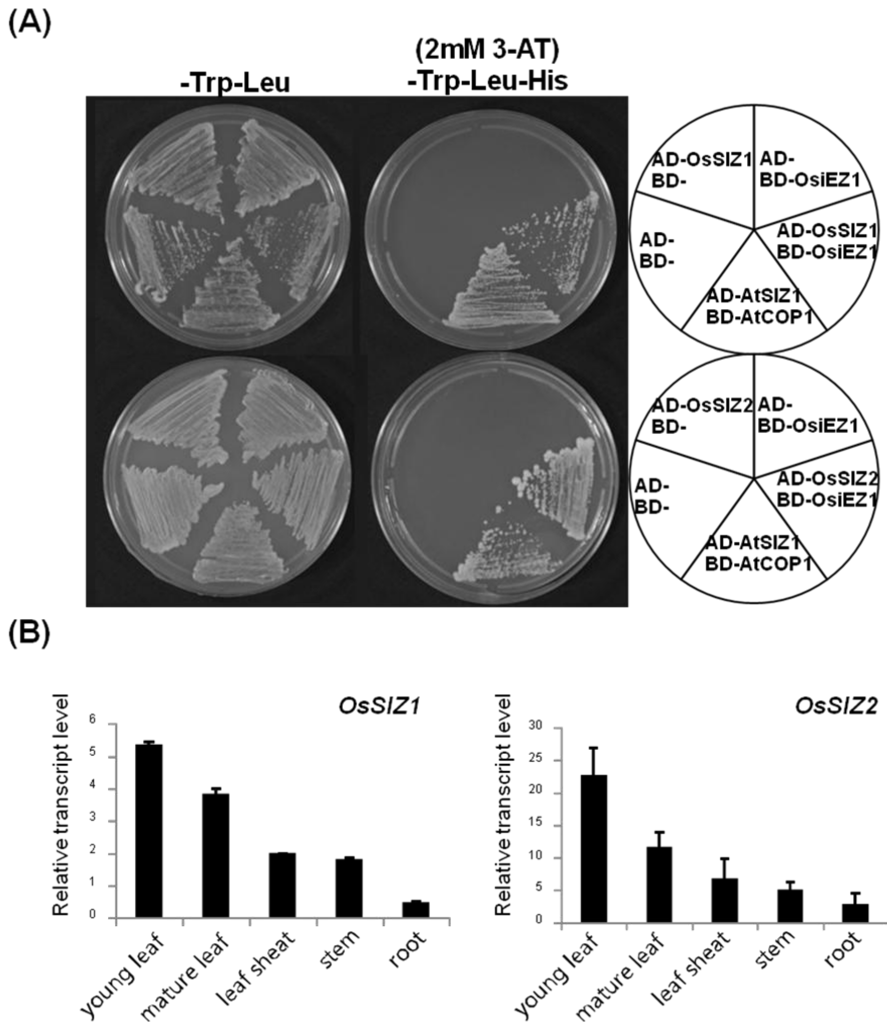

Figure 9. Interaction analyses between OsSIZ1, OsSIZ2 and OsiEZ1

(A)Full-length OsSIZ1 and OsSIZ2 was inserted into region encoding the Gal4 AD in pGAD424. OsiEZ1 cDNAs was inserted into region encoding the Gal4 DNA BD in Pgbt9. Constructs were transformed into AH109 yeast cell strain. Numbers indicate the yeast cells transformed with pGBT9 and pGAD424 vectors or with recombinant plasmids. Transformants were plated on to media, Leu/–Trp and –Leu/–Trp/–His/+ 2mM 3-AT, and incubated for 5 days. AD-COP1 and BD-SIZ1 were used as positive control. (B) Relative transcript levels of *OsSIZ1* and *OsSIZ2*.

Plants were grown under continuous light during 10 days and transferred under short day conditions. After further incubation for 10 days, total RNAs were isolated from leaves of each sample and then the transcript levels of *OsSIZ1* and *OsSIZ2* genes were examined by qRT-PCR.

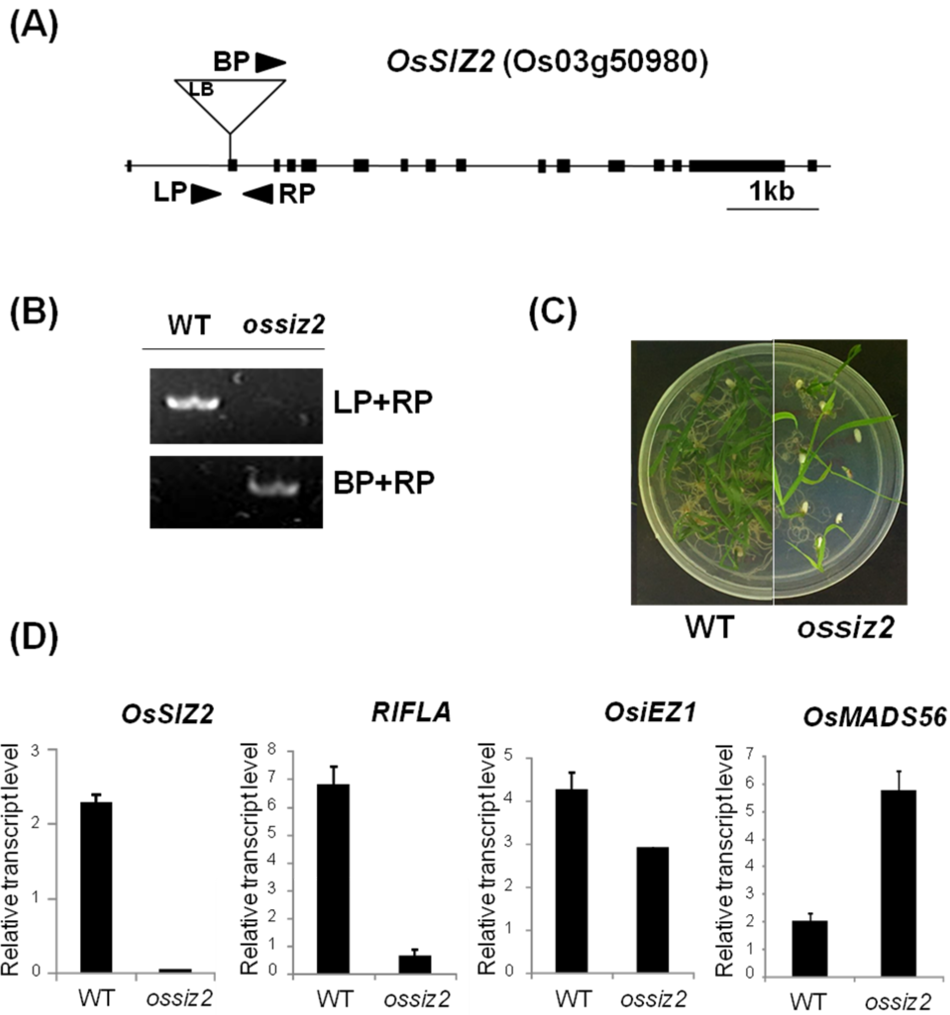

Figure 10. Schematic diagram of *OsSIZ2* genomic structure and the regions of T-DNA insertional mutant alleles and relative transcript levels

(A) Schematic diagram of *OsSIZ2* genomic structure and the regions of T-DNA insertional mutant allele. *OsSIZ2* (Os03g50980) consists of sixteen exons (boxes) and fifteen introns (lines between boxes). T-DNAs were inserted into the second exon in *ossiz2*(Line 3A-13223), respectively. Arrows indicate LP and RP primers used for amplification of gDNA. LB is left border of T-DNA. (B) PCR verification of the homozygous T-DNA insertion alleles of *ossiz2*. (C) Relative transcript levels

of *OsSIZ2, RIFLA*, *OsiEZ1* and *OsMADS56*. Plants were grown under continuous light during 10 days and transferred under short day conditions. After further incubation for 10 days, total RNAs were isolated from leaves of each sample and then the transcript levels of *OsSIZ2, RIFLA*, *OsiEZ1* and *OsMADS56* genes were examined by qRT-PCR.

DISCUSSION

OsMADS56 and _RIFLA_ expression pattern are in complementary relation

RIFLA transcripted from OsMADS56 first intron is necessary regulator. OsMADS56 may regulate *RIFLA* transcription level. We then check the expression level of OsMADS56 in transgenic line(Fig.1C). These results suggest that OsMADS56 and RIFLA role is in complementary relations. Opposite expression pattern between *OsMADS56* and *RIFLA* suggests that *OsMADS56* expression can be regulated by RIFLA. This suggests that OsiEZ1 negatively control *OsMADS56* expression and that *RIFLA* expression is regulated by OsiEZ1.

LncRNA RIFLA acts a linker

In this results, we researched the role of the lncRNA *RIFLA* in interactions with PRC2 and lncRNA binding proteins. There are similar lncRNAs mechanisms known to be interacted with complex and it roles regulator of gene. The 646-823base fragment of RIFLA is necessary for the OsiEZ1 association(Fig. 4) and RIFLA specifically interacts with SANT and CXC domain of OsiEZ1(Fig. 8). Together, OsiEZ1 physically interacted with OsSAP, Histone H3 and OsGlutelin mediated by RIFLA(Fig. 6)

OsiEZ1 can be modulated by sumoylation

E3 SUMO ligase study have been performed because SIZ1 was first identified in *Arabidopsis*. One of the most prominent features of *ossiz2* mutants is their dwarf(Fig. 10C), suggesting impairment of the development and flowering pathways. Therefore, we investigated whether an OsSIZ1 and OsSIZ2 acts as an E3

SUMO ligase on OsiEZ1(Fig. 9). Thus, it strongly suggests that sumoylatied OsiEZ1 acts a positive flowering controler in rice. The present study highlights the notion that flowering repressor *OsMADS56* is mediated by OsiEZ1, lncRNA *RIFLA* and *RIFLA* interacting protein and it is positively regulated by sumoylation. LncRNA-dependent flowering processes is controlled by OsiEZ1 proteins, and OsMADS56 repressor activities are modulated by lncRNA complex via the E3 ligase activity of OsiEZ1(Fig. 10). Such observations have led researchers to focus on OsiEZ1 proteins to elucidate flowering signaling. Therefore, our results indicate that sumoylation is necessary regulation for *RIFLA* function in flowering pathway.

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초 록

후성 유전학를 설명할 수 있는 메커니즘 중 하나는 메틸화이다. 메틸화은 메틸전이인자에 의해 메틸기가 붙는 기질에 따라 크게 두가지로 나눠서 설명할 수 있다. 사이토신에 메틸기가 붙으면 DNA메틸화로 불리며 보통 유전자의 발현을 억제한다. 또 히스톤 아미노산 중 arginine이나 lysine잔기에 메틸기가 붙으면 히스톤메틸화로서 유전자의 발현을 억제하거나 활성화 한다. 이와 같은 메커니즘을 가지는 메틸화는 식물체의 전반적인 생장과 발달에 많은 영향을 끼친다. 그 중 히스톤메틸화와 관련하여 개화조절에 대해서 연구하였다. 애기장대에서 CURLY LEAF (CLF)는 Polycomb Repressive Complex 2 (PRC2)를 이루는 히스톤 메틸전이인자로 histone H3 lysine 27 (H3K27)에 trimethylation을 통해 타겟 유전자의 발현을 조절한다. 하지만, 벼에서 애기장대의 CLF homology인 OsiEZ1(rice enhancer of zeste rice)가 target gene을 메틸화 시키고 gene silencing 시키는 것에 대해선 밝혀지지 않았다. 따라서 저자는 OsiEZ1가 애기장대의 FLC homolog인 rice MADS-box protein gene 56 (*OsMADS56*)의 발현을 조절하는지 보고자 하였다. 먼저 Long noncoding RNA (lncRNA)인 *RIFLA*(RICE FLOWERING-ASSOCIATED) 과다발현체에서 상대적인 유전자 발현량 차이를 통해 *RIFLA*와 *OsMADS56*의 발현이 상반된다는 것을 확인하였다. *OsMADS56*의 첫번째 인트론에서 발현되는 *RIFLA*는 OsiEZ1과 특이적이로 결합 할 뿐 아니라 histone H3, SAP domain 단백질 그리고 glutelin 단백

질과도 특이적 결합을 보였다. 더욱이 OsiEZ1이 각 단백질과의 결합에서 *RIFLA*가 결합력을 높여주는 것을 확인하였다. 그 뿐만 아니라 OsiEZ1는 벼의 E3 SUMO ligases인 OsSIZ1, OsSIZ2와 강하게 결합하는 것을 통해 OsiEZ1와 lncRNA *RIFLA*매개로 target gene을 메틸화시키는 과정에서 OsiEZ1이 E3 SUMO ligase OsSIZ1 and OsSIZ2에 의해 조절 받는 다는 것을 확인 할 수 있었다. 결론적으로, 결과들을 통해 OsiEZ1이 lncRNA *RIFLA*와 직접적인 결합을 통해 OsMADS56발현을 조절한다는 것과 OsiEZ1활성이 수모화에 의해 조절 될 것을 확인 할 수 있었다.

주요어 : FLOWERING LOCUS C (FLC); CURLY LEAF (CLF); Rice enhancer of zeste rice(OsiEZ1); Rice MADS-box protein gene 56 (OsMADS56); Long noncoding RNA (lncRNA) RIFLA

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