A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

E3 SUMO ligase AtSIZ1 regulates FLC-mediated flowering repression

By

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MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY

DEPARTMENT OF PLANT SCIENCE

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UNDER THE DIRECTION OF DR. HAK SOO SEO
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ABSTRACT

Flowering Locus C (FLC), a floral repressor, is antagonistic regulator for transition from vegetative to the reproductive phase. FLC represses expression of several genes involved in floral induction and its transcription is positively or negatively regulated by *FRIGIDA* or by vernalization, respectively. Although several factors affecting the transcription of *FLC* have been described, the post-translational regulation of FLC stability and function has not been clearly characterized. Here, I investigated the mechanisms regulating the activity and stability of the FLC protein.

Sumoylation, a post translational process of attaching small ubiquitin-related modifier (SUMO) to a lysine residue in proteins through an enzyme cascade catalyzed by E1, E2 and E3 enzymes, plays crucial role in protein stability and

biological processes.

Bimolecular fluorescence complementation, co-immunoprecipitation and *in vitro* pull down analysis showed that FLC interacts with the E3 small ubiquitin-like modifier (SUMO) ligase AtSIZ1, suggesting that AtSIZ1 is an E3 SUMO ligase for FLC. *In vitro* sumoylation assays showed that FLC is modified by SUMO in presence of SUMO-activating enzyme E1 and conjugating enzyme E2 but its sumoylation is inhibited by AtSIZ1. In transgenic plants, inducible AtSIZ1 overexpression led to an increase in the concentration of FLC and delayed the post-translational decay of FLC, indicating that AtSIZ1 stabilizes FLC through direct binding. Also, the flowering time in mutant FLC (K154R, a mutation of sumoylation site)-overexpressing plants was comparable to that in wild type, whereas flowering was considerably delayed in FLC-overexpressing plants, supporting the notion that sumoylation is an important mechanism for FLC function. These results indicate that the sumoylation of FLC is critical for its role in the control flowering time and AtSIZ1 positively regulates FLC-mediated floral suppression.

Key words: FLC**,** Flowering, AtSIZ1, Post-translational modification, SUMO, Sumoylation

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LIST OF ABBREVIATIONS

BiFC	bimolecular fluorescence complementation
CHX	Cycloheximide
DTT	Dithiothreitol
EYFP	enhanced yellow fluorescent protein
GST	glutathione *S*-transferase
MBP	Maltose-binding protein
HA	Haemagglutinin
MS	Murashige and Skoog
35S	Cauliflower mosaic virus promoter
AD	Activation domain
BD	DNA binding domain
E.coli	Escherichia coli
NOS	nopalin synthase terminator
IAA4	indoleacetic acid 4
IPTG	isopropyl-β-D-thiogalactoside
PMSF	phenylmethylsulphonyl fluoride
SUMO	small ubiquitin-related modifier
XVE	estradiol-inducible promoter
kDa	KiloDalton

PCR	Polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SAP	Scaffold attachment factor A/B//acinus/PIAS domain
WT	wild type
cDNA	Complementary DNA
RNA	Ribonucleic acid

LITERATURAL REVIEWS

1. SUMOylation

1.1. The SUMOylation pathway

SUMO is a small (~12kDa) protein found in all eukaryotes and involved in several cellular mechanism including nuclear-cytoplasmic shuttling, transcription modulation, maintenance of genome integrity, regulation of cell cycle, protein-protein interaction and diverse biological processes (Hoege *et al*., 2002, Ross et al.,. 2002, Sapetschnig *et al*., 2002, Stade *et al*., 2002, Nacerddine *et al*., 2005, Pfander *et al*., 2005).

Sumoylation is a reversible modification which covalently attaches SUMO to a target protein followed by a cascade of enzyme reactions including the steps of E1-activation, E2-conjugation, E3-ligation and deconjugation (Bayer *et al*., 1998). SUMO is matured through releasing Gly-Gly motif in its C-terminus by the specific Ulps (ubiquitin-like proteases) and then SUMO is activated in the presence of ATP, hetrodimeric E1 (SAE1, SAE2) catalyze the formation of cysteine(C) residue in SAE2 through high-energy thioester bond. In the next step, activated SUMO is transferred to a cysteine residue in Conjugating enzyme E2 (SCE1) through transesterification catalyzed by SCE1. Subsequently, SUMO is transferred to the substrate through an isopeptide linkage between C-terminal glycine residue in SUMO and ε-amino group of the target lysine side chain in the target protein's sumoylation consensus motif

(ψKXE/D, ψ, large hydrophobic residue; K, lysine; X, any amino acid; E/D, glutamic acid or aspartic acid). This step is facilitated by E3 ligase enzyme even if E3-independent transfer is possible. SUMO specific proteases (ULPs) cleave the SUMO-substrate linkages to recycle free SUMO, as well as involved in generating mature SUMO (Figure 1).

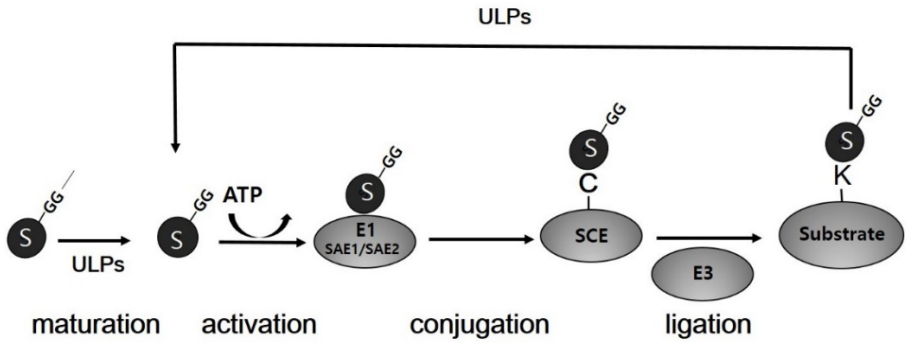


Figure 1. The SUMOylation pathway.

The mechanism of reversible sumoylation. SUMO is synthesized as a precursor and matured by SUMO proteases. Matured SUMO covalently attached to the substrate followed by a cascade of enzyme reactions including E1-activation, E2-conjuation, E3-ligation, in sequence manner. SUMO is deconjugated from substrate by SUMO proteases and recycled to participate in mature step.

1.2. Components of the SUMO pathway in Arabidopsis

Arabidopsis genome analyses identified the genes encoding eight SUMOs (*AtSUMO1-AtSUMO8*) and one SUMO-like psedogene (*AtSUMO9*), but expression of only four paralogues (*SUMO1/2*, *SUMO3* and *5*) has been confirmed (Kurepa *et al*., 2003). The SUMO E1 enzyme is a heterodimer consisting of two small subunit (SAE1a, SAE1b) isoforms and one large subunit SAE2. SAE1 isoforms, SAE1a and SAE1b share 80% identical amino acid sequence, suggesting functional redundancy (Saracco *et al*., 2007).

The SUMO E2 enzyme encoded by single copy of gene though there are many Ub E2. Transcripts of *SAE1a/b* and *SCE1* are highly expressed in many tissue including seedling, cotyledon, root, stem, shoot tip, leaf, flower and Silique. In addition, SCE1 and SUMO are colocalized in nucleus indicating that sumoylation reactions occur in nucleus (Lois *et al*., 2010). Null T-DNA insertion mutants of *SAE2*, *SCE* or both *SUMO1* and *SUMO2* showed embryonic lethal, with arrested in early-stage embryos (globular, heart, torpedo), indicating that sumoylation is essential in *Arabidopsis* (Saracco *et al.*, 2007).

Although there are many E3 ligases in animal, fungi and yeast (Johnson and Gupta 2001, Kahyo *et al*., 2001, Rose and Meier 2001, Pichler *et al.*, 2002, Kagey *et al*., 2003), only two SUMO ligases [AtSIZ1 (PIAS SUMO E3 ligase) and AtMMS21/HPY2 (Mms21 SUMO E3 ligase)] are characterized

in *Arabidopsis*. Loss of function mutants of SIZ1 and HPY2 showed pleiotropic phenotype indicating the importance of E3 ligases in sumoylation pathway (Miura *et al.*, 2005, Catala *et al*., 2007, Jin *et al*., 2008, Huang *et al.*, 2009, Ishida *et al.*, 2009, Miura *et al*., 2010).

Four *Arabidopsis* SUMO specific proteases (AtULPla, AtULP1c, AtULP1d and AtESD4) have been characterized so far (Murtas *et al*., 2003, Colby *et al*., 2006, Conti *et al.*, 2008). These proteases not only have peptidase activity for cleavage of pre-SUMO at the C-terminus to expose the Gly-Gly motif, but also isopeptidase activity for deconjugation of sumoylated protein to recycle pre-SUMO (Hay *et al*., 2007). However, these proteases have different SUMO isoform discrimination and enzymatic activities (Chosed *et al*., 2006, Colby *et al*., 2006).

Name	Chromsome locus	location	Loss-of-fuction allele	Developmental phenotype
SUMO isoforms				
SUM1	At4g26840	cytoplasmic/nuclear	*sum1-1*	Wild type
SUM2	At5g55160		*sum2-1*	Wild type
			sum1-1 sum2-1	Embryo lethal
SUM3	At5g55170	cytoplasmic/nuclear	*sum3-1*	Late flowering
SUM4	At5g48710			
SUM5	At2g32765			
SUM6	At5g48700			
SUM7	At5g55855			
SUM8	N.A			
SUM9	N.A			
SUMO activating enzyme E1				
SAE1a	At3g57870		*sae1a-1*	Wild type
SAE1b	At5g50580			
SAE2	At2g21470		*sae2-1*	Embryo lethal
SUMO conjugating enzyme E2				
SCE	At3g57870	cytoplasmic/nuclear	*sce1-5, sce1-6*	Embryo lethal
E3 SUMO ligase				
SIZ1	At5g60410	nuclear/nuclear foci	*siz1-1, siz1-2, siz1-3*	pleiotropic
MMS21/HPY2	At3g15150	nuclear	*hpy2-1 hpy2-2*	pleiotropic
SUMO protease				
ULP1a/ELS1	At3g06910		*els1-1, els1-2*	slightly smaller
			esd4-2 els1-1	Pleiotropic
ULP1b	At4g00690			
ULP1c/OTS2	At1g10570	nuclear/nuclear foci	*ots2-1*	Wild type
ULP1c/OTS1	At1g60220	nuclear	*ots1-1*	Wild type
			ots1-1 ots2-1	Early flowering
ESD4	At4g15880	nuclear envelope	*esd4-1, esd4-2*	Pleiotropic
ULP2a	At4g33620			
ULP2b	At1g09730			

Table 1. *Arabidopsis* proteins of the SUMO pathway

2. Role of the SUMO in plants

2.1. Responses to abiotic stress

Sumoylation plays crucial role in a variety of abiotic stress responses. The accumulation of SUMO conjugates was induced by heat, cold, drought, salt, exposure to excessive copper, incubation with H_2O_2 or ethanol, implying that sumoylation participates in stress protection and recovery (Kurepa *et al*., 2003, Miura *et al*., 2005, Yoo *et al*., 2006, Catala *et al*., 2007, Saracco *et al*., 2007, Conti *et al*., 2008, Chen *et al*., 2011).

Loss of function SIZ1 mutants showed the phenotypes of short primary roots, increased number of lateral roots, root hairs and greater anthocyanin accumulation sensitively in response to pi starvation. The expression of low-Pi transcripts of *AtIPS1* and *AtRNS1* was reduced in *siz1-2* mutants. PHR1(phosphate starvation response 1), a transcriptional regulator of *AtIPS1* and *AtRNS1*, is sumoylated by AtSIZ1 *in vitro*, indicating that AtIPS1 and AtRNS1 are positively controlled through sumoylation of PHR1 by AtSIZ1 (Miura *et al*., 2005).

SUMO conjugates are accumulated under drought stress. The expression of *P5CS1*, *MYC2*, *COR15A* and *KIN1* genes induced by drought stress was repressed in *siz1-3* mutants. It was suggested that SIZ1 plays a crucial role in drought stress response through the regulation of the genes

expression (Catala *et al*., 2007).

The *siz1-3*, SIZ1 deficient mutant, is sensitive to cold stress, and the cold-induced accumulation of sumo conjugates are reduced in the mutant, suggesting that SIZ1 plays an important role in cold tolerance. The expression of cold-induced genes such as *COR15A, COR47* and *KIN1* is regulated by transcription factor ICE1 (inducer of *CBF/DREB1* expression 1). ICE1 was sumoylated by AtSIZ1 and its modification repressed the expression of MYB15, a negative regulator of *CBF3/DREB1A*. This led to the expression of *CBF3/DREB1A* and its downstream genes, resulted in cold tolerance. SIZ1-dependent sumoylation of ICE1 inhibited its polyubiquitination and led to increase ICE1 stability (Dong *et al*., 2006, Miura *et al*., 2007, Ulrich *et al*., 2008).

Sumoylation of AtHsfA2, heat shock transcription factor, repressed its activity and transcription of *Psf101*, *Hsf17.6* and *Hsf17.4*, *HsfA2* target genes. Moreover, overexpression of SUMO in seedlings reduced tolerance on repeated heat treatment, indicating that sumoylation might act negatively upon acquired thermotolerance (Cohen-Peer *et al*., 2010). Conversely, SIZ1 is a positive regulator of Salicylic acid (SA)-independent basal thermotolerance but not of acquired tolerance. It suggested that the SIZ1-independent pathway involves in the acquired thermotolerance regulatory pathway (Yoo *et al*., 2006).

Previous reports have been suggested that sumoylation is related to salt stress response in *Arabidopsis*. *OTS1/OTS2*, salt stress-related genes,

double mutants showed increased salt sensitivity but not the single *ots1* or *ots2* mutant. Sumo conjugates are accumulated much higher in *ost1ost2* double mutants compared with wild-type plants. OTS1-overexpressing transgenic plants have increased salt tolerance and reduced sumoylation, but mutant overexpressing OST1 (C536S) protein lacking sumo protease activity failed to produce a salt tolerant phenotype. These results indicated that OST1 and OST2 are involved in sumo deconjugation and required for salt tolerance responses (Conti *et al*., 2008).

SIZ1-mediated sumoylation also involved in copper homeostasis and tolerance. *Atsiz1* mutants showed hyposensitivity to excessive copper and accumulated more copper in shoot tissue than wild type. Excessive copper induced more sumo conjugates in wild type than in *siz1* mutant, indicating that sumoylaton might be involved in the response on excessive copper. The expression of metal transporter genes of *YELLOW STRIPE-LIKE 1* (*YSL1*) and *YSL3* was down-regulated under excess copper stress in the *siz1* mutant. The hyposensitivity to excess copper and abnormal distribution of copper are diminished greatly in *siz1ysl3-1* and slightly in the *siz1ysl1* double mutants. These results suggesting that Cu-induced SIZ1 dependent sumoylaion involved in copper distribution and tolerance through transcriptional repression of *YSL1/3* (Chen *et al*., 2011).

2.2 Responses to hormone signaling

Sumoylation involved in modulation of ABA responses. Overexpression of AtSUMO1/2 reduced ABA-mediated root growth inhibition while co-suppression of AtSCE1a showed no significant differences. In addition, AtSUMO1- and AtSUMO2-overexpressing plants increased the expression of stress-responsive genes, *RDA29A* and *AtPLC1*, supporting that SUMO plays a role for ABA response (Lois *et al.*, 2003). *Atsiz1* mutants displayed the ABA hyposensitivity phenotype that germination and seedling primary root growth are inhibited. ABI5 (ABA intensive 5), ABA-responsive transcription factor, is sumoylated on Lys-391 by AtSIZ1. Expression of *ABI5(K391/R)* in *abi5-4* resulted in greater sensitivity to ABA compared with ABI5 expression indicating that SIZ1-mediated sumoylation on ABI5 negatively regulates ABA signaling (Miura *et al.*, 2009).

Auxin signaling is also regulated by sumoylation. The SIZ1 mutation caused the inhibition of primary root (PR) elongation and the promotion of lateral root (LR) formation. However, similar root phenotypes occurred if Pi-deficient wild-type seedlings were supplemented with auxin. N-1-naphthylphthalamic acid (NPA), an auxin efflux inhibitor, reduced the Pi starvation-induced lateral root formation in wild-type and *siz1* seedling. Pi starvation-induced Auxin accumulation in the PRs and LRs is more quickly in *siz1* than in wild-type seedlings. Moreover, expression of auxin-induced genes

is higher in *siz1* compared to wild-type in response to Pi starvation. These results indicating that SIZ1 negatively regulates Pi starvation-induced root architecture remodeling through the control of auxin patterning (Miura *et al*., 2005,2011).

Loss of SIZ1 function leads to accumulation of Salicylic acid (SA) and constitutive Pathogenesis-related (PR) genes expression, resulting in enhanced disease resistance to bacterial pathogens (Lee *et al*., 2007). These results suggesting SIZ1 controls SA-mediated plant defense signaling.

2.3 Control of flowering

SUMO-specific proteases and SUMO E3 ligase, SIZ1 were required for regulating flowering time (Reeves *et al*., 2002, Murtas *et al*., 2003, Xu *et al*., 2007, Conti *et al*., 2008, Hermkes *et al*., 2011). Mutation of SUMO protease ESD4 (early in short day 4) or its cooperative co-factor NUA caused extreme early flowering in short and long day resulting from decrease of mRNA level of a floral repressor *FLC* and increase of expression of downstream flowering-time genes such as *SOC1* and *FT*. The lack of *SIZ1* caused early flowering in short day. In *siz1* mutants, SA was accumulated and expression of PR gene was increased. The introduction of *NahG* gene encoding for salicylic hydroxylase into *siz1* mutant restored phenotypes of the mutant that of WT indicating that SIZ1 regulates flowering mainly through SA-dependent pathway (Lee *et al*.,

2007, Jin *et al.*, 2008). SIZ1 also regulated flowering through SA-independent pathway. Flowering locus D (FLD) is sumoylated by AtSIZ1 and induced deacetylation of histones in the FLC chromatin, leading to repression of FLC. These results indicated that SIZ1 was a negative regulator of flowering, which represses SA accumulation and promotes FLC expression by repressing FLD activity (Jin *et al*., 2008).

2.4. Defense reactions to pathogen attack

Reversible Sumo conjugation involved in plant-pathogen interaction and pathogen defense response. Upon injection of Trichoderma viride into the tobacco and tomato leaf mesophyll, the xylanase induces ethylene formation and cell death. A xylanase (EIX) from the fungus Trichoderma viride interacts with Tomato SUMO. Transgenic plants overexpressing T-SUMO in the sense orientation or co-injection of SUMO together with the xylanase, suppressed EIX induction of ethylene biosynthesis and cell death (Hanania *et al*., 1999). These results suggested that T-SUMO is involved in mediating the signal generated by EIX that leads to induction of plant defense responses.

XopD, an Xcv type III effector protein of pathogen bacteria possesses plant SUMO isopeptidase activity and decreases the level of sumo conjugates in plant extracts *in vitro* (Hotson *et al*., 2003). Similarly, Introduction of AvrXv4 in plant cell leads to decrease in abundance of sumo conjugates,

indicating that AvrXv4 possesses SUMO isopeptidase activity (Roden *et al*., 2004).

2.5. Metabolic regulation

Sumoylation involved in the regulation of nutrient metabolism. Nitrate contents and nitrate reductase activity were low in *siz1* mutants, and its pleotropic phenotype is recovered with ammonium ion treatment but not with nitrate indicating that the pleiotropic phenotype of *siz1* mutants caused by impairment of nitrate reduction due to decreased nitrate reductase activity. Nitrate reductases NIA1and NIA2 are sumoylated by AtSIZ1 which dramatically increases their activity. Thus SIZ1-denpedant sumoylation positively regulates nitrogen assimilation by promoting nitrate reductases activity (Park *et al*., 2011).

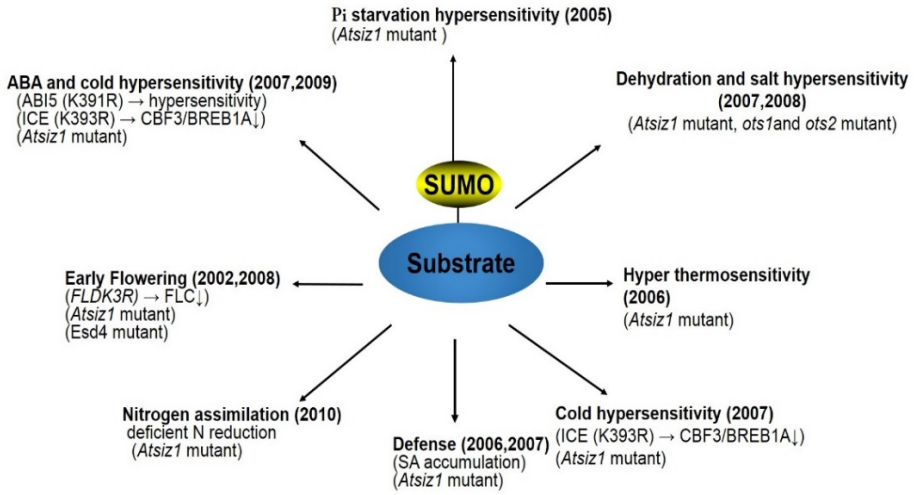


Figure 2. Diverse biological Functions of sumoylation in plants.

3. Regulation of flowering time in Arabidopsis

Flowering is the process of major developmental switch from vegetative to reproductive stage. A variety of environmental and endogenous cues influence the timing of floral induction through multiple genetic pathways (Battey et al., 2000, Simpson and Dean 2002, Izawa *et al*., 2003). In *Arabidopsis*, there are four major genetic pathways including photoperiod, gibberellin, autonomous and vernalization have been identified (figure 3). These pathways activate the expression of floral integrator genes [*FLOWERING LOCUS T* (*FT*), *LEAFY* (*LFY*), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*)] that cause floral transition by regulating the floral meristem identify (FMI) genes [*APETALA1* (*AP1*), *APETALA2* (*AP2*), *FRUITTULL* (*FUL*), *CAULIFLOWER* (*CAL*) and *LFY*] (Nilsson *et al*., 1998, Kardailsky *et al*., 1999, Kobayashi *et al*., 1999, Blázquez and Weigel 2000, Lee *et al*., 2000, Samach *et al*., 2000, Hepworth *et al*., 2002, Moon *et al*., 2003).

3.1. Photoperiod pathway

Arabidopsis is a facultative long day plant that flowering occurs faster under long days than short days. Floral transition of photoperiodic pathway genes was promoted under long day conditions and *CONSTANS* (*CO*),

CRYPTOCHROME2/FHA (*CRY2*), *GIGANTEA* (*GI*), *FT* and *FWA* are involved in this long day promoting pathway (Koornneef *et al*., 1991). *CO* encodes a zinc finger protein and plays a central role in photoperiodic pathway. It has been reported that *FT* and *FW* act downstream of *CO* (Kardailsky *et al*., 1999, Onouchi *et al*., 2000), whereas *CRY2* and *GI* act upstream of *CO* (Guo *et al*., 1998, Suárez-López *et al*., 2001). Also the rhythmic expression of *CO* transcript was controlled by circadian clock oscillators such as *CCA1*, *LHY*, *TOC1* and *ELF4* (Green and Tobin 1999, Strayer *et al*., 2000, Alabadí *et al*., 2001, Kikis *et al*., 2005) and clock- and light-regulated genes such as *GI* and *ELF3* (Fowler *et al*., 1999, Hicks *et al*., 2001). The photoreceptors PHYTOCHROME A (PHYA) and CRYPTOCHROME 2 (CRY2) are essential for the proper stabilization of CO protein, responding to red and blue light, respectively (Valverde *et al*., 2004). On the other hand, PHYTOCHROME B (PHYB) promotes degradation of CO. Accumulation of CO protein under Long day condition triggers the activation of FT expression that promotes floral transition (Hayama and Coupland 2004).

3.2. Gibberellin pathway

It has been extensively reviewed that the hormone gibberellin (GA), promotes flowering of *Arabidopsis* (Wilson *et al*., 1992, Blázquez *et al*., 1998). Mutation of either GA biosynthesis or signaling alters flowering time (Jacobsen

and Olszewski 1993). *GA1* encodes copalyl diphophate synthase, that catalyzes the first committed step in GA biosynthesis (Sun and Kamiya 1994). The *ga1-3* mutant is unable to flower under short days, and results in a slight delay flowering under long days (Wilson *et al*., 1992). GIBERELLIN 20 OXIDASE (GA 20-oxidase) involved in a key regulatory step in GA biosynthesis, that is regulated by environmental or physiological changes (Xu *et al*., 1995) .When plants were transferred from short day to long day conditions, the level of this gene increased and caused early flowering (Xu *et al*., 1997). GA signaling genes *GIBBERELLIC ACID INTENSIVE* (*GAI*), *REPRESSOR OF GA1-3* (*RGA*) and *RGA LIKE 1* (*RGL1*) also control flowering time. *GAI*, *RGA* and *RGL1* inhibit GA response in GA deficient conditions and GA de-represses the GA response by inactivating these genes (Dill and Sun 2001, King *et al*., 2001). In addition, DELLA domain required for the inactivation of GAI, RGA and RGL1 (Peng et al., 1997). GA promotes flowering through increasing the transcriptional activity of genes encoding the floral integrators *SOC1*, *LFY* and *FT* (Langridge *et al*., 1957, Blázquez *et al*., 1998, Moon *et al*., 2003).

3.3. Autonomous pathway

Autonomous pathway acts in parallel to vernalization where it represses *FLC* expression. The autonomous pathway genes *LD*, *FCA*, *FY*, *EPA*, *FVE*, *FLD* and *FLK* were identified from mutants that are late flowering

responsive to day length and vernalization (Martinez-Zapater and Somerville 1990, Koornneef *et al*., 1991). Mutation in these genes leads to an increase the levels of *FLC* transcript and FLC protein (Michaels and Amasino 1999, Sheldon *et al*., 1999). Although all the members of this pathway act to limit *FLC* expression, genetic analysis suggesting that they have different biological processes. *FCA* encodes a protein containing two RNA-binding domains and a WW protein interaction motif at the C terminus (Macknight *et al*., 1997). The *FCA* transcript is alternatively spliced with only one from encoding the entire protein, that functions to promote the floral transition (Macknight *et al*., 1997). *FY* encodes a protein that is highly conserved in eukaryotes, and its homolog in *S. cerevisiae* functions in pre-mRNA 3′ end formation (Simpson *et al*., 2003). FCA-FY interaction is required for controlling flowering time and autoregulation of its expression by modulating the site of 3′ end formation in its own pre-mRNA (Simpson *et al*., 2003). *FPA* also encodes an RNA binding protein containing RNP motifs (Schomburg *et al*., 2001) whereas *FVE* encodes a WD-repeat protein (Blázquez *et al*., 2001) and *FLK* encodes a nuclear KH-type RNA binding protein (Lim *et al*., 2004). Therefore, post transcriptional regulation plays a general role in Autonomous pathway. *LD* encodes a homeodomain which might bind to either DNA or RNA (Dubnau and Struhl 1996). *FLD* encodes a protein that is homologous with a member of a human histone deacetylase complex, acts to deacetylate *FLC* chromatin, preventing the transcription of *FLC* and promoting flowering (He *et al*., 2003).

3.4. Vernalization pathway

Exposure to cold temperature for several weeks strongly down-regulates *FLC* level and so accelerates flowering. *FLC* encodes a MAD box transcription factor, represses flowering by suppressing the expression of the floral integrators, *SOC1* and *FT* (Michaels and Amasino 1999). The presence of an active allele of *FRIGIDA* (*FRI*) cause increased *FLC* level and delayed flowering, which is reversed by vernlaization. Vernalization-mediated *FLC* repression is associated with histone modification. Vernalization leads to the recruitment of *VERNALIZATiON 1* (*VRN1*), *VERNALIZATiON*2 (*VRN2*) and *VERNALIZATION-INSESITIVE 3* (*VIN3*) (Michaels and Amasino 1999, Gendall *et al*., 2001, Levy *et al*., 2002). *VIN3* recognizes the duration of cold exposure and maintain the repressed state of the *FLC* chromatin structure (Sung and Amasino 2004). Subsequently, *VRN1* and *VRN2* are required for the methylation of *FLC* histones and the maintenance of silencing (Gendall *et al*., 2001, Levy *et al*., 2002).

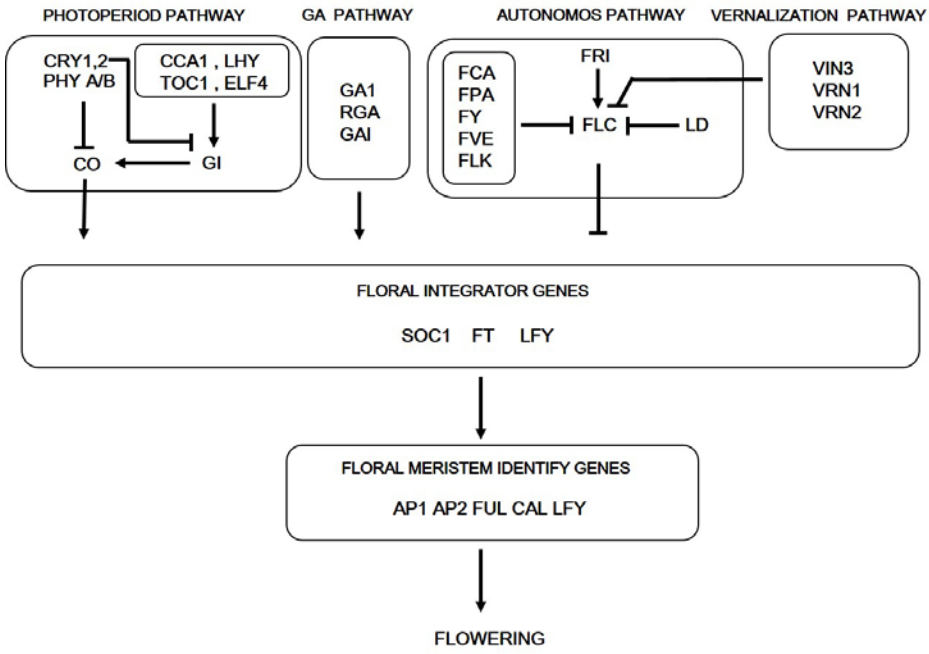


Figure 3. The genetic pathway of flowering in *Arabidopsis*.

INTRODUCTION

In eukaryotic cells, protein function and stability are post-translationally regulated by small and large molecules such as phosphates, carbohydrates, lipids, and small proteins (Castro *et al*., 2012). The post-translational modification of target proteins by small ubiquitin-like modifier (SUMO) is an important regulatory mechanism (Wilkinson *et al*., 2010). The reversible covalent attachment of SUMO to a lysine residue in a target protein is catalyzed by E3 SUMO ligases, although conjugation of SUMO to target proteins can occur without the help of an E3 SUMO ligase (Wilkinson *et al*., 2010). As in other eukaryotes, SUMO modification in plants has been implicated in numerous basic cellular processes, such as stress and defense responses, nitrogen metabolism, and the regulation of flowering (Hotson *et al*., 2003, Kurepa *et al*., 2003, Lois *et al*., 2003, Murtas *et al*., 2003, Miura *et al*., 2005, 2007, Catala *et al*., 2007, Lee *et al*., 2007, Conti *et al*., 2008, Yoo *et al*., 2006, Park *et al*., 2011).

AtSIZ1, a Siz/PIAS (SP)-RING-finger protein, regulates plant responses to nutrient deficiency and environmental stresses and controls vegetative growth and development (Miura *et al*., 2005, 2007, 2010, Catala *et al*., 2007, Lee *et al*., 2007, Yoo *et al*., 2006, Park *et al*., 2011, Garcia-Dominguez *et al*., 2008, Jin *et al*., 2008).

Flowering time is a critical trait in higher plants, as the timing of the

transition from the vegetative to the reproductive phase is essential for reproductive success. Several genes are involved in floral induction in *Arabidopsis*, among which the MADS-box transcription factor flowering locus C (*FLC*) plays an important role in phase transition (Samach *et al*., 2000, Simpson and Dean, 2002). The expression of *FLC* is negatively regulated by vernalization and by components of the autonomous pathway (Michaels and Amasino, 1999, Sheldon *et al*., 1999). Although several factors affecting the transcription of *FLC* have been described, the post-translational regulation of FLC stability and function has not been clearly characterized.

A recent study has shown that FLC is polyubiquitinated by SINAT5 *in vitro* (Park *et al*., 2007), indicating that its stability may be regulated by a specific E3 ubiquitin ligase. This result suggests that the regulation of the floral transition by FLC involves a post-translational mechanism.

In the present study, I show that sumoylation plays a role in the regulation of flowering time by modulating the activity of FLC. AtSIZ1 stabilizes FLC through direct interaction, and it inhibits FLC sumoylation *in vitro*. Overexpression of *mFLC*, a sumoylation site mutant gene, had no effect on flowering time. These findings indicate that FLC is stabilized by the E3 SUMO ligase AtSIZ1, and FLC-mediated flowering repression is stimulated by sumoylation.

MATERIALS AND METHODS

Plant materials and growth conditions

The wild-type *Arabidopsis thaliana* (Columbia-0 ecotype) plants were used in this study. For plants grown in medium, seeds were surface-sterilized in commercial bleach that contained 5% (v/v) sodium hypochlorite and 0.1% (v/v) Triton X-100 solution for 10 minutes, rinsed five times in sterilized water, and stratified at 4^{o}C for 2 days in the dark. Seeds were planted on agar plates that contained MS medium, 2% sucrose (w/v), and 0.8% agar (w/v), buffered to pH 5.7. For plants grown in soil, seeds were directly sown into sterile Vermiculite. All plants including seedlings were grown at 22^{o}C under 16 hour light/8 hour dark cycle condition in a growth chamber.

Construction of recombinant plasmids

To produce the His_6-FLC, the cDNA encoding full-length *FLC* was amplified by PCR and inserted into pET28a (Novagen). To produce glutathione S-transferase (GST)-AtSIZ1 or its deletion mutants, the cDNAs encoding either the full length or the deletion mutants of *Arabidopsis AtSIZ1* cDNA were inserted into pGEX4T-1 vector (Amersham Biosciences). GST-AtSIZ1 (ΔS), GST-AtSIZ1 (ΔSP) and GST-AtSIZ1 (ΔPM) contained amino acids 90–470, 300-470 of AtSIZ1, and 1-100 of AtSIZ1, respectively. For the maltose-binding

protein (MBP)-AtSIZ1-haemagglutinin (HA) fusion, a cDNA encoding full-length *AtSIZ1* was amplified by PCR using a primer tagged with HA and inserted into pMALc2 vector (New England Biolabs).

For His_6-FLC-Myc and GST-FLC-Myc production, cDNA encoding full-length *FLC* was amplified by PCR using primers tagged with Myc and inserted into pET28a and pGEX4T-1, respectively. To produce the FLC mutant protein GST-FLC(K5R)-Myc, GST-FLC(K135R)-Myc, GST-FLC(K154R)-Myc and His_6-FLC(K154R)-Myc (the numbers indicate the positions of the lysines in the FLC that were mutated to Arginine), GST-FLC-Myc and His_6-FLC-Myc were mutated by site-directed mutagenesis using overlapping primers (Table 2). The double mutants, GST-FLC(K5R, K135R)-Myc, GST-FLC(K5R, K154R)-Myc, GST-FLC(K135R, K154R)-Myc, were also generated by site-directed mutagenesis of GST-FLC(K5R)-Myc, GST-FLC(K135R)-Myc and GST-FLC(K154R)-Myc using overlapping primers (Table 2).

The *Arabidopsis SUMO1* full length cDNA was amplified by PCR with gene-specific primers and inserted into pET28a to produce the His_6-AtSUMO1-GG at the 3'end. To produce GST-IAA4, the cDNA encoding full-length *IAA4* was amplified by PCR with gene-specific primers and inserted into the pGEX4T-1 vector. *Arabidopsis* SUMO E1 and E2 enzyme-encoding constructs were kindly provided by Dr H.-P. Stuible. All constructs were transformed into *Escherichia coli* BL21/DE3 (pLysS) cells. The transformed cells were treated with IPTG to induce fusion protein expression. The

Sequences of primers used in this study are listed in Table 2. All the constructs were verified by automatic DNA sequencing to ensure that no mutations were introduced.

Production of transgenic Arabidopsis plants

To produce FLC or FLC (K154R, mFLC)-overexpressing plants, the corresponding full-length cDNAs were amplified by PCR using a forward primer and a reverser primer tagged with $FLAG_3$ and inserted into plant expression vector pBA002. Recombinant plasmids, *35S-FLC-*$FLAG_3$ and *35S-mFLC-*$FLAG_3$, were introduced into *Arabidopsis* by floral dipping (Clough and Bent, 1998). To produce double transgenic plants, the full-length cDNA encoding *AtSIZ1* was amplified by PCR using a forward primer tagged with HA_3 and a reverser primer and inserted into plant expression vector pER8. Resulting recombinant plasmid *XVE-* HA_3*-AtSIZ1* with *35S-FLC-*$FLAG_3$ were also introduced into *Arabidopsis* by floral dipping.

Purification of recombinant proteins

All of the recombinant proteins were expressed in *E. coli* strain BL21 and were purified in accordance with the manufacturer's instructions. Briefly, for His_6-AtSAE1b, His_6-AtSAE2, His_6-AtSCE1, His_6-AtSUMO1, His_6-FLC, His_6-mFLC (K154R), His_6-FLC-Myc and His_6-mFLC-Myc (K154R)

purification, bacteria were lysed in 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 1% Triton X-100, 1 mM imidazole, 5 mM DTT, 2 mM PMSF, and proteinase inhibitor cocktail (Roche), and were purified on Ni^{2+}-nitrilotriacetate (Ni^{2+}-NTA) resins (Qiagen). For GST, GST-AtSIZ1 (S), GST-AtSIZ1 (ΔS), GST-AtSIZ1 (ΔSP), GST-AtSIZ1 (ΔPM), GST-AtSUMO1, GST-FLC-Myc, GST-mFLC-Myc (K154R), GST-FLC (K5R, K135R)-Myc, GST-FLC (K5R, K154R)-Myc and GST-FLC (K135R, K154R)-Myc purification, bacteria were lysed in PBS buffer (pH 7.5) containing 1% Triton X-100, 2 mM PMSF and a proteinase inhibitor cocktail (Roche), and were purified on glutathione resins (Pharmacia). For MBP-AtSIZ1 purification, bacteria were lysed in 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 1% Triton X-100; 2mM PMSF containing a proteinase inhibitor cocktail (Roche), and were purified on amylose resin (New England Biolabs). Protein concentrations were determined by the Bradford assay (Bio-Rad). For MBP-AtSIZ1-HA, bacteria were lysed in 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1% Triton X-100, 5mM dithiothreitol (DTT), 2 mM phenylmethylsulphonyl fluoride (PMSF) and a proteinase inhibitor cocktail (Roche) and purified on amylose resins (New England Biolabs).

In vitro binding assay

For determining the *in vitro* binding of GST-AtSIZ1 to His_6-FLC, 2 μg of full-length GST-AtSIZ1 or deletion mutants baits and 2 μg of full-length

His_6-FLC prey were added to 1 ml of binding buffer [50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1% Triton X-100, 0.2% glycerol, 0.5 mM β-mercaptoethanol]. After incubation at 25°C for 2 hours, the reaction mixtures were incubated with a glutathione resin for 2 hours before washing six times with buffer [50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1% Triton X-100]. Absorbed proteins were analyzed by 11% SDS-PAGE and detected by western blot using an anti-His antibody (Santa Cruz Biotechnology).

To examine dimerization of FLC protein, 2μg of full-length GST-FLC bait and 2μg of full-length His_6-FLC or His_6-mFLC preys were added to 1 ml of binding buffer as described in above. After incubation at 25°C for 2 hours, the reaction mixtures were incubated with a glutathione resin and absorbed proteins were analyzed as described in above.

For determining the *in vitro* binding of FLC mutant protein His_6-mFLC to MBP-AtSIZ1, 2μg of full-length MBP-AtSIZ1 bait and 2μg of full-length His_6-FLC or His6-mFLC preys were added to 1ml of binding buffer as described in above. After incubation at 25°C for 2 hours, the reaction mixtures were incubated with an amylose resin for 2 hours before washing six times with buffer [50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1% Triton X-100]. Absorbed proteins were as described in above.

In vivo Co-immunoprecipitation

To investigate FLC and SIZ1 interaction, different combinations of following constructs, *35S-FLC-FLAG$_3$*, *35S-HA$_3$-SIZ1*, *35S-FLC-FLAG$_3$* and *35S-HA$_3$-SIZ1* were infiltrated into 5-day-old Arabidopsis seedlings using agrobacterium mediated transformation method. After 3 days, total proteins were extracted from each sample in the lysis buffer containing 50mM Tris-Cl (pH7.5), 150mM NaCl, 0.5% NP-40, 1mM PMSF and protease inhibitor cocktail (roche) and then immunoprecipitated with anti-FLAG antibody. The samples were washed, bounded proteins were eluted in SDS sample buffer and detected by western blotting with Anti-Myc antibody.

Sumoylation assay

In vitro sumoylation was performed in 30μl of reaction buffer [20 mM Hepes (pH 7.5), 5 mM MgCl2, 2 mM ATP] with 50 ng of His$_6$-AtSAE1b, 50 ng of His$_6$-AtSAE2, 50 ng of His$_6$-AtSCE1, 8 μg of His$_6$-AtSUMO1-GG, 100 ng of His$_6$-FLC-Myc (or GST-FLC-Myc) with or without 500 ng of MBP-AtSIZ1-HA. After incubation for 3 hours at 30 °C, the reaction mixtures were separated on 10% SDS-polyacrylamide gels. Sumoylated His$_6$-FLC-Myc or GST-FLC-Myc was detected by western blot using an anti-Myc antibody (Santa Cruz Biotechnology).

To identify the sumoylation site on FLC, GST-FLCm1-Myc, GST-

FLCm2-Myc, GST-FLCm3-Myc, and GST-mFLC-Myc were added to the reaction mixtures instead of His_6-FLC-Myc or GST-FLC-Myc, respectively. The reaction and the subsequent steps were as described above.

BiFC assay

To generate constructs for the bimolecular fluorescence complementation protein interaction assay, the cDNAs for *AtSIZ1* and *FLC* were cloned into pDONR201 vector. Next, the cDNAs for *AtSIZ1* and *FLC* were transferred from their respective entry clones to the gateway vector pSAT4-DEST-n(174)EYFP-C1 (ABRC stock number CD3-1089) or pSAT5-DEST-c(175-end)EYFP-C1(B) (ABRC stock number CD3-1097), which contained the N-terminal 174 amino acids of EYFP ($EYFP^N$) or C-terminal 64 amino acids of EYFP ($EYFP^C$). All fusion genes were expressed from a 35S promoter. The fusion constructs encoding nYFP-SIZ1, cYFP-FLC proteins were mixed with 1:1 ratio following different combinations of plasmid and co-bombarded into onion epidermal cells using the helium biolistic gun. Bombarded tissues were incubated at 25 ºC under dark condition for 16 hours and then YFP signal was observed by confocal laser scanning microscope.

Quantitative real-time RT-PCR analysis

To assess the expression level of the *FLC* or *mFLC* transcripts in double transgenic plants carrying *35S-FLC-FLAG$_3$* and *XVE-HA3-SIZ1* or *35S-mFLC-FLAG$_3$* and *XVE-HA$_3$-SIZ1*, two-week-old transgenic plants were treated with β-estradiol and total RNAs were isolated from the plants. First-strand cDNA was synthesized with 5 μg of total RNA using an iScriptcDNA Synthesis Kit (Bio-Rad). An equal volume of cDNA was amplified by quantitative real-time PCR (MyiQ, Bio-Rad) according to the manufacturer's protocol. The specific primers and template cDNA were combined with 25 μL iQ SYBR Green Super Mix (Bio-Rad), and the reactions were performed under the following thermal conditions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s; and 60°C for 1 min. The CT values of target genes were normalized to the CT value of the tubulin gene and analyzed with iCycler IQ software (Bio-Rad). All reactions were repeated three times with separate RNA samples. RT-PCR primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi), and their specificity was verified by cloning into the pGEM T-Easy vector (Promega) and sequencing with an ABI 3730xl DNA Analyzer (Applied Biosystems). Forward primer: 5'-tcatcatgtgggagcagaag-3' and reverse primer: 5'-tccttgtcatcgtcatcctt-3'.

β-estradiol and cycloheximide treatments

Fourteen-day-old light-grown (16 hour light/8 hour dark) plants carrying *35S–FLC–FLAG$_3$* and *XVE–HA$_3$–AtSIZ1* or *35S–mFLC–FLAG$_3$* and *XVE–HA$_3$–AtSIZ1* transgenes on MS medium were treated in the light with or without β-estradiol for 15 hours. Samples were ground in liquid nitrogen and then separated by SDS-PAGE. FLC–FLAG$_3$ and mFLC–FLAG$_3$ levels were examined by western blotting with anti-FLAG antibody. HA$_3$–AtSIZ1 induction was also analyzed by western blot with anti-HA antibody.

Post-translational degradation of FLC was also examined using double transgenic plants of *35S–FLC–FLAG$_3$* and *XVE–HA$_3$–AtSIZ1* or *35S–mFLC (K154R)–FLAG$_3$* and *XVE–HA$_3$–AtSIZ1*. Transgenic plants were incubated in liquid medium with β-estradiol for induction of AtSIZ1 expression for 15 hours, washed and then transferred to MS medium with 100 μM cycloheximide (CHX). Treated plants were then incubated for 4 hours. Proteins were extracted at the indicated time points and analyzed by western blot using anti-HA or anti-FLAG antibodies as described in above.

Cell-free degradation assay

Two weeks old light grown wild type and *siz1-3* mutant plants were

extracted in a buffer (25mM Tris-Cl (pH7.5), 10mM $MgCl_2$, 5mM DTT, 10mM NaCl and 10mM ATP). Cell debris was removed by centrifugation and protein concentration was determined by Bradford assay. 300ug of cell extracts mixed with 3ug of recombinant His_6-FLC-Myc protein and then incubated at room temperature for 2hrs. The samples were taken at the indicated time points and analyzed by western blotting with anti-Myc antibody. To confirm equal loading, BiP was used as a loading control.

Yeast two-hybrid assays

Yeast two hybrid assays was performed using GAL-4-based hybrid system. (clontech). Full-length *AtSIZ1* and *IAA4* cDNAs were cloned into pGAD424 and pGBT8 (clontech) to generate the constructs *AD-AtSIZ1* and *BD-IAA4*. The constructs were transformed into the yeast strain AH109 with the lithium acetate method. The yeast cells were grown on minimal medium (-Leu/-Trp). Transformants were plates onto minimal medium (-Leu/-Trp/-His) to test the interactions between AtSIZ1 and IAA4

Investigation of flowering time

To examine the effect of sumoylation on FLC-mediated flowering, transgenic plants carrying *35S–FLC–FLAG*$_3$ or *35S–mFLC–FLAG*$_3$ were

generated. After selection of FLC-FLAG$_3$ or mFLC-FLAG$_3$-overexpressing transgenic plants, Wild type (WT) and transgenic plants were grown in soil under long day conditions (16 hour light/8 hour dark). Flowering time was assessed by counting the number of rosette leaves present at the time of appearance of inflorescences or was also determined by counting the day to flowering.

Table 2. List of primers used in this study.

purpose	Gene	vector	Sequence
BiFC experiments	FLC	pDONR201	F 5′-AAAAAGCAGGCTGCATGGGAAGAAAAAAACTAGAA-3′ R 5′-AGAAAGCTGGGTTCTAATTAAGTAGTGGGAGAGT-3′
	SIZ1	pDONR201	F 5′-AAAAAGCAGGCTGCATGGATTTGGAAGCTAATTGT-3′ R 5′-AGAAAGCTGGTTTTACTCAGAATCCGAGTCAAT-3′
Gateway cloning Adapter primer	attB1		F 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′
	attB2		R 5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′
Pull-down assay	FLC-MYC	pET28a, pGEX4T-1	F 5′-TCTACGAATTCATGGGAAGAAAAAAACTA-3' R 5′-TCTACCTCGAGttattcattCaagtcctcttcagaaatgagcttttgctccatattaagtagtgggagagt-3'
	SIZ1-HA	pMALC2X	F 5′-TCTACGAATTCATGGATTTGGAAGCTAAT-3′ R 5′-TCTACTCTAGATTAAAGACTAGCATAATCTGGAACATCATAAGGATACATctcagaatccgagtcaat
	SIZ1 △PHD,MIZ	pGEX4T-1	F 5′-TCTACGGATTCATGGATTTGGAAGCTAATTGTAAG-3′ R 5′-TCTACCTCGAGTTACTTAACTTTCAGATTACTGGTATC-3′
	SIZ1 △SAP	pGEX4T-1	F 5′-TCTACGAATTCATGAGTTCAGATACCAGTAATCTGAAA-3′ R 5′-TCTACGTCGACTTAGCTACCATCAGGTGCATGCCACTG-3′
	SIZ1 △SAP,PHD	pGEX4T-1	F 5′-TCTACGAATTCATGAGACTTGTGAAGCGCAGGACTCTA-3′ R 5′-TCTACGTCGACTTAGCTACCATCAGGTGCATGCCACTG-3′
In vitro sumoylation assay	SUMO1-GG	pET28a	F 5′-TCTACGAATTCATGTCTGCAAACCAGGAGGAAGAC-3′ R 5′-TCTACCTCGAGTTAGCCACCAGTCTGATGGAGCAT-3′
Site directed mutagenesis	FLC K5/R-Myc	pGEX4T-1	F 5′-TCTAGAATTCATGGGAAGAAAAAGGCTAGAAAT-3′ R 5′TCTACCTCGAGttattcattcAagtcctcttcagaaatgagcttttgctccatattaagtagtgggagagt-3'
	FLC K135/R-Myc	pGEX4T-1	F 5′-TCTACGAATTCATGGGAAGAAAAAAACTA-3' R 5′-GAGAAAATGCTGAGAGAAGAGAACC-3′ F 5′-CTAGAGCCAAGAGGACCGAACT-3′ R 5′-TCTACCTCGAGttattcattcaagTcctcttcagaaatgagcttttgctccatattaagtagtgggagagt-3'

	FLC K154/R-Myc	pGEX4T-1	F 5′-TCTACGAATTCATGGGAAGAAAAAAA CTA-3' R 5′-GGTTCTCTTCTCTCAGCATTTTC-3′ F 5′-GAGAAAATGCTGAGAGAAGAGAACC-3′ R 5′-TCTACCTCGAGttattcattcaagtcc tcttcagaaatgagcttttgctccatattaag tagtgggagagt-3'
For making Transgenic plants	FLC-3FLAG	pBA002	F 5′-TCTACCTCGAGATGGGAAGAAAAAAAC TAGAAATC-3′ R 5′-TCTACGAGCTCTCACTTGTCATCGTCA TCCTTGTAGTCCTTGTCATCGTCATCCTTGTA GTCCTTGTCATCGTCATCCTTGTAGTCCATAT -3′
	3HA-SIZ1	pBA002	F 5′-TCTACCCCGGGATGGATTTGGAAGCTA AT-3' R 5′-TCTACACTAGTTTACTCAGAATCCGAG TC

Results

AtSIZ1 physically interacts with FLC

Recently, it was reported that FLC directly interacts and colocalizes with the *Arabidopsis* E3 ubiquitin ligase SINAT5 in the nucleus (Park *et al*., 2007). Since the SP-RING motif protein AtSIZ1 also localizes to the nucleus (Miura et al., 2005), I performed BiFC (bimolecular fluorescence complementation) experiments to investigate both the subcellular localization and possible physical interaction between AtSIZ1 and FLC. FLC tagged with the C-terminus of EYFP and AtSIZ1 tagged with N-terminus of EYFP were transiently expressed in onion epidermal cells. It is not know to what extent onion cells reflect the situation in *Arabidopsis* cells. Nevertheless, bright yellow fluorescence signal was detected in the nucleus (figure 4), indicating a physical interaction of the two proteins in the nucleus.

Next I carried out *in vivo* co-immunoprecitation (co-IP) assay to confirm the interaction between two proteins in *Arabiodpsis*. Total protein extracts from *Arabidopsis* seedlings overexpressing FLC-$FLAG_3$, HA_3-SIZ1 alone or both FLC-$FLAG_3$ and HA_3-SIZ1 were immunoprecipitated with Ant-FLAG antibody and examined by western blotting. HA_3-SIZ1 protein was successively coimmunoprecipitated with FLC-FLAG protein (figure 5). Taken together, these results indicated that AtSIZ1 and FLC interact directly *in vivo*.

To confirm the interaction between FLC and AtSIZ1 in an *in vitro* system, pull-down assays were performed by overexpressing the recombinant proteins in *E. coli* and purifying them with affinity columns (Fig. 6-1). Figure 6-2A shows that GST-AtSIZ1, but not GST alone, was able to pull down *Arabidopsis* FLC. Experiments using deletion mutants showed that the N-terminal region containing the SAP domain of AtSIZ1 (GST-AtSIZ1 [ΔPM]) interacts with FLC (Fig. 6-2B). Therefore, these *in vitro* results suggest that the colocalization of FLC and AtSIZ1 in the nucleus likely reflects their direct interaction *in vivo.*

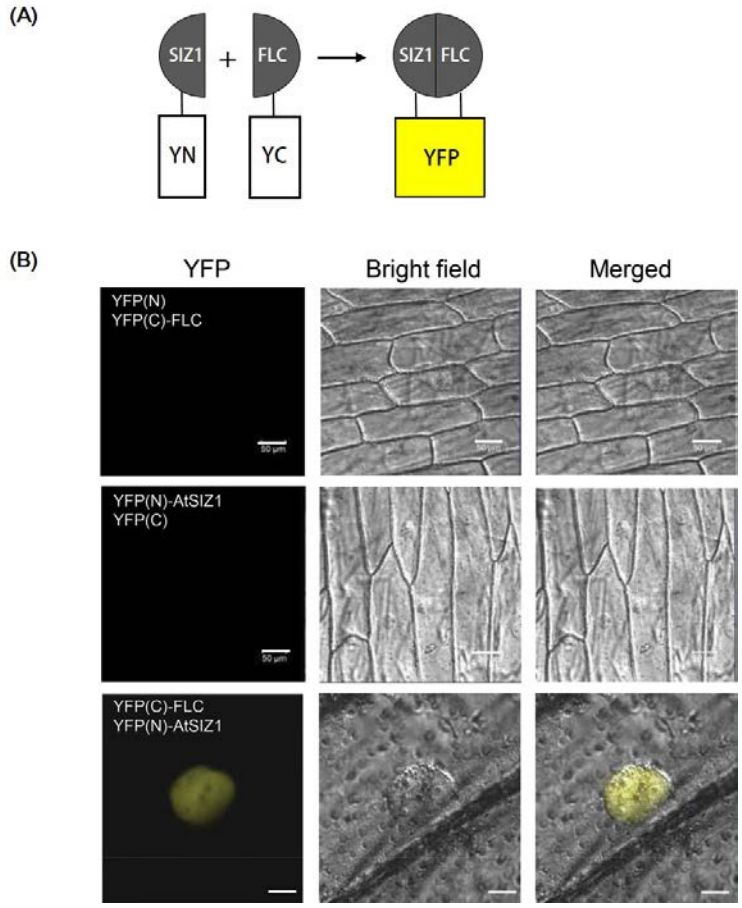


Figure 4. BiFC analysis of interaction between AtSIZ1and FLC. (A) Principle of the BiFC assay. (B) The interaction of AtSIZ1 and FLC was examined by Bimolecular fluorescence complementation (BiFC) assay in onion epidermal cells. AtSIZ1 and FLC cDNAs were fused with YFP N-terminal (N) and C-terminal (C), respectively. Each combination of YFP(N)/35S-YFP(C)-FLC, 35S-YFP(N)-AtSIZ1/YFP(C), and 35S-YFP(C)-FLC/35S-YFP(N)-AtSIZ1 was introduced into onion epidermal cells by particle bombardment, and fluorescence was detected by confocal microscopy. Bar, 50μm.

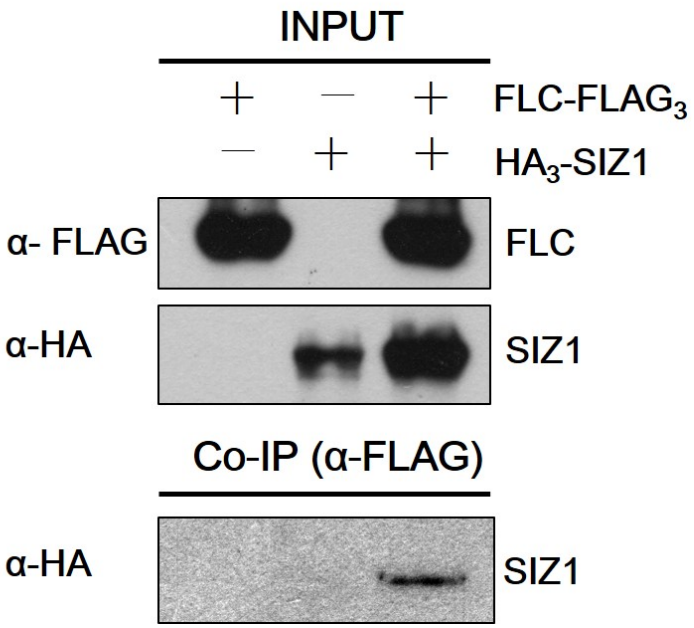


Figure 5. Confirmation of interaction between AtSIZ1 and FLC by co-immunoprecipitation. Extracts of Arabidopsis seedling overexpressing FLC-$FLAG_3$, HA_3-SIZ1, FLC-$FLAG_3$ and HA_3-SIZ1 were immunoprecipitated with anti-FLAG antibody. Input proteins and the immunoprecipitates were separated on 10% SDS-PAGE and detected by western blotting with Anti-HA and FLAG antibodies.

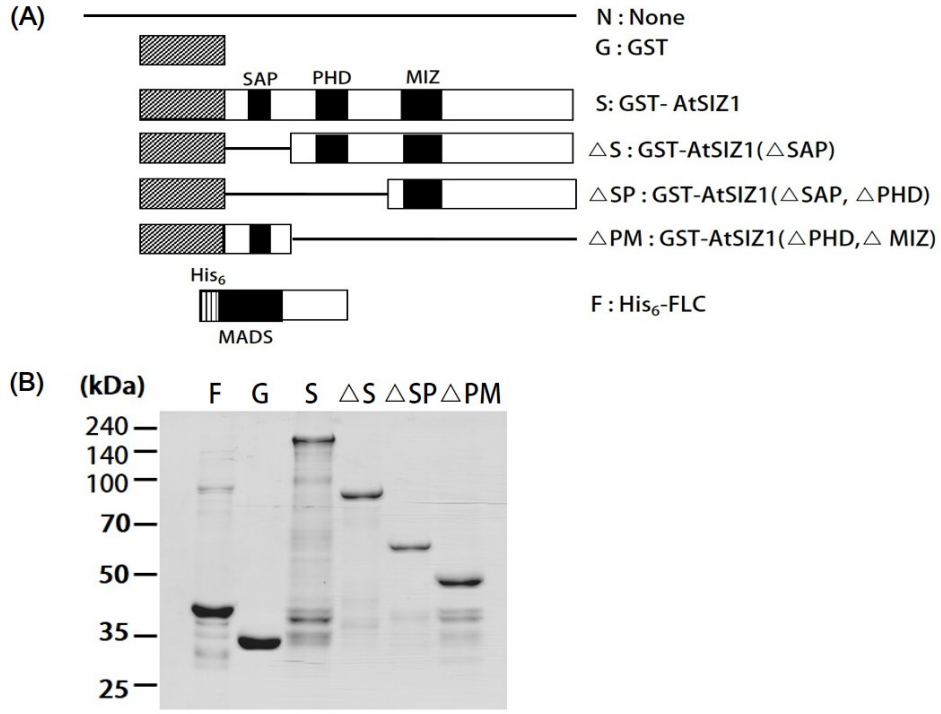


Figure 6-1. Identification of interaction domains between AtSIZ1 and FLC. (A) Schematic diagram of bait [GST (G), GST–AtSIZ1 (S), GST–AtSIZ1 (ΔS), GST–AtSIZ1 (ΔSP), and GST–AtSIZ1 (ΔPM)] and prey [His_6-FLC (F)] proteins. (B) His_6-FLC, full-length AtSIZ1, or its deletion mutants were overexpressed in *E. coli* and purified with Ni^{2+}-NTA or glutathione affinity columns.

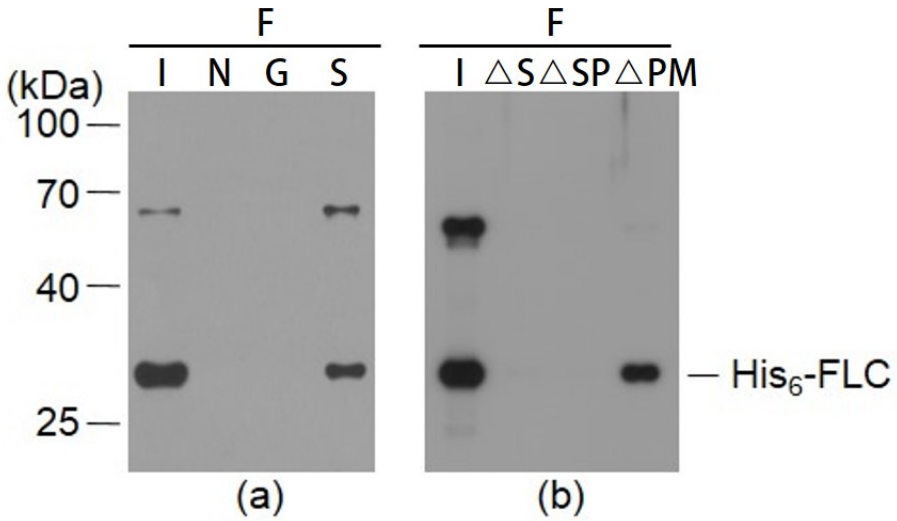
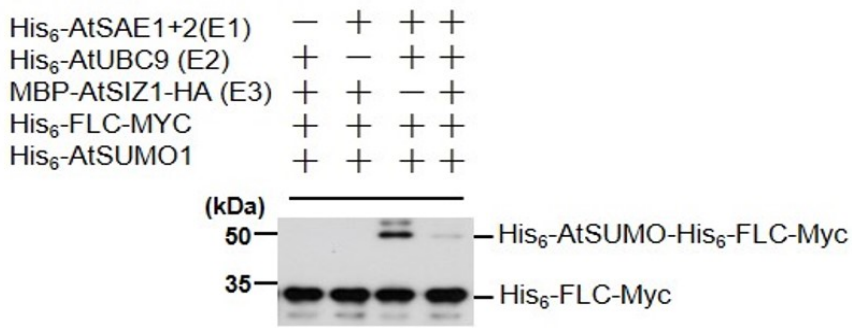


Figure 6-2. *In vitro* pull-down of FLC with AtSIZ1. The His_6-FLC protein was pulled down with full-length AtSIZ1 or its deletion mutant proteins, separated on 11% SDS–polyacrylamide gels, and analysed by western blotting with an anti-His antibody. I, input (His_6-FLC), None(N), GST (G), GST–AtSIZ1 (S), GST–AtSIZ1 (△S), GST–AtSIZ1 (△SP), and GST–AtSIZ1 (△PM)

FLC is sumoylated without AtSIZ1

The direct interaction of FLC and AtSIZ1 indicated by the *in vivo* and *in vitro* results led to the hypothesis that AtSIZ1 may function as an E3 ligase for FLC. Therefore, the recombination proteins GST-AtSIZ1-HA_3 and His_6-FLC-Myc were produced to determine whether AtSIZ1 is E3 ligase for FLC. *In vitro* sumoylation experiments, purified His_6-FLC-Myc was sumoylated in the presence of E1 and E2 activities. (Figure 7A). However, the sumoylation of His_6-FLC-Myc was not induced by AtSIZ1. It was also tested whether another AtSIZ1-interacting protein, IAA4 could be sumoylated by AtSIZ1 (Figure.7B). The results showed that IAA4 was not sumoylated under the reaction conditions employed, including the presence of E1, E2, and E3. (Figure 7C).

(A)



(B)

1: AD/BD
2: AD-AtSIZ1/BD
3: AD/BD-IAA4
4: AD-AtSIZ1/BD-IAA4

(C)

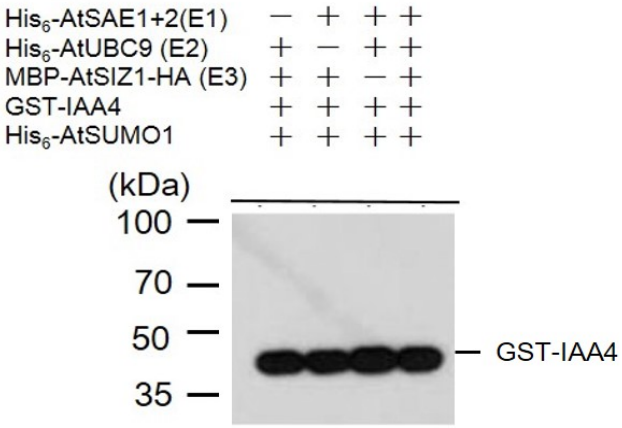


Figure 7. FLC is sumoylated *in vitro*. (A) *Arabidopsis* His_6-AtSAE1b, His_6-AtSAE2, His_6-AtSCE1, MBP–AtSIZ1, His_6-AtSUMO1, and His_6-FLC-Myc were overexpressed in *E. coli* and purified with Ni^{2+}-NTA, glutathione, and amylose affinity columns, respectively. Sumoylation of His_6-FLC-Myc was assayed in the presence or absence of E1 (His_6-AtSAE1b and His_6-AtSAE2), E2 (His_6-AtSCE1), E3 (MBP–AtSIZ1), and His_6-AtSUMO1. After the reaction, sumoylated FLC was detected by western blotting with an anti-Myc antibody. GST–IAA4 was also used for the sumoylation assay as a negative control. (B) AtSIZ1 directly interacts with GST–IAA4 in yeast. Full-length AtSIZ1 and IAA4cDNAs were fused to sequences encoding the Gal4 activation domain (AD) and the Gal4 DNA-binding domain (BD) in pGAD424 and pGBT8, respectively. The constructs were transformed into yeast strain AH109. Each number indicates the yeast cells transformed with a combination of only pGAD424 and pGBT8 vectors or recombinant plasmids. Transformants were plated onto minimal medium –Leu/–Trp or –Leu/–Trp/–His and incubated for 4d. (C) Sumoylation of GST–IAA4 was assayed using the same reaction conditions as above. After the reaction, IAA4 was detected by western blotting with an anti-GST antibody.

AtSIZ1 inhibits FLC sumoylation

Despite the interaction between AtSIZ1 and FLC shown in Figures 4, 5 and 6, the results indicate that AtSIZ1 has no E3 SUMO ligase activity for FLC (Figure 7A). Therefore, experiments were carried out to examine whether AtSIZ1 could block or inhibit the sumoylation of FLC. The addition of increasing amounts of AtSIZ1 protein to the reaction mixture resulted in the gradual inhibition of FLC sumoylation (Figure 8). However, AtSIZ1 was sumoylated under the reaction conditions used here (Figure 8), indicating that AtSIZ1 is active and that it has self-sumoylaiton activity under the reaction conditions used. Since all purified proteins used in this experiment were dialysed prior to the reaction, to confirm the effect of AtSIZ1 on FLC sumoylation, an equal volume of dialysis buffer was added to the reaction; this buffer had no effect of FLC sumoylation (Fig 8B). Therefore, these results indicate that FLC sumoylation is blocked by AtSIZ1 protein.

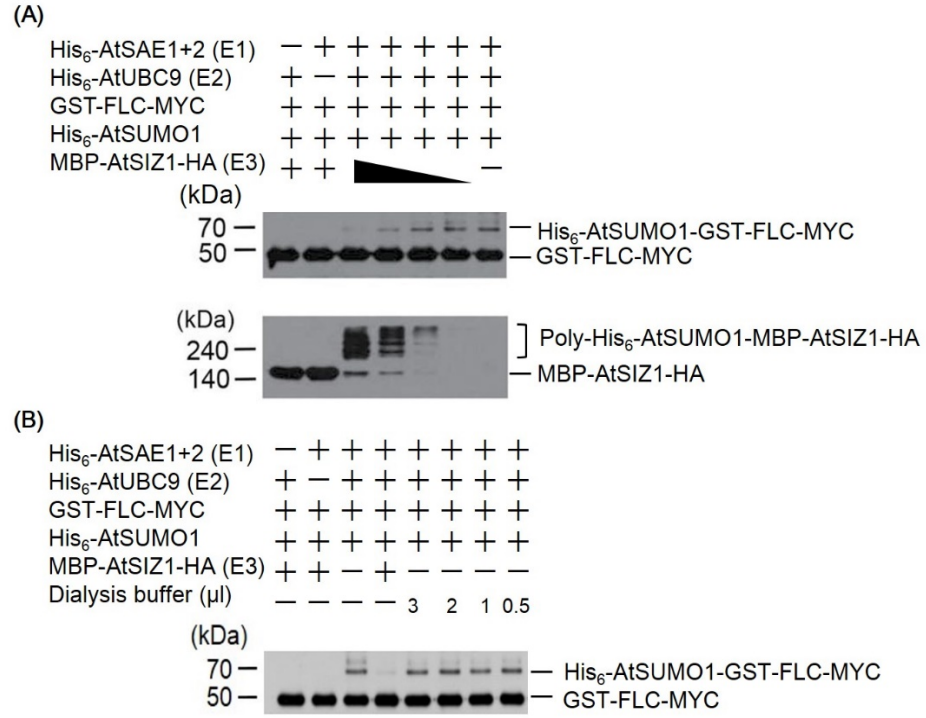


Figure 8. FLC sumoylation is blocked by AtSIZ1. (A) The effect of AtSIZ1 on FLC sumoylation was examined. Purified GST–AtSIZ1-HA was added to the reaction mixture at concentrations ranging from 0.1 μg to 1.0 μg. After the reaction, sumoylated FLC and AtSIZ1 were detected by western blotting with anti-Myc and anti-HA antibodies, respectively. (B) The effect of dialysis buffer on FLC sumoylation was also examined.

Identification of sumoylation sites on FLC

The deduced amino acid sequences of FLC showed three putative sumoylation sites (ΨKXE) located at lysine 5 (K5), lysine 135 (K135), and lysine 154 (K154; Figure 9-1-A and B) . To identify the sumoylation sites on the FLC protein, single or double mutant derivatives were generated with the mutations K154R, K5R/K135R, K5R/K154R, and K135R/K154R. The proteins were overexpressed in *E. coli*, purified with glutathione affinity columns, and used for *in vitro* sumoylation assays. *In vitro* sumoylation with the double mutant proteins GST-FLCm1-Myc (K5R/K135R), GST-FLCm2-Myc (K5R/K154R), GST-FLCm3-Myc (K135R/K154R), and GST-mFLC-Myc (K154R) showed that GST-FLCm1-Myc was sumoylated, whereas GST-FLCm2-Myc, GST-FLCm3-Myc, and GST-mFLC-Myc were not (Figure 9-2A). *In vitro* sumoylation assays including the single mutant protein GST-mFLC-Myc (K154/R) showed that this protein was not modified with SUMO (Figure9-2B), indicating that K154 is the principal site of SUMO conjugation on FLC.

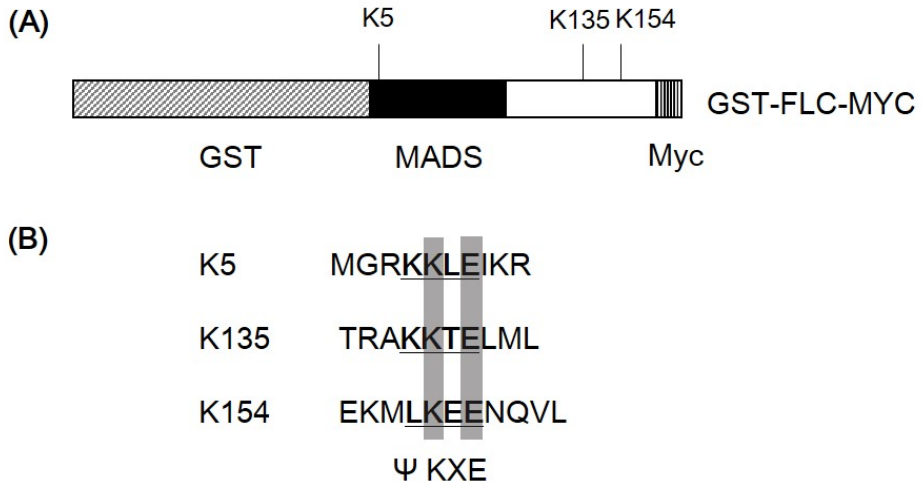


Figure 9-1. Putative sumoylation sites on FLC.

(A) Schematic diagram of the recombinant GST–FLC-Myc protein. The MADS-box and putative sumoylation sites (K5, K135, and K154) are indicated. (B) Three putative sumoylation sites (ΨKXE) identified using the SUMOplot™ Analysis Program are indicated in bold and grey.

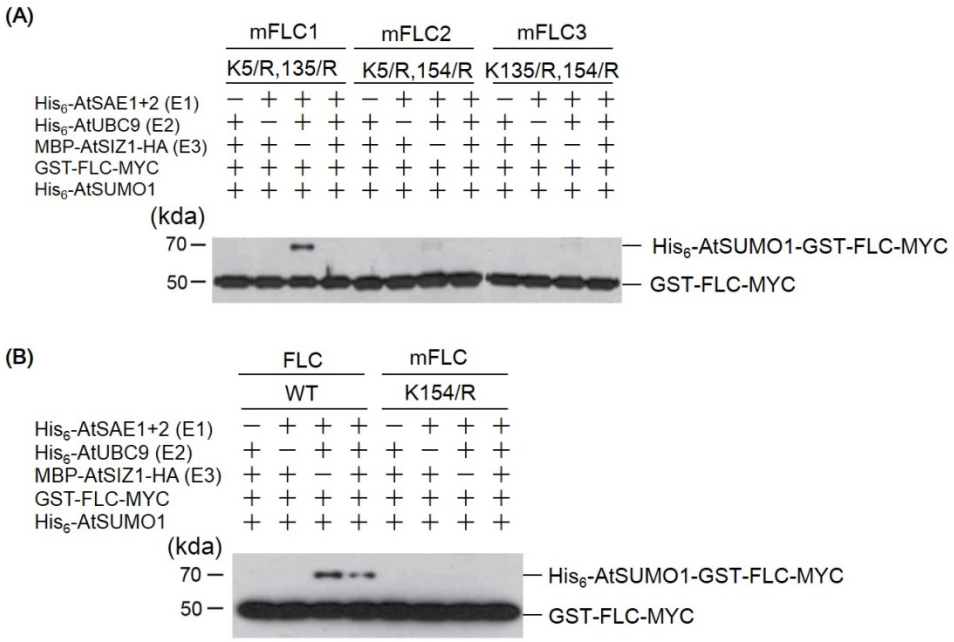


Figure 9-2. FLC is sumoylated at Lys 154**.** (A and B) *In vitro* sumoylation assays. Recombinant GST–FLC-Myc, GST–mFLC-Myc, GST–FLCm1-Myc, GST–FLCm2-Myc, and GST–FLCm3-Myc were overexpressed in *E. coli* and purified using a glutathione affinity column. The reaction mixture contained E1 (His_6-AtSAE1b and His_6-AtSAE2), E2 (His_6-AtSCE1), E3 (GST–AtSIZ1), and His_6-AtSUMO1 without (–) or with (+) a substrate protein. The mutant proteins mFLC, FLCm1, FLCm2, and FLCm3 have amino acid substitutions at residues that are predicted to be SUMO conjugation sites in FLC, as indicated. After the reaction, sumoylated FLC protein was detected by western blotting with an anti-Myc antibody.

FLC is stabilized by AtSIZ1

The AtSIZ1-FLC interaction and the inhibition of FLC sumoylation by AtSIZ1 imply that the concentration of FLC may be regulated by the amount of AtSIZ1 present *in vivo*. FLC concentration were therefore measured in transgenic plants carrying a *35S–FLC–FLAG$_3$* transgene and an estradiol-inducible *XVE–HA$_3$–AtSIZ1* transgene. Induction of the expression of AtSIZ1 increased the FLC concentrations up to 1.5- and 3.3-fold in two independent transgenic plants, respectively (Figure 10-1A). However, the two independent transgenic plants carrying a *35S–mFLC–FLAG$_3$* transgene and an estradiol-inducible *XVE–HA$_3$–AtSIZ1* transgene showed no changes in mFLC concentration in response to AtSIZ1 induction (Fig. 10-1C). It may be possible that the transcript levels of FLC or mFLC can affect the levels of FLC and mFLC proteins in transgenic plants. Thus FLC and mFLC transcript levels were examined by real-time reverse transcription-PCR (RT-PCR) and quantitative real-time RT-PCR after induction of *AtSIZ1* in FLC- or mFLC-overexpressing double transgenic plants. The result showed that the transcript levels of FLC and mFLC were comparable under these conditions (Figure 10-1 B, D and 10-2).

I examined the effect of AtSIZ1 on FLC decay by treating the transgenic plants described above with CHX to block new protein synthesis. The results showed that the degradation of FLC was delayed in plants co-expressing AtSIZ1 (Figure 11A and C). However, the rate of decay of mFLC

was not significantly altered by the expression of AtSIZ1 (Figure 11B and D).

I also measured the degradation kinetics of recombinant His_6-FLC-MYC protein in cell-free extracts of *Arabidopsis* WT and *siz1-3* mutants. The Protein levels were monitored by western blotting with Anti-Myc antibody. The FLC protein level was rapidly decreased over the time in siz1-3 mutant compared to that of the wild type (figure 12). Taken together, these results indicated that FLC is stabilized by AtSIZ1.

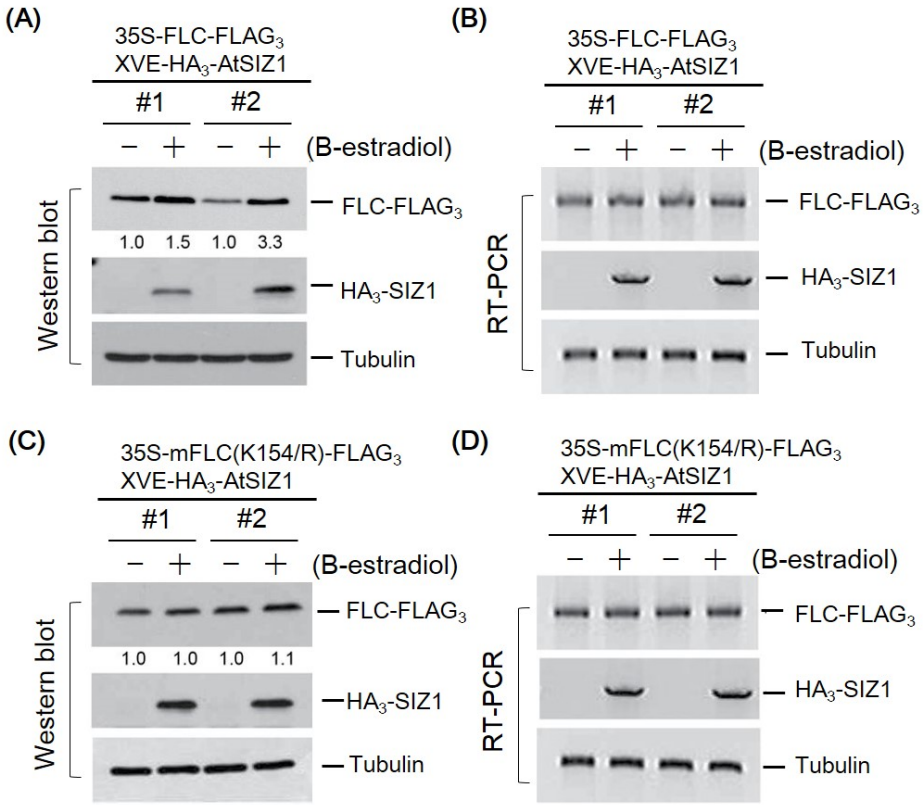

(A)
35S-FLC-FLAG3
XVE-HA3-AtSIZ1
#1
#2
− + − +
(B-estradiol)
Western blot
FLC-FLAG3
1.0 1.5 1.0 3.3
HA3-SIZ1
Tubulin
(B)
35S-FLC-FLAG3
XVE-HA3-AtSIZ1
#1
#2
− + − +
(B-estradiol)
RT-PCR
FLC-FLAG3
HA3-SIZ1
Tubulin
(C)
35S-mFLC(K154/R)-FLAG3
XVE-HA3-AtSIZ1
#1
#2
− + − +
(B-estradiol)
Western blot
FLC-FLAG3
1.0 1.0 1.0 1.1
HA3-SIZ1
Tubulin
(D)
35S-mFLC(K154/R)-FLAG3
XVE-HA3-AtSIZ1
#1
#2
− + − +
(B-estradiol)
RT-PCR
FLC-FLAG3
HA3-SIZ1
Tubulin

Figure 10-1. FLC is stabilized by AtSIZ1 *in vivo*.

Double transgenic plants of *35S-FLC-FLAG$_3$* and *XVE-HA$_3$-AtSIZ1* (A) or *35S-mFLC (K154R)-FLAG$_3$* and *XVE-HA$_3$-AtSIZ1* (C) were incubated in liquid medium with β-estradiol for the induction of *AtSIZ1* expression. After incubation for 15h, HA$_3$-AtSIZ1, FLC-FLAG$_3$, and mFLC-FLAG$_3$ levels were assessed by western blotting with anti-HA or anti-FLAG antibodies. Tubulin was used as a loading control. Numbers under lanes indicate relative intensities. Protein levels were normalized to a value of 1.00 for FLC or mFLC levels in the '−' inducer in both panels. RNA concentrations for *FLC-FLAG$_3$* and *mFLC-FLAG$_3$* were determined by real-time RT–PCR using a FLAG primer and a gene-specific primer. For *HA$_3$-AtSIZ1*, RNA concentration was measured by real-time RT–PCR using an HA primer and a gene-specific primer. *Tubulin* RNA was used as a loading control.

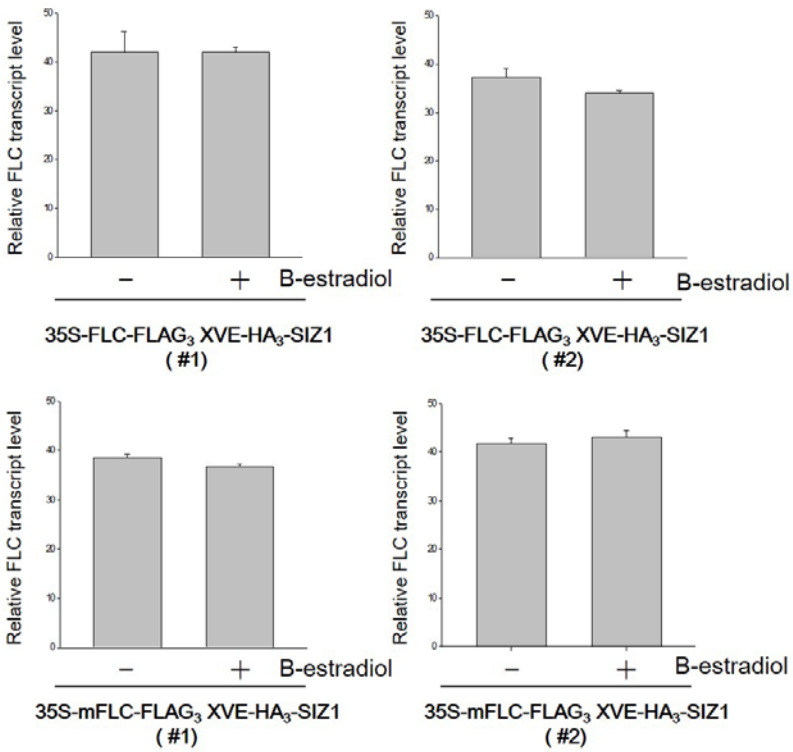


Figure 10-2. The effect of AtSIZ1 on *FLC* transcript levels.

Two independent of Double transgenic plants carrying *35S–FLC–FLAG$_3$* and *XVE–HA$_3$–AtSIZ1* or *35S–mFLC (K154R)–FLAG$_3$* and *XVE–HA$_3$–AtSIZ1* were incubated in liquid medium with β-estradiol for the induction of *AtSIZ1* expression. After incubation for 15 hours, the RNA concentrations for *FLC-FLAG$_3$* and *mFLC-FLAG$_3$* were determined by quantitative real-time RT-PCR using a FLAG primer and a gene-specific primer.

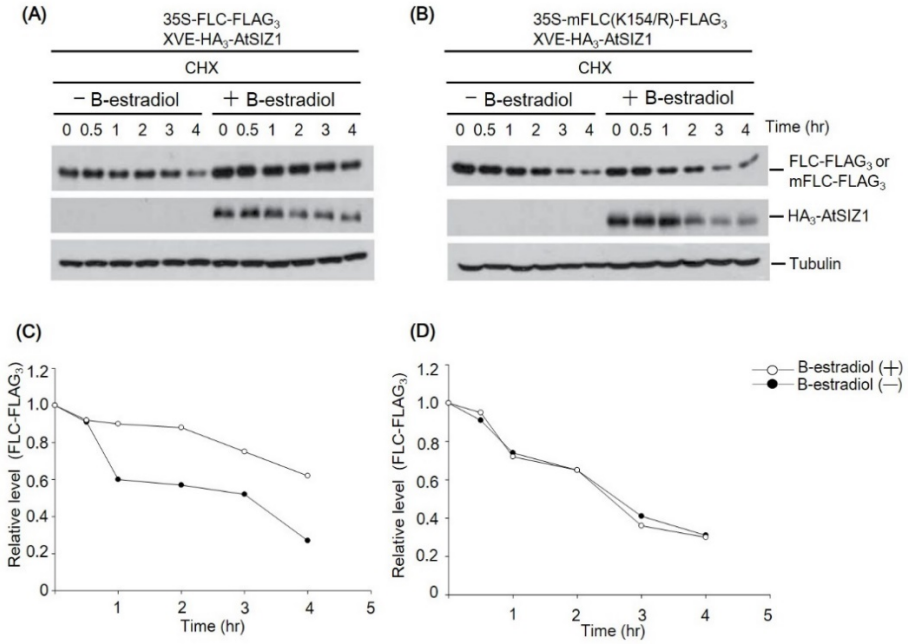

(A)
35S-FLC-FLAG3
XVE-HA3-AtSIZ1
CHX
− B-estradiol
+ B-estradiol
0 0.5 1 2 3 4 0 0.5 1 2 3 4
(B)
35S-mFLC(K154/R)-FLAG3
XVE-HA3-AtSIZ1
CHX
− B-estradiol
+ B-estradiol
0 0.5 1 2 3 4 0 0.5 1 2 3 4
Time (hr)
FLC-FLAG3 or
mFLC-FLAG3
HA3-AtSIZ1
Tubulin
(C)
Relative level (FLC-FLAG3)
Time (hr)
(D)
Relative level (FLC-FLAG3)
Time (hr)
B-estradiol (+)
B-estradiol (−)

Figure 11. Post-translational decay of FLC or mFLC

To assess the degradation of FLC, double transgenic plants of *35S-FLC-FLAG*$_3$ and *XVE-HA*$_3$*-AtSIZ1* (A) or *35S-mFLC (K154R)-FLAG*$_3$and *XVE-HA*$_3$*-AtSIZ1* (B) were incubated in liquid medium with β-estradiol for the induction of *AtSIZ1* expression, washed, and transferred to MS medium with 100 μM cycloheximide (CHX). At the indicated times, protein was extracted and analysed by western blotting with anti-HA or anti-FLAG antibodies. Tubulin was used as a loading control. FLC or mFLC levels during degradation were also expressed in graph form. The relative protein levels of FLC (C) or mFLC (D) were normalized to numerical values based on a value of 1.0 for the protein levels at 0h using the data shown in both (A) and (B). Open circles indicate FLC (or mFLC) with AtSIZ1 and filled circles indicate FLC (or mFLC) without AtSIZ1.

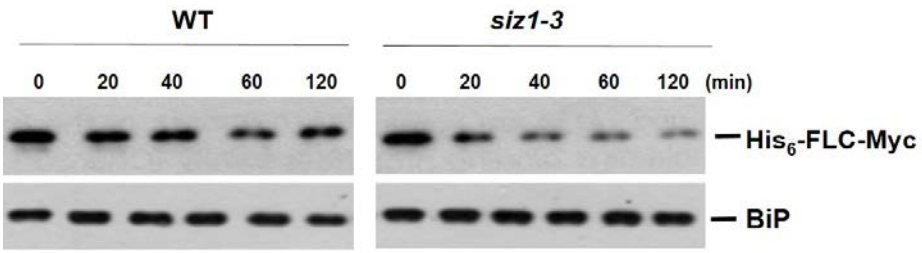


Figure 12. Cell-free degradation of FLC protein. His_6-FLC-Myc was overexpressed in *E.coli* and purified with Ni^{2+}-NTA columns. Total proteins were extracted from wild type and *siz1-3* mutant. His_6-FLC protein was mixed with total protein extracts and then incubated at room temperature. Samples were taken at indicated time point and His_6-FLC was separated on 11% SDS-polyacrylamide gel and then detected by western blot with anti-Myc antibody. BiP was used as a loading control.

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FLC modification by SUMO is necessary for flowering repression

FLC overexpression causes late flowering, and *flc* mutants are characterized by early flowering in *Arabidopsis* (Sanda an d Amasino, 1996). Based on these known effects of FLC and the present sumoylation data, next I examined the effect of sumoylation on the activity of FLC as a repressor of the transition to flowering. *FLC*- and *mFLC*-overexpressing transgenic *Arabidopsis* plants were generated using *35S-FLC-FLAG*$_3$ and *35S-mFLC-FLAG*$_3$ constructs, respectively (Figure 13A). After selecting homozygous lines (Figure 13B and C), the recombinant protein levels of FLC-FLAG$_3$ and 35S-mFLC-FLAG$_3$ were first examined then the transgenic plants were investigated for vegetative growth and flowering time (Figure 14A and B). The relative flowering time of each transgenic plants was assessed by counting the number of rosette leaves. The number of rosette leaves in WT plants was 14.75 ± 0.71, and that of *mFLC*-overexpressing plants was 14.63 ± 1.16, which was comparable with that of the WT. However, in *FLC*-overexpressing plants, the number of rosette leaves was 30.50 ± 4.68, which represented an ~2-fold increase (Figure 14C). The relative flowering time of each transgenic plant was also determined by counting the days to flowering. The number of days before the appearance of inflorescences in WT plants was 28.65 ±1.23, and that of *mFLC*-overexpressing plants was 28.07±0.94, which was comparable with that of the WT. However, in *FLC*-overexpressing plants, the number of days before

the appearance of inflorescences was 52.31±1.57, which represented an approximately 1.85-fold increase (Figure 14D). As a result, the flowering time was significantly delayed in *FLC*-overexpressing *Arabidopsis* plants, while no changes were detected in *mFLC*-overexpressing plants (Figure 14 C and D). However, vegetative growth was not affected in *FLC*- or *mFLC*-overexpressing plants (Fig. 14A), suggesting that sumoylation is an important modification for the regulation of FLC function.

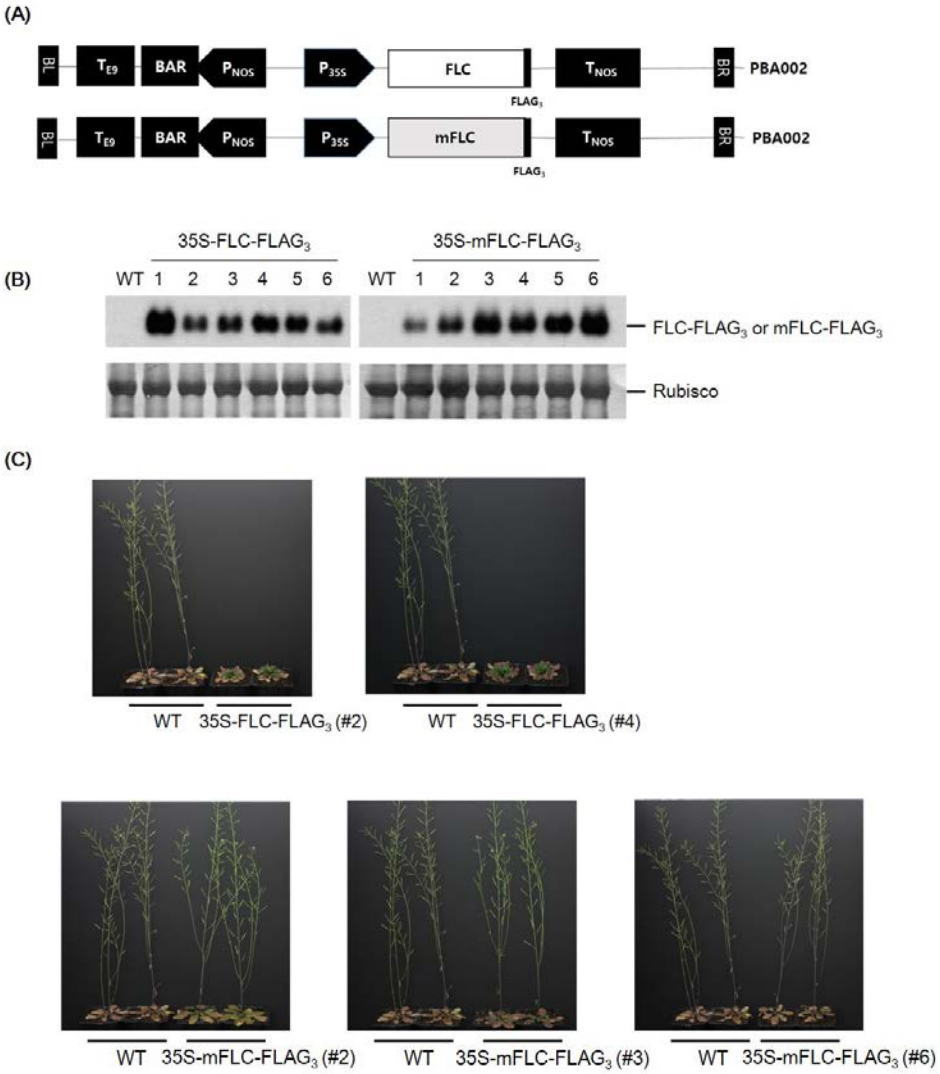
(A)
BL
T_{E9}
BAR
P_{NOS}
P_{35S}
FLC
$FLAG_3$
T_{NOS}
BR
PBA002
BL
T_{E9}
BAR
P_{NOS}
P_{35S}
mFLC
$FLAG_3$
T_{NOS}
BR
PBA002
(B)
35S-FLC-$FLAG_3$
35S-mFLC-$FLAG_3$
WT 1 2 3 4 5 6
WT 1 2 3 4 5 6
FLC-$FLAG_3$ or mFLC-$FLAG_3$
Rubisco
(C)
WT
35S-FLC-$FLAG_3$ (#2)
WT
35S-FLC-$FLAG_3$ (#4)
WT
35S-mFLC-$FLAG_3$ (#2)
WT
35S-mFLC-$FLAG_3$ (#3)
WT
35S-mFLC-$FLAG_3$ (#6)

Figure 13. Selection of *FLC*- and *mFLC*-overexpressing plants.

Arabidopsis was transformed with *35S-FLC-FLAG3* or *35S-mFLC-FLAG3* constructs. Transgenic plants overexpressing *FLC-FLAG*$_3$ or *mFLC-FLAG*$_3$ were selected and homozygous transgenic plants were chosen for further experiment. (A) Recombinant plasmids for *Arabidopsis* transformation. Bar, basta resistance gene; P_{35S}, cauliflower mosaic virus promoter; $T_{E9,}$ 9 copies tandem repeated CArG promoter; BL, left border; BR, right border (B) The protein levels of FLC-FLAG$_3$ and mFLC-FLAG$_3$ were examined by western blotting with anti-FLAG antibody. (C) After flowering, line number 2 and 4 (of transgenic plants overexpressing *FLC-FLAG*$_3$) and line number 2, 3, and 6 (of transgenic plants overexpressing *mFLC-FLAG*$_3$) were photographed.

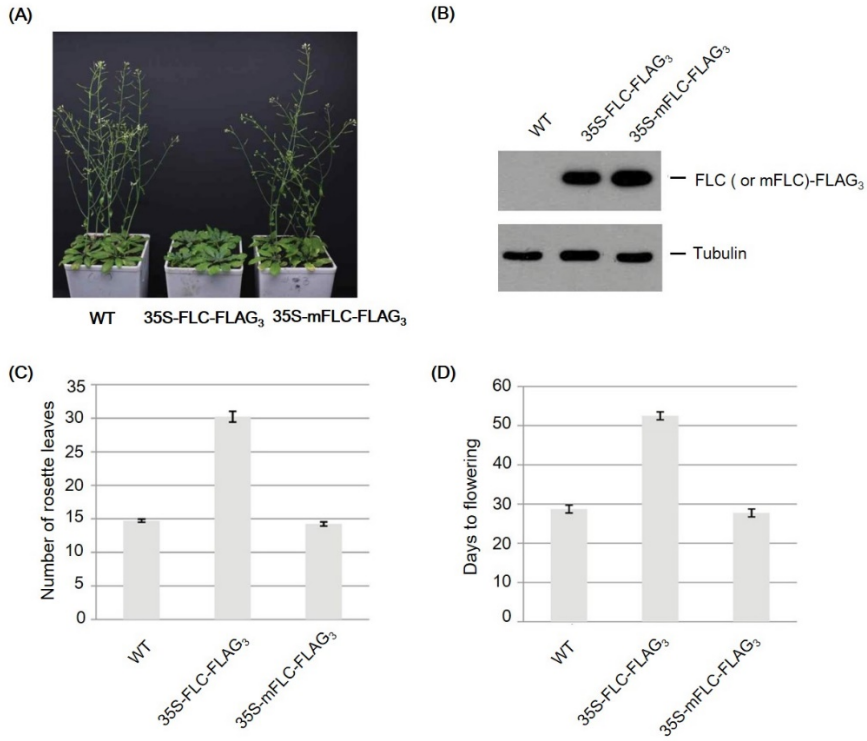

Figure 14. Phenotypes of *FLC* and *mFLC*-overexpressing plants.

(A) Vegetative growth and flowering of transgenic plants overexpressing *FLC-FLAG*$_3$ or *mFLC-FLAG*$_3$ were examined. (B) The protein levels of FLC-FLAG$_3$ and mFLC-FLAG$_3$ were examined by western blotting with anti-FLAG antibody. Tubulin was used as a loading control. (C) Flowering time in transgenic plants was examined by counting the number of rosette leaves. Significant differences were detected between WT and *FLC-FLAG*$_3$-overexpressing plants, whereas WT and *mFLC-FLAG*$_3$-overexpressing plants were almost identical ($P < 0.0001$, *t*-test, $n=12$). (D) The days to flowering were also determined to be identical ($P < 0.0001$, *t*-test, $n=12$). In both cases (C and D), bars indicate standard errors.

Mutant FLC can interact with AtSIZ1 and FLC

The observation that AtSIZ1 stabilizes FLC but not mFLC suggests that mFLC does not interact with AtSIZ1. Therefore, possible interaction between AtSIZ1 and mFLC was examined using an *in vitro* pull down. His_6-FLC or His_6-mFLC, and full-length MBP-AtSIZ1 were purified with Ni^{2+}-NTA or glutathione affinity columns and it was determined whether or not His_6-FLC or His_6-mFLC proteins could be pulled down with AtSIZ1. The results showed that AtSIZ1 interacts with both FLC and mFLC (Figure 15). As *mFLC* overexpression had no effect on flowering time, an experiment was conducted to investigate whether mFLC can form a complex with FLC (Figure 16). To this end, the recombinant proteins His_6-FLC, His_6-mFLC, GST and GST-FLC were overexpressed *in E.coli*, these proteins were isolated with Ni^{2+}-NTA or glutathione affinity columns, and whether His_6-FLC or His_6-mFLC proteins could be pulled down with GST or GST-FLC proteins was examined. As shown in Figure 16, GST-FLC formed a complex with both His_6-FLC and His_6-mFLC.

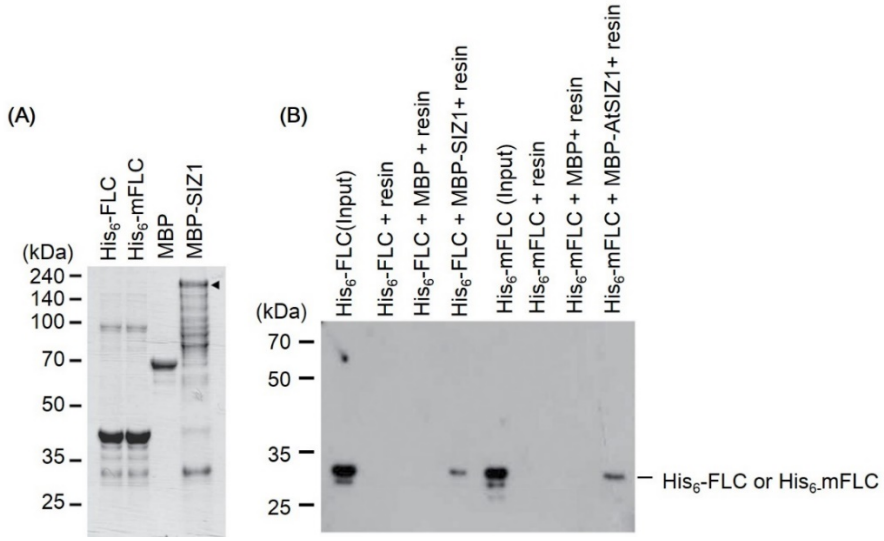

Figure 15. AtSIZ1 interacts with mFLC (K154R). (A) His_6-FLC, His_6-mFLC, and full-length MBP–AtSIZ1 were overexpressed in *E. coli* and purified with Ni^{2+}-NTA or amylose affinity columns. The arrowhead indicates MBP–AtSIZ1. (B) His_6-FLC or His_6-mFLC proteins were pulled down with full-length MBP–AtSIZ1, separated on 11% SDS–polyacrylamide gels, and analysed by western blotting with an anti-His antibody.

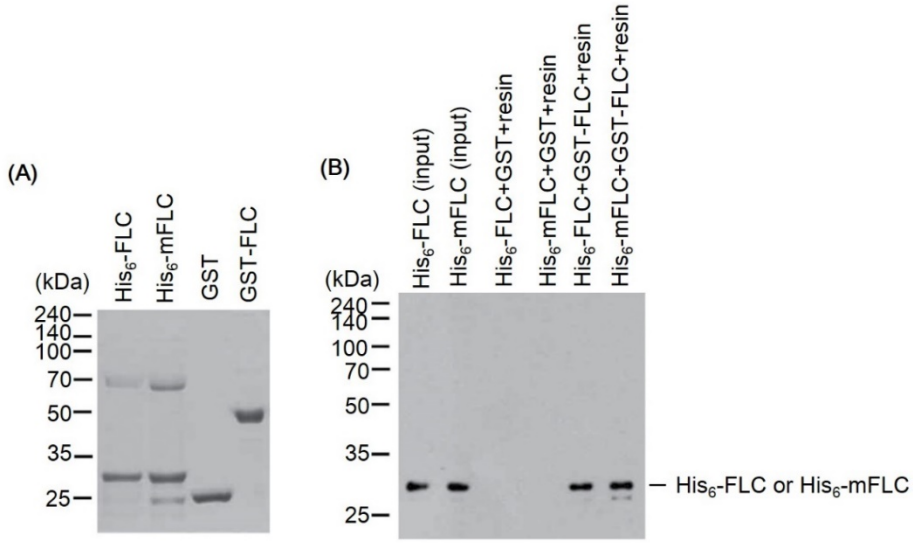

Figure 16. FLC can form a complex. (A) His_6-FLC, His_6-mFLC, GST, and GST–FLC were overexpressed in *E. coli* and purified with Ni^{2+}-NTA or glutathione affinity columns. (B) His_6-FLC or His_6-mFLC proteins were pulled down with GST or GST–FLC proteins, separated on 11% SDS–polyacrylamide gels, transferred onto PVDF membranes, and detected by western blotting with an anti-His antibody.

DISCUSSION

In this study, I showed that FLC-mediated flowering repression is activated by sumoylation and that AtSIZ1 stabilizes FLC.

Eukaryotic cells express SP-RING finger proteins, SAP and Miz-finger domain (Siz) proteins, and protein inhibitor of activated STAT (PIAS) proteins (Hochstrasser, 2001). Recently, SIZ1-type proteins with a SP-RING domain were also identified in plants and were shown to be involved in diverse biological processes (Novatchkova *et al*., 2012; Ishida *et al*., 2012).

The function and stability of transcription factors are modulated by various post-translational modifications. The conjugation of SUMO (a protein modifier) to a target protein regulates its function and stability. FLC is modified by ubiquitin (Park *et al*., 2007), indicating that other post-translational modifications, such as sumoylation, may play a role in the regulation of FLC activity. Therefore I examined whether AtSIZ1 has E3 SUMO ligase activity for FLC. The results of pull-down , BiFC and Co-immunoprecipitation assay showed a strong interaction between FLC and AtSIZ1 (Figure 4,5 and 6), and *in vitro* sumoylation assays showed that FLC is modified by SUMO (Figure 7). However, the results showed that the attachment of SUMO to FLC occurred independently of AtSIZ1 *in vitro* (Figure 7 and 8).

The covalent attachment of SUMO to a lysine residue in the target protein is generally mediated by E3 SUMO ligases. However, direct transfer

from the SUMO-conjugating enzyme Ubc9 can occur through at least two ligase-independent mechanisms. First, Ubc9 can directly recognize the sumoylation motif Ψ-K-x-[D/E] (Ψ, an aliphatic branched amino acid; x, any amino acid) and conjugate the lysine residue (Bernier-Villamor *et al*., 2002). Second, some SUMO substrates contain SUMO-interacting motifs (SIMs) that promote their own conjugation (Meulmeester *et al*., 2008; Zhu *et al*., 2008). These SIMs bind to the SUMO moiety to which Ubc9 is attached, thereby increasing its local concentration and facilitating sumoylation. The results of the present study indicate that FLC is sumoylated by one of these mechanisms in the absence of an E3 SUMO ligase.

Since FLC sumoylation was inhibited by AtSIZ1 (Figure 8), I further examined the mechanisms underlying the binding of AtSIZ1 to FLC and its effect on FLC activity and stability. For this purpose, double transgenic *Arabidopsis* plants were generated through transformation with a *35S–FLC–FLAG*$_3$ transgene an estradiol-inducible *XVE–HA*$_3$*–AtSIZ1* transgene to examine the effect of AtSIZ1 on the stability of FLC. *AtSIZ1* induction with estradiol increased the concentration of FLC but not that of mFLC (Figure 10-1A and C). Furthermore, *AtSIZ1* overexpression retarded the degradation of FLC whereas that of mFLC was not affected (Figure 11A and B). In addition, cell-free degradation assay revealed that the degradation level of recombinant FLC proteins in WT and *siz1-3* mutant extracts. Interestingly, FLC protein was quickly degraded in the *siz1-3* mutants extracts rather than WT in a time dependent manner (figure 12).

In any case, based on the present findings, these data suggest that AtSIZ1 stabilizes FLC through direct binding to FLC before or after FLC sumoylation *in vivo* (Figure 17). Furthermore, the inhibitory effect of AtSIZ1 on FLC sumoylation suggests the possible existence another E3 SUMO ligase for FLC in *Arabidopsis* (Figure 17).

Since FLC is a central regulator of flowering, extensive research has been conducted to elucidate the mechanisms regulating FLC expression at the transcriptional and post-transcriptional levels in association with flowering time (He and Amasino, 2005; Greb *et al*., 2007; Krichevsky *et al*., 2006; Heo and Sung, 2011; Swiezewski *et al*., 2009; Kim *et al*., 2005; Zhao *et al*., 2005; Park *et al*., 2007). In the present study, the role of FLC in the transition to flowering was examined using the sumoylation site mutant mFLC. To characterize the function of FLC in the control of flowering time, I generated *FLC*- or *mFLC*-overexpressing transgenic *Arabidopsis* plants and examined their flowering time by counting the number of rosette leaves. *FLC* overexpression delayed flowering, whereas *mFLC* overexpression had no notable effect on flowering time (Figure 14A and B), indicating that sumoylation is critical for FLC to exert its floral repressor function.

The lack of an effect of *mFLC* overexpression on flowering time may have resulted from an impaired interaction of mFLC with AtSIZ1 or a defect in complex formation with FLC. However, *in vitro* pull-down analysis showed that mFLC interacted with AtSIZ1 and with FLC. From these results, I propose

several possible mechanisms explaining why *mFLC* overexpression does not affect flowering time. First, sumoylation of the FLC protein may be necessary for its activation. As mFLC cannot be modified with SUMO, this protein may not have an effect on flowering time despite its overexpression. Secondly, mFLC may inactivate endogenous FLC. Transgenic mFLC may form a complex with endogenous FLC and act in a dominant-negative form. Thus, a possible reason for the observation that flowering time in *mFLC*-overexpressing plants is comparable to that of WT plants is that the FLC level is originally low in WT plants, although this protein could be scavenged by the overexpressed mFLC through complex formation.

DET1 (De-etiolated 1), a SINAT5-interacting partner, blocks the ubiquitination of LHY (Long Hypocotyl) by SINAT5 through direct interaction with SINAT5 (Park *et al*., 2007). The present data show that AtSIZ1 inhibits the sumoylation of FLC through direct interaction with FLC *in vitro* (Figure 8A). However, AtSIZ1 increased the level of FLC in transgenic plants (Figure 10-1A). Furthermore, the degradation of FLC was delayed in the presence of AtSIZ1 (Figure 11A and 12). These findings suggest that direct binding of AtSIZ1 to FLC protects the protein from degradation induced by its ubiquitination by SINAT5, as shown for DET1, which blocks the ubiquitination of LHY by SINAT5. AtSIZ1 may thus have a protective effect on FLC by antagonizing its ubiquitination (Figure 17).

In conclusion, these results indicate that AtSIZ1 controls the stability

of FLC by directly binding to FLC, but not through its E3 SUMO ligase activity, and that the FLC-mediated floral transition is negatively regulated by SUMO conjugation. In addition, I showed that proteolytic turnover of flowering-associated proteins can be regulated by sumoylation. I elucidated the biochemical mechanisms underlying the regulation of FLC function and stability by sumoylation. Together with previous findings, my results suggest that both the post-translational modification systems, ubiquitination and sumoylation, can regulate flowering by direct modulation of FLC stability and activity.

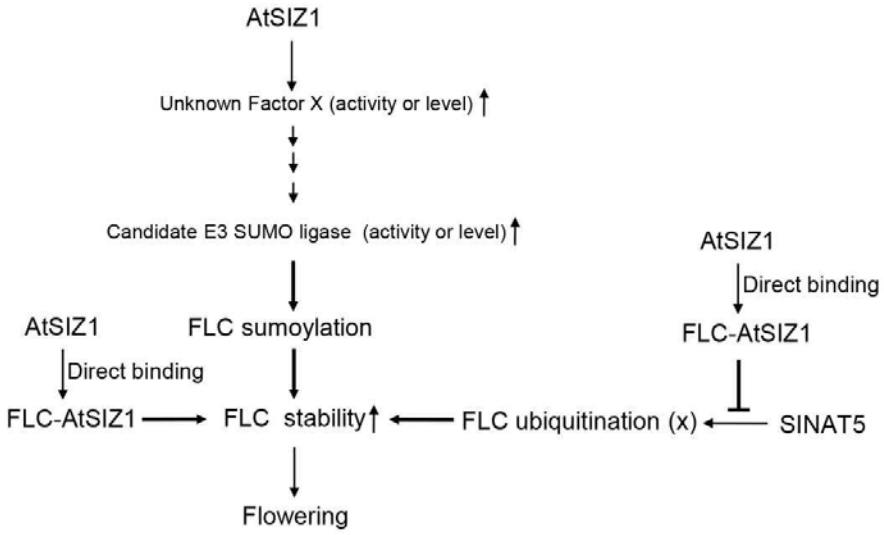

Figure 17. Possible regulatory modes of FLC stability.

Based on sumoylation and ubiquitination data (Park *et al*., 2007), to date, three possible scenarios are suggested to explain how AtSIZ1 can stabilize FLC. Firstly, AtSIZ1 may induce the expression of an unknown factor (a type of transcription factor) or increase its activity, and the activity or level of a candidate E3 SUMO ligase for FLC would then be increased. Subsequently, this factor may sumoylate FLC, resulting in an increase in the stability and activity of FLC. Secondly, AtSIZ1 may directly bind to FLC in vivo, although it inhibits FLC sumoylationin vitro, causing FLC stabilization. Thirdly, the direct binding of AtSIZ1 with FLC may inhibit FLC ubiquitination via SINAT5 and thereby stabilize FLC.

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국문초록

개화억제자인 FLC(Flowering Locus C) 단백질은 영양생장에서 생식생장으로의 전환에 중요한 역할을 한다. *FLC* 는 개화를 유도하는 유전자들의 활성을 억제하여 개화를 억제하며 *FLC* 유전자의 발현은 *FRI*(*FRIGIDA*)에 의해 촉진되고 저온처리에 의해 그 발현이 감소된다. 개화시기 조절의 핵심 유전자인 *FLC* 의 전사에 영향을 주는 몇몇 인자가 알려져 있으나 *FLC* 의 안정성 및 기능의 번역 후 조절기작은 분명하게 밝혀져 있지 않다. 본 연구는 FLC 단백질의 활성이나 안정성 조절을 통한 개화의 메커니즘에 대한 연구이다. 수모화는 수모가 E1, E2, E3 효소의 순차적인 작용에 의해 목적 단백질 내의 라이신 잔기에 결합하는 과정으로 단백질의 기능 및 안정성을 조절하며 다양한 세포학적 과정과 연관되어 있다. 이분자형광상보기법과 시험관 내 풀다운, 면역침강법을 수행하여 FLC 단백질과 애기장대에서 E3 수모 리가아제(E3 SUMO ligase)인 AtSIZ1 단백질이 상호작용을 한다는 것을 확인하였고 이는 FLC 가 수모 리가아제인 AtSIZ1 의 타겟 단백질임을 추측할 수 있었다. 그러나 시험관 내에서 FLC 단백질이 E1 과 E2 효소에 의해 수모화되지만 E3 수모 리가이제인 AtSIZ1 에 의해 FLC 의 수모화는 억제됨을 확인하였다. 애기장대의

FLC 와 AtSIZ1 동시발현 형질전환체 에서는 AtSIZ1 단백질의 발현 유도에 반응하여 FLC 단백질의 함량이 증가하고 분해속도가 느려지는 것을 확인하였는데 이는 AtSIZ1 단백질이 FLC 단백질과의 직접적인 상호작용으로 FLC 단백질의 안정성을 조절함을 보여준다. 또한 FLC 의 과다발현 형질전환체에서는 야생형에 비해 개화시기가 매우 지연되지만 FLC 의 수모화 인지부위의 돌연변이 과다발현 형질전환체는 야생형과 비슷한 개화시기를 보임을 확인하였다. 이는 수모화가 FLC 를 매개로 하여 이루어지는 개화억제 메커니즘에 중요한 역할을 함을 보여준다. 따라서 본 연구는 AtSIZ1 이 상호작용을 통해 FLC 의 수모화 억제 및 안정화에 영향을 주며 FLC 에 의해 매개된 개화시기는 수모화와 FLC 의 활성 및 안정성에 의해 조절 됨을 밝힌 중요한 결과이다.

주요어 : AtSIZ1, FLC, 번역 후 수정과정, 수모, 수모화

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