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Arabidopsis HIGH PLOIDY2 sumoylates and stabilizes FLC through E3 ligase activity

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ABSTRACT

Flowering Locus C (FLC), which is known as a floral repressor, plays an important role in flowering. A large number of studies have been carried out to elucidate the mechanisms of FLC gene expression and its protein function. However, FLC-regulatory mechanism is not clearly identified at post-translational stage yet. Here, we showed that Arabidopsis HIGH PLOIDTY2 (HPY2) functions as a real E3 SUMO ligase for FLC. *In vitro* and *vivo* pull-down analyses showed that FLC physically interacts with HPY2. *In vitro* sumoylation assays showed that FLC sumoylation is stimulated by AtSIZ1 in a dependent manner of SUMO-activating

enzyme E1 and conjugating enzyme E2, indicating that AtHPY2 is an E3 SUMO ligase for FLC. In transgenic plants, inducible *HPY2* overexpression led to an increase in the concentration of FLC, indicating that HPY2 stabilizes FLC through direct sumoylation. The flowering time of *hpy2-2* mutants was delayed compared to wild-type plants under long and short day conditions and its loss effect in flowering is bigger under short day condition than long day condition. In addition, FLC transcript level was also down-regulated in *hpy2-2* mutants. The data indicate that HPY2 regulates FLC function and stability at both transcription and post-translation steps through its E3 SUMO ligase activity.

Key words: E3 SUMO ligase FLC, HPY2, SUMO, sumoylation.

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LIST OF FIGURES

Fig. 1. Interaction of HPY2 with FLC. (A) His_6-FLC and GST-HPY2 were overexpressed in *E. coli* and purified with Ni^{2+}-NTA or glutathione affinity columns. (B) The His_6-FLC protein was pulled down with GST-HPY2 protein, separated on 11% SDS-polyacrylamide gels, and analyzed by western blotting with an anti-His antibody. (C) *In vivo* interaction of HPY2 and FLC. Wild-type plants were infiltrated with different combinations of *35S-Myc$_6$-FLC* or *35S-HPY2-FLAG$_3$* constructs. The total proteins were extracted from each sample and immunoprecipitated with an anti-FLAG antibody. After immunoprecipitation, Myc_6-FLC was detected by western blotting with anti-Myc antibody. Myc_6-FLC and HPY2-$FLAG_3$ expression was also examined by western blotting with anti-Myc and anti-FLAG antibodies, respectively.

Fig. 2. FLC is sumoylated by HPY2 *in vitro*. *Arabidopsis* His_6-AtSAE1b, His_6-AtSAE2, His_6-AtSCE1, GST-HPY2, His_6-AtSUMO1, and GST-FLC-Myc were overexpressed in *E. coli* and purified with Ni^{2+}-NTA and glutathione affinity columns, respectively. Sumoylation of GST-FLC-Myc was assayed in the presence or absence of E1 (His_6-AtSAE1b and His_6-AtSAE2), E2 (His_6-AtSCE1), E3 (GST-HPY2), and His_6-AtSUMO1. After the reaction, sumoylated FLC was detected by

western blotting with an anti-Myc antibody. To identify the sumoylation site on FLC, GST-FLCm1-Myc (K54R), GST-FLCm2-Myc (K135R), and GST-FLCm3-Myc (K154R) 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-HPY2), and His_6-AtSUMO1 without (-) or with (+) a mutant protein instead of GST-FLC-Myc. After the reaction, sumoylated FLC protein was also detected by western blotting with an anti-Myc antibody.

Fig. 3. FLC is stabilized by HPY2 *in vivo*. Double transgenic plants of *35S–FLC–$FLAG_3$* and *XVE–HA_3–HPY2* (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 *HPY2* expression. After incubation for 15 hours, HA_3-HPY2, 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 qRT-PCR using a FLAG primer and a gene-specific primer. *Tubulin* RNA was used as a loading control.

Fig. 4. Flowering time of *hpy2-2* mutants. Early flowering was observed in the *hpy2-2* mutant grown under both long day and short conditions (A). Flowering time was investigated by counting of the number of rosette leaves present when inflorescences appeared (B). The numbers of wild-type and *hpy2-2* plants were significantly different ($P < 0.0001$, t-test, n=20). Bars indicate standard errors.

Fig. 5. Transcript levels of flowering-related genes in *hpy2-2* mutants. Total RNA was isolated from the leaves of wild-type and *hpy2-2* mutant plants grown in soil under long day or short day conditions. Transcript levels were examined using real-time qRT-PCR with gene-specific primers. Results are expressed as means ± S.D. (n = 3). Abbreviations: *FLC*: *FLOWERINBG LOCUS C*; *SOC1*: *SUPPRESSOR OF OVEREXPRESSION OF CO 1*; *FT: FLOWERINBG LOCUS T*; *TSF*: *TWIN SISTER OF FT*.

INTRODUCTION

Post-translational modification is an important mechanism for regulation of protein function and stability. Currently, over 200 types of protein modifications have been reported (Castro *et al*., 2012). One of them is a sumoylation which small ubiquitin-related modifier (SUMO) is covalently attached to lysine residues of target proteins (Wilkinson and Henley, 2010). SUMO is a small peptide which has a *molecular mass* of *approximately* 11 *kDa. As in* yeast and animal systems, it regulates various cellular processes such as stress and defense responses, nitrogen metabolism, hormone signaling, epigenetic gene expression, growth 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, 2007a; Catala *et al*., 2007; Lee *et al*., 2007; Conti *et al*., 2008; Yoo *et al*., 2006; Park *et al*., 2011; Son *et al*., 2014; Kim *et al*., 2015a; Kim *et al*., 2015b).

Modification of target proteins with SUMO is usually catalyzed by E3 SUMO ligases, although conjugation of SUMO to target proteins can occur in the absence of such ligases (Wilkinson and Henley, 2010). So far, four types of E3 SUMO ligases, RanBP2 (RanGAP1-binding protein 2), Pc2 (Polycomb group 2), NES2/MMS21 (non-SMC element/methyl methanesulfonate sensitive 1) and SIZ/PIAS (SAP and MIZ/protein inhibitor of activated STAT), have been identified, (Verger et al., 2003; Johnson and Gupta, 2001; Kahyo et al., 2001; Rose and Meier,

2001; Pichler et al., 2002; Kagey et al., 2003). Among them, SIZ/PIAS family proteins are the largest group of SUMO E3 ligases. They have five structural motifs: an N-terminal SAP (scaffold attachment factor A/B/acinus/PIAS) motif, a PINIT (Pro-Ile-Asn-Ile-Thr) motif, a SP-RING zinc finger domain, a SXS (for serine-X-serine, where X is any amino acid) domain and a PHD motif (plant homeodomain) (Miura et al., 2007a; Sharrocks, 2006; Garcia-Dominguez et al., 2008; Cheong et al., 2009). In Arabidopsis, two PIAS-type SUMO E3 ligases, SIZ1 (Miura et al., 2005; Miura et al., 2007a) and MMS21/HPY2 (NSE2/MMS21-type High Ploidy 2) (Huang et al., 2015; Ishida et al., 2009; Ishida et al., 2012), have been identified.

AtSIZ1regulates plant responses to nutrient deficiency, hormone signaling and environmental stresses and controls vegetative growth and development and flowering (Miura *et al*., 2005, 2007b, 2010a, 2010b; 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; Son *et al*., 2014; Kim *et al*., 2015a; Kim *et al*., 2015b). Several target proteins of AtSIZ1 including the nitrate reductases NIA1 and NIA2, INDUCER OF CBF EXPRESSION 1 (ICE1), an R2R3-type transcription factor MYB30, FLOWERING LOCUS C (FLC), SLEEPY1 (SLY1) and chromomethylase 3 (CMT3) have been identified (Conti et al., 2014; Elrouby and Coupland, 2010; Miller et al., 2010; Miura. and Hasegawa, 2010; Park et al., 2011; Zheng et al., 2012; Son et al., 2014; Kim et al., 2015a; Kim et al., 2015b).

In comparison with AtSIZ1, HPY2 is a very small protein which is composed of 249 amino acids *and* only has a SP-RING zinc finger domain. The role of HPY2 was also very little discovered. So fa, it is known that HPY2 regulates the cell cycle progression and meristem development and auxin signaling (Ishida et al., 2009; Okushima et al., 2014). AtSIZ1 and HPY2 exhibit different expression pattern and their mutants display distinct dwarf phenotype (Ishida et al., 2012). In addition, reciprocal expression of them cannot compliment the single-mutant phenotypes (Ishida et al., 2012).

FLC, a MADS-box transcription factor, 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; He and Amasino, 2005; Krichevsky *et al*., 2006; Greb *et al*., 2007). FLC transcription is also controlled by long noncoding RNAs such as COOLAIR and COLDAIR, (Heo and Sung, 2011; Swiezewski *et al*., 2009). In addition, it is known that FLC is polyubiquitinated by SINAT5 *in vitro* (Park *et al*., 2007) and it is conjugated by SUMO although the E3 SUMO ligase involved was not identified (Son et al., 2014), indicating that a post-translational mechanism is involved in the regulation of the floral transition by FLC. The post-translational modification mechanism of FLC has not been clearly characterized although the transcription al control of *FLC* has been relatively well characterized.

Here, we provide the first evidence that HPY2 has an E3 SUMO ligase for target (substrate) protein. We show that HPY2 functions as a real E3 SUMO ligase for FLC. HPY2 directly interacts with FLC and stimulates FLC sumoylation. Sumoylation stabilizes FLC and HPY2 loss causes early flowering. These findings indicate that HPY2 positively controls FLC-mediated flowering repression through FLC sumoylation.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis thaliana* Columbia-0 ecotype (wild-type; WT) and the T-DNA insertion knock-out mutant *hpy2-2* were used in this study. For plants grown in medium, seeds were surface-sterilized in commercial bleach that contained 5% sodium hypochlorite and 0.1% Triton X-100 solution for 10 minutes, rinsed five times in sterilized water, and stratified at 4°C for 2 days in the dark. Seeds were planted on agar plates containing Murashige and Skoog (MS) medium, 2% sucrose, and 0.8% agar, buffered to pH 5.7. For plants grown in soil, seeds were directly sown into sterile Vermiculite. Plants including seedlings were grown at 22, under a 16 hour light/8 hour dark cycle (long day) or under an 8 hour light/16 hour dark cycle (short day) in a growth chamber.

Construction of recombinant plasmids

To produce His_6-FLC, the cDNA encoding full-length FLC was amplified by PCR and inserted into the pET28a vector (Novagen). To produce GST-HPY2, the cDNA encoding either the full-length HPY2 cDNA was inserted into the pGEX4T-1 vector (Amersham Biosciences). For GST-FLC-Myc production, cDNA encoding full-length FLC was amplified by PCR using primers tagged with Myc and inserted into

pGEX4T-1. The production of FLC mutant proteins, GST-FLC(K5R)-Myc, GST-FLC(K135R)-Myc and GST-FLC(K154R)-Myc (the numbers indicate the positions of the lysines in FLC that were mutated to arginine), were described in previous results (Son *et al*., 2014). 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, containing full-length AtSUMO1 extended with GG at the 3 by PCR. Arabidopsis SUMO E1 and E2 enzyme-encoding constructs were kindly provided by Dr H.-P. Stuible (Colby *et al*., 2006).

All constructs were transformed into *Escherichia coli* BL21/DE3 (pLysS) cells. The transformed cells were treated with IPTG (isopropyl-β-D-thiogalactoside) to induce fusion protein expression. The sequences of the primers used in this study are listed in Supplementary Table S1. All the constructs were verified by automatic DNA sequencing to ensure that no mutations were introduced

Purification of recombinant proteins

All of the recombinant proteins were expressed in *E. coli* strain BL21 and were purified in accordance with the manufacturer instructions. Briefly, for His_6-AtSAE1b, His_6-AtSAE2, His_6-AtSCE1 and His_6-AtSUMO1 and His_6-FLC, bacteria were lysed in 50 mM NaH_2PO_4 (pH8.0), 300 mM NaCl, 1% Triton X-100, 1 mM imidazole, 5 mM DTT, 2 mM PMSF, and a proteinase inhibitor cocktail

(Roche), and purified on Ni^{2+}-nitrilotriacetate (Ni^{2+}-NTA) resins (Qiagen). For GST, GST-FLC-Myc, GST-FLC(K5R)-Myc, GST-FLC(K135R)-Myc, GST-FLC(K154R)-Myc and GST-HPY2 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 purified on glutathione resins (Pharmacia).

***In vitro* binding assay**

To examine the *in vitro* binding of GST-HPY2 to His_6-FLC, 2 μg of full-length GST-HPY2 bait 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 blotting using an anti-His antibody (Santa Cruz Biotechnology).

Sumoylation assays

In vitro sumoylation was performed in 30 μL of reaction buffer [200 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 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,, and 100 ng of GST-FLC-Myc with or without 500 ng of GST-HPY2. After incubation for 3 hours at 30°C, the reaction mixtures were separated on 10% SDS-polyacrylamide gels. Sumoylated GST-FLC-Myc was detected by Western blotting using an anti-Myc antibody (Santa Cruz Biotechnology). To identify the sumoylation site on FLC, GST-FLC-Myc, GST-FLCm1(K5R)-Myc, GST-FLCm2(K135)-Myc and GST-FLCm3(K154R)-Myc, were added to the reaction mixtures instead of GST-FLC-Myc, respectively. The reaction and the following steps were as described above.

***In vivo* interaction of HPY2 with FLC**

To investigate direct interaction between HPY2 and FLC *in vivo*, plant expression plasmids were first constructed. To express FLC, the corresponding full-length cDNA were amplified by PCR using a forward primer and a reverser primer tagged with Myc_6 and inserted into the plant expression vector pBA002. To express HPY2, the corresponding full-length cDNA were amplified by PCR using a forward primer and a reverser primer tagged with $FLAG_3$ and inserted into the plant expression vector pBA002. Secondly, wild-type Arabidopsis plants were infiltrated with different combinations of Agrobacterium transformed with *35S-Myc*$_6$*-FLC* or *35S-HPY2-FLAG*$_3$ constructs. After 2 d, the total proteins were extracted from each

sample, and Myc_6-FLC and HPY2-$FLAG_3$ were detected by western blotting with anti-Myc and anti-FLAG and antibodies, respectively. Finally, the total proteins were extracted from each sample and immunoprecipitated with an anti-FLAG antibody (1 μgml^{-1}, Santa Cruz Biotechnology) in a buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, 1 mM PMSF, and a protease inhibitor cocktail (Promega), and then FLC was detected by immunoblotting with an anti-Myc antibody (0.5 μgml^{-1}, Sigma-Aldrich) after immunoprecipitation.

Production of transgenic Arabidopsis plants

To produce FLC or mFLC (K154R)-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 the 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 HPY2 was amplified by PCR using a forward primer tagged with HA_3 and a reverser primer and inserted into the plant expression vector pET8. The resulting recombinant plasmid *XVE-*HA_3*-HPY2* was introduced into transgenic plants transformed with *35S-FLC-*$FLAG_3$ or *35S-mFLC-*$FLAG_3$ by floral dipping.

Effects of HPY2 overexpression on FLC concentration *in vivo*

Two week-old light-grown (16 hour light/8 hour dark) plants carrying *35S-FLC-FLC*$_3$ and *XVE HA*$_3$*-HPY2* or *35S-mFLC-FLC*$_3$ and *XVE HA*$_3$*-HPY2* transgenes on MS medium were treated in the light with or without β-estradiol for 15 hours. Samples were ground in liquid nitrogen and lysates were separated by SDS-PAGE. FLC-FLAG$_3$ and mFLC-FLAG$_3$ levels were examined by Western blotting with anti-FLAG antibody. HA$_3$-HPY2 induction was analyzed by western blotting with anti-HA antibody.

Investigation of flowering time

Wild-type (Col-0) and *hpy2-2* mutant plants were grown in soil under long day (16 hour light/8 hour dark) or short day (8 hour light/16 hour dark) conditions. Flowering time was investigated by counting of the number of rosette leaves present when inflorescences appeared. Twenty plants of each type (wild-type or *hpy2-2* mutant) were used in the experiment.

Quantitative real-time RT-PCR analysis

The expression levels of the genes encoding *FLC, SOC1*, *FT* and *TSF* were examined by quantitative real-time RT-PCR in WT and *hpy2-2* plants grown in long day or short day conditions as described in above. Total RNAs were isolated from the shoots of WT and *hpy2-2* plants and mRNA levels were analyzed as previously described (Park et al., 2011). Primers for tubulin were added as an internal control together with gene-specific primers. All reactions of this experiment were repeated three times with three independent RNA samples. T**he** primers used are listed in Supplemental Table S1.

Results

AtSIZ1 physically interacts with FLC

We recently reported that FLC directly interacts with the *Arabidopsis* E3 SUMO ligase AtSIZ1 *in vitro* and *vivo* but FLC sumoylation was rather inhibited by AtSIZ1 (Son *et al*., 2014). We thus have tried to identify E3 SUMO ligase which stimulates the sumoylation of FLC. For this experiment, we chose HPY2 characterized in Arabidopsis as E3 SUMO ligase possessing an SP-RING (SIZ/PIAS-RING) domain. We examined the possible physical interaction between HPY2 and FLC by *in vitro* pull-down. We first overexpressed the recombinant proteins GST-HPY2 and His_6-FLC in *E. coli* and purified them with glutathione or Ni^{2+}-NTA resins (Fig. 1A). Pull-down result showed that GST-HPY2, but not GST alone, was able to pull down His_6-FLC (Fig. 1B).

This specific interaction also was examined by immunoprecipitation. *35S-*Myc_6*-FLC* and *35S-HPY2-*$FLAG_3$ constructs were coinfiltrated into the leaves of WT plants. After immunoprecipitation (IP) with an anti-FLAG antibody, FLC was examined by western blotting against an anti-Myc antibody. FLC was detected clearly (Fig.1C), indicating a strong interaction between HPY2 and FLC, similar to the results of the *in vitro* pull-down experiment.

FLC sumoylation is stimulated by HPY2

The direct interaction of FLC and HPY2 indicated by our *in vivo* and *in vitro* results led us to hypothesize that HPY2 may function as an E3 SUMO ligase for FLC. Therefore, the recombinant proteins GST-HPY2 and GST-FLC-Myc were produced to determine whether HPY2 is the E3 SUMO ligase for FLC. His_6-AtSAE1b, His_6-AtSAE2, His_6-AtSCE1, His_6-AtSUMO1, GST-HPY2 and GST-FLC-Myc were overexpressed in *E. coli* and purified with Ni^{2+}-NTA or glutathione resins. Then, the recombinant proteins were applied to the *in vitro* sumoylation reactions. Result showed that FLC sumoylation was stimulated by HPY2 in a reaction that was dependent on E1 and E2 activities (Fig. 2A).

Previous study showed that there are three putative sumoylation sites (ΨKXE) located at lysine 5 (K5), lysine 135 (K135), and lysine 154 (K154) in FLC (Son et al., 2014). Single or double mutant derivatives with the mutations K154R, K5R/K135R, K5R/K154R, and K135R/K154R were produced, and their sumoylation was examined by *in vitro* sumoylation assays (Son et al., 2014). Result showed that K154 is the principal site of SUMO conjugation on FLC (Son et al., 2014). In current study, we thus examined sumoylation of the mutant FLC proteins, GST-FLCm1(K5R)-Myc, GST-FLCm2(K135)-Myc and GST-FLCm3(K154R)-Myc, by HPY2 and found that the sumoylation of GST-FLCm3(K154R)-Myc was not occurred and stimulated in even the presence of HPY2 in the reaction mixture,

confirming that K154 is a real sumoylation site on FLC (Fig. 2B).

FLC is stabilized by HPY2

The direct interaction between HPY2 and FLC and the stimulation of FLC sumoylation by HPY2 imply that the concentration of FLC may be regulated by the amount of HPY2 present *in vivo*. We therefore measured FLC concentrations in transgenic plants carrying a *35S-FLC-FLAG*$_3$ transgene and an estradiol-inducible *XVE-HA*$_3$*-HPY2* transgene. Induction of the expression of *HPY2* increased the FLC concentrations up to 2.3- and 3.6-fold in two independent transgenic plants, respectively (Fig. 3A). 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 *HPY2* induction (Fig. 3B). It may be possible that the transcript levels of *FLC* or *mFLC* can affect the levels of FLC and mFLC proteins in transgenic plants. We thus examined *FLC* and *mFLC* transcript levels by real-time qRT-PCR after induction of *HPY2* in *FLC*- or *mFLC*-overexpressing double transgenic plants. The result showed that the transcript levels of *FLC* and *mFLC* were comparable under these conditions (Fig.3A and B).

hpy2 mutant exhibits early flowering

The stabilization of FLC through FLC sumoylation by HPY2 activity suggests that FLC function may be decreased in *hpy2-2* mutants, which effects on flowering. We therefore examined flowering time of the *hpy2-2* mutant by counting the number of rosette leaves just after bolting under long day and short day conditions. Result showed that flowering time was delayed in the *hpy2-2* mutants (Fig. 4A and B). But, flowering time of the *hpy2-2* mutants was significantly delayed compared to wild-type plants under short day condition while it was slightly delayed compared to wild-type plants under long day condition (Fig. 4A and B).

Expression of flowering-related genes was affected by HPY2

We have shown that HPY2 is an E3 SUMO ligase for FLC, suggesting that the early flowering of *hpy2-2* mutants may be caused in part by low level and activity of FLC. However, it is also possible that early flowering in *hpy2-2* mutants results from a change in gene expression. Therefore, we measured transcript levels of flowering-related genes, *FLOWERINBG LOCUS C* (*FLC*), *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1*), *FLOWERINBG LOCUS T* (*FT*) and *TWIN SISTER OF FT* (*TSF*). Transcript levels of these genes were determined, using real-time qRT-PCR, in samples of total RNA isolated from both wild-type and *hpy2-2* mutants grown in soil under long day or short day conditions. The level of *FLC*

transcript did greatly change in *hpy2-2* mutants, relative to levels in wild-type plants under both conditions (Fig. 5A and B). Expression of *SOC1*, *FT* and *TSF* was very slightly increased in *hpy2-2* mutants compared with wild-type plants under long day condition (Fig. 5A). However, there were obvious differences in expression of *SOC1*, *FT* and *TSF* between wild-type and *hpy2-2* plants under short day conditions (Fig. 5B). Their transcript levels were increase about two folds in the *hpy2-2* plants under short day conditions (Fig. 5B).

Discussion

In this study, we report that HPY2 is a real E3 SUMO ligase which catalyzes FLC sumoylation through direct interaction.

In plants, two SIZ types of E3 SUMO ligases, AtSIZ1 and HPY2, have been identified so far. The functional roles and phenotypic features of AtSIZ1 have been well characterized. But, the functions of HPY2 are less identified. Recently, it was reported that HPY2 has self-sumoylation activity *in vitro* and HPY2 mutation causes the reduction of SUMO-conjugate levels in Arabidopsis (Ishida et al., 2009). In addition, loss of HPY2 function resulted in a premature mitotic-to-endocytic transition, leading to severe dwarfism (Ishida et al., 2009). Besides, the phenotype of *hpy2-1* mutants does not depend on the accumulation of salicylic acid (SA) while the phenotype of *siz1-2* mutants is caused by SA accumulation, indicating that they function through different pathways and their roles are not overlapped (Ishida et al., 2012).

Sumoylation of target proteins by AtSIZ1 has been well characterized (Conti et al., 2014; Elrouby and Coupland, 2010; Miller et al., 2010; Miura. and Hasegawa, 2010; Park et al., 2011; Zheng et al., 2012; Son et al., 2014; Kim et al., 2015a; Kim et al., 2015b). But, target protein of HPY2 was not still identified and E3 ligase activity of HPY2 for target protein was not reported yet, either. In previous study, we showed that AtSIZ1 directly interacted with FLC and stabilized it (Kim et al., 2015a). But,

interestingly, AtSIZ1 inhibited FLC sumoylation. We thus tried to isolate a real E3 SUMO ligase for FLC. Because only two E3 SUMO ligases, AtSIZ1 and HPY2, have been characterized in Arabidopsis so far, we chose HPY2 as a candidate of E3 SUMO ligase for FLC. Pull-down and sumoylation assays showed that HPY2 directly interacts with FLC (Fig.1B and C) and HPY2 has E3 SUMO ligase activity for FLC (Fig. 2A), which is the first report that HPY2 has E3 SUMO ligase activity for target protein.

The covalent attachment of SUMO to a lysine residue in the target protein is generally occurred by two different mechanisms. First mechanism is the direct transfer from the SUMO-conjugating enzyme Ubc9 to target protein (Bernier-Villamor *et al*., 2002; Meulmeester *et al*., 2008; Zhu *et al*., 2008). Second one is the mediation by E3 SUMO ligase (Park and Yun, 2013). Our previous study showed that FLC can be sumoylated without help of E3 SUMO ligase and FLC sumoylation was rather inhibited by E3 SUMO ligase AtSIZ1. By the way, current result showed that FLC sumoylation was increased by addition of HPY2 in the reaction (Fig. 2A), indicating that HPY2 stimulates FLC sumoylation as a real E3 SUMO ligase.

Previous study showed that *AtSIZ1* stabilized FLC although it inhibited FLC sumoylation (Son et al., 2014). In current study, we discovered that HPY2 overexpression increased the concentration of FLC but not that of mFLC (Fig. 3A and B), indicating that FLC sumoylation by HPY2 stabilizes FLC. In most cases,

modification of target proteins by SUMO affects their stability as well as activity in yeast, animal and plants system (Park and Yun, 2013). As reported in those papers, our results also indicate that sumoylation give a positive effect on FLC function.

Since FLC is a central regulator of flowering and HPY2 has an E3 SUMO ligase activity for FLC, we next examined flowering time of the *hpy2-2* mutant and found that it flowered earlier than wild-type (Fig. 4). We previously reported that sumoylation is critical for FLC to exert its floral repressor function because *FLC* overexpression delayed flowering, whereas *mFLC* overexpression had no notable effect on flowering time (Son et al., 2014). Therefore, we guess that early flowering of *hpy2-2* mutants must be caused by lower stability and activity of FLC due to loss of its E3 SUMO ligase activity.

All of these data indicate that early flowering in the *hyp2-2* mutant results from the loss of HPY2 activity, which leads to low FLC levels or activity. But, there is a possibility that early flowering of the *hyp2-2* mutant may be caused by the change of gene expression. Result showed that the level of *FLC* transcript was also decreased in the *hyp2-2* mutant (Fig. 5). This data indicates that HPY2 controls FLC-mediated flowering at transcription and post-translational stages.

In conclusion, our results indicate that HPY2 controls FLC-mediated floral transition by its E3 SUMO ligase activity as well as direct binding to FLC. Together with previous findings, our data indicate that two E3 SUMO ligases AtSIZ1 and

HPY2 involve in the regulation of FLC stability and sumoylation is a critical modification for regulation of FLC function. Elucidation of the mechanism that FLC function is regulated by AtSIZ1 or HPY2 alone and/or both AtSIZ1 and HPY2 will provide the clue about the question how sumoylation system modulates flowering time.

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초록

개화억제인자로 알려져 있는 Flowering Locus C(FLC)는 식물체의 개화에 있어 중요한 역활을 한다. 이러한 FLC의 유전자 발현 및 조절에 대한 연구는 이전부터 많이 수행되어왔다. 하지만 FLC의 번역 후 단계에서의 연구는 거의 진행되지 않았다. 따라서 이 논문을 통해 애기장대의 High Ploidy 2(HPY2)가 FLC의 E3 SUMO ligase로써 작용할 수 있다는 것을 확인하였다. 또한 *in vitro, in vivo* 실험을 통하여 FLC가 HPY2와 물리적으로 상호작용한다는 것을 확인할 수 있었다. 또한 in vitro sumoylation assay를 이용하여 FLC가 HPY2의 SUMO E3 ligase로 작용한다는 것을 확인하였다. 또한 형질 전환식물체를 이용하여 HPY2를 과발현 시켜주었을 때, FLC의 농도가 증가한다는 것을 통하여 HPY2가 FLC를 안정화 시켜준다는 것을 알 수 있었다. 또한 *hpy2-2* 돌연변이의 개화시기는 장일 조건과 단일 조건 모두에서 야생형 식물에 비해 빨라졌으며 단일조건에서 그 차이가 컸다. 또한 FLC의 전사 수준 또한 hpy2-2 돌연변이체에서 감소하였다. 이를 종합하여 볼 때, HPY2가 SUMO E3 ligase의 역할을 통하여 전사 및 번역 후 단계에서 FLC의 기능 및 안정성을 조절한다.

색인어: E3 SUMO ligase FLC, HPY2, SUMO, sumoylation.

학번: 2015-21481

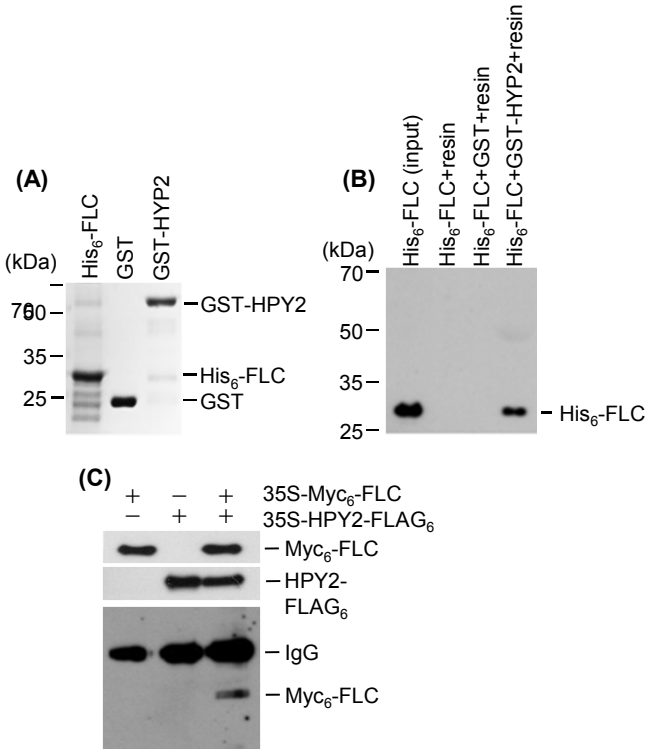
(A)
His6-FLC
GST
GST-HYP2
(kDa)
70
50
35
25
GST-HPY2
His6-FLC
GST
(B)
His6-FLC (input)
His6-FLC+resin
His6-FLC+GST+resin
His6-FLC+GST-HYP2+resin
(kDa)
70
50
35
25
His6-FLC
(C)
+ − + 35S-Myc6-FLC
− + + 35S-HPY2-FLAG6
Myc6-FLC
HPY2-FLAG6
IgG
Myc6-FLC

Figure 1

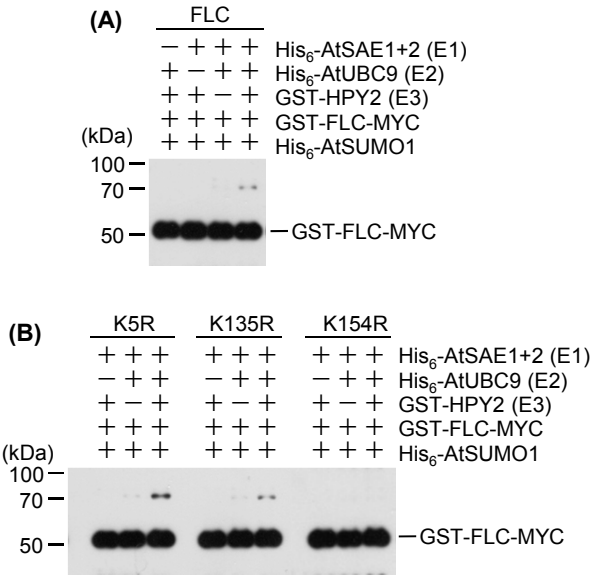
(A)
FLC
− + + + His6-AtSAE1+2 (E1)
+ − + + His6-AtUBC9 (E2)
+ + − + GST-HPY2 (E3)
+ + + + GST-FLC-MYC
+ + + + His6-AtSUMO1
(kDa)
100
70
50
GST-FLC-MYC
(B)
K5R
K135R
K154R
+ + + + + + + + + His6-AtSAE1+2 (E1)
− + + − + + − + + His6-AtUBC9 (E2)
+ − + + − + + − + GST-HPY2 (E3)
+ + + + + + + + + GST-FLC-MYC
+ + + + + + + + + His6-AtSUMO1
(kDa)
100
70
50
GST-FLC-MYC

Figure 2

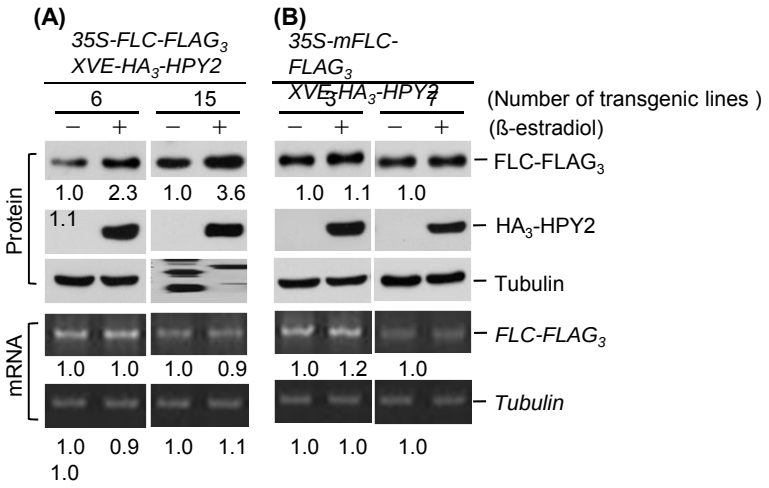
(A)
35S-FLC-FLAG3
XVE-HA3-HPY2
6
15
− + − +
1.0 2.3 1.0 3.6
1.1
Protein
mRNA
1.0 1.0 1.0 0.9
1.0 0.9 1.0 1.1
1.0
(B)
35S-mFLC-FLAG3
XVE-HA3-HPY2
3
7
− + − +
1.0 1.1 1.0
1.0 1.2 1.0
1.0 1.0 1.0
(Number of transgenic lines)
(ß-estradiol)
FLC-FLAG3
HA3-HPY2
Tubulin
FLC-FLAG3
Tubulin

Figure 3

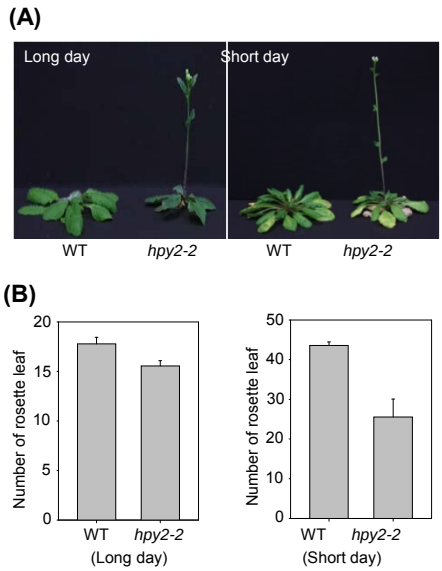
(A)
Long day
Short day
WT
hpy2-2
WT
hpy2-2
(B)
Number of rosette leaf
20
15
10
5
0
WT
hpy2-2
(Long day)
Number of rosette leaf
50
40
30
20
10
0
WT
hpy2-2
(Short day)

Figure 4

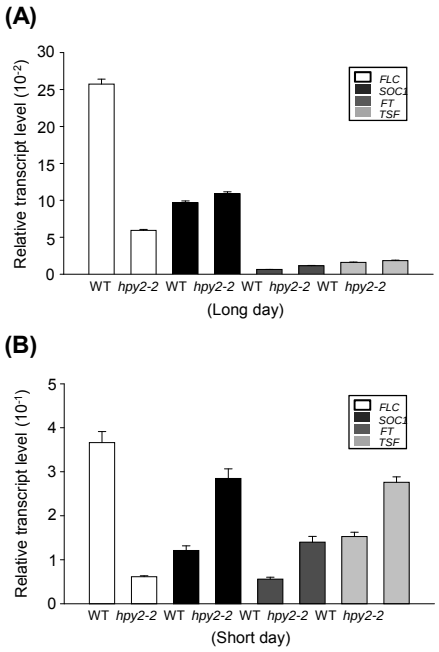
(A)
Relative transcript level (10-2)
30
25
20
15
10
5
0
FLC
SOC1
FT
TSF
WT hpy2-2 WT hpy2-2 WT hpy2-2 WT hpy2-2
(Long day)
(B)
Relative transcript level (10-1)
5
4
3
2
1
0
FLC
SOC1
FT
TSF
WT hpy2-2 WT hpy2-2 WT hpy2-2 WT hpy2-2
(Short day)

Figure 5