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Alternative splicing and expression analysis of *High expression of osmotically responsive genes1 (HOS1)* in *Arabidopsis*

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***High expression of osmotically responsive genes1 (HOS1)*, a key regulator of low temperature response and flowering time, encodes an E3 ubiquitin ligase in *Arabidopsis*. Here, we report characterization of a newly identified splice variant (*HOS1-L*) of *HOS1*. Comparative analyses revealed that *HOS1-L* has a longer 5' nucleotide sequence than that of the previously identified *HOS1* (*HOS1-S*) and that its protein sequence was more conserved than that of *HOS1-S* in plants. *HOS1-L* transcripts were spatio-temporally more abundant than those of *HOS1-S*. The recovery rate of *HOS1-S* expression was faster than that of *HOS1-L* after cold treatment. Diurnal oscillation patterns of *HOS1-L* revealed that *HOS1-L* expression was affected by photoperiod. An *in vitro* pull-down assay revealed that the HOS1-L protein interacted with the ICE1 protein. *HOS1-L* overexpression caused delayed flowering in wild-type plants. Collectively, these results suggest regulation of *HOS1* expression at the post-transcriptional level. [BMB Reports 2012; 45(9): 515-520]**

INTRODUCTION

Alternative splicing is a commonly occurring phenomenon during mRNA processing that involves production of several distinct mRNA molecules from a single gene due to the reconnection of RNA exons in multiple ways during RNA splicing. Alternative splicing can result in the production of different protein isoforms, thereby affecting transcriptome and proteome diversity, and, ultimately, the regulation of protein function and gene expression (1, 2). Recent genome-wide experiments have shown that >40% of *Arabidopsis thaliana* and rice genes can produce multiple diverse mRNA molecules by alternative splicing (3-6). One of the best known examples of alternative splicing in plants is the processing of *FCA* pre-mRNA. In one of the mechanisms that modulates flowering time, *FCA* pre-mRNA is alternatively spliced and subject to alternative polyadenylation, thereby producing two major *FCA* transcripts, *FCA-γ* and *FCA-β*, which encode functional and nonfunctional proteins, respectively (7-9). Furthermore, protein complexes among FCA, FY, PCFS4, and other polyadenylation factors are involved in this process (10, 11). However, the biological functions of alternatively spliced mRNAs are still poorly understood in plants.

Environmental factors, such as light and temperature, regulate gene expression at the post-transcriptional level. Alternative splicing is part of the circadian program that fine-tunes biological clocks. Two circadian clock genes such as *Glycine-rich rna binding protein7 (GRP7)* and *GRP8* are regulated by alternative splicing (5). Furthermore, alternative spliced transcripts of *Late elongated hypocotyl (LHY)* and *Pseudo response regulator7 (PRR7)* are regulated in response to temperature changes (12). These findings suggest that alternative splicing plays an important role in the ability of plants to rapidly adapt to a continually changing environment.

High expression of osmotically responsive genes1 (HOS1) is a key regulator in the response to low temperature (4°C) (13). In *hos1* mutants, low temperature enhances induction of *C-Repeat binding factors (CBFs)*, leading to increased expression of their downstream genes. HOS1 is a RING E3 Ub ligase that physically interacts with Inducer of cbf expression 1 (ICE1) and degrades ICE1 via ubiquitination under cold stress conditions (14). *hos1* mutants also show an early flowering phenotype due to reduced *Flowering locus C (FLC)* expression (13), suggesting that the ubiquitination of target genes by HOS1 plays a role in regulating flowering time. However, the existence of alternative *HOS1* splicing variants has not yet been reported.

In this study, we characterized an alternative spliced form of *HOS1*, which we designated *HOS1-L* (GenBank accession number: BT005517). We found that the HOS1-L protein was the more conserved form, and that *HOS1-L* mRNA levels were more abundant than those of another previously reported alternative spliced form of *HOS1*, designated *HOS1-S* (GenBank accession number: AAB87130), in various tissues and at different ambient temperatures. Rapid recovery of *HOS1-S* expression was observed after 30 min in response to cold treatment. The HOS1-L protein interacted with the ICE1 protein *in vitro*. Furthermore, 35S::*HOS1-L* plants showed more

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delayed flowering than that of *35S*::*HOS1-S* plants. These results suggest that the regulation of *HOS1* expression at the post-transcriptional level may be important in the modulation of plant responses to various environmental conditions.

RESULTS

An additional alternative spliced *HOS1* transcript is present in *Arabidopsis*

We found a *HOS1* expressed sequence tag (EST) [GenBank accession number: BT005517, designated as *HOS1-L* in this study because it was longer than the previously identified EST (13)] that differed from the previously reported *HOS1* cDNA (AAB87130; designated here as *HOS1-S*). The difference between *HOS1-L* and *HOS1-S* was in the 5′-region (Fig. 1A). *HOS1-L* had a slightly longer nucleotide sequence (2,784 bp) than that of *HOS1-S* (2,748 bp), although both contained an identical RING domain. The HOS1-L protein contained 12 additional amino acid residues at the N-terminus. A comparison of their nucleotide sequences with the genomic sequence of *HOS1* revealed that an AUG codon of HOS1-S was located within the first intron of *HOS1-L* (Fig. 1A, B), suggesting that alternative splicing occurs at intron 1 of *HOS1* pre-mRNA to produce two alternatively spliced transcripts.

To exclude the possibility that the *HOS1-L* sequence was incorrectly predicted during annotation of the *Arabidopsis* genome, we compared the deduced amino acid sequences of *HOS1* homologues from other plant species and found that the amino acid sequences of the N-termini of HOS1 homologues from *Arabidopsis lyrata* and *Thellungiella halophila* were almost identical with that of HOS1-L (Fig. 1C). HOS1 homologues identified from other plant species also had longer amino acid sequences than those of HOS1-S, but those from rice and moss had slightly shorter amino acid sequences in their N-termini, suggesting that the HOS1-L protein is more conserved in higher plants.

Two *HOS1* transcripts are differentially regulated at different developmental stages and temperatures

Alternative spliced transcripts are differentially regulated in a spatial and temporal manner (8, 9), and environmental factors, such as light and temperature, influence alternative splicing to regulate gene expression (12, 15). Thus, we examined the expression patterns of *HOS1-L* and *HOS1-S* transcripts in various tissues and in time-course samples grown at two different ambient temperatures (23°C and 16°C). Quantitative reverse transcription (qRT)-polymerase chain reaction (PCR) analyses revealed that both *HOS1* transcripts were ubiquitously expressed in all tissues tested, but that the roots showed relatively low expression (Fig. 2A), suggesting that *HOS1* is mainly expressed in above-ground tissue and that alternative splicing of *HOS1* is a widespread phenomenon. The time-course expression analysis at different ambient temperatures demonstrated that *HOS1* transcripts were detected at both temperatures (Fig. 2B), suggesting that temperature does not influence the alternative splicing of *HOS1* pre-mRNA. *HOS1-L* levels were generally higher than those of *HOS1-S*, demonstrating that the *HOS1-L* transcripts were more abundant than those of *HOS1-S*.

We also examined the expression levels of two *HOS1* transcripts in response to cold treatment. The expression levels of both *HOS1* transcripts decreased rapidly and transiently (Fig.

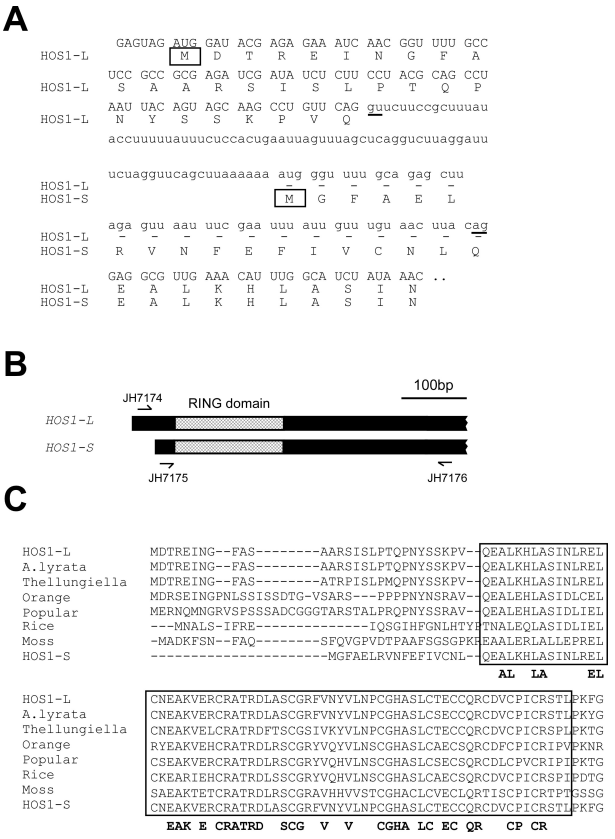

Fig. 1. *HOS1-L* (BT005517) as an alternative splicing variant of *HOS1*. (A) Comparison of nucleotide and amino acid sequences of the N-terminal part of two alternative splicing variants of *HOS1* [BT005517 (designated as HOS1-L) and AAB87130 (designated as HOS1-S)]. Open boxes indicate translational start codons of each EST. Intron sequences identified in *HOS1-L* are denoted in lowercase letters. The canonical splice site (GU and AG) is underlined. (B) Gene structures of the 5′-regions of *HOS1-L* and *HOS1-S*. The hatched box indicates a RING domain. Two different primer sets were used to detect two *HOS1* transcripts, i.e., JH7175 and JH7176 (for *HOS1-S*), and JH7174 and JH7176 (for *HOS1-L*). (C) Amino acid sequence alignment of the HOS1 protein N-termini from other plant species. The RING domain is indicated by the open box. Conserved amino acid sequences are denoted in bold. The GenBank accession numbers used in this analysis are as follows: A. lyrata (*Arabidopsis lyrata*), XP_002879823; Thellungiella (*Thellungiella halophile*), BAJ33739; Orange (*Citrus trifoliate*), ACY 92092; Popular (*Populus trichocarpa*), XP_002304293; Rice (*Oryza sativa*), AAR07079; Moss (*Physcomitrella patens*), XP_001 780235.

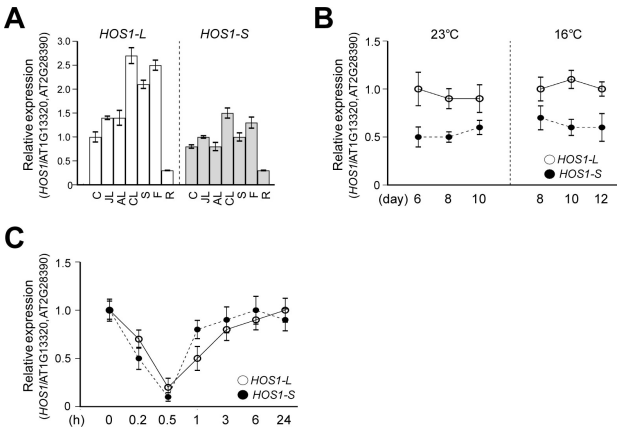

Fig. 2. Relative expression levels of the newly identified splice variant of *HOS1-L* and the previously identified variant *HOS1-S*. (A) Spatial expression patterns of *HOS1-L* and *HOS1-S* determined by qRT-PCR. *HOS1-L* expression levels in the cotyledon of wild-type (Col) seedlings were set to one. C, cotyledons; JL, juvenile leaves (rosette leaves without abaxial trichomes); AL, adult leaves (rosette leaves with abaxial trichomes); CL, cauline leaves; S, stems; F, flowers; R, roots. (B) The effect of ambient temperature on *HOS1-L* and *HOS1-S* expression determined by qRT-PCR. The expression levels of *HOS1-L* in 6- and 8-day-old wild-type seedlings grown at 23°C and 16°C, respectively, also set to one. (C) The effect of cold acclimation on *HOS1-L* and *HOS1-S* expression determined by qRT-PCR. The *HOS1-L* and *HOS1-S* expression levels at the start point of cold acclimation were also set to one. Error bars indicate standard deviation.

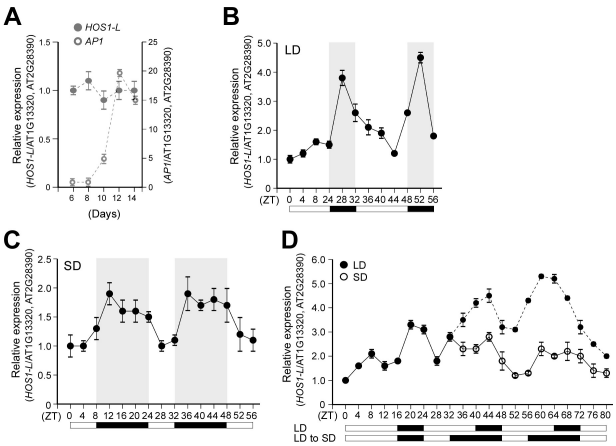

Fig. 3. Expression patterns of the newly identified splice variant of *HOS1-L* in wild-type plants. (A) Time-course expression pattern of *HOS1-L* under LD conditions. *AP1* levels were used as a molecular marker of floral transition (20). (B-D) Diurnal expression of *HOS1-L* in wild-type (Col) seedlings grown for 8 and 9 days at 23°C under LD (B), SD (C), and photoperiod-shift (D) conditions. The expression at ZT 0 was set to one. Open and gray boxes indicate days and nights, respectively.

2C). The expression of *HOS1-S* and *HOS1-L* transcripts started to recover to pre-treatment levels after a 30 min cold treatment. Interestingly, *HOS1-S* transcripts increased more rapidly than that of *HOS1-L* transcripts, suggesting that a major functional HOS1 protein may be produced from *HOS1-S* transcripts under cold stress conditions. Collectively, these results suggest that the expression levels of the two *HOS1* transcripts are differentially regulated in both temporal and spatial manners, and at different temperatures.

Expression patterns of *HOS1-L* transcripts

Time-course expression patterns of *HOS1-L* were determined by qRT-PCR analysis. *HOS1-L* mRNA levels did not show dramatic changes during floral transition under long-day (LD) conditions (Fig. 3A), whereas *APETALA1* (*AP1*) expression increased rapidly around day 10. This was similar to the *HOS1-S* expression pattern (Fig. 2B). This observation indicated that *HOS1-L* expression did not change in a temporal manner. We also examined the diurnal expression patterns of *HOS1-L* to determine whether photoperiod conditions would affect *HOS1-L* expression. This revealed that *HOS1-L* mRNA started to increase after the beginning of the dark period under LD conditions, reaching a peak at the mid-point of the dark period (Fig. 3B). Under short-day (SD) conditions, *HOS1-L* mRNA levels were also higher during the dark period than those during the light period (Fig. 3C). These results indicated that *HOS1-L* expression displayed a diurnal oscillation pattern under different light conditions. This diurnal oscillation pattern was confirmed in a photoperiod-shift experiment in which wild-type (Col) plants shifted from LD to SD conditions showed decreased *HOS1-L* expression (Fig. 3D), indicating that *HOS1-L* expression was higher under LD conditions than that under SD conditions. Taken together, these results suggest that *HOS1-L* expression is regulated by photoperiod.

The HOS1-L protein is functional in *Arabidopsis*

The HOS1 protein interacts with the ICE1 protein to undergo ubiquitination-mediated degradation of the ICE1 protein during the cold response (14). Thus, we performed an *in vitro* pull-down assay to investigate whether the HOS1-L protein is also able to bind to the ICE1 protein. The maltose binding protein (MBP)-HOS1 fusion protein was affinity-purified from *E. coli* (Fig. S1 and Fig. 4A). The ICE1 protein was *in vitro* translated and labeled with ^{35}S (Fig. 4B). Our MBP pull-down assays revealed that the MBP-fused HOS1-L protein bound to the ICE1 protein (Fig. 4C, lane 3); however, the ^{35}S-labeled ICE1 protein did not bind to MBP Sepharose beads (lane 2). The MBP-fused HOS1-S protein also bound to the ICE1 protein (Fig. 4D, lane 3). This result indicated that the HOS1-L protein also interacted with the ICE1 protein *in vitro*.

Because a lesion in *HOS1* results in early flowering (13) (Fig. 4E), we overexpressed *HOS1-L* under control of the *CaMV 35S* promoter in wild-type (Col) plants. About 20 independent transgenic lines were generated, and 15 plants (75%) exhibited late flowering phenotypes in the T_1 generation

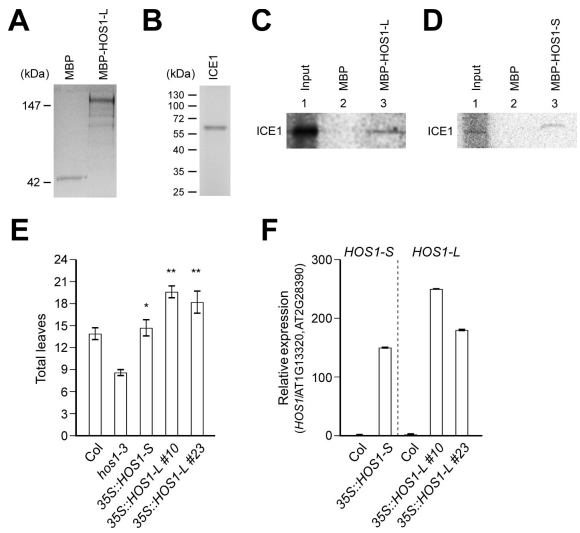

Fig. 4. The newly identified HOS1-L protein is a functional form in *Arabidopsis*. (A) Affinity-purified MBP and MBP-HOS1-L proteins used for *in vitro* pull-down assays. (B) *In vitro* translated ^{35}S-labeled ICE1 protein separated by 10% SDS-PAGE and a dried gel was exposed to an X-ray film. (C, D) *In vitro* pull-down assay. ^{35}S-labeled, *in vitro* translated ICE1 proteins were subjected to MBP pull-down assays using MBP, MBP-HOS1-L (C), or MBP-HOS1-S (D) proteins. Input represents 10% of the *in vitro* translated ICE1 protein. The bands indicate eluted ICE1 protein visualized by autoradiography. (E) Total leaf number of wild-type (Col), *35S*::*HOS1-S*, and *35S*::*HOS1-L* plants grown at 23ºC under long day (LD) conditions. Single and double asterisks indicates significant difference in flowering time between wild-type and *35S*::*HOS1* plants (*35S*::*HOS1-S*, Student's *t*-test, $P < 0.1$; *35S*::*HOS1-L*, Student's *t*-test, $P < 0.01$). (F) Expression levels of *HOS1-S* and *HOS1-L* in respective transgenic plants. Homozygous plants were used. *35S*::*HOS1-S* plants were presented previously (13). Error bars indicate standard deviation.

(data not shown). We randomly selected two transgenic lines (line numbers 10 and 23) showing a Mendelian inheritance pattern (3 : 1 ratio) of antibiotic resistance, and their flowering times were measured again in the T_3 generation. *35S*::*HOS1-L* plants still showed late flowering phenotypes (19.6 and 18.2 leaves in lines #10 and #23, respectively) (Fig. 4E). Furthermore, their flowering times were correlated with *HOS1-L* expression (Fig. 4F). This result indicated that *HOS1-L* overexpression resulted in late flowering. However, *35S*::*HOS1-S* plants that expressed *HOS1-S* at a high level flowered slightly later than that of wild-type plants (14.7 leaves vs. 13.9 leaves) (Fig. 4E, F). This observation suggests that the *HOS1* protein produced from *HOS1-L* transcripts preferentially functions in the control of flowering time at a normal temperature (23^{o}C). Taken together with the *in vitro* pull-down assay results, these data suggest that the *HOS1-L* protein is a functional form in *Arabidopsis*.

DISCUSSION

Ubiquitination and the resulting degradation of components specific to stress signaling pathways are important for plants to successfully adapt to various abiotic stressors (16, 21, 22). Thus, finely tuned regulation of the expression of ubiquitination process-related genes improves the plant's potential to withstand and adapt to various environmental conditions. Here, we demonstrated that two alternative spliced transcripts (*HOS1-L* and *HOS1-S*) were differentially expressed under various conditions, and that the HOS1-L protein acted as a functional form in *Arabidopsis*. Our observations suggest that alternative splicing of *HOS1* pre-mRNA may be an important mechanism for regulating *HOS1* expression.

The regulation of alternative splicing is a key step in the control of gene expression, as splicing variants have different biological functions and regulatory features. We showed that *HOS1-S* expression started to increase rapidly due to pre-cold treatment levels at 30 min under cold stress conditions, compared to that of *HOS1-L* (Fig. 2C). *HOS1-L* overexpressing wild-type plants flowered later than that of *HOS1-S* (Fig. 4E). These results suggest that two alternative spliced transcripts of *HOS1* may have different functions in the regulation of cold stress and flowering time. Furthermore, as the deduced amino acid sequences of both *HOS1* transcripts have a RING domain at the N-terminus (Fig. 1) and a nuclear localization signal at the C-terminus, it is likely that their products may have E3 ubiquitin ligase activities in the nucleus. Thus, the HOS1-L protein may preferentially function in the control of flowering time, whereas the HOS1-S protein may act as a major form under cold stress conditions. However, considering that the HOS1-L protein interacted with the ICE1 protein *in vitro* (Fig. 4C), we cannot dismiss the possibility that the HOS1-L protein may also function in cold acclimation.

In summary, we provided experimental data demonstrating that *HOS1*, an E3 ubiquitin ligase gene, has alternatively spliced transcripts regulated by developmental stage and different temperature regimes. We also showed that the HOS1-L protein affected flowering time in plants. As HOS1 is an important regulator of the CONSTANS (CO) protein in the photoperiod pathway (17), it would be interesting to determine whether balancing the levels of the two different forms of the HOS1 protein produced from alternatively spliced transcripts would serve as a HOS1 regulatory mechanism in response to different environmental stimuli.

MATERIALS AND METHODS

Plant materials and growth conditions

Wild-type *Arabidopsis* plants (Columbia background) were grown in Sunshine Mix 5 (Quincy, MI, USA) or Murashige and Skoog medium at 23^{o}C or 16^{o}C under LD [16/8 h (light/dark)] or SD [9/15 h (light/dark)] conditions with light supplied at an intensity of 120 μmol m^{-2} s^{-1}.

Expression analysis

For the qRT-PCR assay, total RNA was isolated from whole seedlings grown for the indicated number of days or from various tissues using the Plant RNA Purification Reagent (Invitrogen, Carlsbad, CA, USA). Plants were harvested and samples were obtained at Zeitgeber time (ZT) 8. Gene expression levels were analyzed using qRT-PCR methodology, as described previously (18). To detect two different *HOS1* transcripts, we used the following oligonucleotides: for *HOS1-L*, JH7174 (5'-CGCG AGATCGATATCTCTTCC-3') and JH7176 (5'-AGCTAAGGTGC TCCGGAATG-3'); for *HOS1-S*, JH7175 (5'-AAAAATGGGTTTT GCAGAGCTT-3') and JH7176 (5'-AGCTAAGGTGCTCCGGAA TG-3'). Two reference genes (AT1G13320/AT2G28390), which are stably expressed at 23°C and 16°C, were used for quantification. All qRT-PCR experiments were carried out in two or three biological replicates with three technical triplicates, each with similar results. The results from a biological triplicate are shown.

The procedure used for determining the relative abundance of transcripts has been described in detail elsewhere (18).

In vitro pull-down assay

In the *in vitro* pull-down assay, which was designed to test whether the HOS1-L and HOS1-S proteins interact with the ICE1 protein, the open reading frames (ORFs) of *HOS1-L* and *HOS1-S* were cloned into the pMAL C2 vector (New England Biolabs, Ipswich, MA, USA) to create gene fusion with the MBP and then purified from *Escherichia coli* according to the manufacturer's instructions. To generate *in vitro* translated *ICE1* products, we cloned the full-length *ICE1* ORF into the pGEX-5X-1 vector (GE Healthcare, Waltham, MA, USA), and *in vitro* translation products were synthesized using the T7 TNT-coupled Transcription/Translation System (Promega, Madison, WI, USA). MBP-fused HOS1 recombinant proteins (MBP-HOS1-L and MBP-HOS1-S) were incubated with *in vitro* translated ICE1 proteins with gentle rotation for 2-3 h in a cold room, washed three times with washing buffer, and eluted with 10 mM reduced glutathione dissolved in 100 mM NaCl and 20 mM Tris-HCl, pH 7.2. The eluted samples were first analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then subjected to autoradiography.

Preparation of transgenic plants and measurement of flowering time

The *HOS1-L* coding regions were amplified by PCR using Pfusion DNA polymerase (New England Biolabs), cloned into the Gateway entry vector, and subsequently recombined into destination vectors (pGWB11 or pGWB12) harboring the 35S promoter and FLAG tag proteins using Gateway LR Clonase II Enzyme mix (Invitrogen). Plants were transformed using the floral dip method with minor modifications (19), and transformants were selected for kanamycin resistance. At least 20 T_1 seedlings were analyzed, and independent transgenic T_2 plants were used to measure flowering time. *35S*::*HOS1-S* plants have been described previously (13).

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