Histone H2B Deubiquitination Is Required for Transcriptional Activation of FLOWERING LOCUS C and for Proper Control of Flowering in Arabidopsis

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The spectrum of histone modifications at a given locus is a critical determinant for the correct output of gene expression. In Arabidopsis (Arabidopsis thaliana), many studies have examined the relationship between histone methylation and gene expression, but few studies exist on the relationship between other covalent histone modifications and gene expression. In this work, we describe the role of histone H2B deubiquitination in the activation of gene expression and the consequence of a perturbation of histone H2B deubiquitination in the timing of the floral transition in Arabidopsis. A mutation in a H2B deubiquitinase, UBIQUITIN-SPECIFIC PROTEASE26 (UBP26), results in an early-flowering phenotype. In the ubp26 mutant, mRNA levels of the floral repressor FLOWERING LOCUS C (FLC) and other related family members is decreased. Furthermore, this mutant accumulates H2B monoubiquitination, and has decreased levels of H3K36 trimethylation and increased levels of H3K27 trimethylation at the FLC locus. Thus, UBP26 is required for transcriptional activation of FLC through H2B deubiquitination and is consistent with a model in which deubiquitination is necessary for the accumulation of H3K36 trimethylation and the proper level of transcriptional activation.

Flowering is a highly regulated developmental transition. The perception of both internal and environmental cues ensures that plants transition to flowering during optimal times of the year to maximize their reproductive success. Temperature represents one of the many environmental stimuli that influences flowering. For example, a long exposure to cold, which occurs during winter, enhances the ability to flower in several winter-annual, biennial, or perennial varieties of plants. This acquisition of competence to flower that occurs during a prolonged exposure to cold is known as vernalization (Chouard, 1960).

In Arabidopsis (Arabidopsis thaliana), the molecular basis of the vernalization requirement has been well studied. There are both annual (no vernalization required) and winter-annual (vernalization-responsive) accessions of Arabidopsis. A study of the genetic basis of the winter-annual habit identified a dominant locus, FRIGIDA (FRI), that confers a vernalization requirement (Napp-Zinn, 1979). Establishment of the winter-annual habit imposed by FRI also requires the activity of the floral repressor FLOWERING LOCUS C (FLC; Koornneef et al., 1994; Lee et al., 1994). FLC encodes a MADS-box transcription factor that represses the expression of genes required for the floral transition. FRI functions to elevate the mRNA levels of FLC, which results in the repression of flowering in winter-annual Arabidopsis (Michaels and Amasino, 1999; Sheldon et al., 1999).

FLC expression is strongly repressed by vernalization (Michaels and Amasino, 1999; Sheldon et al., 1999). The vernalization-mediated repression of FLC is stable after plants experience warmer temperatures during spring. The mitotic stability of gene repression exhibited by FLC provides an epigenetic “memory of winter” (Bastow et al., 2004; Sung and Amasino, 2004). Epigenetic switches, like vernalization, are often controlled by chromatin remodeling that involves alterations to histone modifications. Prior to vernalization, in winter-annual accessions of Arabidopsis that contain transcriptionally active FLC, histone modifications associated with actively transcribed genes, such as trimethylation of Lys 4 of histone H3 (H3K4me3), di- and trimethylation of H3K36 (H3K36me2/me3), and hyperacetylation of H3, are enriched at the FLC locus (He et al., 2004; Kim et al., 2005; Zhao et al., 2005; Sung et al., 2006b; Oh et al., 2008; Pien et al., 2008; Xu et al., 2008). During and after vernalization, the levels of
these histone modifications are reduced and histone modifications associated with gene repression such as H3K9me3 and H3K27me3 increase (Sung et al., 2006b; Finnegan and Dennis, 2007; Greb et al., 2007). In addition to vernalization-induced histone Lys methylation, histone Arg methylation is also required for the stable repression of FLC by vernalization (Schmitz et al., 2008). The establishment of stable, vernalization-induced epigenetic silencing of FLC requires mitosis (Wellensiek, 1962, 1964; Finnegan and Dennis, 2007). The stability of the vernalized state is largely attributed to the activities of an Arabidopsis Polycomb Repressive Complex 2 and LIKE HETEROCROMATIN PROTEIN1 (Gendall et al., 2001; Mylne et al., 2006; Sung et al., 2006a; Wood et al., 2006). As the FLC locus passes through meiosis and embryogenesis to the next generation, it is reset to an active state (Sung and Amasino, 2006; Sheldon et al., 2008).

In Arabidopsis, several genes have been identified as transcriptional activators of FLC and some of its related family members (the FLC family members includeFLOWERING LOCUS M [FLM]/MADS AFFECTING FLOWERING1 [MAF1] and MAF2–MAF5, and will be collectively referred to as the FLC clade). Among these activators, EARLY FLOWERING7 (ELF7), UBQUITIN-CONJUGATING ENZYMES1/2 (UBC1/2), HISTONE MONOUBIQUITINATION1/2 (HUB1/2), Arabidopsis TRITHORAX1/2 (ATX1/2), and EARLY FLOWERING IN SHORT DAYS/SET DOMAIN GROUP8 have crucial roles in modifying histones at the FLC locus (He et al., 2004; Kim et al., 2005; Zhao et al., 2005; Cao et al., 2008; Pien et al., 2009; Xu et al., 2009). In elf7, ubc1/2, hub1, and atx1 mutants, H3K4me3 is diminished around the transcription start site of FLC, and mRNA levels of FLC are sharply reduced (He et al., 2004; Cao et al., 2008; Pien et al., 2008; Gu et al., 2009; Xu et al., 2009). In yeast (Saccharomyces cerevisiae), the Cdc13 complex is required for monoubiquitination of histone H2B by the E2 ubiquitin conjugation enzyme Rad6 and the E3 ubiquitin ligase Bre1 (Ng et al., 2003; Wood et al., 2003). Monoubiquitination of H2B is a prerequisite for the recruitment of Set1 (Lee et al., 2007). Set1 contains a conserved histone methyltransferase domain, known as the SET domain, which catalyzes the methylation of H3K4 (Briggs et al., 2001). H3K4me3 is essential for the transcriptional activation of a subset of genes in yeast. The Arabidopsis ATX1 gene encodes a protein with a SET domain and is likely homologous to the yeast Set1 protein (Baumbusch et al., 2001). ATX1 is thought to catalyze the methylation of H3K4 at the FLC locus in Arabidopsis (Pien et al., 2008). Recent work has demonstrated that the Arabidopsis E2 conjugating enzymes UBC1/2 and the ubiquitin ligases HUB1/2 are required for H2Bub1 at the FLC locus (Cao et al., 2008; Gu et al., 2009; Xu et al., 2009). These results indicate that H2Bub1 contributes to the recruitment of ATX1 to the transcription start site of actively transcribed genes in Arabidopsis, similar to H2Bub1 and Set1 recruitment in yeast (Lee et al., 2007).

In Mutant Displays an Early-Flowering Phenotype

The ubp26-1 mutant (in the C24 accession) contains a T-DNA in the fifth exon of UBP26. Reverse transcrip-
tion (RT)-PCR analysis revealed no expression of the wild-type UBP26 transcript in ubp26-1 plants (Fig. 1, A and B). ubp26-1 mutants flowered significantly earlier than wild-type C24 in both inductive long days and noninductive short days (Fig. 1, C–E). Consistent with the early-flowering phenotype, the mRNA levels of FLC, MAF2, and MAF3 were quite lower in the ubp26-1 mutant compared to wild type, and the mRNA levels of FLM and MAF4 were also slightly reduced (Fig. 1F). In contrast, the mRNA levels of MAF5 were substantially increased in the ubp26-1 mutant.

In addition to the flowering phenotype, ubp26-1 mutants display a range of other phenotypes such as smaller rosette leaves (Fig. 1, C and D) and weaker apical dominance (only observed after release of secondary inflorescences). To confirm that the ubp26-1 mutation was, in fact, responsible for these phenotypes, we performed complementation experiments. In a subset of the T1 transformant population, all of the ubp26-1 phenotypes were rescued by a transgene containing 2.8 kb of the UBP26 promoter and the entire coding region (Supplemental Fig. S1). Approximately half of the T1 transformants showed severe pleiotropic phenotypes such as multiple vegetative meristems and/or a smaller plant size; these phenotypes were present in both T1 transformants of both the ubp26-1 mutant as well as the wild-type C24 background (Supplemental Fig. S2). Thus, these phenotypes may result from increased UBP26 activity, which indicates that expression of UBP26 in the proper amount and in the appropriate tissues is crucial for normal plant development. Alternatively, these phenotypes could be a result of cosuppression elicited by the transgene.

ubp26 Mutants, in Columbia, Display a High Rate of Seed Abortion

Many studies of the regulation of the floral transition have been performed in the Columbia (Col) and Landsberg genetic backgrounds. The ubp26-1 allele is

Figure 1. ubp26-1 mutants display an early-flowering phenotype. A, A gene structure of UBP26 containing exons (black boxes) and introns (black lines). The T-DNA in ubp26-1 is located in the fifth exon. (Sequence analysis of the T-DNA junction revealed the site of the T-DNA insertion was different from the insertion site published by Sridhar et al. [2007].) B, RT-PCR analysis of UBP26 in C24 and in ubp26-1 (the short fragment indicated below the gene structure in A represents the RT-PCR product). C and D, A picture of wild-type C24 and ubp26-1 plants grown in long days (C) and short days (D). E, Leaf count data of C24 and ubp26-1 in long days and short days. Black portions represent rosette leaves and white portions indicate cauline leaves. Error bars equal one SD. F, RT-PCR analysis of the FLC clade in C24 and in ubp26-1. [See online article for color version of this figure.]

Figure 2. Mutations in ubp26 frequently result in a high rate of seed abortion in the Col genetic background. A, A gene structure of UBP26 containing the positions of the ubp26-4 and the ubp26-3 T-DNA insertions. B, Pictures of a subset of seeds from seed stocks of Col and hemizygotes for the ubp26-4 and ubp26-3 T-DNAs. Arrows indicate shriveled/aborted seeds. The numbers below the picture indicate the frequency of healthy seeds to shriveled/aborted seeds. C and D are pictures of cleared embryos from a single silique of a plant hemizygous for the ubp26-4 T-DNA. In this particular silique, a majority of the cleared embryos were in the heart stage of embryo development (as pictured in C), whereas a minority had arrested at random stages of development (one example pictured in D). E, A picture depicting an example of a seedling lethal ubp26-4 mutant. The numbers indicate the frequency of observed seedling lethality from seed stocks of Col and hemizygotes for the ubp26-4 T-DNA. [See online article for color version of this figure.]
in the C24 genetic background. Therefore, we isolated two additional ubp26 mutant alleles in the Col genetic background (ubp26-4 and ubp26-3; Fig. 2A). However, we were unable to isolate viable plants homozygous for either the ubp26-4 or the ubp26-3 T-DNA allele. We could identify plants hemzygous for the T-DNA, and self-pollination of hemzygous ubp26-4 and/or ubp26-3 plants resulted in an approximately 2:1 ratio of hemzygous:wild type among the progeny. This indicates that the ubp26 lesion results in lethality in the hemzygous state and the effect of the mutation is zygotic.

Lethality occurs throughout seed development and occasionally postgermination. Specifically, among the seed derived from self-pollination of plants hemzygous for either ubp26-4 or ubp26-3 we observed a seed abortion rate of approximately 20% (Fig. 2B). Reciprocal crosses between ubp26-4 and wild-type Col demonstrated that the ubp26-4 T-DNA could transmit through either the male or the female gametes. We cleared embryos from seeds produced by plants hemzygous for the ubp26-4 T-DNA to determine if there were specific stages of embryo development that were affected by the ubp26-4 homozygous mutation. From these plants, we observed stochastic arrest throughout embryo development when compared to wild type (Fig. 2, C and D). We were also able to identify seedlings homozygous for ubp26-4 or ubp26-3 (approximately 4%) when segregating populations were sown on nutrient-rich plates, but seedling growth arrested at an early stage, which led to lethality (Fig. 2E). While this work was in progress, Luo et al. (2008) also reported that loss of ubp26 in Col results in a high percentage of lethality. Furthermore, we examined seed from self-pollination of homozygous ubp26-4 plants kindly provided by Liu et al. (2008) and found a high frequency of aborted seeds and seedling lethality with a low frequency of viable plants (Table 1; Supplemental Fig. S3).

None of the viable ubp26-4 homozygous mutant plants that we obtained from seed provided by Liu et al. (2008) displayed an early-flowering phenotype in long days or in short days. Thus, it appears that, in Col, a subset of mutant plants can escape whatever causes embryo and seedling lethality among their siblings, and those that do escape appear to be relatively normal with respect to visible vegetative phenotypes and flowering time. The basis of this difference in flowering and seed viability between Col and C24 ubp26 mutations is currently under investigation.

The ubp26-1 Mutation Affects Global Levels of H2Bub1, But Not H3 Methylation

UBP26 is a UBP that deubiquitilates H2B, and the ubp26-1 mutant has increased levels of H2Bub1 and can lead to a loss of DNA methylation and heterochromatin formation at transgenes (Sridhar et al., 2007). Using an anti-H2Bub1 antibody we found, similar to Sridhar et al. (2007), an accumulation of H2Bub1 in ubp26-1 compared to wild-type C24 by immunoblot analysis (Fig. 3). We also examined the levels of H2B monoubiquitination in three additional early-flowering mutants: elf7-2, pie1-2, and efs-3 (all from the Col genetic background). These mutants were selected for further analysis because their wild-type products have defined functions in transcription initiation, H2A.Z deposition, and transcription elongation, respectively (Noh and Amasino, 2003; He et al., 2004; Kim et al., 2005). The levels of H2Bub1 were affected in the elf7-2 and pie1 mutants, indicating that these proteins function upstream of H2Bub1 (Fig. 3). In efs-3 mutants, the levels of H2Bub1 remain unchanged, indicating that EFS functions downstream of H2Bub1 (Fig. 3).

The accumulation of H2Bub1 in ubp26-1 mutants raised the issue of whether other histone modifications in the C24 genetic background. Therefore, we isolated two additional ubp26 mutant alleles in the Col genetic background (ubp26-4 and ubp26-3; Fig. 2A). However, we were unable to isolate viable plants homozygous for either the ubp26-4 or the ubp26-3 T-DNA allele. We could identify plants hemzygous for the T-DNA, and self-pollination of hemzygous ubp26-4 and/or ubp26-3 plants resulted in an approximately 2:1 ratio of hemzygous:wild type among the progeny. This indicates that the ubp26 lesion results in lethality in the hemzygous state and the effect of the mutation is zygotic.

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| Genotype | Normal Seedlings | Lethal Seedlings | Aborted/Shriveled |
|----------|-----------------|-----------------|-----------------|
| C24      | 322 (98.5%)     | 3 (0.9%)        | 2 (0.6%)        |
| ubp26-1  | 285 (78.5%)     | 40 (0.9%)       | 38 (10.55%)     |
| Col      | 313 (91.0%)     | 20 (5.8%)       | 11 (3.2%)       |
| ubp26-4  | 282 (37.7%)     | 237 (31.7%)     | 229 (30.6%)     |

Figure 3. Analysis of global levels of histone modifications. Analysis of global levels of monoubiquitinated H2B and histone methylation. Each mutant is compared to its respective wild type. elf7-2, pie1-2, and efs-3 are mutants in the Col genetic background.
might be affected by a change in the status of H2B ubiquitination. We analyzed histone methylation by immunoblot analysis in the same genotypes mentioned above. Lysine 4 trimethylation of histone 3 (H3K4me3) remained unchanged when compared to the respective wild type (Fig. 3). In addition, no changes in Lys 36 methylation of histone 3 (H3K36me) could be observed except in the efs-3 mutants (Fig. 3). In the efs-3 mutant, a decrease in H3K36me3 and an increase in H3K36me1 were observed (Fig. 3). This is consistent with efs-3 functioning as a H3K36 methyltransferase as proposed by Xu et al. (2008).

Analysis of H2Bub1 and H3K4me3 at FLC Chromatin

H2Bub1 was recently found to accumulate in the transcribed regions of highly expressed genes in humans (Minsky et al., 2008). We chose FLC to evaluate the spatial distribution of this histone modification because expression of this gene is decreased in ubp26-1 (Fig. 1F), indicating that H2Bub1 might be required for gene activation. Examination of H2Bub1 throughout the length of FLC chromatin by chromatin immunoprecipitation (ChIP) analysis revealed enrichment of this histone modification within the body of this gene in wild type (Fig. 4, A and B). Furthermore, ChIP analysis demonstrated that this modification also accumulates in FLC chromatin in a ubp26-1 mutant background (Fig. 4B). These results indicate that higher plants, similar to mammalian systems, also contain H2Bub1 in the body of transcribed genes and that UBP26 functions in removal of this covalent modification.

Mutations in components of the Arabidopsis PAF1 complex severely affect the levels of H3K4me3 at FLC chromatin, although there is no discernable difference in the genome-wide levels of this histone modification (Fig. 3; He et al., 2004; Oh et al., 2004, 2008). Therefore, although the accumulation of H2Bub1 had no obvious effect on global levels of H3K4me3, it was of importance to look for local effects of ubp26-1 mutations on H3K4me3 at FLC chromatin. H3K4me3 is a histone modification that facilitates transcription initiation, and as a result it is commonly found around the transcription start site of transcribed genes (Li et al., 2007). ChIP analysis of C24 and ubp26-1 plants revealed a similar spatial distribution of H3K4me3 (Fig. 4C). Furthermore, the amplitude of H3K4me3 variation was similar in wild type and ubp26-1, indicating that perturbation of H2Bub1 did not prevent histone modifications associated with transcription initiation from occurring at FLC chromatin (Fig. 4C).
H3K27me3 is highly enriched in the ubp26-1 mutant compared to the wild-type C24. Black bars indicate wild-type C24 and white bars indicate ubp26-1. Error bars represent SEs.

DISCUSSION

The identification of FLC as a central component in establishing the winter-annual habit has led to a greater understanding of gene activation and gene repression in Arabidopsis. Genetic screens aimed at identifying genes required for FLC activation have revealed three major complexes, the PAF1 complex, the SWR1 complex, and the FRI complex (for review, see Schmitz and Amasino, 2007). Paf1 and SWR1 are required, respectively, for transcription initiation of FLC and histone H2A.Z deposition at FLC (Noh and Amasino, 2003; He et al., 2004; Oh et al., 2004; Choi et al., 2005, 2007; Martin-Trillo et al., 2006; Deal et al., 2007; March-Diaz et al., 2007). The FRI complex activates FLC via an undefined molecular mechanism (Johanson et al., 2000; Michaels et al., 2004; Schmitz et al., 2005; Kim et al., 2006; Kim and Michaels, 2006).

Recent publications and our work presented in this article have identified H2B monoubiquitination as an additional histone modification required for FLC transcription (Cao et al., 2008; Gu et al., 2009; Xu et al., 2009). To our knowledge, our work is the first report of the role of UBP26, a histone deubiquitinase, in facilitating expression of FLC; UBP26 is likely to act by promoting H3K36me3 and by preventing H3K27me3 at FLC chromatin.

The collection of mutants in activators of FLC expression permits exploration into the interplay be-

Figure 6. ChIP analysis of H3K27me3 at FLC chromatin. Shown is ChIP analysis of H3K27me3 at FLC chromatin. H3K27me3 is highly enriched in the ubp26-1 mutant compared to the wild-type C24. Black bars indicate wild-type C24 and white bars indicate ubp26-1. Error bars represent SEs.

Figure 7. A hypothetical model of FLC transcriptional activation by cycling of histone H2B monoubiquitination. A and B. A proposed model for transcriptional activation of FLC through H2B ubiquitination (A) and H2B deubiquitination (B). All factors shown above are essential for proper transcriptional activation of FLC. H2B and H3 indicate histones. K4 and K36 indicate Lys-4 and Lys-36 residues of histone H3. Ub and M indicate ubiquitin and methyl groups on histones, respectively. Solid arrows indicate a positive interaction. Broken arrows predict a positive interaction in Arabidopsis proteins based on experimental evidence from yeast.
tween different histone modifications. Our studies have demonstrated that accumulation of H2Bub1 at FLC chromatin affects H3K36 methylation, and that H3K4 methylation remains unchanged. This is consistent with a model in which H3K4me3 occurs prior to H2B deubiquitination, whereas H3K36me3 occurs afterward. Our work has also helped to better define the hierarchy of molecular functions required for FLC expression. For example, global levels of H2Bub1 were unaffected in efs-3 mutants, although an increase in levels of H3K36me1 was observed at the expense of H3K36me3 in the ubp26-1 mutant (Fig. 5). These data indicate that EFS functions downstream of H2Bub1 in the methylation of H3K36 and the subsequent activation of gene expression. However, there remains much to learn about the hierarchy of histone modifications at FLC and the molecular role of additional regulators of FLC in this hierarchy.

The function of UBP26 in H2B deubiquitination and in gene activation in the regulation of FLC is similar to the function of Ubp8 in yeast. Many of the factors that are involved in the transcriptional activation in yeast are also present in the Arabidopsis genome and play essential roles in FLC activation (Fig. 7). This and other work demonstrates that the floral transition and the regulation of FLC provide a good model for investigating the mechanisms of epigenetic gene regulation in plants.

The seed abortion phenotype of the ubp26 mutant is much stronger in Col than in the C24 background (Supplemental Fig. S3; Luo et al., 2008). One possibility to account for this variation is that other UBPs can provide UBP activity to permit normal embryo development in C24 but not in Col. Indeed, there are at least 27 UBPs present in the Arabidopsis genome (Yan et al., 2000), and UBP26 does not share the highest degree of amino acid sequence identity to yeast Ubp8 or Ubp10. If the reason for the difference in the seed abortion phenotype is an additional UBP that is active in C24 ubp26 mutants but not in Col ubp26 mutants, a genetic analysis of this difference may identify additional H2B deubiquitinases in Arabidopsis.

Although the role of Ubp8 and UBP26 in transcriptional activation is similar between yeast and Arabidopsis, there are certain differences, at least at well-studied loci. For example, H3K4me3 and H3K36me2 levels at the FLC locus are almost the same in wild type and in ubp26-1 (Figs. 4C and 5B). In contrast, H3K4me3 levels at the yeast GAL1 locus are higher and H3K36me2 levels are lower in the ubp8 mutant compared to wild type (Henry et al., 2003). This suggests that, although H2B deubiquitinases are conserved between yeast and Arabidopsis, the downstream biochemical interpretation of histone modifications may be different.

One proposed function for H3K36 methylation in the body of transcribed genes in yeast is to suppress the initiation of intragenic transcription initiation. We tested for the presence of cryptic transcripts in the body of FLC by RT-PCR analysis using primers located throughout the FLC locus. However, we did not observe evidence of intragenic transcription, as defined by this assay, in efs or ubp26 mutants. Thus, intragenic transcription in the ubp26-1 mutant may not be as prevalent as it is in yeast.

H3K27me3 is a histone modification that is commonly associated with repressed loci located in euchromatin in Arabidopsis. We observed an enrichment of H3K27me3 in the body of FLC in ubp26-1 mutants compared to C24. Thus, the presence of H3K27me3 may provide a mechanism for repressing genes that accumulate H2Bub1. This observation contrasts with recent work that shows that UBP26 is required for maintaining H3K27me3 and repression of PHERES1 (Luo et al., 2008). Neither the work presented here nor previous work detailing the function of UBP26 (Sridhar et al., 2007; Luo et al., 2008) has shown a direct interaction between UBP26 and the specific locus under study. Therefore, it is possible that the results in our study or the studies mentioned above are indirect effects of loss of UBP26. Identification of direct targets of UBP26 by ChIP-on-chip or ChIP-sequencing studies will further define the function of UBP26 in gene regulation.

CONCLUSION

In conclusion, there are significant parallels in the activation of gene expression in Arabidopsis and yeast. Additional molecular analyses of genes required for transcriptional activation of FLC in Arabidopsis will further refine our knowledge of the interplay between histone modifications and gene expression in both plants and other eukaryotes.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The ubp26-1 mutant was isolated from the C24 background (Sridhar et al., 2007). T-DNA mutants, ubp26-4 (SALK_024392) and ubp26-3 (SAIL_621_F01) were obtained from the Arabidopsis Biological Resource Center (Alonso et al., 2003).

For the growth condition of plants, we followed the methods described in Schmitz et al. (2008). Seeds were incubated in water at 4°C for 2 d and were directly embedded in soil. Otherwise, seeds were incubated on agar-solidified medium containing 0.65 g/L Peters Excel 15-5-15 fertilizer (Grace Sierra) at 4°C for 2 d and transferred to either long days or short days.

Complementation Analysis

The UBP26 genomic clone and its 2.8-kb native promoter were amplified by PCR with Phusion High-Fidelity DNA polymerase (Finzymes) according to the manufacturer’s protocol (the termination codon was not included in the amplification). The T9C5 bacterial artificial chromosome vector, obtained from the Arabidopsis Biological Resource Center, was used as a template for amplification (Choi et al., 1995). The primers used for amplification were 5′-CACCCACACTGTGATGAAACAGA-3′ and 5′-GCAAGGCTCAGAAGAGATATCCCA-3′. Amplified cDNA was subcloned into pENTR-D-TOPO (Invitrogen) according to the manufacturer’s protocol. Next, the cDNA was subcloned into the pMDC107 binary vector (Curtis and Grossniklaus, 2003) by site-specific recombination with Gateway LR Clonase II enzyme mix (Invitrogen) according to the manufacturer’s protocol. Plasmids were transformed into agrobacterium, which was used for transformation of C24 and ubp26-1.
seeds were selected on the plates with 25 mg/L hygromycin (MP Biomedicala) for 8 d and the resistant seedlings were transplanted to soil.

RNA Analysis and RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis was performed on 2 μg of RNA using the M-MLV system for RT-PCR (Promega) followed by PCR amplification with ExTaq DNA polymerase (Takara Bio) according to the manufacturer's recommendations. Primers were used to amplify the cDNAs and PCR conditions are listed in Supplemental Table S1. PCR products were separated on a 2.5% agarose gel. All RT-PCR results presented are representative data of at least two biological replicates.

Immunoblot Analysis

Protein nuclear extracts were isolated from a chromatin preparation as described in the ChIP protocol in Gendrel et al. (2005). Proteins were separated on a 10% to 20% SDS-PAGE (Bio-Rad Ready Gels catalog no. 1611106) and transferred to a nitrocellulose membrane (Amersham Biosciences). Global levels of histone modifications were determined using the following antibodies from Abcam (H3K4me3 catalog no. 8580, H3K36me1 catalog no. 9048, H3K36me3 catalog no. 9050, H2B catalog no. 1790) and from Millipore (H3K4me3 catalog no. 8580, H3K36me1 catalog no. 9048, H3K36me3 catalog no. 9050, H2B catalog no. 1790) and from Medimabs (H2Bub1 catalog no. MM-0029).

ChIP

Tissue was harvested from seedlings 7 d after germination. Chromatin samples were prepared as described (Gendrel et al., 2005). Immunoprecipitations were performed following a protocol previously described by Johnson et al. (2002). The ChIP antibodies are described in the previous section. The PCR parameters are: one cycle of 10 min at 95°C, 40 cycles of 10 s at 95°C, 10 s at 60°C, and 20 s at 72°C. Regions that cover the promoter, 5’ end, internal, and 3’ end regions of FLC were assayed, and the fold of enrichment was calculated using the retrotransposon Ta3 as an internal control in all experiments except for the H3K27me3 and H2Bub1-ChIPs. In these experiments AGAMOLIS and ACTIN were used as internal controls, respectively. PCR primer sequences are listed in Supplemental Table S1. All ChIP results presented are an average of two biological replicates.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Complementation of the ubp26-1 mutation with a UBP26pro:UBP26-GFP transgene.

Supplemental Figure S2. Pleiotropic phenotypes associated with the UBP26pro:UBP26-GFP transformants.

Supplemental Figure S3. The shriveled seed phenotype is more severe in ubp26-4 than in ubp26-1.

Supplemental Table S1. Primer information for RT-PCR or real-time PCR experiments presented in this article.

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