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# Histone H2B Monoubiquitination in the Chromatin of *FLOWERING LOCUS C* Regulates Flowering Time in *Arabidopsis* [W]

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**Ubiquitination is one of many known histone modifications that regulate gene expression. Here, we examine the *Arabidopsis thaliana* homologs of the yeast E2 and E3 enzymes responsible for H2B monoubiquitination (H2Bub1). *Arabidopsis* has two E3 homologs (HISTONE MONOUBIQUITINATION1 [HUB1] and HUB2) and three E2 homologs (UBIQUITIN CARRIER PROTEIN [UBC1] to UBC3). *hub1* and *hub2* mutants show the loss of H2Bub1 and early flowering. By contrast, single *ubc1*, *ubc2*, or *ubc3* mutants show no flowering defect; only *ubc1 ubc2* double mutants, and not double mutants with *ubc3*, show early flowering and H2Bub1 defects. This suggests that *ubc1* and *ubc2* are redundant, but *ubc3* is not involved in flowering time regulation. Protein interaction analysis showed that HUB1 and HUB2 interact with each other and with UBC1 and UBC2, as well as self-associating. The expression of *FLOWERING LOCUS C* (*FLC*) and its homologs was repressed in *hub1*, *hub2*, and *ubc1 ubc2* mutant plants. Association of H2Bub1 with the chromatin of *FLC* clade genes depended on UBC1,2 and HUB1,2, as did the dynamics of methylated histones H3K4me3 and H3K36me2. The monoubiquitination of H2B via UBC1,2 and HUB1,2 represents a novel form of histone modification that is involved in flowering time regulation.**

## INTRODUCTION

The transition from vegetative to reproductive development is a critical event in the plant life cycle that is coordinated by both endogenous signals and environmental cues (Searle and Coupland, 2004; Baurle and Dean, 2006; Imaizumi and Kay, 2006). Genetic analysis has identified several pivotal genes involved in the control of flowering time in *Arabidopsis thaliana* via multiple pathways (Simpson and Dean, 2002; Searle and Coupland, 2004; He and Amasino, 2005; Imaizumi and Kay, 2006), including *FLOWERING LOCUS C* (*FLC*), which is the central repressor of flowering in *Arabidopsis*. *FLC* inhibits the expression of flowering-time integrators, including *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*, and it indirectly represses such floral meristem identity genes as *LEAFY* and *APETALA1* (He and Amasino, 2005; Baurle and Dean, 2006; Imaizumi and Kay, 2006; Searle et al., 2006). Inactivation of *FLC* leads to flowering; thus, its level of expression is tightly regulated (Michaels and Amasino, 1999; Sheldon et al., 1999). There are five *FLC* homologs, *MADS AFFECTING FLOWERING* (*MAF*s), in the *Arabidopsis* genome, each of which is also involved in the control of flowering time (Ratcliffe et al., 2001, 2003; Scortecci et al., 2001).

The covalent modification of histones is important for chromatin remodeling and proper transcriptional regulation (Jenuwein and Allis, 2001). These modifications include methylation, acetylation, phosphorylation, ADP-ribosylation, and ubiquitination (Turner, 2002; Kouzarides, 2007). The ubiquitination of proteins is performed in a specific manner by E1, E2, and E3 enzymes (Hellmann and Estelle, 2002; Sullivan and Deng, 2003; Pickart, 2004; Smalle and Vierstra, 2004). Two types of protein ubiquitination, multiubiquitination and monoubiquitination, have been described to date. Multiubiquitination targets proteins for degradation by the 26S proteasome, whereas monoubiquitination mediates the localization and activation of the ubiquitinated proteins (Hicke, 2001; Zhang, 2003).

Histone monoubiquitination involves mainly histones H2A and H2B (Zhang, 2003). H2A monoubiquitination, which is mediated by polycomb repression complex 1 in animals, is necessary for the silencing of homeobox genes (Wang et al., 2004). In comparison, monoubiquitinated H2B (H2Bub1) controls the binding of histone H3 lysine methylases, such as COMPASS, to the chromatin in yeast and thus is required for the methylation of H3K4 and H3K36 (Dover et al., 2002; Sun and Allis, 2002; Henry et al., 2003; Wood et al., 2003a; Lee et al., 2007). H2Bub1 worked together with COMPASS, RNA polymerase II associated factor 1 (PAF1) complex, and RNA polymerase II to stimulate transcription elongation (Pavri et al., 2006; Lee et al., 2007; Weake and Workman 2008). In yeast, the monoubiquitination of H2B to H2Bub1 is mainly achieved by two enzymes, a ubiquitin E3 ligase

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encoded by *Brefeldin A-sensitivity protein1* (*BRE1*) and a ubiquitin E2 conjugase encoded by *Radiation sensitivity protein6* (*RAD6*) (Robzyk et al., 2000; Hwang et al., 2003; Kao et al., 2004). H2Bub1 functions in the control of cell size in yeast (Hwang et al., 2003). The homologs of *BRE1* have been found to perform the same biochemical function in animals, *Drosophila* BRE1 is required for wing development (Bray et al., 2005), and human BRE1 is involved in *HOX* gene regulation (Zhu et al., 2005).

In the *Arabidopsis* genome, there are two *BRE1* homologs (*HISTONE MONOUBIQUITINATION1* [*HUB1*] and *HUB2*) and three *RAD6* homologs (*UBIQUITIN CARRIER PROTEIN1* [*UBC1*], *UBC2*, and *UBC3*). Liu et al. (2007) reported that mutations in HUB1 or HUB2 led to defects in seed dormancy and bushy plants with pale green leaves, while Fleury et al. (2007) found that *hub1* or *hub2* plants were abnormally small with narrow leaf laminae and stunted primary roots. Based on these results, it was suggested that HUB1,2 controls seed dormancy and leaf and root growth in *Arabidopsis*. However, the function of UBC1-3 has not been documented.

Recent studies have suggested the involvement of methylation and acetylation of histone H3 in *FLC* expression and flowering time control (He and Amasino, 2005). For example, it was found that FLOWERING LOCUS D (FLD), a homolog of animal lysine-specific demethylase 1, represses *FLC* expression by decreasing the histone acetylation level of *FLC* chromatin (He et al., 2003). In addition, the polycomb group protein and the plant homeodomain protein VERNALIZATION INSENSITIVE3 (VIN3) establishes, while VERNALIZATION2 (VRN2) maintains, the repression of *FLC* expression by modifying the H3K27 and H3K9 methylation state of *FLC* chromatin in the vernalization pathway (Bastow et al., 2004; Sung and Amasino, 2004). Finally, the conserved PAF1 complex is necessary for enhancement of the

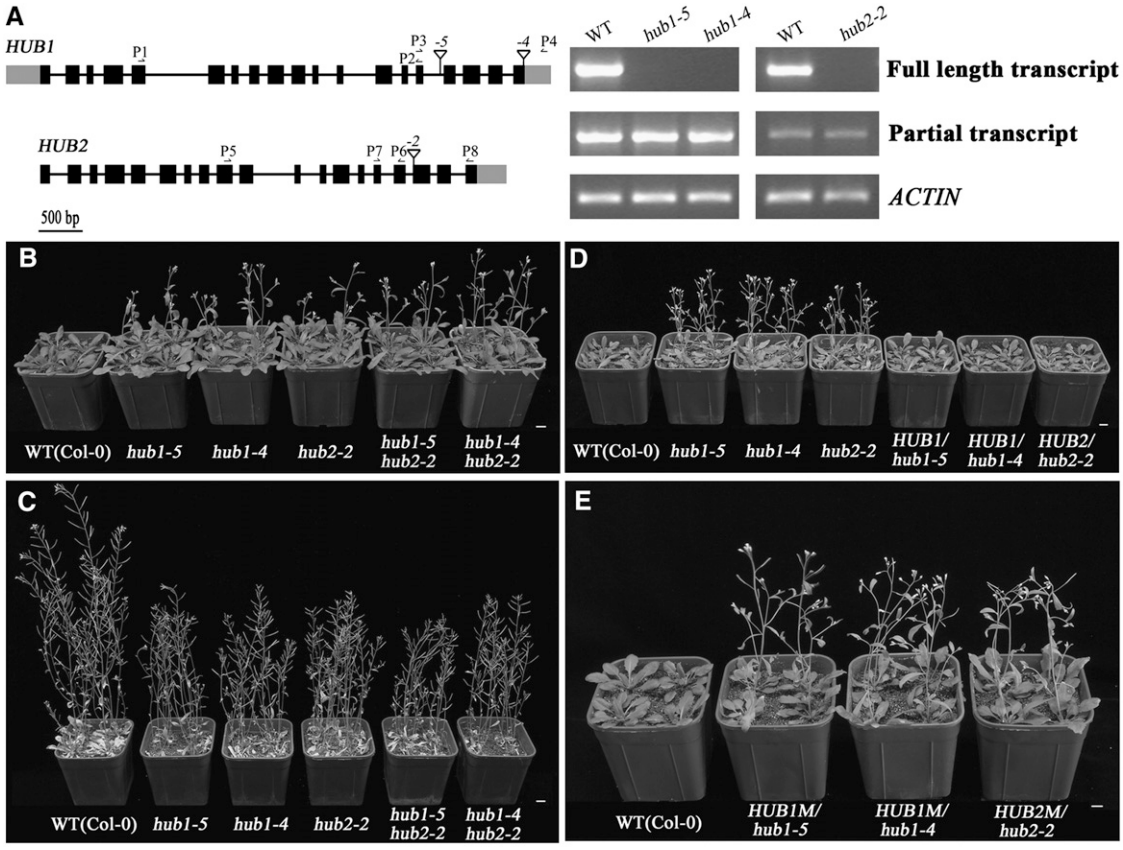



**Figure 1.** Mutation of HUB1 or HUB2 Leads to an Early Flowering Phenotype in *Arabidopsis*.

**(A)** Gene structures and characterization of the steady state mRNA levels of *HUB1* and *HUB2* by RT-PCR in the *hub1* and *hub2* mutants. Exons are represented by filled black boxes, introns by lines, untranslated regions by filled gray boxes, and T-DNA insertions by triangles. The primers used to detect the transcripts are indicated as P1 to P8. Pairs of P3/P4 and P7/P8 were used to detect the full-length transcripts of *HUB1* and *HUB2*, respectively; Pairs of P1/P2 and P5/P6 were used to detect the partial transcripts of *HUB1* and *HUB2*, respectively.

**(B)** and **(C)** Phenotypes of *hub1* and *hub2* single mutants and the *hub1 hub2* double mutant at 26 **(B)** and 42 d old **(C)**.

**(D)** and **(E)** Complementation of *hub1* and *hub2* by wild-type **(D)** (*HUB1*/*hub1* and *HUB2*/*hub2*) or mutant **(E)** (*HUB1M*/*hub1* and *HUB2M*/*hub2*) *HUB1* or *HUB2*, respectively. Thirteen out of 15 and 18 out of 20 single copy insertion lines were rescued by the transformation of *HUB1* into *hub1-5* and *hub1-4*, respectively, whereas 16 out of 19 single copy insertion lines were rescued by the transformation of *HUB2* into *hub2-2*. No line out of 54 lines for each genotype was rescued by the transformation of *HUB1M* into *hub1-5* and *hub1-4*, and no line out of 64 lines was rescued by the transformation of *HUB2M* into *hub2-2*. Bars = 1 cm.

**Table 1.** Summary of Flowering of the Wild Type and Mutants

| Genotype | LD | | SD | |
|---|---|---|---|---|
| | Rosette Leaf No. | *n* | Rosette Leaf No. | *n* |
| Wild type (Col-0) | 12.9 ± 0.8 | 20 | 65.1 ± 3.8 | 18 |
| *hub1-5* | 10.5 ± 0.6 | 21 | 49.8 ± 5.2 | 12 |
| *hub1-4* | 8.4 ± 0.7 | 22 | 45.5 ± 4.2 | 17 |
| *hub2-2* | 10.3 ± 0.8 | 24 | 46.7 ± 4.3 | 15 |
| *hub1-5 hub2-2* | 8.7 ± 0.9 | 23 | 45.8 ± 4.8 | 17 |
| *hub1-4 hub2-2* | 9.0 ± 0.6 | 23 | 47.4 ± 3.7 | 14 |
| *ubc1-1* | 12.5 ± 0.8 | 22 | 67.9 ± 3.5 | 16 |
| *ubc2-1* | 12.7 ± 0.9 | 21 | 65.4 ± 5.2 | 16 |
| *ubc3-1* | 12.8 ± 0.7 | 21 | 63.3 ± 4.2 | 18 |
| *ubc1-1 ubc2-1* | 9.0 ± 0.7 | 23 | 44.3 ± 7.0 | 14 |
| *ubc1-1 ubc3-1* | 12.8 ± 0.7 | 23 | 66.8 ± 4.1 | 15 |
| *ubc2-1 ubc3-1* | 13.0 ± 0.8 | 20 | 62.6 ± 4.0 | 15 |

LD, long-day conditions (16 h light/8 h dark); SD, short-day conditions (8 h light/16 h dark).

H3K4me3 level of *FLC* chromatin and *FLC* expression in *Arabidopsis* (He et al., 2004).

In this article, we show that *Arabidopsis* E2 conjugases UBC1 and UBC2 work together with the E3 ligases HUB1 and HUB2 to monoubiquitinate histone H2B. We also show that the ubiquitinated H2B preferentially associates with the gene body of *FLC* clade genes and that this H2Bub1 is required for the enhancement of H3K4me3 and H3k36me2 and transcriptional activation in *FLC/MAFs* chromatin. Thus, this study provides evidence that H2Bub1 is linked to histone H3 methylation in an H2Bub1-dependent dynamic manner and is specifically involved in regulating the expression of key flowering time genes in *Arabidopsis*.

## RESULTS

### HUB1 and HUB2 Are Necessary for Flowering Time Control in *Arabidopsis*

There are two BRE1 homologs, HUB1 (At2g44950) and HUB2 (At1g55250 and At1g55255), in the *Arabidopsis* genome. To analyze the function of these homologs in *Arabidopsis*, two alleles of *hub1* (*hub1-4* and *hub1-5*) and one allele of *hub2* (*hub2-2*) from the SALK insertion mutant collection were characterized. No full-length transcripts were detected for any of the three alleles; however, partial transcripts were identified in all three cases (Figure 1A).

Mutations in *HUB1* or *HUB2* led to an early-flowering phenotype under both long-day (Figure 1B, Table 1) and short-day (Table 1) conditions. In addition, the small plant size and pale green leaves observed by Fleury et al. (2007) and Liu et al. (2007) using the alleles we used and two additional alleles were evident in the mutants (Figures 1B and 1C). The three alleles of *hub1* and *hub2* produced quite similar phenotypes (Figure 1B, Table 1). Furthermore, the phenotype of the double mutant was very similar to that of each single mutant (Figure 1B, Table 1), suggesting that both *HUB1* and *HUB2* are required for the control of flowering time in *Arabidopsis*.

Next, a complementation assay was conducted by transforming *HUB1* and *HUB2* into *hub1* and *hub2* mutant plants, respectively. Native promoter-driven *HUB1* and *HUB2* coding sequence (CDS) completely rescued the *hub1* and *hub2* phenotypes (Figure 1D, Table 2). By contrast, mutant versions of *HUB1* ($C^{841}A$ and $H^{843}A$) and *HUB2* ($C^{844}A$ and $H^{846}A$) (which alter the two conserved and necessary amino acid residues for E3 activity in yeast; see Supplemental Figure 2B online) were unable to rescue the phenotypes of the *hub1* and *hub2* mutants (Figure 1E), suggesting that the E3 activity of HUB1 and HUB2 is necessary for its function in *Arabidopsis* flowering time control.

Neither HUB1 nor HUB2 was functional if yellow fluorescent protein (YFP) was fused to its C terminus; however, both proteins functioned normally with YFP fused to their N termini. The RING finger domain in HUB1 and HUB2 is located at the very end of the C terminus, which suggests that a free RING finger domain is necessary for proper functionality in HUB1 and HUB2.

### HUB1 and HUB2 Form Homodimers and Heterodimers

Genetic analysis revealed that both *HUB1* and *HUB2* are necessary for flowering time control in *Arabidopsis* (Figure 1B); this observation is also true for HUB1,2-mediated leaf development and seed dormancy (Fleury et al., 2007; Liu et al., 2007). One possible explanation for this result is that the two proteins work together in the same protein complex. Thus, we tested for a physical interaction between HUB1 and HUB2 by yeast two-hybrid assay. HUB1 and HUB2 were able to self-associate and to interact in a pairwise fashion (Figure 2A), suggesting that they form both homodimers and heterodimers.

This result was confirmed in *Arabidopsis* protoplasts using a bimolecular fluorescence complementation (BiFC) assay. HUB1 and HUB2 interacted with both HUB1 and HUB2, whereas neither HUB1 ($YFP^N$-HUB1 or $YFP^C$-HUB1) nor HUB2 ($YFP^N$-HUB2 or $YFP^C$-HUB2) was able to interact with $YFP^C$ or $YFP^N$ (Figure 2B), suggesting that the observed interactions were specific. The interaction between HUB1 and HUB2 took place in the nucleus (Figure 2B), which is consistent with the function (histone modification) of the proteins.

**Table 2.** Summary of Flowering of the Wild Type, Mutants, and Complementation Lines

| Genotype | Rosette Leaf No. | *n* |
|---|---|---|
| Wild type (Col-0) | 13.9 ± 1.1 | 25 |
| *hub1-5* | 10.5 ± 0.8 | 29 |
| *hub1-4* | 9.2 ± 0.6 | 29 |
| *hub2-2* | 10.4 ± 0.9 | 28 |
| *HUB1/hub1-5* | 13.8 ± 0.9 | 24 |
| *HUB1/hub1-4* | 14.3 ± 1.0 | 24 |
| *HUB2/hub2-2* | 14.2 ± 1.0 | 24 |
| *ubc1-1 ubc2-1* | 8.7 ± 0.7 | 20 |
| *UBC1/ubc1-1 ubc2-1*[a] | 13.4 ± 1.5 | 12 |
| *UBC2/ubc1-1 ubc2-1*[b] | 14.0 ± 1.4 | 12 |

[a] Transformant containing *UBC1* genomic DNA in the *ubc1 ubc2* mutant background.

[b] Transformant containing *UBC2* genomic DNA in the *ubc1 ubc2* mutant background.

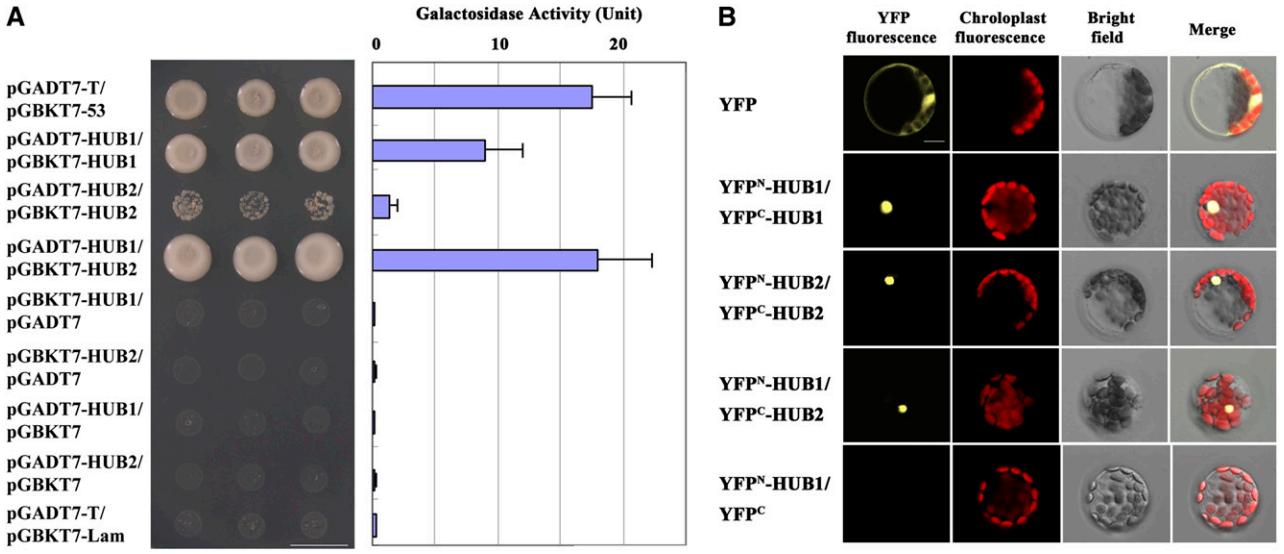



**Figure 2.** Self-Association and Pairwise Interaction of HUB1 and HUB2.

**(A)** Yeast two-hybrid assay. Positive control: pGADT7-T + pGBKT7-53 (encoding the fusions between GAL4 DNA-BD and AD and murine p53 and SV40 large T-antigen, respectively). p53 and large T-antigen interact in a yeast two-hybrid assay. Negative control: pGADT7-T + pGBKT7-Lam (encoding a fusion of the DNA-BD with human lamin C and provides a control for a fortuitous interaction between an unrelated protein and either the pGADT7-T control or AD/library plasmid). Lamin C neither forms complexes nor interacts with most other proteins. The indicated combinations of plasmids were cotransformed into the yeast reporter strain, and the interaction of HUB1 with HUB2 was assessed by growth on plates lacking Leu, Trp, His, and adenine or by analysis of the activation of a second reporter gene (*β-galactosidase*). Bar = 1 cm.
**(B)** BiFC assay to detect the interaction of HUB1 with HUB2 in *Arabidopsis* protoplasts. One ($YFP^N$-HUB1 + $YFP^C$) out of four negative controls ($YFP^N$-HUB1 + $YFP^C$; $YFP^C$-HUB1 + $YFP^N$; $YFP^N$-HUB2 + $YFP^C$; and $YFP^C$-HUB2 + $YFP^N$) is shown. Bar = 20 μm.

### *UBC1* and *UBC2* Are Functionally Redundant in the Control of Flowering Time in *Arabidopsis*

RAD6 is conserved in a number of species, and there are three *RAD6* homologs, *UBC1*, *UBC2*, and *UBC3*, in the *Arabidopsis* genome (see Supplemental Figure 1 online). To determine the function of each of these E2s and whether they are involved in the HUB1,2 pathway, we first characterized T-DNA insertion mutants for the three genes. One T-DNA insertion mutant was available for each of the three genes in the SALK insertion mutant collection (Alonso et al., 2003). Transcript analysis indicated a lack of full-length transcripts in all three mutants. *ubc1* and *ubc2* had partial transcripts, whereas *ubc3* did not, suggesting that it was a null mutant (Figure 3A).

None of the single mutants exhibited obvious defects in flowering time (Figure 3B, Table 1). The *ubc1 ubc2* double mutant flowered early and exhibited a phenotype similar to that of *hub1* or *hub2* (Figures 1B and 3B, Table 1), whereas the *ubc1 ubc3* and *ubc2 ubc3* double mutants did not show visible defects in flowering time under both long- and short-day conditions (Figure 3B, Table 1). The early-flowering phenotype observed in *ubc1 ubc2* double mutant was completely rescued by transformation of the double mutant with *UBC1* or *UBC2* genomic DNA (Figure 3C, Table 2), indicating that UBC1 and UBC2 are functionally redundant and that UBC3 is not involved in the control of flowering time in *Arabidopsis*.

Although *ubc1 ubc3* and *ubc2 ubc3* double mutants did not exhibit an obvious flowering phenotype, the *ubc1 ubc2 ubc3* triple mutant exhibited pleiotropic defects, including dwarfism, an increased number of branches, and sterility (Figure 3D). These results suggest that the three E2s are involved in multiple developmental processes and that they are functionally redundant in processes other than flowering time.

### *UBC1* and *UBC2* Exhibit a Similar but Different Expression Pattern from *UBC3* in the Shoot Apical Meristem

UBC1, UBC2, and UBC3 are homologous proteins with very high identities (see Supplemental Figure 1 online). Expression pattern analysis using promoter-β-glucuronidase (GUS) transgenic plants revealed that the three genes are expressed ubiquitously in seedlings, leaves, roots, and flowers (Figure 4A), which is consistent with the results of a previous report (Thoma et al., 1996), and that they are functionally redundant in the control of multiple developmental processes.

However, the results of the above functional analysis suggested that UBC3 is different from UBC1 and UBC2 in terms of its effect on flowering time in *Arabidopsis* (Figure 3B). As floral organ identity is determined by the genes expressed in the shoot apical meristem (SAM) (Baurle and Dean, 2006), we analyzed the expression pattern of *UBC1*, *UBC2*, and *UBC3* in the SAM. Our efforts to determine the expression level of these genes in the SAM by in situ hybridization were hampered by their apparently weak expression. We therefore examined the expression pattern of the E2s in the SAM using promoter-GUS fusion transgenic

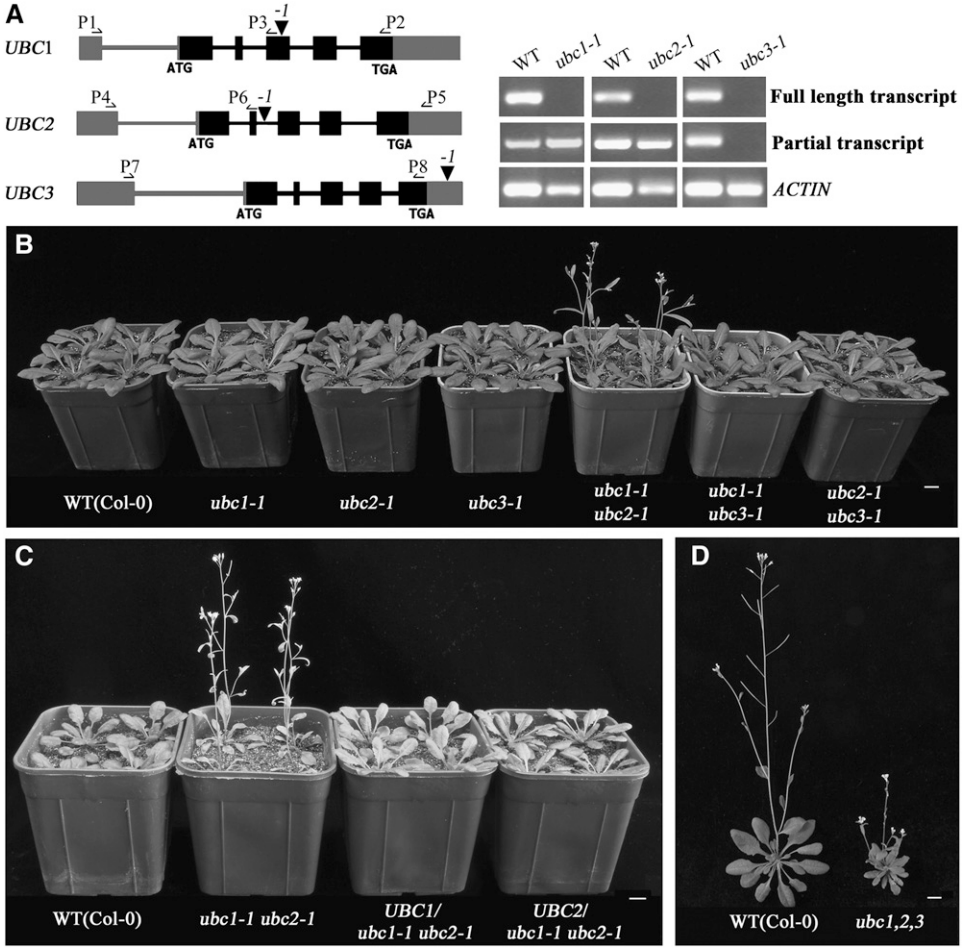



**Figure 3.** Characterization of *ubc1*, *ubc2*, and *ubc3* Mutants.

**(A)** Gene structures and characterization of the steady state mRNA levels of *UBC1*, *UBC2*, or *UBC3* by RT-PCR. Exons are represented by filled black boxes, introns by lines, untranslated regions by filled gray boxes, and T-DNA insertions by triangles. The primers used to detect the transcripts are indicated as P1 to P8. Pairs of P1/P2, P4/P5, and P7/P8 were used to detect the full-length transcripts of *UBC1*, *UBC2*, and *UBC3*, respectively; pairs of P1/P3 and P4/P6 were used to detect the partial transcripts of *UBC1* and *UBC2*, respectively.
**(B)** The phenotype of *ubc1*, *ubc2*, *ubc3*, and each double mutant at 26 d old.
**(C)** Complementation of *ubc1 ubc2* by *UBC1* (*UBC1/ubc1 ubc2*) or *UBC2* (*UBC2/ubc1 ubc2*) genomic DNA (135 of 144 lines were rescued by the transformation of *UBC1*, while 153 of 161 lines were rescued by the transformation of *UBC2*).
**(D)** Phenotype of the *ubc1 ubc2 ubc3* triple mutant. Bars = 1 cm.

lines. As shown in Figure 4B, *UBC1:GUS* and *UBC2:GUS* were ubiquitously expressed in the SAM, whereas no *UBC3:GUS* expression was detected in the central region of the SAM. This suggests that the expression pattern of *UBC3* is different from that of *UBC1* or *UBC2* in the SAM, and this difference in expression may be responsible for the distinct role that each gene plays in the control of flowering time.

### Physical and Genetic Interactions between HUB1 or HUB2 and UBC1 or UBC2

The *hub1* or *hub2* single mutant phenotype is similar to the *ubc1 ubc2* double mutant phenotype (Figures 1B and 3B), indicating that these two groups of proteins may interact and that they may be involved in the same pathway. We found that the expression patterns for *HUB1*, *HUB2*, *UBC1*, and *UBC2* were very similar in seedlings, leaves, roots, flowers, and the SAM (Figure 4). We examined the physical interaction between UBC1/2 and HUB1/2 in yeast. It was found that HUB1 and HUB2 interacted with UBC1 and UBC2 in yeast, as indicated by the growth of yeast strains cotransformed with HUB1 or HUB2 and UBC1 or UBC2 on high-stringency selection medium (Figure 5A). The RING finger domain of HUB1 or HUB2 was shown to be necessary for its interactions with UBC1 and UBC2, due to the fact that each interaction was abolished if the RING finger domain was deleted (Figure 5B). However, the observed interactions were weak, as

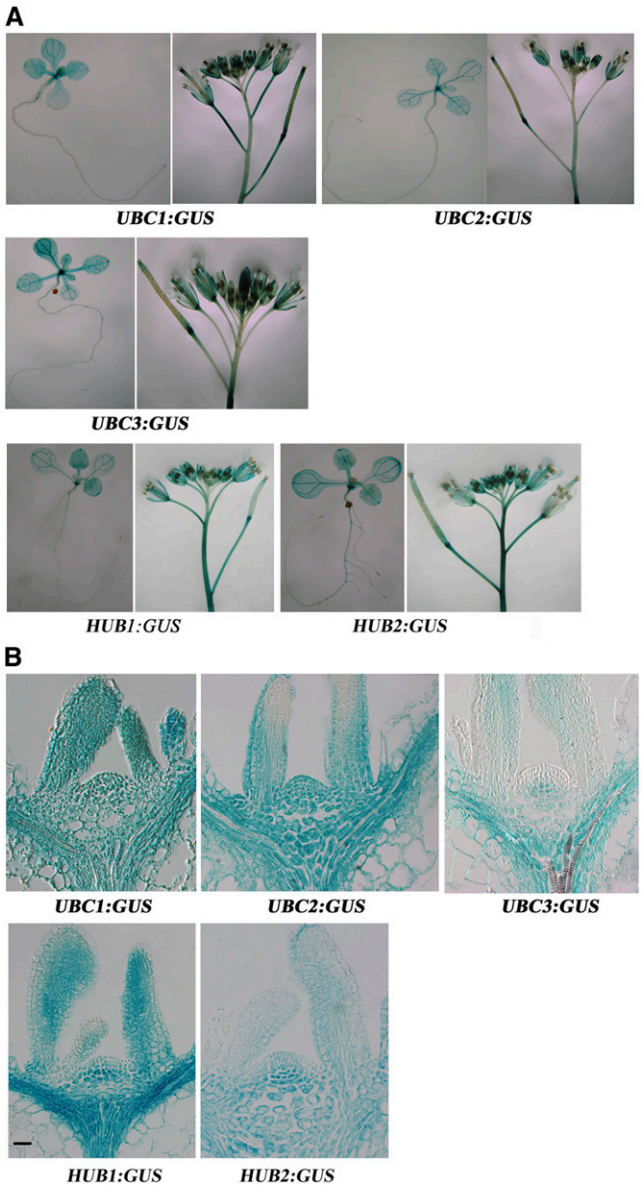



**Figure 4.** Expression Pattern of *UBC1*, *UBC2*, *UBC3*, *HUB1*, and *HUB2* by Promoter-GUS Fusion.

**(A)** Histochemical analysis of *UBC1*, *UBC2*, *UBC3*, *HUB1*, and *HUB2* expression in leaf, root, and flower.

**(B)** Expression pattern of *HUB1*, *HUB2*, and *UBCs* in the SAMs of 8-d-old seedlings. Nine out of 10 (*UBC1*), 10 out of 10 (*UBC2*), 10 out of 11 (*UBC3*), eight out of 10 (*HUB1*), and nine out of 10 (*HUB2*) independent lines exhibited the expression pattern shown. Bar = 10 μm.

indicated quantitatively by a β-galactosidase activity assay (Figure 5A). No interaction was detected for UBC1-UBC1, UBC2-UBC2, or UBC1-UBC2, suggesting that these E2s cannot form homodimers or heterodimers.

To analyze the genetic interaction between HUB1 or HUB2 and UBC1 or UBC2, we generated double and triple mutants for the corresponding genes. The phenotypes of the double and triple mutants were similar to those of the *hub1* or *hub2* single and *ubc1 ubc2* double mutants (Figures 5C and 5D), thus providing genetic evidence of the involvement of the UBC1,2/HUB1,2 pathway in flowering in *Arabidopsis*.

### HUB1/2 and UBC1/UBC2 Are the E3s and E2s Responsible for the Production of H2Bub1 in *Arabidopsis*

To test whether HUB1/2 and UBC1/UBC2 are the E3s and E2s responsible for the production of H2Bub1 in flowering time control in *Arabidopsis* under our conditions, we examined the in vivo H2Bub1 pattern in wild-type and mutant plants. As the anti-H2B antibodies for animal H2B did not work in *Arabidopsis* and anti-H2Bub1 antibody was not available when we conducted the experiment, FLAG-tagged H2B (At3g45980) was transformed into wild-type and *hub1/2* or *ubc1/2/3* plants. Anti-FLAG antibodies detected two bands (upper and lower) in wild-type, *ubc1*, *ubc2*, *ubc3* single mutant, *ubc1 ubc3*, and *ubc2 ubc3* double mutant plants (Figure 6A). However, no upper band was detected in *hub1* and *hub2* single, double, or *ubc1 ubc2* double mutant plants (Figure 6A), indicating that the upper band corresponds to monoubiquitinated H2B. This result further suggests UBC1,2 and HUB1,2 are required for H2Bub1 formation and that the UBC1,2/HUB1,2-mediated production of H2Bub1 is genome-wide and not gene specific.

To obtain additional evidence in support of this notion, we transformed a mutant version of H2B into wild-type plants. Based on the site of ubiquitination in human H2B (Zhu et al., 2005), $K^{146}$ was identified as the likely site of monoubiquitination in *Arabidopsis* H2B. The upper band was absent from transgenic plants expressing mutant ($K^{146}$A) H2B, whereas it was present in transgenic plants carrying wild-type H2B (Figures 6B and 6C). The upper band was recognized by antiubiquitin antibodies as well (Figure 6C). These results confirm that the upper band corresponds to monoubiquitinated H2B and that the site of monoubiquitination in *Arabidopsis* is $K^{146}$.

Moreover, the H2Bub1 patterns in the wild-type and mutant plants clearly indicate that the early-flowering phenotype of the mutants is caused by the loss of H2Bub1 (Figure 6A). In addition, we found that the phenotype of 10 out of 34 independent transgenic lines expressing the mutated version of H2B (H2B$K^{146}$A) was indistinguishable from that of *hub1* and *hub2* single mutants and the *ubc1 ubc2* double mutant (Figures 6D and 6E), and the early flowering phenotype was correlated with the expression level of H2B$K^{146}$A protein (Figure 6E, lines 2 and 30 exhibited phenotype, while lines 8 and 22 did not), providing further evidence that H2Bub1 is required for flowering time control in *Arabidopsis*.

To further confirm that HUB1 and HUB2 are functional homologs of yeast BRE1, *HUB1* or *HUB2* was transformed into yeast *bre1*. Both *HUB1* and *HUB2* rescued the *bre1* phenotype when expressed under the control of the yeast *BRE1* promoter (see Supplemental Figure 2A online). However, the *bre1* phenotype was not rescued if *HUB1* or *HUB2* was driven by the yeast *alcohol dehydrogenase1* (highly and constitutively expressed) promoter (see Supplemental Figure 2C online), suggesting that the precise level of *HUB1,2* is important for its function.

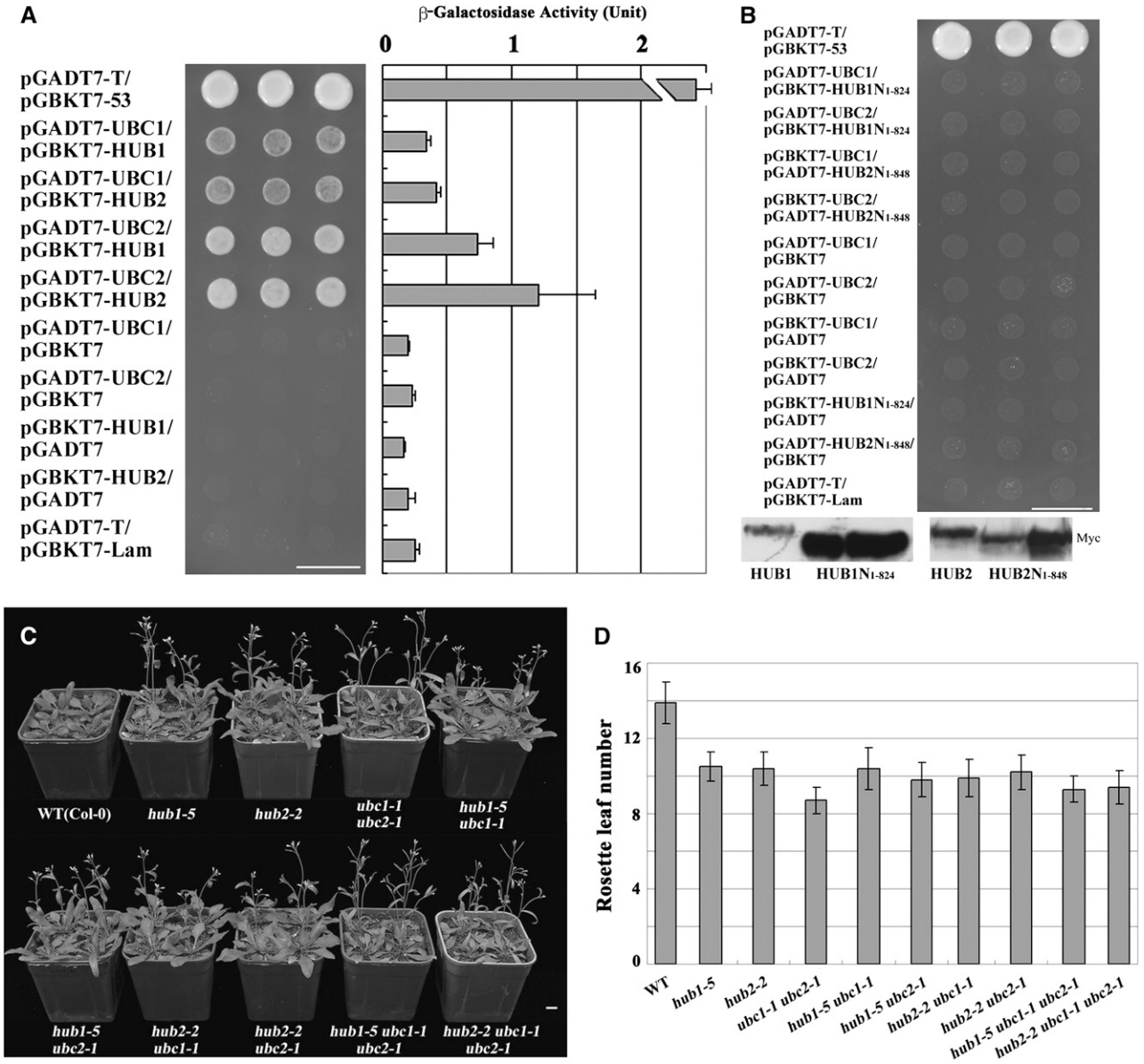



**Figure 5.** Physical and Genetic Interactions between HUB1 or HUB2 and UBC1 or UBC2.

**(A)** HUB1 or HUB2 interacts with UBC1 or UBC2.

**(B)** Deletion of the RING finger domain in HUB1 and HUB2 (constructs pGBKT7- HUB1$N_{1-824}$ and pGADT7-HUB2$N_{1-848}$) abolishes the interaction between HUB1 or HUB2 and UBC1 or UBC2. Positive control: pGADT7-T + pGBKT7-53; negative control: pGADT7-T + pGBKT7-Lam. The indicated combinations of plasmids were cotransformed into the yeast reporter strain, and the interaction between HUB1,2 and UBCs was assessed by growth on plates lacking Leu, Trp, His, and adenine or by analysis of the activation of *β-galactosidase*. The full-length and RING finger domain-deleted HUB proteins expressed in the yeast are shown in the bottom.

**(C)** The early-flowering phenotype of *hub1* or *hub2* and *ubc1,2* double and triple mutants. Bar = 1 cm.

**(D)** The rosette leaf number at flowering of the *hub1* or *hub2* and *ubc1,2* double and triple mutants. The data for wild type and each mutant are from at least 20 plants. Error bars indicate SD.

To further provide evidence that the E3 activity of HUB1,2 is necessary for the rescue of the yeast *bre1* phenotype, a mutated version of *HUB1* ($C^{841}A$ or $H^{843}A$) or *HUB2* ($C^{844}A$ or $H^{846}A$) (see Supplemental Figure 2B online) was transformed into yeast *bre1* cells. The *bre1* phenotype was not rescued by the mutated version of *HUB1* or *HUB2*, even if they were driven by the *BRE1* promoter (see Supplemental Figure 2D online). Thus, HUB1 and HUB2 are functional homologs of yeast BRE1, and the E3 activity of the proteins is necessary for their functionality in yeast.

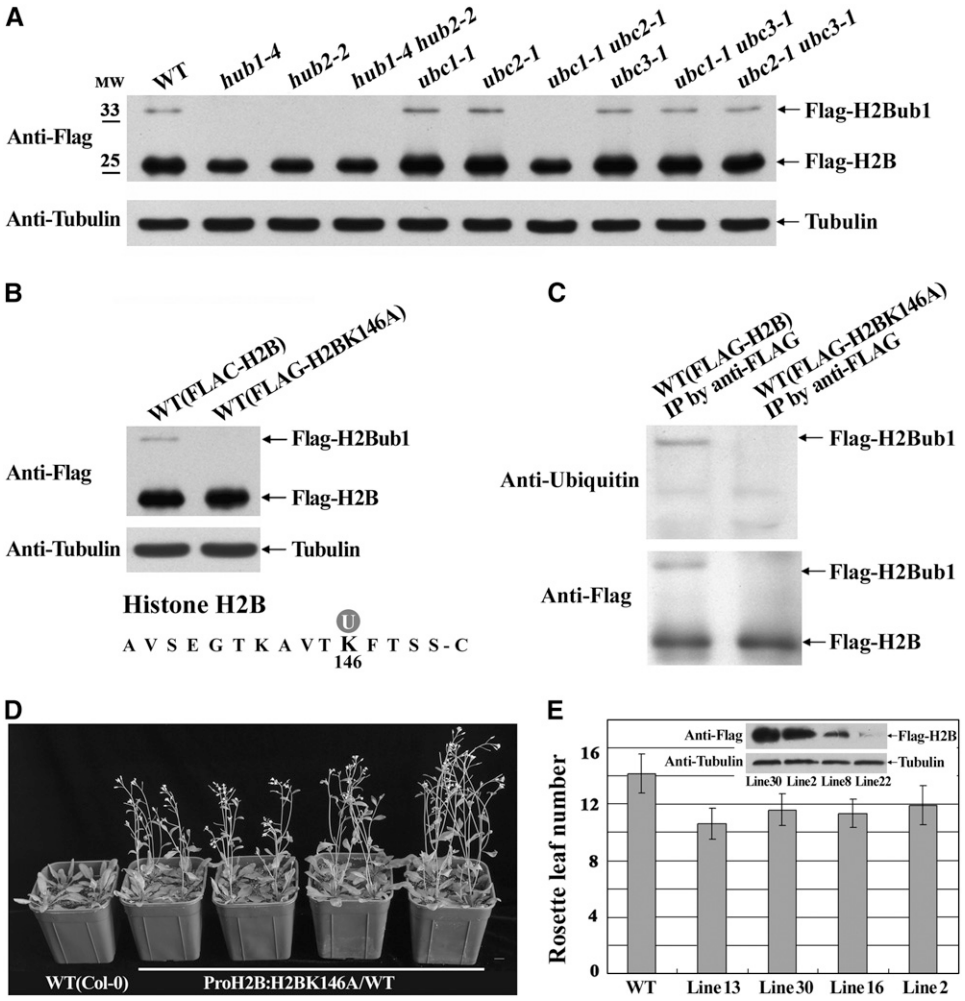



**Figure 6.** Analysis of the H2Bub1 Level in 7-d-Old Seedlings.

**(A)** Detection of H2Bub1 in wild-type and mutant plants. The upper band was monoubiquitinted Flag-H2B, and the lower band was Flag-H2B. The bands were detected by anti-Flag antibody. H2Bub1, monoubiquitinated H2B; MW, molecular weight.

**(B)** No H2Bub1 was detected in wild-type plants transformed with a mutant form of H2B ($K^{146}A$). The U in the circle is ubiquitin, and peptide in the bottom of the panel is the C-terminal sequence of H2B.

**(C)** The H2B-specific upper band was recognized by antiubiquitin antibodies after the immunoprecipitation by FLAG antibodies.

**(D)** and **(E)** Overexpression of the $K^{146}A$ mutant form of histone H2B in wild-type plants (ProH2B:H2BK146A/WT) leads to early flowering. The levels of the mutated form of H2B protein in different lines are shown at the top of the graph in **(E)**. Ten out of 34 independent lines exhibited an early-flowering phenotype (four are shown). Bar in **(D)** = 1 cm.

### Defects in H2Bub1 Inhibit the Expression of *FLC* Clade Genes in *Arabidopsis*

As *FLC* and its homologs are central players in the control of flowering time (Simpson and Dean, 2002; Searle and Coupland, 2004; He and Amasino, 2005), we examined whether the expression of *FLC* and its homologs was altered in *hub1* and *hub2* single mutants and *ubc1 ubc2* double mutants by quantitative real-time PCR. *FLC* expression was repressed in *hub1-4* and *ubc1 ubc2*, but surprisingly not in *hub2-2* or *hub1-4 hub2-2* (Figure 7A). In addition, the expression of some other members of the *FLC* clade was repressed in the mutants, albeit to different extents (Figure 7A). Among them, the expression of *MAF4* and *MAF5* in the mutants was significantly repressed compared with that in wild-type plants (Figure 7A). As the expression of *FLC* was not repressed in *hub2* or *hub1 hub2*, we examined the expression of flower-time integrator gene *FT* and found that *FT* expression was significantly increased in all four mutants (Figure 7B), which is consistent with the early flowering phenotype for those mutants. This result suggests that repression of other *FLC* clade genes, such as *MAF1*, *MAF4*, and *MAF5*, are responsible for the early flowering phenotype in *hub2-2* and *hub1-4 hub2-2*. The reason for this inconsistent repression of *FLC* expression in the different mutants is unclear. There is no obvious change for

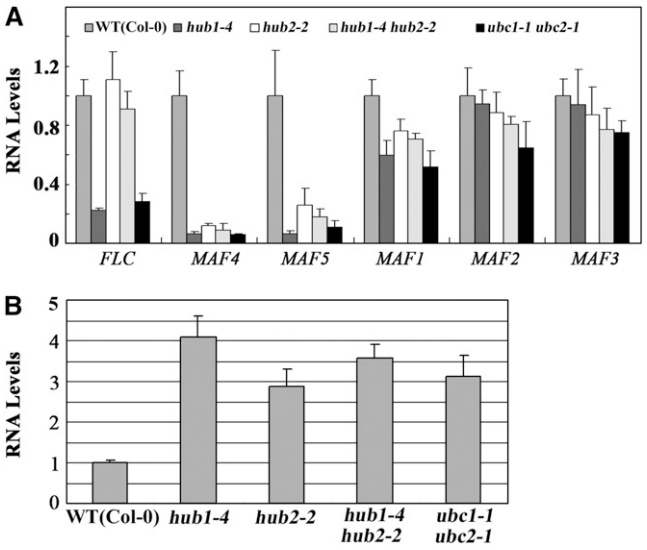



**Figure 7.** H2Bub1 Is Required for the Transcriptional Activation of *FLC*/*MAFs*.

**(A)** Analysis of the relative expression level of *FLC*/*MAFs* by quantitative real-time PCR using RNA from the SAM of 7-d-old seedlings. The levels were normalized to the wild type.

**(B)** Analysis of the relative expression level of *FT* by quantitative real-time PCR using 10-d-old seedlings. The levels were normalized to the wild type. Three biological replications were performed for each experiment. *Actin* was used as an internal control. Error bars indicate SD.

the expressions of *ACTIN*, *TUBULIN*, elongation factor 1 (*eEF-1*), or glyceraldehyde- 3-phosphate dehydrogenase (*GAPDH*) between wild-type and the four mutant plants (see Supplemental Figure 3 online). This result indicates that defects in H2Bub1 specifically lead to the repression of *FLC* and some other *FLC* clade genes, thus promoting flowering.

### Monoubiquitination of *FLC*/*MAF* Chromatin by the UBC1,2/HUB1,2 Pathway

We next examined whether *FLC*/*MAF* chromatin can be monoubiquitinated and whether the UBC1,2/HUB1,2 pathway is responsible for the modification. As our double chromatin immunoprecipitation (ChIP) assay using anti-FLAG(H2B) and -ubiquitin antibodies was unsuccessful, we used a monoclonal anti-H2Bub1 antibody (Minsky et al., 2008) for this purpose. We first examined whether the antibody could be used in *Arabidopsis* and found that the antibody clearly recognized a protein band with a molecular mass of ~28 kD. The band was not detected in *hub1*, or *ubc1 ubc2* mutant plants (Figure 8B), suggesting that the antibody works in *Arabidopsis* and indicating again that the UBC1,2/HUB1,2 pathway is required for H2Bub1.

H2B was monoubiquitinated in the gene body region of *FLC* chromatin; however, it was either not ubiquitinated or monoubiquitinated at a very low level in the promoter region (Figures 8C and 8D). There was a significant reduction in the level of H2Bub1 in the gene body region of *FLC* chromatin in *hub1* and *ubc1 ubc2* plants (Figures 8C and 8D). H2B was also monoubiquitinated in the chromatin of other *FLC* clade genes (Figures 8C, 8E, and 8F), though the level of monoubiquitination in *MAF2* and *MAF3* chromatin was much higher than that in *FLC*, *MAF4*, or *MAF5* (Figure 8F). The level of H2Bub1 in the chromatin of all *FLC* clade genes was significantly reduced in the mutants compared with wild-type plants (Figures 8C and 8E). Thus, *FLC* clade gene-specific H2Bub1 is dependent on the UBC1,2/HUB1,2 pathway. We also detected H2Bub1 in the chromatin of the *ACTIN* gene, and it was determined that the monoubiquitination was dependent on the activity of UBC1,2/HUB1,2 (Figures 8C and 8G).

### H2Bub1 Is Required for the H3K4 and H3K36 Hypermethylation of *FLC*/*MAF* Chromatin

The activation and repression of a gene are controlled by the modification state of its chromatin. In general, histones H3K4me3 and H3K36me2 are necessary for gene activation, while H3K9me2 is associated with gene silencing (Li et al., 2007). Thus, we examined the interaction between H2Bub1 and H3 methylation in *Arabidopsis*. Although no H2Bub1 was detected in *hub1*, *hub2*, or *ubc1 ubc2* mutant plants, there was no obvious difference in the genome-wide level of H3K4me3, H3K4me2, H3K4me1, H3K36me2, or H3K9me2 modification between wild-type and *hub1*, *hub2*, or *ubc1 ubc2* mutant plants (Figure 9A).

Thus, we examined whether H2Bub1 is necessary for gene-specific histone H3 methylation by comparing the levels of H3K9me2, H3K36me2, and H3K4me3 in *FLC* clade gene chromatin between wild-type and *hub1-4* (a representative *hub* mutant) or *ubc1 ubc2* plants. There was no obvious difference between the wild-type and *hub1* or *ubc1 ubc2* plants in terms of their H3K9me2 levels in the chromatin of *FLC* clade genes (Figures 9B and 9H). However, the H3K4me3 level was reduced in the gene body region of *FLC* chromatin, whereas no obvious reduction was detected in the promoter region in the *hub1* single or *ubc1 ubc2* double mutants (Figures 9B and 9C). The H3K4me3 level was reduced in the chromatin of *MAF1*, *MAF4*, and *MAF5* in the *hub1* single and *ubc1 ubc2* double mutants as well (Figures 9B and 9D). The level of H3K4me3 in the chromatin of *MAF2* and *MAF3* was much higher than that in the chromatin of *FLC*, *MAF4*, or *MAF5* (Figure 9E); moreover, there was no obvious change in the H3K4me3 level in the chromatin of *MAF2* and *MAF3* between wild-type and *hub1* or *ubc1 ubc2* plants (Figures 9B and 9D). Similarly, no obvious change in the level of H3K4me3 in the chromatin of *ACTIN* was found between wild-type and *hub1* or *ubc1 ubc2* plants (Figures 8G and 9B).

In terms of H3K36me2, the modification pattern in *FLC* chromatin was similar to that for H3K4me3 in wild-type plants (Figure 9F), and a similar change in the H3K4me3 modification pattern was observed in the chromatin of *FLC* clade genes between wild-type and *hub1* or *ubc1 ubc2* plants (Figures 9B and 9G). There was no obvious change in the level of H3K36me2 in the chromatin of *ACTIN* between wild-type and *hub1* or *ubc1 ubc2* plants (Figures 8G and 9B). These results indicate that the UBC1,2/HUB1,2 pathway is involved in the regulation of H3K4me3 and H3K36me2 levels and that H2Bub1 is required for the enhancement of H3K4 and H3K36 hypermethylation in the chromatin of *FLC* and some other *FLC* clade genes.

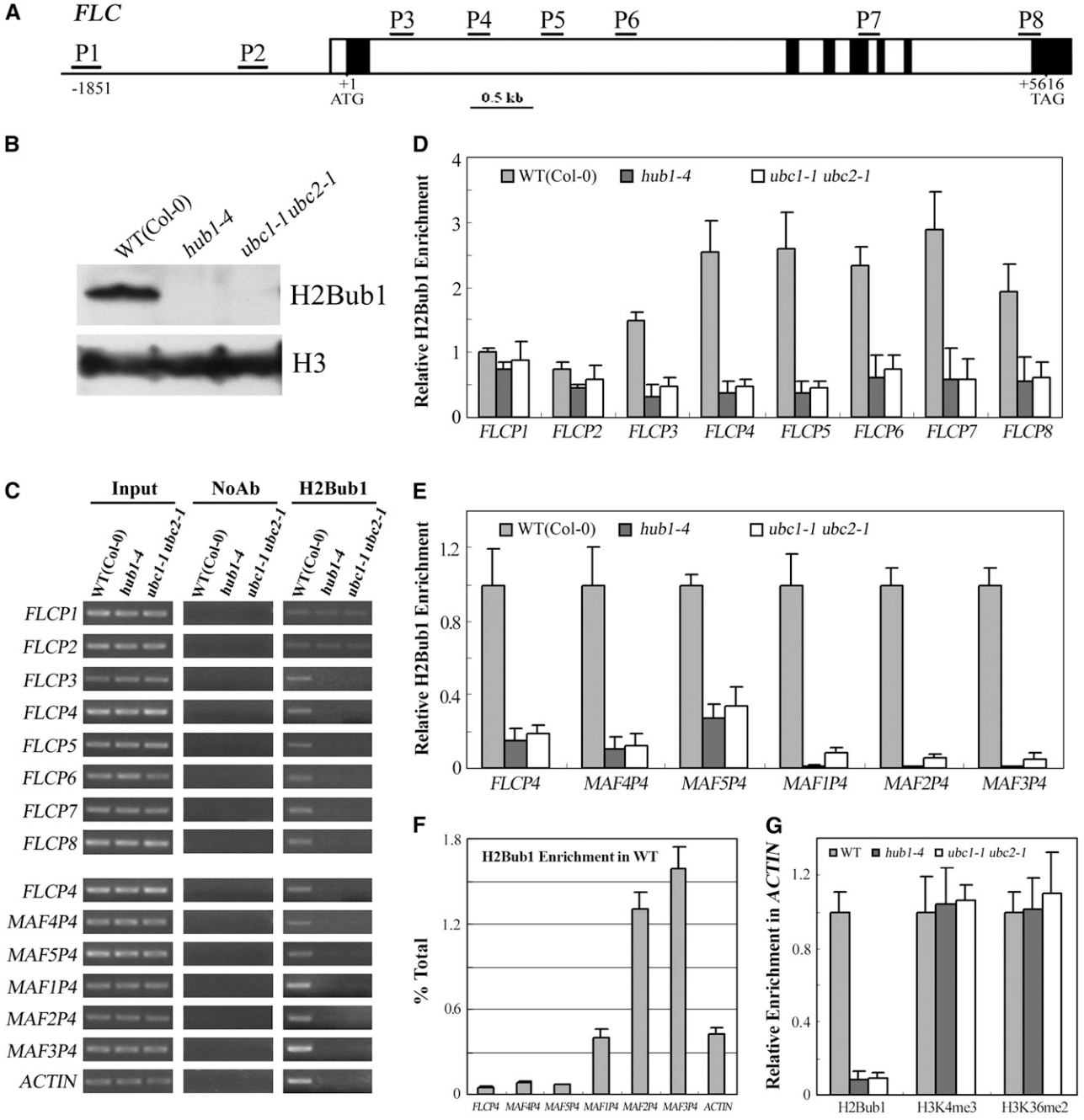



**Figure 8.** Monoubiquitination of *FLC*/*MAF* Chromatin.

**(A)** The structure of *FLC*. P1 to P8 represent the primers used to assess the level of H2Bub1, H3K4me3, or H3K36me2 by ChIP. The filled boxes represent exons, while the open boxes represent introns. +1, the translation initiation point.

**(B)** Detection of H2Bub1 in *Arabidopsis* using a monoclonal antibody against H2Bub1. H3 was used as a loading control.

**(C)** ChIP analysis of the H2Bub1 level in *FLC*/*MAFs* chromatin followed by PCR. Antibodies used for IP are indicated at top, and plant genotypes are indicated above each lane.

**(D)** ChIP was used to analyze the level of H2Bub1 in chromatin across the *FLC* locus (P1 to P8 as in **[A]**), including the promoter and gene body region, from wild-type, *hub1*, and *ubc1 ubc2* seedlings (light gray, dark gray, and white bars, respectively). The amount of immunoprecipitated chromatin as normalized to the total amount of chromatin used in *FLCP1* region in wild-type plants was given as 1.

**(E)** Differences in the level of H2Bub1 in the gene body region of *FLC*/*MAF* chromatin between wild-type and mutant plants as detected by ChIP analysis.

**(F)** ChIP analysis of the H2Bub1 level in *FLC*/*MAF* chromatin from wild-type seedlings. The data represent the amount of immunoprecipitated chromatin

### Genetic Interaction between *HUB1* or *HUB2* and the PAF1 Complex Component Gene *ELF7*

The above results suggested that the UBC1,2/HUB1,2 pathway regulates H2Bub1 to mediate flowering time in *Arabidopsis*. It was previously shown that E2 and E3 involved in H2Bub1 work together with the PAF1 complex in the same pathway in yeast and animals (Ng et al., 2003; Wood et al., 2003b; Zhu et al., 2005). It was also reported that defects in *Arabidopsis* homologs of the components of the PAF1 complex, such as EARLY FLOWERING7 (ELF7), ELF8, and VERNALIZATION INDEPENDENCE4, led to early flowering (Zhang and van Nocker, 2002; He et al., 2004). We therefore examined whether UBC1,2/HUB1,2 and the PAF1 complex function in the same pathway in *Arabidopsis* by characterizing the phenotype of *hub* and *elf7* single and double mutants. The *hub1* or *hub2* and *elf7* single mutants exhibited an early flowering phenotype, while their double mutants exhibited a similar but slightly stronger early flowering phenotype (Figure 10). Moreover, the *elf7* mutants exhibited a stronger phenotype during leaf and flower organ development than the *hub1* or *hub2* mutants (Figure 10; He et al., 2004), suggesting that the function of UBC1,2/HUB1,2 and the PAF1 complex is synergistic, and indicating the action of no identical mechanisms during *Arabidopsis* development.

## DISCUSSION

### H2Bub1 and *Arabidopsis* Development

The covalent modification of histone H2B to H2Bub1 is conserved from yeast to human (Hwang et al., 2003; Wood et al., 2003a; Bray et al., 2005; Zhu et al., 2005). In *Arabidopsis*, mutations in *HUB1,2* have been shown to cause defects in cellular proliferation during early leaf and root development, resulting in plants of reduced size with narrow leaf laminae (Berna et al., 1999; Fleury et al., 2007). In addition, defects in *HUB1* or *HUB2* have been shown to reduce seed dormancy and to result in plants with pale green leaves and a bushy appearance (Liu et al., 2007). Furthermore, a recent report suggested that H2B deubiquitination is critical for non-CpG DNA methylation and transcriptional gene silencing (Sridhar et al., 2007).

In this article, we provide several lines of evidence showing that mutations in *UBC1,2/HUB1,2*, a set of E2/E3 genes, lead to early flowering in addition to the defects in plant size and leaf development in *Arabidopsis* (Figures 1, 3, and 5). Furthermore, we found that the phenotype was highly correlated with the ubiquitination state of H2B (Figures 1, 3, and 6) and that a loss of H2Bub1 leads to early flowering (Figures 1, 3, and 6). Together, these results suggest that a novel form of histone modification (H2Bub1) is involved in the control of flowering time in *Arabidopsis*.

We also found that the monoubiquitination site in H2B is in the C terminus, at $K^{146}$ (Figure 6); however, Sridhar et al. (2007) identified $K^{143}$ as the ubiquitination site of H2B. It should be noted that $K^{146}$ in the C terminus (AVT**K**FTSS) of At3g45980 (the gene used in our experiments) corresponds to $K^{143}$ (AVT**K**FTSS) in another isoform of H2B (At1g07790), suggesting that the modification of H2B is not isoform specific.

### A Conserved E2/E3 Pathway Regulates the Production of H2Bub1 from Yeast to Human and Plant

Protein ubiquitination is achieved by a cascade involving an E1, an E2, and an E3. The E2 and E3 responsible for the production of H2Bub1 were first identified in yeast (RAD6 and BRE1, respectively) (Robzyk et al., 2000; Hwang et al., 2003; Kao et al., 2004). Since then, homologs of BRE1 with the same biochemical function have been identified in animals (Bray et al., 2005; Zhu et al., 2005) and plants (Figure 6; Fleury et al., 2007; Liu et al., 2007). In this study, we provided genetic and biochemical evidence that *Arabidopsis* UBC1 and UBC2 act as E2s together with HUB1,2 to produce H2Bub1 (Figures 1, 3, 5, and 6). Interestingly, several features of the ubiquitination of H2B are conserved in multiple species: (1) homologs of the E2 and E3 responsible for the production of H2Bub1 in yeast (Robzyk et al., 2000; Hwang et al., 2003; Kao et al., 2004) have been identified in plant (this article); (2) the site of ubiquitination in H2B and the primary sequence surrounding the ubiquitination site (AVT**K**FTSS) are highly conserved from yeast to human and plant; (3) histone H2Bub1 is genome-wide, not gene-specific, from yeast to plant; (4) H2Bub1 is associated with transcriptional activation in diverse eukaryotic organisms. Thus, chromatin remodeling via the RAD6/BRE1-mediated production of H2Bub1 has been conserved during evolution.

### H2Bub1, H3K4, and H3K36 Methylation in *Arabidopsis*

We found that a loss of H2Bub1 had no obvious effect on the global level of H3K4me3 or H3K36me2 in *Arabidopsis* (Figure 9A). By contrast, a defect in H2Bub1 led to the loss of genome-wide H3K4me3 in yeast (Hwang et al., 2003), and the knock down of H2Bub1 in human cells significantly decreased the genome-wide H3K4me3 level (Zhu et al., 2005). These findings suggest that H3K4me3 is entirely dependent on H2Bub1 in yeast, but not in plants.

In addition, H2Bub1 has more functions than H3K4me3 in fission yeast, as the loss of H2Bub1 produced stronger defects than the loss of H3K4me3 (Tanny et al., 2007). Furthermore, a defect in the putative histone H3 methyltransferase EARLY FLOWERING IN SHORT DAYS, which decreased the level of H3K4me3 in *FLC* chromatin, produced pleiotropic and stronger phenotypes in *Arabidopsis* than in the *hub1* or *hub2* mutant,

**Figure 8.** (continued).

versus the total amount of chromatin used.

**(G)** Detection of H2Bub1, H3K4me3, and H3K36me2 in *ACTIN* chromatin by ChIP analysis. At least three independent immunoprecipitations were performed for each experiment. Unless otherwise indicated, the immunoprecipitated DNA was quantified by quantitative real-time PCR. The data in **(E)** and **(G)** were normalized to the input chromatin.

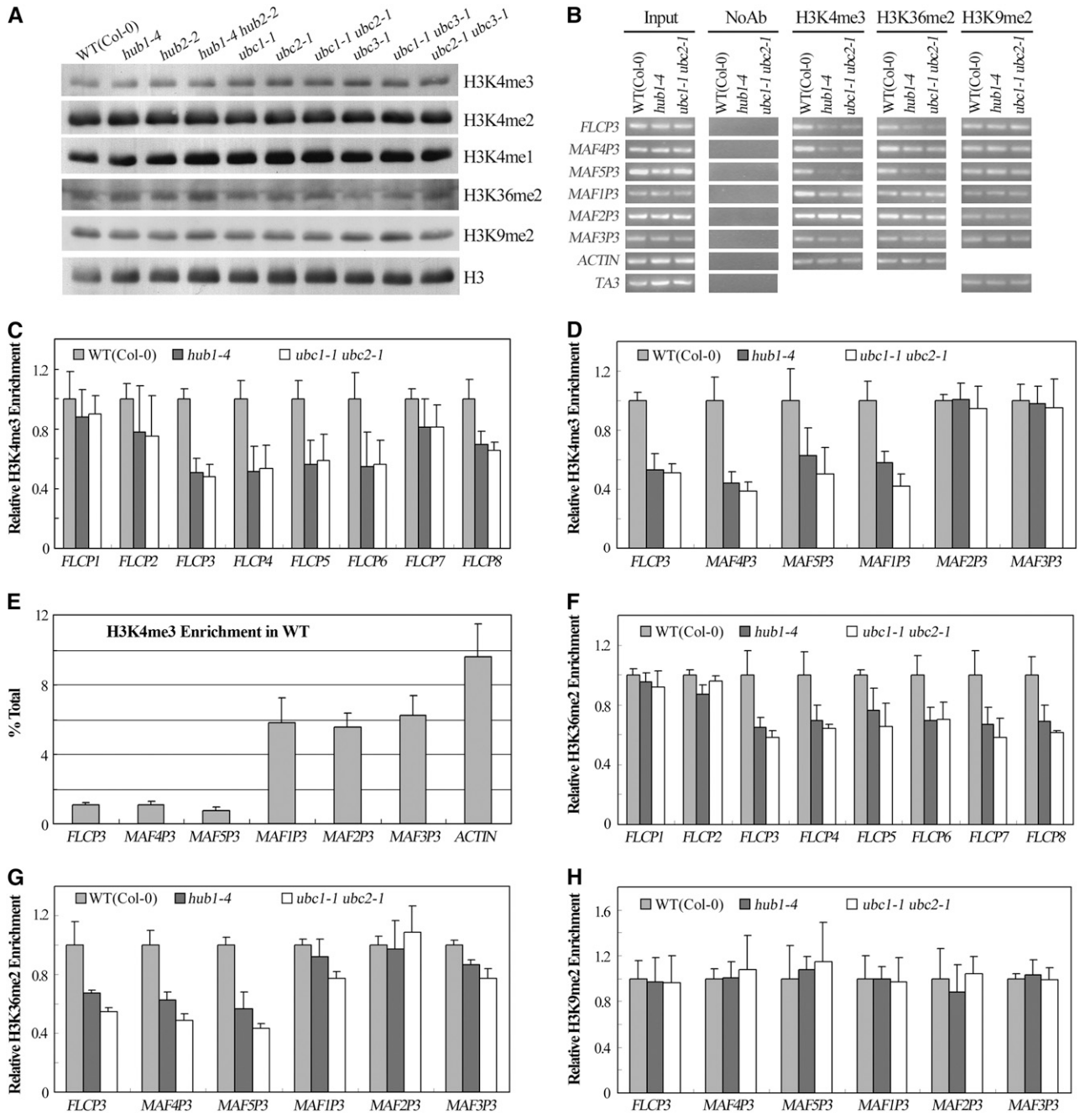



**Figure 9.** H2Bub1 Is Required for H3K4 and H3K36 Hypermethylation in *FLC*/*MAF* Chromatin.

**(A)** Analysis of H3K4, H3K9, and H3K36 methylation on a genome-wide scale by protein gel blotting.

**(B)** Detection of H3K4me3, H3K36me2, and H3K9me2 in the gene body region of *FLC*/*MAF* chromatin from wild-type and mutant plants by ChIP analysis followed by PCR. *ACTIN* was used as an internal control for H3K4me3 and H3K36me2, while *TA3* was used as an internal control for H3K9me2.

**(C)** Detection of H3K4me3 in the promoter and gene body regions of *FLC* chromatin by ChIP analysis.

**(D)** ChIP analysis of the H3K4me3 level in *FLC*/*MAF* chromatin.

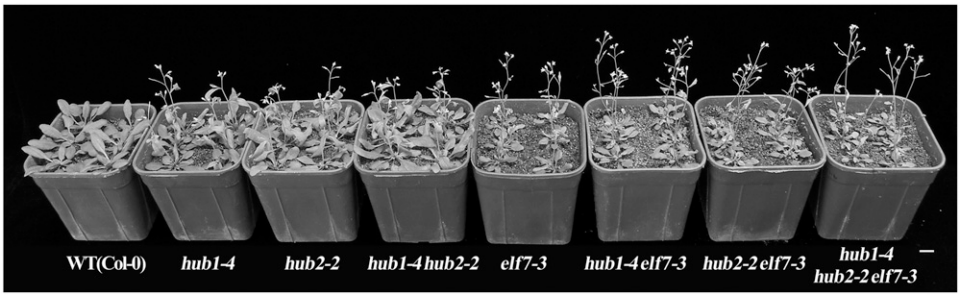
**(E)** ChIP analysis of the H3K4me3 level in *FLC*/*MAF* chromatin from wild-type seedlings. The data represent the amount of immunoprecipitated chromatin versus the total amount of chromatin used.

**(F)** Detection of H3K36me2 in the promoter and gene body regions of *FLC* chromatin by ChIP analysis.

**(G)** ChIP analysis of the H3K36me2 level in *FLC*/*MAF* chromatin.

**(H)** ChIP analysis of the H3K9me2 level in *FLC*/*MAF* chromatin. At least three independent immunoprecipitations were performed for each experiment. Unless otherwise indicated, the immunoprecipitated DNA was quantified by quantitative real-time PCR.

The data in **(C)**, **(D)**, **(F)**, and **(G)** were normalized to *ACTIN*, while the data in **(H)** were normalized to *TA3*.




| Genotype | WT(Col-0) | *hub1-4* | *hub2-2* | *hub1-4 hub2-2* | *elf7-3* | *hub1-4 elf7-3* | *hub2-2 elf7-3* | *hub1-4 hub2-2 elf7-3* |
|---|---|---|---|---|---|---|---|---|
| Rosette Leaf No. | 11.7 ± 1.3 | 7.9 ± 0.8 | 8.5 ± 0.8 | 8.3 ± 0.6 | 8.0 ± 0.8 | 6.7 ± 0.8 | 6.8 ± 0.8 | 6.5 ± 0.9 |

**Figure 10.** Genetic Interaction between *HUB1*, *HUB2*, and *ELF7*.

The plants were grown under long-day conditions for 26 d. The rosette leaf number at flowering of the *hub1* or *hub2* and *elf7* double and triple mutants is shown at the bottom. The data for the wild type and each mutant are at least from 20 plants. Error bars indicate SD. Bar = 1 cm.

which lacks H2Bub1 (Kim et al., 2005; this article). These results indicate that H3K4me3 plays a greater number of roles in *Arabidopsis* than H2Bub1. Therefore, the importance of H2Bub1 appears to have been progressively weakened, while that of H3K4me3 has become strengthened, during evolution.

H2Bub1 is associated with active transcription; thus, it was proposed to be coupled to H3K4me3 and H3K36me2 in yeast and humans (Henry et al., 2003; Zhu et al., 2005; Lee et al., 2007). We found that the H2B in chromatin of *FLC*-clade flowering repressor genes was monoubiquitinated (Figure 8); in particular, the H2Bub1 level in the gene body was higher than that in the promoter region (Figure 8), which is consistent with the role of H2Bub1 in transcriptional elongation (Pavri et al., 2006; Lee et al., 2007). The levels of H2Bub1 and H3K4me3 in the chromatin of several *FLC* clade genes were variable, and the level of H2Bub1 was correlated with the level of H3K4me3 for individual genes (Figures 8F and 9E). A defect in H2Bub1 significantly decreased the level of H3K4me3 and H3K36me2 in the chromatin of *FLC*, *MAF1*, *MAF4*, and *MAF5* (Figure 9) and repressed the expression of those genes (Figure 7), suggesting that H2Bub1 is required for the enhancement of H3K4me3 and H3K36me2 and the increased expression of those genes.

However, there was no obvious change in the steady state level of H3K4me3 or H3K36me2 in the chromatin of *MAF2* and *MAF3* or in the expression of either gene after the removal of H2Bub1 from the chromatin of *MAF2* and *MAF3* in the *hub1*, *hub2*, or *ubc1 ubc2* mutant (Figures 8 and 9). The same results were obtained for the constitutively expressed gene *ACTIN* (Figures 8G and 9B). All three genes showed a higher level of H3K4me3 in their chromatin (Figure 9E), indicating that H3K4me3 and the expression of those genes with a high level of H3K4me3 in their chromatin may not be dependent on H2Bub1. This result is consistent with the lack of an obvious change in the global H3K4me3 level between wild-type and *hub1* or *hub2* mutant plants (Figure 9A).

The reason for the uncoupling between H2Bub1 and H3 methylation in the chromatin of those genes with a high level of H2Bub1 and H3K4me3 is unclear. FLC family proteins show a high level of sequence identity (53 to 87%), and *MAF2* to *MAF5* are arranged in a tight cluster in the *Arabidopsis* genome (Ratcliffe et al., 2003). Significantly greater H2Bub1 was detected in the chromatin of *MAF2*/*MAF3* than in that of other *FLC* clade genes (Figure 8F), suggesting that H2Bub1 in the chromatin of *MAF2* and *MAF3* is more stable, while that in the chromatin of such genes as *FLC*, *MAF4*, and *MAF5* is more dynamic. It was

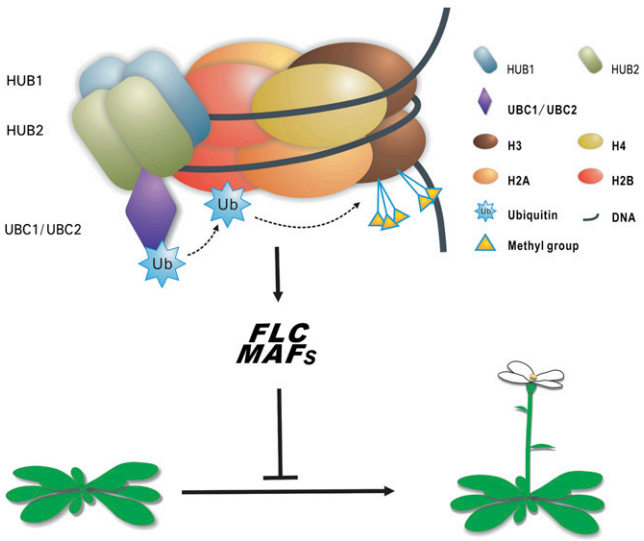



**Figure 11.** Proposed Model for the Modulation of *FLC*/*MAF* Chromatin and Transcription by the UBC1,2/HUB1,2 Pathway in the Control of Flowering Time in *Arabidopsis*.

In this model, HUB1 and HUB2 form a heterotetramer with two HUB1 and two HUB2 molecules to recruit UBC1 or UBC2 to *FLC*/*MAF* chromatin, followed by the transfer of ubiquitin from UBC1 or UBC2 to H2B. The ubiquitinated H2B may then serve as a platform for the recruitment of histone H3 methyltransferase for the enhancement of H3K4 and H3K36 hypermethylation, thus leading to increased *FLC*/*MAF* expression and the delay of flowering in *Arabidopsis*.

reported in yeast and humans that H2Bub1 was highly transient and that the dynamics of H2Bub1 were important for the regulation of transcription (Henry et al., 2003; Minsky et al., 2008). Both the monoubiquitination and deubiquitination of H2B are involved in transcriptional activation. The disruption of either process affects the transient dynamics of H2B ubiquitination, leading to alterations in the levels of H3K4me3 and H3K36me2 (Henry et al., 2003). Thus, the dynamic and transient features of H2Bub1 may depend on a low level of monoubiquitination, and only those genes with a low level of H2Bub1 in their chromatin may couple H2Bub1 and H3 methylation with transcriptional activation. This is consistent with the viable and subtle phenotypes of the *hub1,2* and *ubc1 ubc2* mutants (Figures 1 and 3). The function of H2Bub1 for those genes with a stable level of H2Bub1 in their chromatin remains to be determined.

### UBC1, UBC2, and UBC3 Have Overlapping but Not Identical Roles in *Arabidopsis* Development

UBC1, UBC2, and UBC3 are three highly homologous proteins in *Arabidopsis* (see Supplemental Figure 1 online). The *ubc1 ubc2 ubc3* triple mutant is defective in several developmental processes (Figure 3D), suggesting that these three E2s are involved in both vegetative and reproductive development in *Arabidopsis*. The phenotype of the triple mutant was much stronger than that of the single or double mutants (Figures 3B and 3D), suggesting that UBC1, UBC2, and UBC3 are functionally redundant for the above-mentioned developmental processes.

In terms of floral development, the *ubc1 ubc2* double mutant exhibited an early-flowering phenotype; however, there was no obvious difference between *ubc1 ubc3* or *ubc2 ubc3* double mutants and the wild type in terms of flowering time (Figure 3B), and the early-flowering phenotype of the triple mutant was no stronger than that of the *ubc1 ubc2* double mutant (Figure 3).

This indicates that UBC1 and UBC2 are functionally redundant and that UBC3 is not involved in flowering time control. *UBC1* and *UBC2* were both expressed ubiquitously in the SAM (Figure 4B), whereas *UBC3* was not expressed in the central region of the SAM (Figure 4B), which greatly overlaps with the expression region of *FLC* in the SAM (Searle et al., 2006). The results of our mutant phenotype analysis (Figures 1, 3, and 5), in vivo histone ubiquitination assay (Figures 6A to 6C), and interaction analysis in yeast (Figure 5A) suggest that UBC1 and UBC2 are the E2s responsible for the production of H2Bub1. Thus, UBC1 and UBC2, but not UBC3, work together with the E3s HUB1 and HUB2 to regulate H2Bub1 and flowering time in *Arabidopsis*.

### H2Bub1 Is a Novel Form of Histone Modification Essential for the Enhancement of H3K4 and H3K36 Hypermethylation and *FLC*/*MAF* Transcriptional Activation in *Arabidopsis*

The MADS box transcription factor FLC is a central repressor of the floral transition in *Arabidopsis*, other members of FLC family (MAFs) have similar roles in floral transition, and increases in *FLC*/*MAF* expression delay flowering (Henderson and Dean, 2004; He and Amasino, 2005). It has been shown that the acetylation and methylation of histone H3 is critical for the expression of *FLC* and flowering time control in *Arabidopsis* (He and Amasino, 2005; Zhao et al., 2005). FLD mediates the acetylation of *FLC* chromatin (He et al., 2003). VRN1, VRN2, and VIN3 regulate the level of H3K9me2 (Bastow et al., 2004; Sung and Amasino, 2004), while the H3K9 hyperdimethylation of *FLC* chromatin and the repression of *FLC* expression are maintained by LIKE HETEROCHROMATIN PROTEIN1 in *Arabidopsis* (Mylne et al., 2006; Sung et al., 2006). In this work, we found that H2Bub1 is essential for the enhancement of H3K4 and H3K36 hypermethylation but that it has no effect on the H3K9me2 level in *FLC*/*MAFs* chromatin (Figure 9). Thus, H2Bub1 is required for *FLC*/*MAF* transcriptional activation.

We found that both HUB1 and HUB2 were required, while UBC1 and UBC2 were functionally redundant, for the production of H2Bub1 and the control of flowering time (Figures 1, 3, 5, and 6). Moreover, HUB1 and HUB2 were capable of self-association and pairwise interaction (Figure 2). Thus, HUB1 and HUB2 may form a heterotetramer with two HUB1 and two HUB2 molecules. This E3 complex may then recruit UBC1 or UBC2 to *FLC*/*MAF* chromatin, followed by the transfer of ubiquitin from UBC1/UBC2 to H2B. The UBC1,2/HUB1,2 pathway worked together with H2Bub1 deubiquitylating enzyme (Ubp) to control the dynamics of H2Bub1, and the dynamics of ubiquitinated H2B may then serve as a platform for the recruitment of other components, such as histone H3 methyltransferase, which are responsible for the modification of H3 and the enhancement of H3K4 and H3K36 hypermethylation. Hypermethylation of H3 in *FLC*/*MAF* chromatin leads to increased *FLC*/*MAF* expression and the delay of flowering in *Arabidopsis*; thus, defects in this pathway repress the expression of *FLC*/*MAFs* and promote early flowering (Figure 11). This study shows that a novel form of histone modification (H2Bub1) is required for a key developmental process in *Arabidopsis* (i.e., floral transition), thus providing new insight into the mechanism of floral transition control in *Arabidopsis*.

## METHODS

### Plant Materials and Growth Conditions

All of the *Arabidopsis thaliana* alleles used in this study were in a Columbia background. Seeds of *hub1-5*, *hub1-4*, and *hub2-2*, corresponding to SALK_044415, SALK_122512, and SALK_071289, seeds of *ubc1-1*, *ubc2-1*, and *ubc3-1*, corresponding to SALK_109196, SALK_060994, and SALK_045233, and seeds of *elf7-3*, corresponding to SALK_019433, were obtained from the ABRC. All T-DNA insertions were confirmed by PCR and sequencing.

For the mutant complementation assay, native promoter-driven *HUB1* (2000 bp upstream of ATG; see Supplemental Table 3 online), *HUB2* (2000 bp upstream of ATG; see Supplemental Table 3 online) CDS, *UBC1* (2458 bp upstream ATG + gene body + 1059 bp downstream TAG; see Supplemental Table 3 online), or *UBC2* (2553 bp upstream ATG + gene body + 1439 bp downstream TAG; see Supplemental Table 3 online) genomic DNA was transformed into *hub1* or *hub2* single or *ubc1 ubc2* double mutant plants by the floral dip method using *Agrobacterium tumefaciens* (Clough and Bent, 1998). The seeds of the transformants were sterilized with 2.25% bleach, washed with sterilized water, kept at 4°C for 3 d, and then germinated on Murashige and Skoog medium containing 1% sucrose. After 8 d of growth under continuous white light (160 $\mu$mol $m^{-2}$ $s^{-1}$), the plants were transplanted to soil and grown under long-day (16 h of light [22°C]/8 h of dark [18°C]) or short-day (8 h of light [22°C]/16 h of dark [18°C]) conditions at 65% relative humidity.

### RNA Extraction and Real-Time PCR

Total RNA was isolated from the SAMs of 7-d-old wild-type or mutant seedlings using an RNeasy plant mini kit (Qiagen) according to the manufacturer's protocol and then treated with RNase-free DNase (Promega) to degrade any remaining DNA. A total of 3 μg of RNA was used for cDNA synthesis using an oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was subsequently performed to quantify the cDNA using TaKaRa SYBR Premix Ex Taq in a 7500 real-time PCR instrument (Applied Biosystems). *ACTIN* was included as an internal control, and the level of other housekeeping genes, such as *TUBULIN*, *eEF-1*, and *GAPDH*, among the wild type and mutants were also detected. The primers used for real-time PCR are provided in Supplemental Table 1 online.

### ChIP

ChIP was performed as described by Gendrel et al. (2005) using 7-d-old seedlings, which were grown on Murashige and Skoog medium under continuous white light. Anti-H3K9me2, anti-H3K4me3, and anti-H3k36me2 antibodies were purchased from Upstate Biotechnology, and anti-H2Bub1 monoclonal antibody was purchased from Médimabs. As the H3K4me3 and H3K36me2 levels in *ACTIN* chromatin and the H3K9me2 level in *TA3* chromatin were similar among the *hub1*, *hub2*, *ubc1 ubc2*, and wild-type plants, we used *ACTIN* as internal controls for the H3K4me3 and H3K36me2 levels and *TA3* for the H3K9me2 level, respectively. The primers used for PCR and quantitative real-time PCR are provided in Supplemental Table 2 online.

### GUS Staining

The ~2-kb promoter of *HUB1* (2000 bp upstream of ATG; see Supplemental Table 3 online), *HUB2* (2000 bp upstream of ATG; see Supplemental Table 3 online), or *UBCs* (2007, 1997, and 2069 bp upstream of ATG for *UBC1*, *2*, and *3*, respectively; see Supplemental Table 3 online) was fused with *GUS* and transformed into wild-type plants, and T2 lines were subjected to histochemical analysis. GUS staining was performed as described by Jefferson et al. (1987) and Hemerly et al. (1993); the samples were cleared as described by Malamy and Benfey (1997). To analyze the expression pattern of *HUB1*, *HUB2*, *UBC1*, *UBC2*, and *UBC3* in the SAM, 10 plants each from 10 independent T2 lines were selected for each gene. The level of GUS activity was then assessed by staining as described above, and the seedlings with GUS staining were embedded in Paraplast Plus. Each seedling was then sectioned every 8 μm and observed under a Zeiss light microscope as described by Sessions et al. (1999).

### Protoplast Transient Expression and BiFC Assays

Protoplasts were isolated from 3- or 4-week-old *Arabidopsis* leaves grown under 12 h of light/12 h of dark as described by Asai et al. (2002). To generate BiFC constructs, full-length *HUB1* and *HUB2* CDS were obtained by RT-PCR and cloned into pSY735 and pSY736, respectively. The primers used for amplification of full-length *HUB1* and *HUB2* CDS were as follows: *HUB1* forward 5′-ACGCGTCGACAATGGCGAGCAC-AGGCGAGCCTGAC-3′ and reverse 5′-CGCGGATCCTCATATGTAGAT-AGGTTTAATAT-3′; *HUB2* forward 5′-ACGCGTCGACAATGGAGAATC-AGGAATCGGACGAG-3′ and reverse 5′-CGCGGATCCTTACATTTTGA-CAAGCCGGACGTC-3′. All BiFC plasmids were purified by density gradient centrifugation. The test constructs were then cotransformed into the protoplasts using the polyethylene glycol transformation method (Bracha-Drori et al., 2004; Walter et al., 2004). YFP fluorescence in the transformed protoplasts was detected by confocal laser scanning microscopy (LSM 510 Meta; Zeiss) after 20 to 24 h of incubation in the dark. Roughly 26 to 28% of the protoplasts cotransformed with pSY735-HUB1 or HUB2 and pSY736-HUB1 or HUB2 exhibited YFP fluorescence, whereas YFP fluorescence was observed in <2 to 3% of the negative controls.

### In Vivo H2B Ubiquitination and H3 Methylation Assay

FLAG-H2B (At3g45980) driven by the H2B native promoter was transformed into wild-type and *hub1*, *hub2*, or *ubc* plants as indicated in Figure 6. T2 seedlings were then used for detection of the H2Bub1 level, and total protein was extracted using plant extraction buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, and 0.1% Nonidet P-40, plus 1 mM PMSF and protease inhibitor cocktail). The proteins were then separated by 15% SDS-PAGE and transferred to PVDF membranes for blotting with an anti-FLAG monoclonal antibody (Sigma-Aldrich); tubulin, detected by antitubulin antibodies (Sigma-Aldrich), was used as a loading control. The bound antibodies were visualized using the ECL system (Amersham).

For the confirmation of H2Bub1 using antiubiquitin antibodies, 10-d-old T2 transgenic seedlings, produced by the transformation of FLAG-H2B or FLAG-H2BK$^{146}$A into wild-type plants, were used. Ten grams of plant material were ground in liquid nitrogen to a fine powder then mixed with 8 mL of IP buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, and 0.1% Nonidet P-40, plus 1 mM PMSF and protease inhibitor cocktail). After centrifugation at 14,000 rpm for 20 min, the supernatant was transferred to a fresh cold tube, passed through a 0.45-μm filter, and mixed overnight with 100 μL of anti-FLAG M2 Affinity Gel (Sigma-Aldrich) on a rotator at 4°C. The beads were then collected by centrifugation at 800*g* for 30 s, washed three times with cooled IP buffer, collected by centrifugation at 4500 rpm for 2 min at 4°C, and eluted with 5× SDS-PAGE sample buffer (without β-ME or DTT). The protein samples were then probed with anti-FLAG and antiubiquitin antibodies (Abcam), respectively.

For the detection of global-scale H3 modification and H2Bub1 level among wild-type and mutant plants, protein extracted from 7-d-old seedlings following the chromatin extraction and sonication of the ChIP protocol, chromation solution were diluted with 2× loading buffer and denatured at 100°C for 10 min and then the samples were probed with anti-H3K4me1, anti-H3K4me2, anti-H3K4me3, anti-H3K36me2, anti-H3K9me2 antibodies (all from Upstate Biotechnology), and anti-H2Bub1 antibody (Médimabs), respectively.

### Yeast Complementation Assay

The yeast strains used in this study were kindly provided by Hiter D. Madhani (University of California, San Francisco). YM1740 corresponds to *bre1Δ* in a YM1736 (S288C) background. Yeast complementation plasmids (*BRE1-HUB1* and *BRE1-HUB2*) were generated from *pADH 1-BRE1* (Hwang et al., 2003). The *pADH 1* promoter, which is the promoter of *alcohol dehydrogenase 1*, was replaced by the native promoter of yeast *BRE1*, while *BRE1* was replaced by its *Arabidopsis* homolog, *HUB1* or *HUB2.* Cell size was determined as described previously (Hwang et al., 2003).

### Yeast Two-Hybrid Interaction Assay

All of the genes tested were cloned into two vectors (pGADT7 and pGBKT7) and cotransformed into yeast strain AH109. Transformation, yeast growth, and β-galactosidase quantitative assays were performed as described in the Clontech Yeast Protocols Handbook.

### Sequence Alignment and Phylogenetic Analysis

The amino acid sequences of the open reading frames were initially aligned using the program ClustalX 2.0 with the following parameter value: gap opening = 10, gap extension = 0.2, delay divergent sequences% = 30,

protein weight matrix: gonnet series. The phylogenetic tree was generated using the neighbor-joining method in Phylip3.6 and opened with TreeView 1.6. Numbers at nodes indicate the bootstrap value obtained for 1500 replicates.

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *HUB1* (At2g44950), *HUB2* (At1g55250 and At1g55255), *UBC1* (At1g14400), *UBC2* (At2g02760), *UBC3* At5g62540), *H2B* (At3g45980, At1g07790), *ACTIN* (At5g09810), *TUBLIN* (At1g64740), *TA3* (At1g37110), *eEF-1* (At5g60390), *GAPDH* (At1g42970), *FLC* (At5g10140), *MAF1* (At1g77080), *MAF2* (At5g65050), *MAF3* (At5g65060), *MAF4* (At5g65070), and *MAF5* (At5g65080).

### Supplemental Data

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

**Supplemental Figure 1.** Sequence Alignment and Phylogenetic Tree of the RAD6 Homologs in Different Organisms.

**Supplemental Figure 2.** HUB1 and HUB2 Rescue the Yeast *bre1* Phenotype.

**Supplemental Figure 3.** Relative Expression Level of *ACTIN*, *TUBLIN*, *eEF-1*, and *GAPDH* in Wild-Type and Mutant Plants.

**Supplemental Table 1.** The Primers Used for Real-Time PCR in Gene Expression Assay.

**Supplemental Table 2.** The Primers Used for Real-Time PCR in ChIP Assay.

**Supplemental Table 3.** The Primers Used for Specific DNA Fragment Amplification.

**Supplemental Data Set 1.** Sequence Alignment of *RAD6* Homologs Corresponding to Supplemental Figure 1.

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