Ubiquitin-Specific Proteases UBP12 and UBP13 Act in Circadian Clock and Photoperiodic Flowering Regulation in Arabidopsis[1][W][OA]

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Protein ubiquitination is involved in most cellular processes. In Arabidopsis (*Arabidopsis thaliana*), ubiquitin-mediated protein degradation regulates the stability of key components of the circadian clock feedback loops and the photoperiodic flowering pathway. Here, we identified two ubiquitin-specific proteases, UBP12 and UBP13, involved in circadian clock and photoperiodic flowering regulation. Double mutants of *ubp12* and *ubp13* display pleiotropic phenotypes, including early flowering and short periodicity of circadian rhythms. In *ubp12 ubp13* double mutants, *CONSTANS* (*CO*) transcript rises earlier than that of wild-type plants during the day, which leads to increased expression of *FLOWERING LOCUS T*. This, and analysis of *ubp12 co* mutants, indicates that UBP12 and UBP13 regulate photoperiodic flowering through a CO-dependent pathway. In addition, UBP12 and UBP13 regulate the circadian rhythm of clock genes, including *LATE ELONGATED HYPOCOTYL*, *CIRCADIAN CLOCK ASSOCIATED1*, and *TIMING OF CAB EXPRESSION1*. Furthermore, UBP12 and UBP13 are circadian controlled. Therefore, our work reveals a role for two deubiquitinases, UBP12 and UBP13, in the control of the circadian clock and photoperiodic flowering, which extends our understanding of ubiquitin in daylength measurement in higher plants.

Protein ubiquitination is a critical posttranslational mechanism regulating diverse cellular processes and signal transduction pathways in eukaryotes. Ubiquitin protein is a 76-amino-acid-long polypeptide conserved throughout all eukaryotic organisms. Attachment of ubiquitin to a Lys residue in the substrate protein requires multiple steps catalysis by E1 activating, E2 conjugating, and E3 ligating enzymes (Finley, 2009). Among these enzymes, E3 ligases are responsible for specific substrate recognition. According to their mechanisms of action and subunit composition, four main types of E3s have been identified in plants, including E3-associated protein carboxyl terminus, Really Interesting New Gene (RING), U-box, and cullin-RING ligases (Vierstra, 2009). In Arabidopsis (*Arabidopsis thaliana*), more than 1,400 genes encode components of the ubiquitin-proteasome pathway, and 90% of these genes encode subunits of E3 ligases (Moon et al., 2004). In higher plants, E3 ligases play important roles in hormone responses, photomorphogenesis, senescence, circadian rhythm, and floral development (Moon et al., 2004). For example, CONSTITUTIVE PHOTOMORPHOGENESIS1 (COP1), a RING domain E3 ligase, plays extensive roles in light response and photomorphogenesis by targeting multiple proteins, such as LONG HYPOCOTYL5, Phytochrome A, and LONG AFTER FAR-RED LIGHT1 (Lau and Deng, 2012). Moreover, COP1 also regulates the circadian clock and flowering time by destabilizing GIGANTEA (GI) and CONSTANS (CO; Jang et al., 2008; Liu et al., 2008a; Yu et al., 2008).

Ubiquitination is dynamic and reversible; the enzymatic reaction that opposes ubiquitin conjugation is deubiquitination. In human genome, 79 deubiquitinating enzymes (DUBs) were predicted (Nijman et al., 2005). Most DUBs, roughly 80%, belong to four subfamilies with Cys active sites containing a highly

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conserved catalytic triad; these families are ubiquitin C-terminal hydrolases, ubiquitin-specific proteases (UBPs/USPs), ovarian tumor proteases, and Machado-Josephin domain proteins (Katz et al., 2010). A minor subfamily is the JAB1/MPN/MOV34 metalloenzyme, members of which have a zinc active site (Wing, 2003; Reyes-Turcu et al., 2009). The USP family includes more than 50 members in humans and is the largest family of DUBs. USPs are involved in tumor suppression, DNA repair, neural stem cell progenitor maintenance, immune response, viral replication, and epigenetic control (Katz et al., 2010; Nicholson and Suresh Kumar, 2011; Neutzner and Neutzner, 2012).

Compared with the large numbers of E3 ligases, DUBs in Arabidopsis comprise a relatively smaller group. USPs, the largest subfamily, include 27 proteins with Cys- and His-box signature motifs, which have been predicted to confer deubiquitination activities (Yan et al., 2000). Among these proteins, UBP2 (Yan et al., 2000), UBP3, UBP4 (Chandler et al., 1997), UBP12 (Ewan et al., 2011), UBP14 (Doelling et al., 2001), UBP15 (Liu et al., 2008b), and UBP26 (Sridhar et al., 2007) were shown to be active enzymes in vitro. These UBPs are involved in different signaling pathways and cellular processes. For example, UBP1 and UBP2 are required for the resistance to the amino acid analog Canavanine, but the single and double mutants have no obvious phenotype under normal growth conditions (Yan et al., 2000). UBP3 and UBP4 are homologs and share 93% amino acid sequence identity; they redundantly affect pollen development and transmission (Chandler et al., 1997; Doelling et al., 2007). The *ubp14* mutant shows embryonic lethality at the globular stage (Doelling et al., 2001). The *ubp15* mutant displays leaf developmental defects and other phenotypes, such as early flowering, weak apical dominance, and reduced fertility (Liu et al., 2008b). UBP26 can remove the monoubiquitin at Lys-143 on H2B and controls heterochromatic silencing (Sridhar et al., 2007). The *ubp26* mutant also causes early flowering by repression of *FLOWERING LOCUS C* (*FLC*) transcription and seed developmental defects by activation of the imprinted gene *PHERES1* (Luo et al., 2008; Schmitz et al., 2009). UBP12 in Arabidopsis or NtUBP12 in tobacco (*Nicotiana tabacum*) acts as a negative regulator in plant immunity (Ewan et al., 2011). Therefore, unraveling the biological functions of UBPs and their substrates in Arabidopsis will add another layer to our understanding of the ubiquitination dynamics in plant development.

In this work, we report a novel role for two DUBs, UBP12 and UBP13. These two enzymes are themselves circadian regulated, and the corresponding hypomorphic alleles display a short period of the circadian clock. Study of their roles in flowering time indicates that they repress premature flowering through the photoperiod pathway. Thus, we demonstrate that deubiquitination is also important for circadian clock and photoperiodic flowering regulation.

RESULTS

UBP12 and UBP13 Confer Deubiquitination Activities in Vitro

UBP12 (At5g06600) and UBP13 (At3g11910) are two Arabidopsis homologs of human ubiquitin-specific protease USP7/HAUSP (first identified as a herpes virus-associated cellular factor; Everett et al., 1997). UBP12 and UBP13 share high amino acid sequence similarity, with 91% sequence identity, suggesting that their function may be redundant. They also share 34% amino acid sequence identity with USP7 (Supplemental Fig. S1) and have the conserved Cys- and His-box signature motifs, indicating that they have potential deubiquitination activity. At the N termini, these three proteins contain a meprin and tumor necrosis factor receptor-associated factor homology (MATH) domain, which is not found in other Arabidopsis UBP proteins (Fig. 1A).

To determine whether UBP12 and UBP13 have deubiquitination activity in vitro, we performed enzymatic activity assays using the hexameric polyubiquitin protein UBQ10 and ubiquitin extension protein UBQ1, which bears the 52 amino acid ribosomal protein appended to a single ubiquitin moiety as substrates. When wild-type UBP12 or UBP13 and their mutant forms UBP12C208S and UBP13C207S, in which conserved Cys of the enzymatic active sites were substituted by Ser, were coexpressed with UBQ10 and UBQ1 in *Escherichia coli* as described (Yan et al., 2000), we can detect the cleaved products by immunoblotting analysis with ubiquitin antibody. The wild-type UBP12 and UBP13 were capable of cleaving ubiquitin from both UBQ10 and UBQ1 (Fig. 1B). However, neither of the mutants, UBP12C208S or UBP13C207S, showed any enzymatic activity, indicating that activities of UBP12 and UBP13 were dependent on the conserved Cys residue. This result is consistent with previous findings that UBP12 can remove ubiquitin from Lys-48-linked ubiquitin chain (Ewan et al., 2011). All of these results demonstrate that UBP12 and UBP13 are bona fide DUBs in Arabidopsis.

UBP12 and UBP13 Are Ubiquitously Expressed and Localize to Both Cytoplasm and Nucleus

To figure out the biological functions of UBP12 and UBP13 in plant development, we first determined *UBP12* and *UBP13* expression patterns by examining the GUS signal in transgenic plants with *GUS* expressed under the control of the *UBP12* or *UBP13* promoter. *UBP12* and *UBP13* were both expressed in the hypocotyl, cotyledon, leaf, root, and inflorescence, especially in the vascular part of these tissues (Fig. 2, A–H). However, a few differences were observed. First, *UBP12* was expressed in hypocotyl and cotyledon of 4-d-old plants (Fig. 2B), whereas *UBP13* was mainly expressed in hypocotyl, but not cotyledon, of 4-d-old seedlings (Fig. 2F). Second, in flowers, *UBP12* was expressed in carpel, sepal, and pollen (Fig. 2D),

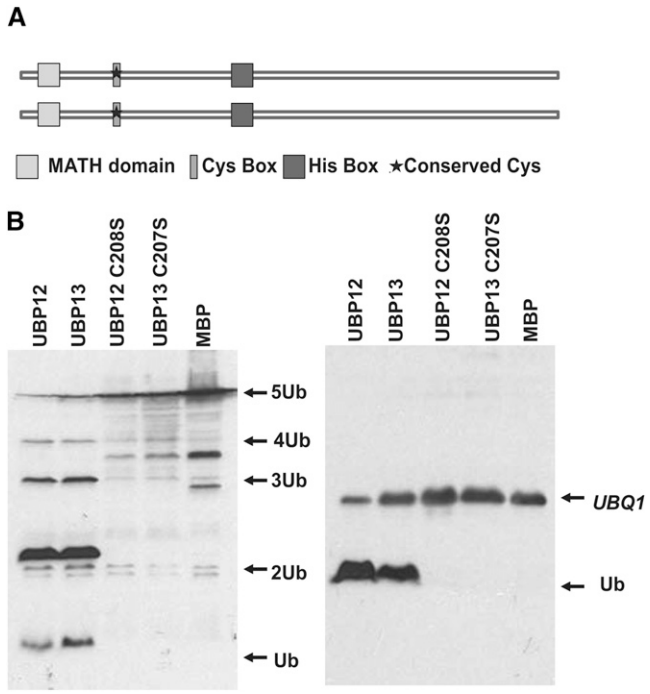

Figure 1. UBP12 and UBP13 have deubiquitination activities. A, Schematic diagram of the UBP12 and UBP13 protein structures. The MATH domain, conserved Cys-box, and His-box are shown as squares or rectangles. The star indicates the Cys residue required for enzymatic activity. B, UBP12 and UBP13 are active DUBs. In vivo cleavage of hexameric polyubiquitin (UBQ10, left) and ubiquitin extension protein (UBQ1, right). UBP12, UBP13, and their mutants UBP12C208S and UBP13C207S were coexpressed with the substrates UBQ1 and UBQ10 in *E. coli*; the cleavage products were detected by immunoblot analyses with anti-ubiquitin antibodies. The positions of the substrates and cleaved products are indicated by arrows.

whereas *UBP13* was mainly expressed only in the pollen (Fig. 2H). The highly overlapping expression patterns of *UBP12* and *UBP13* in Arabidopsis suggest that they may be functionally redundant in regulating plant development.

To examine the subcellular localizations of UBP12 and UBP13 proteins, we generated green fluorescent protein (GFP)- and cyan fluorescent protein (CFP)-tagged UBP12 and UBP13. In UBP12-GFP and UBP13-CFP transgenic plants, we observed that UBP12 and UBP13 were located in both cytoplasm and nucleus (Fig. 2, K and L), which was similar to the GFP (Fig. 2I) and CFP (Fig. 2J) alone in 35S:GFP and 35S:CFP transgenic plants. Moreover, we detected the UBP12/UBP13 protein in separated cytoplasmic or nuclear fractions using UBP antibodies. Consistent with our observation in these transgenic plants, the UBP12/UBP13 can be detected in both cytoplasm and nucleus, though more UBP12/UBP13 can be detected in the cytoplasm (Fig. 2M). These results suggest that UBP12 and UBP13 might affect substrates in both the cytoplasmic and nucleic compartments.

Mutations of *UBP12* and *UBP13* Exhibit Pleiotropic Phenotypes

To investigate the biological functions of *UBP12* and *UBP13*, we isolated mutants from transfer DNA (T-DNA) insertion populations of Arabidopsis. Two insertional mutants of *UBP12* were identified, and the alleles were named as *ubp12-1* (GABI_244E11) and *ubp12-2w* (GABI_742C10; Fig. 3A, top); the alleles contain T-DNA insertions in exons 15 and 28, respectively. Three mutant alleles of *UBP13* were identified and designated as *ubp13-1* (SALK_128312), *ubp13-2* (SALK_024054), and *ubp13-3* (SALK_132368; Fig. 3A, bottom). T-DNAs were inserted in the fifth, 10th, and 21st exons of these three mutants, respectively.

By northern-blot analysis, no accumulation of full-length UBP12 mRNA was detected in *ubp12-1* and *ubp12-2w* mutant plants, and no full-length UBP13 mRNA was detected in *ubp13-1*, *ubp13-2*, and *ubp13-3* (Fig. 3B). However, one smaller segment was found in *ubp12-2w* and *ubp13-3* mutant plants, suggesting that *ubp12-2w* and *ubp13-3* are not null alleles for *UBP12* or *UBP13*. In the *ubp12-2w* mutant, surprisingly, the mRNA level of *UBP13* was also decreased (Fig. 3B), which might result from high transcription of the 3′ primer region of *UBP12* in *ubp12-2w* causing suppression of *UBP13* in trans (Supplemental Fig. S2), indicating that *ubp12-2w* is a weak double mutant, although there is only one T-DNA insertion in the genome (Supplemental Fig. S3). Therefore, we named it as *ubp12-2w*. Different from other single mutants (Fig. 3, C, D, F, G, and H) with no obvious developmental phenotypes, the *ubp12-2w* exhibited distinct phenotypes, including small plants, round leaves, short petioles, dwarfism, and more branches after bolting (Fig. 3, E and N).

The similar expression patterns of *UBP12* and *UBP13* (Fig. 2, A–H) indicate that they could have redundant biological functions in regulating plant development. To test this, we generated double mutants and obtained *ubp12-2w ubp13-1* (Fig. 3, I and O), *ubp12-2w ubp13-2* (Fig. 3, J and P), *ubp12-2w ubp13-3* (Fig. 3, K and Q), and *ubp12-1 ubp13-3* (Fig. 3, L and R). All these double mutants displayed similar but much more severe phenotypes than *ubp12-2w* (Fig. 3, E and N), including smaller plants, rounder leaves, shorter petioles at seedling stage, more severe dwarf statures, and more bushy plants at mature stage. Among these viable double mutants, *ubp12-2w ubp13-3* showed weakest developmental phenotypes in every aspect we observed, which is consistent with the fact that partial transcripts can be detected in *ubp12-2w* or *ubp13-3*. Fertility of all these double mutants was dramatically decreased. Only *ubp12-2w ubp13-3* set enough seeds for further research, whereas *ubp12-1 ubp13-3* was completely infertile. The homozygous *ubp12-1 ubp13-1* and *ubp12-1 ubp13-2* double mutants could not be obtained, suggesting that these two genes are important for Arabidopsis embryo development and/or male/female gametophyte function. Taken together, we conclude that

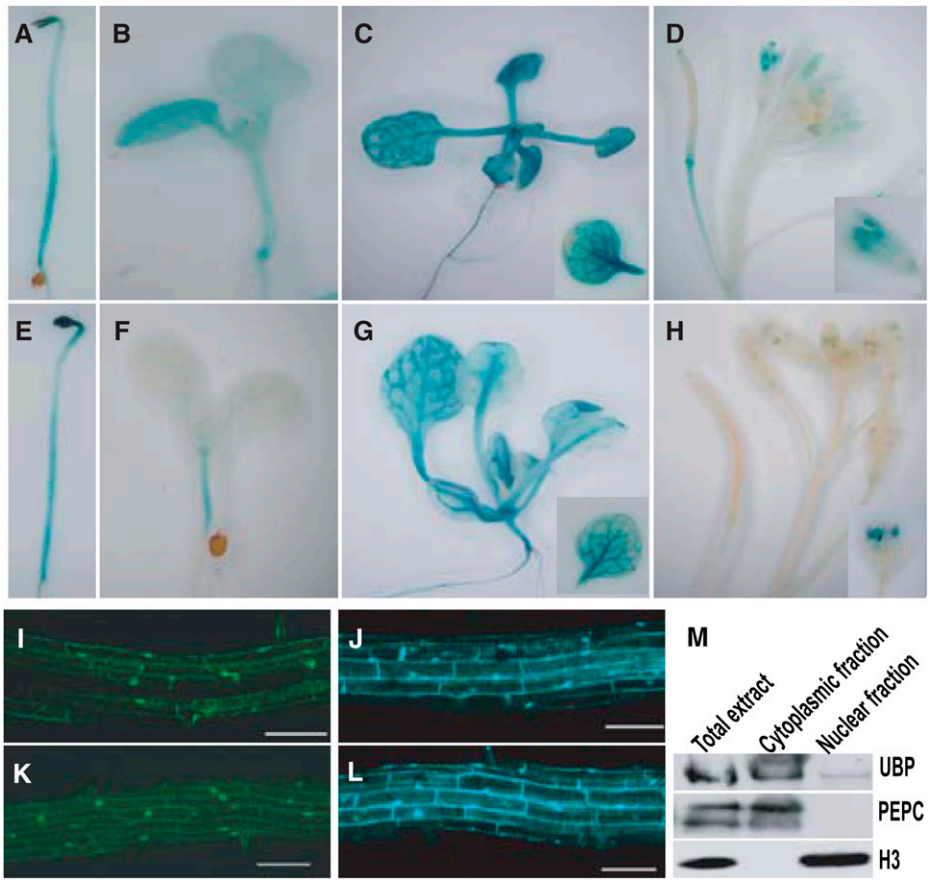

Figure 2. *UBP12* and *UBP13* have similar expression pattern and protein localization. A to D, GUS staining of dark-grown seedlings (A), 4-d-old seedlings under LD condition (B), 14-d-old seedlings under LD condition (C), and inflorescences (D) of the transformants containing *UBP12pro*:GUS. E to H, GUS staining of dark-grown seedlings (E), 4-d-old seedlings under LD condition (F), 14-d-old seedlings under LD condition (G), and inflorescences (H) of the transformants containing *UBP13pro*:GUS. I to L, UBP12 fused to GFP (K) and UBP13 fused to CFP (L) localize to the nuclei and cytoplasm; *35Spro*:GFP (I) and *35Spro*:CFP (J) were used as control. Bar = 200 μm. UBP12/UBP13 protein was detected in both cytoplasmic and nuclear fractions. Histone H3 and phosphoenolpyruvate carboxylase (PEPC) were used as control for nuclear or cytoplasmic fraction, respectively.

UBP12 and *UBP13* are involved in diverse developmental processes.

In addition to the developmental patterning defects of *ubp12-2w* and *ubp12-2w ubp13-3*, the single mutants of *ubp12-1* and *ubp12-2w* and the double mutant *ubp12-2w ubp13-3* also showed early-flowering phenotype under both long-day (LD; 16-h light/8-h dark; Fig. 3S) and short-day (SD; 8-h-light/16-h-dark) conditions (Fig. 3T) compared with wild-type plants. The phenotypes were profounder under SD. The *ubp12-2w ubp13-3* mutant flowered after forming only about 10 leaves under SD, which is similar to the mutants under LD condition, indicating that the double mutant is insensitive to photoperiod. The *ubp12-1 ubp13-3* double mutant also showed early flowering, if only according to leaf numbers (Supplemental Fig. S4, A and B). However, *ubp12-1 ubp13-3* displayed drastic developmental retardation under LD and SD, suggesting that it is not reasonable for us to analyze the flowering time (Supplemental Fig. S4C). Taken together, these results indicate that DUBs UBP12 and UBP13 are involved in the photoperiodic floral transition pathway.

Role of *UBP12* and *UBP13* in Photoperiodic Flowering Requires *CO*

In Arabidopsis, transcriptional regulation of *CO* is crucial for daylength measurement. *CO* is activated under proper daylength and subsequently up-regulates the expression of *FLOWERING LOCUS T* (*FT*) to promote flowering. Changes in *CO* transcription are the key of many daylength-insensitive mutants (Yanovsky and Kay, 2002). To determine if the early-flowering phenotype caused by mutations of *UBP12* and *UBP13* depends on *CO* and *FT*, we measured *CO* and *FT* transcripts by quantitative reverse transcription PCR (qRT-PCR) at 4-h intervals for 24 h under both LD and SD conditions. In *ubp12-2w* and *ubp12-2w ubp13-3* double mutants, the expression level of *CO* started to increase at 4 or 8 h after dawn, which was earlier than that in wild-type plants under both LD (Fig. 4A, left) and SD (Fig. 4B, left) conditions. This led to elevated *CO* expression during the day and then activated *FT* expression (Fig. 4A, right), which was more evident in the SD condition (Fig. 4B). By contrast, the transcription of *FLC* was not affected in *ubp12* or *ubp13* single and double mutants (Supplemental Fig. S5). These results indicate that UBP12 and UBP13 act in the photoperiodic flowering pathway by regulating *CO* and *FT* transcriptions.

Genetic analysis of *ubp12-2w co* double mutants further supported our results. Both the *ubp12-2w co* double mutants and *co* single mutants flowered after forming around 30 leaves, but the *ubp12-2w* mutant flowered with only around 10 leaves under LD condition (Fig. 4C; Supplemental Fig. S6). This indicates that *UBP12* and *UBP13* act upstream of *CO*. *GI* is an

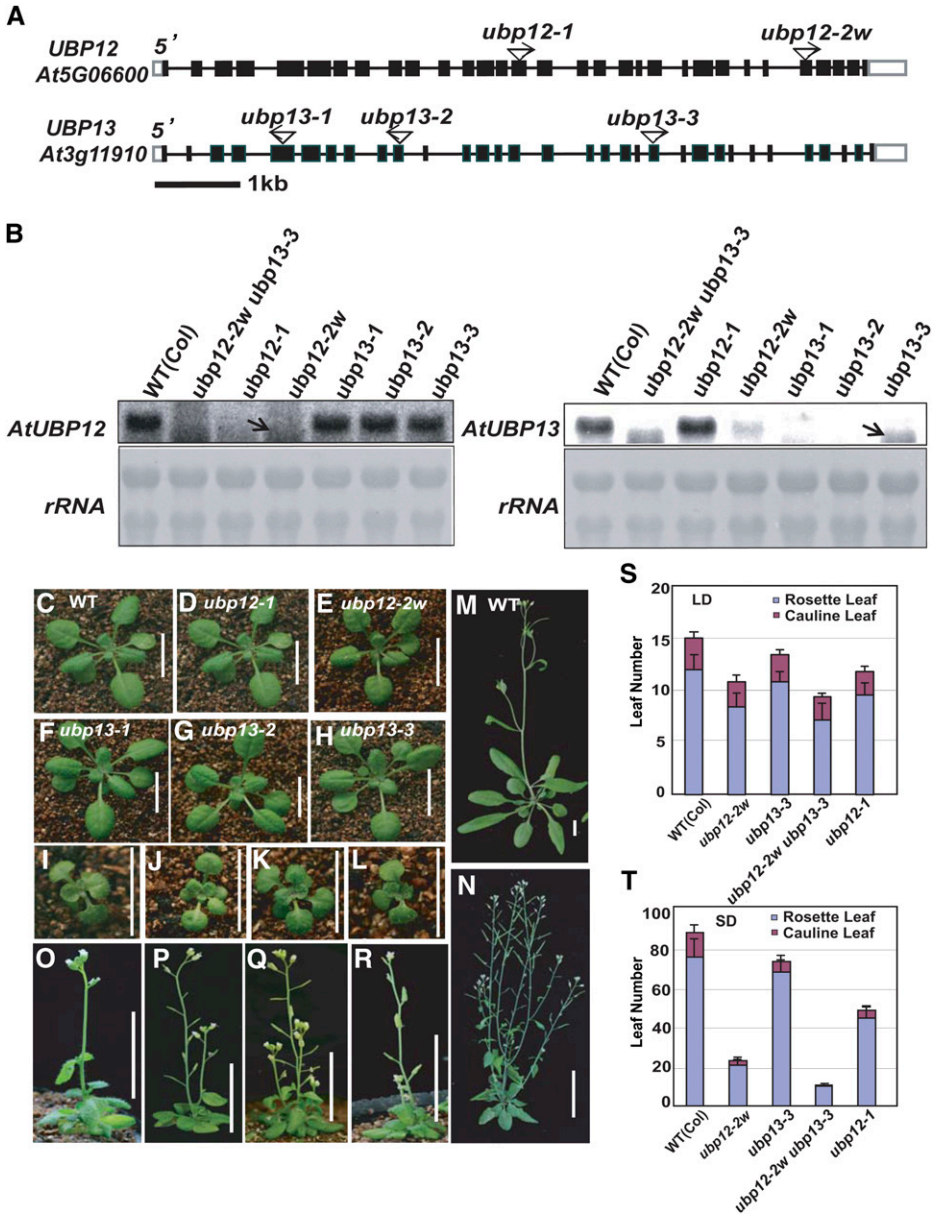

Figure 3. Mutations of *UBP12* and *UBP13* affect plant development and flowering time. A, Schematic diagrams of the *UBP12* and *UBP13* gene structures, with the T-DNA insertion sites indicated. Black boxes indicate exons, white boxes indicate untranslated regions, and lines indicate introns. B, Northern blots showing the expression levels of *UBP12* and *UBP13* in the T-DNA insertion mutants (top). Ribosomal RNA stained with methylene blue was used as loading control (bottom). C to L, Phenotypes of 24-d-old seedlings of the wild type (C), *ubp12-1* (D), *ubp12-2w* (E), *ubp13-1* (F), *ubp13-2* (G), *ubp13-3* (H), *ubp12-2w ubp13-1* (I), *ubp12-2w ubp13-2* (J), *ubp12-2w ubp13-3* (K), and *ubp12-1 ubp13-3* (L). Bar = 1 cm. M to R, Phenotypes of the wild type (M), *ubp12-2w* (N), *ubp12-2w ubp13-1* (O), *ubp12-2w ubp13-2* (P), *ubp12-2w ubp13-3* (Q), and *ubp12-1 ubp13-3* (R) after bolting. Bar = 1 cm. S and T, Statistical analysis of leaf numbers of *ubp12-2w*, *ubp13-3*, *ubp12-2w ubp13-3* double mutants, and *ubp12-1* under LD (S) and SD (T) conditions compared with wild-type plants. Values are means ± SD of at least 20 plants. WT, Wild type.

upstream regulator of *CO* and positively regulates *CO* transcription. So we also analyzed *ubp12-2w gi-4* mutants and found that they flowered as late as *gi-4* plants (Fig. 4C; Supplemental Fig. S6), indicating that *GI* is also downstream of *UBP12* and *UBP13* in regulating photoperiodic flowering. We also tested one MADS box protein involved in photoperiodic flowering regulation, SHORT VEGETATIVE PHASE (SVP), which negatively regulates *FT* expression in a *CO*-independent manner (Kim et al., 2005; Fujiwara et al., 2008; Li et al., 2008). *ubp12-2w svp32* and *ubp12-2w ubp13-3 svp32* showed earlier flowering than *svp32* and *ubp12-2w* or *ubp12-2w ubp13-3* under LD condition (Fig. 4D), indicating that UBP12/UBP13 and SVP regulate photoperiodic flowering in parallel. These genetic interactions suggest that *UBP12* and *UBP13* regulate flowering time through *GI* and *CO*.

Mutations of *UBP12* and *UBP13* Result in Altered Circadian Rhythm

In the photoperiodic flowering pathway, the circadian oscillators take part in the daylength measurement by regulating *CO* expression (Imaizumi and Kay, 2006). The plant circadian clock is composed of multiple feedback loops (Harmer, 2009). In Arabidopsis, two MYB transcription factors, CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), are expressed in the morning. They can bind to the promoter of the evening gene *TIMING OF CAB EXPRESSION1* (*TOC1*) to repress its transcription (Alabadí et al., 2001; Carré and Kim, 2002). In turn, TOC1 regulates the expressions of *CCA1* and *LHY* as a repressor (Gendron et al., 2012; Huang et al., 2012; Pokhilko et al., 2012).

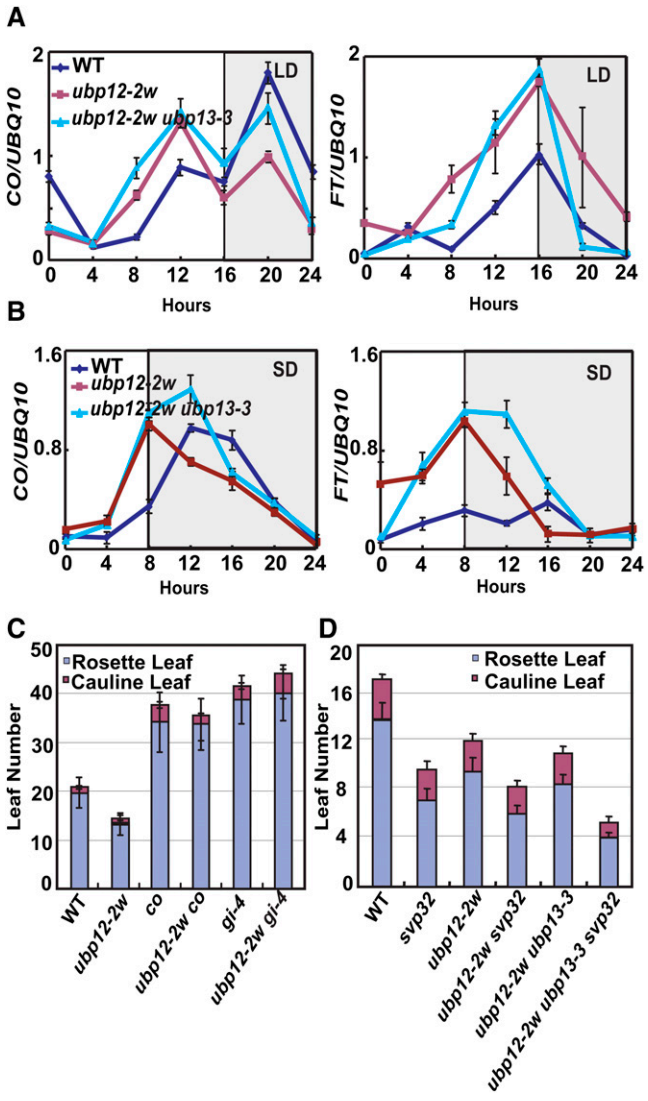

Figure 4. UBP12 and UBP13 regulate photoperiodic flowering. A, Expression patterns and transcript levels of *CO* (left) and *FT* (right) under LD condition in the wild type, *ubp12-2w*, *ubp13-3*, and *ubp12-2w ubp13-3* double mutant. Values are means ± SD of three independent experiments. B, Expression patterns and transcript levels of *CO* (left) and *FT* (right) under SD condition in the wild type, *ubp12-2w*, *ubp13-3*, and *ubp12-2w ubp13-3* double mutant. Values are means ± SD of three independent experiments. C, Statistical analysis of leaf numbers of *ubp12-2w co* and *ubp12-2w gi-4* double mutants under LD condition. Values are means ± SD of at least 20 plants. D, Statistical analysis of leaf numbers of *ubp12-2w svp32* and *ubp12-2w ubp13-3 svp32* plants under LD condition. Values are means ± SD of at least 20 plants. WT, Wild type.

We examined whether mutation of *UBP12* and *UBP13* affects circadian rhythm and the expression patterns of these core clock genes. The rhythmic accumulations of *LHY* and *TOC1* were tested by qRT-PCR in *ubp12-2w* and *ubp12-2w ubp13-3* mutants. Under constant white light (LL) free-running condition, the periods of *LHY* (Fig. 5A) and *TOC1* (Fig. 5B) expression were shortened by 4 h in *ubp12-2w* and *ubp12-2w ubp13-3* double mutants compared with that of wild-type plants. In addition, the transcription level of *TOC1* was obviously increased in *ubp12-2w* and *ubp12-2w ubp13-3* mutants (Fig. 5B). Moreover, the period of *CCA1* promoter:*LUCIFERASE* (*pCCA1*:*LUC*) circadian rhythm was also shortened by 4 h in *ubp12-2w* (n = 23) and *ubp12-2w ubp13-3* mutants (n = 7) compared with the wild type (Fig. 5, C and D). Taken together, these results indicate that *UBP12* and *UBP13* function in the periodic control of the expression of core clock oscillators in Arabidopsis. Then we tested whether *UBP12* and *UBP13* are themselves circadianly regulated. mRNA levels of *UBP12* and *UBP13* oscillated under LL condition (Fig. 5E). The transcripts of *UBP12* and *UBP13* increased and reached their highest level at 4 h (Zeitgeber time [ZT] 28) and then decreased and reached their lowest level around 16 h (ZT 40). Accumulation of UBP12 and UBP13 proteins was also highest at 8 h (ZT 32) and lowest at 16 h (ZT 40), which is similar to their mRNA levels (Fig. 5F). These results demonstrate that UBP12 and UBP13 are under circadian control. Taken together, we show that UBP12 and UBP13 are essential for proper circadian rhythm.

DISCUSSION

The circadian clock coordinates diverse aspects of plant development with daily cycles and promotes their adaption to the environment (McClung, 2011; Nagel and Kay, 2012). In Arabidopsis, proteasomal degradation pathway functions in the circadian clock and photoperiodic flowering by regulating the stability of key components in these pathways. Our research identified two circadian-regulated ubiquitin-specific proteases, UBP12 and UBP13, functioning in photoperiodic flowering and the circadian clock, which broadened our understanding of the proteasomal degradation mechanism in these processes.

UBP12 and UBP13 in Regulating Diverse Aspects of Plant Development

The ubiquitin-proteasome pathway contributes significantly to various aspects of development in Arabidopsis (Moon et al., 2004). As a counterbalance to ubiquitination, DUBs should also regulate diverse developmental processes. UBP12 and UBP13 affect plant development extensively. In addition to their role in the circadian clock and flowering, UBP12 and UBP13 are required for immunity against virulent *Pseudomonas syringae* in tomato (*Solanum lycopersicum*; Ewan et al., 2011). It is very likely that UBP12 and UBP13 target key factors in these processes and regulate protein levels by counteracting ubiquitin-mediated degradation.

Phylogenetic analysis shows that UBP12 and UBP13 are similar to human USP7, which plays crucial roles in diverse cellular processes by deubiquitinating different substrates to regulate protein stability and subcellular

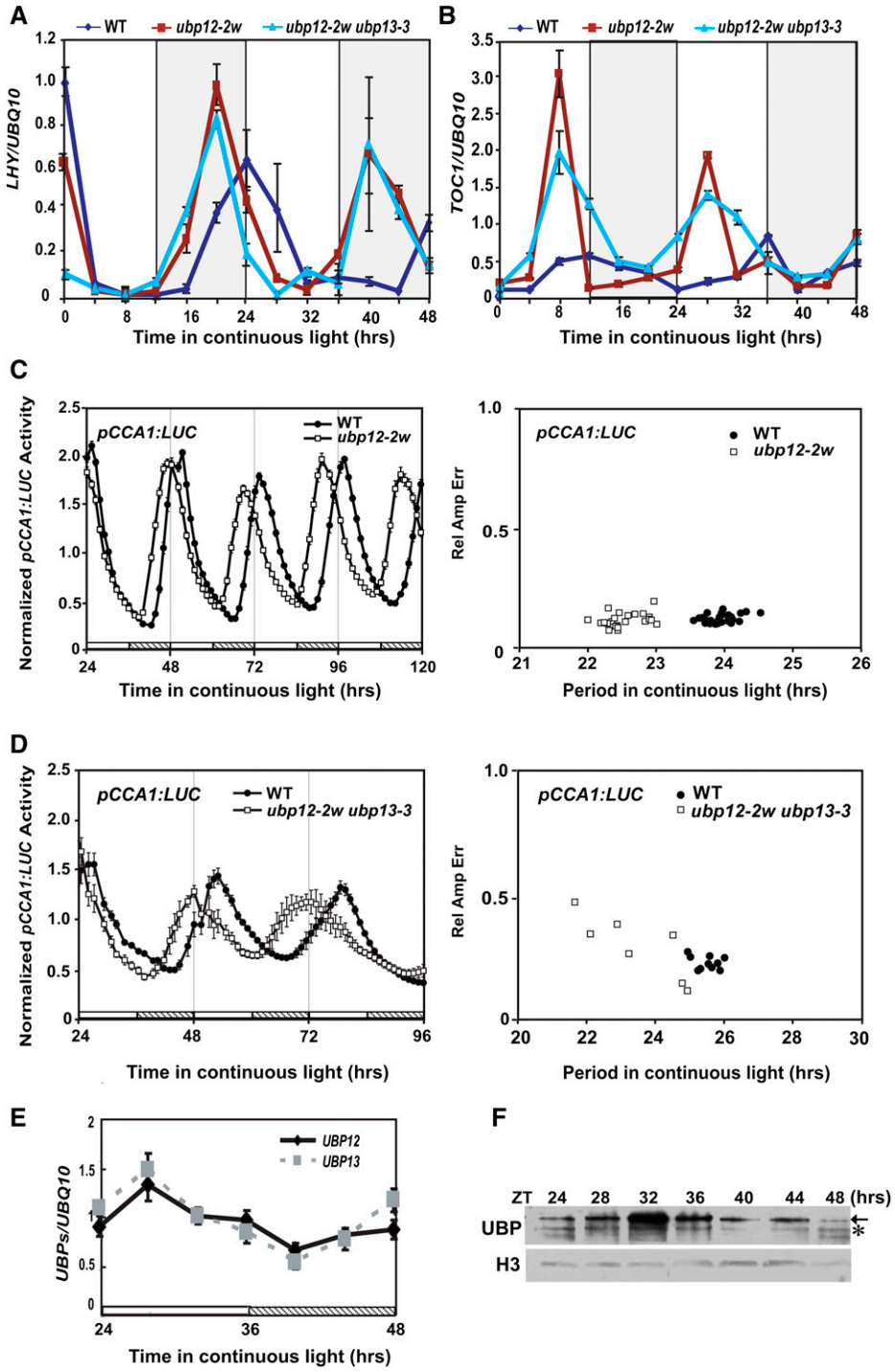

Figure 5. Mutation of UBP12 and UBP13 shortens the circadian period under LL. The expression patterns of *LHY* (A) and *TOC1* (B) under LL condition were analyzed by qRT-PCR. Wild-type, *ubp12-2w*, and *ubp12-2w ubp13-3* plants were grown for 10 d in 12-h-light/12-h-dark cycles before released to LL and sampled every 4 h from ZT0. Circadian rhythm of *pCCA1:LUC* was detected in transgenic seedlings in *ubp12-2w* (n = 23; C) or *ubp12-2w ubp13-3* (n = 7; D). The *pCCA1:LUC* transgenic plants in *ubp12-2w* and *ubp12-2w ubp13-3* were grown for 7 or 15 d, respectively, in 12-h-light/12-h-dark cycles and transferred to LL at ZT 0. Relative amplitude error is a measure of the strength of the oscillation. Statistical analysis of period length showed that in each case, the periods of *ubp12-2w* and *ubp12-2w ubp13-3* were shorter than that of the wild type ($P < 0.001$). *UBP12* and *UBP13* expression levels were tested from ZT 24 to 48 by qRT-PCR in wild-type plants (E). Values are means ± SD of three independent experiments. The protein level of UBP12/UBP13 under LL was tested by immunoblot using UBP12/UBP13 antibodies from ZT 24 to 48 (F). The arrow indicates the UBP protein position. The asterisk indicates the nonspecific band. H3 was used as the loading control. WT, Wild type.

localization (Li et al., 2002; van der Knaap et al., 2005; van der Horst et al., 2006; Song et al., 2008; Daubeuf et al., 2009; Maertens et al., 2010; Huang et al., 2011; Khoronenkova et al., 2012). Therefore, like USP7, UBP12 and UBP13 might have a wide range of targets in different developmental processes. Identifying the substrates of UBP12 and UBP13 in the future will help us to understand how they contribute to various aspects of plant development.

UBP12/UBP13 in Circadian Clock and Photoperiodic Flowering Time Regulation

Daylength measurement plays an essential role in plant growth and development. Plants use endogenous clocks to adjust their physiological and developmental stages according to the change of environment (Harmer, 2009). In Arabidopsis, transcriptional regulation of *CO* is crucial in the daylength measurement. Under SD, the

expression of *CO* is repressed before dusk, and *FT* can only express at a low level, which is insufficient to induce flowering (Imaizumi and Kay, 2006). On the contrary, the *ubp12 ubp13* double mutant shows reduced sensitivity to daylength and results in advance expression of *CO* before dusk. As a result, *FT* is highly expressed in *ubp12 ubp13* double mutants, which leads to the early-flowering phenotype. Moreover, our results indicate that both UBP12 and UBP13 are required to maintain the appropriate expression phases of the circadian genes.

UBP12 and UBP13 are DUBs and might act in circadian and photoperiodic flowering by altering the proteasomal degradation pathway. By now, there are four E3 ligases, FLAVIN-BINDING, KELCH REPEAT AND F-BOX1 (FKF1; Nelson et al., 2000; Imaizumi et al., 2005; Sawa et al., 2007; Fornara et al., 2009), ZEITLUPE (ZTL; Más et al., 2003; Kiba et al., 2007; Kim et al., 2007), LOV KELCH PROTEIN2 (Baudry et al., 2010; Takase et al., 2011; Ito et al., 2012), and COP1 (Jang et al., 2008; Liu et al., 2008a), shown to function in the plant circadian rhythm and photoperiodic flowering pathway.

The mutation of *FKF1* displays late flowering under LD, and the day peak of *CO* appears 3 h later in *fkf1* mutants than in wild-type plants. In contrast with *fkf1*, the *ubp12 ubp13* double mutant is early flowering, and *CO* expression rises earlier in *ubp12 ubp13* double mutants than in the wild type under SD and LD. The *ztl* mutant exhibits long periodicity in LL and decreased amplitude of the circadian genes. By contrast, *ubp12 ubp13* double mutants have short periodicity, and the expression level of the circadian gene *TOC1* is increased. These observations suggest that UBP12 and UBP13 might counteract the functions of these F-box proteins. It would be interesting to test if UPB12 and UBP13 can regulate the ubiquitination and stability of the substrates of these F-box proteins in the future.

The *cop1* mutant shows short periodicity and early flowering under LD and SD conditions by affecting CO protein stability and altering the *CO* and *LHY* expression through impacting on GI degradation (Jang et al., 2008; Liu et al., 2008a; Yu et al., 2008). Mutation of *UBP12* and *UBP13* resulted in similar phenotypes as *cop1* regarding flowering time, *CO* expression, and changes in circadian rhythm. These similarities suggest that UBP12 and UBP13 are not likely to function antagonistically to COP1. It would be interesting to see how these biochemically opposite enzymes contribute the same way to the response to daylength measurement. Thus, understanding the relationship between UBP12/UBP13 and COP1 in the future could help us to understand the circadian clock and photoperiodic flowering regulations better.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia wild-type and mutant plants were grown on vermiculite saturated with water under either LD (16-h-light/8-h-dark) or SD (8-h-light/16-h-dark) conditions with an intensity of 80 to 120 μE m^{-2} s^{-1} of white light at 23°C as described previously (Lu et al., 2011). *ubp12-1* (GABI_244E11), *ubp12-2w* (GABI_742C10), *ubp13-1* (SALK_128312), *ubp13-2* (SALK_024054), *ubp13-3* (SALK_132368), *co* (Liu et al., 2008a), *gi-4*, and *svp32* (Fujiwara et al., 2008) were used for genetics analysis. The primers used for genotyping are listed in Supplemental Table S1.

Alignment Analysis

Amino acid sequences of UBP12, UBP13, and mammalian USP7 were aligned using Jalview software through ClustalW. The domains were analyzed by the Pfam protein families database (http://pfam.sanger.ac.uk/).

GUS Staining and GFP Location

UBP12pro::GUS and *UBP13pro*::GUS transgenic (T3) lines were used to determine the expression pattern of *UBP12/UBP13* via histochemical GUS staining as described (Niu et al., 2008). The promoters of *UBP12* and *UBP13* were amplified by primers CX3019 and CX2973 and CX2971 and CX2972, respectively, and cloned into p1391Z (XF388) vector. The complementary DNAs (cDNAs) of UBP12 and UBP13 were amplified by primers CX2969 and CX2970 and CX2967 and CX2968, respectively, and cloned into vectors pCAMBIA1300-35S-GFP (XF215) or pCAMBIA1300-35S-CFP (XF953). The subcellular localization of UBP12 and UBP13 was performed by observing the roots of transgenic (T3) plants carrying C-terminal fusion of GFP to UBP12 and CFP to UBP13 driven by the 35S promoter and analyzed by confocal microscopy (Leica TCS SP5).

Deubiquitin Activity Assay

The full-length *UBP12* or *UBP13* coding sequences were amplified by primers CX3090 and CX3092 or CX3090 and CX3091, respectively. Their mutant forms, *UBP12C208S* and *UBP13C207S*, were generated by QuikChange II Site-Directed Mutagenesis Kit (Stratagene) using primers CX3287 and CX3288 and CX3285 and CX3286, respectively. These products were cloned into MBP-LIC (XF510) vector and then coexpressed with the substrates UBQ1 and UBQ10 in *Escherichia coli* as described (Yan et al., 2000). Lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes, and detected by immunoblot with anti-ubiquitin antibodies. The primers used for these constructions are listed in Supplemental Table S1.

RNA Gel-Blot Analysis, DNA Gel-Blot Analysis, and Quantitative PCR

Total RNA was extracted using Trizol reagent (Invitrogen), 10 d after germination from whole seedlings grown under the indicated conditions (LD, SD, and LL conditions) on Murashige and Skoog plates. RNA (20 μg per lane) was separated in an agarose gel containing 1% (v/v) formaldehyde, blotted onto Hybond N+ membrane (GE Healthcare), and probed with the PCR-amplified DNA fragments using specific primer pairs (CX3977 and CX3118 for *UBP12* and CX3976 and CX3120 for *UBP13*). Total DNA was extracted using cetyl-trimethyl-ammonium bromide reagent and digested by the restriction enzyme *Eco*RI or *Xho*I, and then digested DNA was separated in an agarose gel, blotted onto a Hybond N+ membrane, and probed with the PCR-amplified 35S promoter using specific primer pairs CX2532 and CX2533. For qRT-PCR, 2.0 μg total RNA was treated with DNaseI (Ambion), and then the first-strand cDNA was synthesized by using a cDNA synthesis kit (Transgen). qRT-PCR was performed using a CFX96 Real-Time PCR Instrument (Bio-Rad) with the SYBR Green reaction mix (Kangwei S-7567). Primers for qRT-PCR can be found in Supplemental Table S1.

Bioluminescence Measurement

For luciferase measurement, the *pCCA1:LUC* transgenic seedlings were entrained for 7 d or 15 d in 12-h-light/12-h-dark cycles at 22°C before transfer to continuous light. Since the first day in continuous light condition, seedlings were transferred to 96-well microplates (Perkin-Elmer) containing 200 μL Murashige and Skoog medium plus 2% (w/v) Suc and 30 μL 2.5 mM luciferin. The bioluminescence production was record with a Packard TopCount luminometer (Xu et al., 2010). Data were assayed using the Biological Rhythms Analysis Software System 2.1.4, which integrates the fast Fourier transform nonlinear least squares analysis for circadian rhythms (Plautz et al., 1997).

UBP Antibody and UBP Abundance in Arabidopsis

UBP12/UBP13-specific antibody was produced using a fragment of the N-terminal 315 amino acids of UBP13 expressed in *E. coli*. Polyclonal antisera was raised in mouse and affinity purified by UBP13 antigen. The specificity of UBP antibody was confirmed by Western blot using the wild type and the double mutant of *ubp12-2wubp13-2*. As showed in Supplemental Figure S7, the full-length band of UBP is present in the wild type but not in the double mutant, whereas the partial UBP12 is present in the mutant only. Total extracts were prepared from wild-type Columbia grown under LL condition every 4 h, and the nuclear and cytoplasmic fractions were separated as described (Weigel and Glazebrook, 2002). Immunoblot assay was performed as described (Lu et al., 2011). Histone H3 was used as control for loading and nuclear fraction. Phosphoenolpyruvate carboxylase was used as control for cytoplasmic fraction; antibodies included anti-H3:ab1791 (Abcam) and anti-PEPC:1004163 (Rockland).

Generation of Double Mutants

The double mutants were generated from the cross of homozygous mutants and identified from the F2 progeny grown on soil by comparing with their parental phenotypes and PCR-based characterization. The primers used for genotyping are listed in Supplemental Table S1.

Supplemental Data

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

Supplemental Figure S1. Amino acid sequence alignment of UBP12, UBP13, and HsUSP7.

Supplemental Figure S2. Schematic diagram of raw unique reads mapped onto the region around the 3′ primer end of *UBP12* in the WT and the *ubp12-2w ubp13-3* double mutant.

Supplemental Figure S3. The number of T-DNA insertions in *ubp12-2w*.

Supplemental Figure S4. Statistical analysis of leaf numbers of *ubp12-2w*, *ubp13-3*, *ubp12-2w ubp13-3*, *ubp12-1*, and *ubp12-1 ubp13-3* mutants under LD and SD conditions compared with wild-type plants.

Supplemental Figure S5. *FLC* expression level in *ubp12* and *ubp13* single and double mutants of 12 DAG seedlings.

Supplemental Figure S6. The phenotypes of *ubp12-2w co* and *ubp12-2w gi-4* double mutants.

Supplemental Figure S7. UBP antibody specificity assay by western blot.

Supplemental Table S1. Primers used for qRT-PCR, constructions, and genotyping.

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