Ubiquitin-specific Proteases UBP12 and UBP13 Act in Circadian Clock and Photoperiodic Flowering Regulation in Arabidopsis

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One sentence summaries: Deubiquitination works in day length measurement to regulate flowering time.
ABSTRACT

Protein ubiquitination is involved in most cellular processes. In Arabidopsis, 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 (UBPs), 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 (FT). 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 HYPOCHOTYL (LHY), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and TIMING OF CAB EXPRESSION 1 (TOC1). 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 day length measurement in higher plants.
INTRODUCTION

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 lysine 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 (HECT), RING, Ubox and cullin-RING ligases (Vierstra, 2009). In Arabidopsis, more than 1400 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 (HY5), Phytochrome A (PHYA) and LONG AFTER FAR-RED LIGHT 1 (LAF1) (Lau and Deng, 2012). Moreover, COP1 also regulates circadian clock and flowering time by destabilizing GIGANTEA (GI) and CONSTANS (CO) (Jang et al., 2008; Liu et al., 2008; 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 cysteine active sites containing a highly 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 Domain subfamily, members of which have a zinc active site (Wing, 2003; Reyes-Turcu et al., 2009). The USPs family includes more than 50 members in
human 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, includes 27 proteins with Cys- and His-box signature motifs, which have been predicted to confer deubiquitination activities (Yan et al., 2000). Among these proteins, AtUBP2 (Yan et al., 2000), UBP3, UBP4 (Chandler et al., 1997), UBP12 (Ewan et al., 2011), UBP14 (Doelling et al., 2001), UBP15 (Liu et al., 2008) 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., 2008). UBP26 can remove the monoubiquitin at lysine 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 PHERESI (Luo et al., 2008; Schmitz et al., 2009). UBP12 in Arabidopsis or NtUBP12 in tobacco 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 deubiquitinating enzymes, 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 MATH [meprin and TRAF (tumour necrosis factor receptor-associated factor) homology] 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 co-expressed 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. Indeed, 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 lysine-48-linked ubiquitin chain (Ewan et al., 2011). All of these results demonstrate
that UBP12 and UBP13 are *bona fide* deubiquitinating enzymes 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 β-glucuronidase (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 to 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), 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 UBP12/13 protein in separated cytoplasmic or nuclear fractions using UBP antibodies. Consistent with our observation in these transgenic plants, the UBP12/13 can be detected in both cytoplasm and nucleus, though more UBP12/13 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 T-DNA insertion populations of Arabidopsis. Two insertional mutants of \textit{UBP12} were identified and the alleles were named as \textit{ubp12-1} (GABI_244E11) and \textit{ubp12-2w} (GABI_742C10) (Fig. 3A, upper panel); the alleles contain T-DNA insertions in exons 15 and 28 respectively. Three mutant alleles of \textit{UBP13} were identified and designated as \textit{ubp13-1} (SALK_128312), \textit{ubp13-2} (SALK_024054) and \textit{ubp13-3} (SALK_132368) (Fig. 3A, lower panel). T-DNAs were inserted in the 5th, 10th and 21th exons of these three mutants, respectively.

By northern blot analysis, no accumulation of full length \textit{UBP12} mRNA was detected in \textit{ubp12-1}, \textit{ubp12-2w} mutant plants, and no full length \textit{UBP13} mRNA was detected in \textit{ubp13-1}, \textit{ubp13-2} and \textit{ubp13-3} (Fig. 3B). However, one smaller segment was found in \textit{ubp12-2w} and \textit{ubp13-3} mutant plants, suggesting that \textit{ubp12-2w} and \textit{ubp13-3} are not null alleles for \textit{UBP12} or \textit{UBP13}. In the \textit{ubp12-2w} mutant, surprisingly, the mRNA level of \textit{UBP13} was also decreased (Fig. 3B), which might result from highly transcription of 3’primer region of \textit{UBP12} in \textit{ubp12-2w} causing suppression of \textit{UBP13 in trans} (Supplemental Fig. S2), indicating that \textit{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 \textit{ubp12-2w}. Different from other single mutants (Fig. 3,C, D, F, G and H) with no obvious developmental phenotypes, the \textit{ubp12-2w} exhibited distinct phenotypes, including smaller plants, round leaves, short petioles, dwarfism and more branches after bolting (Fig. 3, E and N).

The similar expression patterns of \textit{UBP12} and \textit{UBP13} (Fig. 2, A to H) indicate that they could have redundant biological functions in regulating plant development. To test this, we generated double mutants and obtained \textit{ubp12-2w ubp13-1} (Fig. 3, I and O), \textit{ubp12-2w ubp13-2} (Fig. 3, J and P), \textit{ubp12-2w ubp13-3} (Fig. 3, K and Q), and \textit{ubp12-1 ubp13-3} (Fig. 3, L and R). All these double mutants displayed similar but much more severe phenotypes than \textit{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, \textit{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 UBP12 and UBP13 are involved in diverse developmental processes.

Besides the developmental patterning defects of ubp12-2w and ubp12-2w ubp13-3, the single mutants of ubp12-1, 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, the 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 deubiquitinating enzymes, 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 day-length measurement. CO is activated under proper day-length and subsequently upregulates the expression of FT to promote flowering. Changes in CO transcription are the key of many day-length 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 RT-PCR (RT-qPCR) at 4-h intervals for 24 hour (hr) 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 4h or 8h after dawn, which was earlier than that in wild-type plants under both LD (Fig. 4A, left panels) and SD conditions (Fig. 4B, left panels). This led to elevated CO expression during the day and then activate FT expression (Fig. 4A, right panel), 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 and Supplemental Fig. S6). This indicates that UBP12 and UBP13 act upstream of CO. GI is an 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 and 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). The 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 UBP12/13 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 day length 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-ASSOCIATED 1
CCA1) and LATE ELONGATED HYPOCOTYL (LHY), are expressed in the morning. They can bind to the promoter of the evening gene, TIMING OF CAB EXPRESSION 1 (TOC1), to repress its transcription (Alabadi et al., 2001; Carre 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).

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 RT-qPCR 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 hrs in ubp12-2w and ubp12-2w ubp13-3 double mutants compared to 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 (p CCA1:LUC) circadian rhythm was also shortened by 4 hrs in ubp12-2w (n=23) and ubp12-2w ubp13-3 mutants (n=7) compared with 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. Indeed, 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 4hr (ZT28), and then decreased and reached their lowest level around 16 hours (ZT40). Accumulation of UBP12 and UBP13 proteins was also highest at 8hr (ZT32) and lowest at 16hr (ZT40), 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
In Arabidopsis, proteasomal degradation pathway functions in 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 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. Besides their role in the circadian clock and flowering, UBP12 and UBP13 are required for immunity against virulent Pseudomonas syringe pv. in tomato (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 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.

UBP12/13 in Circadian Clock and Photoperiodic Flowering Time Regulation

Day length measurement plays 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 day-length 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 day-length 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 deubiquitinating enzymes and might act in circadian and photoperiodic flowering by altering proteasomal degradation pathway. By now, there are four E3 ligases, FLAVIN-BINDING, KELCH REPEAT AND F-BOX 1 (FKF1) (Nelson et al., 2000; Imaizumi et al., 2005; Sawa et al., 2007; Fornara et al., 2009), ZEITLUPE (ZTL) (Mas et al., 2003; Kiba et al., 2007; Kim et al., 2007), LOV KELCH PROTEIN2 (LKP2) (Baudry et al., 2010; Takase et al., 2011; Ito et al., 2012) and COP1 (Jang et al., 2008; Liu et al., 2008) 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 hours later in fklf1 mutants than in wild-type plants. In contrast with fklf1, ubp12 ubp13 double mutant is early flowering; and CO expression rises earlier in ubp12 ubp13 double mutants than in 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., 2008; 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 day-length 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.

MATERIAL AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) 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 80-120 μEm-2sec-1 of white light at 23 °C as described previously (Lu et al., 2011). The ubp12-1 (GABI_244E11), ubp12-2w (GABI_742C10), ubp13-1 (SALK_128312), ubp13-2 (SALK_024054) and ubp13-3 (SALK_132368), co (Liu et al., 2008), gi-4 and svp32 (Fujiwara et al., 2008) were used for genetics analysis. The primers used for genotyping are listed in Supplemental Table 1.

GUS staining and GFP location

UBP12::GUS and UBP13::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, CX2973 and CX2971, CX2972, respectively, and cloned into p1391Z (XF388) vector. The cDNAs of UBP12 and UBP13 were amplified by primers,
CX2969, CX2970 and CX2967, CX2968, 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, CX3092 or CX3090, CX3091, respectively. And their mutant forms, UBP12C208S and UBP13C207S, were generated by Quick Change II Site Directed Mutagenesis Kit (Stratagene) using primers CX3287, CX3288 and CX3285, CX3286. These products were cloned into MBP-LIC (XF510) vector and then co-expressed with the substrates UBQ1 and UBQ10 in E. coli as described (Yan et al., 2000). Lysates were subjected to SDS-PAGE, transferred to PVDF, and detected by immunoblot with anti-ubiquitin antibodies. The primers used for these constructions are listed in Supplemental Table 1.

RNA Gel Blot Analysis, DNA Gel Blot Analysis, Quantitative-PCR
Total RNA was extracted using Trizol reagent (Invitrogen), 10 days after germination (DAG) from whole seedlings grown under the indicated conditions (LD, SD and LL conditions) on MS plates. RNA (20 µg per lane) was separated in an agarose gel containing 1% 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, CX3976 and CX3120 for UBP13). Total DNA was extracted using CTAB reagent and digested by the restriction enzyme EcoRI or XhoI. And then digested DNA was separated in an agarose gel, blotted onto a Hybond N+ membrane (GE Healthcare), and probed with the PCR-amplified 35S promoter using specific primer pairs CX2532 and CX2533. For quantitative reverse transcriptase-polymerase chain reaction analysis (RT-qPCR), 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, China). RT Quantitative PCR was performed using a CFX96 Real-time PCR Instrument (Bio-Rad) with the SYBR Green reaction mix (KANGWEI S-7567). Primers for RT-qPCR can be found in Supplemental Table 1.

Bioluminescence Measurement

For luciferase measurement, the pCCA1:LUC transgenic seedlings were entrained for 7 days or 15 days in 12-h Light/12-h Dark cycles at 22 ºC 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 MS medium plus 2% sucrose and 30 μl 2.5mM luciferin. The bioluminescence production was record with Packard TopCountTM luminometer (Xu et al., 2010). Data was assayed using BRASS 2.1.4 (Biological Rhythms Analysis Software System), which integrates the fast fourier transform nonlinear least squares analysis for circadian rhythms (Plautz et al., 1997).

UBP antibody and UBP abundance in Arabidopsis

UBP12/13 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 wild type and the double mutant of ubp12-2wubp13-2. As showed in Supplemental Fig. S7, the full length band of UBP is present in wild type but not in the double mutant, whereas the partial UBP12 is present in the mutant only. Total extracts were prepared from WT (Col) grown under LL condition every 4 hours and the nuclear and cytoplasmic fractions were seperated as descried (Weigel D and J., 2002). Immunoblot assay were performed as described (Lu et al., 2011). Histone H3 was used as control for loading and nuclear fraction. PEPC was used as control for cytoplasmic fraction, Antibodies: anti-H3: ab1791
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 Supplementary Table 1.

ACKNOWLEDGMENTS
We thank Mr. Qingbao Zhu for technical help. Dr. Richard D. Vierstra for providing UBQ1 and UBQ10 plasmids. Dr. Hongquan Yang (Shanghai Jiaotong University, China) for providing co (SAIL_H_024) seed; Dr. Chentao Lin (UCLA, USA) for providing gi-4 mutant seed. This work was supported by the National Basic Research Program of China (grant nos. 2011CB915401 to X. Cao, 2013CB967302 to X. Cui), the National Natural Science Foundation of China (grants nos. 30971619 to X. Cui, 30930048 to X. Cao and 30971507 to X. Xu)

FIGURE LEGENDS
Figure 1. UBP12 and UBP13 have deubiquitination activities.
(A) Schematic diagram of the UBP12 and UBP13 protein structures. The MATH domain, the conserved Cys box and His box are shown as square or rectangle, ★ indicates the Cys residue required for enzymatic activity.
(B) UBP12 and UBP13 are active deubiquitinating enzymes. In vivo cleavage of hexameric polyubiquitin (UBQ10, left panel) and ubiquitin extension protein (UBQ1, right panel). UBP12, UBP13 and their mutants UBP12C208S, UBP13C207S were co-expressed 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.

**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. Scale bar equals 200 µm. (M) UBP12/13 protein was detected in both cytoplasmic and nuclear fractions. Histone H3 and PEPC were used as control for nuclear or cytoplasmic fraction, respectively.

**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 UTRs; and lines indicate introns. (B) Northern blots showing the expression levels of *UBP12* and *UBP13* in the T-DNA insertion mutants (upper panel). *rRNA* stained with methylene blue was used as loading control (lower panel). (C) to (L), Phenotypes of 24-d-old seedlings of WT (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), *ubp12-1 ubp13-3* (L). Scale bar equals 1 cm. (M) to (Q), Phenotypes of WT (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. Scale bar equals 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.

**Figure 4.** UBP12 and UBP13 regulate photoperiodic flowering.

(A) Expression patterns and transcript levels of CO (left panel) and FT (right panel) under LD condition in WT, 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 panel) and FT (right panel) under SD condition in WT, 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.

**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 RT-qPCR. Wild-type (Col), ubp12-2w and ubp12-2w ubp13-3 plants were grown for 10 d in 12-h Light/12-h Dark cycles before released to continuous white light (LL), and sampled every 4 hrs from ZT0. Circadian rhythm of CCA1p:LUC was detected in transgenic seedlings and plot the period versus relative amplitude error (RAE) in ubp12-2w (n=23) (C) or ubp12-2w ubp13-3 (D) (n=7). The ubp12-2w and ubp12-2w ubp13-3 mutants were grown for 7d or 15d, respectively, in 12-h Light/12-h Dark cycles and transferred to LL at ZT=0. RAE 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 ZT24 to ZT 48 by RT-qPCR in wild-type plants (E). Values are means ± SD of three independent experiments. The protein level of UBP12/13
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Supplemental Data

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 ubp12-2w ubp13-3 double mutant.

Supplemental Figure S3: The number of T-DNA insertion 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 (A) and SD (B) conditions compared with wild-type plants. Values are means ± SD of at least 20 plants. Phenotypes of developmental retardation of the ubp12-1 ubp13-3 double mutants under LD (C).

Supplemental Figure S5: FLC expression level in ubp12 and ubp13 single and double mutants.

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 Table1: Primers used for RT-qPCR, constructions and genotyping.

LITERATURE CITED


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