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Original Paper

Late flowering phenotype under ultra-short photoperiod (USP) in *Arabidopsis thaliana*

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Abstract Many environmental and endogenous factors affect flowering time of plants. In *Arabidopsis*, there are three major pathways for the control of flowering time; the photoperiod or long-day (LD) pathway, autonomous/vernalization pathway and gibberellic acid (GA) pathway. The flowering regulation under most common photoperiods in *Arabidopsis* involves some floral activators such as CONSTANS (CO) and GIGANTEA (GI) and a circadian clock protein, EARLY FLOWERING 3 (ELF3). In this work, we examined the effect of ultra-short photoperiod (USP) on flowering time of three accessions (L*er*, Col and Ws). All the wild-type plants tested showed remarkable delay of flowering under 3 h or less photoperiods, but different sensitivity to the USP was found in these accessions. Late flowering phenotype of plants with mutations in genes involved in the three major pathways was enhanced under the USP. Expression of *FLOWERING LOCUS T* (*FT*) encoding a florigen decreased under the USP. A mutation in the ELF3 gene in L*er* largely suppressed the delay of flowering under the USP. These results suggest that floral regulation pathway under the USP may be independent of the three well-characterized pathways. ELF3 may play a key role in the USP pathway.

Key words: CCA1, circadian clock, ELF3, FLC, FT, LHY, photoperiodic flowering.

Biological clocks have been shown to mediate the responses of physiological and molecular processes to diurnal changes in environmental conditions such as light quality and quantity, temperature, and humidity. Even in the absence of any environmental time cue, circadian rhythms persist with a period close to 24 h and are generated by an endogenous timing mechanism. The basic principles of circadian clocks have been well studied in many model organisms, including cyanobacteria, *Neurospora*, *Arabidopsis*, mice, and human (Bell-Pedersen et al. 2005; Young and Kay 2001).

In *Arabidopsis*, EARLY FLOWERING 3(ELF3), GIGANTEA (GI), LATE ELONGATED HYPOCOTYL (LHY), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), PSEUDO RESPONSE REGULATOR 1 (PRR1, also known as TOC1), PRR3, PRR5, PRR7, PRR9 and other many proteins play key roles in the control of circadian rhythms (Alabadí et al. 2001, 2002; Mizoguchi et al. 2002).

A transition from the vegetative phase to reproductive phase is called flowering. Flowering time is controlled at least by three distinct pathways, the long-day (LD)/photoperiod pathway, gibberellic acid (GA) pathway and autonomous/vernalization pathway. The LD/photoperiod pathway is largely affected by circadian clock. Two genes GIGANTEA (GI) and CONSTANS (CO) promote flowering in the LD/photoperiodic pathway. GAI and FCA are floral activators of the GA and autonomous/vernalization pathways, respectively. FLOWERING LOCUS T (FT) is a floral hormone, florigen and FT functions a common downstream target of all the three flowering pathways.

For example, mutations in the circadian clock genes *LHY* and *CCA1* (*lhy*;*cca1*) accelerates flowering both under SD and LD (Mizoguchi et al. 2002, 2005), but delays under continuous light (LL; Fujiwara et al. 2008; Yoshida et al. 2009). By contrast, *elf3* flowers earlier than wild-type under SD, LD and LL, and flowering time of *elf3* is almost constant under the photoperiods with 24 h cycles. There have been no reports on environmental conditions that delay flowering time of *elf3*.

Many organisms have internal rhythms with both

Abbreviations: Arabidopsis, *Arabidopsis thaliana*; CCA1, CIRCADIAN CLOCK ASSOCIATED 1; CO, CONSTANS; Col, Columbia; ELF3, EARLY FLOWERING 3; GI, GIGANTEA; L*er*, Landsberg *erecta*; LHY, LATE ELONGATED HYPOCOTYL; LL, continuous light; USP, ultra-short period.

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much shorter and longer periods compared to that of circadian rhythm. These are called ultradian and infradian rhythms, respectively. Ultradian rhythms are recurrent periods repeated throughout a day of 24 h. Ultradian rhythms in sleep, heart rate, thermoregulation, urination, appetite, blood circulation, pulse and hormonal release in human have been well studied. In plants, leaf movement and rotation of root show ultradian rhythms. Compared to molecular mechanisms underlying circadian rhythms, those of ultradian rhythms have not been elucidated. Mutations that disrupt the ultradian rhythms have not been identified even in model organisms.

Circadian clocks of most organisms can be entrained by external stimuli with periods of approximate 17–32 h. Sometimes organisms including plants grown under short cycles of light and dark do not entrain to these ultra short photoperiod (USP) or free-run but instead remain entrained to a 24 h cycle. This occurs when the USP is a multiple of the internal rhythm. For example, it might occur when the USP is light/dark cycles of a frequency of 12 (1L1D), 6 (2L2D), 4 (3L3D), 3 (4L4D), or 2 (6L6D) cycles per 24 h. This phenomenon is called "frequency demultiplication".

In 1931, Garner and Allard reported that such USP with 6 h cycles and less had similar effects of LD and LL on LD and SD plants. In other words, flowering times of the LD and SD plants were almost unaffected and delayed, respectively, under the USP of their conditions. Molecular mechanisms underlying the alteration of flowering time under the USP have not been elucidated. However, here we report that flowering times of three wild-type accessions (Landsberg *erecta*, L*er*; Columbia, Col; Wassileskija, Ws) of *Arabidopsis thaliana* were significantly delayed under the USP with 6, 4 and 2 h cycles. The USP delayed flowering times of plants with mutations in GI or CO that are key floral activators of the LD pathway, indicating that the USP did not simply affect flowering through the photoperiod pathway. The USP had similar effects on other two major pathways for flowering time, the GA and autonomous pathways. The late flowering phenotype caused by the USP was largely suppressed by *elf3* mutation in L*er* background. Our results suggest that the USP pathway might be a novel pathway distinct from the three major pathways, or affect common components of them. ELF3 appears to play a key role in the USP pathway.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana accessions Landsberg *erecta* (L*er*), Columbia (Col) and Wassileskija (WS) were used as the wild-type (WT) plants. The mutant lines *co-2*, *gi-3*, *fca-1*, *gai* (L*er*; Koornneef et al. 1991), *elf3-101* (L*er*; Yoshida et al. 2009), *elf3-1* (Col; Zagotta et al. 1996), *hy3-1* (L*er*; Koornneef et al. 1980) and *lhy-12*;*cca1-101* (L*er*; Fujiwara et al. 2008) were described previously. Seeds were imbibed and cold-treated at 4°C for 3 days in darkness before germination under light. Plants were grown in controlled-environment chambers at 22°C. The light conditions were 24 h light (LL), 18 h light/6 h dark (LD), 3 h light/3 h dark (3L3D), 2 h light/2 h dark (2L2D) and 1 h light/1 h dark (1L1D) with a photon flux density of about 40 μmol m^{-2} s^{-1}.

Measurement of flowering time

Flowering time was scored by growing plants on soil under LD and LL and counting the number of rosette and cauline leaves on the main stem after bolting. Data are presented as means ± S.E. (n=8). Flowering time was measured at least twice, with similar results.

Preparation of RNA and semiquantitative RT-PCR

Plants of wild-type (L*er*) were grown on soil under 16 h light/8 h dark (LD) and 1 h light/1 h dark (1L1D) for 14 days. Aerial parts were used for RNA preparation. RT-PCR was performed with 1 μg total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). cDNA was diluted to 100 μl with TE buffer, and 1 μl diluted cDNA was used for PCR amplification by TaKaRa Extaq (TaKaRa, Shiga, Japan). The primer sequences used were as follows: *FT* forward, 5′-ACA ACT GGA ACA ACC TTT GGC AAT G-3′, *FT* reverse, 5′-ACT ATA TAG GCA TCA TCA CCG TTC GTT ACTCG-3′; *FLC* forward, 5′-TTA GTA TCT CCG GCG ACT TGA ACC CAA ACC-3′, *FLC* reverse, 5′-AGA TTC TCA ACA AGC TTC AAC ATG AGT TCG-3′; *TUB* forward, 5′-CTC AAG AGG TTC TCA GCA GTA-3′, *TUB* reverse: 5′-TCA CCT TCT TCA TCC GCA GTT-3′. The cycles used for amplification were as follows: 38 cycles for *FT*; 35 cycles for *FLC*; and 25 cycles for *TUB*. The PCR products were separated on 1.5% agarose gels, and expression was quantified using a Bio-Rad Molecular Imager (Molecular Imager Fx, 1998, Bio-Rad Laboratories Inc.). The data are represented relative to the maximum value among all data sets after normalization to the *TUB* control. RT-PCR analyses were performed at least twice and usually with independent RNA samples. Similar results were obtained from two experiments.

Results

Wild-type plants showed late-flowering phenotype under ultra-short photoperiod (USP)

To examine the effects of ultra-short photoperiod (USP) on flowering of three wild-type accessions (Landsberg *erecta*, L*er*; Columbia, Col; Wassileskija, WS), these plants were grown under 3 h light/3 h dark (3L3D), 2 h light/2 h dark (2L2D) and 1 h light/1 h dark (1L1D). Photoperiodic conditions of 24 h light (LL) and 16 h light/8 h dark (LD) were also used as controls. Number of cauline (CL) and rosette (RL) leaves when they

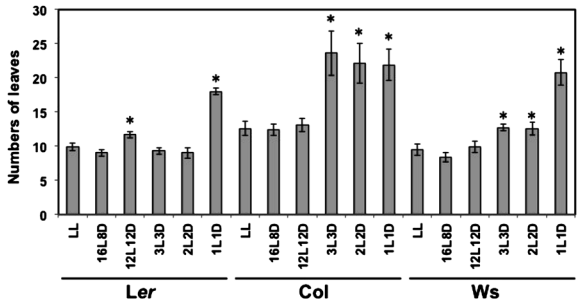

Figure 1. Wild-type plants showed late-flowering phenotype under the USP. Wild-type plants of three accessions (Landsberg *erecta*; L*er*, Columbia; Col, Wassileskija; WS) were grown under 24 h light (LL), 16 h light/8 h dark (LD), 3 h light/3 h dark (3L3D), 2 h light/2 h dark (2L2D) and 1 h light/1 h dark (1L1D). Number of cauline (CL) and rosette (RL) leaves when they flowered were scored. Data are presented as mean±SE ($n=8$). Asterisks (*) represent statistical significance compared to values of the plants grown under LD (Student's *t*-test, $p<0.05$).

flowered were scored and flowering times were compared under various photoperiodic conditions (Figure 1). All the wild-type plants tested showed remarkable delay of flowering under 3 h or shorter photoperiods.

A different sensitivity to the USP was found in these three accessions. Flowering time of wild-type (L*er*) was delayed only under 1L1D. By contrast, wild-type (Col) showed late flowering under all of three USP conditions. Sensitivity of wild-type (WS) appeared to be intermediate between L*er* and Col plants.

Late-flowering phenotype of mutants of the three major flowering pathways was enhanced under the USP

To test whether the USP pathway was common to the photoperiod or long-day (LD) pathway, autonomous/vernalization pathway, gibberellic acid (GA) pathway, plants with mutations in genes involved in the three major pathways were grown under the USP and control conditions. CO and GI play key roles in the LD pathway as floral activators. Loss-of-function of either *CO* or *GI* resulted in late flowering phenotype under LD and LL but not under SD. FCA is a floral activator of the autonomous pathway and GAI is a floral repressor of the GA pathway. The *gai* mutation stabilized GAI protein and the gai mutation slightly delayed flowering under LD and LL.

Late flowering phenotype of these mutants was enhanced under the USP (Figure 2), suggesting that the USP pathway is, at least in part, independent of the LD, autonomous and GA pathways.

The USP pathway decreased FT expression without affecting FLC expression

We next analyzed the expression level of flowering-time genes in two accessions of wild-type (L*er* and Col) under the USP (1L1D) and LD (16L8D). Consistent

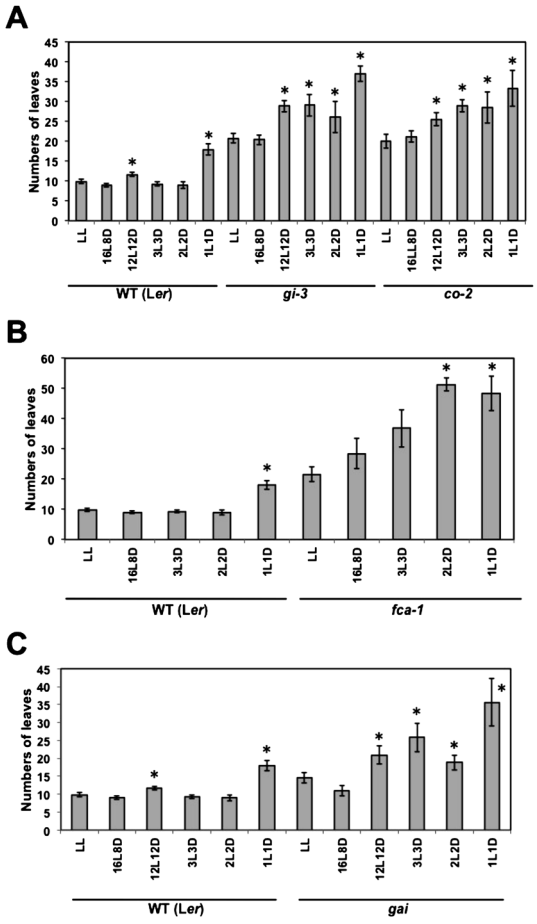

Figure 2. Late flowering phenotype of mutants of the three major floral pathways was enhanced under the USP. Mutants of the long-day pathway (A), autonomous pathway (B), and gibberellic acid pathway (C) were grown under 24 h light (LL), 18 h light/6 h dark (LD), 3 h light/3 h dark (3L3D), 2 h light/2 h dark (2L2D) and 1 h light/1 h dark (1L1D). Number of cauline (CL) and rosette (RL) leaves when they flowered were scored. Data are presented as mean±SE ($n=8$). Asterisks (*) represent statistical significance compared to values of the plants grown under LD (Student's *t*-test, $p<0.05$).

with the delayed-flowering phenotype, *FT* mRNA levels were markedly lower under the USP than LD condition (Figure 3). The USP did not affect the mRNA level of *FLC*, a gene that functions as a repressor of *FT* expression in the autonomous/vernalization pathway. Dark green leaves, semi-dwarf phenotype and decrease of germination efficiency are typical phenotypes observed in mutants with loss-of-function of genes involved in either biosynthesis or signaling of GA. The USP did not affect germination efficiency and cause semi-dwarf phenotype (data not shown). These results suggest that the decrease of the *FT* mRNA level by the USP might be independent of the 3 major flowering-pathways.

The elf3 mutations suppressed the late-flowering phenotype of wild-type under the USP

To explore the molecular mechanisms underlying the USP-dependent late flowering of wild-type plants,

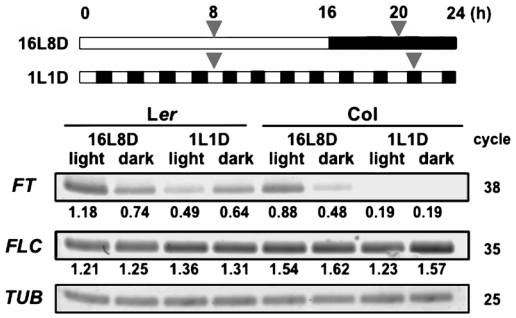

Figure 3. Analysis of *FT* and *FLC* mRNA levels in wild-type (L*er*) grown under 16 h light/8 h dark (LD) and 1 h light/1 h dark (1L1D). Abundance of *FT* and *FLC* mRNA is shown. The plants were grown under LD and 1L1D cycles for 14 days. White and black boxes along the horizontal axis represent light and dark periods, respectively. Dark triangles indicate the sampling time. Quantification was performed as described in the Materials and methods section.

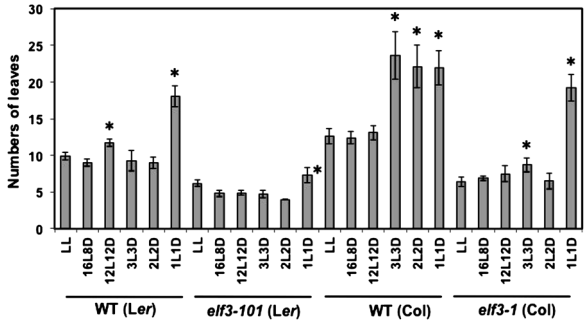

Figure 4. Flowering time of *elf3-101* was almost unaffected by the 1L1D USP. Plants of wild-type (L*er*), *elf3-101* (L*er*), wild-type (Col) and *elf3-1* (Col) were grown under 24 h light (LL), 16 h light/8 h dark (LD), 3 h light/3 h dark (3L3D), 2 h light/2 h dark (2L2D) and 1 h light/1 h dark (1L1D). Number of cauline (CL) and rosette (RL) leaves when they flowered were scored. Data are presented as mean±SE (n=8). Asterisks (*) represent statistical significance compared to values of the plants grown under LD (Student's t-test, $p<0.05$).

plants with mutations in genes that act in the control of flowering time and circadian rhythms were grown under the USPs. *elf3-101* (L*er*) suppressed the late-flowering phenotype of wild-type (L*er*) under all the USPs tested (Figure 4). The late-flowering phenotype of wild-type (Col) under 3L3D and 2L2D but not 1L1D was suppressed by *elf3-1* (Col), suggesting that there might be natural variation(s) of sensitivity to the USP between L*er* and Col. *hy3-1* (*phyB*; L*er*) showed a similar level of early-flowering phenotype to that of *elf3-101* (Ler) under LL and LD. Therefore, the *hy3-1* was also tested as a control (Figure 5). The *hy3-1* did not largely suppress the late-flowering phenotype of wild-type (L*er*) under 1L1D (Figure 4). Alteration of rhythmic expressions of oscillator or clock-controlled genes might be responsible for the delay of flowering of wild-type plants. However, flowering time of *gi* (Figure 2A) and *lhy*;*cca1* (Figure 6) was delayed under 1L1D, indicating that the delay of flowering under the USP would not be simply explained by a change of expression pattern of clock genes. These

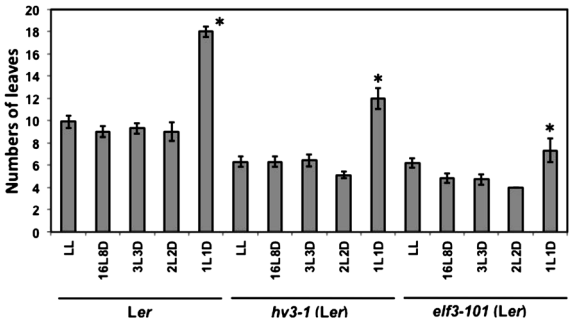

Figure 5. Delay of flowering was not largely affected by *hy3-1* (L*er*) under the 1L1D USP. Plants of wild-type (L*er*), *hy3-1* (L*er*) and *elf3-101* (L*er*) were grown under 24 h light (LL), 16 h light/8 h dark (LD), 3 h light/3 h dark (3L3D), 2 h light/2 h dark (2L2D) and 1 h light/1 h dark (1L1D). Number of cauline (CL) and rosette (RL) leaves when they flowered were scored. Data are presented as mean±SE (n=8). Asterisks (*) represent statistical significance compared to values of the plants grown under LD (Student's t-test, $p<0.05$).

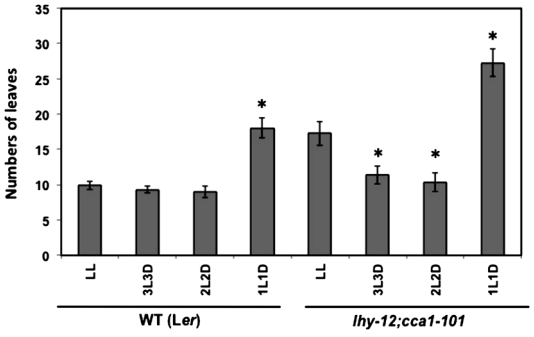

Figure 6. Delay of flowering was enhanced by *lhy-12*;*cca1-101* (L*er*) under the 1L1D USP. Plants of wild-type (L*er*) and *lhy-12*;*cca1-101* (L*er*) were grown under 24 h light (LL), 3 h light/3 h dark (3L3D), 2 h light/2 h dark (2L2D) and 1 h light/1 h dark (1L1D). Number of cauline (CL) and rosette (RL) leaves when they flowered were scored. Data are presented as mean±SE (n=8). Asterisks (*) represent statistical significance compared to values of the plants grown under LL (Student's t-test, $p<0.05$).

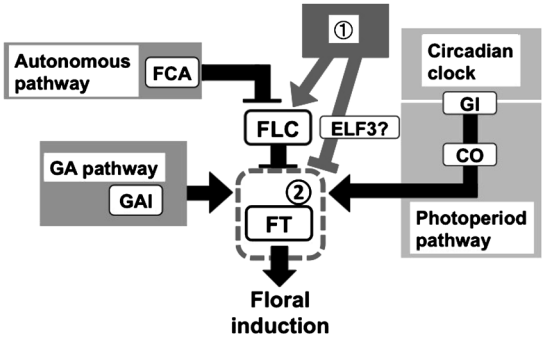

Figure 7. A hypothetical model of floral regulation under the USP. Our results suggest that the USP may regulate flowering independently of the three well-characterized pathways, the Photoperiod, Autonomous and GA pathways (pathway 1). Alternatively, key factors for the USP are likely to function in a hatched box 2. ELF3 was required for the late flowering of Arabidopsis plants under the USPs such as 3L3D, 2L2D and 1L1D (Figure 4). ELF3 may play a key role in mediating between the USP and *FT* expression.

results suggest that ELF3 might be one of key factors involved in the USP pathway (Figure 7).

Discussion

In this work, we tested the effects of artificial photoperiods with short alternations of light and dark. During our studies, we noticed that effects of abnormally long and short alternations of light and darkness on growth and development of a variety of plant species were investigated by Garner and Allard more than 80 years ago (Garner and Allard 1931). The plants involved the long-day, indifferent (day-neutral) and short-day types. One of their primary purposes of these experiments was to find an artificial condition with abnormal photoperiods instead of 12-h alternation for better growth of plants. Flowering time was one of the parameters they tested. Their first experiment in the study involved alternations of light and darkness of 6 h in some instances and 4 h in others, as compared with a 12-h alternation. Flowering times of the Rudbeckia bicolor (long-day type), Manarin soybeans (indifferent type), Peking soybeans (short-day type) and Biloxi soybeans (short-day type) under the alternations of 6 or 4 h were much delayed compared to those under 12 h as a control.

Consistent with their pioneering study, we found that flowering time of a model plant, *Arabidopsis thaliana*, was much delayed under the USPs in the modern experimental conditions (Figures 1–6). So far, we have not identified factors responsible for the different sensitivity of response to the USP among ecotypes (Figures 1, 4). For example, natural variations of sensitivity to the USP among *elf3* in L*er*, Col and Ws ecotypes can be mapped to certain regions of 5 chromosomes in Arabidopsis. This genetic analysis will be helpful to understand a molecular mechanism underlying the different sensitivity of response to the USP among ecotypes. In this work, we revealed an effect of the USPs on the control of flowering via *FT* expression (Figure 3). Actually, we are interested in photoreceptors involved in the UPS pathway. To identify them, effects of the USP with red, far-red and blue light on wild-type and clock-mutants should be tested in the next step. Then we should examine effects of loss- and gain-of-function of the photoreceptor genes on plants. Flowering time of the *hy3-1* (*phyB*) was significantly delayed under 1L1D (Figure 5), suggesting that one of the photoreceptors, Phytochrome B, is unlikely to play a major role in the USP pathway. GI, LHY and CCA1 play key roles in maintaining circadian rhythms in Arabidopsis. Flowering time of *gi* and *lhy*;*cca1* was delayed under 1L1D, suggesting that such clock genes are unlikely responsible for the late flowering phenotype of wild-type plants under 1L1D. However, we are very interested in circadian rhythms under 1L1D and are planning to test them using Arabidopsis plants with *CAB::LUC* or *CCR::LUC*.

We propose that the USP can regulate flowering independently of the three well-characterized pathways, the LD, autonomous/vernalization and GA pathways (pathway 1, Figure 7). Alternatively, our results suggest that key factors for the USP are likely to function in a hatched box 2 in Figure 7. ELF3 was required for the late flowering of Arabidopsis plants under the USPs such as 3L3D, 2L2D and 1L1D (Figures 4, 7). ELF3 may play a key role in mediating between the USP and *FT* expression (Figure 7).

Acknowledgements

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