

WEREWOLF, a Regulator of Root Hair Pattern Formation, Controls Flowering Time through the Regulation of *FT* mRNA Stability^{1[C][W][OA]}

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A key floral activator, *FT*, integrates stimuli from long-day, vernalization, and autonomous pathways and triggers flowering by directly regulating floral meristem identity genes in *Arabidopsis* (*Arabidopsis thaliana*). Since a small amount of *FT* transcript is sufficient for flowering, the *FT* level is strictly regulated by diverse genes. In this study, we show that *WEREWOLF* (*WER*), a MYB transcription factor regulating root hair pattern, is another regulator of *FT*. The mutant *wer* flowers late in long days but normal in short days and shows a weak sensitivity to vernalization, which indicates that *WER* controls flowering time through the photoperiod pathway. The expression and double mutant analyses showed that *WER* modulates *FT* transcript level independent of *CONSTANS* and *FLOWERING LOCUS C*. The histological analysis of *WER* shows that it is expressed in the epidermis of leaves, where *FT* is not expressed. Consistently, *WER* regulates not the transcription but the stability of *FT* mRNA. Our results reveal a novel regulatory mechanism of *FT* that is non cell autonomous.

As for other plants, the correct timing of flowering is essential for reproductive success in *Arabidopsis* (*Arabidopsis thaliana*). Flowering time in *Arabidopsis* is regulated by complex genetic networks monitoring various environmental and endogenous signals. Four major genetic pathways for these signals have been revealed: the photoperiod and the vernalization pathways responding to environmental stimuli; and the autonomous and the GA-dependent pathways monitoring internal conditions (Mouradov et al., 2002; Simpson and Dean, 2002; Boss et al., 2004). These pathways are converged on common downstream target genes, *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), and *LEAFY* (*LFY*), so-called flowering pathway integrators (Simpson and Dean, 2002). Flowering time in *Arabidopsis* is quantitatively controlled by the transcript level of these integrators (Kardailsky et al., 1999; Kobayashi et al., 1999;

Blázquez and Weigel, 2000; Onouchi et al., 2000; Samach et al., 2000; Moon et al., 2003, 2005).

FT, encoding a small approximately 20-kD protein with homology to Raf kinase inhibitor protein, is one of the key floral activators integrating multiple floral inductive pathways (Kardailsky et al., 1999; Kobayashi et al., 1999). *FT* promotes the transition to flowering by activating other floral integrator, *SOC1*, and floral meristem identity genes such as *APETALA1* (*API*), *FRUITFULL*, *CAULIFLOWER*, and *SEPALLATA3* (Ruiz-García et al., 1997; Abe et al., 2005; Teper-Bamnolker and Samach, 2005; Wigge et al., 2005; Yoo et al., 2005). Recently, many laboratories have shown that *FT* protein produced in the leaf phloem moves to the shoot apex and executes its role through the interaction with the bZIP transcription factor *FD*, which is expressed in the shoot apex (Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007). Consequently, *FT* protein is considered a graft-transmissible florigen or at least a component of the floral stimuli.

Although the abundance of *FT* transcripts in the wild type is so low as not to be detected by in situ hybridization, *FT* overexpression plants or loss-of-function alleles show dramatic changes in flowering time (Koornneef et al., 1991; Kardailsky et al., 1999; Kobayashi et al., 1999). This suggests that a small amount of *FT* is sufficient for flowering in wild-type plants, and inappropriate *FT* expression causes disorder in flowering time. Hence, *FT* expression is regulated strictly by a diverse range of regulators. *CONSTANS* (*CO*) directly activates *FT* through the photoperiod pathway (Kobayashi et al., 1999; Samach et al., 2000; Suárez-López

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et al., 2001; Valverde et al., 2004). *CO* expression, of which transcription arises around 12 h after dawn and stays high until the following dawn, is activated in both long days and short days (Suárez-López et al., 2001). However, *CO* mRNA expressed at night does not cause the activation of *FT*, because *CO* protein is degraded in the dark (Valverde et al., 2004). Therefore, flowering is delayed in short days due to the absence of *FT*. In contrast, mutations in genes involved in the photoperiod pathway, such as *gi*, *co*, and *ft*, delay flowering only in long days but not in short days, since the signal from the photoperiod pathway is mainly mediated through *FT* (Koornneef et al., 1991; Suárez-López et al., 2001).

The MADS box transcription factor *FLOWERING LOCUS C (FLC)*, a central floral repressor in autonomous and vernalization pathways, represses *FT* expression. *FT* expression repressed by *FLC* in winter annuals is important because this repression prevents flowering until the following spring (Searle et al., 2006). Another negative flowering repressor, *SHORT VEGETATIVE PHASE (SVP)*, regulates *FT* expression through ambient temperature signaling in the thermosensory pathway (Lee et al., 2007b). Recently, it has been reported that *SVP* acts in a repressor complex together with *FLC* protein, and this complex binds directly to the *CArG* box DNA motifs in the first intron of the *FT* gene (Helliwell et al., 2006; Lee et al., 2007b; Li et al., 2008). Additionally, the chromatin-associated proteins, *TERMINAL FLOWER2* and *EARLY BOLTING IN SHORT DAYS*, repress *FT* transcription by direct binding in *FT* chromatin (Piñeiro et al., 2003; Takada and Goto, 2003). In addition, *CURLY LEAF* and *FERTILIZATION INDEPENDENT ENDOSPERM*, the subunits of Arabidopsis Polycomb Repressive Complex2, strongly repress *FT* during vegetative development (Jiang et al., 2008). Many other genes, such as *PHYTOCHROME AND FLOWERING TIME1 (PFT1)*, *PHYTOCHROME-INTERACTING FACTOR3*, and *TEMPRANILLO* genes (*TEM1* and *TEM2*), which are involved in light signaling and circadian rhythm, are also known to be involved in *FT* regulation (Cerdán and Chory, 2003; Oda et al., 2004; Castillejo and Pelaz, 2008). Although various functions of upstream genes for *FT* regulation are revealed, little is known about the posttranscriptional regulation of *FT* mRNA.

In this study, we show that *WEREWOLF (WER)*, known as a regulator of root hair patterning, is involved in posttranscriptional regulation of *FT*. *WER*, which is classified in the same subgroup with *GLABROUS1 (GL1)* and *AtMYB23 (MYB23)*, encodes an R2R3 MYB transcription factor (Lee and Schiefelbein, 1999; Stracke et al., 2001). In Arabidopsis, root epidermal cells differentiate into either root hair cells or hairless cells in a position-dependent manner: epidermal cells between two cortical cells differentiate into root hair cells (called H-cells), whereas epidermal cells in contact with a single cortical cell usually become hairless cells (called N-cells; Dolan et al., 1994). *WER* is highly expressed in N-cells, whereas it is suppressed in H-cells (Lee and Schiefelbein, 1999; Kwak et al.,

2005; Kwak and Schiefelbein, 2007). In N-cells, *WER* protein forms a transcriptional complex with a WD40 protein, *TRANSPARENT TESTA GLABRA1 (TTG1)*, and a bHLH transcription factor, *GLABRA3 (GL3)*, which functions redundantly with *ENHANCER OF GLABRA3 (EGL3)*. This complex positively regulates *GL2*, which inhibits the generation of root hair and thus makes cells differentiate into N-cells (Lee and Schiefelbein, 1999; Walker et al., 1999; Payne et al., 2000; Bernhardt et al., 2003; Zhang et al., 2003; Koshino-Kimura et al., 2005).

Here, we report the late-flowering phenotype of the *wer* mutant, which has been previously reported to have hairy roots. Delayed flowering occurred in long days but not in short days; thus, *wer* can be classified as a photoperiod pathway mutant. The transcript level of *FT* was reduced in the *wer* mutant in long days, which was independent of *CO* and *FLC*. In addition, such a decrease of *FT* transcript level is due not to the altered transcription but to the reduced stability of mRNA. This study suggests that *WER* in epidermis modulates the *FT* transcript level in phloem through a novel mechanism.

RESULTS

WER Regulates Flowering Time through the Photoperiod Pathway

While exploring if *WER* acts in other developmental processes, we observed that the flowering time of *wer* loss-of-function mutant and *WER* overexpression transgenic plants is changed. The *wer-1* allele in the Columbia (Col) background and the *wer-3* allele in the Wassilewskija (Ws) background have nonsense mutations in the region of the second MYB domain; thus, both alleles apparently produce nonfunctional proteins (Lee and Schiefelbein, 1999). Under long-day (16 h of light/8 h of dark) conditions, both *wer-1* and *wer-3* plants produced more rosette leaves than the wild type at the time of bolting (Fig. 1A). In contrast, the transgenic plants containing *WER* genomic DNA under the control of the strong cauliflower mosaic virus 35S promoter (*35S::WER*) flowered earlier than the wild type (Fig. 1A). To verify that the late-flowering phenotype of *wer-1* was caused by the loss of *WER* function, the *WER* genomic construct containing a 5-kb *WER* genomic fragment that includes a 2.5-kb upstream sequence was transformed into *wer-1*. Most of the resulting transformants showed comparable flowering time to the wild type, indicating that late flowering is caused by the loss of *WER* (Fig. 1B). In addition, the heterozygous lines obtained from the cross between Col and *wer-1* showed similar flowering time with Col, confirming that *wer* is a recessive late-flowering mutant (Supplemental Fig. S1).

The flowering time in Arabidopsis is regulated by four major pathways: photoperiod, vernalization, autonomous, and GA pathways (Boss et al., 2004; Bäurle

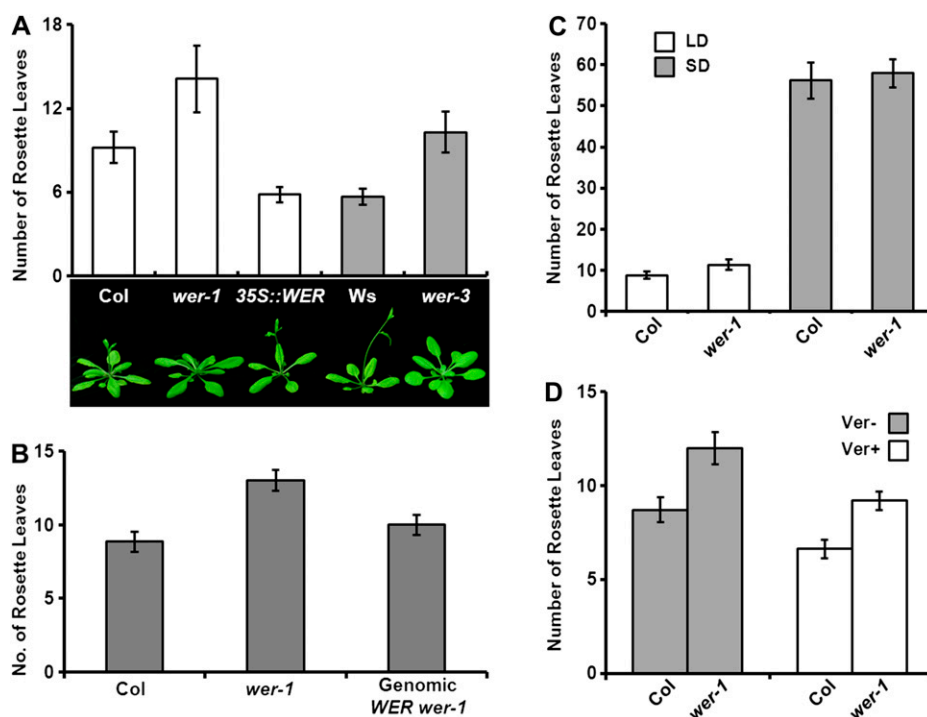


Figure 1. Flowering time of *wer* loss-of-function mutants and *WER* overexpression transgenic plants. A, Comparison of flowering time in wild-type, *wer* mutant, and *35S::WER* transgenic plants under long days. White bars show the flowering time of *wer-1* and *35S::WER* compared with that of Col, whereas gray bars show the flowering time of *wer-3* compared with that of *Ws*. Twenty-five plants were used to measure the flowering time, and the error bars represent sd. Photographs display the phenotype of each plant when *wer-1* or *wer-3* initiated its flowering. B, Complementation analysis of *wer-1*-inserted *WER* genomic DNA. Thirty-six plants were used to measure the flowering time in long days, and the error bars represent sd. C, Flowering time of *wer-1* mutants compared with Col under long days and short days. At least 30 plants were used to measure the flowering time, and the error bars represent sd. D, Flowering time of Col and *wer-1* grown in long days after 0 weeks (Ver-) or 8 weeks (Ver+) of vernalization treatment. At least 25 plants were used to measure the flowering time, and the error bars represent sd. [See online article for color version of this figure.]

and Dean, 2006; Oh and Lee, 2007). To determine in which pathway *WER* regulates flowering, the flowering characteristics of *wer* were checked in response to photoperiod and vernalization. Unlike in long days, *wer-1* flowered similarly to the wild type in short days (Fig. 1C). After 8 weeks of vernalization treatment, all genotypes showed acceleration of flowering compared with the nonvernalization treated control. Although *wer-1* showed slight acceleration of flowering, the responsiveness was weaker than in the wild type (Fig. 1D). These observations demonstrated that loss of function in *WER* results in a delay of flowering only under long days and weak sensitivity to vernalization, which is similar in flowering characteristics to the photoperiod pathway mutants *co* and *gi* (Koornneef et al., 1991).

WER Expression in Root Does Not Affect the Regulation of Flowering Time

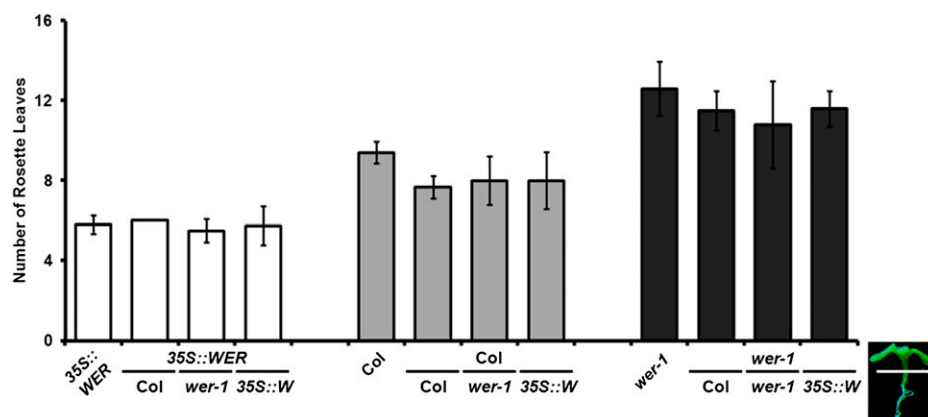
Floral evocation occurs in the shoot apex by inducing floral initiation genes such as *API* and *LFY* (Weigel and Nilsson, 1995; Hempel et al., 1997). However, it

was previously reported that *WER* is expressed mainly in roots (Lee and Schiefelbein, 1999). To determine whether a root-derived signal induced by *WER* affects flowering time, graft chimeras among *wer-1*, *35S::WER*, and the wild type were produced by a transverse-cut grafting method described before (Turnbull et al., 2002). All grafts were denoted as scion/rootstock genotypes. Self-grafted plants, in which the scion and rootstock were from the same genotype, were also produced as controls. These plants appeared to flower slightly earlier than ungrafted plants, probably due to mechanical stress (Fig. 2). The results showed that grafting failed to rescue the late flowering of *wer-1* scions regardless of whether Col or *35S::WER* was used as rootstock. All grafted plants showed similar flowering time as the plants used as scion (Fig. 2). These results suggest that *WER* expressed in root does not affect flowering time.

Expression of WER

To examine the tissue expression pattern of *WER*, reverse transcription (RT)-PCR analysis was per-

Figure 2. Flowering time of grafts among Col, *wer-1*, and *35S::WER*. The graft type is a 90° transverse-cut graft using 5-d-old seedlings grown in long days, shown in the photograph on the right side. Plants on the left in each panel are ungrafted controls. Genotype notation for the stock is shown below the lines at bottom, and that of the scion is shown above. [See online article for color version of this figure.]



formed with total RNA extracted from various tissues. Although *WER* was highly expressed in roots, as reported previously (Lee and Schiefelbein, 1999), transcripts were also detectable in young leaves, shoot apices, adult rosette leaves, stems, and inflorescences, including floral buds and mature flowers (Fig. 3A).

To inspect the spatial pattern of *WER* expression, *WERp::GUS* transgenic plants, in which the *GUS* reporter is driven by the *WER* promoter with a 4-kb DNA fragment upstream of the *WER* coding sequence, were used for histochemical *GUS* staining. This transgenic line was used before for the spatial expression analysis because *GUS* staining faithfully followed the endogenous expression in roots (Lee and Schiefelbein, 1999). In 6-d-old seedlings, *GUS* expression was most notable in the hypocotyl and the shoot apex as well as in the root tip, whereas weak *GUS* expression was detected in the margin of the cotyledons (Fig. 3Ba). *GUS* was also detected in petiole, stem, stigma, and siliques (Fig. 3B, b–g). In shoot and root apices, *GUS* was detected from the embryo stage (Fig. 3Bh). Interestingly, *GUS* expression gradually disappeared while leaves matured (Fig. 3B, b and e). A detailed examination in the shoot apex by longitudinal sectioning of *WERp::GUS* revealed that *WER* is concentrated in the epidermis of leaf primordia (Fig. 3B, i and j). Immunohistochemistry using *WERp::MYC-WER* transgenic plants, in which *WER* protein fused with MYC epitope is driven by the *WER* promoter, showed that *WER* proteins are also expressed along the epidermis (Fig. 3Bk).

The temporal changes in *WER* transcript level were further determined by RT-PCR to investigate how *WER* expression is regulated during the flowering process. *WER* expression in the aerial part of seedlings peaked in 3 d; afterward, it was reduced and became steady under both long days and short days (Fig. 3C). Such temporal expression was not affected by photoperiod. Moreover, *WER* expression did not show any daily rhythm under long days (Fig. 3D).

When the vernalization effect on *WER* expression was checked in Col:*FRI*^{SF2} plants, which have a strong flowering response to vernalization, there was no

change (Fig. 3E). In addition, GA treatment did not change the expression level (Fig. 3F). These results indicate that the vernalization or GA-dependent pathway does not affect *WER* expression.

WER* Regulates *FT* Transcript Level Independent of *CO* and *FLC

WER expression was further studied in various flowering-time mutants to elucidate the possible involvement of *WER* in the previously known flowering pathways. Although *wer* showed flowering characteristics similar to the mutants of the long-day pathway, *WER* expression was unchanged in these mutants, such as *ft-1*, *co-101*, and *gi-2* (Fig. 4A). In addition, the expression was not affected by mutations in the autonomous pathway or *flc*, indicating that *WER* does not act downstream of the previously known flowering pathways.

To examine the molecular basis of the late flowering of *wer*, we checked the expression of genes in the long-day pathway or genes acting on the shoot meristem, such as *CO*, *FT*, *FD*, and *SOC1* (Fig. 4B). Real-time quantitative (q) RT-PCR showed that only *FT* expression was reduced in *wer* mutants under long days, whereas the levels of *CO*, *FD*, and *SOC1* were similar to the wild type. In short days, there was no difference between the wild type and *wer* in the transcript level of all four genes. Such a result is consistent with the flowering characteristics of *wer*, which is late flowering only under long days. It also strongly suggests that *WER* regulates *FT* independent of *CO*. The double mutant analyses also support this hypothesis, such that *35S::FT* completely suppresses but *35S::CO* partially suppresses the late-flowering phenotype of *wer-1* (Fig. 4, C and D). Double mutant analysis also shows that *flc* and *wer* are additive (Fig. 4F), suggesting that *WER* acts independent of *FLC*. It is noteworthy that the molecular basis of the early flowering of *35S::WER* was a little complicated, because ectopic overexpression of *WER* caused increases of both *FT* and *SOC1* (Fig. 4B). Thus, neither *ft* nor *soc1* mutation was epistatic to *35S::WER* (Fig. 4, C and E).

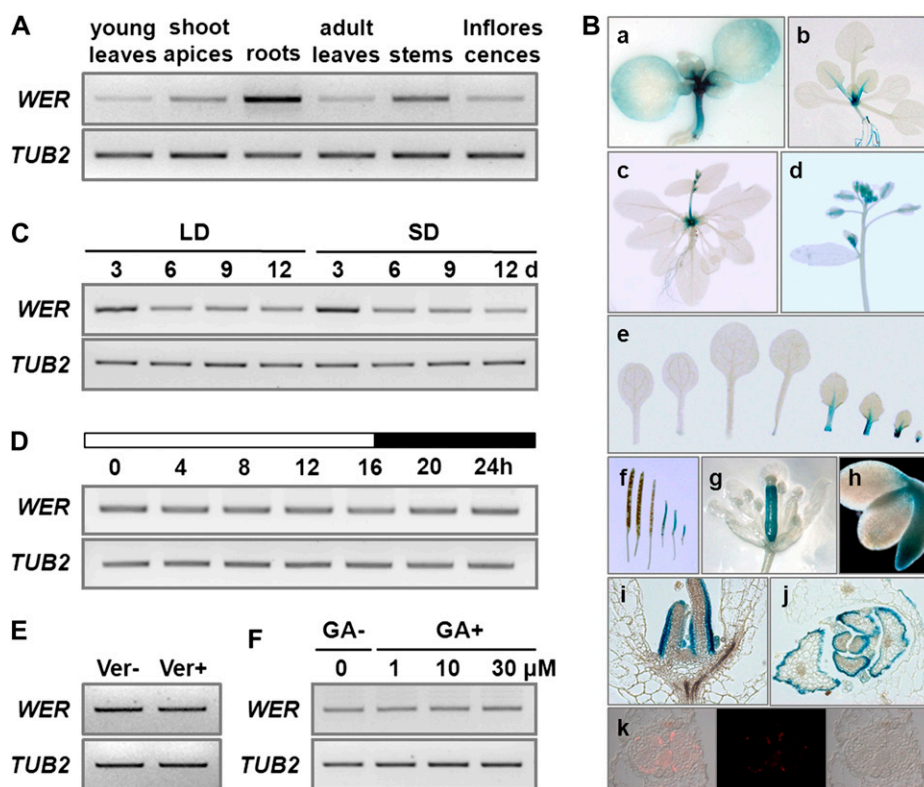


Figure 3. Expression of *WER*. **A**, RT-PCR analysis of *WER* in diverse tissue. RNAs of young leaves, shoot apices, and roots were isolated from 11-d-old Col seedlings grown in long days, while RNAs of rosette leaves, stems, and inflorescences were isolated from 28-d-old Col plants. *TUB2* was used as a quantitative control. **B**, Spatial expression patterns of *WER*. **a** to **i**, GUS staining in *WERp::GUS* transgenic plants: **a**, 6-d-old seedling; **b**, 12-d-old plant; **c**, 22-d-old plant; **d**, an inflorescence with flowers; **e**, leaves obtained from a 12-d-old plant (the leaves are shown in order of production from cotyledon at left); **f**, siliques of different stages; **g**, a mature flower; **h**, a mature embryo; **i**, longitudinal section. **j**, Transverse section through the shoot apex of a 7-d-old seedling. **k**, MYC-*WER* protein expression in *WERp::MYC-WER* transgenic plants, with immunohistochemical data obtained from a 6-d-old seedling. **C**, Temporal expression of *WER* detected by RT-PCR analysis. RNA was isolated from the shoot of Col plants grown for 3, 6, 9, and 12 d in both long days and short days. *TUB2* was used as a quantitative control. **D**, RT-PCR analysis of *WER* expression in Col plants during a 24-h cycle in long days. Shoot of 11-d-old seedlings was harvested every 4 h for RNA isolation. The zero time corresponds to right after dawn, and white or black boxes indicate light on or light off, respectively. *TUB2* was used as a quantitative control. **E**, Comparison of *WER* expression in Col:*FR1*⁶² grown for 11 d in long days with (Ver+) and without (Ver-) vernalization at 4°C for 8 weeks. *TUB2* was used as a quantitative control. **F**, RT-PCR analysis of *WER* expression with (GA+) or without (GA-) exogenous GA₃ treatment. *TUB2* was used as a quantitative control. The whole seedling was used for RNA extraction.

Because *FT* is known to be regulated by the circadian clock, we wondered whether the daily rhythm of *FT* is affected by *wer*. To address this, *FT* expression was analyzed every 4 h over a 24-h period under long days by RT-PCR. The result showed that the daily rhythm of *FT* expression is not affected by *wer*, although the amplitude is lower than in the wild type (Fig. 4, G and H).

wer Mutation Affects the *FT* mRNA Stability

Although *WER* is expressed in the epidermis (Fig. 3B, i and j), *FT* is expressed in the vasculature (Takada and Goto, 2003). Thus, it is not likely that *WER* directly regulates *FT*. However, it is still possible that *WER* acts non cell autonomously through mRNA or protein transport. To check if *wer* mutation affects the tran-

scription of *FT*, the expression of the *GUS* reporter gene driven by the *FT* promoter in *wer-1* was analyzed (Fig. 5A). The result showed that *GUS* expression level in *wer-1* was similar to that in Col, whereas endogenous *FT* level in *wer-1* was less than that in Col. Since initiation of transcription is achieved by binding of RNA polymerase II to the promoter DNA in eukaryotes, we checked if *wer* mutation affects the binding capacity of RNA polymerase II to the proximal region of the *FT* promoter by chromatin immunoprecipitation (ChIP) assay. In *wer-1* mutants, the enrichment of RNA polymerase II at the *FT* promoter was similar to that in Col, demonstrating that the *wer* mutation does not affect *FT* transcription (Fig. 5B). Unlike at the *FT* promoter, RNA polymerase II enrichment was reduced by *wer-1* approximately 40% at promoters of

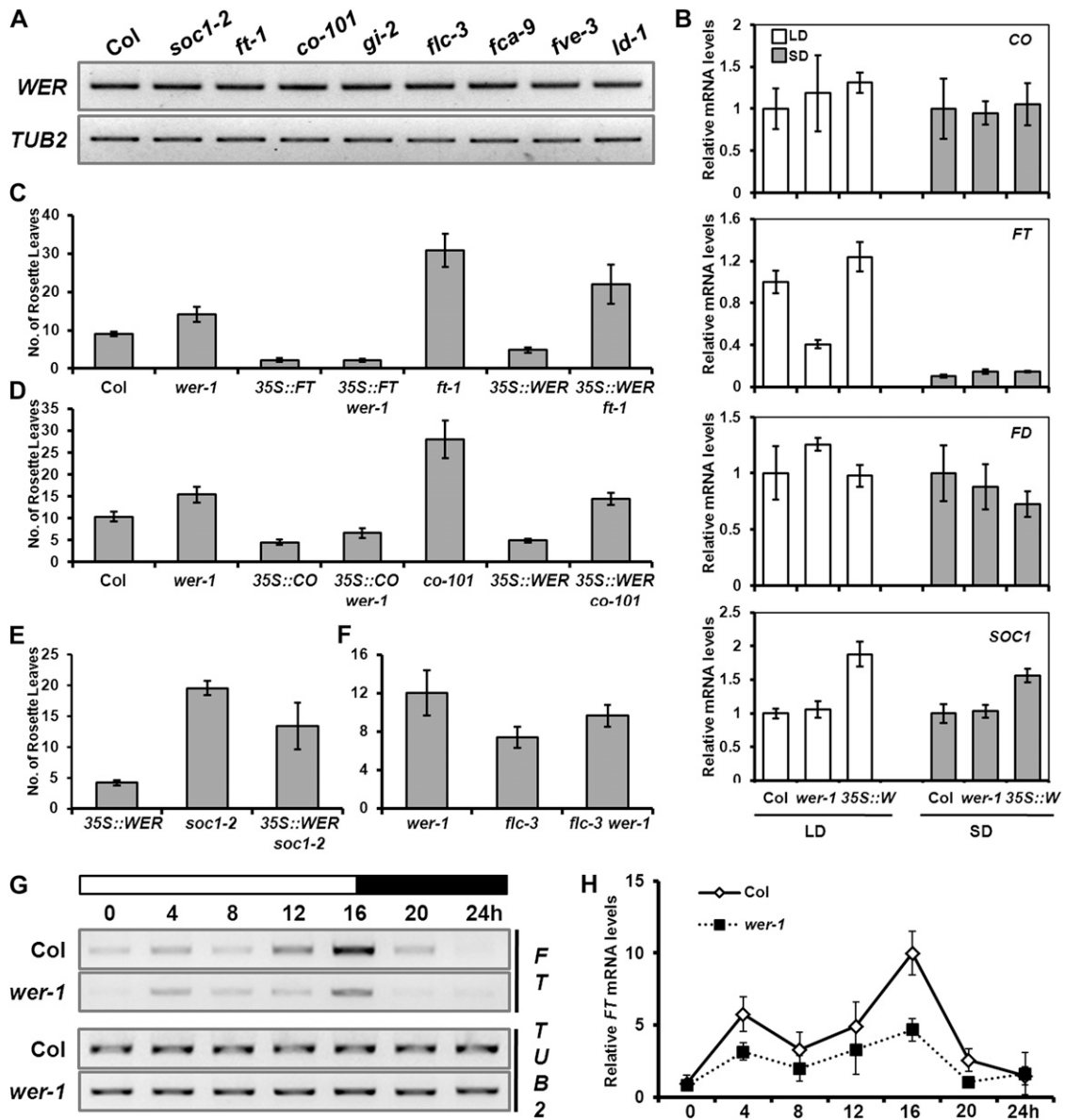


Figure 4. Regulation of flowering-time genes by *WER*. A, RT-PCR analysis of *WER* expression in various flowering-time mutants. For RNA isolation, shoot of seedlings grown for 11 d under long days was harvested. *TUB2* was used as a quantitative control. B, The expression of various flowering-time genes in Col, *wer-1*, and *35S::WER* was detected by qRT-PCR. White bars show gene expression in plants grown for 9 d under long days (LD), while gray bars show gene expression in plants grown for 21 d under short days (SD). The values and error bars represent means and sd, respectively, from three technical replicates. C, Flowering time of Col, *wer-1*, *35S::FT*, *35S::FT wer-1*, *35S::WER*, *ft-1*, and *35S::WER ft-1* grown in long days. D, Flowering time of Col, *wer-1*, *35S::CO*, *35S::CO wer-1*, *co-101*, *35S::WER*, and *35S::WER co-101* grown in long days. E, Flowering time of *35S::WER*, *soc1-2*, and *35S::WER soc1-2* grown in long days. F, Flowering time of *wer-1*, *flc-3*, and *flc-3 wer-1* grown in long days. G and H, Daily rhythm of *FT* in Col and *wer-1* plants grown under long days was detected by RT-PCR analysis (G) or calculated by ImageJ from three independent RT-PCR results (H). Nine-day-old seedlings grown under long-day conditions were harvested every 4 h for RNA isolation. The zero time corresponds to right after dawn, and white or black boxes indicate light on or light off, respectively.

GL2 and *AP1*, which are a direct target of *WER* and a downstream gene of *FT*, respectively. This suggests that the transcription rate of these two genes is reduced in *wer-1*. Taken together, these results strongly suggest that *FT* transcript level is reduced in *wer-1* by posttranscriptional regulation such as mRNA decay.

To directly assess whether the reduced *FT* mRNA level in *wer-1* was a result of altered mRNA stability, the half-life of *FT* mRNA was compared between the wild type and *wer-1* after actinomycin D, a transcription inhibitor, was given. Total RNA of 7-d-old seedlings grown under continuous light was isolated after incubation with

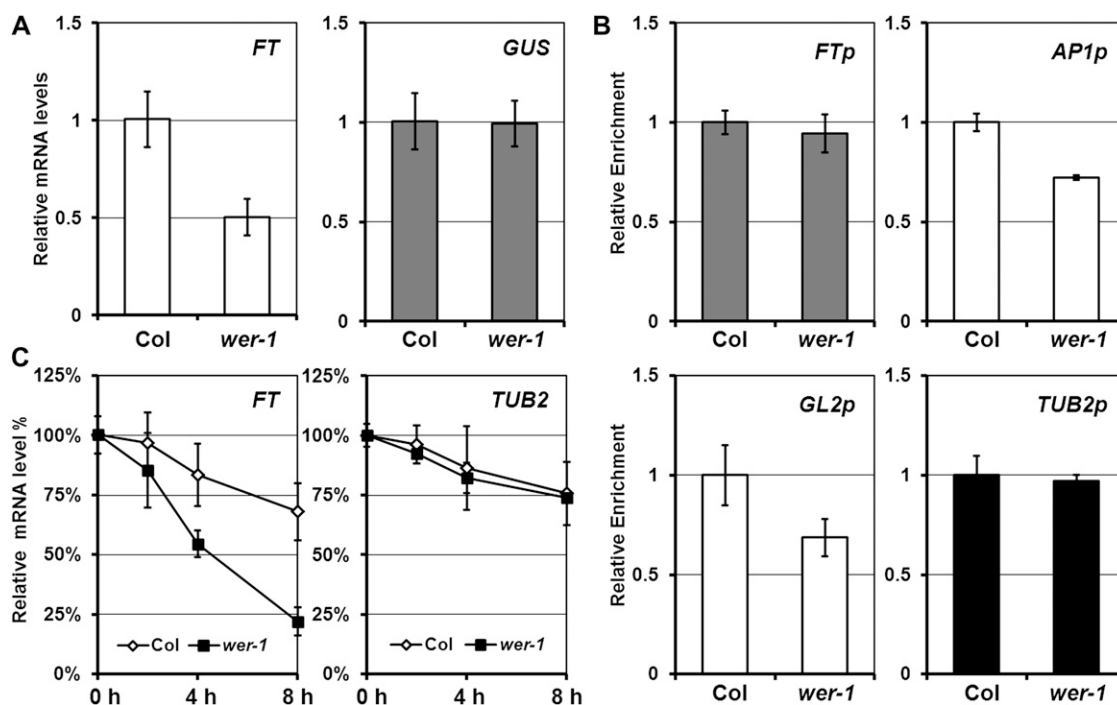


Figure 5. Posttranscriptional regulation of *FT* mRNA. A, Expression levels of the *GUS* gene driven by the *FT* promoter and endogenous *FT* mRNA in *FTp::GUS* Col (Col) and *FTp::GUS wer-1* (*wer-1*) plants detected by qRT-PCR. The values and error bars represent means and SD, respectively, from three technical replicates. B, ChIP assay with RNA polymerase II antibody. Enrichment in each promoter was confirmed by ChIP-qPCR analysis. Values are normalized against Col, and means of triplicate experiments are presented with error bars representing SD. C, RNA was isolated from 7-d-old Col and *wer-1* grown in continuous light (24 h of light) after 200 μ M actinomycin D treatment for 0, 2, 4, or 8 h. Expression of *FT* and *TUB2* in each sample was detected by RT-qPCR, and values are normalized against the expression level of untreated sample (0 h). Mean values from three technical replicates are shown with error bars representing SD.

actinomycin D for 0, 2, 4, or 8 h. After transcriptional block with actinomycin D treatment, *FT* mRNA abundance in *wer-1* was more rapidly reduced than that in the wild type (Fig. 5C). However, the half-life of control mRNA, *TUB2*, was not different in the wild type and *wer-1*, suggesting that the *wer* mutation does not cause a general RNA instability. Therefore, our results suggest that *WER* regulates the stability of *FT* mRNA.

Mutation Affects the *FT* mRNA Stability

For the root hair pattern formation, *WER* forms a transcriptional protein complex with *TTG1* and *GL3/EGL3* and positively regulates the transcription of *GL2* and *CPC* (Lee and Schiefelbein, 2002; Schellmann et al., 2002; Wada et al., 2002; Zhang et al., 2003; Koshino-Kimura et al., 2005; Ryu et al., 2005). We wondered if the genetic tool kit regulating root-hair pattern is also involved in the determination of flowering time. Interestingly, *ttg1-13* and *gl3-2 egl3-1*, mutants of components in the *WER* protein complex, flowered as late as *wer* mutants (Fig. 6A), suggesting that the *WER* complex regulates flowering time also. In contrast, *gl2* and *cpc* showed similar flowering time to the wild type (Fig. 6B). Therefore, our results indicate that *WER* downstream signaling does not regulate flowering

although the *WER* protein complex does, through the regulation of *FT* mRNA stability.

DISCUSSION

Myriad genes are reported to regulate the transcript level of *FT*, since *FT* turns out to be a “florigen” as well as a key integrator of flowering signals. However, in most cases it is not clear if it is regulated at the transcriptional level or the posttranscriptional level. In this study, we clearly show that *wer*, producing ectopic root hairs, is a typical photoperiod pathway mutant and that *WER* regulates *FT* mRNA stability in a non-cell-autonomous way. Therefore, our study provides a novel mechanism regulating *FT* transcript level.

WER Regulates *FT* Non Cell Autonomously

Although *WER* expression was mostly detected in root, grafting analysis showed that flowering time was not affected by *WER* in root (Fig. 2). This result indicates that *WER* expressed in aerial parts are involved in the regulation of flowering time. Consistent with this, *WER* expression was observed in diverse aerial parts such as young leaves, stems, flowers, and siliques (Fig. 3, A and

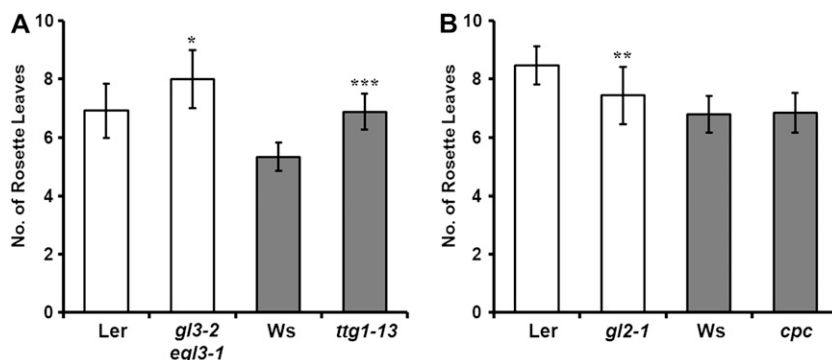


Figure 6. Flowering time of root hair patterning mutants. A, Mutation in components of the same complex with *WER* delayed flowering time. The double mutant *gl3-2 egl3-1* is in the *Ler* background, while the *ttg1-13* mutant is in the *Ws* background. At least 20 plants were used to measure the flowering time in long days, and the error bars represent sd. The asterisks denote statistical significance: * $P < 0.05$, *** $P < 0.0001$ (t test). B, Mutants of *WER* downstream target genes showed similar flowering time with their wild-type plants. The *gl2-1* mutant is in the *Ler* background, while the *cpc* mutant is in the *Ws* background. At least 20 plants were used to measure the flowering time in long days, and the error bars represent sd. The asterisks denote statistical significance: ** $P < 0.01$ (t test).

B). In addition, it is expressed in young, developing leaves where *FT* is expressed (Fig. 3B; Takada and Goto, 2003). The leaf consists of three distinct tissues: mesophyll, vascular bundle, and epidermis. Histological analysis showed that *WER* is expressed in leaf epidermis, whereas *FT* is expressed in vascular bundles (Fig. 3B; Takada and Goto, 2003). Therefore, *WER* is most likely to regulate *FT* non cell autonomously. Alternatively, *WER* may be transported to vascular bundles for the regulation of *FT*. However, this is not likely, because it produces a protein complex, as discussed below, and our preliminary result showed that *WER* protein is located in the leaf epidermis.

It is noteworthy that root hair pattern formation shares the same genetic tool kit with trichome formation in the leaves (Schiefelbein, 2003). The many *glabrous* mutants with no trichome have defects in root hair formation as well (Masucci et al., 1996; Payne et al., 2000; Ohashi et al., 2002; Bernhardt et al., 2003; Zhang et al., 2003). In addition, such genes are expressed in both root and leaf epidermis. Therefore, it provides good evidence indicating that trichome and root hair are evolutionarily homologous organs, as suggested before (Kellogg, 2001). However, our results also show how functional divergence occurs in *WER* activity. Although the same *WER* protein complex regulates root hair pattern formation and flowering in roots and leaves, respectively, the downstream factors involved in each process are different (Fig. 6). Thus, the divergence occurs at the downstream target genes. Future analysis to search for the factors mediating signals between the epidermis and vascular bundles for *FT* regulation would be interesting.

Non-cell-autonomous regulation of *FT* is not unprecedented. It has been reported that phytochrome B located in mesophyll suppresses *FT* expression through a downstream gene, *PFT1* (Cerdán and Chory, 2003; Endo et al., 2005). Interestingly, the *PFT1* mediating phyB signaling also regulates *FT* independent of

CO, similar to *WER*. This may indicate that non-cell-autonomous regulation of *FT* in the leaf is a common process. Therefore, it is possible to identify the inter-tissue signals regulating *FT*, which is critical to understanding florigen entity.

WER Regulates *FT* Posttranscriptionally

CO is known to directly regulate *FT* transcription by binding to the promoter (Samach et al., 2000; Tiwari et al., 2010). Although *CO* protein does not have a conspicuous domain for the transcription factor, much compelling evidence supports that it plays a transcriptional coactivator (Samach et al., 2000; Hepworth et al., 2002; Wenkel et al., 2006). In addition, *FT* is transcriptionally regulated by *FLC*, a central flowering repressor. *FLC* protein binds directly to the first intron of *FT* to prevent the induction of *FT* transcription (Helliwell et al., 2006). Therefore, transcriptional regulation of *FT* is relatively well studied, but posttranscriptional regulation is poorly studied. Here, we revealed that *WER* positively regulates *FT* by controlling mRNA stability at the posttranscriptional level (Fig. 5). This indicates that a *FT* mRNA decay pathway is involved in the regulation of flowering time. Transcript abundance is determined by the equilibrium between the rate of mRNA synthesis and the rate of degradation; yet, in the majority of gene expression analyses, the mRNA decay process has not been considered seriously. However, recent advances provide some knowledge about mRNA decay pathways, such as that the mRNAs involved in regulatory processes have shorter half-lives than those involved in the metabolic pathway in *Arabidopsis* (Gutierrez et al., 2002; Belostotsky and Sieburth, 2009). As was found in yeast and human studies, this implies that a rapid mRNA decay process is required for strict regulation of developmental processes. This is consistent with the result that *FT* transcript was less stable than *TUB2* in this study (Fig. 5C).

Because FT protein is considered a florigen, the FT protein level is directly linked to flowering. Therefore, it is probable that FT transcripts must be carefully monitored to produce FT protein in appropriate amounts. The regulation of FT mRNA stability proposed in this study may provide a new mechanism to control FT transcripts. Since WER encodes a transcription factor, WER would not be directly involved in the regulation of FT mRNA stability. Thus, it is likely that WER activates stabilizing factors or inhibits destabilizing factors for FT mRNA. The genetic components regulating FT mRNA stability will be pursued.

MATERIALS AND METHODS

Plant Materials and Genotyping

Arabidopsis (Arabidopsis thaliana) wer-1 in the Col background and *wer-3* in the Ws background were used (Lee and Schiefelbein, 1999). 35S::WER is a transgenic line with WER genomic DNA (inserting from the start to the stop codon) driven by the cauliflower mosaic virus 35S promoter in *wer-1* mutants (Lee and Schiefelbein, 1999). To confirm that genomic WER rescues the flowering phenotype of *wer-1*, a 5-kb genomic fragment including 2.5 kb of the upstream sequence of WER was cloned into the binary vector pPZP221 and transformed into *wer-1*. The WER::GUS transcriptional reporter construct was reported previously (Lee and Schiefelbein, 1999). The *gl2-1* mutant is in the Landsberg *erecta* (*Ler*) background, and *cpc* and *ttg1-13* are in the Ws background (Masucci and Schiefelbein, 1996; Wada et al., 1997; Walker et al., 1999). The homozygous double mutant *gl3-2 egl3-1* is generated by crossing two single mutants in the *Ler* background (Payne et al., 2000; Zhang et al., 2003; Bernhardt et al., 2005). The 35S::CO and 35S::FT transgenic lines and *ft-1*, *co-101*, *gi-2*, *fca-9*, *fove-3*, *soc1-2*, and *ld-1* are in the Col background, as described before (Lee et al., 1994, 2006; Fowler et al., 1999; Kardailsky et al., 1999; Kobayashi et al., 1999; Page et al., 1999; Takada and Goto, 2003; Ausin et al., 2004). The *flc-3* mutant is the line originally obtained from fast-neutron mutagenesis of Col:FRI^{SF2}, a Col line with FRIGIDA (*FRI*) from San Feliu-2 (SF2), by backcrossing eight times (Michaels and Amasino, 1999; Lee et al., 2000). However, the FRI^{SF2} allele has been eliminated from *flc-3* by backcrossing several times into Col. The FTp::GUS Col line containing an 8.9-kb upstream sequence from the start codon fused to GUS protein was used (Takada and Goto, 2003). FTp::GUS *wer-1* was obtained from the cross between FTp::GUS Col and *wer-1*, and kanamycin-resistant F2 seedlings were genotyped with cleaved-amplified polymorphic sequence (CAPS) markers. To check the genotype of double mutants, the F2 plants were checked using PCR-based markers, simple sequence length polymorphism markers, and CAPS and degenerate CAPS markers, which are described in Supplemental Table S1.

Growth Conditions

Seeds were sterilized by 75% ethanol with 0.05% Triton X-100, then rinsed twice using absolute ethanol and dried. They were seeded on 0.85% plant agar (Duchefa) containing 1% Suc and half-strength Murashige and Skoog (MS; Duchefa) plates and incubated for 3 d at 4°C to break seed dormancy. Afterward, plants were transferred and grown at 22°C with 60% ± 10% relative humidity in long days (16 h of light/8 h of dark) or short days (8 h of light/16 h of dark) under cool-white fluorescent lights (100 μmol m⁻² s⁻¹). For vernalization treatment, seeds on the MS plates were incubated for 8 weeks at 4°C under short-day conditions. For exogenous application of GA, we transferred 7-d-old plants into MS medium with GA₃ and incubated for 2 d under short-day conditions. At least 20 plants were used to measure the flowering time of each genotype. Flowering time was measured as the number of rosette leaves produced when flowering occurs.

Grafting

Transverse-cut grafting was performed as described previously (Turnbull et al., 2002). The grafting experiment was accomplished with a microscope using 5-d-old seedlings grown on Suc-free medium. Horizontal cuts were

made in the upper region of the hypocotyl with small blades (Dorco T-300). For 5 d after grafting, grafts were monitored for whether they formed good unions without bending or any other growth problems. All successful grafts were transplanted to soil. The final proportion of successful grafts, which grow normally until flowering, was over 70%. At least 10 plants were used to measure the flowering time of each graft.

Analysis of Gene Expression

Total RNA extraction and RT-PCR were performed as described before (Lee et al., 2008). The RT-PCR analysis was repeated at least three times using separately harvested samples. The information of each primer for PCR is described in Supplemental Table S2. For semiquantitative RT-PCR analysis, RT-PCR products were analyzed using the ImageJ 1.42 (<http://rsbweb.nih.gov/ij/>) program to quantify the expression level of each gene. qRT-PCR analysis was performed in 96-well format using the 7300 Fast Real-Time PCR System (Applied Biosystems) and iQ SYBR Green supermix (Bio-Rad). Four microliters of cDNA was used in a 20-μL reaction. Primers were designed to amplify DNA fragments shorter than 150 bp. The details of each primer for PCR are described in Supplemental Table S3. Reaction conditions were as follows: 5 min at 95°C, 40 cycles of PCR (30 s at 95°C, 30 s at 60°C, and 30 s at 72°C), and a dissociation from 60°C to 95°C. Data were collected at 72°C in each cycle, and *TUB2* was used as the reference gene. The half-life of mRNA was referenced by 18S rRNA levels. The qRT-PCR analysis was biologically repeated three times, and each consisted of three technical replicates.

Plasmid Construction

To make the chimeric genes of MYC-tagged WER, the DNA fragment for the MYC epitope was inserted in-frame into the 5' end of the PCR-amplified coding region of WER genomic DNA. The insertion of this chimeric MYC-WER between a 2.4-kb 5' flanking region DNA fragment and a 1.1-kb 3' flanking region DNA fragment from the WER gene resulted in the WERp::MYC-WER construct.

GUS Staining and Histological Analysis

GUS staining and histological analysis were performed following standard methods described before (Choi et al., 2007). Embedded samples in paraffin were sectioned at a thickness of 8 μm with a microtome (Leica RM2135). Photographs were taken with a digital microscope (Dimis M) or a digital camera (Photometrics) connected to a microscope (Zeiss Axioskop 2 Plus).

Immunohistochemistry

Immunohistochemistry was performed as described (Paciorek et al., 2006) with some modifications. Tissue samples were fixed in methanol:acetic acid (3:1) fixative solution. The fixed tissue was dehydrated in ethanol, cleared in Neo-clear (Merck), and embedded in ParaplastPlus. Tissue sections were made with 10 μm thickness and mounted on SuperfrostPlus slides. The sections were deparaffinized and rehydrated with 1× phosphate-buffered saline. Antigen unmasking was performed by heating the slides in 10 mM sodium citrate buffer boiled in a microwave oven and incubating the slides in 3% hydrogen peroxide solution. After blocking with MTBS solution (50 mM PIPES [pH 7.0], 5 mM EGTA, and 5 mM MgSO₄) containing 3% bovine serum albumin and 5% goat serum, the sections were incubated with anti-MYC monoclonal antibodies (Calbiochem) at a 1:3,000 dilution in the blocking solution overnight. The slides were washed and incubated with Alexa Fluor 546 goat anti-mouse IgG secondary antibodies (Invitrogen) at a 1:5,000 dilution in MTBS. After washing with MTBS, the fluorescence signal was observed using an AxioImager fluorescence microscope (Zeiss) with a DsRed filter.

Analysis of mRNA Stability

Whole seedlings of the wild type and *wer-1* grown for 7 d in continuous light were harvested and incubated in liquid MS medium with 200 μM actinomycin D (Sigma-Aldrich) for 0, 2, 4, or 8 h. Prior to this treatment, plants were soaked in actinomycin D for 30 min to allow proper distribution of the antibiotic solution.

ChIP Assay

A total of 1 g of Col and *wer-1* seedlings grown under long days for 11 d was used for ChIP. Procedures for ChIP followed the method described before (Lee et al., 2007a, 2008), and antibody for the C-terminal domain of the RNA polymerase II (Abcam AB817) was used. Four microliters of ChIP products resuspended in 100 μ L of Tris-Cl (pH 8.0, 10 mM) and EDTA (1 mM) was used for qPCR. In qPCR analysis, expression levels were normalized against expression in Col. The information of the primer pairs for ChIP-qPCR is presented in Supplemental Table S4.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *WER* (AT5G14750), *FT* (AT1G65480), *CO* (AT5G15840), *SOC1* (AT2G45660), *FD* (AT4G35900), *FLC* (AT5G10140), *API* (AT1G69120), *GL1* (AT3G27920), *MYB23* (AT5G40330), *TTG1* (AT5G24520), *GL3* (AT1G11130), *EGL3* (AT1G63650), *GL2* (AT1G79840), *CPC* (AT2G46410), and *TUB2* (AT5G62690).

Supplemental Data

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

Supplemental Figure S1. Flowering time of heterozygotes.

Supplemental Table S1. Primers for genotyping.

Supplemental Table S2. Primers for RT-PCR.

Supplemental Table S3. Primers for qRT-PCR.

Supplemental Table S4. Primers for ChIP-qPCR.

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