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Rapid paper

Molecular Basis of Late-Flowering Phenotype Caused by Dominant Epi-Alleles of the *FWA* Locus in *Arabidopsis*

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The late-flowering phenotype of dominant *fwa* mutants is caused by hypomethylation in the *FWA* locus leading to ectopic expression of a homeodomain leucine zipper (HD-ZIP) protein. However, little is known about whether *FWA* has any role in regulation of flowering and how ectopically expressed *FWA* delays flowering. Through analysis of *FWA* expression in wild-type seedlings, it was shown that *FWA* is not expressed during the vegetative phase. This suggests that *FWA* has no role in flowering. The previous reports that *fwa* suppressed the precocious-flowering phenotype of plants overexpressing *FLOWERING LOCUS T* (*FT*) suggest that the flowering pathway(s) either at and/or downstream of *FT* is blocked by *FWA*. Comparison of gene expression profiles in three genetic backgrounds ectopically expressing *FWA* and their respective wild types failed to detect common changes, ruling out the possibility that FWA acts through transcriptional misregulation. Yeast two-hybrid analysis and in vitro pull-down assay showed that FWA protein can specifically interact with FT protein. The importance of protein interaction with FT in delaying flowering was supported by studies involving N-terminal and C-terminal truncations of FWA. The C-terminal truncation with abolished interaction did not delay flowering when overexpressed, while the N-terminal truncation, which retains interaction, did. Specific interaction of FWA with FT enabled us to use FWA protein as a specific inhibitor of FT protein function. Through tissue-specific ectopic expression of *FWA*, further support for the shoot apex being the site of action of FT protein was provided.

Keywords: *Arabidopsis* — Epi-mutant — Flowering — FT — FWA — Protein interaction.

Abbreviations: AD, Gal4 activation domain; BD, Gal4 DNA-binding domain; bZIP, basic region/leucine zipper; EMS, ethylmethane sulfonate; GST, glutathione *S*-transferase; GUS, β-glucuronidase; HD, homeodomain; HD-ZIP, homeodomain leucine zipper; LD, long day; ORF, open reading frame; RT–PCR, reverse transcription–PCR; 35S, cauliflower mosaic virus 35S RNA promoter; SD, short day; SINE, short interspersed element; START, StAR-related lipid transfer protein domain; UTR, untranslated region; ZLZ, zipper–loop–zipper.

Introduction

The transition to flowering is controlled by endogenous cues and multiple environmental factors. In the case of *Arabidopsis*, physiological, genetic and molecular studies using flowering time mutants have elucidated several genetic pathways that promote flowering. These include the photoperiod, vernalization, gibberellin and autonomous pathways (Simpson and Dean 2002). These multiple pathways converge on the transcriptional regulation of the floral pathway integrator genes, *FLOWERING LOCUS T* (*FT*), *TWIN SISTER OF FT* (*TSF*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *LEAFY* (*LFY*) (Araki 2001, Simpson and Dean 2002, Michaels et al. 2005, Parcy 2005, Yamaguchi et al. 2005). Of these floral pathway integrators, *FT* is an important direct target of CONSTANS (CO), a key regulator of the photoperiod pathway (Samach et al. 2000). *FT* encodes a protein of the phosphatidylethanolamine-binding protein (PEBP) [also known as Raf1 kinase inhibitor protein (RKIP)] family (Kardailsky et al. 1999, Kobayashi et al. 1999) and is expressed in the phloem tissue of the cotyledons and leaves (Takada and Goto 2003). It has recently been suggested that FT protein acts with a basic region/leucine zipper (bZIP) transcription factor FD in the shoot apex to promote floral transition and floral morphogenesis in part through transcriptional activation of target genes such as *APETALA1* (*AP1*) and *FRUITFULL* (*FUL*)

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(Abe et al. 2005, Wigge et al. 2005). These findings, together with recent reports of possible movement of *FT* mRNA from leaf to the shoot apex (Huang et al. 2005) and the presence of FT protein in the phloem sap of *Brassica napus* (Giavalisco et al. 2006), support a model in which *FT* mRNA and/or FT protein are a part of the long-distance flowering signal(s) called florigen (Corbesier and Coupland 2006, Imaizumi and Kay 2006).

fwa is a dominant late-flowering mutant and is similar to the *ft* loss-of-function mutant in terms of both phenotype and genetic interaction (Koornneef et al. 1991). For example, the flowering time of *fwa* is similar to that of *ft* in the same genetic background (Koornneef et al. 1991); *ft* and *fwa* share similar responses to photoperiods and vernalization treatment (Koornneef et al. 1991); *ft*; *fwa* double mutants showed the same flowering phenotype as the respective single mutants (Koornneef et al. 1998); the flowering time of *fwa* and *ft* is not affected by the presence of sucrose in the media (Roldán et al. 1999, Ohto et al. 2001); *fwa* and *ft* suppress the early-flowering phenotypes of *35S::CO* (Onouchi et al. 2000) and *35S::LFY* (Nilsson et al. 1998); *fwa* and *ft* suppress precocious-flowering phenotypes caused by *emf1* and *emf2* (Haung et al. 1998); and *fwa* and *ft* enhance floral defects caused by *lfy* and *ap1* (Ruiz-García et al. 1997). Based on these observations, it has long been assumed that the same or similar steps are blocked in these mutants and that both genes share a similar role in the promotion of flowering (e.g. Martínez-Zapater et al. 1994, Koornneef et al. 1998).

The findings that dominant late-flowering mutations which mapped very close to *fwa* were induced at a high frequency in a hypomethylated background of a *decrease in DNA methylation1* (*ddm1*) mutant and were stably inherited by the progeny (Kakutani 1997) have led to the elucidation of the nature of dominant *fwa* mutations. In *fwa* mutants, there was no change in the nucleotide sequence of the *FWA* locus, but a severe reduction in cytosine methylation was observed in a 5 Mb region spanning the *FWA* locus including the promoter and the first two non-coding exons which contain two direct repeats derived from the insertion of a short interspersed element (SINE) (Soppe et al. 2000, Lippman et al. 2004). Due to hypomethylation in the promoter, *FWA*, which encodes a class IV homeodomain leucine zipper (HD-ZIP) transcription factor, is ectopically expressed (Soppe et al. 2000, Kinoshita et al. 2007). The gain-of-function nature of ectopic expression explains the dominance of the reported *fwa* mutants (*fwa* epi-alleles or epi-mutants). In the wild-type plants, however, *FWA* may not act as a regulator of flowering. *FWA* expression was not detected during the late vegetative phase, and loss-of-function mutants of *FWA* were indistinguishable from the wild type with regard to flowering time (Soppe et al. 2000). Recent findings have demonstrated that *FWA* displays imprinted expression in endosperm and that this expression depends on alteration of the methylation state of direct repeats in the 5′ region (Kinoshita et al. 2004, Kinoshita et al. 2007). These facts suggest that *FWA* per se may not be a component of the regulatory mechanisms of flowering but rather that ectopically expressed *FWA* may somehow interfere with some important step(s) in the regulatory mechanism of flowering. In addition to the similarity in phenotype and genetic interaction of *ft* loss-of-function mutants and gain-of-function *fwa* epi-mutants mentioned above, it has been shown that the early-flowering phenotype of *35S::FT* was suppressed by *fwa* epi-alleles (Kardailsky et al. 1999, Kobayashi et al. 1999). The flowering pathway at and/or downstream of *FT* is probably the step(s) blocked by ectopically expressed *FWA*.

Although the *FWA* locus provides a unique opportunity to study DNA methylation (e.g. Cao and Jacobsen 2002, Kankel et al. 2003, Chan et al. 2004, Kinoshita et al. 2004, Kinoshita et al. 2007, Zhang et al. 2006), little is understood about the molecular basis of the late-flowering phenotype of the gain-of-function epi-alleles. In this study, we investigated the mechanism by which ectopically expressed *FWA* interferes with the floral transition, expecting that *FWA* will provide a unique tool to dissect pathway(s) from *FT* to flowering. We confirmed that *FWA* is not expressed during the vegetative phase and ruled out the possibility that *FWA* plays a role in the regulation of flowering in wild-type plants. We demonstrated that FWA protein binds specifically to FT protein in yeast cells and in vitro. Overexpression of C-terminally truncated FWA with abolished protein interaction with FT failed to produce the late-flowering phenotype, while removal of the HD did not affect the ability to bind with FT and to produce the late-flowering phenotype by overexpression. Analysis of gene expression profiles failed to show consistent changes in transcription in *FWA*-expressing backgrounds. Therefore, it is unlikely that HD-ZIP protein FWA acts through transcriptional misregulation of target genes. These results together suggest that ectopically expressed FWA delays floral transition by interfering with the FT function through protein–protein interaction. With this mechanism in mind, we further investigated the site of action of FT protein using FWA protein as a specific inhibitor of the FT protein function. We examined the tissue where FWA can exert its negative effect on flowering. FWA expressed in the shoot apex by *FD* promoter delayed flowering, while FWA expressed in the vascular tissues (including the phloem companion cells which express *FT*) did not. These results strongly support the notion that FT protein acts in the shoot apex, as suggested by previous studies (Abe et al. 2005, Wigge et al. 2005).

Results

Isolation of fwa-101D as a strong suppressor of the precocious-flowering phenotype of 35S::FT

To gain clues to the events downstream of *FT* that lead to the floral transition, we screened for mutations that suppress the precocious-flowering phenotype of *35S::FT*. We isolated plants that flowered later than *35S::FT* (a strong line, #11-1) in the M_2 population after ethylmethane sulfonate (EMS) mutagenesis. One of these mutants turned out to be dominant. As expected, this mutation also strongly suppressed the precocious-flowering phenotype of a weak *35S::FT* line (#1-5) (Table 1). In the wild-type background without the *35S::FT* transgene, the dominant late-flowering phenotype (Table 1) and, interestingly, ectopic expression of *FWA* were observed (Fig. 1A). These results are in agreement with the previous reports that *fwa* epi-alleles ectopically expressing *FWA* (*fwa-1* and *fwa-2*) suppress the precocious-flowering phenotype of *35S::FT* (Kardailsky et al. 1999, Kobayashi et al. 1999).

It has been reported that cytosine methylation in two direct repeats in the region of the first two non-coding exons of the *FWA* gene was greatly reduced in *fwa* epi-alleles (Soppe et al. 2000, Cao et al. 2002). To confirm that the mutant is a new *fwa* epi-allele, we analyzed the methylation status of several cytosine residues in the direct repeats by restriction enzyme digestion and DNA gel blot analysis. Genomic DNA was digested with *Cfo*I, which is sensitive for CpG methylation. In the mutant, three digested fragments were detected due to lack of methylation of the two cytosines in the direct repeats, as in the case of *fwa-1* (Fig. 1B). Based on these results, we conclude that the plant carries a new epi-allele of *FWA*, and hereafter refer to it as *fwa-101D*.

Interestingly, it has been reported that *fwa-101D* had no effect on the precocious-flowering phenotype caused by overexpression of *TSF*, the closest homolog of *FT* in *Arabidopsis* (Yamaguchi et al. 2005). Similarly, *fwa-101D* did not affect the precocious-flowering phenotype caused by overexpression of *CiFT*, the *Citrus unshiu* ortholog of *FT* (Endo et al. 2005b), and *Heading-date 3a* (*Hd3a*), the rice ortholog of *FT* (Kojima et al. 2002) (Table 2). These results indicate that ectopically expressed *FWA* somehow discriminates *FT* from its homologs.

Effects of fwa-101D on the early-flowering phenotype caused by overexpression of SOC1 and AP1

It has been reported that dominant *fwa* epi-mutations and *ft* loss-of-function mutations share similar genetic interactions with various flowering-related mutations such as *lfy*, *ap1*, *embryonic flower1* (*emf1*) and *emf2*, or with transgenes such as *35S::LFY* and *35S::CO* (Ruiz-García et al. 1997, Haung and Yang 1998, Nilsson et al. 1998, Onouchi et al. 2000). These reports provide support for the notion that *FT* function and/or step(s) downstream of *FT* is

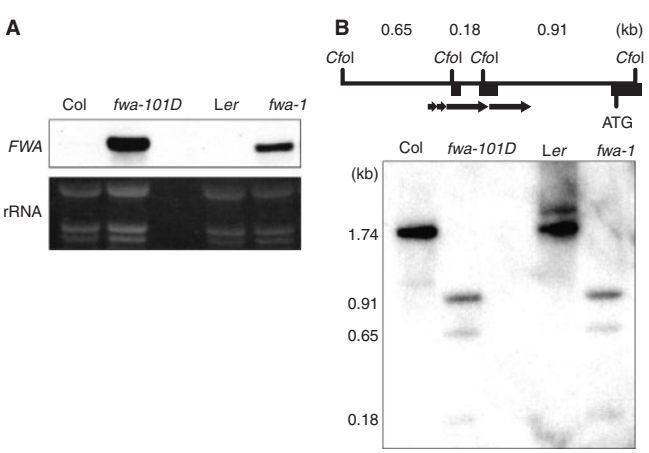

Fig. 1 Ectopic expression and hypomethylation of the direct repeats of the *FWA* gene in *fwa-101D*. (A) RNA gel blot analysis of *FWA* expression in *fwa* mutants and wild types. Total RNA was extracted from 7-day-old seedlings grown under LD conditions and was subjected to analysis. (B) Methylation patterns of the direct repeats in *fwa* mutants and wild types. Genomic DNA was digested with the methylation-sensitive enzyme *Cfo*I and was probed with a 1.74 kb fragment of the 5′ region of the *FWA* gene (shown in the diagram). Two inner *Cfo*I sites are within one of the direct repeats (shown by two larger arrows). Boxes represent exons.

Table 1 Effects of *fwa-101D* mutation on flowering time of transgenic plants overexpressing *FT*, *SOC1* and *AP1*

Genotype	No. of rosette leaves	No. of cauline leaves	*n*
Col	10.5 ± 0.6	2.6 ± 0.7	17
fwa-101D/*FWA*$^+$	19.3 ± 2.1	6.7 ± 0.8	7
35S::FT (#11-1)/–	4.0 ± 0.6	1.1 ± 0.4	7
fwa-101D/*FWA*$^+$; *35S::FT* (#11-1)/–	8.0 ± 1.2	2.6 ± 0.8	10
35S::FT (#1-5)/–	5.0 ± 0	1.8 ± 0.8	5
fwa-101D/*FWA*$^+$; *35S::FT* (#1-5)/–	9.0 ± 1.0	2.0 ± 0.5	17
soc1-101D/*SOC1*$^+$	4.9 ± 0.6	2.3 ± 0.5	8
fwa-101D/*FWA*$^+$; *soc1-101D*/*SOC1*$^+$	9.1 ± 1.5	4.8 ± 0.7	18
35S::AP1/–	7.6 ± 0.5	2.6 ± 0.5	5
fwa-101D/*FWA*$^+$; *35S::AP1*/–	9.4 ± 1.3	5.1 ± 1.4	21

Plants were grown on 1/2 MS medium under LD conditions. The number of leaves is presented as the average ± SD. 'Transgene name/–' indicates hemizygosity.

Table 2 Effects of *fwa-101D* mutation on flowering time of transgenic plants overexpressing *CiFT* or *Hd3a*

Genotype	No. of rosette leaves	No. of cauline leaves	*n*
Col	11.5 ± 1.3	2.6 ± 0.8	16
fwa-101D/*FWA*$^+$	20.4 ± 1.7	6.1 ± 0.9	14
35S::CiFT (#12-4)/–*	4.7 ± 1.0	1.5 ± 0.5	24
fwa-101D/*FWA*$^+$; *35S::CiFT* (#12-4)/–*	4.8 ± 0.9	1.4 ± 0.5	18
Col	11.9 ± 0.9	2.5 ± 0.8	18
fwa-101D/*FWA*$^+$	19.9 ± 2.5	6.2 ± 0.8	9
35S::Hd3a (#5-3)/–**	4.1 ± 0.9	1.0 ± 0.4	23
fwa-101D/*FWA*$^+$; *35S::Hd3a* (#5-3)/–**	4.1 ± 1.1	1.0 ± 0.3	30

Plants were grown on soil under LD conditions. The number of leaves is presented as the average ± SD. 'Transgene name/–' indicates hemizygosity. There was no statistically significant difference between two pairs of genotypes marked with * or ** (Student's *t*-test, $P > 0.6$ or $P > 0.9$), respectively.

compromised in the dominant *fwa* background. To explore the similarity between *fwa* epi-mutations and *ft* loss-of-function mutations further, we investigated the effects of *fwa-101D* on the flowering time of plants overexpressing *SOC1* and *AP1*.

Overexpression of *SOC1*, either by *35S::SOC1* or by an activation tagged allele, *soc1-101D*, produces the early-flowering phenotype which is attenuated in short-day conditions (Borner et al. 2000, Lee et al. 2000, Samach et al. 2000). Similarly, *35S::AP1* plants flower earlier than wild type, and the early-flowering phenotype is greatly attenuated in short-day conditions (Liljegren et al. 1999). Attenuation of the early-flowering phenotype in short-day conditions is probably due to reduction of *FT* expression. Consistent with this finding, *ft-1* partially suppresses the early-flowering phenotype of *soc1-101D* and *35S::AP1* (Yoo et al. 2005, M. Abe and T. Araki, unpublished observation). *fwa-101D* partially suppressed the early-flowering phenotype of *soc1-101D* and *35S::AP1*. Both *fwa-101D*/*FWA*$^+$; *soc1-101D*/*SOC1*$^+$ and *fwa-101D*/*FWA*$^+$; *35S::AP1*/– plants produced a similar number of rosette leaves but approximately twice the number of cauline leaves compared with the wild type (Table 1). These observations provide further evidence that dominant *fwa* mutations and *ft* loss-of-function mutations share a similar genetic interaction with flowering-related transgenes.

Expression of FWA in the wild type and fwa-101D

We examined expression patterns of *FWA* in *fwa-101D* and wild-type seedlings by reverse transcription–PCR (RT–PCR) analysis. In *fwa-101D*, expression was observed in shoot apices, rosette leaves, cotyledons, hypocotyls and roots, while no expression was observed in the wild type (Fig. 2A). We further analyzed *FWA* expression patterns in the shoot apical region of seedlings by in situ RNA hybridization. Strong uniform expression was observed in the shoot apex and leaf primordia in *fwa-101D* (Fig. 2C and D), whereas expression was not detected in the wild type (Fig. 2B). These results suggest that *FWA* is not expressed in the wild-type seedlings. Since a previous report of *FWA* expression in wild-type seedlings suggested a possible role for *FWA* in regulation of flowering (Soppe et al. 2000), we further examined *FWA* expression in wild-type seedlings in detail. Recent findings of *FWA* expression in the endosperm (Kinoshita et al. 2004) prompted us to think that the reported expression of *FWA* in wild-type seedlings was due to mRNA present in endosperm cells in the remnants of seed coats adhering to the seedling. Therefore, we separated the aerial parts of the seedling proper and the seed coat remnants, and analyzed *FWA* expression in these two fractions. Under our conditions of RT–PCR combined with Southern blot analysis, *FWA* expression was not detected in the seedling proper from day 2 to day 12 under either long day (LD) or short day (SD) conditions (Fig. 3A); however, a faint signal was detected in the seed coat fraction. To confirm further the absence of *FWA* expression in wild-type seedlings, we examined expression of the β-glucuronidase (GUS) gene under the regulation of a 3.3 kb genomic sequence upstream of the initiation codon (*FWA::GUS*). As previously reported with a similar construct to express FWA protein fused to green fluorescent protein (GFP) (Kinoshita et al. 2004), strong GUS expression was observed in ovules (Fig. 3B, C). In contrast, GUS expression was not detected in seedlings grown in either LD or SD conditions except for occasional faint staining in a small number of cells at the tip of cotyledons in some lines (Fig. 3D–I, E inset). Based on these observations, *FWA* does not appear to be expressed during the seedling stage in wild-type plants.

Effect of ectopic expression of FWA on gene expression profile

FWA encodes a protein of the HD-ZIP IV family of transcription factors (Soppe et al. 2000, Nakamura et al. 2006). Therefore, it is possible that ectopically expressed *FWA* delays flowering through the transcriptional misregulation of its target genes. Because the *fwa* mutation does not affect expression of *FT* (Fig. 2A, E, F; Kardailsky et al. 1999, Kobayashi et al. 1999) or *TSF* (Yamaguchi et al. 2005), ectopic *FWA* expression may affect expression of genes controlling the floral transition other than *FT* and *TSF*.

To explore this possibility, we performed microarray analysis using two independent *fwa* epi-alleles [*fwa-1* in

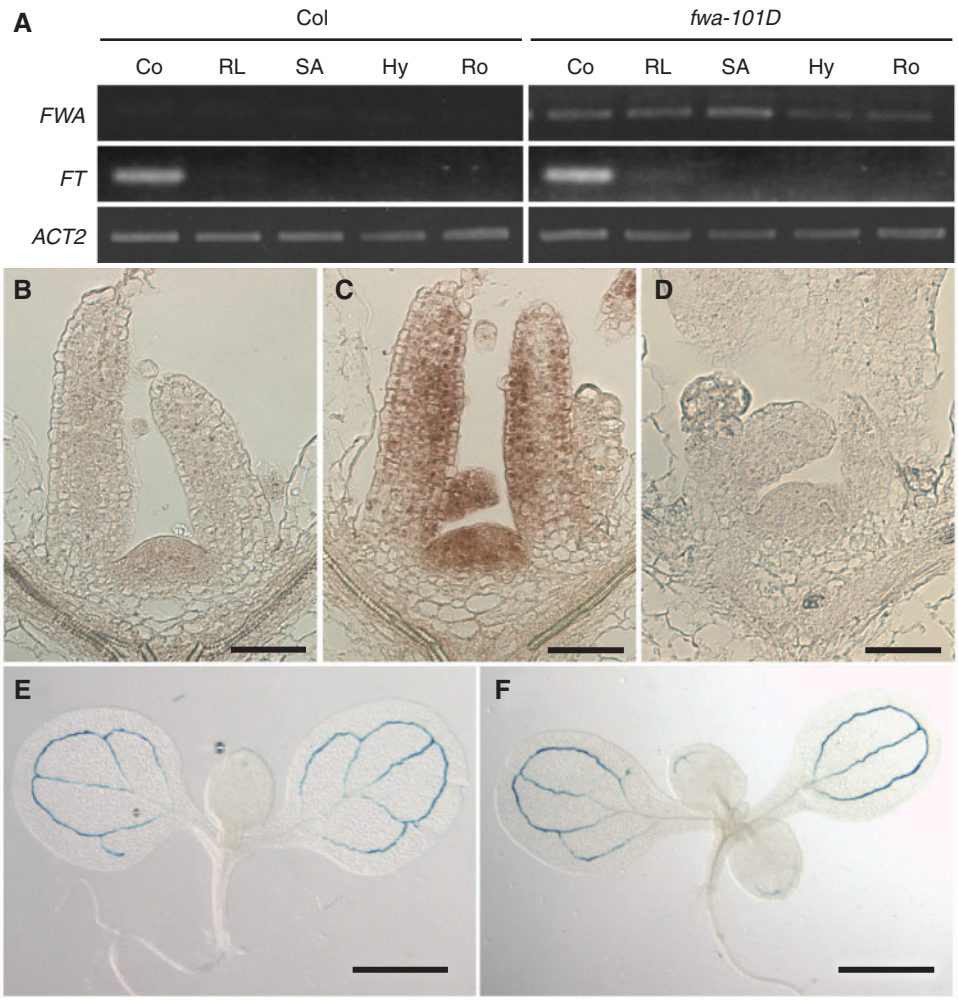

Fig. 2 Expression of *FWA* and *FT* in wild-type and *fwa-101D* seedlings. (A) RT–PCR analysis of *FWA* and *FT* expression in 7-day-old seedlings grown under LD conditions. Co, cotyledons; RL, rosette leaves; SA, shoot apex; Hy, hypocotyls; Ro, roots. A fragment of *ACTIN2* (*ACT2*) transcript was amplified as a control. (B–D) In situ RNA hybridization analysis of *FWA* expression in 6-day-old seedlings grown under LD conditions. Longitudinal sections through the shoot apical meristem of Col (B) and *fwa-101D* (C, D) seedlings were hybridized with antisense (B, C) or sense (D) RNA probes. (E and F) Expression of *FT::GUS* in wild type (E) and *fwa-101D* (F). Six-day-old seedlings grown under LD conditions were sampled at Zeitgeber time 14 and were stained. Scale bars, 50 μm in (B–D) and 100 μm in (E) and (F).

the Landsberg *er* (L*er*) background and *fwa-101D* in the Columbia (Col) background] and an *FWA*-overexpressing transgenic line (*35S::FWA*, line #6-8 in the Col background) and looked for common changes. The gene expression profile of *fwa-1* was analyzed and compared with its parental wild-type L*er*, and those of *fwa-101D* and *35S::FWA* were compared with wild-type Col. Several transcripts including that of *FWA* itself with >4-fold difference in the signal intensity were found for each set of pairs (Supplementary Table S1, Fig. S1). However, none of the known regulators of flowering showed significant differences (Supplementary Table S2, Fig. S2).

Using a 4-fold difference as a criterion, six genes were identified from the comparison between *fwa-1* and L*er* (Fig. 4A; Supplementary Table S1). In *fwa-101D*, 27 genes were up-regulated compared with Col, and one of these (At1g15010) was also up-regulated in *fwa-1* (Fig. 4A; Supplementary Table S1). In *fwa-101D* and *fwa-1*, many (11 out of 33) of the up-regulated genes were categorized as transposon-like elements (Supplementary Table S1). In *35S::FWA*, one gene was up-regulated compared with Col, but it was up-regulated in neither *fwa-1* nor *fwa-101D* (Fig. 4A). Five genes were down-regulated in *fwa-1* (Fig. 4A; Supplementary Table S1). However, there were no common genes whose expression was either increased or

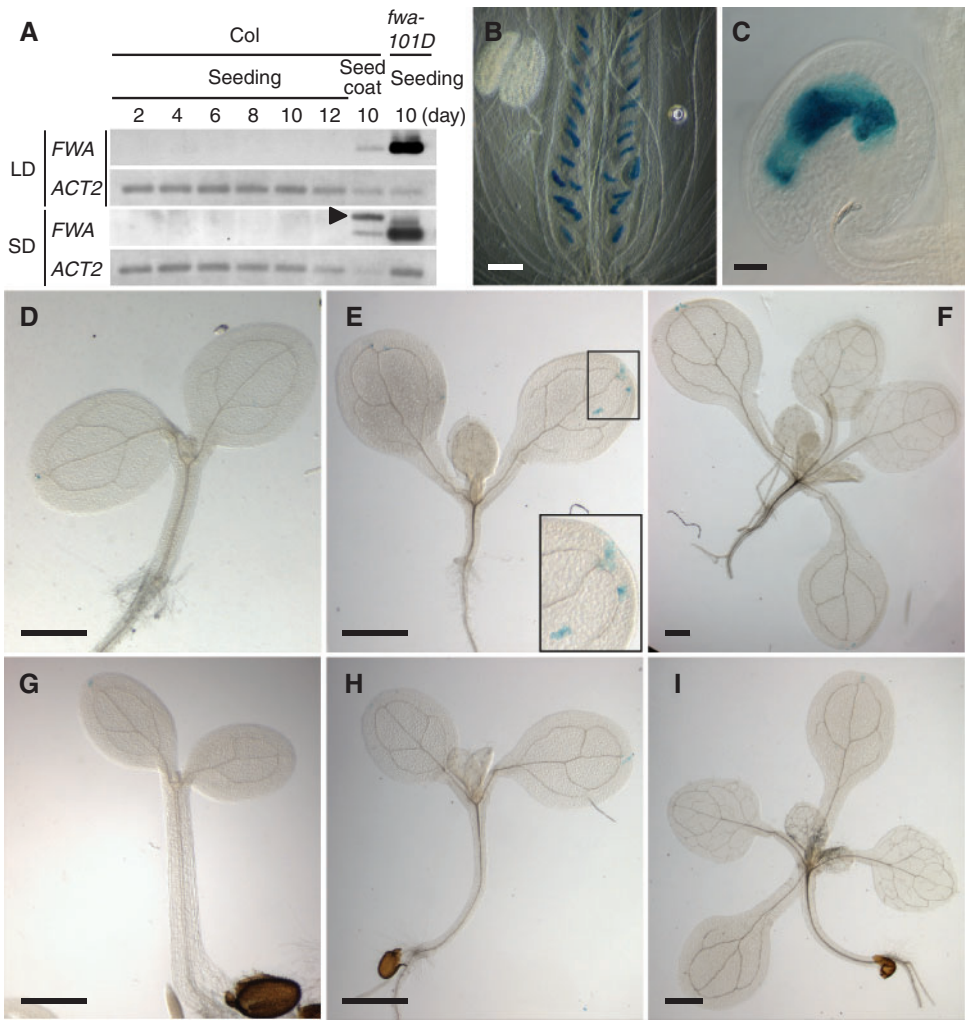

Fig. 3 Expression of *FWA* in wild-type plants. (A) RT–PCR analysis of *FWA* expression in seedlings grown under LD and SD conditions. Wild-type seedlings without seed coats were collected on days 2, 4, 6, 8, 10 and 12. *fwa-101D* seedlings without seed coats were collected on day 10. Remnants of seed coats were collected on day 10. After 22 amplification cycles, expression was detected by DNA gel blot analysis. The arrow indicates the product amplified from the genomic DNA. As a control, a fragment of an *ACTIN2* (*ACT2*) transcript was amplified by 16 cycles and was detected in a similar manner. (B–I) GUS staining of a pistil (B), an ovule (C) and seedlings (D–I) of *FWA::GUS*. Seedlings grown under LD (D–F) and SD (G–I) conditions for 2 (D, G), 6 (E, H) and 10 (F, I) days. Scale bars, 100 μm in (B), 25 μm in (C), 0.25 mm in (D) and (G), and 1 mm in (E), (F), (H) and (I). Inset in (E) is an enlargement of the area of cotyledon with sporadic staining (framed).

decreased in a similar manner in all the three backgrounds with ectopic *FWA* expression (*fwa-1*, *fwa-101D* and *35S::FWA*) as compared with the wild types. Even if we lowered the threshold to a 2-fold difference, there were no changes common to all the three backgrounds (Supplementary Fig. S3).

To confirm the microarray data, we performed RT–PCR analysis. We analyzed the genes with >4-fold difference in at least one of the three pairs. Some of the changes were confirmed by RT–PCR analysis (Supplementary Figs. S4, S5). However, we could not find any genes with confirmed changes observed in all the three pairs (Fig. 4B).

FWA protein can interact with FT protein

To gain clues to the molecular basis of the late-flowering phenotype in *fwa*, we next investigated the possibility of interference with the flowering pathway by misexpressed *FWA* through protein–protein interaction. Based on genetic analysis, it has been suggested that misexpressed *FWA* inhibits the pathway at and/or downstream of *FT*. Therefore, we first examined the interaction between FWA and FT and other regulators acting with and/or downstream of *FT* using a yeast two-hybrid assay. Because FWA conjugated to the DNA-binding domain of Gal4 (Gal4-BD) alone activated reporter expression (Fig. 6A), we used FWA as a prey. Strong interaction

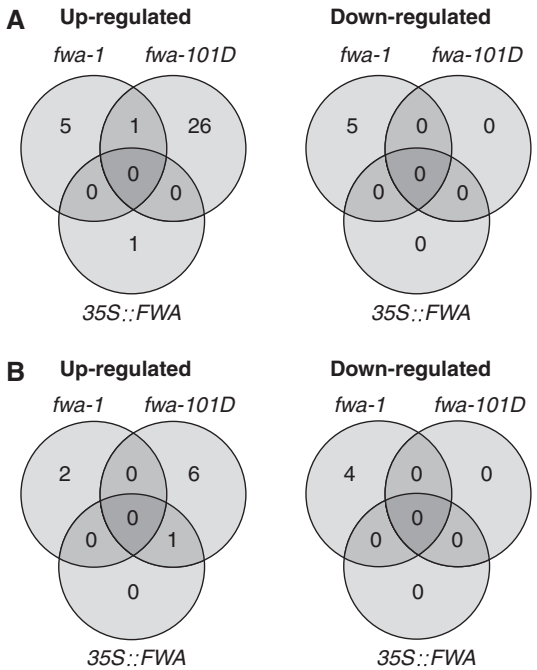

Fig. 4 Microarray and RT–PCR analysis of gene expression profiles in *fwa-101D, fwa-1* and *35S::FWA*. (A) Summary of Agilent Arabidopsis 2 microarray data. The numbers represent those of genes differentially expressed in *fwa-101D, fwa-1* and *35S::FWA* as compared with wild types (excluding *FWA*). Up-regulated and down-regulated genes are those with ratios of >4 and $<4^{-1}$, respectively. Total RNA was extracted from 7-day-old seedlings grown under LD conditions and was subjected to analysis. Scatter-plot graphs of the analysis and a list of the genes with ratios of >4 or $<4^{-1}$ are accessible in the Supplementary materials (Table S1, Fig. S1). (B) Summary of RT–PCR analysis. The genes with ratios of >4 or $<4^{-1}$ in microarray analysis were further analyzed by RT–PCR and, in some cases, the PCR products were sequenced. The numbers represent those of genes with a confirmed change in *fwa-101D*, *fwa-1* and *35S::FWA* as compared with the wild types (excluding *FWA*). The same RNA samples as (A) were used for the analysis. Details of the results of the RT–PCR analysis are accessible in the Supplementary material (Figs. S4, S5).

between FWA and FT was observed (Fig. 5). In contrast, interaction of FWA with TSF, the closest homolog of FT with a redundant role in flowering (Michaels et al. 2005, Yamaguchi et al. 2005), was very weak (Fig. 5A). This finding is in good agreement with the previous observation that *fwa-101D* had no effect on the precocious-flowering phenotype of *35S::TSF* (Yamaguchi et al. 2005). Interaction between FWA and TERMINAL FLOWER1 (TFL1), another homolog of FT with an antagonistic role in flowering (Ratcliffe et al. 1998, Kardailsky et al. 1999, Kobayashi et al. 1999, Ahn et al. 2006), was not detected (Fig. 5B). Since the zipper–loop–zipper (ZLZ) domain of FWA shares homology with the leucine zipper domain of FD (Supplementary Fig. S6) and FD acts with FT to promote floral transition and transcription of *AP1* (Abe et al. 2005, Wigge et al. 2005), we also investigated the interaction of FWA with FD or its paralog FDP (At2g17770; Abe et al. 2005, Wigge et al. 2005). FWA did not interact with either FD or FDP (Fig. 5B). FWA interacted with FWA itself (Fig. 6A), suggesting homodimer formation.

To identify the domain(s) of FWA protein responsible for interaction with FT, we performed domain analysis. FWA protein has an HD (residues 41–98), a ZLZ (residues 104–165), a StAR-related lipid transfer protein domain (START; residues 216–436) (Ponting and Aravind 1999, Schrick et al. 2004) and a C-terminal region (residues 437–686). As shown in Fig. 6A, deletion of the whole C-terminal region (FWAΔC; corresponding to residues 1–436) resulted in the loss of interaction with FT. Deletion of the C-terminal 48 residues (FWAΔ639–686) also abolished this interaction (Fig. 6B), indicating the importance of the C-terminal region in the interaction with FT. However, the C-terminal region alone [FWA(C); corresponding to residues 437–686] was not sufficient for the interaction. In contrast, deletion of the N-terminal region including the HD (FWAΔN; corresponding to residues 99–686) did not abolish the interaction (Fig. 6A). Further deletion of the ZLZ domain [FWA(START+C); corresponding to residues 216–686] did abolish the interaction (Fig. 6A). This implies that the ZLZ domain is also important in the interaction with FT. However, neither the ZLZ domain alone [FWA(ZLZ); corresponding to residues 99–215] nor the ZLZ domain in combination with the C-terminal region [FWA(ZLZ+C); corresponding to residues 99–215 plus residues 437–686] was sufficient for the interaction (Fig. 6A).

An in vitro pull-down assay was performed to confirm the interaction between FWA and FT demonstrated in yeast cells. Various truncated forms of FWA proteins were translated and labeled with [^{35}S]methionine in vitro. Labeled protein was incubated with the purified glutathione *S*-transferase (GST)–FT fusion protein expressed in *Escherichia coli* and was examined for its ability to bind to FT protein. As shown in Fig. 6C, binding of the full-length protein (FWA) and the N-terminally truncated protein (FWAΔN) with FT protein was confirmed. Other truncated forms [FWAΔC, FWA(START+C), FWA(ZLZ), FWA(C) and FWA(ZLZ+C)] did not bind to FT. These results are consistent with those of the yeast two-hybrid assay.

Overexpression of intact or truncated FWA proteins in plants

Given that *FWA* is ectopically expressed in late-flowering *fwa* epi-mutants, overexpression of the full-length *FWA* should confer the late-flowering phenotype in transgenic plants. However, if the interaction between

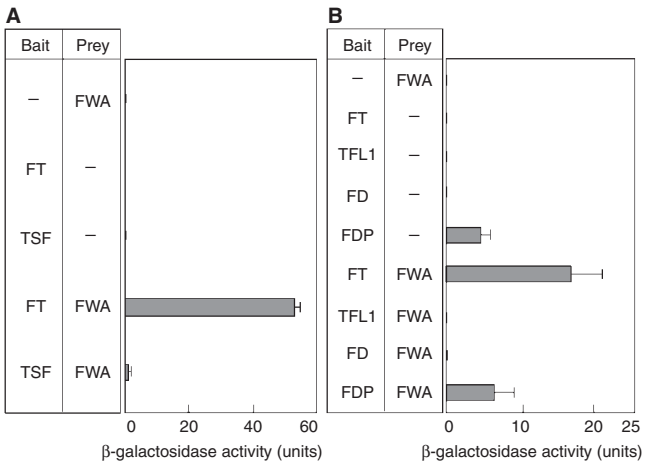

Fig. 5 Analysis of interaction between FWA protein and flowering time regulators by yeast two-hybrid assay. (A) Analysis of protein interaction with FT and TSF. (B) Analysis of protein interaction with FT, TFL1, FD and FDP. Yeast cells expressing combinations of the indicated baits (fusion proteins with the Gal4 DNA-binding domain) and preys (fusion proteins with the Gal4 DNA activation domain) were assayed for β-galactosidase activity. A '–' in the column means no insert in the respective fusion construct. β-Galactosidase activity (units) determined by using *O*-nitrophenyl-β-D-galactopyranoside as a substrate is shown as the average (± SD) of three independent assays.

FWA and FT is the cause of delayed flowering in *fwa* epi-mutants, it is expected that overexpression of FWAΔC with abolished interaction with FT has little effect on flowering time. To test this, we generated plants overexpressing full-length or truncated forms of FWA as the N-terminally myc-tagged protein (*35S::myc-FWA*, *35S::myc-FWAΔN*, *35S::myc-FWAΔC*). By RNA blot analysis, we confirmed high levels of accumulation of the corresponding mRNA in transgenic plants (Fig. 7A). Accumulation of significant amounts of fusion proteins was also observed (Supplemenary Fig. S7; see Supplementary text for details). As expected, *35S::myc-FWA* plants showed delayed flowering (Fig. 7B). Plants overexpressing the N-terminal truncation form of FWA (myc-FWAΔN), which retains interaction with FT in yeast cells and in vitro but lacks a HD, showed delayed flowering. In contrast, plants overexpressing the C-terminal truncation form of FWA (myc-FWAΔC), which is unable to interact with FT in yeast cells and in vitro, did not show the late-flowering phenotype (Fig. 7B). It is interesting to note that *fwa-1R2*, an intragenic suppressor of late-flowering *fwa-1* (Soppe et al. 2000), has a one-base deletion which, if translated, produces a truncated protein. FWA-1R2 protein lacks most of the C-terminal region (see Materials and Methods for the mutation in *fwa-1R2*) and could not interact with FT in yeast cells (Fig. 6A).

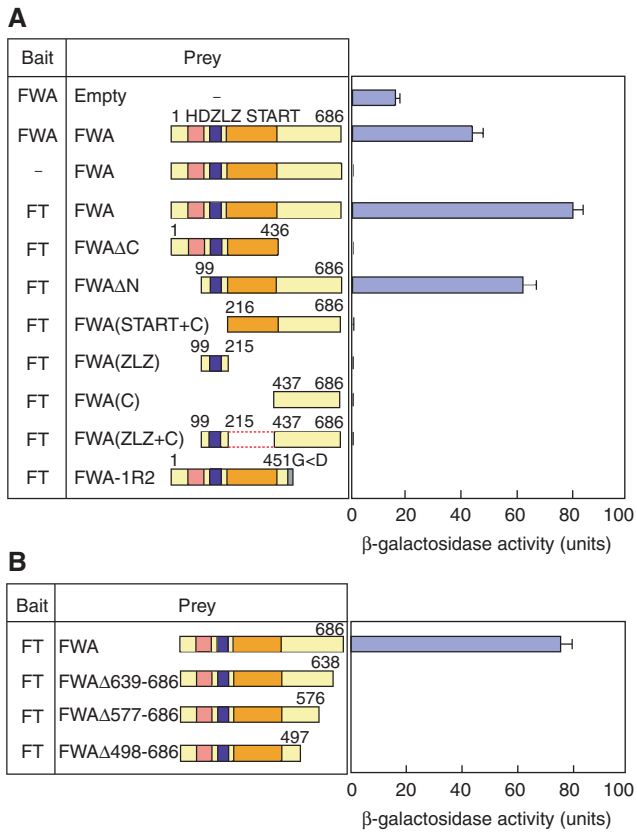

Fig. 6 Domain analysis of FWA protein. (A and B) Interaction between various forms of truncated FWA (shown as diagrams) and intact FT in yeast cells. β-Galactosidase activity (units) determined by using *O*-nitrophenyl-β-D-galactopyranoside as a substrate is shown as the average (± SD) of three independent assays. (C) In vitro pull-down assay of interaction between FWA and FT. Intact and various forms of truncated FWA proteins were translated and labeled with [^{35}S]methionine in vitro, incubated with the purified GST–FT fusion protein (lane 3) or GST (lane 2), and subjected to pull-down reaction with glutathione–Sepharose beads. Ten percent of input was loaded as a control (lane 1). Filled and open arrowheads indicate the position of various forms of FWA protein in the input and pull-down reaction, respectively.

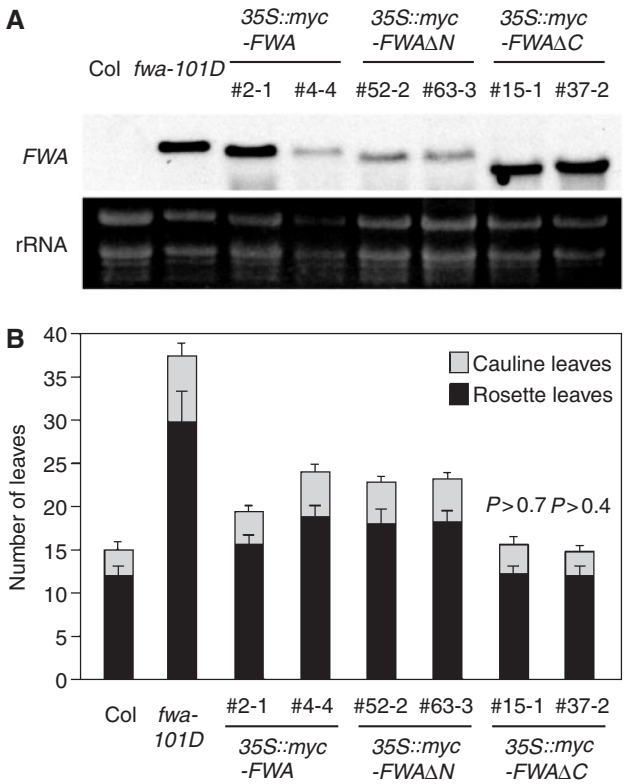

Fig. 7 Phenotypes of plants overexpressing intact or truncated FWA. (A) RNA blot analysis of *FWA* expression in *35S::myc-FWA* (expressing myc-tagged intact FWA), *35S::myc-FWAΔN* (expressing myc-tagged N-terminally truncated FWA: residues 99–686) and *35S::myc-FWAΔC* (expressing myc-tagged C-terminally truncated FWA: residues 1–436) plants. Two representative lines were chosen for analysis. Total RNA was extracted from 7-day-old seedlings grown under LD conditions and was subjected to analysis. (B) Flowering time of *35S::myc-FWA*, *35S::myc-FWAΔN* and *35S::myc-FWAΔC* plants grown under LD conditions. The number of leaves is shown as the average ± SD ($n = 20$–33). There was no statistically significant difference (Student's *t*-test) between Col and two lines of *35S::myc-FWAΔC* (*P*-values are indicated). Other lines are significantly different from Col ($P < 0.0001$).

Flowering was delayed by FWA expressed in the shoot apex but not by FWA expressed in the vascular tissues

Since ectopically expressed FWA protein is likely to act through interaction with FT protein to delay flowering and since interaction is rather specific to FT [little interaction with its close homolog TSF (82% identity with FT in amino acid sequence); Fig. 5A], we reasoned that FWA protein can be used as a kind of specific inhibitor of FT protein. This idea is further supported by the observation that *fwa-101D* had no effect on the precocious-flowering phenotype of *35S::CiFT* and *35S::Hd3a* (Table 2). FWA protein allows us to examine whether inhibition of FT protein function in the vascular tissues, which includes the sites of *FT* transcription, or in the shoot apex, the suggested site of FT protein action (Abe et al. 2005, Wigge et al. 2005), results in delayed flowering.

FWA expressed in the shoot apex by a weak *FD* promoter (Abe et al. 2005) (*FD::myc-FWA*) clearly delayed flowering (Fig. 8A, B, E). Conversely, *FWA* expressed in the vascular tissues by the *SULTR2;1* promoter (Takahashi et al. 2000) (*SULTR2;1::myc-FWA*) did not affect flowering time (Fig. 8A, C, E). These results indicate that FWA protein expressed in the shoot apex is able to interfere with *FT* function, but FWA protein in the vascular tissues is not. This provides further support for the notion that FT protein acts in the shoot apex. In contrast, *fwa-1*, which causes rather ubiquitous expression of FWA (see Fig. 2 for *fwa-101D*), was effective in suppressing the precocious-flowering phenotype caused by *FT* expression either in the shoot apex (*FD::FT* and *PDF1::FT*) or in the vascular tissues (*SULTR2;1::FT*) (Supplementary Table S3).

Discussion

FWA is not expressed during the vegetative phase and has no role in the regulation of flowering

Previous work has not fully ruled out the possibility of *FWA* expression in wild-type seedlings and an authentic role for *FWA* in regulation of flowering (Soppe et al. 2000). The mode of imprinting of the *FWA* locus suggests that *FWA* is likely to remain silent during the vegetative phase (Kinoshita et al. 2004). By detailed RT–PCR analysis of seedlings grown in LD and SD conditions and analysis of *FWA::GUS* transgenic plants (Figs. 2, 3), we confirmed that *FWA* is not expressed during the vegetative phase. It is likely that the reported expression in wild-type seedlings was due to mRNA present in the seed coat debris in seedling preparations (Fig. 3A). It has been reported that one-base deletion or substitution mutants of *FWA* (*fwa-1R1*, *fwa-1R2* and *fwa-1R3*), isolated as intragenic suppressors of the *fwa-1* epi-allele, have no effect on flowering time (Soppe et al. 2000; see Materials and Methods for the nature of the *fwa-1R2* mutation). This, together with the absence of expression during the vegetative phase, indicates that *FWA* has no authentic role in flowering in wild-type plants. Since the hypomethylated and imprinted state of the *FWA* locus has been caused by the insertion of SINE (Lippman et al. 2004), *FWA* may have lost its original function, whatever it may have been, in *Arabidopsis thaliana*. Whether *FWA* orthologs in other Brassicaceae species also have SINE insertions and what kinds of roles the *FWA* orthologs have in these plants is an interesting problem for future investigations.

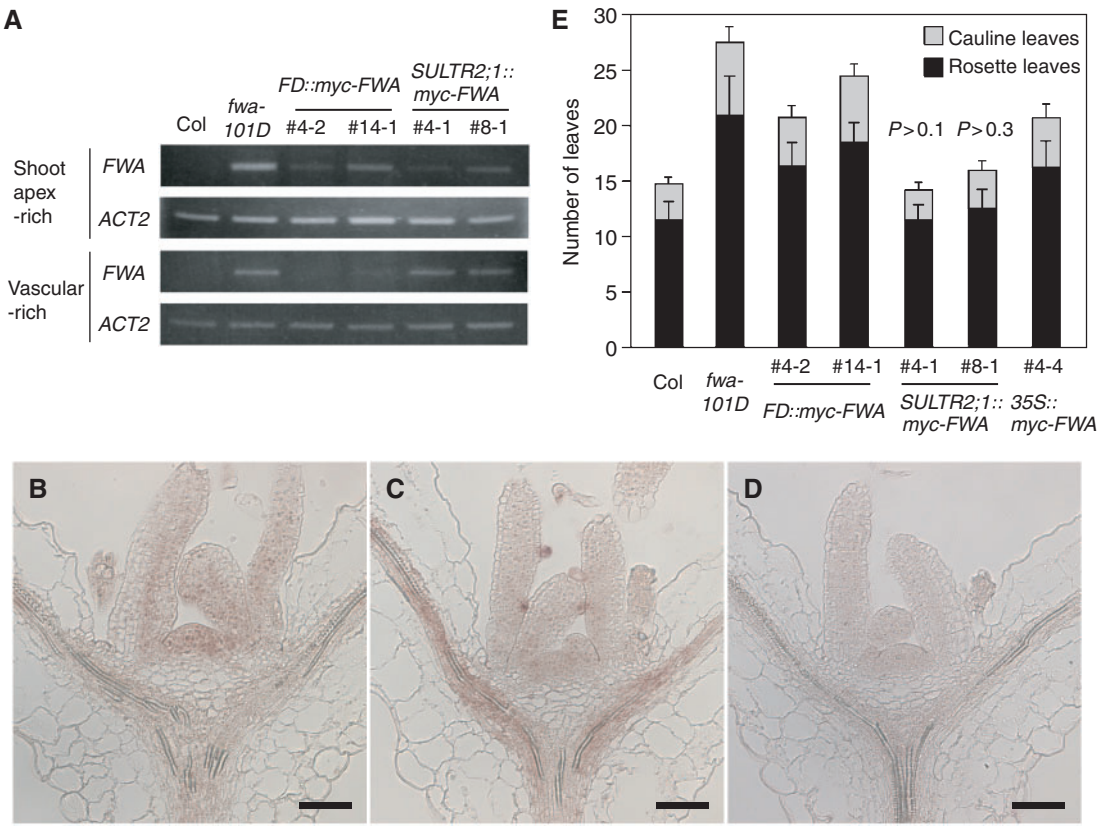

Fig. 8 Phenotypes of plants expressing *FWA* in the shoot apex or vascular tissues. (A) RT–PCR analysis of *FWA* expression in *FD::myc-FWA* and *SULTR2;1::myc-FWA* plants. Two representative lines were chosen for analysis. A shoot apex-rich fraction was prepared by removing cotyledons, hypocotyl and roots from 10-day-old plants grown under LD conditions at positions close to the shoot apex. A vascular-rich fraction was isolated from cotyledons of 10-day-old LD-grown plants essentially as described (Endo et al. 2005a). Total RNA was extracted from each fraction and was subjected to the analysis. (B–D) In situ RNA hybridization analysis of *FWA* expression in *FD::myc-FWA* and *SULTR2;1::myc-FWA* seedlings grown under LD conditions. Longitudinal sections through the shoot apical meristem of 6-day-old *FD::myc-FWA* (B), *SULTR2;1::myc-FWA* (C) and Col (D) seedlings were hybridized with antisense RNA probe. *SULTR2;1::myc-FWA* is expressed in immature trichomes on leaf primordia as well as in the vascular tissues. Note that faint staining in (D) represents background signal. Scale bars, 50 μm. (E) Flowering time of *FD::myc-FWA* and *SULTR2;1::myc-FWA* plants grown under LD conditions. *35S::myc-FWA* (line #4-4) was included as a reference. The number of leaves is shown as the average ± SD ($n = 21$–38). There was no statistically significant difference (Student's *t*-test) between Col and two lines of *SULTR2;1::myc-FWA* (*P*-values are indicated). Other lines are significantly different from Col ($P < 0.0001$).

Specific effect of ectopically expressed FWA on the precocious-flowering phenotype caused by FT overexpression

The finding that *fwa-101D* was isolated as a dominant suppressor of *35S::FT* is in agreement with the previous reports that *fwa* epi-alleles (*fwa-1* and *fwa-2*) suppressed the precocious-flowering phenotype of *35S::FT* (Kardailsky et al. 1999, Kobayashi et al. 1999). *fwa-101D* has no effect on the precocious-flowering phenotype caused by over-expression of *TSF*, which shares 82% amino acid identity with *FT* (Yamaguchi et al. 2005), *CiFT*, the *Citrus unshiu* ortholog of *FT* (Endo et al. 2005b), or *Hd3a*, the rice ortholog of *FT* (Kojima et al. 2002) (Table 4 in Yamaguchi et al. 2005; Table 2). Therefore, the effect of ectopically expressed *FWA* has specificity for *FT* among homologs from *Arabidopsis* and other plants. That ectopically expressed *FWA* can somehow discriminate *FT* from its homologs including *TSF* suggests that *FWA* acts through direct action on *FT* rather than indirectly affecting some common step(s) downstream of *FT* homologs (see below).

In contrast to the lack of suppression of *35S::TSF*, *35S::CiFT* and *35S::Hd3a*, *fwa-101D* suppressed the precocious-flowering phenotype of *35S::SOC1* and *35S::AP1*. Since both SD conditions which repress *FT* expression and *ft-1* mutation greatly attenuate the

phenotypes of *35S::SOC1* and *35S::AP1* (Liljegren et al. 1999, Borner et al. 2000, Lee et al. 2000, Samach et al. 2000, Yoo et al. 2005, M. Abe and T. Araki, unpublished observations), it is likely that the effect on *35S::SOC1* and *35S::AP1* is the result of interference with the *FT* function by direct action on *FT*.

No consistent effect of ectopically expressed FWA on gene expression profiles

Since *FWA* encodes an HD-ZIP transcription factor (Soppe et al. 2000, Nakamura et al. 2006), it seems likely that ectopically expressed *FWA* delays flowering through transcriptional misregulation of its target genes. To explore this possibility, we compared the gene expression profiles of three independent *FWA*-overexpressing backgrounds with those of the respective wild-type backgrounds. However, no genes exhibited common changes in all the three *FWA*-overexpressing backgrounds (Fig. 4; Supplementary Fig. S3). Therefore, it is unlikely that the late-flowering phenotype of *fwa* is due to the misregulation of transcription. The finding that the N-terminal truncation of FWA protein devoid of the DNA-binding HD could still cause the late-flowering phenotype when overexpressed (Fig. 7) also supports this conclusion. Additional support is provided from the fact that *fwa-101D* had no effect on *35S::TSF*, *35S::CiFT* and *35S::Hd3a*, which indicates that *FWA* acts directly on *FT* rather than indirectly on other step(s) downstream of *FT*.

There were unique changes in the gene expression profile in each of the two backgrounds carrying *fwa* epi-alleles (*fwa-1* and *fwa-101D*) (Supplementary Table S1, Figs. S4, S5). These changes include up-regulation of CACTA-like transposable elements (Miura et al. 2001, Miura et al. 2004) and retroelements, none of which was up-regulated in *35S::FWA*. Up-regulated gene elements were distributed throughout the genome and there was no apparent tendency for clustering into characteristic regions such as heterochromatic knobs (data not shown). *fwa-1* and *fwa-101D* were independently isolated after EMS mutagenesis (Koornneef et al. 1991; see Materials and Methods for *fwa-101D*). Hypomethylation in the *FWA* locus may have been caused by an unidentified mutation(s) or genotoxic stress induced by the EMS treatment, and the same agent(s) may have induced stable epigenetic changes in other genes as well. It has been shown that the *ddm1* mutation induced hypomethylated *fwa* epi-allales and activation of CACTA transposons after repeated selfings (Kakutani 1997, Miura et al. 2001). Similarly, a strong loss-of-function mutation (*met1-1*) or antisense suppression of a maintenance methyltransferase gene (*MET1*) induced hypomethylation at *FWA* as well as other genomic sites such as MHC9.7/9.8 (Genger et al. 2003, Kankel et al. 2003). Recently, global loss of DNA methylation and massive reactivation of pseudogenes and transposons in *met1* was reported (Zhang et al. 2006). Although the causative mutation(s) may have been lost during the subsequent backcross with the wild type, some of the epigenetic changes may have been retained and inherited with the *fwa* epi-allele through generations. Therefore, it is likely that the two backgrounds carrying *fwa-1* and *fwa-101D* retain unique sets of epigenetic changes in the genome that result in derepression of silent genes such as transposons and retroelements. It is safe to conclude that the observed changes of gene expression profiles in *fwa-1* and *fwa-101D* are not caused by the activity of ectopically expressed FWA protein per se, but by concomitant epigenetic changes.

Binding of FWA protein with FT protein as a cause of the late-flowering phenotype

If FWA protein does not act through transcriptional misregulation, one of the possible mechanisms of action is via protein interaction with floral regulator(s). We investigated this by yeast two-hybrid analysis and in vitro pull-down assays. FWA protein binds to FT in yeast cells and in vitro (Figs. 5, 6). No interaction was observed with TSF and TFL1 proteins of the same PEBP/RKIP family or with bZIP proteins FD and FDP, which interact with FT. It is important to note that TSF has 82% identity (90% similarity, if conservative substitution is included) in the amino acid sequence with FT (Yamaguchi et al. 2005). Therefore, the interaction of FWA protein seems very specific to FT.

Domain analysis of FWA protein showed that the C-terminal region, but not the N-terminal region containing the HD, is indispensable for the interaction with FT (Fig. 6). The whole C-terminal region seems to be required for the binding to FT, since a small deletion of the C-terminal 48 residues abolished the interaction (Fig. 6B). The C-terminal region is the least conserved part of the FWA protein, and no function has been assigned (Soppe et al. 2000, Nakamura et al. 2006). Our effort to identify the minimal portions of FWA sufficient for binding to FT was not very successful. The combination of the ZLZ domain, the START domain and the C-terminal region is the smallest unit of FWA which retains the ability to bind to FT.

Genger et al. (2003) reported that FWA protein in the C24 accession is different from FWA in L*er* at two positions (phenylalanine in L*er* vs. leucine in C24 at position 257,

and isoleucine in L*er* vs. leucine in C24 at position 311), and these differences may abolish the ability of ectopically expressed FWA in C24 to cause delayed flowering. Since this is of interest from the point of view of protein interaction, we cloned *FWA* from C24 for further analysis. However, we could not confirm the presence of the nucleotide sequence variation corresponding to the phenylalanine vs. leucine difference between L*er* and C24 from various sources. A nucleotide difference corresponding to the isoleucine vs. leucine difference was confirmed, but Col also shares this variation with C24.

Overexpression of the C-terminally truncated FWA protein with abolished binding to FT (Fig. 6) did not cause the late-flowering phenotype, even though high levels of accumulation of mRNA (Fig. 7) and a significant amount of the truncated protein (Supplementary Fig. S7) were observed. In contrast, N-terminal truncation of FWA protein, which may abolish DNA binding but retains the ability to bind to FT (Fig. 6), was effective in delaying flowering when overexpressed (Fig. 7B). These results provide strong support for the importance of protein interaction with FT in the action of FWA.

As discussed above, ectopically expressed *FWA* is likely to act directly on *FT* rather than indirectly through downstream step(s). This, together with the importance of the protein interaction, suggests that ectopically expressed FWA delays floral transition by interfering with the FT function through protein–protein interaction. Since FT protein is present in the nucleus and acts with the bZIP transcription factor FD (Abe et al. 2005), nuclear localization of FWA (Kinoshita et al. 2004) makes it possible for FWA to bind to FT and block its interaction with FD in the nucleus (Supplementary Fig. S8). The finding that the flowering of *35S::FT; fwa-101D* occurs later than that of *35S::FT; fd-1* (Table 1; Abe et al. 2005) indicates that FD is not the only protein whose interaction with FT is blocked by FWA.

It has been reported that overexpression of other genes for class IV HD-ZIP proteins resulted in a late-flowering phenotype similar to that of *fwa*. An activation-tagged *ANTHOCYANINLESS2* (*ANL2*) has a late-flowering phenotype, although *35S::ANL2* failed to show the same phenotype (Weigel et al. 2000). Overexpression of *PROTODERMAL FACTOR2* (*PDF2*) by *35S::PDF2* delays flowering (Abe et al. 2003). Whether these class IV HD-ZIP proteins act in a manner similar to FWA protein, as discussed above, to delay flowering is an interesting problem for investigation.

Shoot apex as the site of action of FT protein confirmed by localized inhibition of FT by FWA protein

It has recently been reported that FT and FD, a bZIP protein preferentially expressed in the shoot apex, are interdependent partners, through protein–protein interaction, in the promotion of floral transition and transcriptional activation of *AP1* (Abe et al. 2005, Wigge et al. 2005). Furthermore, ectopic expression of *FT* in the shoot apex rescued the late-flowering phenotype of *co* (An et al. 2004) and *ft* (Abe et al. 2005), and the late-flowering and severe floral defect phenotype of *ft; lfy* (Abe et al. 2005). Based on these findings, it is now believed that the shoot apex is the site where FT protein exerts its function (Abe et al. 2005, Wigge et al. 2005).

As discussed above, FWA protein seems to discriminate FT protein from its closest homolog, TSF. Therefore, we reasoned that it can be used as a kind of specific inhibitor of FT protein and provides us with a tool for tissue-specific inhibition of FT protein activity. The finding that FWA protein expressed in the shoot apex (*FD::myc-FWA*) delays flowering but FWA expressed in the vascular tissues, including phloem companion cells, (*SULTR2;1::myc-FWA*), had no effect on flowering (Fig. 8) supports the current view that the shoot apex is the site of FT protein function. It is likely that translation of *FT* occurs in the phloem companion cells where it is transcribed. The inability of FWA protein expressed in the vascular tissues to delay flowering suggests that the nuclear functions of FT protein, such as transcriptional regulation with certain transcription factor(s) which can be blocked by nuclear-localized FWA, are not of great importance in promoting flowering. However, this inability does not rule out the possibility that FT protein may have an important cytoplasmic function in phloem companion cells such as facilitation of its long-distance action.

Recently, Huang et al. (2005) have provided evidence for the movement of *FT* mRNA from leaf to the shoot apex. However, the possibility of transport of FT protein (discussed in Abe et al. 2005, Wigge et al. 2005) has not been fully investigated and it is still unclear whether mRNA or protein or both are the transported entity of physiological relevance (see Bäurle and Dean 2006). The finding that FWA protein expressed in the vascular tissues including phloem companion cells (the site of *FT* transcription) does not seem to prevent the *FT* function favors the view that the *FT* gene product is transported in the form of mRNA on which FWA protein may have no effect. Alternatively, FWA protein localized to the nucleus may not prevent cytoplasmic FT protein from being transported and/or facilitating the transport of its own mRNA from the companion cell to the phloem. Therefore, FT protein transport is still a possibility. Identification of the minimal domain of FWA protein sufficient for binding with FT and its use with various subcellular localization signals will provide us with useful tools to analyze FT protein function at tissue, cell and subcellular resolutions.

Materials and Methods

Plant materials and growth conditions

Col and L*er* were used as wild types. All transgenic lines were in the Col background. *fwa-1* and *fwa-2* in the L*er* background were obtained from M. Koornneef (Max Planck Institute, Germany). *fwa-101D* was obtained after mutagenesis of the *35S::FT* (YK #11-1) in the Col background with EMS. *35S::FT* (YK #11-1) and *35S::FT* (YK #1-5C) are strong and weak lines, respectively (Kobayashi et al. 1999). *35S::CiFT* and *35S::Hd3a* transgenic plants were previously described (Kobayashi et al. 1999, Kojima et al. 2002) and strong lines (#12-4 and #5-3, respectively) were chosen for the present work. *soc1-101D* (Lee et al. 2000) and *35S::AP1* (Mandel and Yanofsky, 1992) were obtained from I. Lee (Seoul National University, Korea) and M. Yanofsky (University of California, San Diego, CA, USA), respectively. *FT::GUS* (line #6-16) (Takada and Goto, 2003) was obtained from K. Goto (Research Institute of Biological Sciences, Okayama, Japan).

For expression analysis, plants were grown on 1/2 Murashige and Skoog (MS) medium with 0.5% sucrose containing 0.4% Gellan gum. Seeds were stratified by keeping at 4°C for 3–5 d and then transferred to 22°C; this transfer was defined as day 0. Plants were grown under LD (16 h light/8 h dark) conditions with white fluorescent lights (~60 μmol m^{-2} s^{-1}) or SD (8 h light/16 h dark) conditions with white fluorescent lights (~100 μmol m^{-2} s^{-1}).

For analysis of the flowering-time phenotype, plants were grown on 1/2 MS medium with 0.5% sucrose containing 0.4% Gellan gum (Wako, Osaka, Japan) as described above (Table 1) or on vermiculite with nutrient supplements of Hyponex (1 : 2000 dilution, HYPONex JAPAN Corp., Osaka, Japan) at 22°C under LD conditions with white fluorescent lights (~60 μmo m^{-2} s^{-1}) (Table 2).

Plasmid construction and transgenic plants

To construct *FWA::GUS*, a fragment containing promoter and non-coding exons was amplified from the Col genome by PCR with the following combination of primers: FWApro1, 5′-GAGCCAACAGCATCAGTCAATGAGAAACTC-3′; and FWApro3′XmaI, 5′-TCCcccgggTTTCCAACCGCATCCAAATC ACCTTGTCC-3′. After digestion with *Bam*HI and *Xma*I, a 3.3 kb *FWA* genomic fragment (position –3,322 to +36) was fused to the GUS coding sequence in pBI101.

To construct *35S::FWA*, the *FWA* open reading frame (ORF) in Col was amplified by PCR and cloned into *Bam*HI and *Xba*I sites between the 35S promoter and the nopaline synthase (NOS) gene terminator in a pCGN1547-derivative. To construct *35S::myc-FWA*, the 5′ end of the *FWA* ORF was fused to a c-*Myc* tag and replaced with the GUS coding sequence of pBI121. *35S::myc-FWAΔN* and *35S::myc-FWAΔC* were constructed in the same way as *35S::myc-FWA*. *35S::myc-FWAΔN* and *35S::myc-FWAΔC* contain the region +295 to the stop codon of *FWA* and the region +1 to +1,308 and a stop codon, respectively. To construct *FD::myc-FWA*, the *FD* 5′-untranslated region (UTR) and ORF between the *FD* promoter and the NOS terminator in *FD::FD* was replaced with the *myc-FWA* ORF. *SULTR2;1::myc-FWA* was constructed by replacing the *FT* sequence (ORF and UTRs) of *SULTR2;1::FT* with the *myc-FWA* ORF. *FD::FD* and *SULTR2;1::FT* on the pBIN19 vector used in these constructions were previously described (Abe et al. 2005).

These constructs in binary vectors were introduced into *Agrobacterium* strain pMP90 and transformed into *Arabidopsis* (Col) by the floral dip procedure (Clough and Bent 1998).

Genomic DNA gel blot analysis

Genomic DNA was extracted using Plant DNAzol reagent (Invitrogen, Carlsbad, CA, USA). A 5 μg aliquot of genomic DNA was digested with the restriction enzyme *Cfo*I, separated on a 1% agarose gel and blotted onto a Hybond-N+ nylon membrane (GE Healthcare Bio-Science, Piscataway, NJ, USA). Hybridization was performed in PerfectHyb Hybridization Solution (Toyobo, Osaka, Japan) with a ^{32}P-labeled probe. The probe was made from a PCR-amplified fragment (position +588 to +2,071 in GenBank accession No. AF178688) and synthesized using the Megaprime DNA Labelling System (GE Healthcare Bio-Science) with [α-^{32}P]dCTP (GE Healthcare Bio-Science).

RNA gel blot analysis and RT–PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For RNA gel blot analysis, 10 μg of total RNA was separated on a 1% agarose gel containing formaldehyde and blotted onto a Hybond-N+ nylon membrane (GE Healthcare Bio-Science). Hybridization, washing and detection were performed with the DIG system (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. To prepare *FWA*-specific RNA probe, a PCR-amplified fragment (position +277 to +1,308 relative to the initiation ATG codon whose A is designated +1) was cloned into pCR-Blunt II-TOPO (Invitrogen). The antisense probe was synthesized using SP6 RNA polymerase with digoxigenin-11-UTP (Roche Diagnostics).

For RT–PCR, total RNA (0.5 μg) was treated with RNase-free DNase I (Invitrogen) and reverse-transcribed in a 20 μl reaction mixture using the Superscript III First-Strand Synthesis System for RT–PCR (Invitrogen). Primers used for *FWA*, *FT* and *ACT2* were previously described (Genger et al. 2002, Abe et al. 2005). After amplification, PCR products were electrophoresed on an agarose gel, and visualized by ethidium bromide staining or DNA gel blot analysis. DNA gel blot analysis was performed with AlkPhos Direct (GE Healthcare Bio-Science) according to the manufacturer's protocol. Probes were made from fragments amplified with the same primer sets for each gene.

In situ RNA hybridization

In situ RNA hybridization was performed essentially as described (Heisler et al. 2001). To prepare *FWA*-specific RNA probe, a PCR-amplified fragment (position +886 to +2,043) was cloned into pCR-Blunt II-TOPO (Invitrogen). The antisense probe was synthesized using SP6 RNA polymerase with digoxigenin-11-UTP. The sense probe was synthesized using T7 RNA polymerase. Tissue samples were fixed in FAA (50% ethanol, 5% acetic acid and 3.7% formaldehyde), dehydrated, and embedded in paraffin. Sections (8 μm thick) were hybridized in 50% formamide with 5× SSC at 58°C for 3.5 h and washed in 0.1× SSC at 65°C.

Histological analysis of GUS staining

FWA::GUS line #700-1 was chosen for detailed analysis of *FWA::GUS* expression. Samples were collected from LD- or SD-grown plants. Six-day-old seedlings of *FT::GUS* (line #6-16) in Col and *fwa-101D* backgrounds grown under LD conditions were sampled at Zeitgeber time 14 and were analyzed for *FT::GUS* expression. GUS staining was performed for 48 h as described

(Yamaguchi et al. 2005). After staining, for whole-mount observation, samples were cleared in a mixture of chloral hydrate, glycerol and water solution (8 g : 1 ml : 2 ml).

Microarray analysis

Total RNA was extracted from 7-day-old seedlings grown under LD conditions using TRIzol reagent (Invitrogen), then purified by LiCl precipitation. Samples were subjected to analysis with Agilent Arabidopsis 2 Oligo Microarray (Agilent Technologies, Palo Alto, CA, USA), which is a 60-mer oligo microarray consisting of 21,500 Arabidopsis gene elements. Hybridization and data acquisition were performed by Hitachi Life Science (Saitama, Japan) according to the supplier's manual. Hybridization was performed once per microarray. Results of microarray analysis were further confirmed by RT–PCR analysis for selected genes. Primers used for RT–PCR analysis are shown in Supplementary Tables S4 and S5.

Yeast two-hybrid assay

The HybriZAP-2.1 Two-Hybrid System (Stratagene, La Jolla, CA, USA) was used. ORFs of *FWA*, *FT*, *TSF*, *TFL1*, *FD* and *FDP* were cloned into pBD-GAL4 Cam or pAD-GAL4-2.1. FWA was fused to the Gal4 activation domain (AD) to generate AD:FWA (with a linker of IELGSSASREF) or to the BD to generate BD:FWA (with a linker of EF). TSF was fused to BD with a linker (EFARD) to generate *BD:TSF*. *BD:FT*, *BD:TFL1*, *BD:FD* and *BD:FDP* constructs were previously described (Abe et al. 2005). To prepare various forms of truncated FWA, FWAΔC (corresponding to amino acid residues 1–436 of FWA), FWAΔN (residues 99–686), FWA(START+C) (residues 216–686), FWA(ZLZ) (residues 99–215), FWA(C) (residues 437–686), FWA(ZLZ+C) (residues 99–125 and 437–686), FWA-1R2 (residues 1–450 plus nine foreign residues), FWAΔ498–686, FWAΔ577–686 and FWAΔ639–686 were cloned into pAD-GAL4-2.1. FWA-1R2 was based on the sequence of *fwa-1R2*, an intragenic suppressor of *fwa-1* (Soppe et al. 2000). *fwa-1R2* has a substitution at the splice acceptor site of the ninth exon causing a one-base deletion in the 451st codon (GGA to GA) by mis-splicing and, if translated, produces a truncated protein with the N-terminal 450 residues of FWA and an additional nine amino acid residues. The details of construction are available upon request.

A yeast two-hybrid assay was performed using yeast strain Y187 obtained from Clontech (Mountain View, CA, USA). The appropriate plasmids were transformed into the yeast strain by the lithium acetate method and were selected on SD plates lacking leucine and tryptophan. To measure β-galactosidase activity, yeast cells were grown in 5 ml of liquid SD medium lacking leucine and tryptophan overnight, then transferred to 8 ml of YPD, and cultured until the OD_{600} was 0.5–0.8. The cells were collected and broken by freeze–thawing. The crude extracts were incubated with *O*-nitrophenyl-β-D-galactopyranoside, and then the OD_{420} was measured. The activity is expressed in Miller's units.

In vitro pull-down assay

The *FWA* ORF cloned in pENTR/SD/D-TOPO (Invitrogen) was recombined into Gateway pDEST14 (Invitrogen). Various forms of truncated FWA corresponding to those used in the yeast two-hybrid assay were cloned into pDEST14. In vitro transcription/translation was performed with L-[^{35}S]methionine (GE Healthcare Bio-Science) by TNT Coupled Reticulocyte Lysate Systems (Promega, Madison, WI, USA) according to the manufacturer's instructions. To express the GST–FT fusion protein, pDEST15-FT described previously (Abe et al. 2005) was transformed into *E. coli* BL21-AI (Invitrogen). GST protein was prepared using pDEST15-stop and was used as a control.

For GST pull-down assays, cell lysate was incubated with glutathione–Sepharose 4B beads (GE Healthcare Bio-Science), then 20 μl of the slurry [50% (v/v)] was mixed with 10 μl of ^{35}S-labeled in vitro translation products and 220 μl of NETN Buffer [50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40] and was gently stirred at 4°C for 2 h. The beads were washed extensively with NETN buffer five times, and subjected to SDS–PAGE. After electrophoresis, the labeled protein was detected by the phosphor imager BAS-1000 (Fuji Film, Tokyo, Japan).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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