Published as: *Plant J*. 2008 August ; 55(4): 543–554.

Wheat FT protein regulates *VRN1* transcription through interactions with FDL2

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SUMMARY

A precise regulation of flowering time is central to plant species survival. Therefore, mechanisms have evolved in plants to integrate different environmental cues to optimize flowering time. In this study we show that the wheat gene *Ta*FT, which integrates photoperiod and vernalization signals promoting flowering, interacts with bZIP proteins *Ta*FDL2 and *Ta*FDL6. We also show that *Ta*FDL2 can interact *in vitro* with five ACGT elements in the promoter of the meristem identity gene *VRN1*, suggesting that *TaFDL2* is a functional homologue of Arabidopsis *FD*. No direct interactions between the *Ta*FT protein and the *VRN1* promoter were detected. Transgenic wheat plants overexpressing *TaFT* showed parallel increases in *VRN1* transcripts, suggesting that *Ta*FT provides transcriptional activation to *VRN1*, possibly through interactions with the *Ta*FDL2 protein. The same transgenic plants also showed increased transcript levels of *TaFT2* (a *TaFT* paralogue) indicating that *TaFT2* acts downstream of *TaFT*. The fact that *Ta*FT2 interacts with the different bZIP protein *Ta*FDL13, which lacks the ability to interact with the *VRN1* promoter, suggests that *TaFT* and *TaFT2* have different gene targets. This observation agrees with the functional divergence observed for the *TaFT* and *TaFT2* orthologous genes in rice. The temperate cereals analyzed so far show *VRN1* transcripts in the leaves, a characteristic not observed in Arabidopsis or rice. The high levels of *TaFDL2* transcripts observed in wheat leaves provide a simple explanation for this difference. We present a hypothesis to explain the conservation of *VRN1* expression in the leaves of temperate cereals.

Keywords

wheat; flowering; FT; FD; VRN1; AP1

INTRODUCTION

The precise control of flowering is central to plant reproductive success and species survival. Optimization of flowering time to maximize grain yield is also an important target in cereal breeding programs. Plants have evolved mechanisms to integrate different environmental signals, including photoperiod and vernalization, to flower under conditions that optimize seed production. These different environmental signals are perceived by separate parts of the plant and their integration requires a precise spatial and temporal

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coordination. Photoperiod, for example, is perceived by the leaves, whereas, cold temperature is perceived directly by the shoot apical meristem (SAM) (Bernier, 1988).

The photoperiod signal perceived in the leaves is transmitted to the SAM to induce flowering, as demonstrated by grafting experiment (reviewed in Zeevaart, 1976). This transmissible substance named “florigen” (Chailakhyan, 1937) was not identified until recently (Corbesier and Coupland, 2006). Studies in Arabidopsis and rice have now demonstrated that this flowering signal is the FLOWERING LOCUS T (FT) (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Lin, *et al.*, 2007; Mathieu, *et al.*, 2007; Tamaki *et al.*, 2007), a small globular protein similar to the RAF kinase inhibitors found in animals (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). Over-expression of *FT* is associated with early flowering in a wide range of plant species suggesting a highly conserved function (Böhlenius *et al.*, 2006; Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Kojima *et al.*, 2002; Lifschitz *et al.*, 2006; Lin *et al.*, 2007; Yan *et al.*, 2006).

FT is the primary target of *CONSTANS* (*CO*), a B-box zinc finger CCT protein that plays a central role in the photoperiod pathway (Putterill *et al.*, 1995; Robson *et al.*, 2001; Wigge *et al.*, 2005). When Arabidopsis plants are exposed to long days (LD), *CO* activates *FT* in the leaf vascular tissue (phloem) (An *et al.*, 2004). The FT protein moves then through the phloem to the SAM, where it interacts with the bZIP transcription factor FD to activate the expression of the MADS-box meristem identity gene *APETALA 1* (*AP1*) (Abe *et al.*, 2005; Corbesier *et al.*, 2007; Wigge *et al.*, 2005). The FT-FD protein interaction is spatially regulated by the preferential expression of *FD* in the SAM, and temporally by the integration of different environmental signals that converge to regulate *FT* (Wigge *et al.*, 2005). According to this model, FD provides specificity in the recognition of the DNA target and FT acts in concert with FD to transcriptionally activate *AP1* (Wigge *et al.*, 2005).

In the temperate cereals, the role of the *FT* homologues seems to be similar to the one described above for Arabidopsis. The over-expression of *TaFT* (*Ta* indicates *Triticum aestivum* L.) in transgenic wheat plants significantly accelerates flowering relative to the non-transgenic controls, suggesting a conserved role as flowering promoter (Yan *et al.*, 2006). In addition, both *TaFT* and *HvFT* (*Hv* indicates *Hordeum vulgare* L.) are up-regulated by long days, and increased transcript levels correlate with accelerated flowering times (Turner *et al.*, 2005; Yan *et al.*, 2006). Arabidopsis and the temperate cereals differ in the spatial transcription profile of the main target of *FT*, *AP1* in Arabidopsis and the homologous *VRN1* in temperate cereals. In wheat and barley *VRN1* is normally expressed in the leaves at high levels (Schmitz *et al.*, 2000; Yan *et al.*, 2003) whereas in Arabidopsis, *AP1* transcripts are either not detected in the leaves or present at very low levels (e.g. in the vascular tissues of cotyledons, Abe *et al.*, 2005). Interestingly, the ectopic expression of *FD* in 35S::FD transgenic Arabidopsis plants results in high expression of *AP1* in the leaves (Wigge *et al.*, 2005). Based on this result we hypothesized that a wheat functional homologue of Arabidopsis *FD*, designated as *TaFD*-like (*TaFDL*, hereafter), should be normally expressed in leaves.

The understanding of the role of *TaFT* in the regulation of flowering in the temperate cereals is complicated by the existence of a similar (78% identical) paralogous copy in both wheat

(*TaFT2*) and barley (*HvFT2*) (Faure *et al.*, 2007; Yan *et al.*, 2006). Since the *TaFT-TaFT2* duplication occurred after the divergence between the grasses and Arabidopsis, this duplication is independent of the *FT-TSF* (*Twin Sister of FT*) duplication in Arabidopsis.

In this study we provide evidence that *TaFT2* is regulated by *TaFT* and that these two genes interact with different *TaFDL* partners. We also show that only one of the *Ta*FDL proteins that interact with *Ta*FT was able to interact with the *VRN1* promoter. The expression of this particular *TaFDL* gene in the leaves provides a simple explanation for the presence of *VRN1* transcripts in the leaves of temperate cereals. Finally, we discuss a hypothesis for the putative role of *VRN1* expression in the leaves of temperate cereals.

RESULTS

FD-like genes are transcribed in wheat leaves

Using cDNA samples from leaves of hexaploid wheat Chinese Spring at the 5th-leaf stage and primer pairs for 16 *TaFDL* genes (Table S1) we were able to amplify five different *FDL* genes. The cDNA sequences of these genes and their predicted protein sequences were deposited in GenBank: *TaFDL2* (EU307112), *TaFDL3* (EU307113), *TaFDL6* (EU307114), *TaFDL13* (EU307115) and *TaFDL15* (EU307116). This result confirmed that at least some *TaFDL* genes are transcribed in the leaves.

A multiple sequence alignment of the five predicted wheat FD-like proteins and the Arabidopsis proteins FD and FDP is presented in Fig. 1. The alignment shows a conserved region of 69 amino acids without gaps including perfectly conserved bZIP domains (N-x_7-R-x_9-L-x_6-L-x_6-L). The regions outside this 69 amino acids region were not well conserved among the different proteins and were excluded for the tree construction (Fig. 1).

The *Ta*FT protein interacts with the bZIP proteins *Ta*FDL2 and *Ta*FDL6

Yeast two-hybrid assays were used to test the interactions between *Ta*FT and the different *Ta*FDL proteins. We first performed auto-activation tests and confirmed that *Ta*FT was not able to activate the reporter genes when used alone as bait or prey. The *Ta*FT bait construct was then co-transformed into yeast with individual prey constructs for *Ta*FDL2, *Ta*FDL3, *Ta*FDL6, *Ta*FDL13, and *Ta*FDL15.

The two proteins used as positive controls, Murine p53 and SV40 large T-antigen (Iwabuchi *et al.*, 1993; Li and Fields, 1993), showed the expected interaction on the SD –Leu –Trp –His –Ade medium (Figure 2B). Under the same selective conditions, *Ta*FDL2 and *Ta*FDL6 also showed very strong interactions with the *Ta*FT bait (Figure 2B). A *Ta*FDL6 truncation construct carrying only the bZIP domain showed a weaker interaction with the *Ta*FT bait than the full-length protein (data not shown). These interactions were validated using reverse bait / prey constructs. When used as bait, both *Ta*FDL2 and *Ta*FDL6 showed strong interactions with co-transformed *Ta*FT prey construct (data not shown).

*Ta*FT2 protein interacts with *Ta*FDL13

We also investigated the interactions between *Ta*FDL proteins with *Ta*FT2. Auto-activation tests confirmed that *Ta*FT2 was not able to activate the reporter genes when used alone as bait or prey or in combination with *Ta*FT (Fig. 3).

Interestingly, co-transformation of *Ta*FT2 bait with *Ta*FDL2, *Ta*FDL3, *Ta*FDL6, *Ta*FDL13, and *Ta*FDL15 prey constructs showed different results from those observed with *Ta*FT. Whereas *Ta*FT showed strong interactions with *Ta*FDL2 and *Ta*FDL6, *Ta*FT2 interacted only with *Ta*FDL13 among these five FD-like proteins (Fig. 3). This interaction was also validated using the reverse bait / prey combination, in which *Ta*FDL13 was used as a bait construct and *Ta*FT2 as a prey (data not shown).

*Ta*FDL2 protein binds to bZip binding sites in the *VRN1* promoter *in vitro*

Members of the bZIP transcription factor family exhibit DNA binding specificity to DNA motifs with an ACGT core sequence including the A box (TACGTA), G box (CACGTG), C box (GACGTC) (Foster *et al.*, 1994; Izawa *et al.*, 1993), and hybrid C-box/G-box motifs (Martinez-Garcia *et al.*, 1998). In Arabidopsis, a C-box present in the *AP1* promoter has been suggested as the binding site for the interaction between the FT-FD protein complex and the *AP1* promoter (Wigge *et al.*, 2005). Since this is a critical step in the initiation of the flowering cascade, we investigated if the same interaction can occur in wheat.

The *VRN1* (the *AP1* homologue) promoter region in *T. monococcum* most likely does not extend beyond 2.3 kb upstream from its start codon, since an un-interrupted 67-kb stretch of nested retroelements was found upstream from this region (AY188331). Within this 2.3 kb region we found a G box and four putative hybrid boxes (Fig. 4).

Four segments of the *VRN1* promoter, each including one or two of the five putative binding sites (presented at scale in Fig. 4) were radiolabeled as DNA probes. The purified bZIP proteins *Ta*FDL2, *Ta*FDL6 and *Ta*FDL13, which showed interactions with either *Ta*FT or *Ta*FT2 in the yeast two-hybrid assays, were used with the radiolabeled DNA probes in the EMSA experiments. Among these three bZIP proteins, only the *Ta*FDL2 protein was able to bind to the putative bZIP binding sites in the *TaVRN1* promoter, and none of them was able to bind to the CArG box located approximately 160 bp upstream from the *VRN1* start codon (Fig. 4). To determine the binding specificity of *Ta*FDL2 oligonucleotides including either four copies of the wild type bZIP binding sequence or a mutant version in which the ACGT core sequences were replaced by AATT (Table S3) were tested in binding assays and competition experiments. As shown in Fig. S1A, *Ta*FLD2 binds specifically to the wild-type bZIP sequence in a concentration dependent manner, and is out competed by an excess of un-labeled cold oligonucleotides. Furthermore, the mutation of ACGT to AATT in the bZIP binding sequence completely abolishes the interactions (Fig. S1B).

In a separate EMSA experiment, five overlapping segments covering the complete (2.3-kb) *VRN1* promoter region were used as probes in binding reactions with *Ta*FT protein. No interactions were observed (data not shown).

Spatial and temporal localization of *TaFDL2* transcripts

Our yeast two-hybrid assays and EMSA experiments confirmed that the wheat protein *Ta*FDL2 interacts with *Ta*FT and with the *VRN1* promoter in a similar way as described for the homologous FT and FD proteins in Arabidopsis. Therefore, to understand the spatial and temporal regulation of *VRN1* in wheat it is important to know the spatial and temporal transcription profiles of *TaFDL2*. For comparative purposes we also tested *TaFDL6* and *TaFDL13* transcript levels.

Abundant *TaFDL2* transcripts were detected by RT-PCR in the RNA samples extracted from leaves of vernalized and unvernalized plants of common winter wheat variety Jagger (Fig. 5). Two RNA samples from vegetative and early reproductive SAM (including a small portion of the crown) extracted from Chinese Spring plants, also showed abundant *TaFDL2* transcripts. RT-PCR analyses for the other *TaFDL* genes showed that *TaFDL6* transcript levels were even more abundant than *TaFDL2* in all these tissues; whereas *TaFDL13* transcripts were more abundant in the apices than in the leaves (Fig. 5).

A quantitative RT-PCR analysis of cDNA samples from leaves further confirmed the high transcript levels of *TaFDL2*. We used available cDNA samples from spring wheat lines Chinese Spring (CS) and CS (Hope 7B) chromosome substitution line (dominant *TaFT* allele), previously characterized for *TaFT* and *VRN1* (Yan *et al.*, 2006). In all the samples, we found high levels of *TaFDL2* transcripts (close to those of *ACTIN*). We detected no significant differences in *TaFDL2* transcript levels between the two lines, or during the six weeks of vernalization at 4°C (Fig. S2). Plants kept at room temperature showed an approximately 2-fold increase in *TaFDL2* transcript levels during the six weeks, but the differences were not significant (Fig. S2). In our previous study, these same samples showed significant increases in *TaFT* and *VRN1* transcript levels with development (in both vernalized and unvernalized plants), and higher transcript levels in CS (Hope 7B) than in CS (Yan *et al.*, 2006)

Transcript levels of *TaFT* and *VRN1* are correlated

We have shown that *TaFDL2* transcripts are abundant in both leaves and apices, and do not seem to be a limiting factor in the induction of *VRN1*. Therefore, if the interactions described above between *Ta*FDL2, *Ta*FT and the *VRN1* promoter also occur *in planta*, modifications of the transcription levels of *TaFT* should be paralleled by the *VRN1* transcript levels. To test this prediction we used two independent transgenic hexaploid wheat lines Tr-1 and Tr-2, generated in a previous study in the winter variety Jagger (Yan, et al. 2006). These transgenic plants exhibit increased levels of *TaFT* as a result of the insertion of a dominant *TaFT* allele for spring growth habit from the variety Hope (Yan, et al. 2006).

TaFT transcript levels in the leaves of unvernalized plants from both transgenic lines (LD photoperiod) were significantly higher than in the non-transgenic sister-lines or Jagger, at the two developmental stages tested (Figure 6). Transcript levels at the 5th leaf stage were approximately 5-fold higher than at the 1st leaf stage and more than 8-fold higher than *ACTIN*. The *VRN1* transcript levels paralleled the *TaFT* ones, with significantly higher *VRN1* transcript levels in transgenic plants than in the non-transgenic controls (Figure 6),

and higher transcript levels at the 5th leaf-stage than at the 1st leaf stage. In this experiment, non-transgenic control plants took approximately 124 days from sowing to heading, whereas Tr-1 and Tr-2 plants headed on average 46 and 44 days after sowing, approximately 80 days earlier than the non-transgenic control.

A 40-fold (1st leaf stage) to 500-fold (5th leaf-stage) increase in *TaFT2* transcript levels was observed in the *TaFT* transgenic lines relative to the controls, suggesting that *TaFT2* may be regulated directly or indirectly by *TaFT*. In the non-transgenic plants the transcript levels of *TaFT2* were 20- to 100-fold lower than those of *TaFT*.

DISCUSSION

bZIP transcription factors

The bZIP transcription factors are characterized by a basic region that binds DNA and a leucine zipper dimerization motif. This family is found in all eukaryotes but has more members in plants than in humans (*Homo sapiens*) or worm (*Caenorhabditis elegans*) (Riechmann *et al.*, 2000). Approximately 75 different bZIP transcription factors involved in pathogen resistance, light and stress signaling, flower development, and seed maturation have been described in Arabidopsis (Jakoby *et al.*, 2002).

The bZIP transcription factor FD plays a central role in the regulation of flowering in Arabidopsis (Wigge et al. 2005, Abe et al. 2005). FD is expressed in the SAM before floral induction, and its transcript levels increase with time after germination under short or long day photoperiods (Abe et al. 2005). Under inductive photoperiod, the FT protein is expressed in the phloem and travels to the shoot apex where it interacts with FD (Corbesier *et al.*, 2007; Jaeger, *et al*., 2007; Mathieu, *et al.*, 2007; Tamaki *et al.*, 2007). The interaction between these two proteins *in planta* has been demonstrated in Arabidopsis by both bimolecular fluorescent complementation (Abe *et al.*, 2005) and chromatin immunoprecipitation (ChIP) using FT antibodies in plants overexpressing FD (Wigge *et al.*, 2005).

The ChIP experiment showed enrichment for the C-box region in the *AP1* promoter only under conditions promoting the expression of FT, suggesting that a stable FT-FD-*AP1* complex was formed in Arabidopsis (Wigge et al 2005). The presence of a similar interaction in wheat is indirectly supported by the *in vitro* interactions (*Ta*FT –*Ta*FDL2 and *Ta*FDL2-*VRN1* promoter) described before and by the spatial correlation between the expression of *TaFDL2* and *VRN1*. High levels of *TaFDL2* and *VRN1* are observed in the leaves of wheat plants expressing *TaFT*, paralleling the induction of *AP1* in the leaves of Arabidopsis when *FD* is expressed ectopically in this tissue (Wigge *et al.*, 2005).

To explore further if a similar *Ta*FT-*Ta*FDL2-*VRN1* complex could be also detected in wheat we included both *Ta*FT and *Ta*FDL2 proteins in the same binding reaction with the *VRN1* promoter segments (SOM). Unfortunately, we failed to observe any supershift relative to the *Ta*FDL2 protein used alone (SOM). These results suggest that either there is no stable *Ta*FT-*Ta*FDL2-*VRN1* complex in wheat, or more likely that the conditions used in the binding assays were not conductive to the formation of a stable complex. It is also possible

that additional proteins are necessary to stabilize this complex. ChIP studies will be necessary to confirm the existence of a *Ta*FT-*Ta*FDL2-*VRN1* complex in wheat.

The identification of wheat FD homologue was not a trivial task due to the limited sequence conservation among bZIP transcription factors, which is generally limited to the bZIP motif (Fig. 1). Therefore, we relied on the simultaneous interaction of the *Ta*FDL candidates with *Ta*FT in yeast-two-hybrid tests and with the *VRN1* promoter in EMSA experiments to select the best candidate. Only *Ta*FDL2 fulfilled both selection criteria among the five *TaFDL* genes identified in wheat leaves, making it the best candidate for a functional homologue of Arabidopsis FD.

Expression results showed that *TaFDL2* transcripts accumulate in the vegetative and reproductive apices (Fig. 5) suggesting that the *Ta*FDL2 protein is present in the correct tissue and developmental stage to be involved in flowering induction once *Ta*FT becomes available.

Duplication and functional differentiation of *FT* and *FT2* genes

Phylogenetic and comparative mapping analyses have shown that orthologues of wheat *TaFT* and *TaFT2* are present in barley and rice, indicating that the duplication that originated these paralogous genes predated the divergence of the grasses (Faure *et al.*, 2007; Yan *et al.*, 2006). The same phylogenetic analyses also showed that the *TaFT-TaFT2* duplication occurred after the divergence of the grass species from the dicots. Therefore, this duplication is independent from the *FT-TSF* (*Twin Sister of FT*) duplication in the Arabidopsis lineage (Faure *et al.*, 2007; Yan *et al.*, 2006). Arabidopsis *tsf* mutations delay flowering and enhance the phenotype of *ft* mutants; whereas *TSF* over-expression causes precocious flowering under short day (Michaels *et al.*, 2005; Yamaguchi *et al.*, 2005).

Unfortunately, similarities and differences between Arabidopsis *FT* and *TSF* (Michaels *et al.*, 2005; Sung *et al.*, 2006; Yamaguchi *et al*., 2005) cannot be used to infer the functions of *TaFT* and *TaFT2* in the grasses because independently duplicated genes may have different sub-functionalization. Therefore, the specific roles of the *TaFT* and *TaFT2* genes need to be studied directly in the grass species. Detailed microcolinearity studies between barley *HvFT* gene region on chromosome arm 7HS and the rice region on rice chromosome arm 6S including *Hd3a* (the functional orthologue of Arabidopsis *FT*) have confirmed that these genes are true orthologues (Yan *et al.*, 2006). However, this relationship is complicated by a recent tandem duplication that occurred only in the rice lineage resulting in closely linked genes *Hd3a* (=*OsFTL2*) and *RFT1* (=*OsFTL3*) (Izawa *et al.*, 2002; Kojima *et al.*, 2002; Yan *et al.*, 2006). Comparative mapping studies also confirmed that *HvFT2*, located in the short arm of barley chromosome 3H, is orthologous to rice *OsFTL1*, which is located on the colinear region of the short arm of rice chromosome 1 (Faure *et al.*, 2007).

In rice, a short day plant, *Hd3a* transcript levels are rapidly induced under SD, but this effect can be suppressed by short exposures to light in the middle of the night (night breaks). These characteristics are not observed for other rice *FT*-like genes (including *OsFTL1* and *OsFTL3*) suggesting that *Hd3a* is the central gene in the photoperiod pathway in rice (Ishikawa *et al.*, 2005). Recent studies have confirmed that the Hd3a protein moves from the

leaves to the apices where it induces flowering, confirming that it is the functional homologue of Arabidopsis *FT* (Tamaki *et al.*, 2007).

Whereas over-expression of *Hd3a* results in early flowering in rice (Kojima *et al.*, 2002), over-expression of *OsFTL1* produces more complex phenotypes including elongation of internodes, loss of apical dominance, and a terminal tissue at the apical meristem (Izawa *et al.*, 2002). This terminal tissue is composed of multiple glumes, and occasionally a terminal floret at the tip instead of a panicle, suggesting that *OsFTL1* may be involved in the regulation of panicle and floral development rather than in the regulation of flowering initiation, which is mainly regulated by *Hd3a* (Izawa *et al.*, 2002).

The different functions described above for *Hd3a* and *OsFTL1* may also apply to the orthologous wheat *TaFT* and *TaFT2* genes. The finding that these wheat paralogues interact with different FD-like proteins provides a putative molecular explanation for a functional differentiation. *Ta*FT interacts with the *Ta*FDL2 and *Ta*FDL6 proteins, whereas *Ta*FT2 interacts with *Ta*FDL13, which belongs to a different clade of FD-like proteins (Fig. 1). We showed here that the interaction between *Ta*FT and *Ta*FDL2 likely confers *Ta*FT the ability to regulate *VRN1* transcription and therefore, to affect the fate of the SAM. On the contrary, *Ta*FDL13 (the *Ta*FT2 partner) showed no interactions with the *VRN1* promoter. Since *Ta*FDL13 is mainly expressed in the apical region, it may provide *TaFT2* specificity for targets expressed in this tissue.

A role of *TaFT2* in processes occurring after the SAM reproductive differentiation is also supported by the transcription profiles of *HvFT2*. The proteins coded by *TaFT2* and *HvFT2* are 98% identical (excluding 7 initial amino acids) suggesting that they may also share similar structural and functional characteristics. Faure et al. (2007) found that *HvFT2* is upregulated by LD, but this up-regulation occurs two to three weeks after the differentiation of the SAM. Our results in wheat exhibit a similar trend. The transcript levels of *TaFT* in the transgenic plants overexpressing *TaFT* are very high from the first leaf, whereas *TaFT2* transcript levels were low at the first-leaf stage and reached high levels at the fifth–leaf stage (Fig. 6, E–F), suggesting a delayed induction relative to *TaFT*. In the non-transgenic control plants the transcript levels of *TaFT* were 40–70 fold higher than those of *TaFT2*, also suggesting a more central role of *TaFT*. More importantly, the strong upregulation of *TaFT2* in the two *TaFT* overexpressing transgenic wheat lines relative to the control suggests that *TaFT2* may be regulated directly or indirectly by *TaFT* (Fig. 6, E–F).

Similarities between the Arabidopsis and wheat flowering pathways

FT transcripts in Arabidopsis and wheat are rapidly upregulated upon transfer of plants to LD (Imaizumi *et al*., 2003; Faure *et al.*, 2007; Turner *et al.*, 2005; Yan *et al.*, 2006). In addition over-expression of *FT* in transgenic plants results in precocious flowering confirming the conserved role of this gene as a promoter of flowering (Kobayashi, *et al*., 1999; Yan *et al.*, 2006). The observed protein-protein interaction between *Ta*FT and *Ta*FDL2, and protein-DNA interaction between *Ta*FDL2 and the *VRN1* promoter suggest that the molecular interactions downstream of *FT* are also conserved between wheat and

Arabidopsis. These interactions are incorporated into our current model of flowering regulation in temperate cereals (Fig. 7).

The model presented in Fig. 7 shows the interaction between *Ta*FDL2 and the G-box and hybrid boxes in the *VRN1* promoter (Fig. 4). A similar protein-DNA interaction has been previously identified in Arabidopsis, where FD can bind to a 130-bp region of the *AP1* promoter including a C-box motif (Wigge *et al.*, 2005).

In this study, we observed a close correlation between *VRN1* and *TaFT* transcript levels in transgenic wheat plants overexpressing *TaFT*. Lower transcript levels of *TaFT* in the transgenic plant Tr-1 relative to Tr-2, and in the 1st leaf stage relative to the 5th leaf stage (Fig. 6, A–B) were paralleled by similar differences in *VRN1* transcript levels (Fig. 6, C–D). More importantly, *VRN1* transcript levels were 500- to 1000-fold higher in the transgenic wheat plants over-expressing *TaFT* than in the non-transgenic control plants. Additional experiments in Arabidopsis showed that the addition of a strong transcriptional activation domain to FT increases its flowering promoting activity (Wigge *et al.*, 2005). Taken together, these results suggest that *TaFT* is a limiting factor in the activation of *VRN1*.

Since we detected no binding between the *Ta*FT protein and the *VRN1* promoter by EMSA (data not shown), it is unlikely that *Ta*FT can regulate the transcription of *VRN1* through a direct protein-DNA interaction. These results, combined with the observed yeast two-hybrid interaction between *Ta*FDL2 and *Ta*FT (Figure 2) and the *TaFDL2* interaction with the *VRN1* promoter, suggest a model in which *Ta*FT and *Ta*FDL2 act in concert to regulate the transcription of *VRN1* (Figure 7).

Differences between the Arabidopsis and wheat flowering pathways

Although the conservation of the last steps of the photoperiod pathway suggests that their origin predates the divergence of the monocot and dicot lineages, the differences in the vernalization pathways between Arabidopsis and the temperate cereals suggest independent origins. This is consistent with the view that the temperate Pooideae grasses are a specialized monophyletic group that evolved from subtropical bambusoid-like species (Clayton and Renvoize, 1986; Preston and Kellogg, 2008).

The central repressor in the vernalization pathway of Arabidopsis, the MADS-box gene *FLOWERING LOCUS C* (*FLC*) (Michaels and Amasino, 1999; Sheldon *et al.*, 1999), has not been found in the grass species; whereas *VRN2*, the central repressor of flowering in the vernalization pathway of the temperate cereals, has not been found in Arabidopsis (Yan *et al.*, 2004b). *FLC* delays flowering by interacting with regulatory regions of *FT* in the leaves and *SOC1* in the meristems (Helliwell *et al.*, 2006; Searle *et al.*, 2006; Wigge *et al.*, 2005). Vernalization permanently down-regulates *FLC* releasing *FT* and *SOC1* to promote the transcription of *AP1* (Michaels and Amasino, 1999).

In the temperate cereals, *VRN2* is also down-regulated by vernalization (Yan *et al.*, 2004b), but in contrast to *FLC*, it also can be down-regulated by short days (Dubcovsky *et al.*, 2006; Trevaskis *et al.*, 2006) (Fig. 7). The position of *VRN2* upstream of *VRN1* and *TaFT* in the model is supported by results from transgenic winter wheat plants with reduced *VRN2*

transcript levels (Yan *et al.*, 2004b) and isogenic lines for *VRN2* (Yan *et al.*, 2006). Plants with reduced or non-functional *VRN2* transcripts showed up-regulation of *TaFT* and *VRN1* and a significant acceleration of flowering (Yan *et al.*, 2004b, 2006). The increase in *VRN1* transcript levels is then followed by down-regulation of *VRN2* (Fig. 7) suggesting the existence of a regulatory feedback loop that has not been described in Arabidopsis (Loukoianov *et al.*, 2005).

Arabidopsis and the temperate cereals also differ in the genes that show natural variation in vernalization requirement. In Arabidopsis, most of the natural mutants with reduced or non-vernalization requirement are concentrated in *FRI* and *FLC* (Gazzani *et al.*, 2003; Michaels, *et al.*, 2003); whereas in the temperate cereals they were detected in *VRN1* (Fu *et al.*, 2005; Yan *et al.*, 2004a; Yan *et al.*, 2003), *VRN2* (Cockram *et al.*, 2007; vonZitzewitz *et al.*, 2005; Yan *et al.*, 2004b), and *TaFT* (Yan *et al.*, 2006). The known natural mutations for *TaFT* and *VRN1* are dominant for spring growth habit and are located within regulatory, rather than coding regions of these genes, suggesting the disruption of recognition sites for a flowering repressor, likely *VRN2*.

An additional characteristic that seems to be unique for the temperate cereals is the high level of expression of *VRN1* in the leaves (Schmitz *et al.*, 2000; Yan *et al.*, 2003). In contrast, *AP1* transcripts are abundant in the induced SAM and floral primordia in Arabidopsis, but are undetectable or present at much lower levels in some vegetative tissues (e.g. vascular tissues of cotyledons, Abe *et al.*, 2005). Interestingly, transgenic 35S:FD Arabidopsis plants with ectopic expression of *FD* show high levels of *AP1* transcripts in the leaves (Wigge *et al.*, 2005), suggesting that the spatial differences between *AP1* and *VRN1* transcription profiles are the result of differences between the spatial transcription profiles of Arabidopsis *FD* and its wheat homologue (*TaFDL2*). The results presented here support this hypothesis. We first identified *Ta*FDL2 as the functional homologue of Arabidopsis FD by its ability to interact with *Ta*FT and the *VRN1* promoter, and then showed that *TaFDL2* exhibits high transcript levels in the leaves of both vernalized and unvernalized winter wheat plants from very early developmental stages (Fig. 5). The simultaneous presence of *TaFDL2* and *TaFT* in the leaves of spring or vernalized winter wheat plants provides a simple explanation for the simultaneous presence of *VRN1*. The regulation of *TaFT* by different environmental cues provides temporal specificity to this interaction.

Although the results presented above explain the molecular mechanism by which *VRN1* transcription is regulated in the leaves, they do not explain why such a complex regulation of a meristem identity gene has been conserved in the leaves of the temperate grasses analyzed so far. Upregulation of *VRN1* transcripts in the leaves of winter genotypes has been confirmed in wheat (Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003), barley (Trevaskis *et al.*, 2003), *Lolium* (Petersen *et al.*, 2004), and oats (Preston and Kellogg, 2008) suggesting conservation across several tribes of temperate cereals (Triticeae, Poeae, and Aveneae).

An interesting observation is that *VRN1* transcript levels are negatively associated with the accumulation of *COR* (cold responsive) genes and with the degree of frost tolerance (Danyluk *et al.*, 2003). Frost tolerance increases during the acclimation of wheat plants to

cold but non-freezing temperatures, but decreases after the transition between the vegetative and reproductive apices. Near isogenic lines for the *VRN1* gene carrying the recessive *vrn1* allele (winter growth habit) can tolerate 11°C lower freezing temperatures than lines carrying the dominant *Vrn1* allele (spring growth habit) (Limin and Fowler, 2006). Similarly, spring lines grown under SD, which down-regulates *VRN1* transcript levels, can tolerate 8.5°C lower temperatures than the same lines under LD (Limin and Fowler, 2006). A similar result has been reported for barley. In a double-haploid population segregating for *VRN-H1* lines carrying the recessive *vrn-H1* allele showed higher *CBF* (C-repeat binding factors) transcript levels than those carrying the dominant *Vrn-H1* allele (Stockinger *et al.*, 2007). In addition, lines grown under SD (reduced *VRN-H1* levels) showed higher *CBF* transcript levels than lines grown under LD. *CBF* transcription factors are rapidly upregulated by cold temperatures, inducing the expression of *COR* genes and playing a critical role in cold acclimation and frost tolerance in temperate cereals (Francia *et al.*, 2004; Knox *et al.*, 2008; Miller *et al.*, 2006; Skinner *et al.*, 2005; Vágújfalvi *et al.*, 2003). Based on these results, it is tempting to speculate that the temperate cereals have developed the ability to use the presence of VRN1 in the leaves as a signal to down-regulate the cold-tolerance regulatory network. Since *VRN1* is upregulated upon the arrival of the spring, its presence would prevent the upregulation of the frost tolerance genes in the spring but not in the fall, when cold temperatures are an indication of future frost events.

CONCLUSION

In summary, we have identified the wheat functional homologue of Arabidopsis *FD*, *TaFDL2* and confirmed that the protein coded by this gene interacts with *Ta*FT and the *VRN1* promoter. Transcription of *TaFDL2* in the leaves provides a simple explanation for the characteristic expression of *VRN1* in the leaves in temperate cereals. We also showed here that *TaFT2* is partially regulated by *TaFT* and that the proteins coded by these two genes interact with different bZIP proteins providing the molecular basis for the sub-functionalization of these paralogous genes.

EXPERIMENTAL PROCEDURES

Wheat *FD*-like genes

The database used for the searches is available at The Gene Index (TGI) website (http://compbio.dfci.harvard.edu/tgi/). Starting with Arabidopsis FD protein sequence At4g35900, a TBLASTN search was carried out against wheat (*Triticum aestivum*) TGI Gene Index release 10.0 to search for *TaFD*-like genes in wheat. The top 16 gene sequences producing high-scoring segment pairs were chosen and further investigated. Primers were designed to amplify cDNAs for each of the top 16 *TaFD*-like genes (Table S1). Restriction sites (underlined) were included in the oligos to facilitate the cloning of the PCR products into yeast vectors.

A multiple sequence alignment and a Neighbor Joining phylogenetic tree were constructed using Mega v.4 (Tamura *et al.*, 2007). The tree was based on a conserved un-gapped 69 amino acid region including the bZIP domain (Fig. 1). Bootstrap confidence values for the nodes were based on 1000 iterations.

Yeast two-hybrid assays

The yeast cloning vectors pGBKT7 and pGADT7, control vectors pGADT7-T and PGBKT7-53, and the yeast strain AH109 used in the yeast two-hybrid assays were obtained from Clontech (Mountain View, CA, USA). The yeast two-hybrid assays were performed according to the manufacturer's instructions. Wheat full-length *TaFT* and *TaFT2* cDNAs were fused to GAL4 DNA binding domain of pGBKT7 or the GAL4 activation domain of pGADT7 to generate bait and prey constructs, respectively. Full-length coding regions of *TaFDL2*, *TaFDL6*, *TaFDL13* and *TaFDL15*, and a 375bp cDNA fragment of *TaFDL3* (including the putative bZIP binding domain) were cloned in frame with GAL4 activation domain into the prey vector pGADT7.

Table S2 describes the primers and restriction sites used to generate yeast bait and prey constructs. The appropriate plasmids were transformed into yeast strain AH109 using the lithium acetate method and selected on SD medium lacking leucine (Leu) and tryptophan (Trp). After 4 days of incubation at 30°C, yeast cells were re-plated out on selection plates containing SD medium lacking Leu, Trp, histidine (His) and adenine (Ade) for the interaction test.

For validation, coding regions of the three FDL proteins that showed interactions with either *Ta*FT or *Ta*FT2 bait constructs were switched from prey constructs to the bait vector pGBKT7 using restriction sites *Eco*RI and *Pst*I for *Ta*FDL2, *Bam*HI and *Pst*I for *Ta*FDL6, *Eco*RI and *Bam*HI for *Ta*FDL13. The new constructs were retested by yeast two-hybrid assays with *Ta*FT or *Ta*FT2 prey constructs.

Electrophoretic Mobility Shift Assays (EMSA)

TaFDL2, *TaFDL6* and *TaFDL13* cDNA fragments (Table S3) were cloned in frame with GST coding region into the corresponding sites of pGEX-6p-1 (GE Healthcare, Piscataway, NJ http://www4.gelifesciences.com/) to generate *Ta*FDL2-GST, *Ta*FDL6-GST and *Ta*FDL13-GST fusion constructs. Four *VRN1* promoter fragments containing one or two putative bZIP binding sites were generated by PCR (Table S3) and cloned in pGEM-T easy vector (Promega, Madison, WI, USA). Detailed EMSA protocols are described in the SOM.

Quantitative PCR and RT-PCR analyses

RNA samples were extracted from leaves and apices using the TRIZOL method (INVITROGEN). *ACTIN* was used as endogenous control for both the RT-PCR and the quantitative PCR SYBR GREEN® Applied Biosystems, http://www3.appliedbiosystems.com experiments, with primers described before (Dubcovsky *et al.*, 2006). SYBR GREEN® systems for *VRN1* and *TaFT* were developed in the previous study (Yan *et al.*, 2006). The primers for the new SYBR GREEN® systems and RT-PCR experiments for *TaFT2*, *TaFDL2*, *TaFDL6*, and *TaFDL13* are listed in Table S4. Quantitative PCR experiments were performed in an ABI7000. The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) was used to normalize and calibrate transcript values relative to the endogenous controls. For each experiment, the same calibrator was used across replications, genotypes and environmental conditions to make units comparable. However, different calibrators were used for different experiments and therefore, their units are not comparable.

RNA samples for the RT-PCR analysis of the different *TaFDL* transcript levels were extracted from leaves of unvernalized and vernalized (6 weeks at 4°C and LD) winter wheat variety Jagger. The apical region samples were extracted from the spring wheat variety Chinese Spring at the vegetative and early reproductive stages. Thirty plants were pooled to generate sufficient tissue. The apical region includes the SAM and a small portion of the crown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the United States Department of Agriculture Cooperative State Research, Education, and Extension Services National Research Initiative competitive grant 2007-35301-17737 and 2007-35301-18188. The authors thank Ann Blechl (USDA-Agricultural Research Service Albany) for the transgenic wheat plants.

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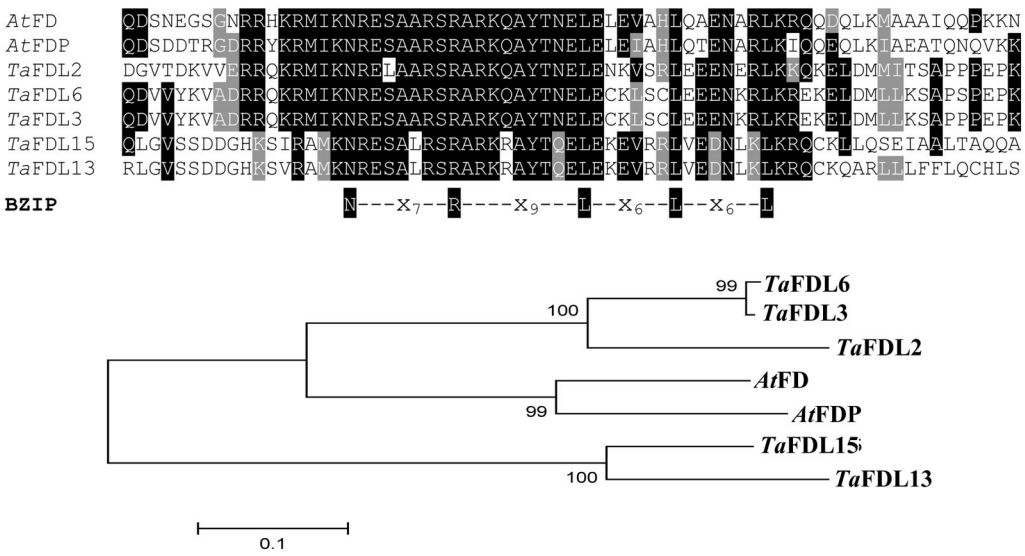

Figure 1.

Comparison among wheat and Arabidopsis proteins using MEGA4 (Tamura et al. 2007). Top: ClustalW multiple sequence alignment of Arabidopsis FD and FDP with five wheat FD-like protein sequences based on the bZIP domain and conserved un-gapped flanking regions. Sequences used in the alignment: *At*FD (At4g35900), *At*FDP (At2g17770), *Ta*FD-like protein sequences *TaFDL2* (EU307112), *TaFDL3* (EU307113), *TaFDL6* (EU307114), *TaFDL13* (EU307115) and *TaFDL15* (EU307116). Bottom: Neighbor joining tree based on the previous alignments. Numbers in the nodes indicate bootstrap confidence values based on 1000 iterations.

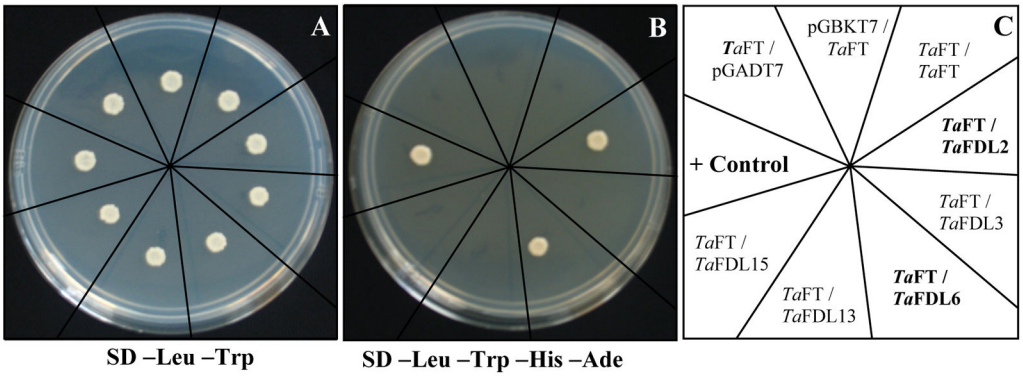

Figure 2.

Yeast two-hybrid assays using *Ta*FT bait. A) Yeast cells plated on SD-LEU-TRP; B) Equal amount of yeast cells as in A, but plated on SD-LEU-TRP-HIS-ADE; C) Diagram indicating the different bait / prey combinations used in the assay (baits are indicated first in each pair): Positive control: Murine P53 / SV40 T-antigen.

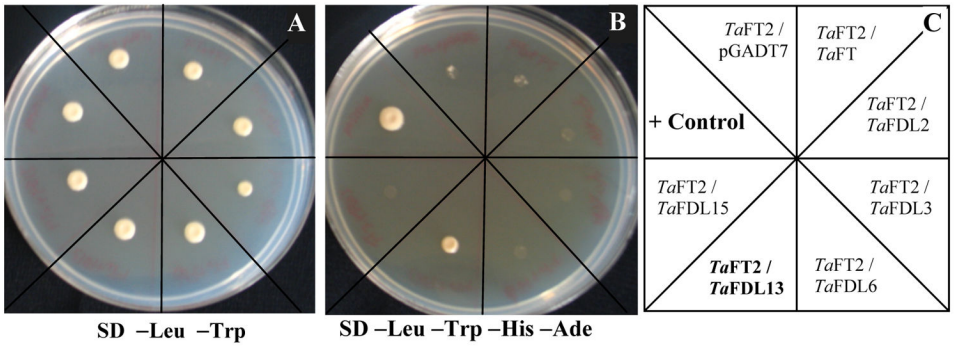

Figure 3.

Yeast two-hybrid assays using *Ta*FT2 as bait. A) Yeast cells plated on SD-LEU-TRP; B) Equal amount of yeast cells as in A, but plated on SD-LEU-TRP-HIS-ADE; C) Diagram indicating the different bait / prey combinations used in the assay (baits are indicated first in each pair): Positive control: Murine P53 / SV40 T-antigen.

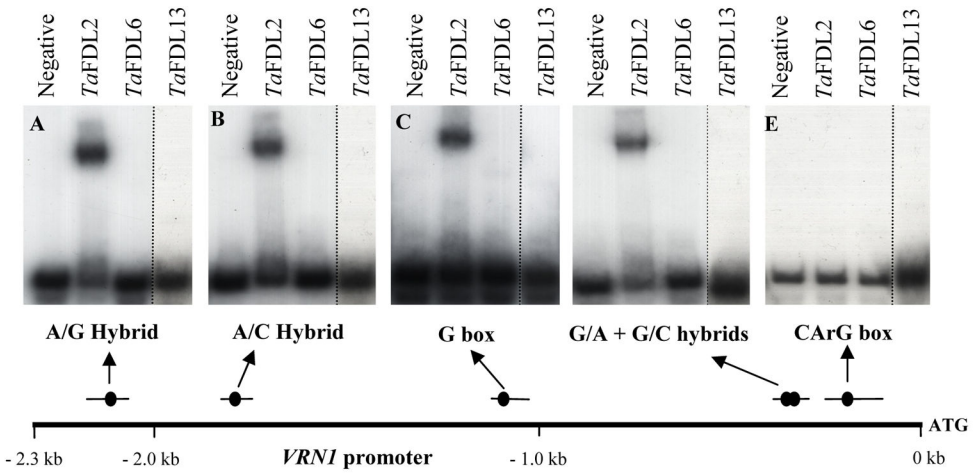

Figure 4.

Electrophoresis Mobility Shift Assays (EMSA). Potential bZIP binding sites present in the *VRN1* promoter were radiolabeled and used as probes in the binding reactions with purified recombinant proteins *Ta*FDL2, *Ta*FDL6, and *Ta*FDL13. The first lane in each panel labeled as 'Negative' contains the probe alone. *Ta*FDL13 was tested in a separate experiment. The four probes included the following potential bZIP binding sites: A) A/G hybrid of A- and G-boxes (TACGTG), B) A/C hybrid of A and C-boxes (TACGTC), C) G box (CACGTG), D) G/A hybrid of G and A-boxes (CACGTA) plus a G/C hybrid of G and C-boxes (CACGTC), E) CArG box. The bottom scheme indicates the position and size of the different probes and potential bZIP binding sites.

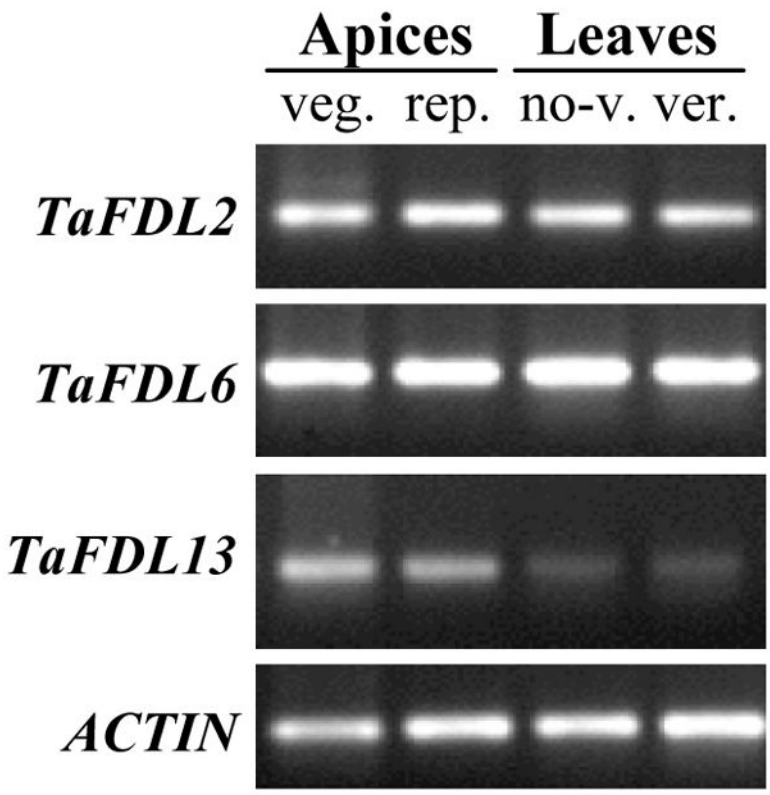

Figure 5.
Transcript levels of *TaFDL2*, *TaFDL6*, and *TaFDL13* in apices (spring wheat Chinese Spring) and leaves (winter wheat Jagger). *ACTIN* is included as endogenous control. Abbreviations: veg.= vegetative apex, rep.= reproductive apex, no-v= non vernalized leaves, ver.= vernalized leaves.

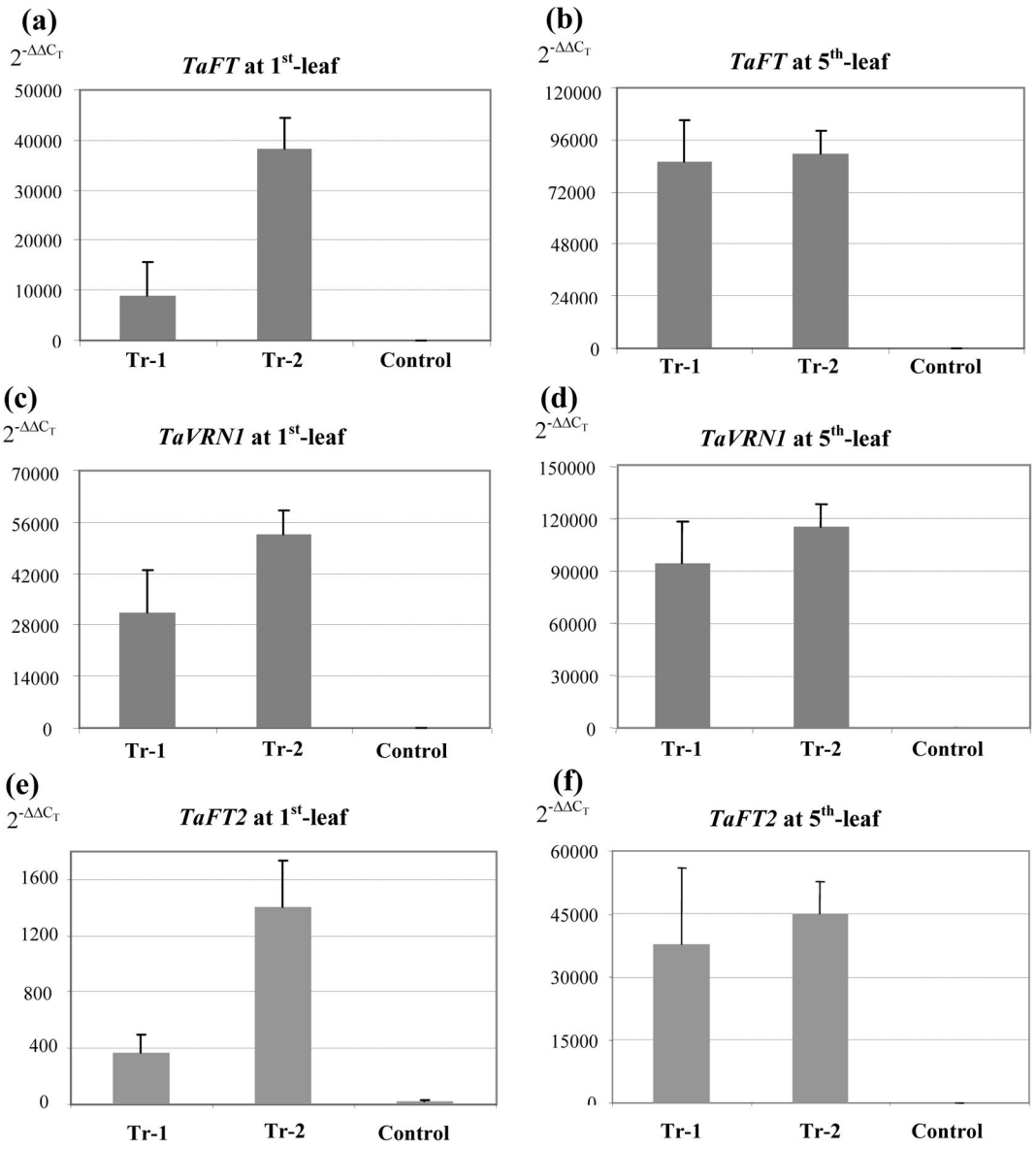

Figure 6.

Q-PCR of *TaFT*, *VRN1* and *TaFT2* in transgenic plants overexpressing *TaFT*. Tr-1 (average of 6 plants) and Tr-2 (average of 13 plants) are two independent transgenic lines. The controls include 9 non-transgenic sister plants and 10 Jagger plants. Plants were grown at 20–24 °C under LD (16light / 8 dark). Transcript levels for *TaFT* (A–B), *VRN1* (C–D), and *TaFT2* (E–F) were examined at the 1st-leaf stage (A, C, E) and the 5th-leaf stage (B, D, F). The same calibrator was used for *TaFT* and *VRN1*, so their units are comparable. A different corrector was used for *TaFT2* because of its low transcript levels relative to *TaFT* and *VRN1*, therefore their scales are not comparable. When using the same calibrator, *TaFT2* transcript levels were 40–70 fold lower than *TaFT* in the non-transgenic control plants.

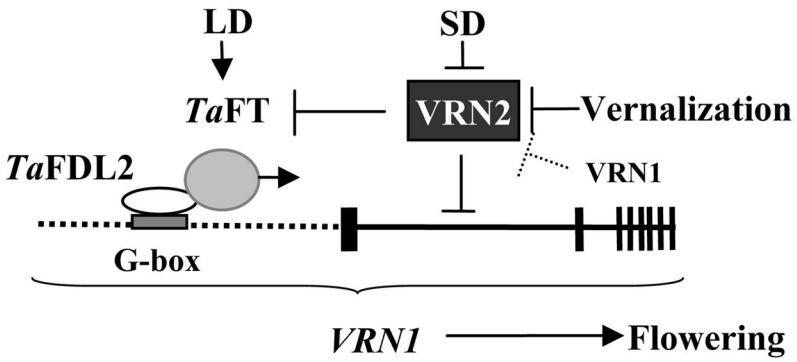

Figure 7.

Model for the regulation of flowering time in temperate cereals. The horizontal dotted line represents the *VRN1* promoter and the vertical boxes indicate the eight exons of *VRN1*. The white oval represents the *Ta*FDL2 protein interacting with the G-box (or hybrid boxes, grey rectangle) in the *VRN1* promoter. The grey circle represents the *Ta*FT protein interacting with *Ta*FDL2. "→" indicates induction and "⊥" indicates repression. The doted line from VRN1 to *VRN2* indicates a negative regulatory feedback loop. LD= long days, SD= short days.