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The *FLOWERING LOCUS T*-Like Gene Family in Barley (*Hordeum vulgare*)

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ABSTRACT

The *FLOWERING LOCUS T* (*FT*) gene plays a central role in integrating flowering signals in Arabidopsis because its expression is regulated antagonistically by the photoperiod and vernalization pathways. *FT* belongs to a family of six genes characterized by a phosphatidylethanolamine-binding protein (PEBP) domain. In rice (*Oryza sativa*), 19 PEBP genes were previously described, 13 of which are *FT*-like genes. Five *FT*-like genes were found in barley (*Hordeum vulgare*). *HvFT1*, *HvFT2*, *HvFT3*, and *HvFT4* were highly homologous to *OsFTL2* (the *Hd3a* QTL), *OsFTL1*, *OsFTL10*, and *OsFTL12*, respectively, and this relationship was supported by comparative mapping. No rice equivalent was found for *HvFT5*. *HvFT1* was highly expressed under long-day (inductive) conditions at the time of the morphological switch of the shoot apex from vegetative to reproductive growth. *HvFT2* and *HvFT4* were expressed later in development. *HvFT1* was therefore identified as the main barley *FT*-like gene involved in the switch to flowering. Mapping of *HvFT* genes suggests that they provide important sources of flowering-time variation in barley. *HvFTI* was a candidate for *VRN-H3*, a dominant mutation giving precocious flowering, while *HvFT3* was a candidate for *Ppd-H2*, a major QTL affecting flowering time in short days.

THE timing of flowering during the year is an important adaptive trait throughout the angiosperms. Correct flowering ensures the greatest chance of pollination, seed set, and dispersal, and therefore reproduction of the species. Flowering is regulated by environmental and internal cues and the genetic basis of this control is best understood in Arabidopsis where the photoperiod, vernalization, giberellic acid, and autonomous pathways have been defined (recently reviewed by Boss *et al.* 2004; Jack 2004; Bäurle and Dean 2006). The pathways' major point of convergence are genes called pathway integrators, which in Arabidopsis are *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) [also called *AGAMOUS-LIKE 20* (*AGL20*)], and, to a lesser extent, *LEAFY* (*LFY*) (Boss *et al.* 2004; Jack 2004; Moon *et al.* 2005; Bäurle and Dean 2006).

Each pathway does not have the same influence on each pathway integrator. In the case of the photoperiod pathway, which is our primary interest, *FT* is predominant. It was first identified in Arabidopsis (Kardailsky *et al.* 1999; Kobayashi *et al.* 1999) and was shown to be a direct target of the nuclear protein CONSTANS (CO) (Samach *et al.* 2000; Wigge *et al.* 2005). *CO* transcription is regulated by the circadian clock and peaks ~16 hr after dawn. This peak of expression has to correspond to a period of exposure to light for the CO protein to be stable and to induce *FT* expression, and by this process *FT* expression is restricted to long days (Suarez-Lopez *et al.* 2001; Searle and Coupland 2004; Valverde *et al.* 2004). Although CO was also shown to induce *SOC1* expression (Samach *et al.* 2000), recent data show that this is through the action of *FT* (Yoo *et al.* 2005). *FT* expression can be detected in the leaves, mainly in the vascular tissues (An *et al.* 2004), but also at the apical meristem where the FT protein interacts with FD, a bZIP transcription factor, to promote flowering (Abe *et al.* 2005; Wigge *et al.* 2005). It has been suggested that the *FT* mRNA itself moves from the leaves, where photoperiod is perceived, to the apex where flowering is promoted (Huang *et al.* 2005).

The genetic basis of photoperiod response has also been studied extensively in rice (*Oryza sativa*), a monocot in which flowering is promoted by short days. Despite the evolutionary separation from Arabidopsis and the contrasting flowering response, photoperiod pathway genes are well conserved, with *OsGI*, *Hd1*, and *Hd3a* being orthologous to Arabidopsis *GIGANTEA* (*GI*), *CO*, and *FT*, respectively (Yano *et al.* 2000; Hayama *et al.* 2002; Kojima *et al.* 2002). It was shown that *Hd1* represses *Hd3a* (*FT*) expression in long days (Hayama and Coupland 2004) but promotes *Hd3a* (*FT*) expression in short days, leading to flowering. These results show that variation in the *CO*–*FT* interplay is at the center of the long-day/short-day difference, but in both situations the induction of *FT* expression consistently promotes flowering.

The above data make *FT* of central interest to the photoperiodic regulation of flowering. However, interpreting

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession nos. DQ297407, DQ411319, DQ411320, and EF012202.

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the exact role of *FT* is complicated by variation in the structure of the *FT* family in different plants. In Arabidopsis, *FT* encodes a protein similar to a phosphatidylethanolamine-binding protein (PEBP), such as Raf kinase inhibitor (RKIP) from mammals (Kardailsky *et al.* 1999). It is a member of a small gene family, which includes five other genes: *TERMINAL FLOWER 1* (*TFL1*), *TWIN SISTER OF FT* (*TSF*), *ARABIDOPSIS THALIANA CENTRORADIALIS* (*ATC*), *BROTHER OF FT AND TFL1* (*BFT*), and *MOTHER OF FT AND TFL1* (*MFT*) (Kobayashi *et al.* 1999). *BFT* has not been implicated in flowering (Yoo *et al.* 2004), but constitutive expression of *FT*, *TSF*, and, to a lesser extent, *MFT* accelerates flowering (Kobayashi *et al.* 1999; Yoo *et al.* 2004; Yamaguchi *et al.* 2005). Constitutive expression of *TFL1* or *ATC* delays flowering (Mimida *et al.* 2001). However, FT and TFL1 proteins share ~59% amino acid identity and it was shown that swapping a single amino acid in the PEPB domain is sufficient to convert TFL1 to FT function and vice versa (Hanzawa *et al.* 2005).

Thirteen *FT*-like sequences have been found in the rice genome. These have been designated *OsFTL1*–*OsFTL13* with *Hd3a* corresponding to *OsFTL2* (Izawa *et al.* 2002; Chardon and Damerval 2005; Zhang *et al.* 2005). Chardon and Damerval (2005) show that the higher number in rice can be attributed, in part, to duplication of chromosome regions within the rice genome. These duplications are thought to predate the divergence of the major grass lineages (Paterson *et al.* 2003; Salse *et al.* 2004) and so eight *FT*-like genes were suggested to have been present in the grass ancestral genome. At least three *FT*-like genes (*OsFTL1*, *OsFTL2*, and *OsFTL3*) are known to be active and capable of promoting flowering in rice (Izawa *et al.* 2002).

Barley (*Hordeum vulgare*) is more closely related to rice than to Arabidopsis but resembles the latter in photoperiod response. Several photoperiod pathway gene homologs have been identified in barley, such as *HvGI*, *HvCO1*, and *HvCO2* (Griffiths *et al.* 2003; Dunford *et al.* 2005), and four *FT*-like EST consensus sequences were identified in barley through a database search (Chardon and Damerval 2005). Because of the importance of *FT* in flowering, it is important to define the structure of the family in temperate grasses. This article discusses the cloning, sequencing, and gene expression analysis of *FT*-like genes in barley and their phylogenetic relationship to rice and Arabidopsis *FT*-like genes. Our emphasis is on characterizing the genes most closely related to *FT* and likely to have a role as pathway integrators activated by photoperiod.

MATERIALS AND METHODS

Searches of The Institute for Genomic Research rice pseudomolecules and the Barley Gene Index for *FT*-like genes: All the databases used for the searches are available at The Institute for Genomic Research (TIGR; http://www.tigr.org/) (Yuan *et al.* 2005) and at Gramene (http://www.gramene.org/) (Jaiswal *et al.* 2006). Starting with the Arabidopsis FT protein sequence FT_ARATH (Q9SXZ2), a BLASTP search was carried out against the predicted proteins from the TIGR rice pseudomolecules release 4. In addition, a TBLASTN search was carried out against all rice bacterial artificial chromosome (BAC) and P1-artificial chromosome (PAC) sequences in GenBank (http://www.ncbi.nlm.nih.gov/) to search for genes not present or not correctly annotated in the TIGR gene models.

A multiple sequence alignment of the TIGR gene models was made using CLUSTAL W (http://www.ebi.ac.uk/) (Thompson *et al.* 1994) against the conserved PEBP domain (PF01161). New gene predictions were made using FGENESH+ and PROT_MAP (http://sun1.softberry.com) for FT-like genes showing incorrect alignment within the PEBP domain and those not predicted by TIGR (supplemental Table S1 at http://www.genetics.org/supplemental/).

Starting with the rice *OsFTL2* (Os06g06320) encoded protein (11976.m05358), a TBLASTN search was carried out against the Barley TIGR Unique Gene Indices release 9.0 (Lee *et al.* 2005). Gene index mapping information available from TIGR was used to align the barley sequences to the rice *FT*-like genes. Representative clones were selected from the ESTs used to construct the TIGR Gene Indices for the screening of a barley BAC library.

BAC library analysis: High-density filters of a Morex barley BAC library with approximately sixfold genome coverage were purchased from Clemson University Genomics Institute (CUGI; http://www.genome.clemson.edu). Three EST clones containing full-length cDNAs were also obtained. HVSMEh0101D16 (BE602964) corresponding to the *FT*-like unigene sets TC143893 and TC143873 was obtained from CUGI, while baak20f16 (BJ448552) for TC151142 and bahl1n08 (AV937451) for TC152179 were provided by the Barley Germplasm Center, Okayama University (Kurashiki, Japan). Hybridization probes were amplified from the three EST clones using sequence-specific primers (HVSMEh0101D16: forward—5′-gacgtggtggacccgttc-3′ and reverse—5′-cagtcggtggatcccgag-3′; baak20f16: forward—5′-ccgttccattaaggatagcc-3′ and reverse—5′-ccatccggtggataccag-3′; bahl1n08: forward—5′-ctcagtgcctctaactgtgatg-3′ and reverse—5′-cagctgctggtacagaac-3′). An *OsFTL2*-specific probe was amplified from Nipponbare rice genomic DNA using primers designed for the *OsFTL2* sequence as described in Turner *et al.* (2005). After hybridization screens with all four probes using the Church and Gilbert (1984) method, five barley BACs, each containing one of five *FT*-like genes, were investigated in detail. Primer pairs (supplemental Table S2 at http://www.genetics.org/supplemental/) amplifying overlapping segments of the all *FT*-like genes were designed from the barley EST sequences used as probes. Two BACs (236M13 and 440G4) were subcloned using the TOPO Shotgun subcloning kit (Invitrogen, San Diego) to obtain 5′- and 3′-end sequences. Additionally, coding regions and intervening introns for *HvFT1*, *HvFT3*, and *HvFT4* were amplified from the barley cultivars Igri and Triumph. The absence of intron 3 in *HvFT1* was investigated in wild barley (*H. spontaneum*) accessions and in the wheat cultivar Chinese Spring using the following primers: forward—5′-gttggtgacagatatccgg-3′ and reverse—5′-ccctggtgttgaagttctgg-3′.

Phylogenetic analysis of the PEBP domain of Arabidopsis, rice, and barley *FT*-like genes: Multiple sequence alignment with CLUSTAL X of the PEBP domain (Pfam PF01161) for the two Arabidopsis proteins AtFT (FT_ARATH, Q9SXZ2) and AtTSF (TSF_ARATH, Q9S7R5), 13 rice FT-like proteins, and five barley FT proteins, together with the outgroup sequence OsMFTL1 (encoded by the gene Os06g30370), was used to generate input files for phylogenetic analysis. A tree was constructed using PHYLIP 3.5 (Felsenstein 1993). Bootstraps with 100 replicates were performed to assess node support. Multiple sequence alignment data were read in using SEQBOOT to

TABLE 1

Rice and barley *FT*-like genes

Rice			Barley			
Gene name/ other name	TIGR locus	BAC[a]	TIGR TC[b]	ESTs[c]	Gene name	Sequence accession no.
OsFTL1/FTL	Os01g11940	AP002745	TC143893 TC143873	HVSMEh0101D16	*HvFT2*	DQ297407
OsFTL2/Hd3a	Os06g06320	AP005828			*HvFT1*	DQ100327
OsFTL3/RFT1	Os06g06300	AP007223				
OsFTL4/osFT	Os09g33850	AP006756				
OsFTL5		AP004124				
OsFTL6	Os04g41130	AL662946				
OsFTL7	Os12g13030	AL831806				
OsFTL8	Os01g10590	AP003105				
OsFTL9	Os01g54490	AP003076				
OsFTL10	Os05g44180	AC130603	TC151142	baak20f16	*HvFT3* *HvFT5*	DQ411319 EF012202
OsFTL11	Os11g18870	AC136448				
OsFTL12	Os06g35940	AP003682	TC152179	bah11n08	*HvFT4*	DQ411320
OsFTL13	Os02g13830	AP004070				

[a] BAC sequence used to construct TIGR pseudomolecule release 4 (http://www.tigr.org/tdb/e2k1/osa1/).
[b] TIGR tentative consensus sequence accession.
[c] TC constituent EST clone used for hybridization.

produce multiple data sets for bootstrap resampling to, in turn, produce 100 data sets. PROTDIST (Dayhoff PAM matrix) using 100 data sets computed a distance measure using maximum-likelihood estimates. NEIGHBOR was used to produce an unrooted tree using the neighbor-joining method with one outgroup species (OsMFTL1). CONSENSE was used to draw the tree by majority rule, including bootstrap values. The tree was viewed using ATV: A Tree Viewer (Zmasek and Eddy 2001).

Genetic mapping: A probe specific for each barley *FT*-like gene was hybridized to wheat/barley telosomic addition lines (Islam 1983) to assign the barley genes to chromosome arms. Subsequently, *HvFT1*, *HvFT2*, *HvFT4*, and *HvFT5* were mapped in an F_6 RI population from an Igri × Dairokkaku cross. All four were mapped using single-strand conformation polymorphism (Martins-Lopes *et al.* 2001) after amplification using the primers described in supplemental Table S2 at http://www.genetics.org/supplemental/. *HvFT3* was mapped in an Igri × Triumph doubled haploid (DH) population (Laurie *et al.* 1995) as a presence/absence polymorphism using the cDNA probe used to screen the BAC library.

Collinearity of the *HvFT1*, *HvFT2*, *HvFT3*, and *HvFT4* regions with rice was investigated by identifying additional closely linked genes. Sequences of rice BAC clones carrying the respective homologous *OsFTL* genes were used for BLASTN searches to identify corresponding barley ESTs, which were used to design PCR primers (supplemental Table S3 at http://www.genetics.org/supplemental/). These primers were used to amplify from the parents of the two mapping populations described above and from the BAC clones containing the respective *HvFT* genes. PCR reactions were as follows: in a total reaction volume of 20 μl, 50 ng of DNA, 0.2 μm of primers, 0.2 μm of dNTPS, 1× Taq polymerase buffer (Roche), and 0.4 unit of Taq polymerase (Roche). The PCR conditions were an initial denaturation at 94° for 2 min, followed by 40 cycles of denaturation at 94° for 30 sec, annealing at 55° for 30 sec, and extension at 72° for 1 min, followed by a final extension of 5 min at 72°. Single-strand conformation polymorphism (Martins-Lopes *et al.* 2001) was used to genetically map polymorphic markers.

Gene expression analysis: Gene-specific primers (supplemental Table S2 at http://www.genetics.org/supplemental/) for each *HvFT* gene were used to assay expression in mature embryos (after stratification) and in plants grown for 7, 14, 21, and 28 days in long-day (LD; 16 hr light) and short-day (SD; 8 hr light) conditions (136 μmol m^{-2} sec^{-1}, 22° during the day, 18° during the night). The genotype used was Triumph into which a functional *Ppd-H1* allele has been introgressed as described in Turner *et al.* (2005). This isogenic line [Triumph(*Ppd-H1*)] is therefore responsive to photoperiod but lacks any vernalization requirement. Reports in Arabidopsis suggested that *FT* expression was relatively high 4 hr after the start of the dark phase (Kardailsky *et al.* 1999; Kobayashi *et al.* 1999, Suarez-Lopez *et al.* 2001). Plants were therefore sampled 4 hr after dusk with each sample comprising six plants. RNA was extracted, cDNA was synthesized, and samples were processed using an Opticon 2 real time PCR instrument (http://www.mjr.com) as described in Turner *et al.* (2005).

RESULTS

Database searches for *FT*-like genes in rice and barley: A BLASTP search of peptides (TIGRv4 gene models) using FT_ARATH identified 12 *FT*-like genes from rice corresponding to all the *FT*-like genes described by Chardon and Damerval (2005) except *OsFTL5*. A TBLASTN search of all rice BAC and PAC sequences found an additional *FT*-like gene on BAC AP004124 corresponding to *OsFTL5*. This gene is not annotated as a predicted gene by the TIGR annotation project (Yuan *et al.* 2005). The chromosome position and BAC location of the genes was used to verify that the 13 *FT*-like genes identified by Chardon and Damerval (2005) corresponded to the correct TIGR loci (Table 1).

The protein sequences for all the rice *FT*-like genes except *OsFTL5* were downloaded from TIGR and a

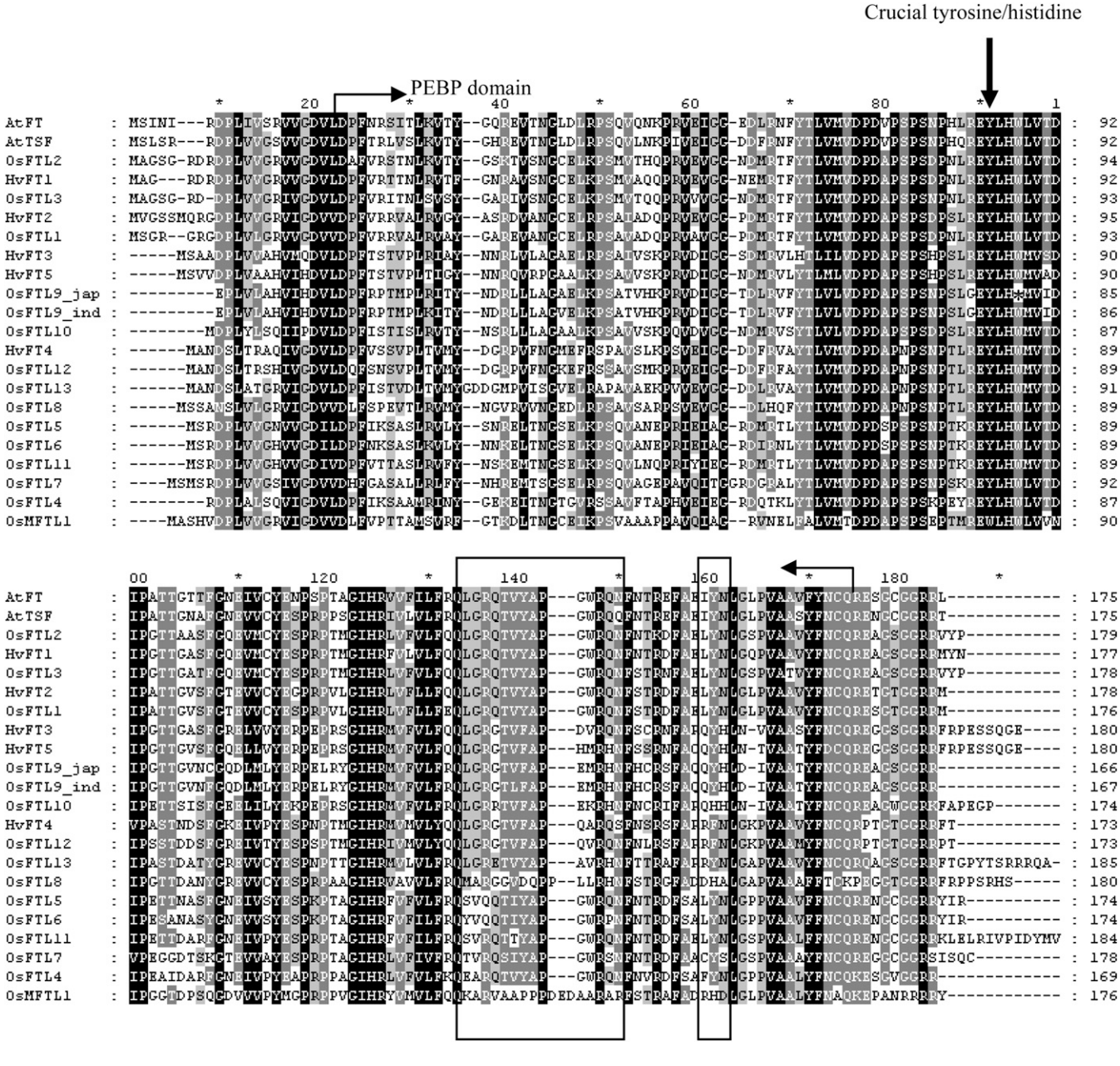

FIGURE 1.—ClustalW multiple alignment of the complete protein sequences of Arabidopsis, rice, and barley FT family proteins. OsMFTL1, used for construction of the phylogenetic tree (Figure 2A), is also included. The PEBP domain boundaries are marked by horizontal arrows. The residue distinguishing FT- and TFL1-type functions (HANZAWA *et al.* 2005) is marked by a vertical arrow. All the FT-like proteins have a tyrosine at this position. The 14-amino-acid stretch and the LYN triad identified by AHN *et al.* (2006) as diagnostic of true *FT* genes are boxed. The *indica* OsFTL9 is predicted to be functional but the *japonica* sequence should be nonfunctional because of a stop codon at position 95.

multiple alignment was performed using ClustalW. This showed that predicted protein sequences for *OsFTL4*, *-8*, *-9*, and *-10* did not align with the PEBP domain. New protein predictions were carried out for these genes and for *OsFTL5* (Figure 1; supplemental Table S1 at http://www.genetics.org/supplemental/). The *OsFTL9* protein from Nipponbare (subspecies *japonica*) contained a stop codon in the PEBP domain and is predicted to be nonfunctional. The OsFTL9 protein from 93-11 (subspecies *indica*) was intact (Figure 1).

A TBLASTN search of the barley TIGR Gene Indices database using *OsFTL2* (*Hd3a*) as a template retrieved four barley tentative consensus (TC) sequences as described by CHARDON and DAMERVAL (2005) (Table 1).

BAC library screens: No barley ESTs closely matched the rice *OsFTL2* (*Hd3a*) sequence. To identify this gene, a nucleotide probe derived from *OsFTL2* was used to screen a Morex barley BAC library. Subclones from BAC clone 440G4 were identified as containing a *Hd3a* homolog and were sequenced, revealing an *FT*-like gene

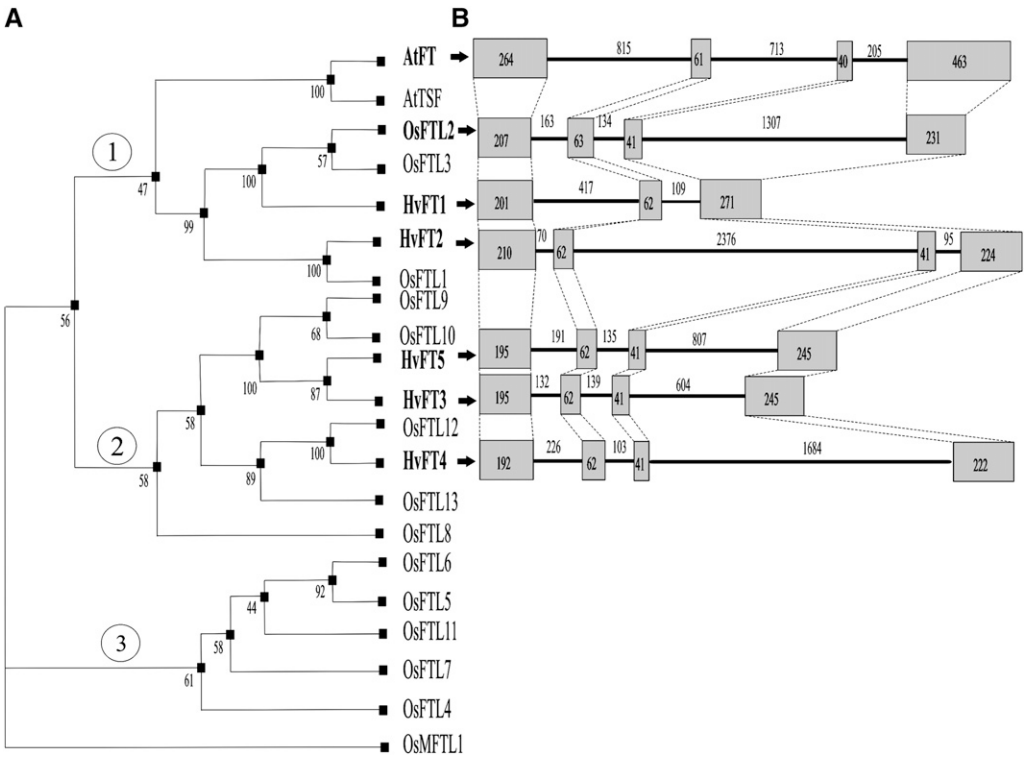

FIGURE 2.—Phylogenetic relationships of FT-like proteins from Arabidopsis, rice, and barley. (A) Phylogenetic tree of PEBP domain protein sequences. Major groups are marked 1–3. Bootstrap support values are shown at each node. (B) Exon/intron structure of Arabidopsis *FT*, rice *Hd3a*, and the five barley *FT* family genes. Exon and intron sizes are in base pairs.

highly homologous to *OsFTL2* (*Hd3a*) and *FT*. This gene was designated *HvFT1*. The BAC library was also screened with probes specific to each barley EST contig (Table 1). A total of 32 positive BACs were obtained and, after fingerprinting and hybridizing with the same probes, they were classified into four groups. Two distinct classes corresponded to *HvFT2* and *HvFT4* while cross-hybridization suggested that two other genes (*HvFT3* and *HvFT5*) were closely related and might be the result of a recent duplication. Full gene sequence was obtained for each of these genes from a representative BAC clone (Table 1; Figure 1).

Phylogenetic analysis of the PEBP domain of Arabidopsis, rice, and barley *FT*-like genes: The evolutionary relationship between the *FT*-like genes in Arabidopsis, rice, and barley was investigated by constructing a phylogenetic tree using neighbor-joining of the PEBP domain protein sequences (Figure 2A). Protein sequence homology measured as the percentage of identical protein residues is shown in supplemental Table S4 at http://www.genetics.org/supplemental/. The *FT* family fell into three groups. The seven genes in group 1 were the most *FT*-like and comprised *AtFT*, *AtTSF*, *OsFTL1-3*, and *HvFT1-2* (Figure 2A). Rice and barley proteins had 71–73% identity with AtFT and there is 79–89% identity between rice and barley proteins. HvFT1 was most similar to OsFTL2 while HvFT2 was highly homologous to OsFTL1.

Group 2 proteins (58–65% identity with AtFT) included HvFT3, HvFT4, and HvFT5. HvFT4 was very similar to OsFTL12, while HvFT3 and HvFT5 were highly homologous to each other and are related to the pair of rice proteins OsFTL9 and OsFTL10. Group 3 contained rice sequences with 63–71% homology with AtFT but no barley proteins. CHARDON and DAMERVAL (2005) also found no barley ESTs homologous to these rice genes. If present in barley, as seems likely, they were too diverged in sequence to be detected in the BAC library hybridization screen.

HANZAWA *et al.* (2005) showed that the tyrosine (Y) amino acid at position 85 in AtFT is critical to FT function and that replacing this with a histidine (H) was sufficient to convert FT to TFL1 in terms of function. The reciprocal substitution converted TFL1 to FT. All the *FT*-like genes in Figure 1 have the critical Y consistent with being true *FT*-like genes and likely to have activator-type roles in flowering. A barley TFL1 homolog (DQ539338) has the expected H residue at this position.

More recently, AHN *et al.* (2006) identified a 14-amino-acid stretch in which 11 amino acids were invariant in all *FT*-like proteins that they analyzed compared to 4 in all TFL1-like proteins. A single residue unambiguously distinguished between *FT* and *TFL1* homologs: Gln140 in FT and Asp144 in TFL1. Group 1 proteins show the same invariance (boxed in Figure 1), further suggesting that they are the most FT-like. Group 2 and group 3 proteins have some variation in this stretch. HvFT5 has a histidine in place of the Gln140 and HvFT3; HvFT4 and HVFT5 have at least 3 amino acids varying of the 11 amino acids characterized as invariant. Furthermore, AHN *et al.* (2006) also observed an essentially invariant triad starting at residue 150 with a leucine or isoleucine, followed by a tyrosine and an asparagine. This triad is

present only in the group 1 and in some of the group 3 proteins (boxed in Figure 1). Taken together, these observations confirm that proteins from group 1 are the most FT-like, while group 2 and group 3 proteins are more diverged.

The exon/intron structure of the barley *FT*-like genes: In Arabidopsis, *FT* and *TSF* have four exons and three introns (Figure 2B). The first and fourth exons are the largest (264 and 463 bp, respectively), while exons 2 and 3 are smaller (61 and 40 bp, respectively). Intron 1 and 2 are similar in size (815 and 713 bp, respectively), but intron 3 is smaller (205 bp). This structure is well conserved across all the *FT*-like genes found in the rice genome, although some variation is found in the relative sizes of the introns. In barley, *HvFT2*, *HvFT3*, *HvFT4*, and *HvFT5* also have this structure, but *HvFT1* is distinct in lacking the third intron, perfectly merging exons 3 and 4 together (Figure 2B). The sequence was obtained from a BAC clone derived from the spring barley cultivar Morex. The loss of the intron was confirmed in cultivars Igri and Triumph, in *H. spontaneum* accessions, and in the three group 7 chromosome wheat nullisomic/tetrasomic lines derived from the variety Chinese Spring, showing that the intron is absent in all three wheat genomes (supplemental Figure S1 at http://www.genetics.org/supplemental/).

Expression profiles under LD and SD in a *Ppd-H1* genetic background: Gene expression was studied in Triumph (*Ppd-H1*) plants, which do not require vernalization but are highly responsive to a long-day photoperiod (Turner *et al.* 2005). Expression levels of the five *HvFT* genes were compared in plants grown under LD (16 hr light) or SD (8 hr light) conditions. For *HvFT1*, *HvFT2*, and *HvFT4*, no or extremely low expression was detected under SD conditions at all time points (Figure 3a). In LD conditions, *HvFT1* was rapidly induced and was detected after 1 week, while *HvFT2* and *HvFT4* expression was significantly induced after 3–4 weeks (Figure 3a). Dissection of developing apices showed that the appearance of the double ridge stage, the first visible sign of the switch from vegetative to floral development (Kirby and Appleyard 1981), occurred during the second week under LD and during the fourth week under SD conditions (Figure 3b). Therefore, *HvFT1* was the only *FT*-like gene highly expressed at the transitional phase under LD conditions.

HvFT3 was unusual in being strongly expressed under SD (noninductive) conditions from week 1, but being very weakly expressed under LD (inductive) conditions (Figure 3a). Barley plants will flower in SD conditions but the apical transition in SDs occurred while *HvFT1* expression remained very low, suggesting that this is not due to a low-level induction of genes normally involved in the long-day response. Possibly a second mechanism using a different *FT* gene is involved. *HvFT3* may be a candidate for this, but *HvFT3* expression increased well before the apical transition in SDs, suggesting that

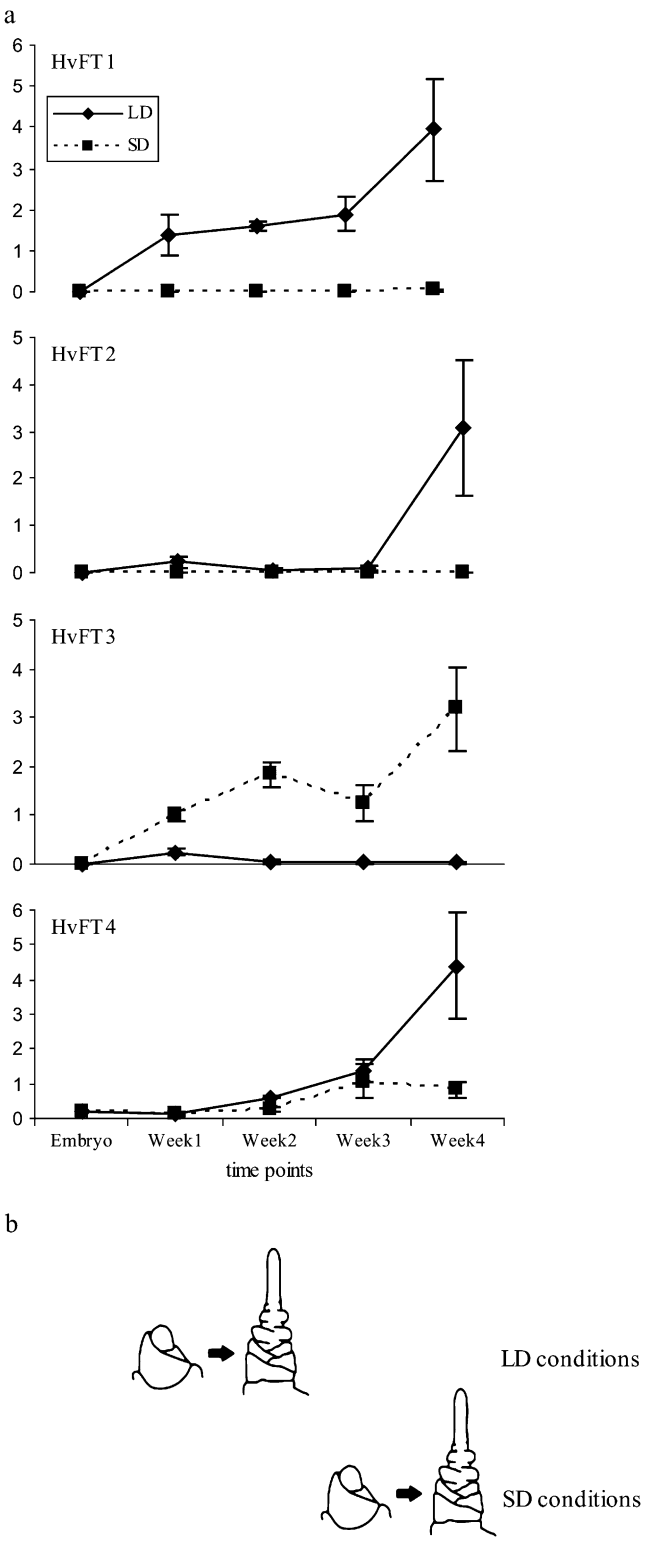

Figure 3.—Expression of *HvFT* genes under short-day and long-day conditions. (a) Levels of gene expression in arbitrary units normalized against 18s rRNA. (b) Timing of the transition of the developing apex from vegetative to reproductive growth (extension of the apical dome and appearance of primordia with a double-ridge structure). The transition was observed between weeks 1 and 2 in LD conditions and between weeks 3 and 4 in SD conditions.

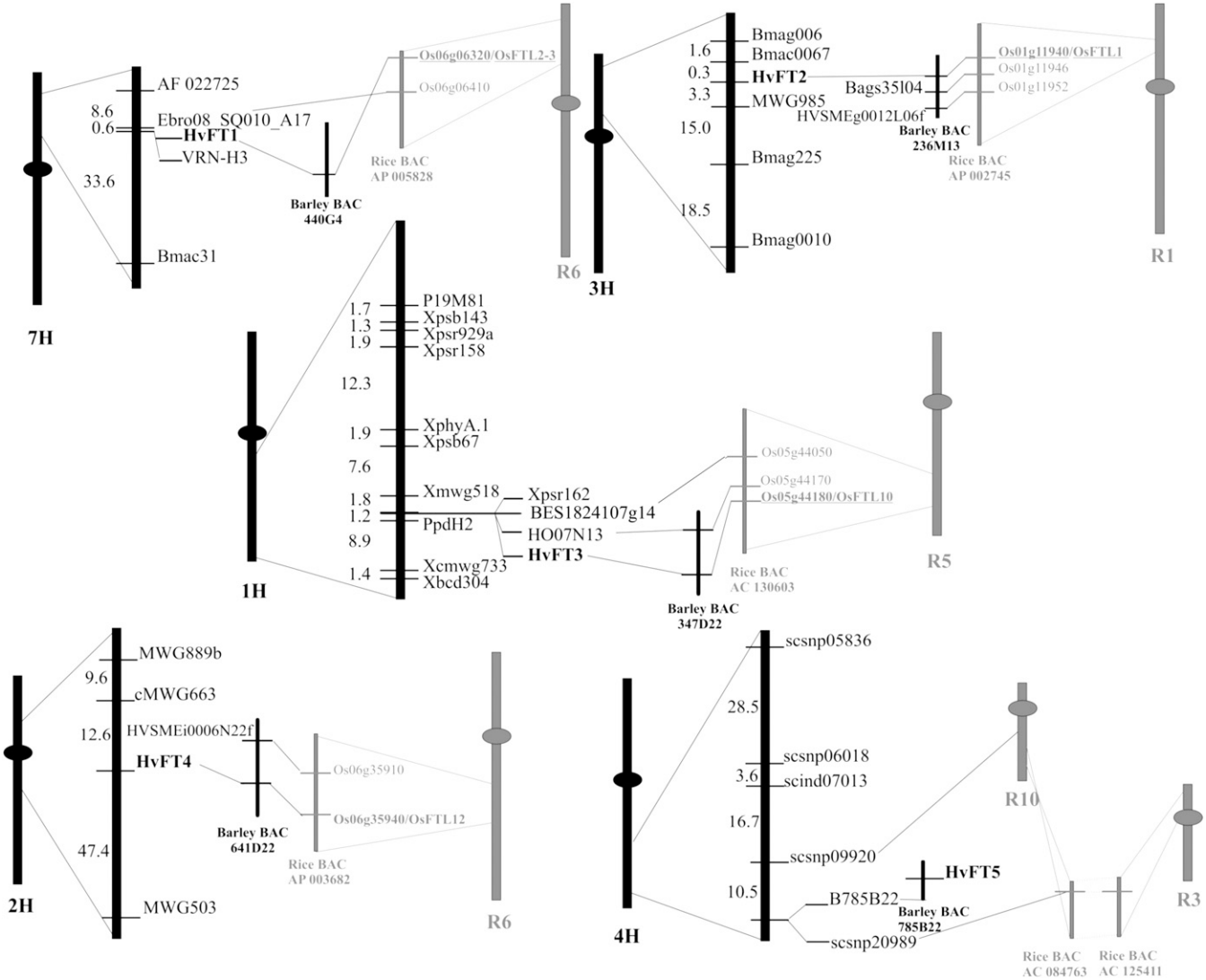

FIGURE 4.—Genetic map positions of barley *HvFT* genes and their relationships to rice. Solid lines show the mapped segments in relation to the approximate size of complete barley linkage groups (H). Barley BACs are shown in boldface type. Shaded lines show homologous sequences from rice (R) physical maps and individual BAC clones. Barley clones on chromosomes 7H, 3H, 2H, and 4H were mapped using an Igri × Dairokkaku (*Vrn-H3*) population and clones on 1H using an Igri × Triumph population.

HvFT3 expression is not sufficient to induce the transition. *HvFT4* was weakly expressed during the transitional phase in SD conditions and may play a role. *HvFT3* was also unusual in being expressed in the embryo after stratification, but in this case the transcript was unspliced (supplemental Figure S2a at http://www.genetics.org/supplemental/) and is predicted to give a nonfunctional protein.

Expression of an unspliced *HvFT5* transcript was detected at all time points in LD and SD conditions (supplemental Figure S2b at http://www.genetics.org/supplemental/). The predicted protein had a stop codon at residue 69 and is likely to be nonfunctional. The correctly spliced form was detected after 4 weeks in SDs, but the expression level was very low.

Comparative mapping of the five barley *FT*-like genes: Phylogenetic analysis of the PEBP domain of barley and rice FT-like protein sequences showed that the five barley genes could each be associated with one or two different rice genes (Figure 2A). Comparative mapping of barley with rice was used to provide additional information on the relationships among genes. First, hybridizations of probes specific to each of the five *HvFT* genes to wheat/barley telosomic addition lines were used to assign individual barley genes to chromosome arms. *HvFT* genes were then genetically mapped (Figure 4). For additional comparative mapping, rice genes present on the same BAC clones as the putative homologous *OsFT*-like genes were used for BLASTN searches (http://www.ncbi.nlm.nih.gov/) to identify corresponding barley ESTs. Matching barley ESTs were used to develop markers for genetic and physical mapping in barley.

HvFT1 was mapped to the short arm of chromosome 7H, between microsatellite markers AF022725 and

Bmac31. The HvFT1 protein was highly homologous to the OsFTL2 and OsFTL3 proteins. These two rice genes are ~12 kb apart and are likely to be the result of a recent duplication. Among the 39 other genes predicted on the same BAC (AP005828), Os06g06410 was highly homologous to barley EST Ebro08_SQ010_A17. This sequence was mapped in barley by SSCP to the short arm of chromosome 7H, 0.3 cM distal to *HvFT1*. This shows that the region carrying *HvFT1* is orthologous to the *OsFTL2/3* region in rice. There was no evidence of a tandem duplication in barley from sequencing or from Southern hybridizations, which showed single-band profiles.

HvFT2 was mapped to the short arm of chromosome 3H, between microsatellite marker *Bmac67* and STS marker *MWG985*. *HvFT2* was most similar to *OsFTL1* (Os01g11940). A rice BAC (AP002745) containing *OsFTL1* also contained two genes (Os01g11946 and Os01g11952) with matching barley ESTs (Bags35l04 and HVSMEg 0012L06) that were present on the *HvFT2* barley BAC 236M13.

HvFT4 was mapped to the short arm of chromosome 2H, proximal to marker cMWG663. *HvFT4* was highly homologous to *OsFTL12* (Os06g35940), located on BAC AP003682 from rice chromosome 6. Barley EST HVSMEi0006N22 was homologous to Os06g35910, present on the same rice BAC. HVSMEi0006N22 was present on barley BAC 641D22, which also carried *HvFT4*, confirming that *HvFT4* is the ortholog of *OsFTL12*. *OsFLT12* and *-13* are likely to derive from duplication within the rice genome. No equivalent duplication was found in barley, and Southern hybridization using an *HvFT4* probe yielded a single band.

HvFT3 was mapped to the long arm of chromosome 1H, cosegregating with marker *Xpsr162*. The HvFT3 protein was highly homologous to OsFTL10 encoded by the rice gene Os05g44180 present on a rice chromosome 5 BAC (AC130603). Two rice genes also present on this BAC (Os05g44050 and Os05g44170) were homologous to the barley ESTs BES1824107g14 and HO07N13, respectively. BES1824107g14 was mapped in barley by SSCP and cosegregated with *HvFT3*, while HO07N13 was both mapped by SSCP, co-segregating with *HvFT3*, and found on barley BAC 347D22, which also carried *HvFT3*. *HvFT3* was therefore confirmed as the ortholog of *OsFTL10*. *HvFT5* was also highly homologous to *OsFTL10*, but was mapped to the long arm of chromosome 4H, cosegregating with marker scsnp20989. Markers in the region of *HvFT5* have homologous sequences at the distal ends of rice chromosome 3S and 10L, which do not contain any *FT*-like genes. The lack of an equivalent rice gene to *HvFT5* and the high sequence homology of *HvFT3* and *HvFT5* suggest that the latter is the result of a duplication that occurred after the divergence of barley and rice. Therefore, while *OsFTL9*, *OsFTL10*, *HvFT3*, and *HvFT5* were related (Figure 2A), they are likely to derive from independent duplications. Absence of the *OsFTL9* equivalent from barley was supported by hybridizing *OsFTL9* and *OsFTL10* probes amplified from rice to wheat/barley telosomic addition lines. *OsFTL10* showed strong hybridization to both wheat and barley while *OsFTL9* hybridized only weakly to the same fragments as *OsFTL10* and detected no additional band.

Associations of *HvFT* genes with flowering-time QTL: Three of the *HvFT* genes are in regions previously shown to contain flowering-time QTL in an Igri × Triumph cross (Laurie *et al.* 1995). *HvFT4* mapped to the region of barley chromosome 2 where Laurie *et al.* (1995) mapped *eps2S*, and *HvFT1* was in the same region as *eps7S*. Both QTL are earliness factors with no obvious relationship to photoperiod response (Laurie *et al.* 1995). No direct evidence was found for these being the underlying genes as there was no polymorphism in the *HvFT4* or *HvFT1* coding regions between Igri and Triumph. However, this does not exclude the possibility of differences in expression. An *Hd3a*-like *FT* gene was also associated with a QTL for early flowering that was found on chromosome 7 in *Lolium perenne* in a collinear position to *OsFTL2* in rice (Armstead *et al.* 2002). Interestingly, the Igri × Dairokakku population used to map *HvFT1* also segregated for *VRN-H3*, a vernalization locus previously mapped to 1HL (Takahashi and Yasuda 1971). However, *VRN-H3* cosegregated with *HvFT1* on chromosome 7H in our population, consistent with recent findings by Yan *et al.* (2006) who showed that spring habit mutations in *Vrn-B3* lines of wheat and *Vrn-H3* lines of barley are due to altered expression of *HvFT1* (in our nomenclature). No sequence polymorphism was observed in the coding regions of *HvFT1* between Igri and Dairokakku, but, as found by Yan *et al.* (2006), nine SNP and three indels were found in the 648 bases of the promoter region upstream of the start codon, three SNPs were present in the first intron, and a polymorphic SSR was observed in the second intron (supplemental Figure S3 at http://www.genetics.org/supplemental/).

HvFT3 was also interesting because it was closely associated with *Photoperiod-H2* (*Ppd-H2*). This major QTL affected flowering time in a short-day glasshouse experiment (10 hr light) and in an autumn-sown field experiment but was not detectable in long days (Laurie *et al.* 1995), consistent with the observed expression pattern of HvFT3 (Figure 3). In short days, Igri contributed the late-flowering allele. The Triumph sequence for *HvFT3* was identical to Morex but no fragment could be amplified from Igri. A presence/absence result from Southern hybridizations showed the gene to be at least partly deleted from Igri. Reanalysis of the SD glasshouse experiment suggested that at least one line (DH48, Figure 5) showed recombination between *HvFT3* and *Ppd-H2*. However, analysis using JoinMap 4.0 (Plant Research International B.V.) showed additional QTL to be present. By using the *Ppd-H2* region as a cofactor,

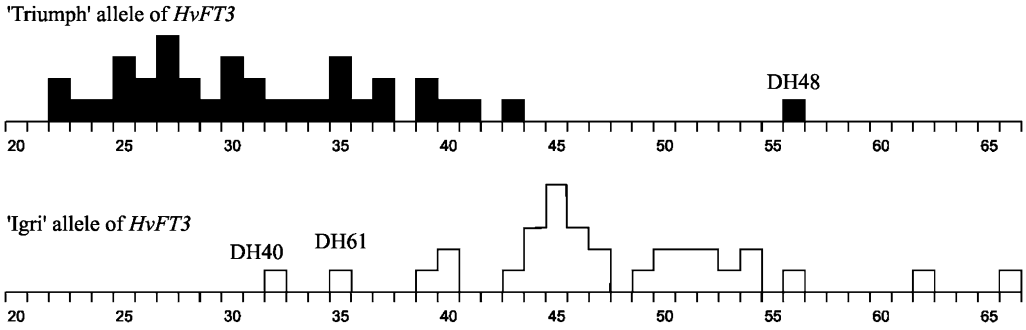

FIGURE 5.—Flowering times (days to awn emergence from the flag leaf) of DH lines from an Igri × Triumph population showing the association of early flowering with the Triumph allele of *HvFT3*. The effect of additional QTL is shown in supplemental Figure S4 at http://www.genetics.org/supplemental/.

additional significant QTL were localized on 2H, 5H, and 6H and the additive effects of these QTL could account for the range of flowering times in DH lines 48, 40, and 61 (Figure 5; supplemental Figure S4 at http://www.genetics.org/supplemental/). This ambiguity means that *HvFT3* is a sufficiently good candidate for *Ppd-H2* to warrant further study.

To test whether Igri was unusual in having a deletion of *HvFT3*, we used the presence/absence PCR polymorphism to analyze a sample of 60 spring and 40 winter barley cultivars from Europe (supplemental Table S5 at http://www.genetics.org/supplemental/). The Triumph allele was prevalent in spring types (46/60, 78%) and the Igri allele in winter types (36/40, 90%), and this association was highly significant (χ^2 1, d.f. 43.7, $P < 0.001$). Both alleles could be found in two-row and six-row types. The prevalence of the deletion in winter types suggests that this may have been selected to enhance the suppression of flowering in overwintering plants.

DISCUSSION

Our main interest was the identification of barley genes most similar to Arabidopsis *FT* and hence most likely to be significant as floral pathway integrators. The barley genes that we identified correspond to rice genes in groups 1 and 2 of Figure 2. Group 3 genes may exist in barley, but no ESTs have been found and no clones were identified in our library screens, probably because the nucleotide sequence is too diverged.

Differences in gene number between rice and barley are primarily attributable to differences in the fate of duplicated genes. *HvFT1* corresponds to *OsFLT2* and *-3*, which are likely to be a recent duplication. *OsFTL9-10* and *OsFLT12-13* are pairs resulting from duplications within the rice genome (PATERSON *et al.* 2003; SALSE *et al.* 2004), but for the former we detected only an equivalent of *OsFLT10* (*HvFT3*) and for the latter only the equivalent of *OsFLT12* (*HvFT4*), suggesting that two genes have been lost from barley. *HvFT3* and *HvFT5* are likely to derive from a more recent duplication in barley. *HvFT2* corresponds to a single gene in rice, *OsFTL1*.

In rice, *OsFTL2* was identified by positional cloning of a QTL for flowering time (*Hd3a*), showing significant natural variation at this locus (KOJIMA *et al.* 2002). The significance of this gene was confirmed by experiments on the effect of night break on the expression of rice *FT*-like genes. Only *OsFTL2* expression was affected by night break, pointing to a key role for this gene in photoperiod response (ISHIKAWA *et al.* 2005). In barley, *HvFT1* is highly homologous to *OsFTL2* (89% in the PEBP domain) and maps to a collinear position on chromosome 7H. *HvFT1* was the gene most rapidly upregulated by long days and the only *HvFT*-like gene to be upregulated at the time of apical transition. Expression of this gene was also found to be significantly reduced in the barley *ppd-H1* mutant, which is late flowering in long days (TURNER *et al.* 2005). Furthermore, YAN *et al.* (2006) have shown that allelic variation in *HvFT1* is associated with large differences in flowering time. These data suggest that this gene is a prime candidate for a floral pathway integrator in barley, but this does not exclude roles for the other *FT*-like genes.

An unusual feature of *HvFT1* was the absence of the third intron present in *AtFT*, all rice *FT*-like genes, and all other *HvFTs*. Reverse transcription of spliced mRNAs followed by homologous recombination of the cDNA with the genomic copy of the gene has been suggested as a mechanism for precise intron loss (ROY and GILBERT 2006), and this process has been shown to occur in unicellular eukaryotes (BON *et al.* 2003; MOURIER and JEFFARES 2003; SVERDLOV *et al.* 2004; ROY and GILBERT 2005). The loss of the intron was found in several barley cultivars, wild barley accessions, and wheat, suggesting that it may be a general feature of temperate grasses.

A surprising finding was the potential role of an *FT* gene in the control of flowering time under noninductive (SD) conditions. Under SD conditions, *HvFT3* was expressed from the first week at a higher level than under LD conditions, while *HvFT4* was very weakly expressed after 3 and 4 weeks, and *HvFT1* and *HvFT2* were not expressed at all. The switch at the apex from vegetative to floral meristem occurred between the third and the fourth weeks, suggesting that *HvFT4* could be involved in the promotion of flowering under SD or that an additional pathway is in place to promote flowering under SD in barley. A priority for understanding flowering in SDs is to resolve the relationship between *HvFT3*

and *Ppd-H2*, which can be done by further mapping and tests of gene function using transgenic approaches.

Barley and rice diverged from an ancestral grass ~60 MYA (Gale and Devos 1998) and have contrasting photoperiodic responses. This is achieved using photoperiod pathway genes that are well conserved with more distantly related plants like Arabidopsis. Furthermore, the photoperiod response in barley and rice has been modified considerably during domestication. A challenge for cereal biology is to reconcile this diversity with the conservation of the underlying pathway. To do this, it is important to understand the pathway components and to develop models that accommodate variation in the structure of gene families.

Hayama and Coupland (2004) showed that although both *CO* and *FT* are conserved between rice and Arabidopsis, their relationship is different. In Arabidopsis, *CO* promotes *FT* expression under LD conditions only, while in rice, *CO* represses *FT* under LDs and promotes it under SDs. Barley is a LD plant, like Arabidopsis, but is phylogenetically closer to rice, a SD plant. Barley differs from rice and Arabidopsis in having two *CO*-like genes (*HvCO1* and *HvCO2*) of which the former is unusual in having lost key residues in a normally highly conserved zinc-finger domain (B-box 2) (Griffiths *et al.* 2003). Characterization of the roles and interactions of the various *CO* and *FT* genes in barley is now feasible because of the increased understanding of the gene families and the availability of new resources for functional analysis, including efficient transformation methods (Travella *et al.* 2005) and a TILLING population (Caldwell *et al.* 2004).

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