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ORIGINAL PAPER

FT genome A and D polymorphisms are associated with the variation of earliness components in hexaploid wheat

Isabelle Bonnin · Michel Rousset · Delphine Madur · Pierre Sourdille · Céline Dupuits · Dominique Brunel · Isabelle Goldringer

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Abstract The transition from vegetative to floral meristems in higher plants is determined by the coincidence of internal and environmental signals. Contrary to the photoperiod pathway, convergent evolution of the cold-dependent pathway has implicated different genes between dicots and monocots. Whereas no association between natural variation in vernalization requirement and Flowering time locus T (FT) gene polymorphism has been described in *Arabidopsis*, recent studies in Triticeae suggest implication of orthologous copies of FT in the cold response. In our study, we show that nucleotide polymorphisms on A and D copies of the wheat FT gene were associated with variations for heading date in a collection of 239 lines representing diverse geographical origins and status (landraces, old or recent cultivars). Interestingly, polymorphisms in the non-coding intronic region were strongly associated to flowering variation observed on plants grown without vernalization. But differently from VRN1, no epistatic interaction between FT homeologous copies was revealed. In agreement with the results of association study, the A and D copies of FT were mapped in regions including major QTLs for earliness traits in hexaploid wheat. This work, by identifying additional homeoalleles involved in wheat vernalization pathway, will contribute to a better understanding of the control of flowering, hence providing tools for the breeding of varieties with enhanced adaptation to changing environments.

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I. Bonnin (✉) · M. Rousset · D. Madur · I. Goldringer
UMR de Génétique Végétale, INRA/CNRS/UPS/INA-PG,
Ferme du Moulon, 91190 Gif/Yvette, France
e-mail: bonnin@moulon.inra.fr

P. Sourdille
INRA, Amélioration et Santé des Plantes, 234 Avenue du Brézet,
63100 Clermont-Ferrand, France

C. Dupuits · D. Brunel
UR Etude du Polymorphisme des Génomes Végétaux (EPGV),
INRA, Centre National du Génotypage, 2 rue Gaston Crémieux,
91057 EVRY cedex, France

Introduction

The transition from vegetative to floral meristems in higher plants is programmed by the coincidence of internal and environmental signals. Extensive genetic analyses in the model plant species *Arabidopsis thaliana* have revealed four interacting genetic pathways, a light-dependent, a cold-dependent, a hormonal and an autonomous pathway (see Ausin et al. 2005; Bernier and Périlleux 2005; for a review). In the flowering regulatory network, all pathways ultimately regulate a common set of key integrator genes called Suppressor of Over-expression of Constans1 (SOC1), Leafy (LFY) and Flowering time locus T (FT), which act on the floral meristem identity genes to initiate flowering. FT belongs to a larger group of plant proteins that share structural similarities to mammalian phosphatidylethanolamine binding proteins (PEBPs), which have also been found in yeast and bacteria (Chautard et al. 2004; Yang et al. 2004). Based on these similarities, the plant PEBPs were predicted to play a role in the regulation of signalling cascades controlling growth and differentiation as in mammals (Badiani and Arthur 1995; Schleiff and Soll 2005). As demonstrated in *A. thaliana*, related genes from

this family as Terminal Flower1 (TFL1) and FT may have an opposite effect on the flowering time, a single amino acid change being sufficient to modify potential binding sites for protein interaction (Hanzawa et al. 2005; Ahn et al. 2006). Comparative genetic approaches show that the functionality of plant PEBPs is conserved between *Arabidopsis* and a large range of species including woody perennial species such as grapevine (Carmona et al. 2006) or poplar (Hsu et al. 2006), and crop species such as legumes (Hecht et al. 2005) or cereals (Laurie et al. 2004). Like Constans (CO), a key component of the photoperiod pathway, the FT gene was originally defined by an allelic series of mutants that flower late in long days and do not respond to photoperiod (Koornneef et al. 1991). Further studies in *Arabidopsis* (Samach et al. 2000), rice (Kojima et al. 2002), and barley (Turner et al. 2005) showed that FT is a direct regulatory target of CO, the difference between long day and short day plants being due to alterations in the way CO interacts with FT.

Like *A. thaliana*, most temperate grasses require a prolonged period of low temperatures, i.e. vernalization, followed by an increase in day length to induce flowering. Contrary to the photoperiod pathway, convergent evolution of the cold-dependent pathway has implicated different genes between dicots and monocots. The two major genes Frigida (FRI) and Flowering Locus C (FLC) controlling vernalization response in *Arabidopsis* do not seem to exist in monocots. In both diploid (*Triticum monococcum*) and hexaploid (*T. aestivum*) wheat, the major gene VRN1 was found to be homologous to the *Arabidopsis* meristem identity gene Apetala 1 (AP1), while a second epistatic gene observed in *T. monococcum* (Tm VRN2) and functioning as FLC, was related to CO like gene family (Yan et al. 2003, 2004a). Orthologous genes to VRN1 and VRN2 were also identified in barley (Dubcovsky et al. 2005) and perennial ryegrass (Andersen et al. 2006), suggesting that causative polymorphism could be conserved within grasses.

In *Arabidopsis*, no association between natural variation in vernalization requirement and FT has been described so far, even if FT acts downstream of FLC. Very recently, Yan et al. (2006) showed that VRN2 can modulate the quantity of FT in *T. monococcum*, invoking the presence of a common peptide domain to VRN2 and CO (CCT domain), known for the latter, to be involved in the regulation of FT transcript levels in *Arabidopsis* (Suarez-Lopez et al. 2001). They described the barley FT gene (called VRN-H3 or HvFT) and its orthologous copy on the B genome of hexaploid wheat (called VRN-B3 or TaFT), suggesting strong epistatic interactions for vernalization response among VRN1, VRN2 and VRN3 in Triticeae. In hexaploid wheat, the causal polymorphism appeared as a retroelement inserted in the TaFT promoter. Although conferring early flowering of transformed winter plants, the retrotransposon was not found in a collection of 125 polyploid wheats, leading the authors to propose that this recent insertion could be used in commercial varieties to modulate flowering time. In our study, complementary to Yan et al. (2006), we show that nucleotide polymorphisms or insertions–deletions on A and D copies of the wheat FT gene can also explain variations for heading date in a collection of 239 lines representing diverse geographical origins and status (landraces, old or recent cultivars). Using different conditions of daylength and vernalization time, earliness was split up into its three components (Worland 1996): the vernalization requirement, the daylength sensitivity (DLS) and the narrow-sense earliness, allowing us to determine the effect of FT gene polymorphism on this complex character. In agreement with the results of association study, the A and D copies of FT were mapped in regions including major QTLs for earliness traits in hexaploid wheat.

Materials and methods

Plant material and DNA extraction

Genome-specific primers were tested on a set of six genotypes: one hexaploid AABBDD accession (*T. aestivum* var. Recital), one tetraploid AABB accession (*T durum* var. Langdon) and four diploid accessions: *T. urartu* and *T. monococcum* (AA genome), *T. speltoides* (BB genome, selfed progeny), *T. tauschii* (DD genome). A core set of 40 genotypes representing the highest allelic richness found at 42 SSR loci (Roussel et al. 2004) was used to screen for sequence polymorphism at FT genome A, B and D loci (Supplementary Table 1). For each identified SNP (except singletons), genetic association with earliness was examined on an extended sample of 239 lines [kindly provided by F. Balfourier, INRA Clermont Ferrand (France)], representing diverse geographical origins and status (landraces, old or recent cultivars) (list of genotypes available upon request). This sample was chosen because it showed a high level of diversity for field heading date in a preliminary unpublished study. All genomic DNA were extracted from fresh leaves using DNAeasy kit (QIAGEN, Basel, Switzerland).

In order to assign the three copies of FT gene to deletion bins, we used a set of 17 wheat deletions lines from group 7 chromosomes (lines 7AS1, 5, 8; 7AL1, 16, 17, 18, 21; 7BS1; 7BL2, 7, 10; 7DS4, 5; 7DL2, 3, 5), characterized by terminal deletions (Sourdille et al. 2004). They were kindly provided by John Raupp (Kansas State University, USA). The genetic mapping of the markers and genes was obtained using a set of 187 doubled haploid lines from the

inter-varietal mapping population CtCS derived from a F1 cross involving the French variety Courtot (Ct) and Chinese Spring (CS) (Cadalen et al. 1997). In each case, plant DNA was extracted from young leaves using the CTAB method (Rogers and Bendich 1985).

Isolation of the three copies FTA, FTB and FTD (genome-specific) of FT gene (PCR conditions, cloning and sequencing) in hexaploid wheat

As wheat is a hexaploid species, genes are usually encountered at the three homeologous genomes, requiring the design of genome-specific PCR primers to avoid co-amplification of the different copies. Amplification of the six wheat described in plant material was used to check genome specificity. All sequences were analysed using the Staden software package (Staden et al. 2000).

Triticum aestivum partial mRNA for putative PEBP protein [Hd3a gene (Kojima et al. (2002)] was available from Ciaffi et al. (2005) (Genbank accession number AJ577367). BLASTN search at the web site of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) revealed three exons by comparison to the rice Hd3a (Genbank accession numbers AB052942, AB052944, AB062675 and AB062676 in Kojima et al. 2002).

Gene-specific primers (FTex1U and FTex2L in Supplementary Fig. 1) were designed using Oligo 6 software (Medprobe, Oslo, Norway). PCR reactions were realized in a final volume of 25 µl containing 25 ng of genomic DNA, 100 µmol/l of each dNTP, 0.5 µmol/l of each primer, 1 U of Taq polymerase (Qiagen, Valencia, CA, USA), 1× Qiagen buffer and 1× Taq polymerase buffer (protocol A). Cycling consisted of a touch-down profile No.1 as follows: 1 cycle at 94°C for 4 min; 10 cycles at 94°C for 1 min, decreasing annealing temperature by 1°C per cycle starting from 64 to 54°C for 1 min each temperature, and 72°C for 2.5 min; 21 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 2.5 min; a final extension at 72°C for 4 min. Amplification products were separated on a 1% agarose gel and visualized under UV light following ethidium bromide staining. Each genomic copy was then isolated by cloning amplification products from hexaploid into pGEM-T Vector system I (Promega, Madison, WI, USA) and sequencing (Genome Express, Meylan, France).

From the sequence alignment, three upstream genome specific primers (FTint1gAU, FTint1gBU and FTint1gDU) were designed in the first intron, and were coupled with two downstream not genome specific degenerated primers localised in the third exon of the published cDNA sequence (R2-FTex3L for A and B genomes and R4-FTex3L for D genome in Supplementary Fig. 1). Genome-specific PCR reactions were performed in a final volume of 28 µl containing 25 ng of genomic DNA, 100 µmol/l of each dNTP, 0.4 µmol/l of each primer, 1 U of Taq polymerase (Qiagen, Valencia, CA, USA), 1.8 mM MgCl2 and 1× Taq polymerase buffer (protocol B). Touch-down profile No.2 was similar to profile No.1 except for decreasing annealing temperature (from 66 to 58°C) and for the number of cycles (30 cycles) using 58°C as annealing temperature.

After sequencing and alignment, three new downstream genome specific primers were designed (FTex3gAL, FTex3gBL and FTex3gDL in Supplementary Fig. 1) in couple with FTex1U, allowing us to study most of the FT gene after the ATG codon (Supplementary Fig. 1). Final PCR conditions were achieved using protocol A modified with 0.5 U of Taq polymerase (Qiagen, Valencia, CA, USA). Touch-down profile No.3 was derived from profile No.1 except for decreasing annealing temperature starting from 74 to 64°C for A and B genomes, from 72 to 62°C for D genome, and for the annealing temperature applied during the 21 following cycles (64°C for A and B genomes and 62°C for D genome). The subsample of 40 hexaploid lines was amplified using these primers and PCR conditions. All PCR products were purified and sequenced with the Big Dye Sequencing kit according to the manufacturer's specifications (Applied Biosystems, Courtaboeuf, France), then loaded onto ABI3700 96 capillary sequencers. Mutations detected in only 1 line (singleton) were checked by resequencing an independent amplification product.

Nucleotide diversity and linkage disequilibrium in hexaploid wheat

To describe nucleotide diversity for each copy of FT gene in the panel of 40 wheats, we used DnaSp version 4.1 (Rozas et al. 2003). The number of polymorphic sites (S), the number of haplotypes (h), and the average number of nucleotide differences per site between two sequences π (or nucleotide diversity by Nei 1987) were calculated. Linkage disequilibrium (LD) between variants of different polymorphic sites within each FT copy was also estimated in DnaSp.

Relationship between FT from hexaploid wheat and FT from other species

In order to study phylogenetic relationship between FT-like proteins in *A. thaliana* (At), *Oryza sativa* (Os), *Hordeum vulgare* (Hv), *T. monococcum* (Tm) and *T. aestivum* (Ta), a Neighbour Joining tree was constructed by using pairwise deletions and 1,000 bootstrap iterations in MEGA version

3.0 software (Kumar et al. 2004). Variation in wheat peptide structure was examined using the following web site (http://www.predictprotein.org/newwebsite/) (Rost et al. 2003).

Genetical and physical mapping in hexaploid wheat

For genetic mapping, the last update of the Courtot (Ct) × Chinese Spring (CS) map was used (Sourdille et al. 2003). Segregation distortion for all the loci was tested using a Chi-square test. Mapmaker/exp version 3.06 (Lander et al. 1987) was used to construct the maps for biased or unbiased markers separately. Linkage groups were established using LOD and θ thresholds of 5 and 0.25, respectively. The genetic distances were calculated using the Kosambi (1944) mapping function. Anchor loci defining the backbone of the map were chosen according to the following criteria: absence of segregation distortion, minimum of missing data, and optimal spacing along the chromosomes.

Physical and genetical mapping information for the FT genes was integrated in an outline of the three *T. aestivum* group 7 chromosomes based on the consensus genetic map of Somers et al. (2004). Putative limits of the bins were drawn based on the assignment to physical bins of some of the markers ordered as in the consensus map. In addition, the estimated positions of earliness traits QTLs detected in the literature were projected on the consensus map either by meta-analysis (Hanocq et al. 2007) or manual projection (Ahmed et al. 2000; Gervais et al. 2003; Shindo et al. 2003; Schmolke et al. 2005; Huang et al. 2006; Kuchel et al. 2006; Quarrie et al. 2006).

Phenotypic, genotypic scoring and association study in hexaploid wheat

Genetic association was performed using 239 accessions of bread wheat. Phenotypic traits related to earliness were assessed in field and nursery experiments at Le Moulon in a 3-year experiment (2004, 2005 and 2006). In the field experiment, the 239 genotypes were sown in early November and grown in a two-replicate plot design where they received natural vernalization during winter. In the nursery experiments, the 239 genotypes were planted in April (long days) after 0, 4 or 8 weeks of vernalization treatment in controlled conditions (4°C). Experimental design, traits measurement (heading date in the field experiment HdField, heading dates after 0, 4 and 8 weeks vernalization, HdNV, HdV4 and HdV8) and transformation into developmental variables (partial vernalization sensitivity PVS = HdV4 − HdV8, narrow-sense earliness HdV8 and DLS = HdField − HdV8) are described in Goldringer et al. (2006). For all the treatments, heading dates were scored either on five to six individuals per line or on two single-row plots in the nursery experiment and in the field experiment, respectively. Contrary to the core set, the 199 remaining lines (239-40) were genotyped for polymorphic sites only by downstream sequencing only with FTex3gAL and FTex3gDL primers (Supplementary Fig. 1).

In order to avoid spurious associations, the genetic structure among lines was inferred from 82 microsatellite loci (about 4 per chromosome) using an admixture model parameter inferred from the EM-algorithm devised by Tang et al. (2005). For each value of K (from 2 to 10) the number of underlying ancestral populations, ten runs of the EM-algorithm with random start have been done, and we kept only the one with the highest log likelihood. Finally, a K value was chosen by using a PCA-based approach devised by Veyrieras et al. (personal communication). This procedure provides the minimal number of populations K that best explains the LD due to structuration. Four polymorphisms were studied, three on the A genome and one on the D genome (cf. "Results"). Genotypic classes with less than five individuals were discarded, so that heterozygous were not taken into account. Association between the four nucleotide polymorphisms and phenotypic traits was tested with the GLM procedure of the SAS software package (SAS Institute 2000) using the following linear model:

$$Y_{ijk} = \mu + \mathrm{yr}i_i + \sum_k G_j^k + P^1 + P^2 + P^3 + P^4 + R_{ijk}$$

with yr the fixed year effect, $\sum_k G^k$ the contributions of the k ancestor groups to a given line j, P^1, P^2, P^3, P^4 the effect of the four polymorphisms, and R the residual. For each polymorphism significantly associated to a trait, least-square-adjusted means (LSM) was also estimated. The global effect of the four SNPs was also assessed by comparing the determination coefficient (R^2) of the above model with the R^2 of the same model without any polymorphism effects. When the low frequency classes and the missing data were removed, 224 genotypes were left in the analysis. Broad sense heritability of the variables was assessed overall using a simple ANOVA model including a year and a genotype effect.

GenBank accession numbers for nucleotide sequences described in the manuscript are:

EF428113 for TAFTDh1 "*Triticum aestivum* var Chinese spring FT genome D haplotype 1"
EF428114 for TAFTDh2 "*Triticum aestivum* var Courtot FT genome D haplotype 2"
EF428115 for TAFTAh1 "*Triticum aestivum* var Chinese spring FT genome A haplotype 1"

EF428116 for TAFTAh2 "*Triticum aestivum* var Chyamtang FT genome A haplotype 2"
EF428117 for TaFTAh3 "*Triticum aestivum* var Renan FT genome A haplotype 3"
EF428118 for TAFTAh4 "*Triticum aestivum* var Recital FT genome A haplotype 4"
EF428119 for TaFTBBT21 "*Triticum aestivum* var M708G25N163 FT genome B"

Results

Supplementary Fig. 1 shows the alignment of consensus sequences (obtained from the alignment of the 40 sequences) for each FT copy with the cDNA published by Ciaffi et al. (2005). In our study 791, 1064 and 828 bp were sequenced for genomes A, B and D, respectively. Intron length was variable depending on the genome and on the position in the gene. Intron 1 varied from 390 (400) bp for genome A (D) to 626 bp for genome B due to an insertion of 263 bp present in the latter only. The smaller intron 2 was also less variable in length among the genomes, varying from 90 bp for genome D to 105 bp for genome A. When comparing the consensus sequence found for genome B with those published by Yan et al. (2006) [Genbank accession numbers DQ890162 and DQ890165 for Chinese spring (CS) and CS(Hope7B), respectively], perfect alignment was obtained with CS, whereas as previously mentioned by the authors, three SNP and two deletions were detected in intron 1 in comparison to CS(Hope7B). Copy from genome B appeared poorly variable in the panel of 40 wheats. Polymorphism was detected in one line (BT21 GenBank accession number EF428119 in Supplementary Table 1) only and the five affected sites were all present in the insertion of the first intron. This result was confirmed by re-sequencing new PCR products. As a consequence, further analyses of nucleotide diversity and association study were not conducted for FTB. The highest level of variability was observed for the genome A ($S = 3$ polymorphic sites, $h = 4$ haplotypes with GenBank accession numbers EF428115, EF428116, EF428117, EF428118 and $\pi = 1 \times 10^{-3}$), and was mainly located in the non-coding regions (S equal to 1 and 2, and π equal to 0.5×10^{-3} and 1.3×10^{-3} for coding and non-coding regions, respectively). Both introns were concerned, with an SNP Y (EUB code) and a variable microsatellite motif $(TG)_{8or9}$-$(CG)_3$ encountered within the first and the second introns, respectively (Supplementary Fig. 1). The latter polymorphism was analysed as an SNP Y by DnaSp [$(TG)_8$ and $(TG)_9$ being treated as a C and a T, respectively]. The coding polymorphism consisted in a SNP K (EUB code) localised within exon 3 of FTA (Supplementary Fig. 1). Whatever the estimation used in DnaSp, no LD was found between FTA polymorphisms (227 bp at maximum between SNP Y from intron 1 and SNP K from exon 3). For genome D, a single polymorphism was observed, consisting in an insertion–deletion of one G in a poly G [noted as $(G)_3$ or $(G)_4$ and present in 29 and 11 lines, respectively] which was located in the third exon (Supplementary Fig. 1). Two haplotypes were then found overall (GenBank accession numbers EF428113 and EF428114). A low but significant level of LD was observed between FTA K and FTD $(G)_{3or4}$ [$r^2 = 0.25$ and P value (Fisher's exact test) = 0.01].

Phylogenetic relationships of wheat FT peptide obtained after FTA, FTB and FTD copies translation are shown in Fig. 1. Ta FTABD peptide results from the translation of: haplotypes 1, 3 and 4 for FTA (37 wheat out of 40), unique coding sequence for FTB (40 wheat), and haplotype 1 for FTD (11 wheat out of 40). As expected, Ta FTABD was found to be more related to Hv FT from barley than to other species, and was closer to FT genes than to FT-like genes. Similarly to Hv FT gene, Ta and Tm FT genes showed three exons, whereas Os and At genes presented four exons. This difference was generated by the fusion of exons 3 and 4 in the former. Ta FTABD protein was strictly identical in sequence to CS(Hope7B) peptide from Yan et al. (2006) (Fig. 2). Differently from this study, two peptide variants were generated by mutations in exon 3 of genomes A and D (haplotype 2 for FTA and FTD present in 3 and 29 lines out of 40, respectively), which did not modify the NJ tree structure (data not shown). All modifications occurred outside the phosphatidylethanolamine-binding protein (PEBP) domain composed by 23 amino acids (Fig. 2). The G substitution for the FTA-K SNP conducted to replace a methionine (M) by an arginine (R) without consequences on the protein site function. Conversely, the $(G)_3$ repetition for FTD, by modifying the

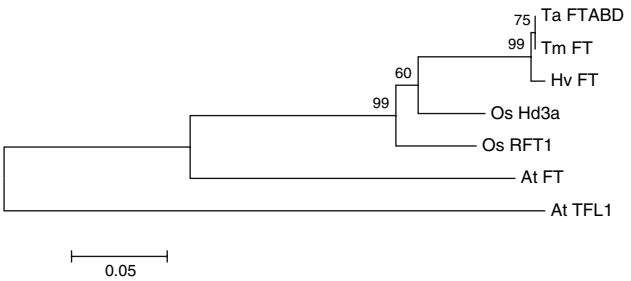

Fig. 1 Phylogenetic relationship between FT-like proteins from this study, *Triticum aestivum* (*Ta* FTABD, and *T. monococcum* (*Tm* FT), and from the literature using following Genbank accession numbers: *Hv* FT (DQ100327) for *Hordeum vulgare*, *Os* Hd3a (AB052944) and *Os* RFT1 (BAB78480) for *Oriza sativa*, *At* FT (AB027504) and *At* TFL1 (NP196004) for *Arabidopsis thaliana*. Bootstrap values are indicated close to their respective nodes

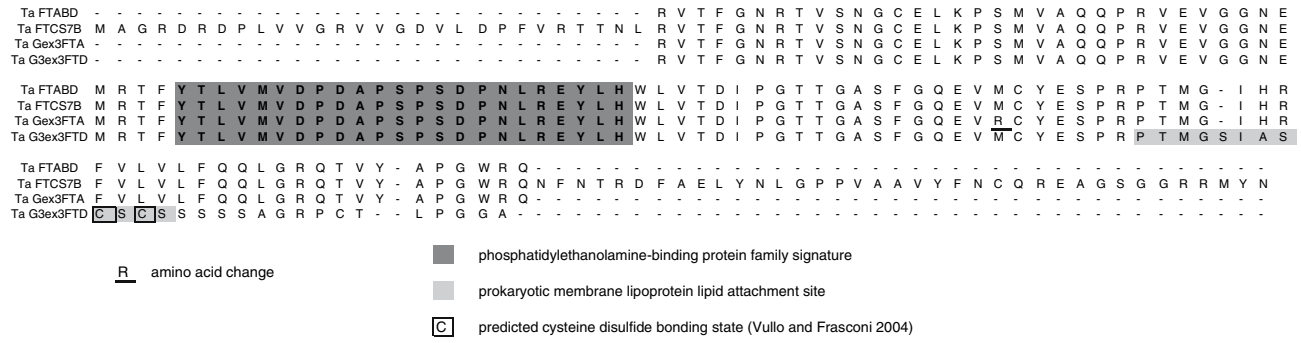

Fig. 2 Wheat FT peptides alignment and results from the website "Predictprotein" (see "Materials and methods"). Ta FTCS7B is the Chinese Spring (Hope7B) peptide of 179 amino acids from Yan et al. (2006); Ta FTABD is the main peptide found in this study whatever the genome; Ta Gex3FTA is the peptide due to the G substitution in exon 3 of FTA (translation of FTA haplotype 2); Ta G3ex3FTD is the peptide resulting from the G3 repetition in exon 3 of FTD (translation of FTD haplotype 2)

amino acid sequence, revealed a new lipoprotein attachment site with two disulfide bonding sites (Fig. 2).

Figure 3 shows physical and genetical mapping informations for the FT genes. In some cases, the ordering of the markers on the consensus genetic map was not consistent with their assignment to the bins. This might be due to inaccuracy in the genetic map, duplications or inversions of chromosome fragments between the different genotypes used for genetic mapping and physical assignation. For the three chromosomes, our FT copies were located close to meta-QTLs or QTLs of earliness traits (Fig. 3). For chromosomes 7B and 7D, respectively, FT genes were physically assigned to the distal bins of the short arms (7BS1-0.27-1.00 and 7DS4-0.61-1.00, respectively, Fig. 3). This was consistent with the position found by Yan et al. (2006) for the TaFT gene (Vrn-B3) on chromosome 7B. FT genome A was assigned to the 7AS8-0.45-0.59 bin (Fig. 3), which appeared difficult to delimit on the genetic map. Genetic mapping was possible for the polymorphic copies FTA and FTD only. But since they were mapped on the Courtot × CS map, their positions were estimated by homothetic projection on the map from Somers et al. (2004). Consistently with the physical assignation, the estimated position of the FTA copy was near the marker *barc154* (Fig. 3). One QTL for flowering time (Quarrie et al. 2006) was possibly located in the same bin as FTA, given the uncertainty on the bin boundaries. One meta-QTL for heading date and earliness per se (Hanocq et al. 2007) and one single QTL for heading date (Kuchel et al. 2006) were also located in a 30 cM region around *barc154*. For chromosome 7B, one meta-QTL for heading date and earliness per se (Hanocq et al. 2007) and one QTL for heading date (Schmolke et al. 2005) were located in the same bin as FT, although the boundary was also uncertain. The estimated mapping position of FTD on chromosome 7D was near the *gwm44* marker. One QTL for heading date (Börner et al. 2002) and one QTL for the number of days to maturity (Huang et al. 2006) were located in the same bin as FTD. One other QTL for heading date (Sourdille et al. 2000) was located on another bin but close to the FT gene [$\sim$10 cM based on the mapping position on the consensus map of Somers et al. (2004) provided by E. Hanocq, personal communication].

Three out of four FT polymorphisms were found significantly associated with the variation at some of the earliness traits studied (Table 1). While FTA $(TG)_{8or9}$ was significantly and highly associated with all variables, the other SNPs in the A genome were seldom, if ever, significant in the analyses of variance (Table 1). Polymorphism in the D genome was found associated with heading date in the field (HdField) and heading date under long days with 0, 4 and 8 weeks of vernalization (HdNV, HdV4, HdV8). Second-order interactions between SNPs were always far from significance indicating that their effects were highly additive. The effect of the SNP FTA $(TG)_{8or9}$ was always the most significant, eventhough the differences between the two genotype adjusted means of FTA $(TG)_{8or9}$ and FTD $(G)_{3or4}$ were about the same order for HdField, HdV4 and HdV8. The largest difference between adjusted SNP means (221 dd) was obtained for HdNV between FTA $(TG)_8$ and FTA $(TG)_9$, indicating the strong association between the SNP and heading date under non-vernalized condition but also the considerable range of variation for plant development without vernalization. Only HdField was influenced by three different SNPs from both genomes A and D, but all variables were partly controlled by at least one of these SNPs, indicating that their effects (or associated effects) were not specific but rather global. While some of these traits are highly correlated (for instance, the phenotypic correlation between HdField and HdV8 is +0.87), other earliness components that are only slightly correlated ($r = -0.34$ between HdNV and DLS, and $r = 0.35$ between HdV8 and PVS) or not linked at all (HdField and DLS) are also associated to the same FT polymorphisms.

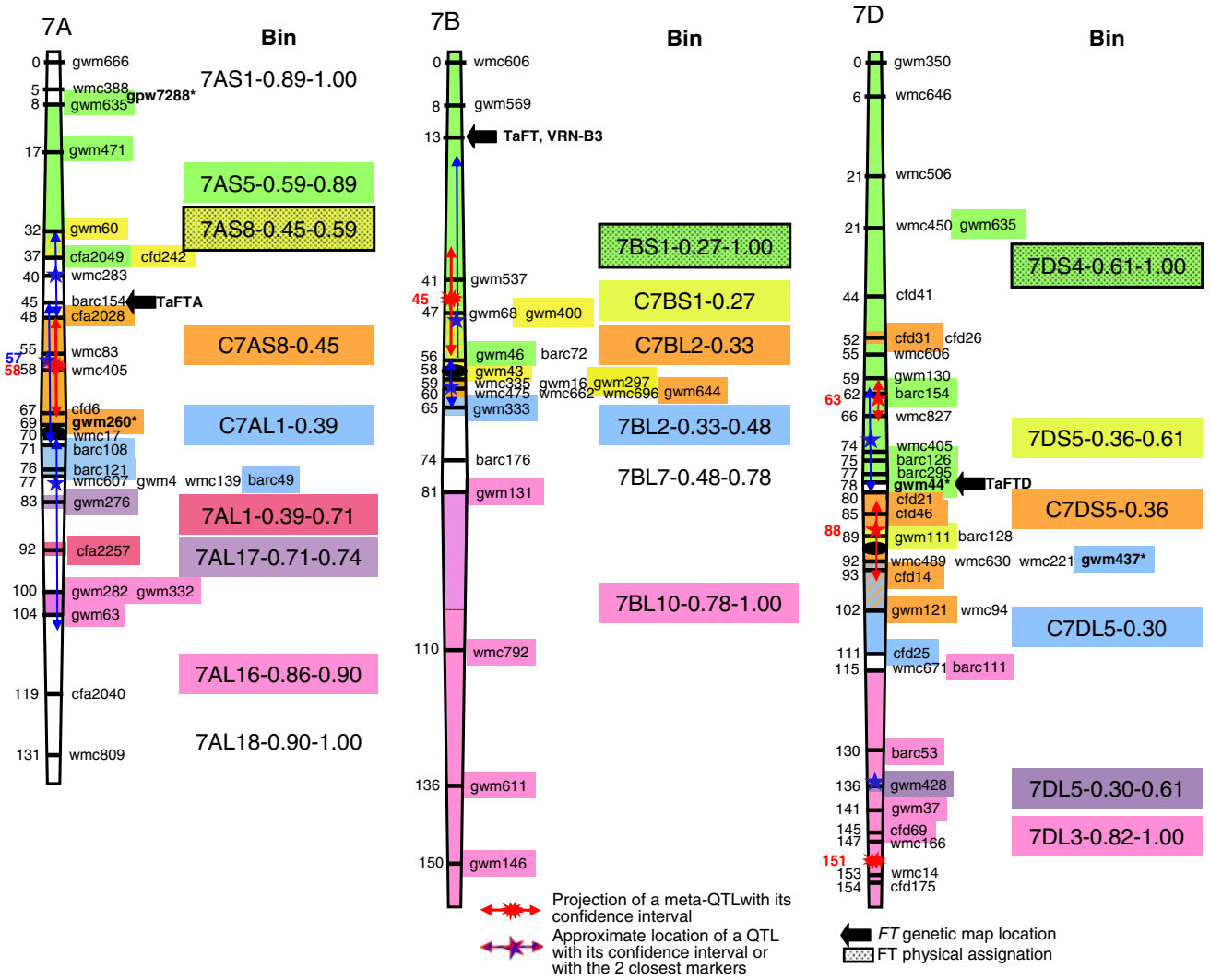

Fig. 3 Outline of the three *T. aestivum* group 7 chromosomes integrating both the physical map (Sourdille et al. 2004), the consensus genetic map of Somers et al. (2004) and projections of earliness traits QTLs positions with their confidence intervals (or with the two closest markers when IC were not available) on the consensus map (meta-analysis by Hanocq et al. (2007); manual projection for the other QTLs). Meta-QTLs and individual QTLs that have been projected on the Somers et al. (2004) map by Hanocq et al. (2007) appear in *red*, while all QTLs from others studies appear in *blue*. Different bins are represented with *different colours*. The markers that were assigned to a given bin are indicated with the *corresponding colour*. Position of FT genes is indicated by *black arrows*. FTA and FTD were mapped on the Courtot × CS map and their positions were then estimated by homothetic projection on the map from Somers et al. (2004). Marqueurs indicated in *bold* with *asterisk* were genotyped on the set of 239 accessions to assess the genetic structure

The comparison of R^2 values between full and partial models (with and without the SNPs effects, respectively) showed that 2% (for DLS) to 6% (for narrow-sense earliness, HdV8) of the total phenotypic variation was explained by the polymorphism effect. Whereas the broad sense heritability of HdField, HdNV, HdV4 and HdV8 was very high, indicating the major contribution of genetic variation to the phenotypic variability of these traits in this experiment, the heritability was somewhat lower for PVS and quite weak for DLS. This was consistent with the lowest SNPs contribution obtained for DLS variation (Table 1).

Among the parental lines used for the QTL detection experiments, only Renan and Recital (Gervais et al. 2003; Hanocq et al. 2004; 2007), Synthetic W7984 and Opata85 (Börner et al. 2002; Hanocq et al. 2007), Courtot and Chinese Spring (Sourdille et al. 2000; Hanocq et al. 2004) were common to the set of 239 accessions used for the association tests. For these genotypes, we assessed the concordance between genetic effects from QTL analyses and association mapping. Using Renan–Recital population, three QTL for earliness were detected: 1 on the 7A (at position 58 cM in Fig. 3, that is 13 cM from FTA polymorphisms) and 2 on the 7D (meta-QTL at position

Table 1 Association tests between the four FT polymorphisms and variation at earliness components

		N	HdField	HdNV	HdV4	HdV8	PVS	DLS
h^2_{SL}			0.96	0.97	0.89	0.91	0.76	0.28
R^2 (yr + G)			0.52	0.26	0.24	0.39	0.12	0.12
R^2 (full model)			0.55	0.31	0.29	0.45	0.15	0.14
FTA Y	*F* (SNP)		0.16	0.99	1.34	0.00	3.24	0.15
	P value*		NS	NS	NS	NS	NS	NS
Lsmeans (dd)			–	–	–	–	–	–
FTA $(TG)_{8or9}$	*F* (SNP)		21.82	32.04	19.25	18.41	12.64	8.42
	P value*		<0.0001	<0.0001	<0.0001	<0.0001	0.0004	0.004
Lsmeans (dd)	$(TG)_8$	80	1,179	1,639	1,168	968	217	243
	$(TG)_9$	153	1,145	1,418	1,059	920	126	263
FTA K	F (SNP)		5.58	1.29	0.34	1.37	0.55	0.04
	P value*		0.02	NS	NS	NS	NS	NS
Lsmeans (dd)	G	21	1,139	–	–	–	–	–
	T	215	1,185	–	–	–	–	–
FTD $(G)_{3or4}$	*F* (SNP)		13.64	7.50	8.26	10.93	1.77	1.79
	P value*		0.0002	0.006	0.004	0.001	NS	NS
Lsmeans (dd)	$(G)_3$	181	1,179	1,599	1,161	968	–	–
	$(G)_4$	54	1,145	1,458	1,064	920	–	–

PVS partial vernalization sensitivity, *DLS* daylength sensitivity

h^2_{SL} is the broad sense heritability in the experiment; R^2 (yr + G) and R^2 (full model) are the determination coefficients values issue from the ANOVA model restricted to the group and year effects and from the full model, respectively; Lsmeans (dd) are the least square means expressed in °C days of the genotypes sharing a given SNP. Earliness traits: HdField, heading date in the field experiment; HdNV, HdV4 and HdV8 heading dates after 0, 4 and 8 weeks of vernalization, respectively

* Only *P* values lower than 0.05 are given, else "NS" is specified

151 cM in Fig. 3, that is 53 cM from FTD polymorphism). These parental lines were polymorphic at the FTA $(TG)_{8or9}$ locus only (genotypes 88 and 99, respectively). Differently from Hanocq et al. (2007) but consistently with Gervais et al. (2003) QTL detection study, earliness was associated with Recital genotype in our association study. Synthetic W7984–Opata85 population and Courtot–Chinese Spring population allowed for the detection of one QTL for earliness on the 7D each (at position 63 and 88 cM, respectively, in Fig. 3, that is 15 and 10 cM from FTD polymorphism, respectively). Whereas no polymorphism was found between Synthetic W7984 and Opata85 at the FTD $(G)_{3or4}$ locus, Courtot and Chinese Spring lines were polymorphic with genotypes 33 and 44, respectively. Moreover, in agreement with Sourdille et al. (2000), the allele associated to earliness came from Chinese Spring.

Discussion

In wheat, previous mapping and segregation analyses have shown that vernalization requirement and photoperiod sensitivity are mainly regulated by four and three major genes, respectively, whereas narrow-sense earliness is more polygenic (see Law and Worland 1997; for review). Overriding the problem of the ploidy level, recent advances in molecular biology and genomics of wheat have allowed some of these genes to be isolated and sequenced, especially those involved in vernalization requirement such as VRN1 or VRN3 (previously called VRN5 or VRN-B4) (Yan et al. 2003, 2006). As shown in barley for VRN-H2 (Dubcovsky et al. 2005) and *Arabidopis* for FT, LFY, SOC1, and AP1 (Bernier and Périlleux 2005), causal polymorphism in wheat (i.e. discriminating winter and spring vernalization-independent plants) was found within regulatory regions of the genes (Yan et al. 2004b, 2006; Fu et al. 2005). However, contrary to VRN1, the causal polymorphism published for VRN3 (retroelement inserted in the promoter) was detected in one line only, the Hope variety (Yan et al. 2006). Because this allele was found in the progeny of a cross between the substitution line CS(Hope7B) and CS developed by C Law (Law 1966), the real presence of a major gene in this region has been challenged for a long time. It has been suggested that VRN3 had arisen as a small translocation during the development of the substitution line and that the gene could be originated from a group 5 chromosome (Law and Worland 1997). Here, we confirm the presence of FT in the short arm of the group 7 chromosomes, but still there is no indication that the Hope allele was common to any other

wheat genotypes, whereas the three FTA and FTD polymorphisms associated to variation in earliness components were largely shared within the collection of 239 wheats (see Table 1). Our paper, by using both association study and QTLs mapping synthesis is complementary to the approach of Yan et al. (2006) which relies on both transformation and expression study. Although false positive associations resulting from historical linkage disequilibria within collections might lead one to misjudge the causal polymorphism in association studies, a possible modified transcriptional regulation in the transgenic genotype relative to the wild genotype has to be taken into account in drawing conclusion from transformation studies. This restriction was pointed out by the authors in their supplementary material.

As previously shown for VRN1, depending on the genomic copy, different regions of the same gene can be involved in phenotypic variation (Fu et al. 2005). Differently from Yan et al. (2006) for FTB (VRN3), we did not examine the promoter region of FTA, FTB and FTD copies. However, in accordance with their results, only singleton polymorphism was observed inside the FTB gene. Conversely, both copies from genomes A and D showed polymorphism in non-coding and coding regions, respectively. The estimation of molecular diversity obtained for FTA was very close to the values derived from literature (Ravel et al. 2006a; Haudry et al. 2007). Considering 21 gene fragments and the same 40 *T. aestivum* lines as in our study, Haudry et al. (2007) found only three polymorphic sites per locus on average (from 0 to 18 depending on the gene) and a mean value of nucleotide diversity π equal to 0.8×10^{-3} (from 0 to 2.46×10^{-3} depending on the gene). As shown by these authors, compared to wild wheat, diversity was reduced by 69% in bread wheat, with considerable differences between loci, due to both selective and demographic events, which occurred during its domestication and subsequent selection.

In our study, a low level of LD was observed at the inter genomic level only (between FTA K and FTD $(G)_{3or4}$). Estimation of LD between FT polymorphisms and physically linked SSR markers among those used for the genotyping of the collection (two markers around FTA and two around FTD in Fig. 3) was not significant. But except for gwm44 near FTD, these markers were rather far from SNP positions (Fig. 3). In selfing species, inbreeding drives lineages to homozygosity and hitchhiking occurs quickly between unliked loci (Hedrick 1980). Moreover, in bread wheat, the range of LD varies depending on the chromosome and on the germplasm studied. LD may extend over less than 1 cM for genetically linked loci pairs (Ravel et al. 2006b; Haudry et al. 2007; Chao et al. 2007) to several cM (Breseghello and Sorrells 2006; Somers et al 2007), and LD can also be detected between independent loci, or it may disappear between closely linked markers (Chao et al 2007). Testing genetic association for gwm44 alone or with FTD revealed significant effects of the SSR marker, that however, remained lower than those of FTD (Table 2). Because the large difference between the number of alleles at the microsatellite locus and at the SNPs may influence both the LD pattern (Breseghello and Sorrells 2006) and the effects detected in association studies, it is hard to discriminate conclusively whether FTD is indeed the causative locus or is only in LD with another locus/gene physically linked that controls heading date traits. A third possibility is that a second locus within this region of chromosome 7D and closer to gwm44 is also associated with heading date variation in wheat. Testing the three hypotheses would require further investigations.

In our study, polymorphisms associated with variation in earliness components consisted in a variable repeat number of a dinucleotide microsatellite motif for FTA and a poly G motif for FTD, the latter by modifying the end of the FT peptide revealed a probable new site for protein regulation. Both polymorphisms had a significant effect on earliness components variation, especially for heading date in the treatment without vernalization, which is in agreement with the role of wheat FT gene proposed by Yan et al. (2006). Assuming that wheat FT is an integrator gene as in *Arabidopsis* and rice, FT polymorphisms are expected to affect both vernalization and photoperiod pathways. In a hypothetical model, Yan et al. (2006) suggested that long days might up-regulate the promoter of FTB (VRN3). Our results (see Table 1) showed that the dinucleotide microsatellite motif for FTA only is associated to DLS. However, this estimation of DLS is poorly heritable (0.28), probably because it is based on both nursery and field heading date records. As a consequence, it is difficult to conclude that FT A polymorphism only and not FT D is

Table 2 Testing genetic association for earliness components using SSR marker close to FTD alone (analysis 1) or with FTD (analysis 2)

SNP/marker	HdField	HdNV	HdV4	HdV8	PVS	DLS
Analysis (1)						
R^2 (full model)	0.57	0.31	0.28	0.48	0.16	0.17
gwm44						
P value	0.0003	NS	NS	NS	NS	NS
Analysis (2)						
R^2 (full model)	0.61	0.34	0.30	0.53	0.17	0.17
gwm44						
P value	<0.0001	0.01	0.04	0.02	NS	NS
FTD $(G)_{3or4}$						
P value	<0.0001	<0.0001	<0.0001	<0.0001	0.05	NS

The group and year effects were included into the ANOVA model (full model)

associated with the variation of this character. Plants should be grown with artificially controlled photoperiod to better estimate DLS.

Interestingly, non-coding intronic region was highly involved in phenotypic variation, but differently from VRN1, for which epistatic interactions among genomes were demonstrated (Fu et al. 2005), no epistatic interaction between FT homeologous copies was revealed. Partial R^2 associated with the SNPs (2–6%) were generally lower than those of QTLs detected for earliness traits in classical bi-parental populations designs (e.g. 8.7–20.9% in Ahmed et al. (2000); 5–31.6% in Huang et al. (2006); 5–41% in Kuchel et al. (2006); 6–38.9% in Hanocq et al. (2007) meta-analysis). This was probably due to the much wider genetic variation involved in our study (239 genotypes in the sample), which enabled us to reveal variations at numerous earliness loci. Assessing whether the genetic effects from QTL analyses were consistent with those estimated in the association study was not easy because most parental lines of the QTL detection populations from the literature had not been genotyped for FT gene polymorphisms. Information was available for parents involved in the detection of five QTLs: (1) for the QTL detected on chromosome 7A using one population (Hanocq et al. 2004), opposed effect was found in our association study, earliness being associated with Recital parental line, as it was the case in Gervais et al. (2003); (2) for the four QTLs detected on chromosome 7D using three populations, only the parents of the closest QTL to FTD $(G)_{3or4}$ locus were polymorphic and the genetic effects were in agreement with those from our association study. Discrepancy might be due to multiple linked QTLs located in this region (Hanocq et al. 2007) or to false-positive in either the QTL or the association study.

While our results do not prove that the FTA and FTD polymorphisms studied are truly involved in the variation of earliness traits, we provide some clues of that, as well as the tools for further expression or transformation study. Further work will also investigate the respective contributions of FT and VRN1 genes on earliness components variation in bread wheat. Studying FT and VRN1 allelic distribution in experimental populations of wheat differentiated for heading date in response to local climatic conditions (Goldringer et al. 2006) will provide valuable insights, such as the identification of combinations of alleles favouring adaptation to specific environments. Predicted climate change as for example increasing summer temperatures in Europe, will probably enhance the use of early flowering varieties, requiring less vernalization than previously, but conserving a high seed production. This work, by identifying additional homeoalleles associated with variation in wheat vernalization requirement, will contribute to a better understanding of the control of flowering, hence providing tools for the breeding of varieties with enhanced adaptation to changing environments.

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