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RESEARCH PAPER

Flowering time variation in oilseed rape (*Brassica napus* L.) is associated with allelic variation in the *FRIGIDA* homologue *BnaA.FRI.a*

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Abstract

Oilseed rape (*Brassica napus* L.) is a major oil crop which is grown worldwide. Adaptation to different environments and regional climatic conditions involves variation in the regulation of flowering time. Winter types have a strong vernalization requirement whereas semi-winter and spring types have a low vernalization requirement or flower without exposure to cold, respectively. In *Arabidopsis thaliana*, *FRIGIDA* (*FRI*) is a key regulator which inhibits floral transition through activation of *FLOWERING LOCUS C* (*FLC*), a central repressor of flowering which controls vernalization requirement and response. Here, four *FRI* homologues in *B. napus* were identified by BAC library screening and PCR-based cloning. While all homologues are expressed, two genes were found to be differentially expressed in aerial plant organs. One of these, *BnaA.FRI.a*, was mapped to a region on chromosome A03 which co-localizes with a major flowering time quantitative trait locus in multiple environments in a doubled-haploid mapping population. Association analysis of *BnaA.FRI.a* revealed that six SNPs, including at least one at a putative functional site, and one haplotype block, respectively, are associated with flowering time variation in 248 accessions, with flowering times differing by 13–19 d between extreme haplotypes. The results from both linkage analysis and association mapping indicate that *BnaA.FRI.a* is a major determinant of flowering time in oilseed rape, and suggest further that this gene also contributes to the differentiation between growth types. The putative functional polymorphisms identified here may facilitate adaptation of this crop to specific environments through marker-assisted breeding.

Key words: Association mapping, *B. napus*, flowering time, *FRI*, growth type, QTL, vernalization requirement.

Introduction

Oilseed rape or rapeseed (*Brassica napus* L.) is one of the most important oilseed crops worldwide. *B. napus* is an allotetraploid species with $2n=38$ chromosomes and two genomes, AA derived from *B. rapa* and CC from *B. oleracea*. Because of its high seed oil and protein content, rapeseed is mainly grown as animal feed, for the production of vegetable oil for human consumption, and for biodiesel production. The yield potential of rapeseed to a large extent depends on flowering time, and flowering time adaptation is a major breeding goal. Three different growth types can be

distinguished. Spring types flower early without vernalization and are grown in geographical regions such as Canada with strong winters which do not allow the cultivation of winter types due to unsatisfactory winter hardiness (Thomas, 2003), or in subtropical climates such as in parts of Australia. By contrast, winter types have an obligate requirement for prolonged periods of cold temperatures for flowering to occur and are grown in moderate temperate climates such as Western Europe. Semi-winter types are sown before winter and flower after winter, but lack frost hardiness and are therefore grown in geographical regions with moderate winter temperatures (>0 °C) such as central China. In addition to cold temperatures, the length of the daily light period (photoperiodism) is a second major environmental cue affecting floral induction, with longer days promoting flowering in *B. napus*. Increasing demands for plant oil with altered quality traits and a changing climate impose new challenges for rapeseed breeding. Crossings between different rapeseed types (e.g. spring× winter) are generally avoided because the offspring are non-adapted to the respective growing conditions, particularly with respect to disease resistance traits and flowering time (Qian *et al.*, 2006). For example, the introduction of genes into European rapeseed from different geographical origins is hampered by non-adapted flowering time traits and requires extensive backcrossing (Qian *et al.*, 2007). Thus, functional markers for flowering time traits are desirable for the selection of adapted material (Jung and Müller, 2009). Because of the low genetic diversity within *B. napus* (Gómez-Campo, 1999) knowledge of the mechanism underlying flowering time variation is expected to have considerable impact on breeding strategies.

In the model species *Arabidopsis thaliana*, several floral transition pathways have been identified which regulate the timing of flowering in response to different environmental and endogenous cues (Bäurle and Dean, 2006; Albani and Coupland, 2010; Amasino, 2010; Amasino and Michaels, 2010). The key regulator in the photoperiod pathway, which mediates the effect of day length on flowering time, is the CCT (*CONSTANS*, *CONSTANS-LIKE*, *TIMING OF CAB EXPRESSION 1*) domain transcription factor gene *CONSTANS* (*CO*). Under long-day conditions, CO protein accumulates at the end of the light phase and promotes flowering by activating the expression of *FLOWERING LOCUS T* (*FT*), a central integrator of input signals from different pathways whose protein product is a major component of the mobile signal that moves to the shoot apical meristem and initiates flowering (Turck *et al.*, 2008). The key regulator of vernalization requirement and response is the MADS box transcription factor gene *FLOWERING LOCUS C* (*FLC*), which is a repressor of *FT* but is down-regulated by vernalization, thus enabling promotion of flowering by *FT*. Both *FLC* and its upstream regulator *FRIGIDA* (*FRI*) are major determinants of natural variation in flowering time (Johanson *et al.*, 2000; Le Corre *et al.*, 2002; Gazzani *et al.*, 2003; Michaels *et al.*, 2003; Caicedo *et al.*, 2004; Hagenblad *et al.*, 2004; Stinchcombe *et al.*, 2004; Le Corre, 2005; Shindo *et al.*, 2005; Werner *et al.*, 2005). Dominant alleles of *FRI* confer a vernalization requirement causing plants to overwinter vegetatively, and several loss-of-function mutations are associated with early flowering (Johanson *et al.*, 2000). *FRI* was suggested to up-regulate *FLC* expression through interaction with the histone methyltransferase *EARLY FLOWERING IN SHORT DAYS* (*EFS*), which results in the modification of *FLC* chromatin (Ko *et al.*, 2010), and interaction with a nuclear cap-binding complex (CBC) with concomitant effects on *FLC* transcription and splicing (Geraldo *et al.*, 2009).

In *B. napus* and related *Brassica* species, many flowering time quantitative trait loci (QTL) with large effects and high heritabilities have been mapped, and several studies reported the identification of flowering time gene homologues, including *FLC* homologues in *B. napus* (*BnFLC1–5*; Tadege *et al.*, 2001), *B. oleracea* (*BoFLC1, BoFLC2*/*BoFLC4, BoFLC3*, and *BoFLC5*; Schranz *et al.*, 2002; Lin *et al.*, 2005; Okazaki *et al.*, 2007; Razi *et al.*, 2008), and *B. rapa* (*BrFLC1, BrFLC2, BrFLC3*, and *BrFLC5*; Schranz *et al.*, 2002; Lin *et al.*, 2005; Kim *et al.*, 2007), *CO* homologues in *B. napus* and *B. nigra* (Robert *et al.*, 1998; Osterberg *et al.*, 2002), and *FT* homologues in *B. napus* (Wang *et al.*, 2009). A comprehensive QTL analysis of a DH population (TNDH) derived from a cross between the European winter cultivar Tapidor and the Chinese semi-winter cultivar Ningyou7, which flowered 9 d or 13 d earlier than the winter type parent under winter and semi-winter conditions, respectively (Shi *et al.*, 2009), revealed a total of 32 unique flowering time QTL, corresponding to five to 13 QTL in each of 11 winter or spring crop environments (Long *et al.*, 2007). Of the 32 QTL, 25 could be aligned to syntenic regions in the *A. thaliana* genome containing known flowering time genes. The largest-effect QTL, which corresponds to a major vernalization-responsive flowering time QTL (*VFN2*) identified by Osborn *et al.* (1997), explained up to 50% of the phenotypic variation for flowering time in the spring crop environments and co-localized with the *FLC* homologue *BnFLC1* (Tadege *et al.*, 2001) on linkage group N10. In addition, several other QTL in the TNDH and other populations are located in regions where other *FLC* homologues (*BnFLC2–5*) were mapped (Osborn and Lukens, 2003; Udall *et al.*, 2006; Long *et al.*, 2007). Similarly, three *FLC* homologues in *B. rapa* (*BrFLC1, BrFLC2, BrFLC5*) co-segregate with flowering time loci, including two alleles which affect flowering time in a completely additive manner (Schranz *et al.*, 2002; Zhao *et al.*, 2010), while in *B. oleracea* thus far there is only evidence for the co-localization of a single *FLC* homologue (*BoFLC2*) with a flowering time QTL (Okazaki *et al.*, 2007; Razi *et al.*, 2008). Constitutive expression of *BnFLC1–5* and *BrFLC1–3* in *A. thaliana*, and *BrFLC3* in *B. rapa*, significantly delayed flowering (Tadege *et al.*, 2001; Kim *et al.*, 2007), thus further supporting the notion that, at least in *B. rapa* and *B. napus*, the role of *FLC* homologues as floral regulators is largely conserved.

Functional conservation of flowering time control by *CO* homologues in *Brassica* species was shown by

complementation analysis in *A. thaliana co* mutants with the *B. napus* allele *BnCO*a1 (Robert *et al.*, 1998) and two alleles of the *B. nigra* gene *Bni COa* (Osterberg *et al.*, 2002). In addition, a naturally occurring InDel variation in the *B. nigra CO*-like gene *Bni COL1* gene is associated with variation in flowering time (Osterberg *et al.*, 2002). *Bni COa* and *Bni COL1* are located adjacent to each other (Osterberg *et al.*, 2002) and co-localize with a major QTL that explains 53% of the phenotypic variation for flowering time under long-day conditions (Lagercrantz *et al.*, 1996). Among the six *B. napus* homologues of *FT*, three (*BnA2.FT*, *BnC6.FT.a*, and *BnC6.FT.b*) co-localize with major QTL clusters identified in the TNDH population on chromosomes A02 and C06 (Wang *et al.*, 2009). Interestingly, a consensus QTL for seed yield co-localized precisely with consensus QTL for the yield-associated traits flowering time, maturity time, and plant height (Shi *et al.*, 2009). Because the respective quantitative trait values are more easily measurable than seed yield, these QTL were named 'indicator QTL'. The co-localization of flowering time QTL with yield and yield-related QTL in *B. napus* has also been reported in other studies (Udall *et al.*, 2006; Long *et al.*, 2007; Basunanda *et al.*, 2010; Chen *et al.*, 2010). Finally, a major flowering time QTL in *B. napus* (*VFN1* on linkage group N2) was found to be located in a region of fractured collinearity with several chromosomal regions in *A. thaliana*, including the region carrying the *FRI* locus (Osborn *et al.*, 1997; Osborn and Lukens, 2003). Similar to the QTL co-localizing with *BnFLC1* (*VFN2*; Osborn *et al.*, 1997) and a third vernalization-responsive QTL (*VFN3* on linkage group N3; Osborn *et al.*, 1997; Butruille *et al.*, 1999), this QTL was detected in non-vernalized populations derived from crosses between winter and spring type accessions, but not in vernalized populations, suggesting that the QTL may contribute to the regulation of vernalization requirement. However, despite these earlier results and the prominent function of *FRI* in flowering time regulation in *A. thaliana*, thus far *FRI* homologues have not been identified and further analysed in *B. napus*.

Linkage disequilibrium (LD)-based association analysis (also known as association mapping or LD mapping) has attracted the attention of plant researchers due to its high resolution and because there is no need for producing structured populations (e.g. F_2 or DH; Gupta *et al.*, 2005). As a result of the limited number of crossover events during meiosis, recombination-based linkage analysis typically localizes QTL to 10 to 20 cM intervals (Doerge, 2002; Holland, 2007). By contrast, association mapping which is based on large germplasm collections can localize the genetic basis of phenotypic variation to very small regions of the genome or even single genes. However, spurious associations can occur and QTL with small phenotypic effects can go undetected. Here, a combination of association analysis and recombination mapping is used to unravel the contribution of a candidate gene to phenotypic variation of a complex quantitative trait. Four *B. napus* homologues of *FRI* were identified, one of which (*BnaA.FRI.a*) co-localizes with a cluster of major flowering time QTL. Furthermore, evidence is provided that allelic variation at the gene locus is associated with variation in flowering time in a worldwide panel of *B. napus* accessions. Together, the data suggest that *BnaA.FRI.a* is a major determinant of flowering time and contributes to the differentiation between growth habits of rapeseed.

Materials and methods

Plant material and growth conditions

The *B. napus* TNDH population encompassing 202 lines (Qiu *et al.*, 2006) was used as a mapping population. The parents of this doubled-haploid (DH) population are *B. napus* cv. Tapidor and Ningyou7, with the former being a European winter type rapeseed which flowers late and the latter being a Chinese semi-winter type which flowers early under winter field conditions in central China (Long *et al.*, 2007). Flowering time data for the TNDH population grown at five different locations in China in successive field trials over a period of four years (2002–2006), which included both winter and spring growing conditions, were recorded by Long *et al.* (2007).

A collection of 248 *B. napus* accessions was used for association mapping (see Supplementary Table S1 at *JXB* online). These accessions included registered varieties and breeding lines from all over the world, with emphasis on European and Chinese material. Seeds were obtained from the Leibniz Institute of Plant Genetics and Crop Plant Research (Gatersleben, Germany), Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (Hohenlieth, Germany), and from breeding material available at the College of Agronomy and Biotechnology, Southwest University (Chongqing, China). Plants were grown in three-row plots with 30 plants plot^{-1} in a semi-winter growth environment in Chongqing, China (29°31' N, 106°35' E) in 2007/2008 and 2008/2009 (referred to in the following by the year in which flowering time was recorded, i.e. 2008 and 2009, respectively). Under these growth conditions, in central China both semi-winter and winter types are generally sufficiently vernalized to flower after winter. Spring type accessions grown under these conditions do not usually start flowering before the onset of winter. During the winter months, plant growth slows down or comes to a halt, but the relatively mild winter temperatures at the chosen location in central China generally allows the spring types to survive as well. Flowering time in both field trials was recorded as the days from sowing to the date when the first flower had opened on half of the plants of an accession. Furthermore, accessions were classified as spring, semi-winter or winter types according to the annotation of the original cultivars (Chinese Crop Germplasm Information System (CGRIS), http://icgr.caas.net.cn/cgris_english.html) or their general performance in the field (see Supplementary Table S1 at *JXB* online; W Qian, unpublished data). Genomic DNA was extracted from a pooled sample of approximately equal amounts of leaves from all 30 plants of a plot.

For gene expression experiments, three different *B. napus* accessions were used, the German winter type variety Express, the Chinese semi-winter type variety Ningyou7, and the spring type variety Haydn. Seeds were sown on 3 August 2009, and plants were grown in the greenhouse under long-day conditions. For vernalization, 30-d-old plants were transferred to a climate chamber, grown at 5 °C for 42 d, and returned to the greenhouse. Leaves, stems, floral buds, and flowers were harvested at the flowering stage 63–65 according to the BBCH scale (Meier, 2001). Non-vernalized Haydn plants flowered at 50 d, non-vernalized Ningyou7 plants and vernalized Haydn plants at 88–89 d, vernalized Ningyou7 plants at 95 d, and vernalized Express plants

at 127–136 d after sowing. Biological replicates were obtained by harvesting samples from three different plants.

Identification of FRIGIDA *homologues in* B. napus

The genomic, cDNA and protein sequences of *A. thaliana FRI* (At4g00650) were used as queries for BLASTN and TBLASTN homology searches of the nt/nr and EST sections of GenBank (as of 27 November 2005), which identified a *B. napus* EST (GenBank accession number CD836567, derived from *B. napus* cv. Jet Neuf) with 74% sequence identity over 578 bp of *FRI* exon 1. A genomic fragment corresponding to the EST was amplified from *B. napus* cv. Express genomic DNA using an EST sequence-derived primer pair (A102–A103; see Supplementary Table S2 at *JXB* online). The PCR product of 614 bp had 99% sequence identity to the EST and was used as probe to screen a *B. napus* cv. Express BAC library (obtained from Deutsches Ressourcenzentrum für Genomforschung GmbH (RZPD), Berlin, Germany) according to standard protocols for radioactive hybridization (Sambrook *et al.*, 1989). DNA of positive BAC clones was extracted using the NucleoBond® PC 500 plasmid DNA purification kit (Macherey & Nagel GmbH & Co. KG, Düren, Germany). Sequence identity or homology to the probe fragment was confirmed by PCR amplification and sequencing of BAC DNA using primer pair A102–A103. The BAC clones were grouped into three sequence groups according to a multiple sequence alignment using the AlignX module of the Vector NTI software package (Invitrogen Corporation, Carlsbad, USA). To identify the full-length genomic sequences, one BAC of each group was selected for primer walking using primers A127, A133, A201, A248, A249, A250, A260, A261, A262, A372, and A378 (see Supplementary Table S2 at *JXB* online). The genes were annotated using pairwise sequence alignments (BLAST2; http://www.ncbi.nlm.nih.gov) with *FRI* and the FGENESH+ and FGENESH_C gene prediction programs (http://linux1.softberry.com/berry.phtml). Orthology was inferred by bidirectional best hit analysis (Overbeek *et al.*, 1999) using the *B. napus* genes as queries for BLASTX searches against the *A. thaliana* protein database at TAIR (http://www.arabidopsis.org). Following the nomenclature for *Brassica* genes (Ostergaard and King, 2008), the three *FRI* homologues were designated as *BnaA.FRI.a*, *BnaX.FRI.b*, and *BnaX.FRI.c*.

A primer pair derived from the exon 3 region of the *B. napus* genes (A249–A374), was used for PCR amplification from *B. napus* cv. Express genomic DNA. PCR products were cloned into the pGEM-T vector (Promega Corporation, Madison, USA) according to the manufacturer's protocol. Twenty positive clones were selected and sequenced. A multiple sequence alignment identified four sequence groups, three of which corresponded to *BnaA.FRI.a*, *BnaX.FRI.b*, and *BnaX.FRI.c*. The fourth sequence was designated as *BnaX.FRI.d*. *BnaX.FRI.d*-specific primers N010 and A156 were designed (see Supplementary Table S2 at *JXB* online). By using primer A102 in combination with the *BnaX.FRI.d*-specific primer N010, and primer A157 together with the *BnaX.FRI.d*-specific primer A156, the 5' and 3' regions of *BnaX.FRI.d* were obtained. The sequences of *BnaA.FRI.a*, *BnaX.FRI.b*, *BnaX.FRI.c*, and *BnaX.FRI.d* were deposited at GenBank (http://www.ncbi.nlm.nih.gov/genbank).

Genetic mapping and QTL detection

Specific primers were designed for each of the four *B. napus FRI* homologues. The following primer combinations covered the respective genes: N036–A385 and N053–N020 (*BnaA.FRI.a*), A379–A386 and N030–N032 (*BnaX.FRI.b*), A370–N004 (*BnaX.FRI.c*), and A102–N010 (*BnaX.FRI*.d; see Supplementary Table S2 at *JXB* online). The primers were used to amplify genomic DNA of the two parents of the DH mapping population, and the PCR products were sequenced. Upon detection of polymorphisms between Tapidor and Ningyou7 in the promoter region of *BnaA.FRI.a*, a new primer (N016) was designed which is specific for the Tapidor allele and was used in combination with A385 to genotype all 202 DH lines by PCR amplification. Genotypes were scored according to the presence or absence of the target fragment after agarose gel electrophoresis.

BnaA.FRI.a was mapped onto the framework map constructed by Long *et al.* (2007) using JoinMap 3.0 (Van Ooijen and Voorrips, 2001) and a LOD score of 2.5. The resulting genetic map and the flowering time data for 11 environments recorded by Long *et al.* (2007) were used for detection of QTL for flowering time by composite interval mapping (CIM) with Windows QTL cartographer 2.5 (Wang *et al.*, 2011).

SNP detection and inference of haplotypes in a collection of B. napus *accessions*

According to the flowering time data of 248 accessions from two growth seasons (see Supplementary Table S1 at *JXB* online), a subset of 95 accessions including similar numbers of early, intermediate, and late flowering accessions were selected for a first genotypic analysis. The *BnaA.FRI.a*-specific primer combinations N036–A385 and N053–N020 were used to genotype these accessions by PCR amplification and sequencing. Two primers (N018 and A385) were used for sequencing the N036–A385 PCR products, and three primers (N020, N054, and N057) were used for sequencing the N053–N020 products. Whole gene sequences were obtained by assembling the sequence data from these sequencing reactions for each accession. Sequences were aligned using *VectorNTI 10.0* (Invitrogen Corporation, Carlsbad, USA) or *CLC main workbench 5* (CLC bio, Aarhus, Denmark), and alignments were edited manually using the *BioEdit* software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Haplotypes were inferred using the software *HAP* at default parameters (http://research.calit2.net/hap). Three primers (N069, N076, and N078; see Supplementary Table S2 at *JXB* online) were designed to differentiate between the two nucleotides for each of the three haplotype tagging SNPs (htSNPs) detected by *HAP* in haplotype block 2. All 248 accessions were genotyped at these htSNP positions by PCR with primer combinations N069–N020, N053–N076, and N053–N078. Haplotypes were scored according to the presence or absence of the respective bands after agarose gel electrophoresis of the PCR products (see Supplementary Table S1 at *JXB* online).

Population structure evaluation

28 publicly available SSR markers (see Supplementary Table S3 at *JXB* online) were selected from Cheng *et al.* (2009) which were distributed equally across the genetic map of *B. napus*. To reduce costs, an M13-tailing PCR technique was used (Schuelke, 2000). For each SSR primer combination, the forward sequence of the M13 primer was added to the 5′ end of the forward SSR primer and the reverse sequence of the M13 primer was added to the 5' end of the reverse SSR primer. PCR reactions were performed with four primers, the M13F-forward SSR primer, the M13R-reverse SSR primer, an IRD700-labelled M13F primer (MWG Operon, Ebersberg, Germany) and an unlabelled M13R primer. The 28 SSR markers were used to assay the 95 accessions which were selected for the first round of genotyping. The DNA analyser system Li-Cor 4300 (Li-Cor Biosciences, Lincoln, Nebraska, USA) was used for electrophoresis and detection of PCR products. The presence or absence of PCR product bands was scored as 1 or 0, respectively. The software *STRUCTURE 2.2.3* was used to estimate population structure (http://pritch.bsd.uchicago.edu/structure.html) (Pritchard *et al.*, 2000). *K* values ranging from 1 to 10 were tested with a burn-in of 100 000 iterations and 100 000 Markov Chain Monte Carlo (MCMC) iterations according to the software's instructions.

The effect of population structure on flowering time was tested using *SAS* GLM (SAS Institute, Cary, North Carolina, USA). The

model included two components of the Q matrix (Q1 and Q2) obtained with *STRUCTURE 2.2.3*. R^2 (variance explained by the model) was considered as an estimate of the proportion of phenotypic variation explained by population structure.

Linkage-disequilibrium and association analysis

TASSEL 2.01 was used to calculate linkage disequilibrium (LD) based on the parameter R^2 (Bradbury *et al.*, 2007). Only SNPs with frequencies larger than 0.05 were included for LD calculation. To avoid redundancy, each InDel was considered as a single polymorphic site.

For SNP/InDel-based association analysis, a structure-based model which takes population structure into account to reduce spurious associations was used. Associations between markers and the total trait variation were tested using a general linear model (GLM) which was implemented in *TASSEL 2.01*. A Q matrix which was obtained with *STRUCTURE 2.2.3* was used to account for population structure. The null hypothesis of this study was that the occurrence of polymorphisms in *BnaA.FRI.a* is independent of the variation in flowering time, whereas the alternative hypothesis was that polymorphisms are associated with flowering time variation. Only polymorphisms with frequencies above 0.05 were included and 1000 permutations were performed. An adjusted P value $\leqslant 0.05$ was considered as a criterion for association. For haplotype association analysis, block 1 and block 2 were regarded as two polymorphic loci. Different haplotypes in the same block were regarded as allelic variants at one locus. For further analysis of association between haplotype and flowering time variation in a larger panel which included 248 accessions, the software *SAS 9.0* (SAS Institute, Cary, North Carolina, USA) was used to conduct an analysis of variance (ANOVA) and multi-comparison analyses with least significant differences (LSD).

Transcript and expression analysis

Total RNA was extracted using the RNeasy kit (QIAGEN, www.qiagen.com) according to the manufacturer's protocol. RNA concentration was determined by spectrometry (NanoDrop; Thermo Scientific, Wilmington, USA) and quality was checked by agarose gel electrophoresis. Total RNA was treated with DNAse I (Fermentas Inc., Maryland, USA). First-strand cDNA was synthesized using Oligo$(dT)_{18}$ primers and the M-MuLV Reverse Transcriptase (Fermentas). cDNA was diluted to produce approximately equal amounts of products by RT-PCR with the reference gene primers.

For sequencing of PCR products and RT-PCR, cDNA was amplified with DreamTaq polymerase (Fermentas). For cloning of full-length coding sequences, the proof-reading polymerases Pfu (Fermentas) or Phusion (Biozym Inc., Los Angeles, USA) were used. The coding sequences of *BnaA.FRI.a* and *BnaX.FRI.d* were amplified from cDNA of vernalized floral buds of cultivar Express using primers FRI3_nested_fwd and FRI3_nested_rev or FRI4_fwd2 and FRI4_rev, respectively. *BnaX.FRI.b* and *BnaX.FRI.c* were amplified from cDNA of non-vernalized roots of *B. napus* cv. Express sampled 50 d after sowing at BBCH 19 (nine or more leaves unfolded) using primers A459 and A454 or A458 and A455, respectively, and reamplified using nested primers FRI1_nested_fwd and FRI1_nested_rev or FRI2_nested_fwd and FRI2_nested_rev, respectively. PCR products were cloned into the pGEMT-vector (Promega Corporation, Madison, USA) according to the manufacturer's protocol.

For semi-quantitative RT-PCR of *BnaA.FRI.a* and *BnaX.FRI.d*, cDNA was amplified with primers FRI34_RT_fwd_2 and N008. The resulting product sizes differ by 30 bp. For semi-quantitative RT-PCR of *BnaX.FRI.b* and *BnaX.FRI.c*, cDNA was amplified with primers FRI12_RT_fwd_2 and FRI12_RT_rev in a final volume of 25 µl, followed by restriction enzyme digestion of 15 µl of the resulting PCR product with *Xho*I (Fermentas) in a final volume of 20 µl to distinguish between the two genes. The expected fragment sizes are 418 bp (*BnaA.FRI.a*), 617 bp (*BnaX.FRI.b*), 374 bp and 246 bp (*BnaX.FRI.c* digested by *Xho*I), and 388 bp (*BnaX.FRI.d*). As a reference gene, a *B. napus* tubulin gene (GenBank accession number AF258790) was amplified with primers BnTubulin_fwd and BnTubulin_rev, resulting in PCR products of 224 bp for cDNA templates and 0.3 kb for genomic DNA templates.

Quantitative real-time RT-PCR (RT-qPCR) was performed with SYBR qPCR Supermix w/ROX (Invitrogen Corporation, Carlsbad, USA) using a CFX96 Real-Time System (Bio-Rad Laboratories GmbH, München, Germany). Reactions were performed in a total volume of 15 µl containing 100 nM of each primer and 2 µl of diluted cDNA templates, and amplified using the following cycling conditions: 95 °C for 3 min, 40 cycles of 95 °C for 10 s, 61.4 °C for 30 s, and 72 °C for 30 s, followed by 95 °C for 10 min. A melting curve was generated using a temperature range from 65 °C to 95 °C with increments of 0.5 °C every 5 s. *BnaA.FRI.a* cDNA was amplified with forward primer FRI34_RT_fwd_2, which spans the junction of exons 2 and 3, and the gene-specific reverse primer FRI3_RT_rev. As a reference gene, a *B. napus* actin gene (GenBank accession number FJ529167) was amplified with primers N062 and N063. For each sample at least three technical replications were performed. For data analysis, the mean Ct value of the target gene was normalized against the average Ct value of *B. napus* actin, using the ΔCt method implemented in the *CFX manager* software (Bio-Rad Laboratories GmbH, München, Germany). Primer efficiencies for the different targets (95% for *BnaA.FRI.a*, 104% for *B. napus* actin) were determined using four 2-fold serial dilutions of cDNA and were included in the calculation of relative expression levels in *CFX manager*. Melting curves showed a single peak for each target with PCR products of *BnaA.FRI.a* and *B. napus* actin peaking both between 82.5 °C and 83.0 °C. 'No template controls' (NTCs), included in triplicates for each target in each run, were either not amplified (Ct values below baseline threshold) or had very high (>36–39) Ct values, with melting curves peaking at clearly distinguishable temperatures (76.0–78.0 °C and 75.5–77.0 °C) from the respective PCRs with templates. Normalized expression was averaged over three biological replicates in each case.

Results

FRIGIDA *homologues in* B. napus

BAC library screening and PCR amplification from genomic DNA of *B. napus* identified four putative rapeseed orthologues of *FRI*, which were designated as *BnaA.FRI.a, BnaX.FRI.b, BnaX.FRI.c*, and *BnaX.FRI.d.* The predicted protein sequences range from 569 to 596 amino acids and are thus somewhat shorter than *FRI* (609 amino acids for the functional allele in the *A. thaliana* accession H51; Johanson *et al.*, 2000). All four genes are predicted to carry a conserved, centrally located 'Frigida' domain (Fig. 1; http://pfam.sanger.ac.uk, accession number PF07899; Finn *et al.*, 2010) which is characteristic for the superfamily of *FRI* and *FRI-LIKE* genes (Risk *et al.*, 2010). Furthermore, on the basis of sequence conservation within a 37 amino-acid region in the N-terminal part of the protein (Fig. 1), all four *B. napus* genes can be assigned to the FRI family I as defined by Risk *et al.* (2010), which includes *FRI* but not *FRI-LIKE* genes. In contrast to *FRI* in *A. thaliana*, where this region and a second region at the C-terminal end of the Frigida domain are predicted to form coiled-coil structures (Johanson *et al.*, 2000; Schläppi, 2006), only the C-terminal

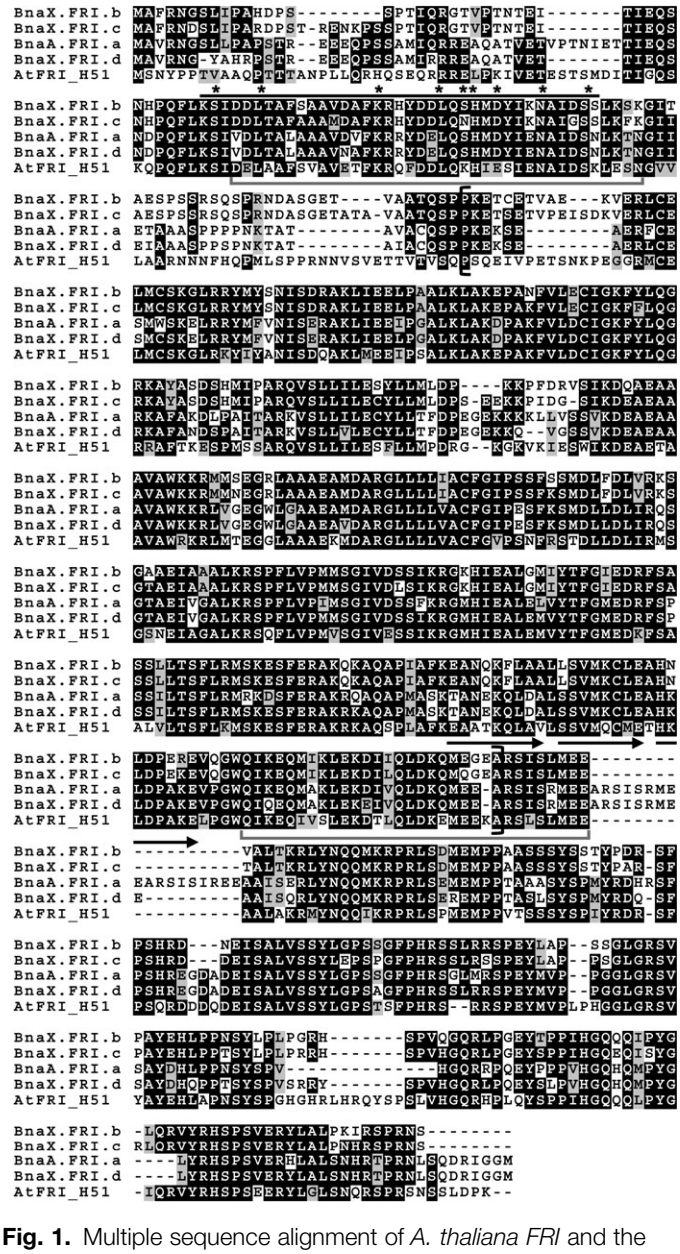

Fig. 1. Multiple sequence alignment of *A. thaliana FRI* and the *B. napus* homologues *BnaA.FRI.a*, *BnaX.FRI.b*, *BnaX.FRI.c*, and *BnaX.FRI.d*. The functional *FRI* allele in the *A. thaliana* accession H51 (GenBank accession number AF228500; Johanson *et al.*, 2000) was used for the alignment. The conserved 'Frigida' domain according to Pfam 24.0 (http://pfam.janelia.org) is indicated by vertical brackets. The regions corresponding to the two coiled-coil domains in FRI are marked by the grey horizontal brackets below the alignment. The nine residues within a 37 amino acid N-terminal region (marked by the black bar above the alignment) which are typical for family I proteins among FRI homologues (Risk *et al.*, 2010) and are conserved in all four *B. napus* genes are indicated by asterisks. The arrows mark the three eight amino acid (MEEARSIS) repeats in *BnaA.FRI.a.*

coiled-coil structure is also predicted for all four *B. napus* genes at a stringent probability threshold (90% for a window size of 28 residues; http://www.ch.embnet.org/software/coils/COILS_doc.html; Lupas *et al.*, 1991). In two genes, *BnaA.FRI.a* and *BnaX.FRI.d*, the N-terminal coiled-coil domain is predicted with lower probabilities (~50% and ~80%, respectively), whereas this domain is not predicted for *BnaX.FRI.b* and *BnaX.FRI.c* (probabilities <5%). The largest of the *B. napus* genes, *BnaA.FRI.a*, was found to carry an additional 17 amino acids in a region of three eight-amino-acid repeat elements (MEEARSIS; Fig. 1). Only two of these repeat elements are present in *BnaX.FRI.d.*, whereas *BnaX.FRI.b*, *BnaX.FRI.c*, and *A. thaliana FRI* each carry only a single copy of this or a closely related motif, respectively. The repeat region is located at the C-terminal end of the Frigida domain and overlaps with the coiled-coil domain. The overall amino acid identities of *BnaA.FRI.a, BnaX.FRI.b, BnaX.FRI.c*, and *BnaX.FRI.d.* to *A. thaliana FRI* are, respectively, 58%, 61%, 61%, and 59%.

B. napus FRI *genes are differentially expressed*

The exon–intron structure of all four *FRI* homologues was identified by RT-PCR and sequencing and was found to be conserved between the *B. napus* genes and functional *FRI* alleles in *A. thaliana* (Fig. 2A). Gene expression was analysed in leaves, stems, floral buds, and flowers of three different *B. napus* accessions, the winter type Express, the semi-winter type Ningyou7 (with or without prior vernalization), and the spring type Haydn (with or without prior vernalization), using primer combinations specific for either the gene pair *BnaA.FRI.a*/*BnaX.FRI.d* or *BnaX.FRI.b*/*BnaX.FRI.c*. Individual genes of a gene pair were distinguished by gel electrophoresis on the basis of size differences (*BnaA.FRI.a*/*BnaX.FRI.d*), or by gel electrophoresis following *Xho*I restriction (*BnaX.FRI.b* and *BnaX.FRI.c*). RT-PCR fragments of the expected sizes were obtained in most samples from all three accessions, demonstrating that all four *B. napus FRI* genes are expressed in accessions of different growth types (Fig. 2B). Expression levels were similar between the different ecotypes and between vernalized and non-vernalized plants. However, while *BnaX.FRI.b* and *BnaX.FRI.c* transcripts were ubiquitously and relatively evenly expressed across all tissues analysed, *BnaA.FRI.a* and *BnaA.FRI.d* exhibited marked differences in expression, which ranged from barely detectable in leaves and stems to very high in flowers (Fig. 2B). For *BnaA.FRI.a*, differential spatial expression was confirmed by transcript-specific RT-qPCR in Ningyou7 (Fig. 2C).

BnaA.FRI.a *co-localizes with a major flowering time QTL cluster*

The four *B. napus FRI* genes in the parents of the TNDH mapping population (*B. napus* cv. Tapidor and Ningyou7) were sequenced to detect sequence polymorphisms for mapping. Whereas *BnaX.FRI.b*, *BnaX.FRI.c*, and *BnaX.FRI.d* are monomorphic across the entire gene sequences, several polymorphisms were identified for *BnaA.FRI.a* (see Supplementary Fig. S1 at *JXB* online). A marker (primer combination N016–A385; see Supplementary Table S2 at *JXB* online) was developed and used to genotype the 202 DH lines of the mapping population. The newly developed

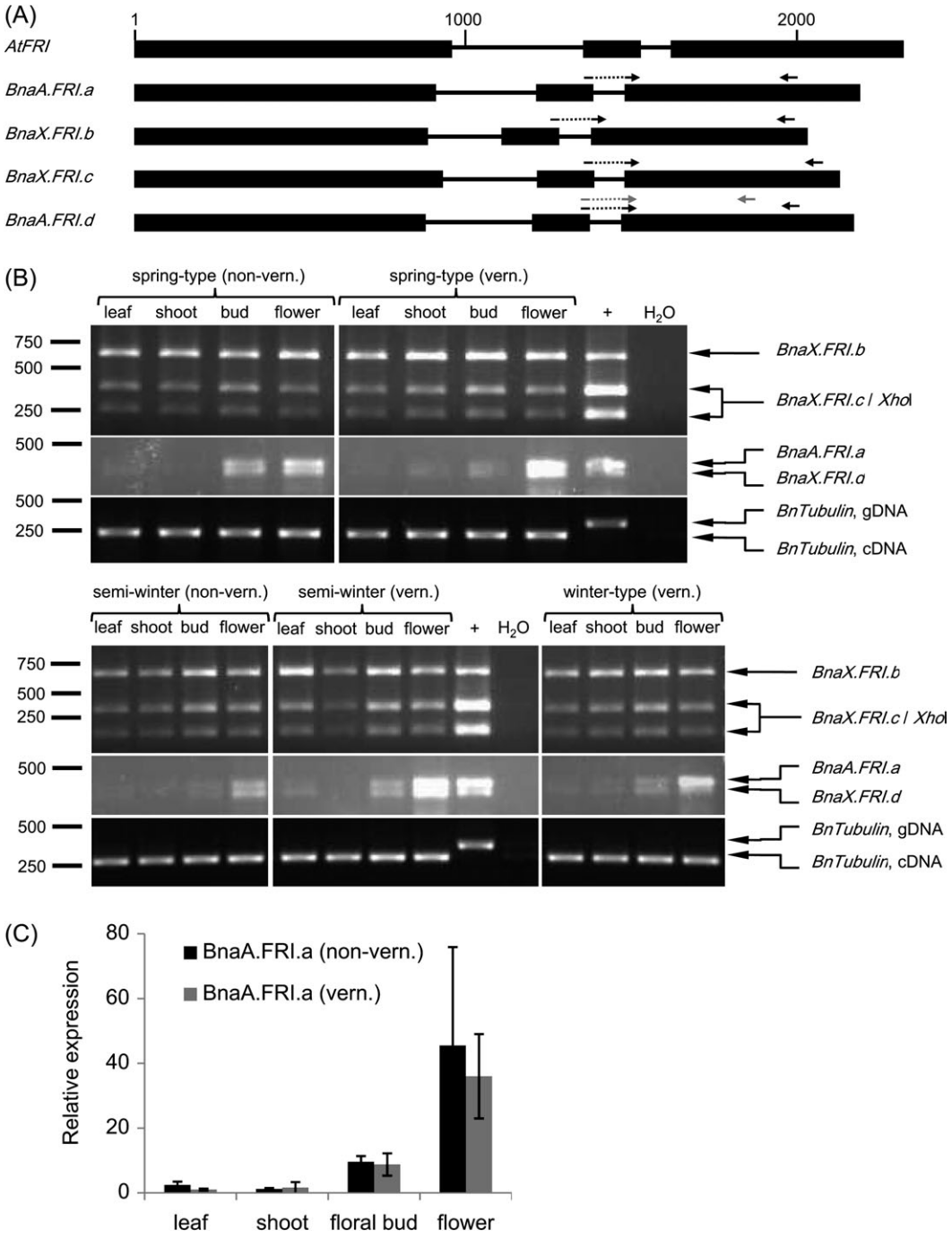

Fig. 2. Exon–intron structure and expression analysis of *B. napus FRI* homologues. (A) Exon–intron structure of *BnaA.FRI.a, BnaX.FRI.b, BnaX.FRI.c*, *BnaX.FRI.d*, and *AtFRI* (accession H51, accession number AF228499) between start and stop codons. Exons are indicated as black rectangles. Numbers indicate base pair positions relative to the start codon. Arrows mark the positions of primers used for semi-quantitative RT-PCR (black) and real-time qPCR (grey), with the forward primers spanning across splice sites (dotted lines). (B) Semi-quantitative RT-PCR of *B. napus FRI* homologues in aerial parts of three different growth types of rapeseed at the flowering stage. (Top panel) Spring type accession Haydn (non-vernalized or vernalized as indicated). (Bottom panel) Semi-winter type accession Ningyou7 (non-vernalized or vernalized) and winter type accession Express (vernalized). '+', Positive control, i.e. PCR on pGEM-T plasmids carrying cloned full-length coding sequences of the respective *B. napus* genes as template DNA, or PCR on genomic DNA from rapeseed for the *B. napus* tubulin gene. H_2O, negative control. Numbers on the left indicate the fragment length in base pairs of a DNA size marker (GeneRuler™ 1 kb DNA Ladder; Fermentas Inc., Maryland, USA). (C) RT-qPCR analysis of tissue-specific expression of *BnaA.FRI.a* in non-vernalized and vernalized Ningyou7 plants at the flowering stage. Expression was normalized using *B. napus* actin as a reference gene.

BnaA.FRI.a marker was integrated into the genetic map generated with the TNDH population (Long *et al.*, 2007) and was mapped on chromosome A03 at position 49.8 cM between markers S15M04-2-85 and RA2E11. Using the genetic map containing the *BnaA.FRI.a* marker, the QTL map for flowering time data from 11 environments (Long *et al.*, 2007) was recalculated. A flowering time QTL cluster with two major peaks was detected on chromosome A03 between 10 cM and 60 cM (Fig. 3). *BnaA.FRI.a* is centrally located within the narrow confidence intervals (48.8-50.9 cM) of QTL detected in seven environments at the position of one of the two major QTL peaks (Table 1; Fig. 3). Interestingly, the flowering time QTL co-localizing with *BnaA.FRI.a* were detected in both winter (Fig. 3A) and spring environments (Fig. 3B). The QTL explained up to 12.0% of the phenotypic variation in winter environments and up to 8.1% in spring enviroments. The early-flowering allele is derived from the semi-winter type parent Ningyou7.

Linkage disequilibrium at the BnaA.FRI.a *locus*

A panel of 95 *B. napus* accessions was genotyped at the *BnaA.FRI.a* locus by PCR amplification from genomic DNA with primer pairs N036–A385 and N053–N020 and sequencing of the products with primers A385, N018, N020, N054, and N057 (Fig. 4). The sequenced fragments covered 2179 bp from the start codon to the stop codon and ~140 bp of the 5′ UTR. In total, 35 SNPs and five InDels with frequencies >0.05 were identified (Table 2). Of these 40 polymorphic sites, four were located within the 5′ UTR, five were located within introns, and 31 were located within the coding sequence.

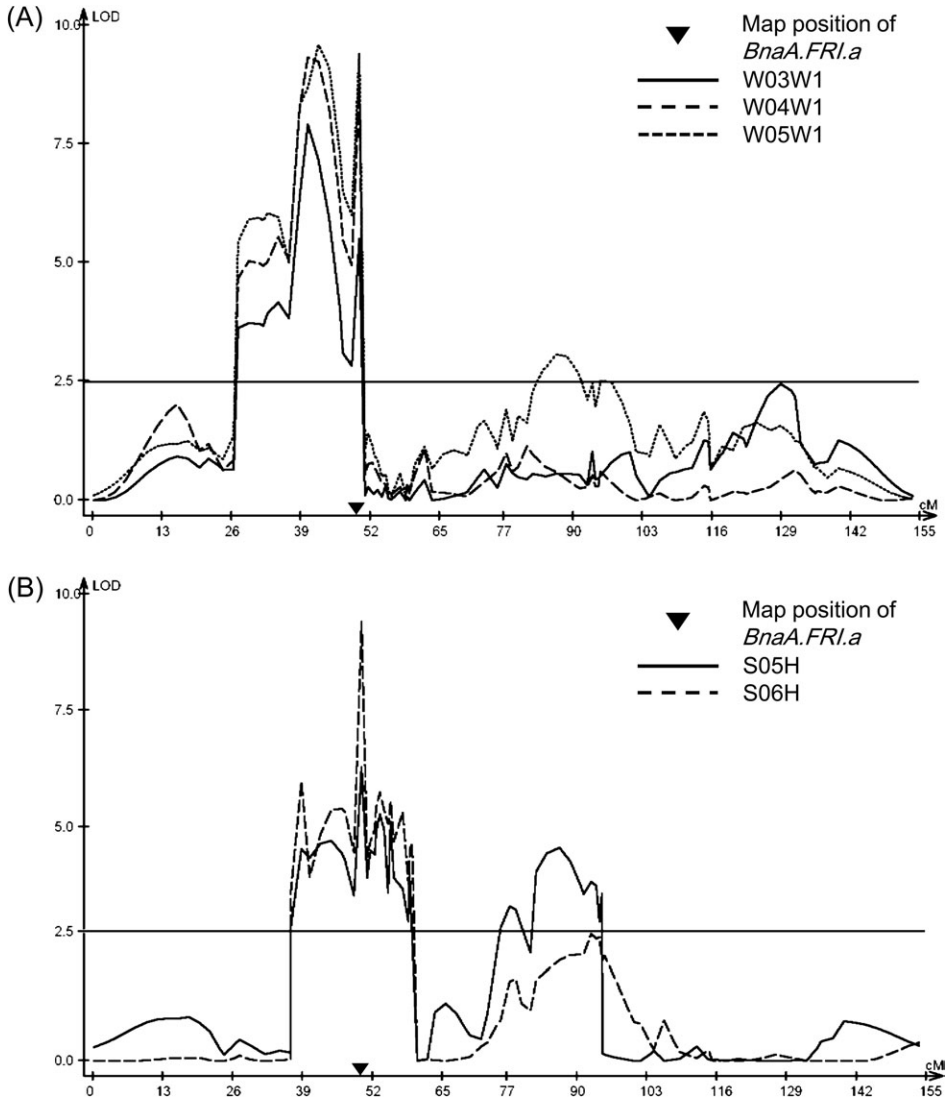

Fig. 3. *BnaA.FRI.a* co-localizes with major flowering time QTL on chromosome A03 in both winter (A) and spring environments (B): *y*-axis, LOD values; *x*-axis, genetic map positions on chromosome A03 (in centiMorgan, cM). The black horizontal line indicates a LOD value of 2.5. The arrowhead marks the genetic map position of *BnaA.FRI.a* (49.8 cM), which was integrated into the map developed by Long *et al.* (2007). In (A), the LOD profiles are only shown for the winter environments in which the three most significant QTL were detected. The numerical QTL data are given in Table 1.

Table 1. Flowering time QTL co-localizing with *BnaA.FRI.a* on chromosome A03

No.	QTL[a]	Environment[b]	LOD	Position (cM)	QTL confidence interval (95%)	R^2
1	W03D	Winter	2.6	49.8	48.8–50.9	3.1%
2	W04D	Winter	<2.5	n.a.[d]	n.a.	n.a.
3	W03W1	Winter	5.5	49.8	49.3–50.0	8.2%
4	W04W1	Winter	8.8	49.8	49.4–49.9	10.9%
5	W05W1	Winter	9.4	49.8	49.4–49.9	12.0%
6	W05W3	Winter	<2.5	n.a.	n.a.	n.a.
7	S05H	Spring	5.7	49.8	49.2–50.3	6.0%
8	W06W1	Winter	<2.5	N/A	n.a.	n.a.
9	W06W2	Winter	6.3	49.8	49.3–50.2	6.8%
10	W06D	Winter	<2.5	n.a.	n.a.	n.a.
11	S06H	Spring	8.5	49.8	49.5–50.0	8.1%

[a] The first letter (W or S) indicates winter or spring environments, the number in the middle indicates the year of harvest, and the last letter or letter and number (D, W1, W2, or H) indicate different locations in China (Long *et al.*, 2007).
[b] In winter environments, plants were grown from October to May; in spring environments, plants were grown from May to September.
[c] Phenotypic variation explained by this QTL.
[d] Not applicable; values are only given for QTL with LOD ⩾2.5.

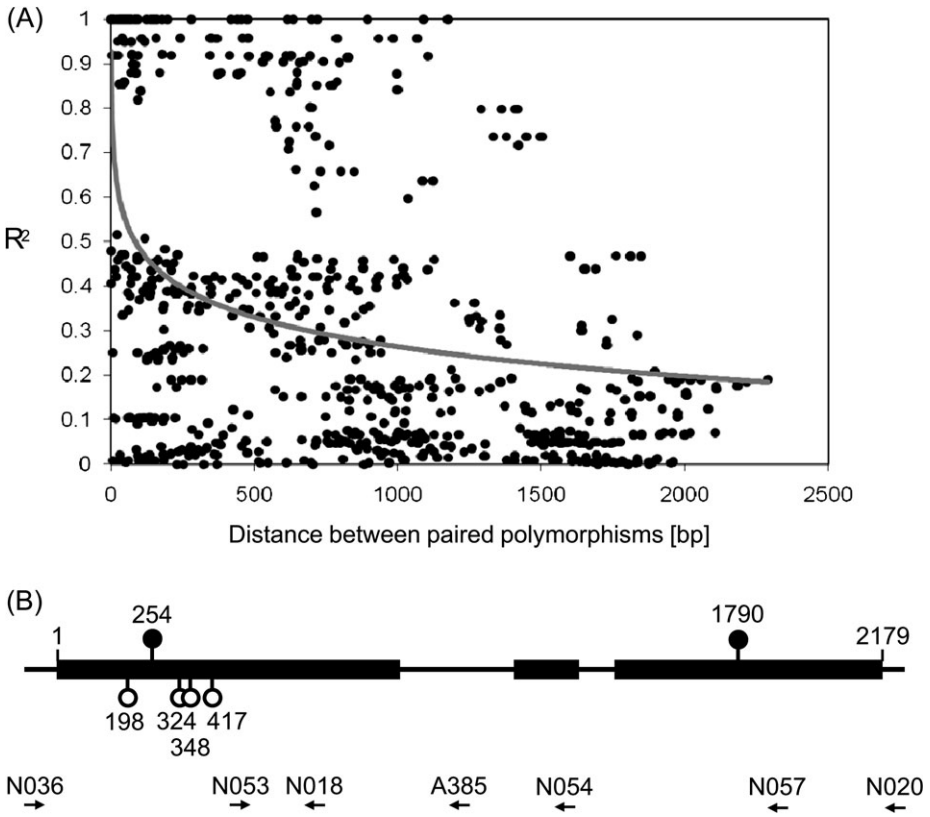

Fig. 4. Linkage disequilibrium and polymorphisms at the *BnaA.FRI.a* locus. (A) 40 polymorphisms (SNPs and InDels) in *BnaA.FRI.a* were used to calculate their paired R^2 values (*y*-axis) with *TASSEL*. The *x*-axis indicates distances in base pairs between paired polymorphisms. The logarithmic trend line is shown in grey. (B) The position of polymorphisms in *BnaA.FRI.a*. Only polymorphisms showing association with flowering time are included in the figure. Black circles indicate amino acid substitutions, whereas open circles indicate synonymous SNPs. Exonic regions between the translational start (1) and stop positions (2179) are indicated as black rectangles. Arrows mark the position and orientation of primers used for LD and association analysis.

To determine the degree of LD within *BnaA.FRI.a*, *TASSEL 2.01* (Bradbury *et al.*, 2007) was used to calculate R^2 values for each pair of polymorphisms. Out of 780 pairs of polymorphic sites, 646 showed significant linkage with P <0.05. LD decay for *BnaA.FRI.a* is illustrated in Fig. 4A. For distances larger than approximately 2 kb, the R^2 value is lower than 0.2. This indicates that LD decays rapidly within this gene, suggesting that *BnaA.FRI.a* is suitable for an analysis of gene–trait associations.

Table 2. SNPs and InDels within the *BnaA.FRI.a* gene and their association with flowering time as determined in two consecutive years in a panel of 95 accessions.

SNPs and InDels with frequencies >0.05 are listed in the table. The probability of association with flowering time is given for *P* values ⩽0.05.

Position within gene	Polymorphism	Location	Predicted amino acid change	Association with flowering time (*P* value)	
				2008	2009
–133[a]	30/0[b]	5' UTR			
–28	T/C[c]	5' UTR			
–26	C/T	5' UTR			
–25	11/0	5' UTR			
99	21/0	exon1	VETVPTN/deletion[d]		
137	A/G	exon1	G46E		
138	A/G	exon1			
140	A/C	exon1	Q47P		
189	A/T	exon1			
198	G/A	exon1			0.046*[f]
206	T/C	exon1	V69A		
254	A/G	exon1	E85G		0.005**
282	C/T	exon1			
323	C/A	exon1	P108L		
324	G/A	exon1			0.013*
343	G/A	exon1	V115I		
348	G/T	exon1			0.050*
417	T/C	exon1			0.029*
762	C/A	exon1			
801	C/G	exon1			
854	C/A	exon1	A285D		
900	C/G	exon1			
958	A/G	intron1			
971	A/G	intron1			
972	4/0	intron1			
994	T/G	intron1			
1042	T/A	intron1			
1224	T/A	exon2	F309I		
1276	T/A	exon2	M325K		
1615	C/T	exon3			
1702	C/T	exon3			
1790	G/A	exon3	A467T	0.017*	
1796	G/T	exon3	A469S		
1824	3/0	exon3	H/deletion[e]		
1888	C/T	exon3			
1949	G/T	exon3	V520F		
1974	G/C	exon3			
1980	C/A	exon3			
2084	G/C	exon3			
2158	A/G	exon3			

[a] Basepair positions are given relative to the ATG start codon. '–' indicates positions upstream of the start codon. At sites of InDel polymorphisms, the basepair positions refer to the start of the respective insertion in the *BnaA.FRI.a* allele in *B. napus* cv. Tapidor.
[b] At sites of InDel polymorphisms, the numbers preceding the slash indicate the size of the insertion in the Tapidor allele.
[c] Letters preceding the slash indicate the nucleotide at the respective position in the Tapidor allele.
[d] The 7 amino acid tract VETVPTN is present in Tapidor, but deleted in some accessions.
[e] The additional H residue is present in Tapidor, but deleted in some accessions.
[f] *$P \leqslant 0.05$, **$P \leqslant 0.01$.

Population structure and impact on flowering time variation

Using 28 SSR markers which are distributed equally across the whole genome (Cheng *et al.*, 2009; see Supplementary Table S3 at *JXB* online) to evaluate population structure in the panel of 95 *B. napus* accessions, 52 polymorphisms were detected in total with frequencies larger than 0.05. Four SSR primer combinations amplified more than one locus according to Cheng *et al.* (2009). These four primer combinations and 12 additional SSR markers revealed multiple allelic diversity

among accessions. The population structure was calculated using these SSR markers and the software *STRUCTURE 2.3.2* (Pritchard *et al.*, 2000). The highest likelihoods were calculated for subpopulation numbers (K values) of K=3 and K=6, which were almost equally likely [*Ln p (D)*= –2198 for K=3; *Ln p (D)*= –2168 for K=6], whereas the variance for the respective *Ln p (D)* values differed considerably (152.7 for K=3; 376.5 for K=6) (Table 3). Because the variance was lower for K=3, this K value was used to select the Q matrix. By variance analysis of flowering time data from 2008 and 2009, population structure was found to explain 53.6% of the variation. This indicates that population structure had a large effect on flowering time.

Association of BnaA.FRI.a *with flowering time variation*

Because a strong population structure was observed and a large percentage of the observed phenotypic variation could be explained by population structure, association analysis may identify spurious associations. Therefore, a structure-based association analysis was performed. This analysis revealed an association of SNP1790 with flowering time variation in 2008, and an association of SNP198, SNP254, SNP324, SNP348, and SNP417 with flowering time variation in 2009 ($P \leqslant 0.05$; Table 2). All of these six SNPs were located within exons, including five (SNP198, SNP254, SNP324, SNP348, and SNP417) in exon 1 and one (SNP1790) in exon 3 (Fig. 4B). Two of these SNPs, SNP254 and SNP1790, are predicted to result in amino acid changes.

As there was no SNP with significant association to flowering time in both years, a haplotype-based association analysis was performed. Using the software *HAP* (http://research.calit2.net/hap) to infer haplotypes for *BnaA.FRI.a* among all accessions, two haplotype blocks were found within this gene. 27 SNPs or InDels were located in block 1 (position –140 to 1223, including exon 1) and 13 SNPs or InDels in block 2 (position 1224 to 2182, including exons 2 and 3). Three common haplotypes (represented by more than two accessions each) were identified in block 1, while four common haplotypes were found in block 2. Three polymorphic sites (InDel99, SNP282, and SNP348) in block 1 and three polymorphic sites (SNP1224, SNP1615, and SNP1790) in block 2 were identified as haplotype tagging SNPs (htSNPs) or InDels (Table 4; see Supplementary Fig. S2 at *JXB* online). Taking population structure into account, block 2 showed association with flowering time for both years (P <0.05).

To analyse the effects of different haplotypes in block 2 on flowering time variation further, htSNPs of this block were genotyped in a larger panel with 248 accessions including the 95 accessions described above. In total, 52 accessions were inferred as haplotype 1, 54 accessions as haplotype 2, 74 accessions as haplotype 3, and 46 accessions as haplotype 4. 22 accessions were inferred as rare haplotypes and were excluded from further analysis. To analyse the possible differential effects of haplotypes on flowering time, a one-way ANOVA and a pair-wise comparison by LSD analysis were performed. In the year 2008, haplotypes 1, 2, and 3 were significantly different with regard to the respective means of days to flowering (P <0.001), whereas haplotype 1 (163.9±8.7) and haplotype 4 (162.2±5.4) were not different (Fig. 5). In 2009, the flowering times of all haplotypes (haplotype 1, 150.7±9.7; haplotype 2, 156.9±13.3; haplotype 3, 164.1±11.6; and haplotype 4, 144.7±7.1) were significantly different from each other. The two alleles in the parents of the mapping population, Tapidor and Ningyou7, correspond to haplotype 3 and haplotype 4, respectively. The difference between the averages of flowering time in these two groups was 12.6 d in 2008 and 19.4 d in 2009. In addition, to estimate association between haplotypes and the three growth types in *B. napus*, the 226 accessions represented by haplotypes 1–4 were classified as spring (27 accessions), semi-winter (104 accessions) or winter type (95 accessions; see Supplementary Table S1 at *JXB* online), and a three by four Chi square analysis was performed (Table 5). The results suggest that *BnaA.FRI.a* haplotypes are non-randomly distributed among the different growth types. Whereas the 'late-flowering' haplotype 3 occurs predominantly in winter accessions, the 'early-flowering' haplotype 4 is common among semi-winter and spring types. Finally, ANOVA was used again to test whether flowering time differs between haplotypes among the accessions of a given growth type (Table 6). While there was no significant effect of haplotype on flowering time among spring type accessions, which constituted the smallest group, the ANOVA indicated a significant effect at least in one year (2008) among semi-winter types. Furthermore, although the means of flowering time were higher for each of the haplotypes in winter rapeseed than the respective means in the 226 accessions encompassing all growth types, the analysis indicated a highly significant effect of haplotype on flowering time among winter type accessions in both years. As observed for the whole panel of accessions, winter type accessions carrying haplotype 4 flowered earliest, whereas accessions with haplotype 3 flowered the latest.

Table 3. Analysis of population structure: estimation of *k* (number of populations) for 95 *B. napus* accessions

k	*Ln p (D)*[a]	Variance
1	–2519.0	25.6
2	–2274.7	92.6
3	–2198.2	152.7
4	–2275.9	405.2
5	–2204.6	341.5
6	–2168.2	376.5
7	–2421.2	914.7
8	–2268.7	661.4
9	–2401.9	931.3
10	–2760.8	1668.2

[a] *Ln p (D)*, Natural logarithm of the probability of data.

Discussion

In *A. thaliana*, *FLC* and *FRI* are key regulators of vernalization requirement and life cycle adaptation to

Table 4. Haplotypes and their association with flowering time in 95 *B. napus* accessions.
Only SNPs and InDels with frequencies >0.1 are included.

Haplotype block	Position within gene	Haplotype	Number of accessions	htSNPs/InDels			Association with flowering time (*P* value)[b]	
				99[a]	282	348	2008	2009
1	–140 to 1123	1	44	T	C	G	0.639	0.091
		2	23	T	T	T		
		3	15	A	C	T		
				1224	**1615**	**1790**		
2	1224 to 2182	1	10	A	C	A	0.002**	0.021*
		2	13	A	C	G		
		3	45	T	C	G		
		4	21	T	T	G		

[a] The polymorphism at position 99 in haplotype block 1 is an Indel. The nucleotide 'A' corresponds to the first position 3′ of the 21nt sequence tract which is missing in the sequence of haplotype 3.
[b] *$P \leqslant 0.05$, **$P \leqslant 0.01$.

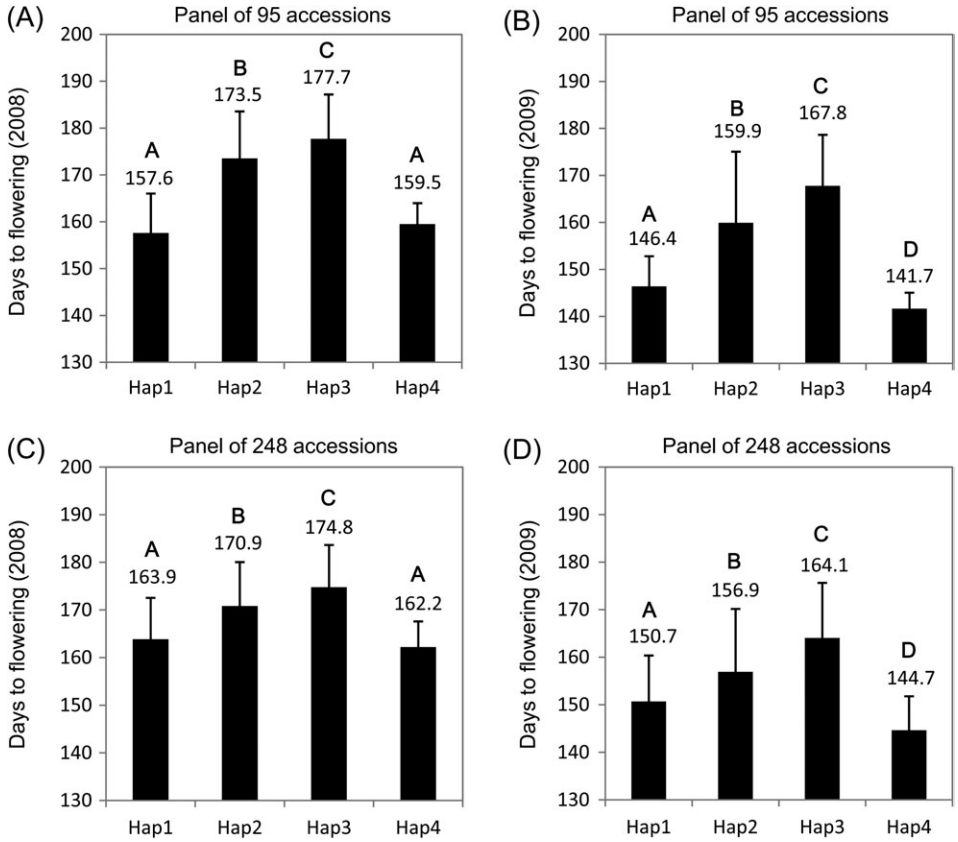

Fig. 5. Flowering time variation among 95 (A, B) or 248 (C, D) *B. napus* accessions grouped according to haplotypes in *BnaA.FRI.a* haplotype block 2. The means of days to flowering in 2008 (A, C) and 2009 (B, D) are shown. Error bars indicate standard deviations. Capital letters indicate significant differences according to LSD multi-comparison analysis (α=0.01).

climate, with allelic variation at these loci differentiating between summer annual and winter annual growth habits. While there is evidence for *FLC* homologues in *Brassica* species being important determinants of vernalization requirement, homologues of *FRI* and their role in flowering time regulation have not yet been described in *Brassica*. The main results of the current study are (i) the identification of four *FRI* homologues in *B. napus*, (ii) evidence for expression and differential regulation of these genes, (iii) the finding that *BnaA.FRI.a* co-localizes with a major

Table 5. Chi-square association analysis of *BnaA.FRI.a* haplotypes in haplotype block 2 and *B. napus* growth types.

3×4 Chi-square test for panel of 95 accessions

	Hap1[a]	Hap2	Hap3	Hap4	Rare haplotypes	Total
Spring	5	0	4	1	1	11
Semi-winter	5	4	4	20	3	36
Winter	0	9	37	0	2	48
Total	10	13	45	21	6	95
Chi-square value	*P* value	Level	df[b]	Threshold	Contingency coefficient	
70.15	3.82×10^{-11}	0.05	6	12.59	0.67	

3×4 Chi-square test for panel of 248 accessions

	Hap1	Hap2	Hap3	Hap4	Rare haplotypes	Total
Spring	8	0	8	11	3	30
Semi-winter	17	40	17	30	11	115
Winter	27	14	49	5	8	103
Total	52	54	74	46	22	248
Chi-square value	*P* value	Level	df	Threshold	Contingency coefficient	
61.493	2.24×10^{-11}	0.05	6	12.59	0.46	

[a] Hap 1-Hap 2, haplotypes 1-4.
[b] df, degrees of freedom.

Table 6. Analysis of variance of flowering time within each growth type

Haplotype in block 2	Spring (*n*=27)		Semi-winter (*n*=104)		Winter (*n*=95)	
	DTF±SD[a] 2008	DTF±SD 2009	DTF±SD 2008	DTF±SD 2009	DTF±SD 2008	DTF±SD 2009
Haplotype 1	161.0±9.1 (*n*=8)	154.2±6.1 (*n*=8)	161.5±5.2 (*n*=17)	143.6±4.0 (*n*=17)	176.0±3.9 (*n*=27)	167.2±4.0 (*n*=27)
Haplotype 2	n.a. (*n*=0)	n.a. (*n*=0)	162.2±3.3 (*n*=40)	144.0±4.3 (*n*=40)	177.3±6.6 (*n*=14)	165.9±10.9 (*n*=14)
Haplotype 3	161.3±10.2 (*n*=8)	153.1±9.2 (*n*=8)	165.0±5.4 (*n*=17)	146.4±8.0 (*n*=17)	180.3±5.2 (*n*=49)	170.7±5.9 (*n*=49)
Haplotype 4	160.1±6.3 (*n*=11)	149.5±7.5 (*n*=11)	160.7±3.7 (*n*=30)	142.2±3.6 (*n*=30)	174.0±4.8 (*n*=5)	158.6±14.0 (*n*=5)
Degrees of freedom	3	3	4	4	4	4
P value	0.9505	0.3857	0.0117*[c]	0.0556	0.0013**	0.0009**
LSD[b]			Hap1–Hap2		Hap1–Hap2	Hap1–Hap2
			Hap1–Hap3**		Hap1–Hap3**	Hap1–Hap3**
			Hap1–Hap4		Hap1–Hap4	Hap1–Hap4**
			Hap2–Hap3**		Hap2–Hap3	Hap2–Hap3**
			Hap2–Hap4		Hap2–Hap4	Hap2–Hap4**
			Hap3–Hap4**		Hap3–Hap4**	Hap3–Hap4**

[a] Days to flowering ±standard deviation.
[b] Fisher's Least Significant Difference analysis.
[c] *$P \leqslant 0.05$, **$P \leqslant 0.01$.

QTL cluster for flowering time on chromosome A03, and (iv) the finding that allelic variation within *BnaA.FRI.a* is associated with natural variation for flowering time, thus providing independent support for a role of *BnaA.FRI.a* in flowering time control in *B. napus*.

The QTL co-localizing with *BnaA.FRI.a* explained up to 12% of the phenotypic variation for flowering time (Table 1) and thus has a somewhat intermediate effect among the 32 flowering time QTL detected in the TNDH population (Long *et al.*, 2007). The QTL also explains less of the phenotypic variation than the maximal effects of two major flowering time QTL in the same population that previously have been shown to co-localize with flowering time genes, i.e. a QTL on chromosome A10, which co-localizes with *BnFLC1* and explains up to 50% of the phenotypic variation in a spring environment (Long *et al.*, 2007), and

a QTL on chromosome C06 which colocalizes with two *FT* homologues (*BnC6.FTa* and *BnC6.FTb*) and explains up to 52% of the phenotypic variation in winter environments (Long *et al.*, 2007; Wang *et al.*, 2009). The fact that cultivation over winter (and thus vernalization) abolishes the effect of allelic variation at the QTL co-localizing with *BnFLC1* (Osborn *et al.*, 1997; Long *et al.*, 2007) suggests that *BnFLC1* is involved in the regulation of vernalization requirement, similar to the role of *FLC* in *A. thaliana*. Interestingly, in contrast to *BnFLC1*, *BnaA.FRI.a* was found to co-localize with a QTL which occurs in both spring and winter environments and at comparable maximal LOD (8.5 and 9.4, respectively) and R^2 values (8.1% and 12.0%, respectively; Table 1). This suggests that *BnaA.FRI.a* regulates flowering time both with and without prior vernalization and is consistent with our observation that *BnaA.FRI.a* is expressed both in non-vernalized and in vernalized plants. Similarly, allelic variation of *FRI* in *A. thaliana* is not only associated with variation in flowering time of non-vernalized plants, with loss-of-function mutations accelerating flowering (in accessions from Northern latitudes; Stinchcombe *et al.*, 2004), but also affects flowering time after vernalization (Caicedo *et al.*, 2004; Stinchcombe *et al.*, 2004; Le Corre, 2005). The co-localization at the *BnaA.FRI.a* locus of QTL in both spring and winter environments also distinguishes this locus from the QTL co-localizing with *BnC6.FT.a/b*, which only occurs in winter environments and thus may contribute to the regulation of flowering time in response to vernalization, but in apparent contrast to *BnFLC1* and *BnaA.FRI.a*, may not control the vernalization requirement. The map position of *BnaA.FRI.a* on chromosome A03 precludes the possibility of co-localization with the vernalization-responsive flowering time QTL *VFN1* on linkage group N2 (Osborn *et al.*, 1997; Osborn and Lukens, 2003), whereas *BnaA.FRI.a* cannot be ruled out as a candidate gene for *VFN3* on linkage group N3 (Osborn *et al.*, 1997; Butruille *et al.*, 1999). The presence of *FLC* homologues on this linkage group and previous QTL co-localization studies in *B. rapa* and *B. napus* suggest that, besides *BnaA.FRI.a*, *FLC* homologues may also contribute to QTL effects on chromosome A03 (Schranz *et al.*, 2002; Osborn and Lukens, 2003; Udall *et al.*, 2006).

With regard to a role of *BnaA.FRI.a* in the regulation of vernalization requirement, it is interesting to note that 43 DH lines did not flower under spring cultivation conditions in both years, possibly due to insufficient exposure to cold temperatures. Of the 42 of these lines which were genotyped at both the *BnaA.FRI.a* locus (this study) and the *BnFLC1* locus (Long *et al.*, 2007), all carried at least one allele derived from the winter type parent Tapidor at either of these loci, and the majority (32/42) of the non-flowering lines carried the Tapidor alleles at both loci (data not shown; see Supplementary Table S4 at *JXB* online). Among 30 additional DH lines which did not flower in one year, all except two carried the Tapidor allele at least at one of the two loci, and 11 carried the Tapidor alleles at both loci. Among the remaining 17 lines which carry the Tapidor allele only at one locus, only two carry the Tapidor allele at the *BnFLC1* locus, whereas 15 carry the Tapidor allele only at the *BnaA.FRI.a* locus. Furthermore, the distribution of Tapidor and Ningyou7 alleles at the *BnaA.FRI.a* locus among the non-flowering plants of the TNDH population differs significantly from random (see Supplementary Table S4 at *JXB* online). These data are consistent with a role for *BnaA.FRI.a* in the regulation of vernalization requirement in rapeseed, and tend to suggest further that the winter type alleles at both loci may act together to increase the vernalization requirement.

The data obtained with the structured population are supported by our association analysis with a worldwide panel of accessions encompassing winter, semi-winter, and spring type rapeseed. Time to flowering varied substantially and ranged from 144 to 192 d in 2008 and from 137 to 182 d in 2009. In the earliest flowering haplotype group (haplotype 4) compared with the latest flowering haplotype group (haplotype 3) the number of days to flowering was reduced by 12.6 (in 2008) and 19.4 (in 2009), respectively. Taking population structure into account, flowering time variation was significantly associated with haplotype variation in both years. Evidence for population structure was obtained by genotypic analysis using SSR markers. Selection for different growth habits of oilseed rape in different geographical environments may contribute to population structure, and growth habit is generally thought to affect flowering time strongly. In particular, winter type rapeseed generally flowers late, whereas spring and semi-winter type rapeseed flower early in the field, which is consistent with our own data (see Supplementary Table S1 at *JXB* online). Consistent with these observations, haplotype variation of *BnaA.FRI.a* is not only associated with flowering time variation, but the different haplotypes are also non-randomly distributed among the three growth habits (Table 5). Because the non-parametric test applied does not control for population structure, it is possible that population structure contributes to the observed non-random distribution. However, as discussed above with regard to the QTL co-localization data, *BnaA.FRI.a* may indeed contribute to differential regulation of vernalization requirement and thus the differentiation between growth types. Finally, our finding that haplotype variation correlates with flowering time variation not only among all the accessions analysed but also among the accessions of a given growth type, at least for the winter type accessions (Table 6), further supports the notion that *BnaA.FRI.a* has dual, though possibly overlapping, roles in the regulation of vernalization requirement and flowering time.

Consistent with the phase relationships in the QTL population, the *BnaA.FRI.a* allele in the parental accession Ningyou7 corresponds to the earliest flowering haplotype 4, whereas the Tapidor allele corresponds to the latest flowering haplotype 3. Although there are several non-synonymous SNPs within haplotype block 2, including the htSNP at position 1790 which shows a SNP–trait association in one year (P=0.017 in 2008; Table 2) and could possibly affect protein function, the protein sequence in this region is identical between the two extreme haplotypes 3

and 4. However, the two haplotypes differ at two positions at the nucleotide sequence level, i.e. the htSNP at position 1615 and the SNP at position 1702, which are both located within the coding sequence. In addition to the SNPs observed between haplotypes 3 and 4, the two alleles in the TNDH population carry multiple additional polymorphisms, including several SNPs and a 3 bp indel in the promoter region, two indels in the 5′ UTR and six non-synonymous SNPs in exon 1 (see Supplementary Fig. S1 at *JXB* online). Among the six non-synonymous SNPs, two are located within the Frigida domain, but not at positions which are conserved among FRI family I proteins in *A. thaliana* and other rosid species (Risk *et al.*, 2010), and two are located in the non-conserved region between the Frigida domain and the N-terminal putative coiled-coil domain. The remaining two non-synonymous SNPs are located within the N-terminal putative coiled-coil domain. One of these SNPs (SNP254) results in the substitution of a charged residue (glutamate) by an uncharged amino acid (glycine). Charged residues are present at high percentages in coiled-coil domains and are thought to stabilize the domain through electrostatic interactions (McFarlane *et al.*, 2009). Interestingly, a glutamate to glycine substitution in the winter type allele in Tapidor (in an otherwise unchanged, theoretical molecule) would reduce the probability of a coiled-coil domain in the N-terminal region from ~50% to <5%, and the probability of a coiled-coil domain in the semi-winter type allele in Ningyou7 is also low (<10%; predictions according to COILS for a window size of 28 residues; http://www.ch.embnet.org/software/coils/COILS_doc.html; data not shown). The glutamate residue is conserved between *A. thaliana* and the Tapidor allele and corresponds to the position with the most significant *P* value for SNP–trait association within the entire gene (0.005 in 2009; Table 2). Furthermore, it was recently demonstrated that the N-terminal region including the coiled-coil domain of FRI in *A. thaliana* is required for protein–protein interaction with FRL1 as part of a transcriptional activator complex which establishes an active chromatin state of FLC (Choi *et al.*, 2011). It is conceivable that the polymorphism within the N-terminal coiled-coil domain in *BnaA.FRI.a* contributes to functional differences between the segregating alleles in the TNDH population and may also play a part in natural variation at least in some environments.

The relatively high number of polymorphisms in exon 1 compared with the number of SNPs in exons 2 and 3, which together are of comparable size to exon 1, between the parents of the mapping population as well as in our panel of natural accessions (Table 2) is consistent with data for *A. thaliana*, which indicate that exon 1 is hypervariable and may be under different selection constraints than exons 2 and 3 (Le Corre *et al.*, 2002). Interestingly, these regions largely coincide with the two haplotype blocks that were identified in *BnaA.FRI.a*, with one including exon 1 and the other including exons 2 and 3. The notion of a hypervariable region and the existence of two haplotype blocks in *BnaA.FRI.a* is consistent with the observed rapid decay of LD within this gene. Furthermore, the recent data from *Arabidopsis* (Risk *et al.*, 2010; Choi *et al.*, 2011) suggest that N-terminal and C-terminal regions of FRI differentially affect protein function and are involved in different protein–protein interactions which could, at least partially, explain why different parts of the gene appear to be subject to different evolutionary forces. Conceivably, functional heterogeneity may also account for differences between haplotype blocks 1 and 2 as well as between SNPs in the corresponding regions of the gene in regard to statistical significance of their association with flowering time in the two growth seasons investigated (Tables 2, 4). The average winter temperatures differed substantially between the growth seasons, with temperatures in the winter 2008/2009 (average temperature 7 °C) being two degrees colder, on average, than in 2007/2008, and a low temperature of –1 °C in 2008/2009 and +2 °C in 2007/2008 (temperature data provided by the Chongqing city government). These observations may suggest that temperature differences of a few degrees between years differentially affect the functionality of the N-terminal part (represented by haplotype block 1) and the C-terminal part of the gene (represented by haplotype block 2). Genotype×environment interactions are indeed apparent when comparing the flowering time data for both growth seasons. While the winter type accessions always flowered latest, as expected, semi-winter types flowered slightly later than spring types in 2008, but earlier in 2009 (see Supplementary Table S1 at *JXB* online). Although both spring and semi-winter types (as well as winter types) flowered earlier in 2009 than in 2008, the promotive effect of a decrease in temperature in the semi-winter environments under study appears to be strongest in semi-winter types. It is tempting to speculate that *BnaA.FRI.a* is involved in the fine-tuning of vernalization requirement and response, and that this may be achieved at the molecular level by differential interaction of the N- and/or C-terminal regions of the protein with other components of a transcriptional activator complex. The SNPs at positions 254 and 1790, which are located in the respective N- (Choi *et al.*, 2011) or C-terminal regions (Risk *et al.*, 2010), could thus be candidate sites for functional analyses, for example, through site-directed mutagenesis and for the development of functional markers for flowering time in rapeseed.

Previous studies in *Arabidopsis* have focused mainly on phenotypic differences between functional and loss-of-function alleles of *FRI*, whereas, to our knowledge, there is little known about phenotypic variation within populations or between accessions with different functional alleles. Several lines of evidence suggest that the winter type alleles (represented by the identical alleles in Express and Tapidor) and the semi-winter type allele (in Ningyou7) analysed in the current study are functional orthologues of *FRI*. First, *BnaA.FRI.a* contains an uninterrupted open reading frame, a conserved central Frigida domain, and the nine residues within an N-terminal sequence tract which are characteristic of the members of the FRI family I (Risk *et al.*, 2010). Second, the gene is expressed and differentially regulated,

with the spatial expression profiles being similar for both the winter and semi-winter type alleles. Interestingly however, while *FRI* in *Arabidopsis* is ubiquitously expressed and the levels of expression are similar in leaves and flowers (Risk *et al.*, 2010), our data suggest elevated expression in floral buds and flowers. The two observations differentiate both the winter and semi-winter type alleles from the majority of mutant alleles or sequence variants which have been correlated with phenotypic effects in *Arabidopsis*, and comprise mainly frameshift mutations or deletion alleles including a promoter deletion which abolishes *FRI* expression (Johanson *et al.*, 2000; Le Corre *et al.*, 2002; Gazzani *et al.*, 2003; Shindo *et al.*, 2005). By comparison with the data for *Arabidopsis*, the fact that the winter type rapeseed accessions have a strong vernalization requirement (Qiu *et al.*, 2006; Long *et al.*, 2007), together with our QTL co-localization data for *BnaA.FRI.a*, suggest that, at least, the winter type parental accession carries a functional *BnaA.FRI.a* allele. Although our finding that the *BnaA.FRI.a* locus co-localizes with a QTL indicates that the two alleles differ in their functionality, it is conceivable that the Ningyou7 allele is not a complete loss-of-function allele but retains at least part of its function. A relatively weak activity could contribute to confer the low vernalization requirement generally found in semi-winter rapeseed accessions, and may provide a selective advantage in regions with mild winters. The contribution of the additional three members of the FRI family I in *B. napus*, which were identified in the present study and also all contain uninterrupted open reading frames and are expressed, to the regulation of flowering time and/or vernalization requirement remains to be determined. Among these three genes, *BnaX.FRI.d* shares the highest degree of homology to *BnaA.FRI.a* (89% amino acid identity) and is most similar to *BnaA.FRI.a* with regard to sequence features and spatial expression profiles, and thus may be the closest functional relative.

Association mapping has become increasingly important to analyse quantitative traits in crop plants, and can involve candidate gene and genome-wide approaches. As the cost of genome-wide genotyping is high, candidate gene analysis is often more suitable for crops where complete sequence information is not yet available. In contrast to linkage mapping approaches which focus on trait variation resulting from allelic differences between the two parents of a segregating population and generally have relatively low resolution (10–20 cM), association mapping has a high resolution (sometimes at the level of or even within single genes) but can also identify spurious associations (Nordborg and Weigel, 2008). In the current study, the results from linkage and association mapping are highly consistent with each other, thus demonstrating the high potential of combining these two types of quantitative research techniques to assess the contribution of a gene to phenotypic variation.

Flowering time is a quantitative trait that is crucial for an appropriate timing of reproduction so as to obtain a high seed or biomass yield, as has been demonstrated for *A. thaliana*, rice, and maize (Izawa, 2007; Buckler *et al.*, 2009). Moreover, flowering time control can prevent plants from suffering from adverse growth conditions as they occur during drought, frost, or in the presence of pests (Blum, 1996; Jung and Müller, 2009). An understanding of the molecular basis of flowering time variation offers the possibility of direct selection for favourable alleles. One example is gene introgression from non-adapted varieties such as Chinese semi-winter types into European rapeseed material to increase heterosis (Qian *et al.*, 2007). The SNPs and haplotypes in *BnaA.FRI.a* which showed significant association with flowering time variation could be used as functional markers to assist in the breeding of cultivars with different growth habits and flowering times. In addition, other studies have reported other genes which affect flowering time in *B. napus* and can be used as functional markers, such as *CO*, *FLC*, and *FT* homologues (Robert *et al.*, 1998; Tadege *et al.*, 2001; Wang *et al.*, 2009). The combination of these markers with those discovered in the present study is expected to facilitate the selection of oilseed rape breeding material with distinct flowering times to help improve adaptation to specific local environments or changing climatic conditions.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Sequence alignment of the winter type allele in Tapidor and the semi-winter type allele in Ningyou7 at the nucleotide (A) and amino acid sequence level (B).

Supplementary Fig. S2. Multiple sequence alignment of *BnaA.FRI.a* nucleotide (A) and amino acid (B) sequences of the four haplotypes in block 2.

Supplementary Table S1. Phenotypic and genotypic data for 248 *B. napus* accessions.

Supplementary Table S2. Primer sequences.

Supplementary Table S3. SSR primers used for evaluating population structure.

Supplementary Table S4. Distribution of *BnFLC1* and *BnaA.FRI.a* alleles among non-flowering plants of the TNDH population.

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