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 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 oblige 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* (Gomez-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 (Baurle 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*; Tadeg *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* (Tadeg *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 (Tadeg *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 *BnCOa1* (Robert et al., 1998) and two alleles of the *B. nigra* gene *Bn COa* (Osterberg et al., 2002). In addition, a naturally occurring InDel variation in the *B. nigra* CO-like gene *Bn COL1* gene is associated with variation in flowering time (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 (*BnaA.FT*, *BnaC6.FT.a*, and *BnaC6.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. F2 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 *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...
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/rf and EST sections of GenBank (as of 27 November 2005), which identified a *B. napus* EST (GenBank accession number CD36567, derived from *B. napus* cv. Jet Neuf) with 74% sequence identity over 578 bp of accession number CD836567, derived from *At4g00650*. 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.pt/ml). 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 further 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 detecting the N036–A385 PCR products, and three primers (N020, N054, and N057) were used for sequencing the N053–N020 products. Whole genome 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 $\leq 0.05$ 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 approximatively 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 FRI12_RT_fwd and FRI12_RT_rev in a final volume of 25 $\mu$l, followed by restriction enzyme digestion of 15 $\mu$l of the resulting PCR product with *Xho*I (Fermentas) in a final volume of 20 $\mu$l to distinguish between the two genes. The expected fragment sizes are 418 bp (*BnaA.FRI.a*), 617 bp (*BnaX.FRI.b*), 246 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 AF255890) was amplified with primers *BrTubulin_fwd* and *BrTubulin_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 $\mu$l containing 100 nM of each primer and 2 $\mu$l 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 FRI13_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
The 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). 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 XhoI 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. H2O, 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 environments. 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.

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**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.
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 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.$^d$      | 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.$^d$      | 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.
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

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.

| Position within gene | Polymorphism | Location | Predicted amino acid change | Association with flowering time (P value) |
|---------------------|--------------|----------|-----------------------------|------------------------------------------|
| -133a               | 30/0b        | 5' UTR   |                             |                                          |
| -28                 | T/Cc         | 5' UTR   |                             |                                          |
| -26                 | C/T          | 5' UTR   |                             |                                          |
| -25                 | 11/0         | 5' UTR   |                             |                                          |
| 99                  | 21/0         | exon1    | VETVPTN/deletiond           |                                          |
| 137                 | A/G          | exon1    | G46E                        |                                          |
| 138                 | A/G          | exon1    |                             |                                          |
| 140                 | A/C          | exon1    | Q47P                        |                                          |
| 189                 | A/T          | exon1    |                             |                                          |
| 198                 | G/A          | exon1    |                             | 0.046e                                    |
| 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/deletione                 |                                          |
| 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 Letters preceding the slash indicate the nucleotide at the respective position in the Tapidor allele.
c At sites of InDel polymorphisms, the numbers preceding the slash indicate the size of the insertion 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 < 0.05, **P < 0.01.
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 \[ Ln p (D) = -2198 \text{ for } K=3; \text{ and } -2168 \text{ for } K=6 \], 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 (\( P \leq 0.05 \); 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   | \( Ln p (D) \) | 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
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 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 | Haplotype block Position within gene | Haplotype | Number of accessions | hSNPs/InDels | Association with flowering time (P value) |
|-----------------|-----------------------------------|-----------|---------------------|--------------|-----------------------------------------|
|                 |                                    |           |                     |              | 2008 | 2009 | 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        |       |      |
| 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 < 0.05, **P < 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).
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

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

| Haplotype | Spring | Semi-winter | Winter |
|-----------|--------|-------------|--------|
| Hap1      | 5      | 17          | 27     |
| Hap2      | 0      | 40          | 14     |
| Hap3      | 4      | 17          | 49     |
| Hap4      | 1      | 17          | 17     |
| Total     | 10     | 54          | 74     |

Chi-square value: 70.15; P value: 3.82×10⁻¹¹; Level: 0.05; df: 6; Threshold: 12.59; Contingency coefficient: 0.67.

### 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 2008 | DTF ± SD 2009       | DTF ± SD 2008 | DTF ± SD 2009 |
| Haplotype 1          | 161.0 ± 9.1   | 154.2 ± 6.1         | 161.5 ± 5.2  | 143.6 ± 6.0  |
| Haplotype 2          | n.a.          | n.a.                | 162.2 ± 3.3  | 144.0 ± 4.3  |
| Haplotype 3          | 161.3 ± 10.2  | 153.1 ± 9.2         | 165.0 ± 5.4  | 146.4 ± 8.0  |
| Haplotype 4          | 160.1 ± 6.3   | 149.5 ± 7.5         | 160.7 ± 3.7  | 142.2 ± 3.6  |
| Degrees of freedom  | 3              | 3                   | 4              | 4              |
| P value              | 0.9505         | 0.3857              | 0.0117c       | 0.0556         |
| LSDb                 | 0.345         | 0.3857              | 0.0117c       | 0.0556         |

a Days to flowering ± standard deviation.
b Fisher’s Least Significant Difference analysis.
c *P < 0.05, **P < 0.01.
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.FTa, 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 predicts the possibility of co-localization with the vernalization-responsive flowering time QTL VFN3 on linkage group N2 (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 TNHD 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 later, 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 hSNP 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 hSNP 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 coding sequence. In addition to the SNPs positions 1615 and the SNP at position 1702, which are both located within the coding sequence. 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 located within 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 located within the N-terminal putative coiled-coil domain. The two haplotypes differ at two positions located within the coding sequence. In addition to the SNPs positions 1615 and the SNP at position 1702, which are both located within the coding sequence. 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 located within 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 located within 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 located within 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 located within the N-terminal putative coiled-coil domain.
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 (Nödberg 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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