Nucleotide polymorphism affecting FLC expression underpins heading date variation in horticultural brassicas

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SUMMARY

Variation in flowering time and response to overwintering has been exploited to breed brassica vegetables that can be harvested year-round. Our knowledge of flowering time control now enables the investigation of the molecular basis of this important variation. Here, we show that a major determinant of heading date variation in Brassica oleracea is from variation in vernalization response through allelic variation at FLOWERING LOCUS C.C2 (BoFLC4). We characterize two alleles of BoFLC.C2 that are both functional and confer a requirement for vernalization, but they show distinct expression dynamics in response to cold. Complementation experiments in Arabidopsis thaliana revealed that the allelic variation results from cis polymorphism at BoFLC.C2, which quantitatively influences the degree of cold-induced epigenetic silencing. This results in one allelic variant conferring consistently later heading under both glasshouse and field conditions through reduced environmental sensitivity. Our results suggest that breeding of brassica varieties for commercially valuable variation in heading date has been achieved through the selection of cis polymorphism at FLC, similar to that underpinning natural variation in A. thaliana. This understanding will allow for the selection of alleles with distinct sensitivities to cold and robust heading dates under variable climatic conditions, and will facilitate the breeding of varieties more resistant to climate change.

Keywords: vernalization, FLC, Brassica oleracea, environmental sensitivity, accession numbers KUS21322/3.

INTRODUCTION

The transition to reproductive development significantly influences the production of horticultural brassicas: for broccoli and cauliflower the reproductive inflorescence is harvested, whereas for cabbage, kale and Brussels sprouts it is the vegetative tissue that is harvested. Understanding of the molecular control of this developmental transition has come predominantly from studies in Arabidopsis thaliana. Mutations in many loci influence flowering time, but the majority of the natural variation in A. thaliana flowering time maps to two loci, FRIGIDA (AtFRI) and FLOWERING LOCUS C (AtFLC). Allelic variation at AtFRI is the major determinant of vernalization requirement (Johanson et al., 2000; Shindo et al., 2005), whereas allelic heterogeneity at AtFLC explains a large proportion of the variation in vernalization response: namely, how much cold is required to satisfy the vernalization requirement (Shindo et al., 2006; Strange et al., 2011; Coustham et al., 2012; Li et al., 2014). Analysis of approximately 1300 A. thaliana accessions worldwide identified five major AtFLC haplotype groups, defined by non-coding sequence polymorphism that alters AtFLC expression and the degree of epigenetic silencing by cold exposure (Li et al., 2014). At least some of these haplotypes appear to confer flowering responses adapted to the local environment (Duncan et al., 2015; Li et al., 2015a).

The close evolutionary relationship of A. thaliana and Brassica oleracea facilitates the identification of functionally equivalent loci (Parkin et al., 2005; Gao et al., 2007; Bancroft et al., 2011). Analysis of flowering time/heading date in Brassica initially identified quantitative trait loci (QTLs) in Brassica rapa and Brassica napus in genomic regions that are co-linear with Arabidopsis chromosome 5, containing AtFLC, AtFY and AtCO, and chromosome 4, containing AtFRI (Osborn et al., 1997). In B. oleracea, the major QTLs for heading date also map to linkage groups C2, C3 and C9, which include this co-linear region (Rae et al., 1999;
Axelsson et al., 2001; Okazaki et al., 2007; Razi et al., 2008). A loss-of-function mutation in one of these, BoFLC2, is associated with earliness in annual cauliflower varieties (Ridge et al., 2014), supporting an equivalent role of FLC in Brassica and Arabidopsis. Allelic diversity at AtFRI has also been associated with heading date variation in B. oleracea (Irwin et al., 2012; Fadina et al., 2013).

To date, there has been little analysis of the variation in vernalization sensitivity between different B. oleracea cultivars. Here, through analysis of commercially important variation in broccoli, we show that allele variation at one B. oleracea FLC locus alters vernalization sensitivity. Nucleotide polymorphism between functional BoFLC.C2 alleles leads to changed vernalization sensitivity under a range of winter conditions. This polymorphism alters the dynamics of BoFLC.C2 expression, both the cold-induced BoFLC repression and subsequent reactivation on return to warmer conditions. Thus, in the same way as Arabidopsis FLC cis polymorphism influences the degree of epigenetic silencing in response to cold. We propose that mining cis polymorphism at BoFLC will provide a rich source of variation for breeding Brassica crops with an extended season and robust heading dates in response to variable climate.

RESULTS

A QTL at BoFLC.C2 is associated with late flowering under both glasshouse and field conditions

We investigated heading date in four genotypes of purple sprouting broccoli (B. oleracea subsp. italica; Figure S1a). This variation is used commercially to ensure continuous production of the crop. We chose two genotypes, E5 and E9, for further study. Both have an obligate requirement for cold, but E9 requires longer cold periods than E5 to head. This difference in vernalization response was observed in duplicate field trials at independent UK sites with contrasting winters in Lincolnshire (relatively hard winters) and Cornwall (relatively mild winters; Figure S1b). In these trials the heading date of the E5 parent line significantly differed between years and locations (P < 0.001), whereas that of the E9 parent was the same in Lincolnshire in both 2007/8 and 2011/12, and in Cornwall in 2011/12 (P = 0.227). Only in Cornwall in 2007/8, with a milder winter in which the mean weekly temperature did not fall below 6°C, was the heading date of the E9 parent significantly later (P < 0.001; Figure S1b,c).

A proprietary Doubled Haploid (DH) mapping population was available from a cross between these genotypes, and we used this to map QTLs controlling vernalization response under both controlled and field conditions. Up to eight QTLs of varying levels of significance were identified, four of which were common across at least four of the seven growth conditions (Figure S1d). One QTL was mapped on all seven occasions. This common QTL, accounting for between 5 and 25% of the heading date variation, mapped to linkage group C2 (Figure 1a). This region of B. oleracea chromosome 2 shows conserved synteny with the top of A. thaliana chromosome 5, and includes one of the five B. oleracea orthologues of AtFLC, BoFLC4 (Lin et al., 2005), also known as BoFLC2 (Ridge et al., 2014), and hereafter referred to as BoFLC.C2. Individuals homozygous for the E5 and E9 alleles at the marker most significantly associated with heading variation (Figure 1b) show a consistent and robust difference in heading date (on average 14 days) under all conditions tested; with later heading always conferred by the E9 allele (one-way ANOVA, P = 0.0195). Thus, variation at this locus appears to result in differential heading and environmental sensitivity.

BoFLC.C2 alleles confer similar phenotypic variation in Arabidopsis

To investigate whether allelic variation at BoFLC.C2 accounts for this QTL we undertook heterologous complementation experiments. Genomic regions carrying BoFLC.C2E5 and BoFLC.C2E9 (6 kb, including the native promoter and terminator) were transformed into the A. thaliana genotype Columbia FRI flc2 (Michaels and Amasino, 1999) that carries a loss-of-function mutation within AtFLC, but has an active AtFRI. These experiments aimed to determine whether BoFLC.C2 could complement the flc2 mutation, and whether the two alleles would induce different degrees of late flowering. Multiple (48 BoFLC.C2E5 and 31 BoFLC.C2E9) independent transgenic plants were generated for each allele and flowering time was analysed in the T3 generation. Homozygous BoFLC.C2E5 transgenic plants flowered consistently later than those carrying BoFLC.C2E9 (with a mean of 62 ± 1.8 days compared with 34 ± 0.87 days after 4 weeks of vernalization at 5°C; Figure 2a,b). The results of the heterologous complementation therefore support our hypothesis that molecular variation at BoFLC.C2 contributes to the QTL underlying the variation in vernalization response.

Two common functional BoFLC.C2 alleles exist within cultivated B. oleracea germplasm

BoFLC.C2 was previously cloned by Lin et al. (2005), and shows similar genomic organization to AtFLC with seven exons of similar sizes to those in Arabidopsis and a smaller first intron (approximately 1.1 kb, compared with 3.5 kb in Arabidopsis). We sequenced BoFLC.C2 from the E5 and E9 genotypes and found that the two alleles encode predicted open reading frames of 198 and 197 residues, respectively (Figure 3a). Three single-nucleotide polymorphisms in exon 2 confer three non-synonymous amino acid changes, V66I, I67V (both aliphatic) and E72K. A 3-bp deletion in E9 results in the loss of an aspartic acid in position 78, compared with the original BoFLC.C2 identified by Lin et al.
(2005) and that in E5. An additional 43 polymorphisms are located in introns 1, 4, 5 and 6 (Figure 3b,c), none of which match the polymorphisms reported in the AtFLC haplotypes (Li et al., 2014).

The high level of non-coding polymorphism between the BoFLC.C2 alleles prompted us to sequence a 1300-bp fragment (covering exon 1 to the end of exon 2) in 20 genotypes from the cultivated B. oleracea Diversity Foundation set (described in Irwin et al., 2012). These are representative of the six major Brassica crop types (Figure S2a). Nine putatively functional BoFLC.C2 alleles were identified (Figure S2a). Analysis of polymorphism between the nine allelic classes revealed a network divided into three broad groupings, with BoFLC.C2E5 and BoFLC.C2E9 the most frequently occurring alleles within the main two groups, BoFLC.C2E7 and BoFLC.C2E9, respectively (Figure S2b). Once genotypes carrying the previously identified loss-of-function single nucleotide polymorphism (SNP) in exon 4 (Okazaki et al., 2007; Ridge et al., 2014) are removed from the analysis, there is an over-representation of biennial types carrying the BoFLC.C2E9 allele (Figure S2a).

BoFLC.C2 alleles show distinct expression dynamics following cold exposure

To determine how polymorphism at FLC leads to the phenotypic differences observed, we analysed BoFLC.C2E5 and
expression in the *B. oleracea* E5 and E9 genotypes by quantitative real-time RT-PCR. Both BoFLC.C2 alleles are repressed by 10 weeks of vernalization at either 5°C or 10°C (Figures 4a,b and S3a). On return to warm conditions, however, the alleles reactivate to differing degrees.

To investigate these differential expression dynamics further, we analysed *A. thaliana* transgenic lines homozygous for the BoFLC.C2E5 and BoFLC.C2E9 alleles. Three BoFLC.C2E5 and BoFLC.C2E9 T3 homozygous transgenic lines were selected with flowering times that matched the mean of all the transgenic lines (Figure 2a). These BoFLC.C2E5 and BoFLC.C2E9 lines showed differences in flowering time after 4, 6 and 8 weeks at 5°C, consistent with the BoFLC.C2E9 allele being less sensitive to cold exposure (Figure S4a, b). Following 4 weeks at 5°C the expression of both the BoFLC.C2E5 and BoFLC.C2E9 alleles reactivated after plants were returned to warm conditions; however, following 8 weeks at 5°C, expression of the BoFLC.C2E5 allele was almost completely repressed when plants were returned to the warm, whereas expression of the BoFLC.C2E9 allele reactivated (Figures 2c and S5).

These data support the hypothesis that cis polymorphism at BoFLC.C2 accounts for the differential silencing dynamics between the two alleles.

Given the differential responses of the BoFLC.C2 alleles in the Arabidopsis transgenic lines, we analysed BoFLC.C2 expression in the E5 and E9 Brassica parents following an 18-week vernalization treatment at 5°C. This confirmed that even after this extended cold exposure the two alleles show different silencing dynamics: BoFLC.C2E5 reactivated to approximately 18% of the expression level found in non-vernalized plants, whereas BoFLC.C2E9 reactivated to approximately 52% of that observed in non-vernalized plants (Figure 4a).

**Activation of BoFT expression is attenuated in E9**

We assayed the expression of the two *B. oleracea* FLOWERING LOCUS T loci (BoFT.C2 and BoFT.C6) in the E5 and E9 genotypes (Figures 4c,d and S3b). It has previously been reported that only one of these, BoFT.C6, is expressed in *B. oleracea* and *B. napus* (Wang et al., 2012), BoFLC.C2E9 expression in the *B. oleracea* E5 and E9 genotypes by quantitative real-time RT-PCR. Both BoFLC.C2 alleles are repressed by 10 weeks of vernalization at either 5°C or 10°C (Figures 4a,b and S3a). On return to warm conditions, however, the alleles reactivate to differing degrees.

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Similarly, dynamics of the two BoFLC.C2E9 alleles post-vernalization were recapitulated in the transgenic lines. Thus, cis variation between two functional BoFLC.C2 alleles confers quantitative variation in heading date, via an influence on the epigenetic silencing of FLC expression. Therefore, the two BoFLC.C2 alleles show differential environmental sensitivity, an important factor in commercial broccoli production (Figures S1 and S4).

Which of the cis polymorphisms is responsible for BoFLC.C2 expression variation is yet to be determined. The fact that the exon-2 polymorphisms do not appear to segregate with crop type (Figure S2a) points to non-coding intronic polymorphism at BoFLC.C2 underpinning quantitative variation in cold-induced BoFLC silencing and reactivation. There are therefore parallels between this molecular variation and the natural variation in vernalization response in A. thaliana (Coustham et al., 2012). The conservation of COOLAIR (Swiezewski et al., 2009), the AtFLC antisense RNA in Arabis alpina (Castaings et al., 2014) and in B. rapa (Li et al., 2015b), also points to strong commonality in the regulation of FLC across the Brassicaceae. Non-coding sequence polymorphism has recently been shown to define a small number of major AtFLC haplotype groups varying in AtFLC expression and degree of epigenetic silencing (Li et al., 2014). These haplotypes can be grouped as RV (rapid vernalization) and SV (slow vernalization) types. Accessions within the SV groups require longer periods of cold before AtFLC is epigenetically silenced, and in at least two cases (SV2 and SV4) appear to flower at a similar time following the same number of weeks of cold at temperatures ranging between 2°C and 12°C (Duncan et al., 2015). This similarity in heading date across a range of different growth
conditions suggests parallels with the observed decreased environmental sensitivity of the BoFLC.C2E9 allele.

Repression of the different functional BoFLC.C2 alleles activates the expression of *B. oleracea* FLOWERING LOCUS T, as previously reported for annual cauliflower under field conditions (Ridge et al., 2014). BoFT.C2 and BoFT.C6 were both activated once FLC expression was reduced, but to very different levels (Figures 4c,d and S3b), and only transiently. This transient activation was not observed in the Arabidopsis transgenic lines (Figures 2d and S6). The reason for this difference is unknown. In Arabidopsis, AtFLC is thought to act as an inhibitor of AtSOC1 and AtFT by binding to cis-regulatory elements in intron 1 of AtFT and the AtSOC1 promoter (Helliwell et al., 2006; Searle et al., 2006). It may be that FLC in the monocarpic *A. thaliana* becomes functionally irrelevant once this repression has been released and AtFT is activated. The expression dynamics of BoFT.C2 and BoFT.C6 resemble those of AhFT in *Arabidopsis halleri*, a perennial relative of *A. thaliana*, where AhFT expression increases after prolonged cold, but is then repressed in subsequent warm temperatures concomitant with the reactivation of AhFLC (Aikawa et al., 2010). A similar pattern of expression is seen in the orthologue of AtFT in perennial poplar (*PdFT2*; Hsu et al., 2011) that is associated with reproductive onset and bud break; *PdFT2* transcription was promoted by warm temperatures and long-day conditions, and was repressed by cold temperatures and short days. The parallels in the quantitative, inverse correlation of FLC and FT expression between *B. oleracea* and *A. halleri* reinforce the previously reported view that *B. oleracea* crops are derived from a wild ancestor with a short-lived perennial life cycle (Raybould et al., 1999). They also support the conclusion that quantitative differences in BoFLC silencing are the cause of the heading date variation between the E5 and E9 BoFLC.C2 alleles.

This analysis provides another important example of cis-regulatory variation driving a major adaptive trait under...
both natural selection and domestication. Evidence has previously been reported in Zea mays (maize) of domestication resulting in a variation bottleneck caused by the removal of cis-acting polymorphisms, as a consequence of manmade selection (Hufford et al., 2012). In the case of BoFLC this cis variation opens up the possibility of exploiting allelic diversity for predictive breeding for vernalization response and cold sensitivity, aiming for the consistent, continuous scheduling of brassica vegetables. BoFLC.C2$^{E9}$ is an example of a strong BoFLC allele that is less sensitive than others to cold temperatures. With increased variability in winter temperatures, mining germplasm for allelic variation at this and other Brassica FLC loci should help to breed less environmentally sensitive varieties and reduce crop losses.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana seeds were grown as described previously (Irwin et al., 2012). Flowering time was recorded as the number of days to flowering, when the inflorescence stem was 3-cm tall.

Brassica oleracea plants were germinated in long-day greenhouse conditions (16-hour photoperiod) at 18°C (lit by 400-W HQI metal halide lamps where supplementary lighting was required). Seedlings were pricked out after 11 days and grown for 70 days in experiment 1 and 90 days in experiment 2, so the plants were no longer in the juvenile phase and were responsive to vernalization treatment. Plants were then vernalized for 10 or 18 weeks under a short-day photoperiod at 5°C or 10°C in a controlled environment room. Plants were transferred to warm conditions in April 2010 and 2011, potted on into 2-L pots and grown in an unheated glasshouse with a minimum 14-hour natural photoperiod. Plants were scored for buds visible at the apex, apical head at approximately 12 mm (the size of a 10-pence coin in the UK) and date of opening of first flower.

Field experiments

A total of 120 and 82 Doubled Haploid (DH) lines from the proprietary mapping population were transplanted into the field at independent sites in Cornwall (50°12′45.97″N, 5°17′41.19″W) and Lincolnshire (52°47′13.52″N, 0°09′5.08″E) in three randomized, replicated blocks in July 2007 and 2011, respectively. The plots were netted to prevent damage from rabbits and pigeons. Plants were scored for buds visible at the apex, apical head at approximately 12 mm (the size of a 10-pence coin in the UK) and date of opening of first flower.

QTL analysis

Single-marker regression analysis in which each marker was individually regressed against the phenotypic data was conducted in GENEPOP (Payne et al. 2009) using mean values for heading date. Potential QTL locations were estimated as genomic regions where loci were statistically significant at $P < 0.05$, with the marker with the highest $F$ probability in the analysis defined as the QTL peak and therefore closest to any putative candidate genes.

Functional analysis of BoFLC.C2 alleles

A 6-kb fragment containing BoFLC.C2 was isolated from genomic DNA of lines E5 and E9 by PCR with primers FLC4-Xhol-F and FLC4-Xhol-R, using PhuUltra II Fusion HS DNA Polymerase (Agilent Technologies, http://www.agilent.com) following the manufacturer’s conditions, and was then sequenced (primers are given in Table S1). The constructs were ligated into binary vector pSlL1711 (a gift from Prof. Jonathan Jones, Jones et al., 1992). The constructs were transferred into Agrobacterium by triparental mating and transformed into A. thaliana accession Columbia FRI f1c2 by a floral-dipping method (modified from Bechtold et al., 1993). T1 transformants were isolated by selection for Kanamycin resistance. T2 and T3 seeds were collected, homogenous lines were identified and flowering time was determined by ‘days to flower’, excluding the period of vernalization treatment.

Sequencing BoFLC.C2 in BolDFS

The B. oleracea Diversity Foundation set (BolDFS) is a core collection of lines that represent the genetic variation across the morphologically diverse crops of this species (http://www.brasica.info/resource/plants/diversity_sets.php). DNA was isolated as described in Irwin et al. (2012). A 1300-bp fragment of BoFLC.C2 was then amplified from DNA of 20 genotypes of the BolDFS by PCR with primers FLC4_F13 and FLC4_R13, using AMPLITaq GOLD TAQ DNA Polymerase (Life Technologies Ltd, now Thermo-Fisher Scientific, http://www.thermofisher.com). Sequences were aligned using AlignX in Vector NTI (Invitrogen) and analysed in SplitsTree4 (Huson and Bryant, 2006) using the median-joining method (Bandelt et al., 1999).

Expression analysis

Total RNA from three biological replicates was extracted from leaf material sampled at the same time of day as described previously (Etheridge et al., 1999; Box et al., 2011). RNA was DNase-treated to remove contaminating DNA using an Ambion Turbo DNase kit according to the manufacturer’s instructions. cDNA was synthesized using the Invitrogen Superscript III First Stand Synthesis System (18080-051; Invitrogen, now Thermo-Fisher Scientific, http://www.thermofisher.com) and analysed by qPCR using an orthologue of the Arabidopsis gene UBC (At5g25760, GENBANK EU593895), with primers UBC-F and UBC-R. BoFT.C2 and BoFT.C6 were similarly assayed using the following primer combinations: BoFT.C2-F1 and BoFT.C2-R1; BoFT.C6-F2 and BoFT.C6-R2.

Expression analysis in Arabidopsis transgenics was conducted on pooled samples of homozygous T3 seedlings from two T2 lines derived from three independent T2 lines selected from the median of the flowering time distribution of 48 E5 and 31 E9 T3 families, respectively. RNA was extracted for each pool, DNase-treated, and cDNA synthesis conducted and analysed by qPCR, as described above. BoFLC.C2 expression was assayed using the BoFLC-F and BoFLC.C2-R primers. Expression was normalized using an orthologue of the Arabidopsis gene UBC (At5g25760, GENBANK EU593895), with primers UBC-F and UBC-R. BoFT.C2 and BoFT.C6 were similarly assayed using the following primer combinations: BoFT.C2-F1 and BoFT.C2-R1; BoFT.C6-F2 and BoFT.C6-R2.

Expression of endogenous Arabidopsis FT was assayed with primers FT_UPL#138_F and FT_UPL#138_R. Primers are listed in Table S1.
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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Heading date variation in purple sprouting broccoli.

Figure S2. Allelic variation at BoFLC.C2.

Figure S3. Expression analysis in E5 and E9 parent lines.

Figure S4. Flowering time of Arabidopsis BoFLC.C2 transgenics.

Figure S5. Expression of BoFLC.C2 alleles in transgenic Arabidopsis.

Figure S6. Expression of AtFT.C2 in transgenic Arabidopsis.

Table S1. Primers used in this study.

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