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Genetic and physical mapping of flowering time loci in oilseed rape (*Brassica napus* L.)

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Abstract

We identified quantitative trait loci (QTL) underlying variation for flowering time in a doubled haploid (DH) population of vernalisation - responsive canola (*Brassica napus* L) cultivars Skipton and Ag-Spectrum and aligned them with physical map positions of predicted flowering genes from the *Brassica rapa* genome. Significant genetic variation in flowering time and response to vernalisation were observed among the DH lines from Skipton/Ag-Spectrum. A molecular linkage map was generated comprising 674 markers, including simple sequence repeat, sequence tagged site, Diversity Array Technology, and CAPS loci. QTL analysis indicated that flowering time is a complex trait and is controlled by at least 20 loci, localized on ten different chromosomes. These loci each accounted for between 2.4% and 28.6% of the total genotypic variation for first flowering, and response to vernalisation. However, identification of consistent QTL was found to be dependant upon growing environments. We compared the locations of QTL with the physical positions of predicted flowering time genes located on the sequenced genome of *B. rapa*. Some QTL associated with flowering time on A02, A03, A07, and C06 may represent homologues of known flowering time genes in *Arabidopsis*; *Vernalisation Insensitive3, APETALA1, CAULIFLOWER, FLOWERING LOCUS C, FLOWERING LOCUS T, CURLY LEAF, SHORT VEGETATIVE PHASE, GA3 oxidase,* and *LEAFY*. Identification of the chromosomal location and effect of the genes influencing flowering time may hasten the development of rapeseed varieties having an optimal time for flowering in target environments such as for low rainfall areas, via marker assisted selection.

Key words: Genetic mapping; *in silico* physical mapping; *Brassica napus*; flowering time, QTL

Introduction

Canola (rapeseed, *Brassica napus* L., 2n = 4x = 38; genome AACC) is the one of most important oilseed crops in the temperate regions of the world, with production of approximately 57.2 million tonnes of seed and over 21 million tonnes of oil in 2009-2010 (http://www.worldoil.com/, www.agricommodityprices.com/). It is predominantly grown in North and South America, Europe, Australia, and East and South Asia, for the production of vegetable oil for human consumption, bio-fuel, and as a high quality protein additive for poultry and animal stockfeed. In Australia, canola accounted for more than 95% of the total national oilseed crop of 1.8 million tonnes in 2009 (http://www.abs.gov.au/AUSSTATS).

Brassica napus is an amphidiploid member of the Brassicaceae and evolved as a result of spontaneous hybridisation between *B. rapa (*genome AA*,* 2n = 20*)* and *B. oleracea* (genome CC, 2n = 18) followed by chromosome doubling (U 1935). In Australia, canola breeding commenced in late 1960's, more recently than in many European and Asian countries (Salisbury and Wratten 1999). During the last 40 years, more than 100 cultivars have been released for commercial cultivation in Australia using both spring and winter type germplasm (Diers and Osborn 1994). However, knowledge of the loci controlling genetic variation for flowering time and response to vernalisation is limited.

Flowering time is a major determinant for the evolution, domestication and local adaptation of various crops, including canola, and is regulated by a number of genes as well as environmental cues. Genes controlling flowering time and their networks have been extensively studied in *Arabidopsis* (Koornneef et al. 1991), and at least 80 genes have been shown to affect flowering (Levy and Dean 1998b). Photoperiod and vernalisation play a major role in shaping flowering time diversity in different crop plants. Photoperiod regulates the expression of many genes, including *GIGANTEA* (Fowler et al. 1999; Park et al. 1999), *CONSTANS* (Putterill et al. 1995) and *FLOWERING LOCUS T - FT* (Kardailsky et al. 1999; Kobayashi et al. 1999; Samach et al. 2000), while *FLOWERING LOCUS C (FLC)* is involved in the autonomous flowering

and vernalisation pathways to repress floral transition in a dose dependent manner in *Arabidopsis* (Schmitz and Amasino 2007; Sheldon et al. 1999; Sheldon et al. 2000).

Comparative genetic analysis between *Arabidopsis* and *Brassica* that diverged around 14.5 to 20.4 million years ago (Bowers et al. 2003; Yang et al. 2006) has identified numerous regions of homeology and triplication in the diploid and amphidiploid *Brassica* species (Lagercrantz et al. 1996; Lysak et al. 2005; Parkin et al. 2003; Rana et al. 2004; Schranz et al. 2006). These studies have assisted the isolation of homologoues of *FLC*; *BrFLC1, BrFLC2, BrFLC3, BrFLC4* and *BrFLC5,* in *Brassica rapa, BoFLC1, BoFLC2, BoFLC3, BoFLC4, BoFLC5* in *B. oleracea* and *BnFLC1, BnFLC2, BnFLC3, BnFLC4* and *BnFLC5* in *B. napus,* and the association of variation in these genes with flowering time variation (Lagercrantz et al. 1996; Lin et al. 2005; Okazaki et al. 2007; Schranz et al. 2002; Tadege et al. 2001; Yuan et al. 2009; Zhao et al. 2010). A direct relationship between flowering time and *BnFLC* gene expression level has been reported in spring and winter rapeseed cultivars (Tadege et al. 2001). This study concluded that vernalisation dramatically reduces the abundance of *BnFLC* transcripts and restores early flowering in the winter cultivar 'Columbus'. However, recent studies have shown that sequence polymorphisms in *FLC* genes may not correlate with phenotypic variation in flowering time in *B. oleracea* (Okazaki et al. 2007; Razi et al. 2008). To dissect the complexity of flowering genes, genetic mapping approaches have been used to identify and map loci and estimate their allelic effects in populations of *B. rapa, B. nigra B. oleracea,* and especially of *B. napus* that were developed between annual (spring/semi-spring) and biennial (winter) cultivars (Ferreira et al. 1995; Kennard et al. 1994; Long et al. 2007; Osborn et al. 1997; Schranz et al. 2002; Teutonico and Osborn 1994; Zhao et al. 2010).

In this study, we aimed to identify genomic regions associated with flowering time in a doubled haploid (DH) population from a cross between the Australian vernalisation responsive cultivars Skipton and Ag-Spectrum, and align them with the recently sequenced reference *B. rapa* genome. The mapping of loci for flowering time in Australian canola populations may allow breeders to develop new germplasm by selecting advanced breeding lines having desirable allelic combinations controlling flowering time, which exhibit an optimal time of flowering to maximise yield under

Australian growing conditions. Molecular markers linked with flowering-time loci can also be used to trace the domestication and selection history in rapeseed germplasm.

Materials and methods

Plant material

A DH population comprising 186 lines was developed from the cross between the Australian cultivars: Skipton and Ag-Spectrum at the Wagga Wagga Agricultural Institute (WWAI), New South Wales, Australia, as described previously (Raman et al. 2012b). DH lines as well as their parental lines were characterised for flowering time and response to vernalisation. During the course of experiment, it was established that both parental lines are responsive to vernalisation.

Evaluation for flowering time under field conditions and experimental design

Flowering time was evaluated over two years at the Agricultural Institute research farm located at Wagga Wagga, NSW (latitude 36.06, longitude 147.22, 182 m above sea level). The DH population was grown in three field trials; two experiments in 2008 (first sowing on 18th June and second sowing on 29th July) and a third in 2009 (sown on 18th June). Details on environmental conditions are shown in Supplementary Fig 2. The 2008 trials were partially replicated with 160 DH lines sown in two plots, eight DH lines sown in single plots and the two parental lines each sown in 6 plots. Two blocks of 5 ranges by 34 rows were replicates augmented with 4 unreplicated genotypes. The placement of genotypes was optimised using the DiGGer design package (Coombes 2002). Mean temperatures of 15 to 31°C for the period from sowing to flowering were recorded (Supplementary Fig 2).

The 2009 experiment was a two replicate trial with additional replication of the parental lines. The trial was set out in 12 ranges by 30 rows with additional Australian cultivar ‘Tarcoola’ buffer rows. Each block of 6 ranges held 160 DH lines and the remaining plots had 23 replicates of Skipton and 17 replicates of Ag-Spectrum. The

genotypes were spatially optimised using DiGGer (Coombes 2002). Plants within each row were counted and scored when they reached to 5% flowering in 2008 and 2009.

Evaluation for flowering time under glasshouse conditions and experimental design

The vernalisation trial was designed as a split-plot set out on 8 benches arranged in a 4 row by 2 column array, with each column of benches holding a replicate of 186 DH lines by 2 vernalisation treatments. In each replicate two benches held 6 rows by 15 columns of pots and two benches held 6 rows by 16 pots. A DH line was allocated to each main plot of two rows by 1 column of pots and the vernalisation treatments were randomised within. The allocation was made in a two stage spatial optimisation using DiGGer.

Seeds were initially sown in Petri-dishes at 18°C for 48 hr and then germinated seedlings of equal vigour were sown in plastic tray (7×8 wells) containing pre-soaked Jiffy discs (Jiffy Products International B.V. Moerdijk, Netherland). Sixteen seedlings per genotype were raised as per the experimental design in the glasshouse (18 ± 2°C) and were watered daily and fertilised once a week with Thrive complete fertilizer (Yates Australia, Padstow). After 2 weeks, plants were thinned to one seedling per well and only seven seedlings per genotype were raised to flowering. Plants were given two treatments: no vernalisation and 8 weeks of vernalisation. The latter was accomplished in a cold room maintained at 4 ± °C, and illuminated with cool white fluorescent tubes with an irradiance of 150µM/m^2/s, with a 16-h photoperiod. For the control treatment (non-vernalisation), two independent sowings (after 5th wk and 6th wk of vernalisation) were performed to match the growth of the vernalised plants. Both vernalised and unvernalised plants of each DH line having an equal vegetative growth stage (after 6th wk of vernalisation) were grown further for 7 days before transplanting to 4 plants per pot (25 cm diameter) containing commercial potting mix, and were grown to maturity in the glasshouse. Plants were fertilized monthly with Thrive and sprayed with Confidor and Rose Shield (Yates Australia) to control aphids and powdery mildew, respectively, when required.

The days to first flowering (DTF) were determined when the 25% of the plants of a given DH line showed the first open flower calculated from day of transplanting. The response to vernalisation (RV) was the calculated difference between 50% flowering in vernalised and non-vernalised plants.

Molecular marker analysis and map construction

Genomic DNA was isolated from the leaves harvested from 4-5 week-old glasshouse-grown seedlings using a standard phenol-chloroform method. A genetic linkage map, consisting of 216 SSR, SRAP, SCAR and EST-SSR markers, that was developed previously using a DH population from Skipton/Ag-Spectrum (Raman et al. 2012b) was further saturated with *B. napus* Diversity Array Technology (DArT) based markers (Raman et al. 2012a). Genotyping and scoring were carried out at the DArT P/L (http://www.diversityarrays.com., Canberra, Australia) as described previously.

PCR analysis of flowering time alleles associated with *FLC* homologues, *FT* and *FRIGIDA* was carried out using the following primers:

BnFLC1.A10: forward (FLC1F4) 5'-CTT GAG GAA TCA AAT GTC GAT AA-3' and reverse 5'-CGG AGA TTT GTC CTG GTG AG-3' which amplifies *BnFLC1* (AY036888) designated as *BnFLC-10* (Long et al. 2007),

BnaA.FRI.a: forward (N016): 5'' GTT GCA ATT TCT CAG CCC-3', and reverse (A385): 5'- TGT GCA GCT TTA CAA CTT GTC-3' as described by Wang et al (2011a)

BnFT-2a were obtained from published literature (Long et al 2007, see supplemental Table 2b at http://www.genetics.org/supplemental/).

BnFLC.A3a: forward 5'-GTG CAT CTG GTC TTT CAG GGA TGA-3'and reverse 5'-TGT GCA AGT ATA AGA TGC AAG AAG TG-3'

BnFLC.A3b: forward 5'- TAA TTT GTT GCA GGC AGA ACT-3' and reverse 5'- TGC AAC ATC CCT AAT AGA CAA G-3'

BnFLC.A3a primer-pairs were designed to identify allelic differences in intron 5 between the parental lines of mapping populations from Tapidor/Ningyou7, and were developed by aligning full length sequence of *BnFLC.A3*a (*BnFLC3*), obtained from a BAC clone 'JBnB 50A15' of *B. napus* cv. Tapidor and the *BnFLC.A3a* sequence of *B. napus* cv.

Ningyou7 (Zou *et al*, unpublished). The 5' end of forward primer sequence was tailed with a nineteen-nucleotide-long M13 sequence (5'-CAC GAC GTT GTA AAA CGA C-3') as described previously (Raman et al. 2005). PCR amplification and further allele sizing was carried either on a CEQ 8000 genetic analysis system (Beckman Coulter Inc) as described previously (Raman et al. 2005) or on agarose gel electrophoresis in TAE buffer, according to standard conditions.

An integrated genetic linkage map was produced using Map Manager version QTL20b (Manly et al. 2001) using the Kosambi mapping function, as described previously (Raman et al. 2009). Allele segregation ratios for goodness of fit were was determined using χ^2 tests to determine if segregation ratios were more consistent with one locus (1:1 allelic ratio) or two loci (1:3 or 3:1 allelic ratio) models. The accuracy of marker order and genetic distances within and between linkage groups was checked using the R/qtl statistical analysis package R (Broman et al. 2003). The linkage groups were assigned to their respective chromosomes by aligning them with previously published maps (Choi et al. 2007; Lowe et al. 2004; Piquemal et al. 2005; Suwabe et al. 2008; Suwabe et al. 2006).

Statistical and QTL Analyses

An integrated map consisting of 674 markers was subsequently employed to identify quantitative trait loci (QTL) associated with flowering time using the whole genome average interval mapping (WGAIM) approach (Verbyla et al. 2006) using the original data sets for each experiment. QTL analyses were conducted using the ASREML-R package (Butler et al. 2007). A minimum LOD-score of 2 was used to identify markers (marker-interval) linked to flowering time and vernalisation responsive loci, as a QTL with low LOD score in one experiment may be higher in another experiment. The percentage of genetic variation accounted for by a QTL was calculated as described previously (Raman et al. 2009). The chromosome map was drawn using Mapchart (Voorrips 2002) using linkage distances calculated by Map Manager (Manly et al. 2001). QTL identified were named using a standard 'designation' system (http://www.shigen.nig.ac.jp/wheat/komugi/genes/macgene/supplement2007, validated

on 1st July 2008) as described previously (Raman et al. 2012b). For example *Qdtf(f).wwai-A2* represents a QTL associated with days to first (5%) flowering (*dtf*) under field (*f*) conditions that is mapped on chromosome A2 at the WWAI (*wwai*). An additional suffix (*a, b, c, d,* and *e*) was used if either more than one QTL affecting the trait was identified on the same chromosome or multiple segregating loci were detected by a primer-pair.

Physical mapping of genetic markers and flowering time genes on the *B. rapa* genome

A total of 59 genes that regulate flowering time in *Arabidopsis*, *B. rapa*, *B. oleracea*, *B. napus*, *B. nigra* and other species (Blackman et al. 2011) were compiled from the literature (Table S3). Corresponding sequences were retrieved from The *Arabidopsis* Information Resource (TAIR, http://www.arabidopsis.org/), the *Brassica* Database (BRAD, http://brassicadb.org/brad/index.php) and NCBI GenBank (http://www.ncbi.nlm.nih.gov/Genomes/) and used to search the sequence of *B. rapa* (Wang et al. 2011b) using BLASTN (Altschul et al. 1997). PCR primers for markers based on PCR assays and sequences of DArT clones (unpublished) were also used to search the assembled *B. rapa* genome using a custom perl script and the predicted marker positions were compared with the genetic mapping positions and the physical positions of the predicted flowering time genes.

Results

Genetic analysis of flowering time

Histograms of the predicted means of the first flowering in the four testing environments revealed continuous distributions close to normality (Fig. 1), which is typical of traits with polygenic inheritance. For first flowering, transgressive segregation was apparent, with a substantial number of lines exhibiting earlier flowering than Ag-Spectrum and later flowering than Skipton, indicating that the both parents carry genes that contribute to flowering time variation (Fig1a-c). However, the response to vernalisation and

flowering time (days to flowering) was skewed toward Ag-Spectrum under glasshouse conditions. Variation in flowering time across different environments (experiments) was observed (Fig 2) and this indicates that different flowering time genes responded to different environments. For example, the DH population flowered more quickly in the second sowing compared to first sowings. The approximate mean line heritability is provided in Table 1. The heritability estimate for days to first flower was very high (89%), while a moderate heritability (60%) was observed for response to vernalisation.

The parental lines of the DH population, Ag-Spectrum and Skipton, flowered after an average of 128.5 ± 6.1 days, and 134.6 ± 5.5 days in control (non-vernalised), and 37.8 ± 0.5 and 30.3 ± 0.5 days after 8 wk of vernalisation, respectively. The range of mean flowering time of 52.6 to 166.7 DTF in the control, and 10.4 to 94.1 DTF in the vernalisation treatment of the DH population was beyond the values of the parental lines, suggesting transgressive segregation. Both parental lines responded to vernalisation (90.7 to 104.3 days), however the DH population showed a much broader range of response varying from a 'strong' response (18.7 days) to a 'weak' response (121 DTF) to vernalisation (Table 1, Fig 1d-f).

Integrated linkage map construction

A total of 796 markers based upon DArT, SSRs, SRAP, candidate genes and SCARs were genotyped utilizing 186 DH lines of Skipton/Ag-Spectrum. This includes four hundred and ninety five new DArT markers that were used to incorporate into the genetic map of the Skipton/Ag-Spectrum population constructed previously (Raman et al. 2012b). This population exhibited extensive degree of segregation distortion as only 33% of the markers (253/796) showed 1 (Skipton alleles):1 (Ag-Spectrum alleles) segregation ratio (Table S1). Segregation distortion occurred towards both the parents. One hundred and twenty two markers that showed extensive segregation distortion (more than 2 locus model) and either remained unlinked or formed small linkage groups were excluded for estimation of the linkage map length. An integrated framework molecular map of 674 markers was constructed comprising 24 linkage groups representing at least 17 chromosomes of *B. napus*. Generally, the markers were well-distributed across the

genome with an average marker density of one marker per 6.7 cM. However, some markers especially the DArTs, exhibited co-segregation and therefore mapped to the same loci (Fig 3, Table S2). Several marker loci amplified homeoalleles from the A and C genomes, such as *Xbrms287a* (A1), *Xbrms287b* (C1), *Xol12-d04a* (A2), *Xol12-d04d* (C2), *Xbrms269a* (A3) and *Xbrms269b* (C3). Thirty-four SSR markers exhibited locus duplication within the same chromosome and/or across different chromosomes. The frequency of locus duplication ranged from two to five (26 primer-pairs detected two loci, six detected three, and one detected 4 loci). For example, SSRs *Xna14-f11 (Xna14-f11a, Xna14-f11b)* and *Xra3-e05 (Xra3-e05a, Xra3- e05b, and Xra3-e05c)* were localized on chromosome A1. In contrast, five marker loci of the CB10079: *Xcb10079e, Xcb10079a, Xcb10079b* and *Xcb10079c* and *Xcb10079d* were mapped on chromosomes A1, A3, C8 and A10 respectively (Table S2). The *Xcb10079a* accounted for a significant genetic variation for first flowering on chromosome A3 under glasshouse and field conditions (Table 2, 3). Homologues of the CB10079 and BRMS008 marker loci map closely to the different copies of *FLC*. This suggests that chromosomal arrangements such as duplications and translocations may have occurred within genomic regions for flowering time in the Skipton/Ag-Spectrum population.

QTL analysis of flowering time

WGAIM indicated that flowering time is a complex trait and is controlled by at least 20 loci, localized on chromosomes A2, A3, A4, A6, A7, C2, C3, C5, C6, and C8 (Table 2, Fig 3). These loci each accounted for between 2.4% and 28.6% of the total genotypic variation. Some of these QTL were clustered in genomic regions on chromosomes A2, A3, A4, A6, C2, and C3.

(i) First flowering

Four significant QTL: *Qdtf(f).wwai-A2, Qdtf(f).wwai-A3, Qdtf(f).wwai-A4,* and *Qdtf(f).wwai-C3a* were identified for first flowering, consistent over the 2008 and 2009 field trials (Table 2, Fig. 3). These QTL were located on chromosomes A2, A3, A4 and

C3 respectively (Table 2) and accounted for up to of 56.3% of the genotypic variance. The most significant QTL for first flowering *Qdtf(f).wwai-A2* with a LOD score of 9.86 was located on chromosome A2 between *Xna10-c01a* and *Xol12-d04a* markers (Fig. 3). This QTL accounted from 9.4 to 12.6% of the genotypic variance. The QTL *Qdtf(f).wwai-C3b*, flanked by markers *Xpbcessrna7b* and *XbrPb-839739* was only identified in year 2009. The late-flowering parent, Skipton contributed alleles for delaying flowering time on chromosomes A2, while Ag-Spectrum alleles located on chromosome A3, A4 and C3 promoted early-flowering (Table 2).

In the second sowing of 2008, nine genomic regions localised on A2, A3, A4, A7, C3, and C8 chromosomes were significantly associated with flowering time (Table 2). However, additive effects of only two QTL: *Qdtf(f/s).wwai-A2b*, flanked with *XbrPb-660784* and *Xna10-c01a* on chromosome A2, and *Qdtf(f/s).wwai-A4b* on chromosome A4were identical under field conditions in 2008 (first sowing) and 2009 (Table 2). In this experiment, the additive effects of Ag-Spectrum and Skipton in promoting and delaying flowering time respectively were consistent between year 2008 (first sowing) and 2009.

Under glasshouse conditions, we identified six QTL for unvernalised first flowering time on chromosomes A3, A6, C2, C3 and C6 (Table 3). However, at least one of these QTL, *Qdtf(g).wwai-A3*, detected on chromosome A3 (r^2 = 6.02%), was identified under field conditions (2008 and 2009). This QTL region was delimited by marker interval *XbrPb661557-Xcb10079a* (Fig 3) and was localised in the vicinity of *Qdtf(f/s).wwai-A3* that was identified under field conditions in 2008 (second sowing).

(ii) Response to vernalisation

Seven QTL were detected for response to vernalisation, which together accounted for 59.4% of the total genotypic variance (Table 3). The highly significant QTL, *Qrv(g).wwai-C3* (LOD = 7.1, r^2 = 16.7%) was identified on chromosome C3. Two QTL *Qrv(g).wwai-A3* and *Qrv(g).wwai-C2* were located in the same marker-intervals where first flowering QTL: *Qdtf(g).wwai-A3* and *Qdtf(g).wwai-C2* were identified under glasshouse/field conditions (Fig 3, Table 3). Ag-Spectrum contributed alleles for early flowering on chromosomes A3, A6, C3, and C5, whereas Skipton contributed alleles on

A7, C2, and C5 for delaying flowering time.

In order to determine whether the *FLC* and *FRIGIDA* paralogues of *Arabidopsis* control variation for flowering time on chromosome A3, we mapped *BnFLC.A3a (FLC3), BnFLC.A3b (FLC5)* and *BnA.Fri.a* (*BnFRI.A3*) gene-specific markers. Linkage analysis showed that the *BnFLC.A3a* gene is co-localised with the QTL (*Qdtf(g)-wwai-A3, Qdtf(f)-wwai-A3* and *Qrv(g)-wwai-A3*) for flowering time. The *BnFLC.A3b* was localised on the proximal end of chromosome A3 delimited with marker *XbrPb-658100* that map 1.2 cM apart from *BnFRI.A3* gene (Fig. 3). Under field and glasshouse conditions, an additive effect was contributed from the early flowering parent Ag-Spectrum (1.05 to 10.11 days). Among the QTL identified, the most significant marker-interval was *XbrPb-660999/XbrPb-661396* (LOD = 15.14, r^2 = 22.4%) on chromosome C2 that was responsible for delaying flowering by 12.5 days (Table 3). The Ag-Spectrum allele at *Qdtf(g).wwai-C3* (LOD = 11.99, r^2 = 6.71), delimited by the *XbrPb-839739/XbrPbXbrPb-658284* marker interval accounted for early flowering by 10.11 days. After vernalisation the DH lines, we identified two QTL for flowering time, *Qdtf(g).wwai-A2* and *Qdtf(g).wwai-C2,* with LOD score of 2.5 and 8.2 respectively (Table 3). The major QTL, *Qdtf(g).wwai-C2* was flanked by *XbrPb-661529/XbrPb-671282* markers and accounted for 28.6% of the genetic variance. The early-flowering allele at *Qdtf(g).wwai-A2* was derived from Ag-Spectrum and this QTL was not identified in any other experiments conducted in this study.

Physical mapping of flowering genes in *B. rapa*

We identified several genomic sequences related to flowering genes using the public *Brassica rapa* genome sequence. The predicted physical locations of these genes were compared with the location of the genetic markers and flowering time QTL loci. The location of some of the known *B. napus* genes for flowering time such as *BnFLC1* (A10), *BnFLC2* (A2), *BnFLC.A3a* (A3), *BnFLC.A3b* (A3), *BnA.Fri.a/BnFRI.A3* (A3) and *BnFT.A2* (A2) on the *B. rapa* sequenced genome was consistent with genetic linkage map positions of Skipton/Ag-Spectrum population. Several flowering genes such as *Floral*

homeotic protein APETALA 1 (AP1, NM_105581.2), CAULIFLOWER (*CAL, NM_102395.2*) and *GIBBERELLIN 2-OXIDASE 1* on A2; *VERNALISATION INSENSITIVE 3 (VIN3, NM_125121.3*), *FLOWERING LOCUS C (BnFLC.A3a*, *BnFLC.A3b)*, *FRIGIDA (BnFRI.A3),* and *TERMINAL FLOWER (TFL1, AY271513.1)* on A3; ABC transporter-like protein (DQ296184.1), putative *AP2/EREBP* transcription factor (AY560867.1), ethylene-responsive transcription factor *ERF024* (*HRD*, NM_129202.1), *CURLY LEAF* (*CLF*, NM_127902.5), and *SHORT VEGETATIVE PHASE (SVP)* on A4, and dehydrin (ERD10, AY376669.1), *LEAFY* (LFY, NM_125579.1), and *Ent-kaurene oxidase (GA3)*/*Cytochrome P450 (*NM_122491.2), on A6 were mapped within and or in the vicinity of QTL associated with flowering time (Table S3, Fig. S1).

Alignments between genetic regions that showed significant association with flowering time in the Skipton/Ag-Spectrum population with the sequenced genome of *B. rapa,* revealed that several flowering genes involved in autonomous, photoperiod (light sensing and circadian rhythm), vernalisation, and gibberellic acid-response pathways and in the transition to flowering in *Arabidopsis*, are localised in clusters and or in close proximity to each other in the *B. rapa* genome such as *PHYTOCHROME DEFECTIVE E/AGAMOUS* on chromosome A1; *AGAMOUS LIKE 8, LEAFY*, *A AGAMOUS LIKE 42* on chromosome A2; *FLOWERING LOCUS C* (*BnFLC.A3*) and *FRIGIDA* (*BnFRI.A3*) on chromosome A3; *CURLY LEAF* and *SVP* on chromosome A4; *SPINDLY/PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1* and *PHYTOCHROME B* (*RAFL)/PHYTOCHROME DEFECTIVE D* on chromosome A5; *APETALA1* and *CAULIFLOWER* on chromosome A7 and A8, (Fig. S1). Some of the markers such as *XbrPb-809660* on chromosome A5 showed the tight linkage with flowering time genes such as *PHYTOCHROME B/PHYTOCHROME DEFECTIVE D* (in *B. rapa*) and therefore may represent to the sequence of flowering genes.

Discussion

Significant progress has been made in identifying loci that control flowering time in both model and key food crops such as Arabidopsis, rice, wheat, maize and barley. At least 80

genes have been shown to regulate flowering time in *Arabidopsis* (Koornneef et al. 1998; Levy and Dean 1998a). In rice, 23 major genes and numerous QTL affecting flowering time have been reported (Yamamoto et al. 2000). In rapeseed, Long et al., (2007) identified a large number of 'statistically significant' and 'micro-real' QTL (up to 42) associated with flowering time in the DH and RC-F_2 populations derived from a cross between Tapidor (winter type) and Ningyou7 (semi-spring type) that were evaluated in 14 environments. In the present study, we identified 20 QTL for flowering time (Table 2-3, Fig 3). Significant differences were observed between QTL, their effect and size under different environments. This strongly suggests that flowering time is controlled by quantitative loci in the DH population from Skipton/Ag-Spectrum. In the previous studies, loci controlling flowering time and vernalisation requirement were mapped in populations derived from spring/semi-spring and winter cultivars (Ferreira et al. 1995; Long et al. 2007). Herein, we localised loci for flowering time and response to vernalisation in a DH population derived from two semi-spring cultivars which are both responsive to vernalisation. QTL detection was largely dependent upon the phenotyping environment. For example, the majority of QTL and their allelic effects were consistent in both years with early sowing, whereas only a few consistent QTL were identified in the later sowing (Table 2), and under both vernalisation and non-vernalisation treatments (Table 3). Trial conducted in 2008 (second sowing) was late-sown and grown under water-limited and high temperature conditions (Fig. S2). This suggests that different genes respond to flowering time under different environments. Such effects on flowering time have been reported previously in a mapping and in reconstituted populations derived from Tapidor and Ningyou7 crosses (Long et al. 2007). Transgressive segregation occurred in our population suggesting that there is a potential to develop very early flowering derivatives of rapeseed from Ag-Spectrum and Skipton, which are likely to be important in the drought-prone Australian environment, which is predicted to become more water-limited due to climate change.

The majority of the flowering time QTL that we identified in this study partially overlap with QTL identified in Tapidor/Ningyou7 DH (TN-DH) and its reconstructed RC-F_2 population on chromosomes A2, A3, C2, C6, and C8 (Long et al. 2007; Wang et al. 2009) and with circadian period QTL on chromosomes A2, A3, and A7 (Lou et al.,

2011). For example, we detected *Qdtf(f).wwai-C3a* for flowering time on chromosome C3 that explained 20.1% of the genotypic variation for flowering time in the Skipton/Ag-Spectrum population. Long et al (2007) detected a highly significant QTL for flowering time *SL-qFT* accounting for 24% of variation in a TN-DH population on chromosome C3. Similarly, a QTL on chromosome C6, *Qdtf(f).wwai-C6* may be the same as detected in TN-DH and in French populations (Delourme et al. 2006; Long et al. 2007) as they map on the same genetic positions. However, no QTL for flowering time was identified on chromosome A10, suggesting that *BnFLC.A10* [*BnFLC1,* (Pires et al. 2004)], does not control genetic variation for flowering time in the DH population from Skipton/Ag-Spectrum under the environmental conditions that were used here. Previously, Long et al. (2007) detected a major QTL; *qFT10-4* on chromosome A10, explaining 50% of the phenotypic variation for flowering time in a spring-environment. This QTL corresponds to the *BnFLC.A10* gene (Hou et al., unpublished).

Various factors complicate the identification of consistent QTL across populations and environments such as the large diversity of flowering genes and their interactions, the different genetic backgrounds of populations, the system of scoring traits, the method of data analysing for QTL detection, the prevalence of chromosome rearrangements (reciprocal and non-reciprocal translocations and *de novo* non-reciprocal translocations) in the genome under investigation. We identified 7 QTL for vernalisation response under glasshouse conditions (Table 3). However, Ferreira et al., (1995) reported three QTL associated with vernalisation requirement in the DH rapeseed population derived from Major (a biennial type)/Stellar (an annual type) and used discrete data for flowering time (flowering or not flowering) to test association with molecular markers. A single major QTL on linkage group 9 (A2) and minor QTL effects, on linkage groups LG12 (A10) and LG 16, associated with vernalisation requirement and flowering time were identified using MAPMAKER/QTL computer software. In contrast, in this study, we have not identified any genetic effect on chromosomes A2 and A10 that are associated with response to vernalisation. We used WAGIM for mapping QTL using raw data instead of predicted phenotypic means. Moreover, this procedure detects a much higher number of genuine QTL that composite interval mapping approach (Verbyla et al. 2006).

We detected allelic effects for flowering time on group 2 and 3 chromosomes (A2, C2, A3 and C3). Both of these homeologous chromosomes harbour multiple copies of *FLC* genes that are known as repressing flowering in many plants requiring vernalisation, including in *Brassica* (Schranz et al. 2002). With vernalisation treatment, the allelic effects on A3 and C3 chromosomes were not detected in this study (Table 3), unlike that reported previously in the DH population from Major/Stellar and different rapeseed genotypes (Osborn and Lukens 2003; Tadege et al. 2001). This may be due to the downregulation and stable repression of *FLC* during and after vernalization (Bastow et al. 2004; Michaels and Amasino 1999; Sheldon et al. 1999), which subsequently induces the flowering by activating the *FLOWERING LOCUS T* and other floral integrator genes such as *FLOWERING DURATION* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (Helliwell et al. 2006; Kim et al. 2009).

In this study, the genomic region spaning approximately 2 megabase pair of chromosome A3, delimited by *XbrPb-658157/Xbrms008* marker loci, harbours key flowering repressor gene *BnFLC.A3a* (*FLC3*) and may be responsible for variation in flowering time and response to vernalisation at the *Qdtf(f).wwai-A3* and *Qdtf(g).wwai-A3* and *Qrv(g).wwai-A3.* Functionality of *BnFLC.A3a* gene needs to be tested to demonstrate its role in shaping variation for flowering time. However, the presence of multiple of copies of *FLC* and its complex interaction with other repressors such as *FRIGIDA* and other flowering activators remains a challenge to demonstrate its precise role in modulating flowering time. The *BnFLC.A3a (FLC3)* was mapped 50.4 cM apart from a gene cluster of *BnFLC.A3b (FLC3')* and *BnFRI.A3, - FRI* orthologue in *A. thaliana* that showed association with flowering time variation in diverse rapeseed germplasm (Wang et al. 2011a). However, no allelic effect of either *BnFLC.A3b (FLC3')* and or of *BnFRI.A3* was found to contribute flowering time difference in the DH population from Skipton/Ag-Spectrum. Both *BrFLC.A3a (FLC3)* and *BrFLC.A3b (FLC3')* are linked with SSR marker BRMS008, and have been shown to be recently duplicated in *B. rapa* genome using linkage mapping and *in situ* hybridisation (Kim et al. 2006; Yang et al. 2006). The BRMS008 marker was associated with flowering time differences at the *Qdtf(f/s).wwai-A3* (this study) and has also been mapped in close proximity of *FLC3* gene *(BoFLC3)* and *FLC2 (BrFLC2)* controlling flowering time in an F_2 population of *B.*

oleracea on the chromosomes O3, and in the RIL population of *B. rapa* on chromosome A2 respectively (Lou et al. 2011; Okazaki et al. 2007).

We identified another major QTL, *Qdtf(g).wwai-C2* that account for 28.6% of the genotypic variance (Table 3) and mapped approximately 18 cM away from *Xbrms215*. Previously, the largest QTL for flowering time [LOD = 20.0, r^2 (phenotypic variance) = 36.8%) corresponding to *BoFLC2* was mapped in an F_2 population from *B. oleracea* cv. Green Comet (spring type)/*B. oleracea* cv. Reiho (spring type), in vicinity of SSR marker BRMS215 on chromosome C2 (Okazaki et al. 2007). This genomic region showed colinearity with the top of Arabidopsis chromosome 5 region which harbour *FLC, CO, FY, LFY, EMF1* and *TFL1* genes involved in flowering time (Koornneef et al. 1994). Given that *FLC* is the key repressor for flowering time and is responsible for vernalisation requirement in *Brassica* and *Arabidopsis* (Li et al. 2009; Zhao et al. 2010), *BnFLC.C2* is likely to be one of the candidate genes for flowering time differences in the Skipton/Ag-Spectrum population. Our results suggested that the homologues of *FLC, AP, CAL, TFL1* (At5g03840)*, CLF, SVP, GID1B, LFY* and *GA REQUIRING 3* are likely the major determinants that control variation for flowering time in this DH population from Skipton/Ag-Spectrum. In *Arabidopsis* and other members of Brassicaceae, several copies of *FLC* in *B. rapa, B. oleracea,* and *B. napus* genomes have been associated with variation in flowering time in *Brassica* species (Butruille et al. 1999; Kim et al. 2006; Kole et al. 2001; Lagercrantz et al. 1996; Lin et al. 2005; Long et al. 2007; Okazaki et al. 2007; Osborn et al. 1997; Pires et al. 2004; Tadege et al. 2001; Udall et al. 2006; Yang et al. 2006; Yuan et al. 2009; Zhao et al. 2010). However, the precise role of other genes has not been established yet in rapeseed. Several genes involved in vernalisation such as *VIN3* (Sung and Amasino 2005), and genes involved in autonomus pathway such as *FCA, FVE* and *FLK* repress *FLC* in *Arabidopsis* (He and Amasino 2005). However, *FRI, VIP3, ELF7* and *PIE1* genes positively regulate *FLC* (Rouse et al. 2002).

The presence of several flowering genes that control flowering time differences in *B. rapa* /*B. napus* genomes, and within the QTL regions that account variation in flowering time (in this study), their interaction with environment, existence of functional multiple copies (such as presence of the *Arabidopsis* meristem identity gene *AP1,* responsible for the transition from the vegetative to the reproductive structure and *CAL*

cluster on chromosomes A2, A7, and A8, Table S3) and their diverse roles (Deng et al. 2011) imply that identifying candidate genes for flowering time in *B. napus* is a challenging exercise. Therefore, genetic dissection of flowering time is necessary for any given germplasm.

Occurrence of multiple gene copies and gene clusters suggests that these genes may have experienced expansion through polypolidisation and chromosomal rearrangements and have experienced intense selection pressure during its evolution, domestication and breeding for target environments. Duplicated flowering genes might offer adaptive advantages when present in multiple copies, and novel patterns of gene expression might evolve after gene duplication (Lynch and Conery 2000). In this study, we could not align QTL regions for flowering time that we identified in this study with the sequenced C /AC genomes, as their sequence information is not in public domain yet. The future availability of new genomic tools, such as access to sequenced Brassica genomes, sequencing technologies, and bioinformatics tools, will make it easier to detect and sequence clusters of flowering genes from rapeseed in order to identify the structure of functional genes and estimate the evolutionary forces that led to clustering of flowering genes and their sequence divergence.

SUPPLEMENTARY DATA

Table S1: Genotyping of the DH population from Skipton/Ag-Spectrum population with different molecular marker systems. Position of markers was calculated with MapManager software. Some DH lines with few markers excluded from phenotypic evaluation. Markers that showed no linkage and highly distortion are indicated as 'bad markers'.

Table S2: Segregation of Skipton and Ag-Spectrum alleles in a doubled haploid population of Skipton/Ag-Spectrum.

Table S3: Physical map positions of flowering time genes on different pseudomolecules (chromosomes) of *Brassica rapa.*

Figure S1: Alignments between genetic regions that showed significant association with flowering time in the Skipton/Ag-Spectrum population with the sequenced genome of *B. rapa*. For clarity we dropped 'X' suffix from DArT markers. Detailed description of candidate genes is given in Table S3

Figure S2: Daylengths, rainfall and screen and soil temperatures during the growing seasons in 2008 and 2009.

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Fig. 1: Frequency distributions for days to flowering and response to vernalisation in the DH lines from Skipton/Ag-Spectrum: (a) days to flowering under field conditions in 2008 (first sowing), (b) days to flowering under field conditions in 2008 (second sowing), (c) days to flowering under field conditions in 2009, (d) days to flowering under glasshouse conditions (non-vernalisation), (e) days to flowering under glasshouse conditions (vernalisation for 8 wks), and (f) response to vernalisation under glasshouse conditions. Days to flowering was calculated from date of transplanting.

Fig 2: Box-plot showing genetic variation for days to flowering in the DH population from Skipton/Ag-Spectrum grown under four different environments (2008-1st sowing, 2008-2nd sowing, 2009, non-vernalised and vernalised plants under glasshouse conditions in 2009).

Fig 3: Molecular linkage groups of *Brasica napus* L showing QTL associated with days to flowering and response to vernalisation in a DH population from the Skipton/Ag-Spectrum. Genetic distances (cM) are located to the left of the linkage groups and locus names are listed to the right. The loci that showed significant association with target traits are indicated in bold. Markers with *XbrPb*-suffix are Diversity Array Technology based. Vertical bars *at right* represent the genomic regions (QTL) significantly associated with different components of flowering time.

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