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R. A. Teutonico · T. C. Osborn

Mapping loci controlling vernalization requirement in *Brassica rapa*

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Abstract *Brassica* cultivars are classified as biennial or annual based on their requirement for a period of cold treatment (vernalization) to induce flowering. Genes controlling the vernalization requirement were identified in a *Brassica rapa* F_2 population derived from a cross between an annual and a biennial oilseed cultivar by using an RFLP linkage map and quantitative trait locus (QTL) analysis of flowering time in F_3 lines. Two genomic regions were strongly associated with variation for flowering time of unvernalized plants and alleles from the biennial parent in these regions delayed flowering. These QTLs had no significant effect on flowering time after plants were vernalized for 6 weeks, suggesting that they control flowering time through the requirement for vernalization. The two *B. rapa* linkage groups containing these QTLs had RFLP loci in common with two *B. napus* linkage groups that were shown previously to contain QTLs for flowering time. An RFLP locus detected by the cold-induced gene *COR6.6* cloned from *Arabidopsis thaliana* mapped very near to one of the *B. rapa* QTLs for flowering time.

Key words *Arabidopsis* · *Brassica* · Flowering · Quantitative trait loci · Vernalization

Introduction

The transition from vegetative to reproductive growth is a major developmental control point in *Brassica* and is strongly influenced by both genetic and environmental factors (Friend 1985). Biennial *Brassica* cultivars require a period of cold treatment, (vernalization) to induce flowering whereas annual *Brassica* cultivars do not require cold treatment to flower, although some annuals can respond to vernalization by flowering earlier (Medham and Scott 1975; Evans and Ludeke 1987). Individual cultivars within each classification also vary in the flowering time, suggesting that genes regulate both a qualitative component (growth habit) and a quantitative component of flowering time.

Molecular-marker linkage maps have been used recently to identify and map genes controlling the vernalization requirement and flowering time in segregating populations from crosses of annual and biennial types of *Brassica*. In a doubled haploid population of *B. napus*, Ferreira et al. (1995) found one region with major effects on vernalization requirement and flowering time and two other regions with minor affects on flowering time. In an F_2 population from cabbage×broccoli, Kennard et al. (1994) found two regions that had large effects on the vernalization requirement and showed significant epistatic interaction. Genetic studies with *B. rapa* have suggested that a few genes regulate bolting resistance (Mero and Honma 1984 a, b); however, linkage maps have not been used to identify flowering-time genes in this species.

Many genes conditioning late flowering have been identified and mapped in the related crucifer *Arabidopsis thaliana*, some of which are responsive to vernalization treatment (Koornneef et al. 1991; Martinez-Zapater et al. 1994). The biochemical pathways affected by these genes have not been elucidated, and efforts are underway to clone late-flowering genes in order to determine their mode of action (Bernier et al. 1993; Lee et al. 1994). These *A. thaliana* genes may be homologous to *Brassica* genes that confer the vernalization requirement. However, *Brassica* species may contain very different homologs, since these species include types with obligate vernalization requirements whereas *A. thaliana* does not (Napp-Zinn 1985).

In the present study, loci affecting the vernalization requirement were mapped in *B. rapa* using a RFLP linkage map that allowed comparison to flowering-time genes in *B. napus* (Ferreira et al. 1994, 1995; Teutonico and Osborn

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R. A. Teutonico[1] · T. C. Osborn (✉)
Department of Agronomy, University of Wisconsin, Madison, WI 53706, USA

Present address:
[1] DNA Plant Technology Corp., 6702 San Pablo Ave., Oakland, CA 94608, USA

1994). In addition, cloned genes from other *Brassica* species and *A. thaliana* which are either cold-regulated or flowering-related were used to detect homologous RFLPs in the *B. rapa* map. This allowed comparison of the map positions of these candidate genes with the vernalization genes identified in *B. rapa* to determine if they are potentially related.

Materials and methods

Plant population and RFLP linkage maps

A *B. rapa* F_2 population was generated by self-pollination of a single F_1 plant from a cross of oilseed cultivars Per (biennial) and oilseed cultivars R500 (annual). F_3 families from self-pollination of 85 F_2 plants were used for this study. RFLP analysis and map construction were conducted as described previously for a set of 91 F_2 genotypes from the same cross which had 62 genotypes in common with the 85 F_2 plants (Teutonico and Osborn 1994). The map used for the present study had 143 of the 145 marker loci used previously, including eight loci detected by cold-induced genes from *B. napus* (BN59, BNC24A, PEP/4) or from *A. thaliana* (COR6.6, COR15). One additional marker locus detected by PMA5, a cloned *A. thaliana* sequence of *ld* (Lee et al. 1994), was included in the map used for the present study. Except for linkage group (LG) 2, which contained the additional locus and was re-ordered, the two maps had similar distances and identical locus orders.

Measurement of flowering time

Nine plants of 85 F_3 families, self-pollinated progeny of the two parent plants, and the F_1 hybrid were grown from seed in a soil mix (3:2:1 soil:Jiffy mix:sand) in 10-cm^2 pots in a greenhouse. Plants were grouped according to family and families were randomized on greenhouse benches. Natural lighting was supplemented by metal halide lamps with a 14-h day length and the temperature was maintained at 22–24°C. Plants were observed daily for 199 days after sowing and the number of days to the first fully opened flower on each plant was recorded. Plants that did not flower after 199 days showed no sign of flower initiation and were assigned a value of 220 for the number of days to flower because flowering took approximately 3 weeks from the first sign of transition to flowering. The trait 'days-to-flowering' (DTF) was calculated for each F_3 family, parental line and F_1 hybrid as the mean number of days to flowering after sowing. A second experiment was conducted in a similar manner using 62 of the F_3 families except that 4 weeks after planting all plants were moved to a cold room (4°C), under fluorescent lights emitting 80–100 E/m^2 with a 24-h photoperiod, for 6 weeks and then returned to the greenhouse for flowering evaluation.

Identification of quantitative trait loci and statistical analysis

Quantitative trait loci (QTLs) involved in the control of flowering time were estimated by interval mapping (Lander and Botstein 1989) using Mapmaker/QTL 1.1 (Lincoln et al. 1992). A LOD (log of the likelihood odds ratio) threshold of 2.0 was used to identify marker intervals containing putative QTLs for flowering time. A multi-locus model was developed (Zeng 1994) by including the effects of putative QTLs that increased the LOD score for the model by at least 2.0. The percentage variation explained by each QTL was calculated as the decrease in variation explained when each QTL was removed from the complete model. All QTLs in the model were fixed and the genome re-scanned for additional marker intervals associated with flowering time using a 2.0 increase in LOD as the threshold (Lincoln et al. 1992). Epistasis was evaluated by selecting the marker locus from each QTL region that was most significantly associated with flowering time and performing a two-factor analysis of variance using the SAS PROC GLM procedure (SAS 1988).

Results

Flowering time

In the experiment with unvernalized plants, the annual parent 'R500' was the earliest to flower (44 DTF), while no plants flowered from the biennial parent 'Per', the F_1 hybrid, and 10 of the 83 families (220 DTF). At least one plant flowered from 73 F_3 families and these families had a range of flowering times between 71 days and 219 days (Fig. 1a). The distribution of F_3 families for DTF did not deviate significantly from normality (skewness=–0.88, kurtosis=–0.05). The 6 weeks of vernalization treatment had a pronounced effect on flowering times. Both the biennial parent 'Per' and the F_1 flowered, while only one F_3 family did not flower. Compared to the unvernalized treatment, most of the F3 families flowered much sooner and the population had a distribution with a lower mean and variance than the unvernalized population.

Identification of QTLs

Two regions containing putative QTLs with large effects on DTF were identified for the unvernalized population:

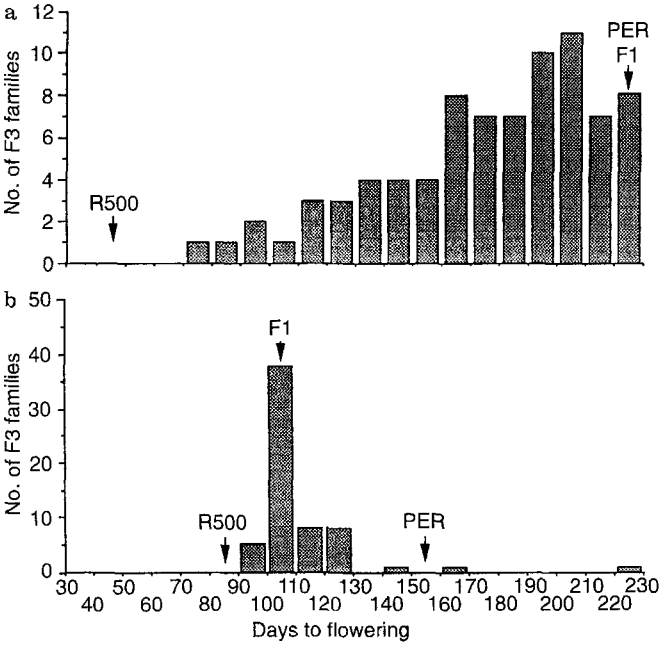

Fig. 1 Distribution of *B. rapa* F_3 families derived from the cross of 'Per'×'R500' for mean number of days from sowing to first open flower (days-to-flowering) with no vernalization (**a**) and 6 weeks of vernalization (**b**). The days-to-flowering for the parents and F_1 are indicated by *arrows*. Families in which no plants flowered were assigned a value of 220 days to flowering. Days-to-flowering for families in **b** include the 42 days for vernalization

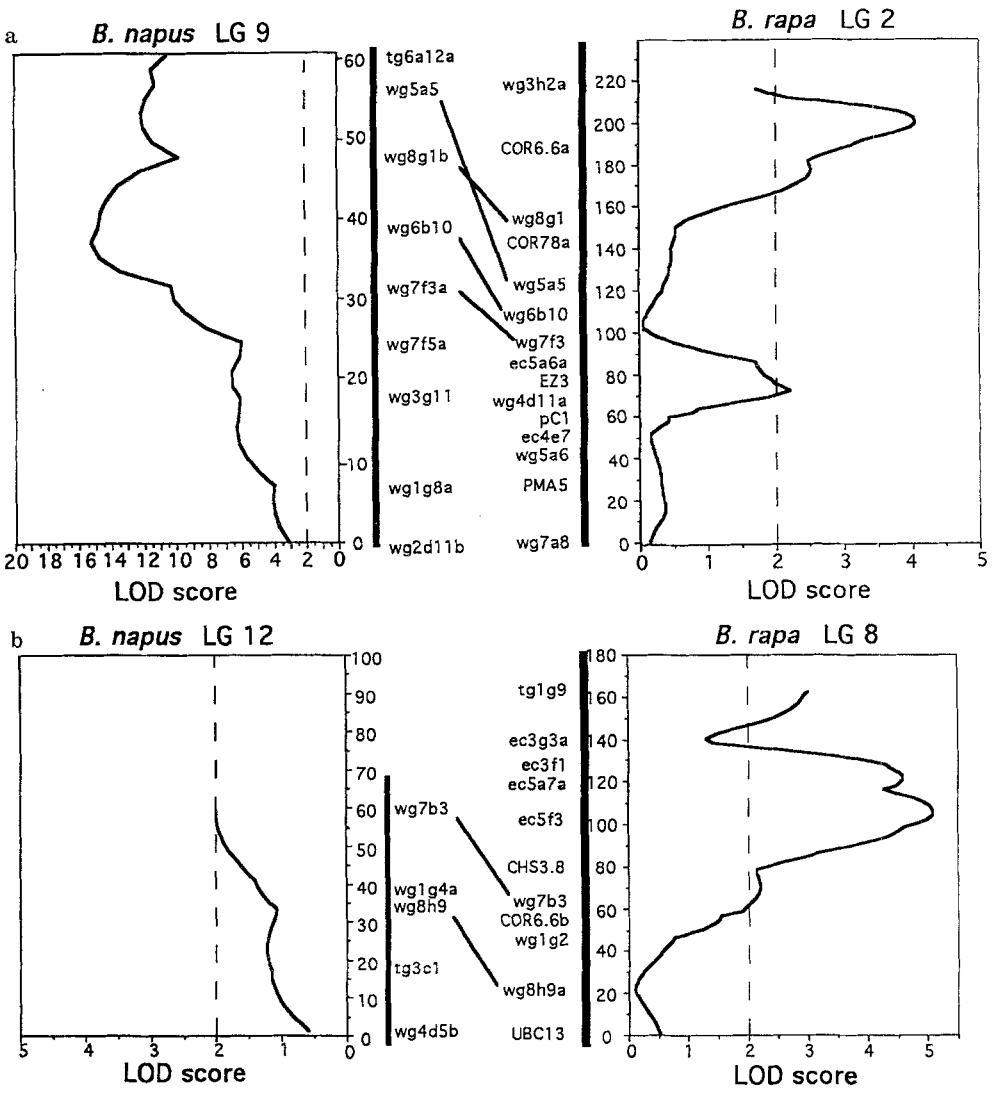

Fig. 2a, b Comparison of linkage groups containing putative QTLs associated with flowering time in segregating populations of *B. rapa* and *B. napus*. LOD plots and map comparisons are shown for *B. napus* LG 9 and *B. rapa* LG 2 (**a**) and for *B. napus* LG 12 and *B. rapa* LG 8 (**b**). RFLP locus designations and map distances in cM are on the vertical axes and RFLP loci detected by the same probe are connected by *lines*. The LOD threshold of 2.0 used to identify QTLs is indicated by a *dashed line*

COR6.6a - *wg3h2a* on LG 2 and *ec5f3* - *ec5a7a* on LG 8 (Fig. 2). When the effects of the QTLs on LG 2 and LG 8 were fixed and the genome re-scanned only one additional region with a small effect (increased LOD 2.3) was detected between marker loci *tg1f8* - *Eru* on LG 1, but inclusion of this additional region in the multilocus model did not add significantly to the LOD score of the model.

The two-QTL model had a LOD value of 12.26 and explained 75.2% of the variation in DTF, with the QTL on LG 2 accounting for 44.6% of the variation and the QTL on LG 8 accounting for 21.7% of the variation. At both QTLs, 'Per' alleles increased flowering time. 'Per' alleles on LG 8 exhibited primarily additive effects, with the heterozygous (PR) genotypic value (149.8 DTF) intermediate to the homozygous RR (126.5 DTF) and PP (189.8 DTF) genotypic values. The QTL on LG 2 exhibited greater dominance, with the PR genotypic value (159.6 DTF) shifted towards the PP genotype value (171.1 DTF) (RR=126.7 DTF). No epistasis was detected between these QTLs. Significant QTL effects were not detected on LG 2 and LG 8 for the second experiment in which plants were given 6 weeks of vernalization, and only one region with a minor effect was detected: *COR160b* - *wg1g4* on LG 3 (LOD 2.5).

The locations of the flowering-time QTLs in the unvernalized *B. rapa* population were compared to those identified in a *B. napus* population (Ferreira et al. 1995) using an RFLP map constructed with some of the same marker loci (Teutonico and Osborn 1994). *B. rapa* LG 2 had four marker loci in common with *B. napus* LG 9, which contained the largest effect on flowering time and vernalization requirement detected in *B. napus* (Fig. 2a). A much smaller effect on flowering time was identified on LG 12 of *B. napus* and this LG had two marker loci in common with LG 8 of *B. rapa* (Fig. 2b).

Discussion

Vernalization requirement has often been studied as a qualitative trait in which plants are classified as annual or bi-

ennial based on their ability to flower without a vernalization treatment. This type of classification is dependent on the conditions and duration of the experiment and the type of population studied. In our *B. rapa* population, not all families could be classified as annual (all plants flowering) or biennial (all plants not flowering), and the population distribution suggested that more than one gene may control flowering time. Therefore, we analyzed vernalization requirement as a quantitative trait and measured the mean number of days from sowing to flowering for each F_3 family.

The unvernalized plants of the annual parent 'R500' flowered while the biennial parent 'Per' and the F_1 hybrid did not flower during the 199 days of the experiment, suggesting that vernalization requirement was a dominant trait in *B. rapa*. QTL analysis identified two regions of the genome with major effects on DTF. A dominance effect was detected on LG2, but not on LG8. However, the magnitude of the dominance effects of these QTLs are largely dependent on the DTF value assigned to plants that did not flower and this value would increase if the duration of the experiment were extended. Also, the F_1 hybrid may have flowered if the duration of the experiment was extended. Therefore, it is difficult to make claims about the dominance of the trait and QTL alleles. Vernalization greatly reduced the mean and variance of the population, and the effects of the two major QTLs were not detected. These results suggest that the effects of late-flowering alleles at these loci were negated by the vernalization treatment, and therefore that they affect flowering time through the requirement for vernalization.

Results from previous studies have suggested there are from one to a few genes responsible for regulating the vernalization requirement in *Brassica* species, depending on the species, the cross, and the environmental conditions used for the analysis. In *B. rapa* spp. *pekinensis*, vernalization requirement was reported to be a dominant trait controlled by one or two major genes with additive effects (Mero and Honma 1984 a, b). In *B. oleracea*, growth habit has been reported to be either a monogenic character (Wellensiek 1960; Walkof 1963) or a polygenic trait (Baggett and Wahlert 1975; Baggett and Kean 1989; Kennard et al. 1994), and vernalization requirement was recessive (Baggett and Kean 1989; Kennard et al. 1994). Vernalization requirement was also recessive in *B. napus* and one major locus was detected that controlled most of the variation (Ferreira et al. 1995).

Some of the loci for vernalization requirement identified in these different studies could encode the same genes which are conserved across different *Brassica* species. One way to obtain evidence for this is to compare the position of trait loci in different populations using RFLP linkage maps constructed with a common set of DNA probes. We conducted this type of comparison between our *B. rapa* population and a *B. napus* population (Ferreira et al. 1994, 1995) for which conserved RFLP linkage arrangements were previously reported (Teutonico and Osborn 1994). The two *B. rapa* LGs with major effects on DTF had marker loci in common with *B. napus* LGs that also contained flowering-time QTLs. For *B. rapa* LG 2 and B napus LG 9, the peak effects were not in the same position relative to the common marker loci; and for *B. rapa* LG 8 and *B. napus* LG 12, comparisons of QTL positions are difficult to make due to the small number of common marker loci. However, these observations suggest the possibility of gene conservation and warrant further investigation by fine structure mapping using additional marker loci in common between the two species.

One of the probes we used for RFLP mapping (PMA5) is an *A. thaliana* DNA clone of the flowering-time gene *ld* which is responsive to vernalization (Lee et al. 1994). This probe detected one segregating RFLP locus on LG 2, but it was over 100 cM from the flowering-time QTL on this LG. However, homology between *ld* and the QTL we detected cannot be ruled out since PMA5 also detected two monomorphic restriction fragments which could represent additional loci but were not mapped in our population.

Seven of the probes we used are cloned, cold-induced genes and these genes could have roles in the vernalization response since they are regulated by cold temperature. One RFLP locus detected by COR6.6 mapped near the flowering-time QTL on LG 2. A QTL associated with variation in the freezing tolerance of plants after a cold treatment was also identified in the same interval (*COR6.6a - wg3h2a*) on LG 2 (Teutonico et al. 1995) as the flowering-time QTL. In barley, researchers have found vernalization requirement to be associated with winter hardiness (Doll et al. 1989; Hayes et al. 1993), but they could not determine whether this was due to pleiotropy or the presence of linked genes. The potential role of COR6.6, or another linked gene, in both the vernalization requirement and freezing tolerance of *B. rapa* could be further investigated by fine structure mapping of this region.

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