Theor Appl Genet (1995) 90: 727–732

M. E. Ferreira · J. Satagopan · B. S. Yandell
P. H. Williams · T. C. Osborn

Mapping loci controlling vernalization requirement and flowering time in *Brassica napus*

Received: 9 March 1994 / Accepted: 22 September 1994

Abstract Rapeseed cultivars (*Brassica napus* L.) can be classified into annual and biennial groups according to their requirement for vernalization in order to induce flowering. The genetic control of these phenotypic differences is not well understood, but this information could be valuable for the design of breeding approaches to accelerate rapeseed improvement. In order to map loci controlling this variation, a doubled haploid population, derived from a cross between annual and biennial cultivars, was evaluated for vernalization requirement and days-to-flowering in a replicated field experiment using three treatments: no vernalization, 4 weeks of vernalization and 8 weeks of vernalization. A linkage map of 132 RFLP loci was used to locate loci controlling these traits. Marker segregation in one region of linkage group 9 was strongly associated with the annual/biennial growth habit in the unvernalized treatment and with days-to-flowering in all three treatments. Two other regions with smaller effects on days-to-flowering were also identified.

Key words *Brassica napus* · Restriction fragment length polymorphism · Vernalization · Days-to-flowering Linkage map

Communicated by G.E. Hart

M. E. Ferreira[1], T. C. Osborn (✉)
Department of Agronomy, University of Wisconsin, Madison, WI 53706, USA

J. Satagopan, B. S. Yandell
Department of Statistics, University of Wisconsin, Madison, WI 53706, USA

P. H. Williams
Department of Plant Pathology, University of Wisconsin, Madison, WI 53706, USA

Present address:
[1] EMBRAPA/CENARGEN, C.P. 0.2372, CEP 70.770, Brasilia DF, Brazil

Introduction

Within *Brassica* species there are genotypes which require a period of low temperature (vernalization) to induce flowering (biennials), and those that flower without vernalization (annuals). Biennial forms of rapeseed (*Brassica napus* L.) are planted in the fall and require vernalization during the winter to flower the following spring (winter types), whereas annual forms are planted in the spring and flower during the summer (spring types). These two forms have been bred mainly by inter-crossing plants within the groups, and the two groups seem to represent distinct gene pools (Diers and Osborn 1994). Heterosis has been observed in hybrids of annual and biennial forage rape cultivars (Johnston 1970), but it has not been exploited in commercial rapeseed cultivars. Understanding the genetic mechanisms controlling flowering may be of value for the full exploitation of both gene pools in breeding programs, and might also have implications for other crop species with genotypes that respond to vernalization.

Restriction fragment length polymorphisms (RFLPs) can be used to construct detailed genetic linkage maps and to locate genes controlling traits of interest (see review by Tanksley et al. 1989). RFLP maps have been developed for *B. oleracea* (Slocum et al. 1990; Kianian and Quiros 1992; Landry et al. 1992), *B. rapa* (Song et al. 1991; Chyi et al. 1992), and *B. napus* (Landry et al. 1991; Ferreira et al. 1994). In *B. oleracea*, genes controlling the annual/biennial growth habit were mapped with respect to RFLP markers in a cabbage × broccoli F_2 population (Kennard et al. 1994). Markers in two regions of the genome were significantly associated with the trait and explained about one-half of the phenotypic variation in a multi-locus model.

In *B. napus*, we have constructed an RFLP linkage map using an F_1-derived doubled haploid (DH) population from a cross of biennial and annual cultivars (Ferreira et al. 1994). The lines in this population can be used in replicated trials to accurately measure the genetic component of variation for traits of interest and to map genes controlling these traits with respect to RFLP markers. The objec-

tives of the present study were to identify genomic regions associated with vernalization requirement and flowering time in the segregating DH population, and to estimate the magnitude of effects, gene action, and the parental contributions of alleles, in these regions.

Materials and methods

Plant material

A single plant of the *B. napus* cv Major (a biennial rapeseed cultivar) was crossed as a female to a doubled haploid line derived from cv Stellar (an annual canola cultivar) and a segregating population was obtained by microspore culture of a single F_1 hybrid plant (Ferreira et al. 1994). One-hundred-and-four F1-derived doubled haploid lines (DH), progeny from self-pollination of the parent plants (Major and Stellar), the F_1 hybrid, and a backcross population of the F_1 to the parent Major (BCM) were evaluated in the field for flower initiation.

Evaluation of flowering

Seeds from all accessions were sown in Com-pack D806 trays filled with compost soil:sand:Jiffy-Mix (1:1:1). The seedlings were kept in a greenhouse environment under minimum light irradiance of 220 $\mu E/m^2/s$ at 22°C and a 16-h photoperiod, watered daily and fertilized with Osmocote pellets and Hoagland's solution. Plants from all accessions were given three treatments: no vernalization, 4 weeks of vernalization and 8 weeks of vernalization. The plants treated with 4 and 8 weeks of vernalization were transferred to a cold room (4–6°C with cool white fluorescent irradiance of 180 $\mu E/m^2/s$ and a 16-h photoperiod) when they were 4-weeks old. The unvernalized plants were maintained in the greenhouse conditions described previously, and were transplanted to the field 4 weeks after seeding. Sowings for each treatment were staggered so that plants in all treatments were transplanted to the field on May 24, 1992. Plants were kept in a semi-shaded area in the field for 5 days before transplanting.

The experimental design was a split-plot with two replications, where treatments were main plots and DH lines were subplots. Each plot consisted of a single row composed of ten plants of a DH or parental line, the F_1 hybrid, or the BCM population. The distance between plants was 30 cm within rows and 120 cm between rows. Irrigation, fertilizer and pesticide were applied to maintain vigorous plants. The discrete classification of each accession for vernalization requirement was recorded by scoring lines in the unvernalized treatment as having 50% of the plants flowered or not flowered after 83 days in the field. Plots were observed daily and the date of anthesis of each plant was recorded throughout the season. The trait 'days-to-flowering' was calculated as the day when 50% of the plants in the plot flowered, using the date of transplanting to the field as day zero.

RFLP data analysis

A genetic map of RFLP loci was constructed for the DH population. This map includes 132 RFLP marker loci on 22 linkage groups (LGs) plus six pairs of linked loci, covering 1016 cM of the *B. napus* genome with an average locus distance of 7.7 cM. In addition, six unlinked RFLP marker loci were used in the analysis of vernalization requirement. Segregating RFLP loci were scored as homozygous M/M for RFLP alleles coming from the parent Major or S/S if coming from Stellar. Procedures for DNA clone selection, probe hybridization, RFLP segregation analysis, and map construction are described elsewhere (Ferreira et al. 1994).

Genetic and statistical analysis

Discrete classification of the DH lines as having flowered or not flowered in the unvernalized treatment was used to test the associations of segregation at 144 RFLP marker loci with vernalization requirement using an analysis of chi-square values by orthogonal functions (Mather 1951). A marker locus was considered associated with vernalization requirement if the chi-square value was greater than 9.0 ($P< 0.001$).

For days-to-flowering, analysis of variance using generalized linear models (SAS 1988) was performed for each vernalization treatment to test for significant differences between DH lines and for replication effects. Loci involved in the control of flowering time were located in a *B. napus* genetic map by interval-mapping analysis (Lander and Botstein 1989) using the MAPMAKER/QTL computer program (Lincoln and Lander 1990). The means of the two replications for days-to-flowering were used and DH lines that did not flower were assigned a value of 100 days-to-flowering for these analyses. A LOD ($\log_{10}$ of the likelihood odds ratio that a QTL is present vs absent)-score threshold of 3.0 was used to initially identify regions containing putative loci associated with days-to-flowering. Only the additive component effect was estimated since there were no heterozygous loci in the DH population. Searches for additional marker loci associated with flowering time were performed by fixing the locus with the highest LOD score and re-scanning the genome (Lincoln and Lander 1990). Additional loci were considered associated with flowering time if the LOD score exceeded the LOD score of the fixed locus by two units. Tests for digenic epistatic interactions were conducted by selecting pairs of marker loci significantly associated with days-to-flowering and performing a two-factor analysis of variance using the SAS GLM procedure (Edwards et al. 1987).

Results

Vernalization requirement

The annual parent Stellar and the F_1 hybrid flowered during the course of the experiment in the unvernalized treatment, whereas the biennial parent Major did not flower. This suggests that the requirement for vernalization to flower is a recessive trait. However, none of the BCM plants flowered without vernalization. The discrete classification of the DH lines as having flowered (74 lines) or not flowered (30 lines) indicated that the segregation ratio for vernalization requirement was significantly different from the 1:1 ratio expected if a single locus controlled the trait (χ^2=18.6, $P< 0.001$) . Tests for association of the 144 RFLP marker loci with vernalization requirement revealed seven linked loci significantly associated with the trait that mapped to LG 9 of *B. napus* (Table 1). Locus *wg6b10* was the most strongly associated with a vernalization requirement (χ^2=38.4, $P< 0.001$).

Days-to-flowering

The analysis of variance indicated significant differences between the DH lines ($P< 0.0001$) but no significant replication effects ($P>0.05$) for each of the vernalization treatments. Most of the DH lines were homogeneous for days-to-flowering. For some lines, all plants flowered with 3 days after the first plant in the plot flowered. In the 8-week ver-

Table 1 Associations of RFLP marker loci located in *B. napus* linkage group 9 with vernalization requirement

RFLP marker loci	n[a]	χ^{2b}
wg3g11	94	18.77***
wg7f5a	96	16.67***
wg7f3a	94	28.77***
wg6b10	100	38.44***
wg8g1b	87	25.39***
wg5a5	97	28.96***
tg6a12a	88	22.00***

[a] Number of DH lines used in the comparison
[b] Chi-square values calculated by orthogonal functions (Mather 1951), ***$P< 0.001$

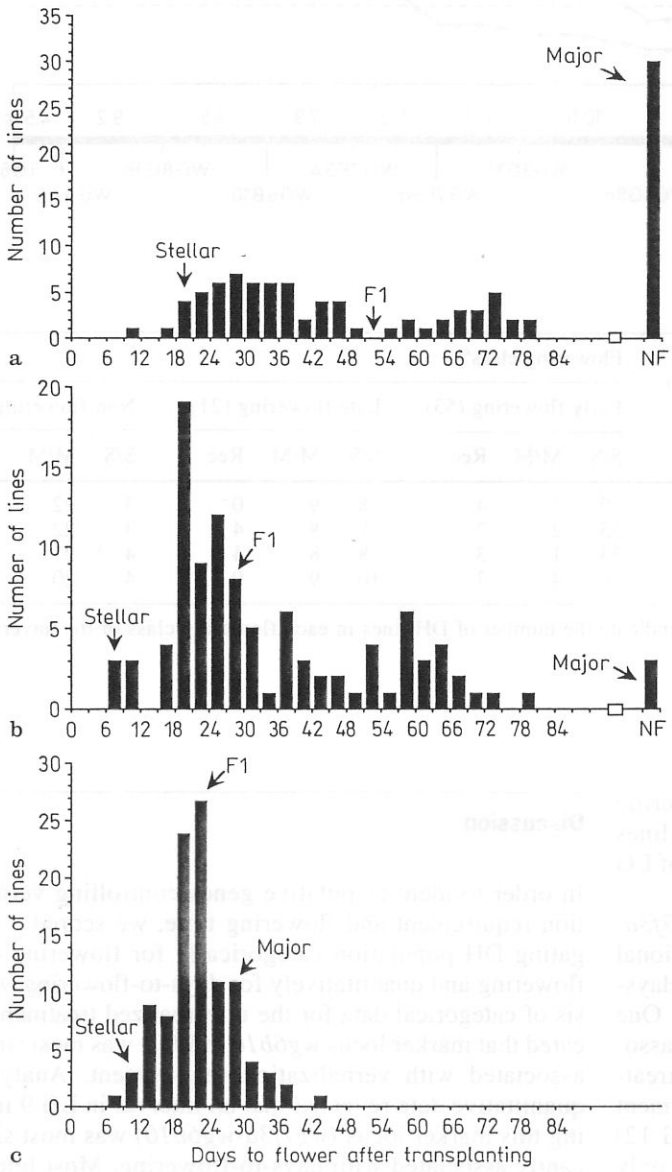

Fig. 1 Frequency distribution for days-to-flowering of 104 DH lines obtained from a cross between annual (Stellar) and biennial (Major) cultivars of *B. napus* that were transplanted to the field after no vernalization (**a**), 4 weeks of vernalization (**b**) and 8 weeks of vernalization (**c**). Positions of the parents and F_1 in the distributions are shown. *NF* indicates lines that did not flower

nalization treatment, the average number of days between flowering of the first plant and the fifth plant was 5.7±3.9. Since each of the ten plants per plot were genetically identical, the within-plot variation was due to environmental causes.

Flowering time of the DH population was normally distributed for the 8-week vernalization treatment, but deviated significantly from normality for the 4-week and unvernalized treatments (Fig. 1). The unvernalized DH population could be roughly subdivided into three groups based on the distribution of days-to-flowering (Fig. 1 a): early flowering lines (53 lines that flowered within 48 days after transplanting), late flowering lines (21 lines that flowered 54–80 days after transplanting), and non-flowering lines (30 lines that had not flowered after 83 days from transplanting and showed no signs of bud formation). The parent Stellar flowered soon after being transplanted, whereas the selfed progeny of Major flowered only after being vernalized for 8 weeks (Fig. 1 c). The F_1 hybrid was intermediate to the early and late flowering groups in the unvernalized treatment (Fig. 1 a). The backcross population flowered only after 4 weeks of vernalization. Most of the lines that did not flower in the unvernalized treatment were induced to flower after 4 weeks of vernalization, and all lines flowered after 8 weeks of vernalization (Fig. 1). An increase in the period of vernalization resulted in a decrease in the number of days-to-flower. For example, lines that flowered late without vernalization (69.0±6.9 days), flowered after 36.1±10.2 and 23.4±4.5 days with 4 weeks and 8 weeks of vernalization, respectively. A similar response was observed for the early flowering and non-flowering lines.

Interval-mapping analysis of days-to-flowering was performed separately for each treatment. We analyzed the original data sets and data sets with various transformations and different values assigned to the non-flowering lines. All these results were similar and therefore, we report only results from an analysis of the original data. In the unvernalized treatment, a strong association (LOD=15.2) was found in the *wg7f3a-wg6b10* marker interval of LG 9 (Fig. 2). The same interval was strongly associated with days-to-flowering (LOD=18.2) in the 4-week vernalization treatment, and also in the 8-week vernalization treatment (LOD=7.4) (Fig. 2). For the 8-week vernalization treatment, this interval explained approximately 28% (R^2) of the variation in days-to-flowering. The estimated additive effect of alleles in this interval was approximately –6.8, indicating that the substitution of M/M alleles by S/S alleles decreased flowering time about 1 week for this treatment. Genetic effects in the other treatments were not estimated due to large deviations from normal distributions.

In all treatments, the allelic contribution to reduction of days-to-flowering in the *wg7f3a-wg6b10* interval came from the annual parent Stellar. Most of the early flowering DH lines were homozygous for alleles from Stellar in the *wg7f3a-wg6b10* interval, and most of the non-flowering lines were homozygous for alleles from Major in this interval (Table 2). This was also observed for intervals near the

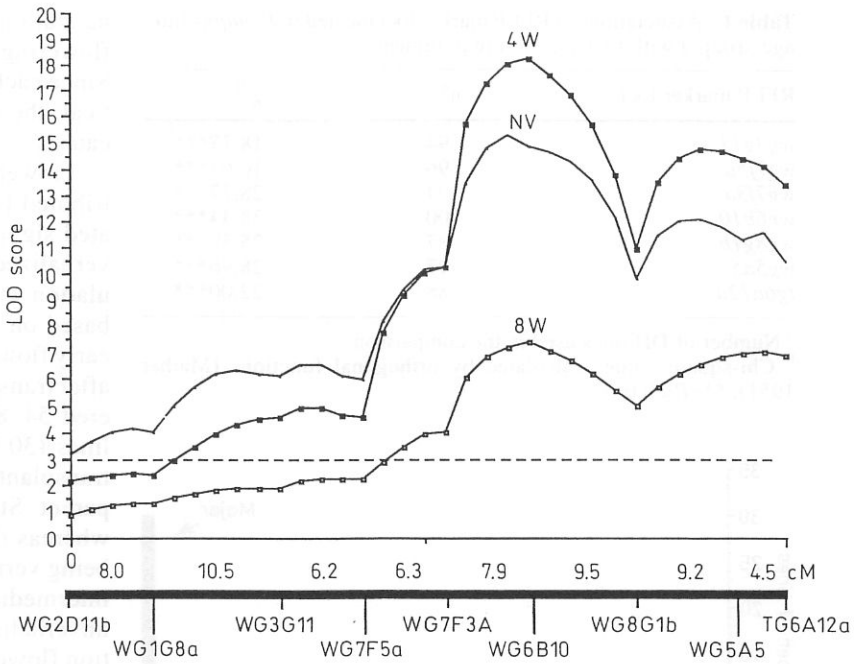

Fig. 2 LOD profiles for loci controlling days-to-flowering in LG 9 of *B. napus*. The Y axis gives LOD scores ($\log_{10}$ of the likelihood odds ratio that a QTL is present vs absent), and the X axis shows the position of marker loci and their interval distances in centiMorgans (cM)

Table 2 Frequency of Stellar (S/S), Major (M/M) and recombinant (Rec) genotypes at RFLP marker locus intervals in a segment of *B. napus* linkage group 9

RFLP marker locus interval	Size (cM)	Flowering class[a]								
		Early flowering (53)			Late flowering (21)			Non-flowering (30)		
		S/S	M/M	Rec	S/S	M/M	Rec	S/S	M/M	Rec
wg7f3a-wg6b10	7.9	39	3	4	8	9	0	3	22	3
wg6b10-wg8g1b	9.5	35	2	2	8	8	4	3	22	1
wg8g1b-wg5a5	9.2	33	1	3	8	8	4	4	21	1
wg5a5-tg6a12a	4.5	36	4	3	10	9	0	4	20	1

[a] Numbers in parenthesis indicate the number of DH lines in each flowering class of the unvernalized treatment

wg7f3a-wg6b10 interval. However, 1–4 early flowering lines with M/M genotypes and 3–4 non-flowering lines with S/S genotypes were observed for each interval of LG 9 (Table 2).

After including trait-locus effects for the *wg7f3a-wg8g1b* interval in the statistical model, two additional intervals were found associated with the control of days-to-flowering in the three vernalization treatments. One interval (*wg6b2-wg9c7* in LG 16) was significantly associated with days-to-flowering in the unvernalized treatment (LOD=2.7) and in the 4-week vernalization treatment (LOD=3.1). Another interval (*wg1g4a-wg7b3* in LG 12) was significantly associated with days-to-flowering only in the unvernalized treatment (LOD=2.0). The M/M genotypes at these marker intervals were associated with increased days-to-flower. There was no evidence for digenic epistasis between any of the marker loci in intervals significantly associated with days-to-flowering.

Discussion

In order to identify putative genes controlling vernalization requirement and flowering time, we scored a segregating DH population categorically for flowering or not flowering and quantitatively for days-to-flowering. Analysis of categorical data for the unvernalized treatment indicated that marker locus *wg6b10* in LG 9 was most strongly associated with vernalization requirement. Analysis of quantitative data revealed that an interval in LG 9 including this marker locus (*wg7f3a-wg6b10*) was most significantly associated with days-to-flowering. Most lines that either flowered early or did not flower differed for genotypes in this marker interval; early lines had alleles predominantly from the annual parent (Stellar) and non-flowering types from the biennial (Major). These results suggest that a major locus in LG 9, or closely linked loci,

affected flowering time through the requirement for vernalization.

Vernalization for 4 weeks resulted in flowering of most of the DH lines, and all lines flowered after 8 weeks of vernalization. Also, as the period of vernalization increased, a decrease in time-to-flowering was observed even in the annual lines. A decreased LOD score was observed in the *wg7f3a-wg6b10* interval for the 8-week treatment, suggesting that full vernalization reduced the genetic effect in this region; however, this region was still strongly associated with days-to-flower. Some differences in days-to-flowering among fully vernalized DH lines were probably caused by differences in the annual-biennial habit among the lines, because annual types developed towards flowering both before and during the vernalization period, whereas the transition to flowering of biennials began only after some vernalization.

Two additional intervals associated with days-to-flowering were located in LGs 12 and 16, but their effects on flowering time were small compared to the *wg7f3a-wg6b10* interval. Genotypes in these regions with minor effects appeared to at least partially explain the early or late flowering of 12 DH lines with M/M genotypes in the *wg7f3a-wg6b10* interval. Eleven of these lines had S/S or recombinant genotypes for at least one of the intervals with minor effects and seven had S/S or recombinant genotypes in both intervals. Only one of 22 non-flowering lines with M/M genotypes in the *wg7f3a-wg6b10* interval had S/S or recombinant genotypes in both intervals with minor effects.

In wheat, major loci controlling annual versus biennial habit have been identified and localized to homoeologous chromosomes (Pugsley 1971; Roberts and MacDonald 1984). *B. napus* LG 9 shares some homology with LG 1 (Ferreira et al. 1994), but there was no evidence for marker intervals in LG 1 associated with either vernalization requirement or days-to-flowering. Although the loci we detected explained much of the observed variation, there may be other loci controlling variation for vernalization requirement and days-to-flowering that were not detected in this population, perhaps due to incomplete coverage of the entire genome by molecular markers.

In this *B. napus* population, annual habit seemed to be dominant over biennial habit since all F_1 hybrid plants flowered without vernalization. None of the 20 BCM plants flowered; however only 20 individuals were assayed and flowering might have been observed in a larger sample size. Studies with *B. oleracea* indicated dominance of annual over biennial habit (Dickson 1968; Pelofske and Baggett 1979; Baggett and Kean 1989; Kennard et al. 1994). Thurling and Vijendra Das (1979 b) hypothesized the presence of two recessive genes in the *B. napus* cv Bronowski and two different recessive genes in the cv Isuzu controlling the vernalization requirement. Some of these genes may be the same as the ones we have detected in this study; however, the vernalization-requiring parent (Major) has a very different genetic background to that employed by Bronowski and Isuzu (Diers and Osborn 1993).

There was no evidence of additive × additive epistatic effects for days-to-flowering in the population used in the present study. Digenic epistasis has been considered important for the flowering time of some *Brassica* species. In *B. juncea*, significant digenic interactions of linked pairs of genes were reported in two of three crosses between early and late flowering types (Sachan and Singh 1985). Likewise, in *B. oleracea*, two marker loci associated with flowering time showed significant additive × additive and additive × dominant epistasis in a cross of cabbage and broccoli (Kennard et al. 1994). However, there was no evidence for non-allelic interaction in a diallel analysis involving early and late annual rapeseed cultivars (Thurling and Vijendra Das 1979 a).

Flowering is an important adaptive characteristic for oilseed production, and the identification and understanding of genes controlling flowering would be valuable for breeding programs. For example, crosses between annual and biennial forms would broaden the genetic base of each group, allowing the exploiation of heterotic effects between the two groups. In the progenies of these crosses, lines with annual or biennial growth habit could be developed by selecting for alleles at loci controlling vernalization requirement, followed by the selection of other desirable traits for these groups, such as winter survival in biennial forms and earliness in annual forms. The resulting progenies would have new gene combinations from the two groups that could be useful for improving rapeseed cultivars.

Acknowledgements The authors thank Deb Prutel and Robert Vogelzang for technical assistance. This research was funded by support from 15 companies to T.C. Osborn for research on molecular markers in oilseed *Brassica*, and from the College of Agricultural and Life Sciences University of Wisconsin-Madison.

References

Baggett JR, Kean D (1989) Inheritance of annual flowering in *Brassica oleracea*. HortScience 24:662–664

Chyi Y-S, Hownecke ME, Sernyk JL (1992) A genetic linkage map of restriction fragment length polymorphism loci for *Brassica rapa* (syn. *campestris*). Genome 35:746–757

Dickson MH (1968) Eight newly described genes in broccoli. Proc Am Soc Hort Sci 93:356–359

Diers BW and Osborn TC (1994) Genetic diversity of oilseed *Brassica napus* germplasm based on restriction fragment length polymorphisms. Theor Appl Genet 88:662–668

Edwards MD, Stuber CW, Wendel JF (1987) Molecular-marker-facilitated investigations of quantitative trait loci in maize. I. Numbers, genomic distribution, and types of gene action. Genetics 116:113–125

Ferreira ME, PH Williams, TC Osborn (1994) RFLP mapping of *Brassica napus* using F_1-derived doubled haploid lines. Theor Appl Genet 89:615–621

Johnston TD (1970) A comparison of inbred lines and their F_1 hybrids in forage rape (*Brassica napus L.*). Euphytica 20:81–85

Kennard WC, Slocum MK, Figdore SS, Osborn TC (1994) Genetic analysis of morphological variation in *Brassica oleracea* using molecular markers. Theor Appl Genet 87:721–732

Kianian SF, Quiros CF (1992) Generation of a *Brassica oleracea* composite RFLP map: linkage arrangements among various populations and evolutionary implications. Theor Appl Genet 84:544–554

Lander ES, Botstein D (1989) Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185–199

Landry B, Hubert N, Etoh T, Harada JJ, Lincoln SE (1991) A genetic map of *Brassica napus* based on restriction fragment length polymorphism detected with expressed DNA sequences. Genome 34:543–552

Landry B, Hubert N, Crete R, Chang MS, Lincoln S, Etoh T (1992) A genetic map of *Brassica oleracea* based on RFLP markers detected with expressed DNA sequences and mapping of resistance genes to race 2 of *Plasmodiophora brassicae* (Woronin). Genome 35:409–420

Lincoln SE, Lander ES (1990) Mapping genes for quantitative traits using MAPMAKER/QTL. A Whitehead Institute for Biomedical Research Technical Report, Cambridge, Massachusetts

Mather K (1951) The measurement of linkage in heredity. John Wiley and Sons, New York

Pelofske PJ, Baggett JR (1979) Inheritance of internode length, plant form, and annual habit in a cross of cabbage and broccoli (*Brassica oleracea* var. *capitata* L. and var. *italica* Plenck). Euphytica 28:189–197

Pugsley AT (1971) A genetic analysis of the spring-winter habit of growth in wheat. Aust J Agric Res 22:21–31

Roberts DWA, MacDonald MD (1984) Evidence for the multiplicity of alleles at the *Vrn1*, winter-spring habit locus in common wheat. Can J Genet Cytol 26:191–193

Sachan JN, Singh B (1987) Generation-mean analyses for flowering and maturity in Indian mustard [*Brassica juncea* (L.) Czern & Coss]. Theor Appl Genet 73:571–574

SAS Institute Inc (1988) SAS procedures guide, release 6.03 Edn. Cary North Carolina

Slocum MK, Figdore SS, Kennard WC, Suzuki JY, Osborn TC (1990) Linkage arrangement of restriction fragment length polymorphism loci in *Brassica oleracea*. Theor Appl Genet 80:57–64

Song KM, Suzuki JY, Slocum MK, Williams PH, Osborn TC (1991) A linkage map of *Brassica rapa* (syn. *campestris*) based on restriction fragment length polymorphism loci. Theor Appl Genet 82:296–304

Tanksley SD, Young ND, Paterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding: new tools for an old science. BioTechnology 7:257–264

Thurling N, Vijendra Das LD (1979 a) Genetic control of the preanthesis development of spring rape (*Brassica napus* L.). I. Diallel analysis of variation in the field. Aust J Agric Res 30:251–259

Thurling N, Vijendra Das LD (1979 b) Genetic control of the preanthesis development of spring rape (*Brassica napus* L.). II. Identification of individual genes controlling developmental pattern. Aust J Agric Res 30:261–271