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Study of Pod Length Trait in Doubled Haploid *Brassica napus* Population by Molecular Markers

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ABSTRACT

Pod length is one of the effective components on canola yield selection on the basis of which can increase not only the seed yield but also the oil yield. Molecular markers associated with long pod loci were identified in a doubled haploid population derived from a cross between the canola lines 'Quantum' (long pod) ×'China A' (short pod) using RAPD and bulked segregant analysis. A molecular marker linkage map of 37 loci for this population was used to identify quantitative trait loci (QTL) controlling pod length of which two markers in two unlinked loci were selected by using an interval mapping model which explained 22% of phenotypic variation for pod length in this population. Selection for markers at two loci for increasing pod length resulted in a group of doubled haploid lines with an average 112 mm pod length that increased 15% of whole population mean. This shows that using these markers in the breeding program will enhance the breeding of long pod canola varieties.

Keywords: *Brassica napus,* Bulked segregant analysis, Doubled haploid, Molecular marker, RAPD, Quantitative trait loci (QTL) analysis.

INTRODUCTION

The elements of brassica seed yield are directly constructed by seed number, seed size and unit of seed weight [1, 14]. However, these entire elements are associated with pod length, as the longer pod usually contains more and bigger sized seed than the shorter one [13]. Undoubtedly, a breeding program to develop a long pod cultivar can effectively improve the seed yield in the canola industry, as the current cultivars are mostly the short pods [4]. Information on the genomic and genetic background of pod length therefore becomes important for modern breeding techniques [21]. Recently, research efforts have focused on studies of several yield components including pod and seed size which may significantly affect the oil and protein content as well as the seed yield [4].

Several studies on the physiological activities of rapeseed pods have demonstrated that the pod not only functions as a phytosynthetic organ but also exports phytosynthate to the developing seeds contained therein, with approximately 30% of dry matter of pods and seeds being attributable to pods [2, 3].

A possible alternative to selecting for seed yield improvement *per se* is to increase the pod length and the seed number retained. Indeed, selection for long pods increased seed number by at least five seeds per pod [9]. Long podded genotypes, if coupled with large seeds, may also have a high oil and protein content since large seeds generally have a high seed weight and a reduced proportion of hull, resulting in elevated oil and protein contents [20]. Field studies on the inheritance of pod length revealed that pod

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length appears to be a multigenic trait controlled by genes with additive effects and the genes responsible for the long pod trait are largely located in the nucleus; the approximate number of genes for this trait was 7 [20].

The introduction of marker-assisted selection (MAS) techniques into canola breeding programs might speed up the breeding of long pod cultivars. With this technique, selections in segregating generation of crosses are based on the presence or absence of molecular markers that are closely linked to the long pod trait and which are not influenced by the environment. Also, molecular selection can be automated and made inexpensive when polymerase chain reaction (PCR)-based techniques are employed, such as random amplification of polymorphic DNA (RAPD) analysis or sequence characterized amplified regions (SCARs) [19]. Most of the important agronomic characters like yield and yield components (grain number, grain weight), plant height and days to flowerings are controlled by several genes [16]. The earliest attempt to establish linkage of an individual QTL to morphological mutations was done by Sax [18] and it was fulfilled 60 years later when saturated linkage maps of DNA markers became available. A number of methods for mapping QTLs, and estimating their effects have been suggested and investigated [5, 7, 8, 9, 10 and 12] but the simplest QTL detection method relies on one-way analysis of variance [17].

In the study presented here, two unlinked loci were identified with RAPD. The effectiveness of the markers from the two loci in selecting lines with long podded characteristic resulting in a long pod genotype from an experimental DH population is discussed.

MATERIALS AND METHODS

Plant Material

A cross between a canola-type, long pod source of *Brassica napus* cv 'quantum' and a canola-type, short pod line china A was used to derive 50 doubled haploid lines from F1 microspores. The doubled haploid lines were grown at the University of Alberta experimental farm in 1999 and 2000. The field evaluation in each of two years used reserve seed from the original selfed? doubled haploid plants.

The DNA analysis of this population used reserve DH seed from the original selfed greenhouse-grown doubled haploid plants. Five doubled haploid plants from each of 50 lines and 5 inbred plants of each parent grown in a controlled environment chamber for 3-4 weeks. Equal amounts of leaf tissue from four plants of each line were bulked, lyophilized, ground to a fine powder and then stored at -20°C.

DNA Extraction and PCR

Brassica napus DNA was extracted from 30-50 mg of dry tissue in a 2 ml microtube based on a modified CTAB method [6] by adding 600 µl of hot (65°C) 2X CTAB buffer (100 mM Tris-Hcl PH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% CTAB, 20 mM β-mercaptoethanol). After extraction with one vol. of chloroform/isoamyl alcohol (24:1) the aqueous phase was transferred into a clean tube and 0.1 vol. of 10% CTAB solution (0.7 M NaCl, 10% CTAB) was added to the aqueous phase and the extraction was repeated. The aqueous phase was removed and mixed with an equal vol. of hot (65°C) CTAB precipitation buffer (50 mM Tris-Hcl PH 8.0, 10 mM EDTA, 1% CTAB). The resulting DNA/CTAB complex was immediately platted by centrifugation for 10 minutes at 12000 rpm at 20°C. The resulting pellet was resuspended in 650 µl high salt buffer (10 mM Tris-Hcl PH 8.0, 1 mM EDTA, 1 M NaCl) and the DNA was precipitated by the addition of two vols. of cold (-20°C) 100% ethanol. The DNA was platted with centrifugation at 12000 rpm for 10 minutes and washed three times with 1 ml of cold (-20°C) 70% ethanol. The DNA was resuspended in 100 µl of ddH2O (PH 8.0), then quantified by Gene Quant. Typical

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yields were 20-30 µg of DNA sample, and all of the DNA samples were diluted to 5 ηg/µl. The bulked segregant analysis [15] (BSA) included DNA of the Parent ('quantum' and 'china A') and the DNA from long and short pod lines. The bulk segregants were prepared by combining equal amounts of DNA from each of seven long and seven short pod lines. A total of 412 random, 10-base pair (bp) oligonucleotide primers from the University of British Colombia (Vancouver, Canada) and the Applied Biosystem Company were screened using bulked segregant analysis.

Each PCR reaction contained 10 ηg DNA as a template. Amplification reactions contained 2 U of Taq DNA polymerase (Gibco BRL), 2.5 mM $MgCl_2$, 250 µM each dNTP and 0.2 µM primer. The DNA amplification was done in MJ PTC-100 thermocycler in 21 µl volume. The used protocol was 5 minutes at 95°C, followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 36°C, 1 minute at 72°C and the final cycle followed by 10 minutes at 72°C.

All PCR products were resolved in 2% (w/v) agarose gel in 1X TAE by electrophoresis at 120 V for 105 minutes. Gels were stained in ethidium bromide and photographed on a digital gel documentation system (Stratagene).

Data Analysis

The RAPD banding pattern and particularly the marker band were shown to be reproducible by repeating the PCR reactions at least four times. Genetic segregation data and QTL analysis for RAPD markers associated with pod length trait by BSA among the 50 doubled haploid lines were analyzed using Map Manager Version 0.25. The grouping and genetic linkage map was generated using a minimum LOD of 3.0 and a maximum recombination fraction of 0.4. Map distances were converted to centimorgan using the Kosambi function [11]. The ANOVA was performed using SAS V6.12 (SAS Institute, Cary, N.C.) for each selected marker and for a combination of unlinked markers.

RESULTS

A total of 412 primers were screened by BSA in search of polymorphisms. In total, 305 (74%) amplified an intense and reproducible PCR banding pattern. There were 37 primers (9%) that showed qualitative polymorphism between parents, and 6 primers (2%) that showed polymorphism between the long and short pod bulked DNA samples. These putative pod length RAPD markers were next screened against the 14 individuals used in the bulked DNA samples, and the best markers were finally tested against the entire 50 individuals in the DH population.

The RAPD analysis using the BSA strategy identified a total of two amplified fragments that were all associated with loci controlling pod length. Figure 1 shows the PCR profiles of these markers amplified from the 14 individuals of the bulked DNA samples. Primer PL2 (band 470 bp) and PL18 (band 640 bp) amplified DNA fragments that were associated with long and short pods, respectively. In both RAPD marker profiles, recombinant lines were identified within the bulk individuals (Figure 1). The markers that showed one or two recombinants were studied at this stage of the BSA.

Table 1 lists the six RAPD markers which showed an association with long and short pod bulked DNA samples. There were 3 markers dominant for long pods and 3 markers dominant for short pods. All of the markers segregated in a 1:1 ratio among the 50 DH lines as demonstrated by Chi-square analysis. Regression analysis was used to describe the amount of phenotypic variation explained by each marker (Table 1). Marker PL2 (r^2= 14.6%) and PL18 (r^2= 9.6) explained the highest amount of variation in pod length content. For these two markers, the mean of pod length between the genotypic class for each marker was significantly different (Table 1).

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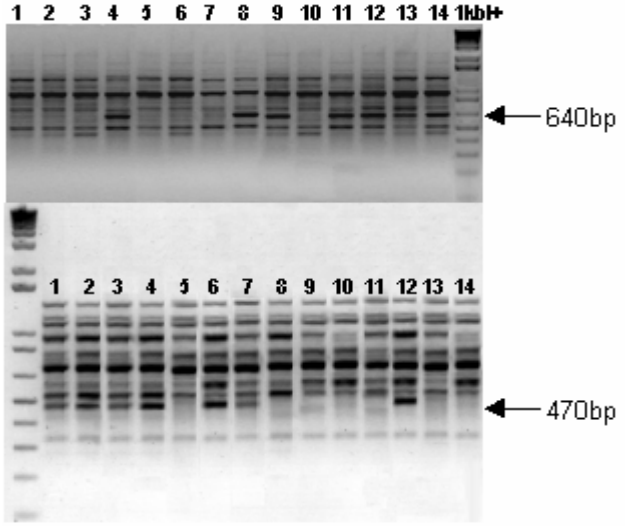

Figure 1. PCR profiles produced by RAPD analysis in *B. napus* with PL 18 (A) and PL 2 (B) primers.

The set of 37 markers was assigned to seven arbitrary linkage groups and one unlinked group by analysis of segregation data with Map Manager Version 0.25 (Figure 2). A QTL analysis using an interval mapping model revealed a pick on the PL2 marker region, which explained 15% of the phenotypic variation in pod length (Figure 3). The QTL analysis also showed another pick on the PL18 marker region, which explained 10% of phenotypic variation in pod length. The interaction between markers at two unlinked loci (PL2 and PL18) explained 22% of the phenotypic variation based on ANOVA. Addition of the r^2 value for the second marker was 7.8% as well.

The pod length of 'quantum' was 137 mm and 'China A' was 82. The mean pod length of the DH population was 97.5 mm while that of the long pod bulk was 133.1 and the short pod was 80.04 mm. The mean pod lengths of the long and short classes are indicated for the group of two interacting loci (Table 1). Among 50 DH lines, 9 lines were shown to carry the two 'quantum' loci for markers PL2 and PL18 which agreed with the expected number of lines for this genotypic class (12.5) in a DH population of this size (χ^2= 4.46, p= 0.25). Selection of 'quantum' loci for both markers resulted in a mean pod length of 112.23 mm (n=9) and a maximum pod length of 137.9 mm, which

Table 1. Analysis of variance for pod length marker (s).

Marker (primer)	MS	P	R^2	Mean ± SE	
				Long	Short
PL2	2202.4	0.006	14.60	105.5 ± 22.01	92.10 ±10.03
PL9	712.42	0.130	4.70	101.6 ± 20.33	94.10 ±13.27
PL 14	863.62	0.094	6.00	102.2 ± 20.22	93.90 ±13.56
PL 17	522.39	0.196	3.40	101.7 ± 18.98	95.14 ± 16.12
PL 18	1468.3	0.028	9.70	105.2 ± 19.46	93.94 ±12.24
PL 26	1027.4	0.067	7.80	100.6 ± 18.98	89.94 ± 8.19
PL 2× PL 18	1129.6	0.008	22.40	112.2 ± 21.86	89.23 ± 5.23

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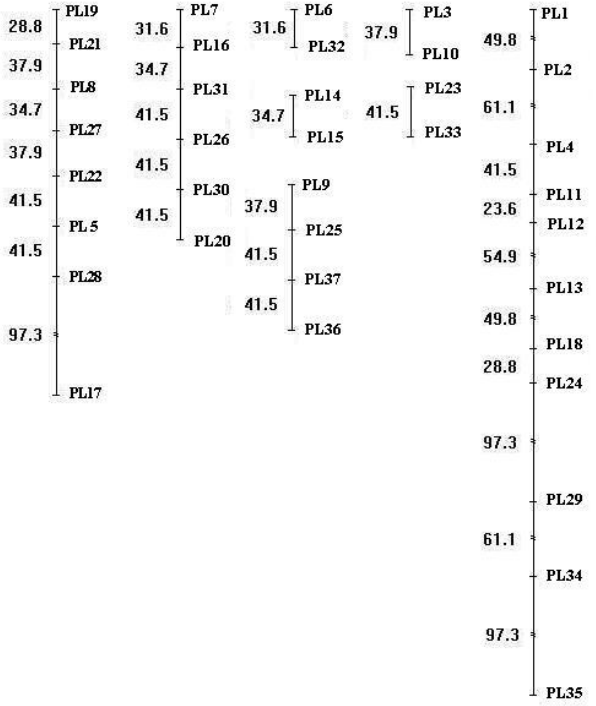

Figure 2. A linkage map of RAPD markers in a population drived from 'Quantum' X 'China A' cross.

was 6.5% higher than the pod length from lines picked with marker PL2 alone. A further seven out of nine individuals in this selected group had more than a 100 mm-pod length.

DISCUSSION

The random primer screening was moderately efficient, resulting in 74% of the primers tested amplifying informative RAPD profiles. Through BSA, 2% of the primers were found to show polymorphisms between the bulked DNA samples. This ultimately resulted in two primers, which amplified a band associated with pod length. Two RAPD marker bands proved to be significantly associated with pod length loci in this population based on ANOVA (Table 1). These markers clearly segregated the population into two significantly different phenotypic classes based on the mean pod length of each genotypic class (Table 1).

An important finding in this study was the identification of two loci with RAPD markers. The pod length in the long pod parent 'quantum' was almost two times more than the short pod parent 'China A', which established a wide range in pod length levels in the DH population. These wide ranges of phenotype likely facilitated the identification of the two genes in this study.

The mapping of markers placed 37 markers on 7 linked groups and one unlinked group and in the unlinked group (Figure 3) the markers PL1-PL2, PL4-PL11-PL12, and PL18-PL24 were linked together closely (Figure 1). A QTL analysis of these data placed the peak of the marker within 5 cM of marker PL2 and another peak within 4 cM of marker PL18 (figure 2). The marker PL2 showed a dominant DNA fragment for a long pod and the marker PL18 was recessive for this trait. These attributes will give this set of markers an excellent potential in

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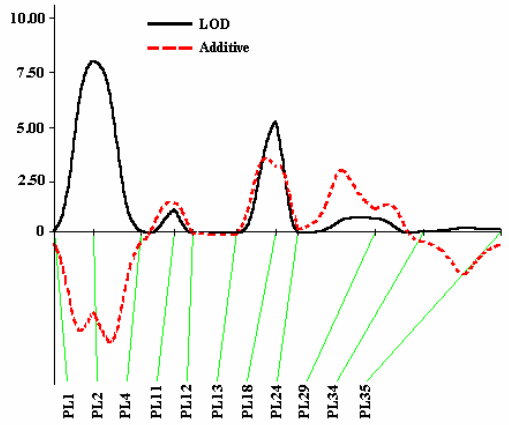

Figure 3. A QTL scan of unlinked group showing the position of The LOD peak.

MAS of long pod at this locus, given their proximity to the QTL peak and the availability of positive tests.

The mean pod length of selected lines from this population demonstrated the effectiveness of the markers at two loci. The best marker alone, based on ANOVA, was PL2, which explained 14.6% of the phenotypic variation. This 'quantum'-derived marker alone selected lines that were 105.5 mm in mean pod length. When both loci were used for selection of 'quantum' loci, the mean pod length was increased to 112.2 mm and the two loci explained 22.4% of the phenotypic variation (Table 1). Similarly, the QTL analysis indicated that the two loci could explain 18% of the variation. The selection for markers at two loci assorting independently among 50 DH lines should result in 12 lines on average. There were only nine lines carrying 'quantum' markers at both loci in this data set, and seven of the nine lines averaged 119.6 mm pod length. This high mean pod length in selected lines is noteworthy since 'quantum' has a pod length of 137 mm. These data emphasize the effectiveness of the suit of markers developed in this study for selection of long pod lines from breeding programs. When both QTLs are considered, the observed pod length of the selected line was 112.2 mm. The marginal deviation from 'quantum' can be explained by (1) undetected QTLs, (2) marker density and the recombination fraction around the identified QTLs and (3) the size of the DH population.

The usefulness of the set of markers identified in this study will be the subject of a concurrent project. The markers will be tested for the amplification of a similar sized DNA fragment in a variety of germplasm including: (1) interspecific crosses, (2) alternative (not 'china A'-derived) *B. napus* long pod mutant lines and (3) standard short pod canola cultivars. Since Double RAPD markers were identified in the study, the chances of introgressing the 'Quantum' genes into different genetic backgrounds is increased. It is proposed to convert the suit of markers into SCARs for a more efficient and automated selection of both 'Quantum' loci controlling long pod.

Comparatively, theoretical information related to the pod length is so limited [20]. No report associated with pod length inheritance via molecular marker approach has been found yet. This report is the first time of using RAPD method to screen out the pod length QTLs.

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مطالعه طول غلاف کلزا در جمعیت دابلد هاپلوئید کلزا با استفاده از مارکر مولکولی

ح. سمیع زاده ، ب. یزدی صمدی ، م. ر. بی‌همتا، ع. ر. طالعی، گ. استرینگام

چکیده

طول غلاف یا خورجین یکی از اجزاء موثر بر عملکرد کلزا می باشد که با انتخاب بر روی این صفت بطور غیر مستقیم میتوان نسبت به افزایش عملکرد و به تبع آن افزایش عملکرد روغن دست یافت. بدین جهت مارکرهای مولکولی جهت انتخاب سریع و مطمئن ژنوتیپهای با طول غلاف بلند در برنامه های به نژادی مورد جستجو قرار گرفتند و مارکرهای مولکولی همبسته با طول غلاف در یک جمعیت هاپلوئید دوبل شده ناشی از تلاقی بین لاینهای کلزای رقم کوانتوم (با طول بلند غلاف) × لاین چایناای (طول غلاف کوتاه) با استفاده از متد تجزیه توده متفرق(BSA) تشخیص داده شدند. یک نقشه لینکاژی مارکر مولکولی حاوی ۳۷ مکان ژنی RAPD برای این جمعیت جهت تشخیص مکانهای ژنی صفت کمی(QTLs) کنترل کننده صفت طول غلاف مورد بررسی قرار گرفت که دو مارکر در دو مکان ژنی ناپیوسته با استفاده از روش اینتروال مپینگ انتخاب شدند که دو مارکر ۲۲٪ تنوع فنوتیپی برای طول غلاف را در جمعیت مورد بررسی تخمین زدند. گزینش با این مارکرها در جهت افزایش طول غلاف موجب انتخاب گروهی از لاینهای هاپلوئید دوبل شده با میانگین طول غلافی معادل ۱۱۲ میلیمتر گردید که افزایشی معادل ۱۵٪ را نسبت به میانگین جمعیت اولیه باعث شد. این نشان میدهد که استفاده از این مارکرها در برنامه‌های به نژادی موجب پیشرفت اصلاح جهت تهیه ارقام با طول غلاف بیشتر میگردد.

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