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Study of microspore-culture responsiveness in oilseed rape (*Brassica napus* L.) by comparative mapping of a F_2 population and two microspore-derived populations

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Abstract RFLP segregation analyses were performed on a F_2 population and two F_1 microspore-derived populations from the same cross between a microspore culture-responsive parent ('Topas') and a non-responsive parent ('Westar'). A total of 145 loci were detected with 87 cDNA clones. Eighty-two markers were common across all three populations. A total of 66 markers was assembled into 18 linkage groups and 16 markers remained unlinked. Segregation distortions were significant for 29% of the markers in the F_2 population and 23% and 31% in microspore-derived populations M3 and M5, respectively. An equivalent number of markers showed biased segregation towards each parental allele in the F_2 population while more markers showed a significant deviation from the expected Mendelian ratio towards the responsive parent in both microspore-derived populations. Different subsets of markers showed segregation distortions in the three populations indicating that the selective pressures leading to microspore-derived plants are different from those acting during selfing of the F_1. Linkage groups 1 and 18 were identified as putative chromosomal regions associated with microspore-culture responsiveness.

Key words *Microspore culture* · Responsiveness genes · Comparative mapping · Doubled haploid populations · *Brassica napus* · Segregation distortion

Introduction

Homozygous lines can be obtained through microspore or anther culture in only one generation, whereas a minimum of six to eight generations of selfing is required to reach a sufficient level of homozygosity. The genetic purity of the plant material and the time saved in producing the homozygous lines has rendered the in vitro system very attractive to plant breeders (Beversdorf and Kott 1987). Doubled haploid plants can be obtained in many plant species. High frequency pollen embryogenesis and plant regeneration can be induced in *B. napus* and it is now possible to produce large populations of homozygous doubled haploid plants segregating for important agronomic traits (Chuong et al. 1988). Response to microspore culture is, however, genotype dependent, therefore limiting its use to responsive genotypes (Chuong et al. 1987).

To be useful in breeding programs, doubled haploid populations must compare advantageously to other conventional breeding populations in terms of fitness and genetic variability (Snape et al. 1986). Very little information, however, is available about the amount of genetic variability released in F_1-derived doubled haploid populations of oilseed *B. napus*, as well as the genetic make-up of such populations in comparison to the traditionally used selfed lines (Siebel and Pauls 1989; Chen and Beversdorf 1990). From a breeders perspective, gametic selection occurring during androgenesis must not lead to reduced performance. On the other hand, in vitro selection can be used as a tool to select desired genotypes (Hormaza and Herrero 1992).

The development of DNA markers has made possible detailed studies on the segregation of large numbers of markers in various segregating populations. Detailed linkage maps based on restriction fragment length polymorphisms (RFLPs) and polymerase chain reaction (PCR) markers have been produced in a number of species, therefore providing the necessary tools to examine if not the entire genome, at least large portions thereof.

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In most of these studies, however, a single population was used to derive the often sole linkage map of a species. In barley, maize and rice, maps derived from more than one cross and more than one population type have been generated (Shin et al. 1990; Beavis and Grant 1991; Graner et al. 1991; Heun et al. 1991; Bentolila et al. 1992; Jena et al. 1993; Kleinhofs et al. 1993; Murigneux et al. 1993; Nagamura et al. 1993; Wang et al. 1994). Comparative mapping of different populations of the same species was performed in maize and barley and proved to be a powerful tool to assess the heterogeneity between genomes (Beavis and Grant 1991; Graner et al. 1991; Bentolila et al. 1992; Murigneux et al. 1993).

The objective of our study was to identify putative *B. napus* chromosomal regions that may be associated with microspore culture responsiveness using comparative RFLP analyses. We wanted to examine the genetic make-up of microspore-derived populations and to compare it with more traditionally used selfed populations to assess their usefulness as breeding populations. We therefore studied the segregation of 145 RFLP markers in two populations derived from a microspore culture of F_1 individuals and one F_2 population from the same cross between a responsive and a non-responsive cultivar of *B. napus*. In particular, the extent and the direction of segregation distortions were examined.

Materials and methods

Plant materials

Two cultivars of *B. napus* were chosen as parents on the basis of their known differential response to microspore culture and the level of polymorphism between them (Landry et al. 1991). The microspore culture-responsive cultivar 'Topas' was crossed to the non-responsive cultivar 'Westar' and two F_1 individuals were used as donor parents for the production of two microspore-derived populations M3 and M5 (provided by Dr. L. Kott and Dr. W. D. Beversdorf of the Department of Crop Science, University of Guelph, Ontario, Canada). The microspore culture method used to produce these lines has been described previously (Kott et al. 1987). Another F_1 individual from the same cross was selfed to produced an F_2 population (Landry et al. 1991).

The regenerated haploid and spontaneous diploid plants were grown in the greenhouse under the same conditions as previously described for the F_2 population (Landry et al. 1991). Leaf tissues of haploid plants were harvested and immediately frozen until DNA extraction. Spontaneous diploid plants were bagged for selfing and dihaploid lines were grown and processed in the same way. Lines that were not 100% homozygous according to their RFLP patterns were discarded for mapping purposes.

The microspore-derived population M3 was composed of eight haploid plants and 36 dihaploid lines, whereas population M5 was composed of three haploid plants and 40 dihaploid lines. The third mapping population was composed of 90 F_2 plants.

Source of probes

The choice of probes was based on the polymorphism detected between the parental cultivars 'Topas' and 'Westar' and within the F_2 segregation (Landry et al. 1991). Eighty cDNA clones, designated 1ND1 to 4NG11, six seedling-specific cDNA clones (pCa15, pCot1, pCot39, pCot44, pGs43 and pAx92) provided by Dr. J. J. Harada, and a cruciferin clone (pC1) provided by Dr. M. Crouch, were used as probes on all three segregating populations. Plasmids were prepared using the Magic minipreps DNA purification system (Promega, USA) and cDNA inserts were purified with a Geneclean^{TM} kit (Bio 101 Inc, USA), or with a Sephaglas band prep kit (Pharmacia, Canada), prior to labeling with ^{32}P.

Southern blot analysis

Protocols for DNA isolation, purification and restriction-enzyme digestion have been described previously (Landry et al. 1991). The restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV and *Hin*dIII were used and several duplicates of each restriction digest were made to perform up to ten hybridizations simultaneously. Five micrograms of restricted DNA were loaded per lane. Following electrophoresis, DNA was transferred onto Hybond$^{TM}-N^+$ membranes (Amersham, UK) by capillarity (Southern 1975). The alkaline transfer procedure (Reed and Mann 1985) with 0.4 M sodium hydroxide was also performed as recommended by the membrane manufacturer (Amersham, UK).

Purified cDNA inserts were labeled with ^{32}P with a T7 Quick Prime^{TM} kit (Pharmacia, Canada) and used as probes. Hybridization conditions were as described by Landry et al. (1991) with the exception that 10% (w/v) dextran sulfate was added to the hybridization solution.

Identification of polymorphic clones and nomenclature of loci

Polymorphic cDNA probes were identified by comparing the DNA hybridization patterns between the parental lines. The same 'probe × restriction enzyme' combinations was then used in all three segregating populations. When the restriction fragments were consistent and clearly readable in at least two populations, association of a specific RFLP to a locus was possible among populations because the same 'probe × enzyme' combinations were used across the populations. Duplicated loci detected by a single clone were designated by the same probe name followed by a different lower case letter.

Segregation ratios and linkage analyses

A Chi-square test to confirm the hypothesis of equal allele frequency over all loci in a population was performed for each of the three populations. Data were also pooled for all co-dominant marker loci of the F_2 population and were statistically tested for deviation from the overall 1:2:1 expected segregation ratio. Goodness-of-fit to expected Mendelian segregation ratios were calculated for each locus in each population using Linkage-1 software (Suiter et al. 1983). For co-dominant loci of the F_2 population, the two degrees of freedom were partitioned to test for equal representation of the parental alleles (gametic selection) and an equal number of homozygote to heterozygote genotypes.

Maps were constructed on a MacIntosh LC III using Mapmaker version 2.0 software (Lincoln et al. 1990). The group function with $LOD > 4.0$ and a maximum recombination fraction of 0.30 was used to first assign markers to linkage groups. Markers common to all three populations and forming consistent linkage groups in all of them were used to build framework comparative maps. The 'big LODs' command with $LOD > 6.0$ and a minimum recombination fraction of 0.05 was used to select the most informative markers in each linkage group. Marker order in each linkage subgroup was determined by comparing all possible orders and choosing the order that was the most consistent in all three populations. Other markers assigned to a linkage group with $LOD > 4.0$ were subsequently added by trying them in each interval. Finally, markers assigned to a linkage group with $LOD > 2.0$ in at least two of the three populations were added in the same manner.

Results and discussion

Polymorphic and monomorphic loci

B. napus cultivars ‘Topas’ and ‘Westar’ are not pure lines. The level of residual heterozygosity has been estimated to be between 11 and 23% (Cloutier et al. submitted). The F_1s produced by crossing these two parents have the potential to represent a large number of different genotypes because each locus showing residual heterozygosity in one of the parental cultivars can generate two genotypes. It is therefore possible to observe polymorphism between the two parental cultivars at a locus while observing a monomorphic pattern in the segregating population depending on the genotype of the F_1 plant used to generate the segregating population. The reverse can also be true if a single individual of each cultivar was used to determine polymorphism between parental lines. Because three different F_1 individuals were used to generate the three segregating populations (F_2, M3, M5), only 82 of the 145 loci detected were polymorphic and comparable in all three populations. A total of 119 loci segregated in the F_2 population, 115 loci segregated in microspore-derived population M3, and 113 segregated in microspore-derived population M5.

Comparative mapping

A total of 118 markers out of the 145 detected could be assembled into 16 linkage groups of three or more markers and eight pairs of linked markers. Twenty-seven markers remained unlinked. The total length covered by these markers has been estimated to correspond between 65% to 80% of the total length of the genome. Comparative mapping was done exclusively with the 82 markers polymorphic and comparable in all three populations. These markers were assembled into ten linkage groups of more than three markers and eight pairs of linked markers (Fig. 1). Sixteen markers were unlinked in all populations. The total length covered by the 66 mapped markers was 863, 524 and 614 cM (Kosambi) for the F_2, M3 and M5 populations respectively.

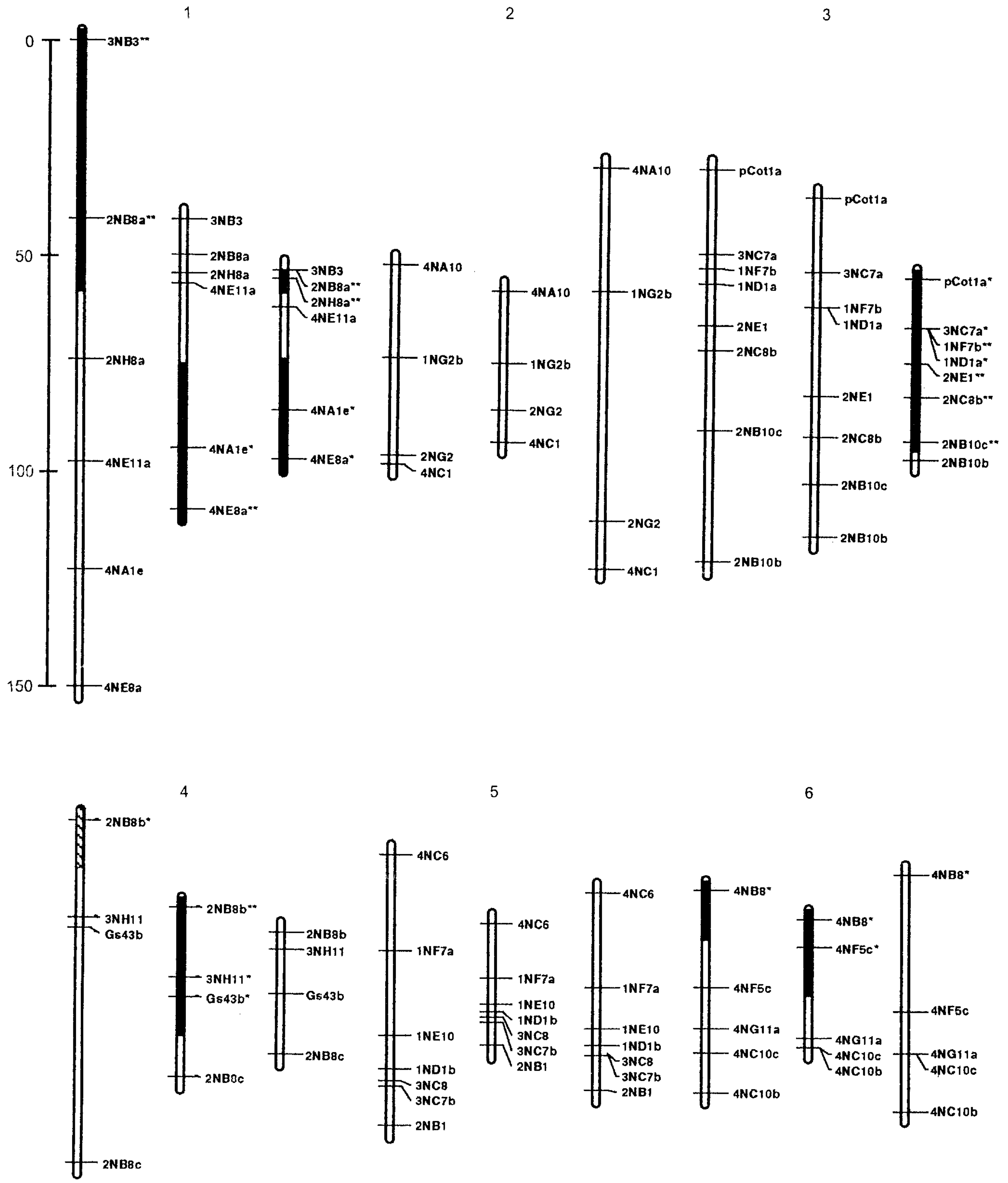
For mapping purposes, an F_2 population is more informative than a doubled haploid population of the same size because the first represents twice the number of meiotic events compared to the second. Heterozygous hybridization patterns, however, cannot be distinguished from homozygous patterns with dominant markers in the F_2 population. Less information per RFLP is available surrounding such markers. Co-dominant and dominant markers are equivalent in doubled haploid populations and dominant markers are not associated with relatively less information with this population type. The heterogeneity of recombination has not been measured between the F_2 population and the microspore-derived populations because, even though we had the prerequisite for a comparable subset of common markers, the differential informativity of the populations would have greatly hindered the conclusions that could be drawn from such a comparison.

Segregation distortions

The overall ratio of ‘Topas’ to ‘Westar’ RFLP alleles estimated for the three populations over the 145 loci is shown in Table 1. Co-dominant markers of the F_2 population showed a significant under-representation of heterozygotes. The entire F_2 population, however, displayed an equivalent proportion of both parental alleles. Microspore-derived population M3 also showed a random assortment of the parental alleles. By contrast, population M5 contained a significant excess of alleles from the microspore culture responsive parent ‘Topas’ with a total of 52.3% (2233/4269) of all scored alleles from ‘Topas’.

Goodness-of-fit to the expected Mendelian segregation ratio for each locus revealed that 29% of the markers showed distorted segregation in the F_2 population, 23% in microspore-derived population M3, and 31% in microspore-derived population M5 (Table 2). Deviations from Mendelian segregation ratios could be the result of viability factors at various stages of development during gametogenesis, zygote formation (F_2), or subsequent plant development. Sampling error alone cannot explain 23–31% of the markers showing distorted segregation. In maize, populations derived from anther culture of a F_1 from a cross between a responsive parent and a non-responsive parent had 73% and 52% of markers showing biased segregations as opposed to 1% in the comparative F_2 population and 7% in the comparative single seed-descent (SSD) population, respectively (Bentolila et al. 1992; Murigneux et al. 1993). In barley, 44% of the markers showed biased segregation compared to 10% in the F_2 population (Graner et al. 1991). A nearly random genetic array of the variability of the cross was observed in these F_2 and SSD populations, versus a large proportion of the genomes being under selection pressure in the populations derived from anther culture. In our *B. napus* populations, selection not only occurs in the androgenetic populations but also, and to the same extent, in the F_2 population, suggesting that selection did not only take place during microspore culture as suggested for maize and barley.

In the F_2 population, 15/119 markers showed a significant excess of ‘Topas’ alleles and 14/119 showed a significant excess of ‘Westar’ alleles. Gametic and zygotic selection occurred at a number of loci but, overall, an equivalent number of each parental allele was positively selected. For a total of 15 markers, including five markers showing as many homozygous ‘Topas’ as homozygous ‘Westar’, heterozygous genotypes were under-represented suggesting that, at the zygotic level, some allelic combinations may be selected against in



favor of the homozygous genotypes. Markers showing under-representation of heterozygotes were clustered (linkage group 10, Fig. 1). The heterozygous state at these loci, however, is not lethal because 5 of the 90 F_2 plants were heterozygous at all five loci.

In microspore-derived population M3, significant deviations from the 1:1 expected segregation ratio were observed at 16 loci in favour of 'Topas' alleles and at 11 loci in favor of 'Westar' alleles. The ratio was 21 loci in favor of 'Topas' alleles to 14 loci in favor of 'Westar' alleles in microspore-derived population M5 (Table 2). The gametic selection operating in microspore-derived populations resulted in more loci showing a significant segregation distortion in favor of the alleles from the

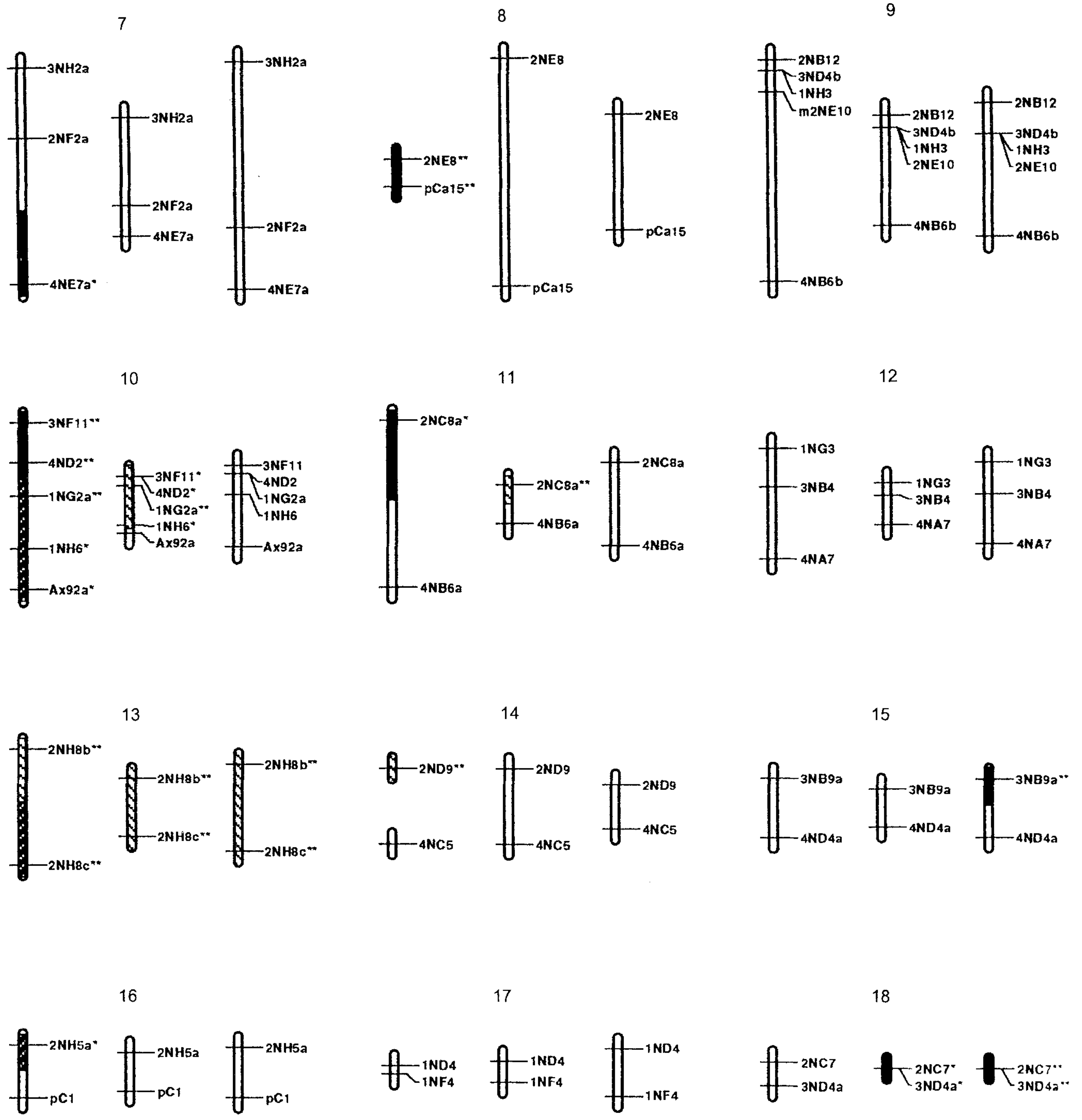


Fig. 1 Comparative linkage maps of *Brassica napus* generated by a set of 82 common markers in the F_2 population (left) and microspore-derived populations M3 (center) and M5 (right). Linkage group number are indicated above the linkage groups. The scale is in recombination units. Unlinked markers: 1NH4, 1NH12, 2NB4, 2NB7, 2NC10, 2ND12, 3NC3a, 3NF2, 3NG3a, 3NH3a, 3NH12a, 4NA1d, 4NB12, 4NE11b, 4NF4a, 4NF4b. ■ segregation distortion towards 'Topas' parental alleles. ▨ segregation distortion disfavoring heterozygous genotypes. □ segregation distortion towards 'Westar' parental alleles. * $P < 0.05$, ** $P < 0.01$

microspore culture responsive parent 'Topas'. Our results, again, parallel those obtained in maize and barley with populations derived from a cross between a responsive parent and a non-responsive parent (Graner et al. 1991; Thompson et al. 1991; Bentolila et al. 1992; Murigneux et al. 1993). A similar number of loci showing segregation distortion towards each parental allele was observed in a maize doubled haploid population derived from a cross between two responsive parents (Murigneux et al. 1993). The percentage of distortion, and the direction of the distortion towards the responsive parent alleles, in microspore-derived populations is indicative of gametic selection. The hypothesis of linkage between markers showing distorted segregation and genetic factors involved in culture responsiveness is again supported (Foisset and Delourme 1995). Finally, a different subset of markers showed significant segregation distortions in each of the three populations (Fig. 1).

Table 1 Overall distribution of RFLP genotypes/alleles for a total of 145 loci in the F_2 population and microspore-derived populations M3 and M5

Population	Marker type	Number of RFLP genotypes/alleles			χ^2	Genotype/allele under selection
		Topas	Heterozygotes	Westar		
F_2	Co-dominant Dominant	8394	–	8188	2.55	
	Co-dominant only	1417	2174	1376	77.81**	H –
M3	Co-dominant	2228	–	2116	2.89	
M5	Co-dominant	2233	–	2036	9.09**	T +

** $P < 0.01$

H –, significant under-representation of heterozygous genotypes

T +, significant excess of 'Topas' alleles

Table 2 Number of loci showing significant segregation distortion and the genotype/allele under selection in the F_2 population and microspore-derived populations M3 and M5

Population	Genotype/allele under selection					
	T +	T + H –	H –	W + H –	W +	Total
F_2	10	5	5	5	9	34/119 (29%)
M3	16	–	–	–	11	27/115 (23%)
M5	21	–	–	–	14	35/113 (31%)

T +, significant excess of 'Topas' alleles

H –, significant under-representation of heterozygous genotypes

W +, significant excess of 'Westar' alleles

If the allele frequency resulted from a viability factor it would not affect both selfing and androgenesis (Murigneux et al. 1993).

Microspore-culture responsiveness

As for other species, microspore culture responsiveness in canola is dependent on the genotype of the donor plant (Chuong et al. 1987). Plants derived from microspore culture of a F_1 from a cross between a responsive parent and a non-responsive parent could accumulate favorable alleles from the responsive parent (Wan et al. 1992). DNA markers linked to these selected alleles will display an excess of alleles from the responsive parent in the microspore-derived populations but will show a Mendelian segregation in the F_2 population. Markers positioned on linkage groups 1 and 18 showed significant segregation distortion towards 'Topas' alleles in both microspore-derived populations, and segregated according to expected Mendelian ratios in the F_2 population (Fig. 1). In addition, markers associated with linkage group 1 also showed segregation distortions against the 'Westar' alleles in three other F_1-derived doubled haploid populations where cv 'Westar' was one of the parental lines (unpublished results).

We also measured the embryo production of 16 randomly chosen microspore-derived lines from populations M3 and M5. Some of the lines produced an average of only one or two embryos per plate, while others were highly embryogenic (data not shown). Interestingly, only the non-embryogenic lines displayed 'Westar' alleles at loci 2NC7 and 3ND4a of linkage group 18. All the highly embryogenic lines displayed the 'Topas' allele at the same loci of linkage group 18, and at loci 4NE11a, 4NA1e, 4NE8a of linkage group 1. Other markers not common to all three populations and positioned in this interval of linkage group 1 also showed segregation distortion towards 'Topas' alleles. These observations, while still preliminary, reinforce our hypothesis of microspore culture responsiveness genes from 'Topas' being located on these two linkage groups. QTL studies with more individuals are needed to confirm the position of these genes.

In this respect, our results support previous observations on maize. RFLP analysis was applied to establish a genetic basis for the formation of embryo-like structures and the subsequent formation of a regenerable callus. A genetic model including four chromosomal regions explained 57% of the phenotypic variance for the production of embryo-like structures (Cowen et al. 1992). Armstrong et al. (1992) found that a set of five markers (three of them linked) also explained 82% of the phenotypic variance observed for the percentage of immature embryos forming an embryogenic callus in maize. The same chromosomal regions were found to be associated with segregation distortion towards the responsive parents in other crosses of maize (Wan et al. 1992).

Conclusion

Doubled haploid populations are suitable as breeding populations because they can represent a random sample of the genetic variability of the cross. Segregation distortions were present in all three populations but the DNA markers and the direction of the segregation distortion differed among populations. Selection pressures, however, were different when the plants were derived from selfing or from microspore culture of an F_1.

Putative genes associated with responsiveness to microspore culture were mapped to linkage groups 1 and 18. Further studies are needed to confirm the involvement of these QTLs. In the future, marker-assisted selection could be used to introgress the chromosomal regions containing the putative responsiveness genes into non-responsive elite lines. This would facilitate the development of germplasm resources for responsive genotypes (Murigneux et al. 1993). Finally, crosses between two responsive parents would generate F_1 donors with the genetic variability necessary for plant breeding and would also minimize gametic selection.

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