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Philip Marshall · Marie-Claude Marchand
Zenon Lisieczko · Benoit S. Landry

A simple method to estimate the percentage of hybridity in canola (*Brassica napus*) F_1 hybrids

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Abstract We have developed an efficient PCR-based system that uses RAPD markers for the certification of F_1 hybrids of canola. These markers were selected by screening five parental lines used in three crosses X, Y and Z with 131, 131 and 322 primers respectively. Stable DNA fragments that were homozygous and specific to the male inbreds were used to certify F_1 hybrid populations. The hybrid production system was based on self-incompatibility (SI) alleles that prevent self-pollination of the female parent. The efficiency of two S-alleles was compared under both field and greenhouse conditions. The percentage of hybridity was estimated in different F_1 populations. We found a significant difference between the two alleles for their efficiency in controlling selfing; both alleles were stable under greenhouse conditions, one allele appeared less reliable under field conditions.

Key words Polymerase chain reaction
Random amplified polymorphic DNA
Self-incompatibility · *Brassica campestris*
Brassica napus

Introduction

In recent years, marker-assisted selection has been integrated into several plant breeding programs to select traits of agronomical importance. Isozymes were initially sought for this purpose but their use was hindered by the low amount of variability detected between closely related genotypes such as those used for commercial production. Advances in molecular biology, however, have focused the attention of plant breeders on DNA markers such as DNA restriction fragment length polymorphisms (RFLP) (Botstein et al. 1980) and random amplified polymorphic DNA (RAPD) (Williams et al. 1990). RAPD markers are now preferred to RFLP because they are easy to analyse, require very little DNA and do not need radioactivity handling facilities. Studies on many crops have shown that RAPD markers are also convenient for targeted mapping of genes responsible for monogenic or polygenic (quantitative) traits which are often difficult to map using classical methods. One application has coupled the use of RAPD and nearly isogenic lines (NIL) of tomato to identify genes that confer resistance to *Pseudomonas* (Martin et al. 1991, 1993), while another identified markers linked to mildew resistance by the combined use of RAPDs and DNA pools that were constructed from homozygous individuals, of a lettuce F_2 population, that were either sensitive or resistant to mildew (Michelmore et al. 1991; Paran et al. 1991). Although many research groups have questioned the reliability of RAPD markers, reproducible amplification of RAPD markers has been demonstrated in flowering plants that belong to the genera *Datisca*, *Yucca* and *Helianthus* (Fritsch et al. 1993) as well as in many crop species including corn (Heun and Helentjaris 1993), wheat (Devos and Gale 1992; Vierling et al. 1992), barley (Tinker et al. 1993), rice (Heun et al. 1994) and others (Hu and Quiros 1991). Other studies have used RAPD markers to determine the genotype of microspores derived from *Brassica napus* embryos, which suggests that this assay may be used for the identification of microspore-derived plants (Horn and Rafalski 1992; Cloutier and Landry 1994).

In the present study, we demonstrate how RAPD markers can be used to certify F_1 hybrids of canola (*B. napus*). Recently, King Agro has utilized a method based on self-incompatibility to control the pollination of parental lines of highly performing F_1 hybrids. Self-incompatibility (SI) alleles S_1 and S_3 (Banks et al. 1988) of *Brassica campestris* (Dwyer et al. 1990; Nasrallah et al. 1991; Toriyama et al. 1991) were transferred into breeding lines of oilseed, *B. napus*, to prevent self-pollination and to facilitate the production of highly performant F_1 hybrid seeds (Banks

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P. Marshall · M.-C. Marchand · B. S. Landry (✉)
Station de recherche Agriculture Canada, 430 Boul. Gouin, St-Jean-sur-le-Richelieu, Québec J3B 3E6, Canada

P. Marshall · M.-C. Marchand · Z. Lisieczko
King Agro, Research Center, Highway 23 south, R.R #2, Listowel, Ontario N4W 3G7, Canada

1988). The sensitivity of S-alleles to environmental factors and genetic background, however, can cause fluctuations in the efficiency of this pollination control system and generates variations in the hybridity levels between F_1 seed populations (Banks 1988). We selected highly repeatable dominant DNA fragments amplified only in the male parent and developed a RAPD-based diagnostic to monitor the level of hybridity in experimental F_1 hybrids. This study also allowed us to compare the efficiency of different S-alleles both under field and greenhouse conditions.

Materials and methods

Plant materials

Parental inbreds as well as F_1 hybrid populations were from King Agro (Listowel, Ontario). Their pedigrees are described in Table 1. Male parental lines #1, #6 and #11 are self-compatible and female parental lines #3 and #8 are self-incompatible since they carry *B. campestris* alleles S_1 and S_3 respectively (Banks 1988). F_1 hybrid seed populations #4, #10 and #12 were produced in the field from crosses between parental lines #3 and #6, #8 and #1 and #8 and #11 respectively. F_1 hybrid seed populations #5 and #9 were produced in the greenhouse from crosses between parental lines #3 and #6 and #8 and #1 respectively.

Micro-extraction of DNA and RAPD reactions

Plants were grown until the first true leaf emerged and their DNA extracted as described by Cheung et al. (1993) with the following modifications. DNA was pelleted at 12000×*g* for 30 min at room temperature and resuspended in 50 µl of an RNAse solution (10 µg/ml) to a final concentration of approximately 1 ng/µl as determined by fluorescence in the presence of bisbenzimide (Hoechst dye 33258, Kodak) using a fluorescence spectrophotometer (model LS5, Perkin-Elmer; excitation, 365 nm; emission, 460 nm). For each RAPD reaction, 2 ng of genomic DNA was incubated with 0.2 µM of DNA primer (Operon Technologies Inc.), 200 µM of each dNTPs (Pharmacia LKB Biotechnology), 0.5 U of Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus) in a final volume of 25 µl of polymerase chain reaction buffer containing: 10 mM TRIS-HCl pH 8.2, 50 mM KCl, 2 mM $MgCl_2$, 0.02% gelatine. PCR reactions were performed on the Perkin Elmer thermocycler, model 9600. A denaturation period of 2 min at 95°C was followed by 35 cycles of: denaturation at 95°C (ramping 60 s), annealing at 40°C for 25 s (ramping 100 s) and polymerization at 72°C for 60 s (ramping 75 s). A final cycle of denaturation at 95°C for 30 s, annealing at 40°C for 30 s, DNA synthesis at 72°C for 3 min was performed prior to separation of the amplification products by agarose (1.4%)-gel electrophoresis in 1×TBE buffer (90 mM TRIS-borate; 2 mM EDTA, pH 8.0).

Table 1 Pedigree of different spring-type canola (*B. napus*) lines analysed in this study. Self-incompatible and self-compatible lines are designated "si" and "sc" respectively. P1, P2 and P3 represent the genetic backgrounds of the parental lines used for the production of F_1 hybrids. For example, P1 sc and P1 si are nearly isogenic lines differing at the S-locus

Line #	Pedigree	S-allele
1	P1 sc	–
2	P1 si	1
3	P1 sc×P1 si	1
4	(P1 si×P1 sc)×P2 sc	1
5	(P1 si×P1 sc)×P2 sc	1
6	P2 sc	–
7	P2 si	3
8	P2 si×P2 sc	3
9	(P2 si×P2 sc)×P1 sc	3
10	(P2 si×P2 sc)×P1 sc	3
11	P3 s	–
12	(P2 si×P2 sc)×P3 sc	3

Heterozygosity, stability and hybridity tests

The stability of male-specific RAPD markers was verified by performing 30 RAPD reactions using the same DNA preparation. Residual heterozygosity within parental lines was estimated with the stable RAPD markers by performing 30 RAPD reactions on DNA of different plants from each parental line. Hybridity tests were performed on 120 individuals of each F_1 population by using the stable and homozygous RAPD primers that were diagnostic (male-specific). The percentage of hybridity for all five F_1 hybrid seed populations (#4, #5, #9, #10 and #12) was determined as the percentage of plants that contained the selected polymorphic DNA band.

Southern-blot analysis

RAPD reaction products were separated by agarose-gel electrophoresis and transferred to a nylon membrane (Hybond N^+; Amersham) as recommended by the manufacturer. The membrane was removed from the gel and rinsed in 2 x SSC, prehybridized in a solution containing: 5 x SSC (750 mM NaCl; 75 mM sodium citrate, pH 7.0), 1% SDS, 1 x Denhardt, 50% formamide, 10% Dextran and salmon sperm DNA (10 mg/ml) for 4 h and hybridized to a RAPD fragment labelled with [^{32}P]-αdCTP (3000 Ci/mmol; ICN) using a T7 Quick prime labelling kit (Pharmacia LKB Biotechnology) overnight at 42°C. Membranes were prewashed in 2 x SSC, 1% SDS at 42°C for 10 min and at higher stringency (0.1 x SSC, 1% SDS) at 65°C for 30 min before being exposed to Kodak X- Omat AR films.

Results and discussion

Residual heterozygosity

To maintain the genetic stability in F_1 hybrids during several years, parental lines must be homozygous. Although the homozygosity of parental lines was confirmed by classical genetics (Lisieczko, unpublished results), all five parental lines that were used in this study were monitored for residual heterozygosity by screening 30 individuals of each parental line with 30 random primers; residual heterozygosity would be revealed as RAPD fragments segregating within parental lines. Our results showed, however, that all five parental lines were homozygous at the 167 loci detected with the 30 primers with the exception of two loci in line #6 and six loci in line #8 that were found to segregate. These results suggest that all five parental lines are suitable for the production of genetically stable F_1 hybrids.

Identification of stable polymorphic DNA bands between parental lines

Stable male-specific RAPD markers are required to certify F_1 hybrids. We, therefore, screened five parental inbreds

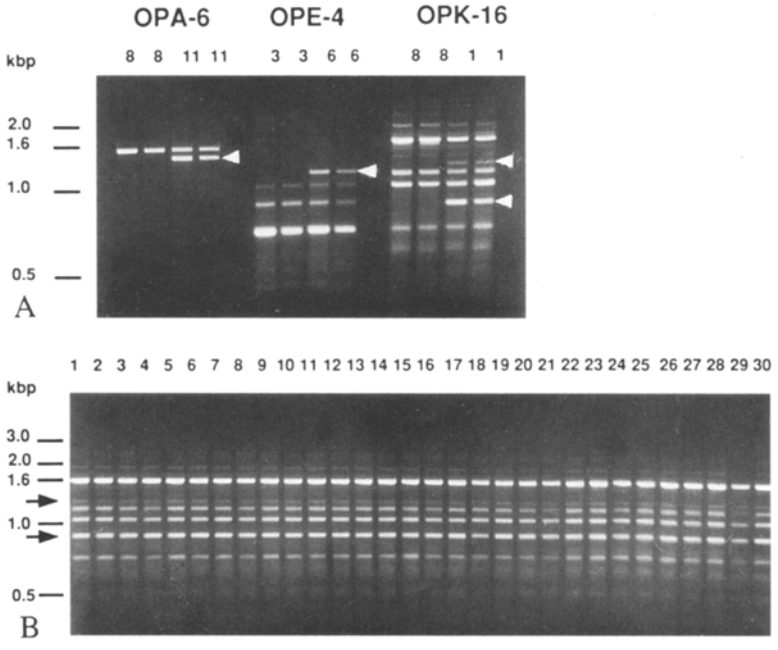

Fig. 1a, b Identification of male-specific RAPD fragments between parental lines used for crosses X, Y, and Z. **a** Male-specific RAPD markers (*open arrows*) between lines #8 and #11, #3 and #6 and #8 and #1 were identified by performing RAPD reactions with primers OPA-6, OPE-4 and OPK-16 respectively. **b** The stability of RAPD marker OPK-16 (*dark arrow*) was confirmed by performing 30 RAPD reactions using the same DNA preparation

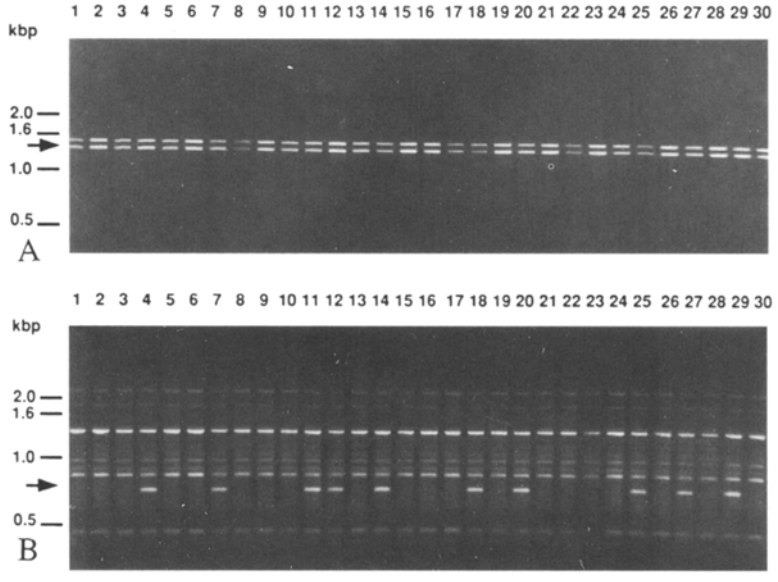

Fig. 2a, b Heterozygosity level of polymorphic DNA bands within parental lines. **a** RAPD reactions were performed on 30 individuals of parental line #11 with primer OPA-6. The homozygosity of the polymorphic DNA fragment (*arrow*) was confirmed throughout all individuals. **b** Residual heterozygosity revealed by RAPD marker OPE-7 (*arrow*) segregating in parental line #8

involved in three crosses, X, Y and Z, for the presence of DNA bands specific to the three male parental lines #6, #11 and #1 (Fig. 1A). A total of 131, 131 and 322 RAPD primers were tested respectively in each of the X, Y and Z crosses. Only 17 male-specific DNA fragments were identified in cross X, 16 in cross Y, and 43 in cross Z. The low number of polymorphic loci detected between parental inbreds is explained by the narrow genetic distance between parental inbred lines, the unidirectional selection of the polymorphisms, and the selection of only highly intense RAPD band. As a consequence, the number of primers needed to identify polymorphic loci between parental inbreds is much greater than required in other canola lines and other crops (Hu and Quiros 1991; Landry et al. 1991, 1992; Demeke et al. 1992; Tinker et al. 1993; Mailer et al. 1994).

The stability of the 76 male-specific RAPD markers that were identified between parental lines used in crosses X, Y and Z was thoroughly tested by performing 30 RAPD reactions on the same DNA preparation from each of the lines (Fig. 1B). Male-specific RAPD fragments which were not present in all reactions were eliminated. We found that polymorphic DNA fragments corresponding to major amplification products are less ambiguous and more reproducible than DNA fragments corresponding to amplification products of weaker intensity, as was also found in corn (Heun and Helentjaris 1993). After this highly stringent selection, only two (from crosses X and Y) and four (from

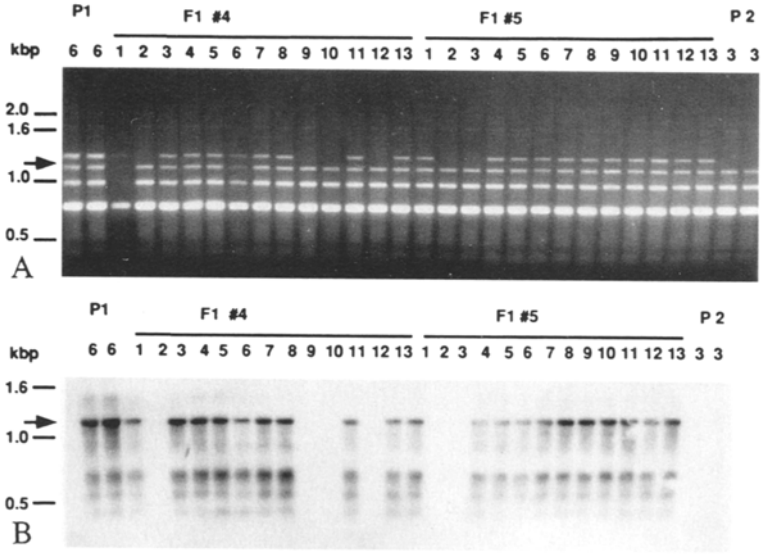

Fig. 3a, b Southern analysis of DNA samples from F_1 populations #4 and #5. **a** Amplification profiles obtained by primer OPE-4 of parental lines #6 (*P1*), #3 (*P2*) and F_1 populations #4 (*F_1 #4*) and #5 (*F_1 #5*). **b** Southern hybridization profile of the above gel using as probe the polymorphic DNA fragment generated by RAPD marker OPE-4

cross Z) male-specific RAPD fragments were retained as potential markers to certify F_1 hybrids on a routine basis.

Identification of diagnostic RAPD markers

The eight male-specific RAPD markers that were selected for the diagnostic of F_1 hybridity must also be homozygous; they must be present in every individual of the male parent and absent in the corresponding female parent. RAPD reactions on 30 individuals of parental lines used in the crosses X, Y and Z were performed and confirmed that all eight RAPD markers were consistently present in male individuals and absent from the corresponding female lines (Fig. 2a and b).

When the the same markers were tested on their respective F_1 individuals, however, we found that three of the four RAPD markers identified as stable when tested on DNA of parental lines of cross Z became less stable in the corresponding F_1 population; RAPD reactions repeated on the same F_1 DNA sample were not always reproducible and showed a broad range of intensity of the diagnostic DNA fragment. The instability of PCR products in F_1 populations of cross Z is difficult to explain, although a previous study on corn F_1 hybrids showed that RAPD amplification is affected only slightly by the genetic background surrounding the sequences to be amplified (Heun and Helentjaris 1993). A possible explanation could be the formation of unstable heteroduplexes from the DNA of alternate alleles in heterozygotes. This phenomenon was observed in RAPD reactions performed on heterozygous diploid honey bee DNA (Hunt and Page 1992). In contrast, the five remaining markers (OPE-4, OPC-9, OPA-6, OPE-16 and OPK-16) that were specific to male parental lines #1, #6 and #11 yielded DNA fragments of major intensity that were unambiguous and easily visualized on agarose stained gels. Only markers OPE-4, OPK-16 and OPA-6 were retained for further analyses.

To confirm the presence or absence of male-specific DNA markers amplified by primers OPE-4, OPK-16 and OPA-6 in corresponding F_1 populations, RAPD reaction products were separated by electrophoresis (Fig. 3A), transferred to a nylon membrane and hybridized to the radioactively labelled polymorphic DNA bands that are specific to male parents of each cross (Fig. 3B). Male-specific probes OPA-6, OPE-4 and OPK-16 hybridized to RAPD fragments of identical size in addition to other fragments that were found only in male parental lines #6, #11 and #1 and their corresponding F_1 hybrids. Although none of the probes detected the male-specific RAPD markers in either the female parental lines or their selfed progenies, the probe OPA-6 hybridized to additional fragments that were common to both parental lines #8 and #11 (data not shown). Individuals that were certified as F_1 hybrids by agarose-gel electrophoresis were the same as those identified by Southern hybridization. Therefore, RAPD markers OPA-6, OPE-4 and OPK-16 can be used for the certification of F_1 hybrids produced from crosses X, Y and Z respectively.

Determination of the percentage of hybridity

Hybridity was estimated as the percentage of individuals within F_1 populations that contained the male-specific RAPD marker. Diagnostic RAPD fragments obtained with primers OPE-4, OPA-6 and OPK-16 were scored on 120 randomly chosen individuals in each of the F_1 populations of crosses X (F_1 populations #4 and #5), Y (F_1 population #12) and Z (F_1 populations #9 and #10) (Table 2). The percentages listed in Table 3 show important variations in the degree of hybridity between different F_1 populations. These variations reflect previously observed differences in the efficiency of the self-incompatibility (SI) alleles S_1 and S_3 to control pollination under field or greenhouse conditions (Lisieczko, unpublished observations). Under green-

Table 2 Diagnostic RAPD primers that were used for the certification of F_1 hybrid seed populations produced from crosses X, Y and Z

Cross	Primer	Polymorphic loci	Nucleotide sequence
X (#3×#6)	OPE-4	1	5′-GTGACATGCC
Y (#8×#11)	OPA-6	1	5′-GGTCCCTGAC
Z (#8×#1)	OPK-16	2	5′-GAGCGTCGAA

Table 3 Variations of the percentage of hybridity of five different F_1 hybrid populations produced from crosses X, Y and Z

F_1 population	S-allele	Cross	Hybridity (%)	Environment
4	1	X (#3×#6)	77.5–90.3	Field
5	1	X (#3×#6)	81.4–92.9	Greenhouse
9	3	Z (#8×#1)	72.0–86.2	Greenhouse
10	3	Z (#8×#1)	18.1–33.4	Field
12	3	Y (#8×#11)	27.0–43.9	Field

house conditions, the two crosses X and Z produced a high percentage of true F_1 seeds, indicating that both S-alleles are capable of controlling pollination for F_1 hybrid production under this environment (Table 3). Under field conditions, however, the percentage of hybridity of F_1 populations of crosses Y and Z are much lower than in cross X, indicating that pollination control was more efficient with the S_1 allele than the S_3 allele. Nevertheless, we cannot infer from these results that the S_3 allele is more susceptible to the environment than the S_1 allele since the efficiency of the two S-alleles varies with the genetic background of the parents (Lisieczko, unpublished results).

Identification of markers potentially linked to the S-locus

Because we are also interested in identifying single-copy DNA markers linked to the S-locus, we selected RAPD markers that were polymorphic between lines that are isogenic with the exception of the self-incompatibility locus. Lines #6 and #8 and lines #1 and #3 were compared by using 131 primers. We selected DNA fragments that were specific to the female parent since the active S-allele was introgressed from *B. campestris*, a diploid self-incompatible species closely related to *B. napus*. None of the other lines amplified these fragments. Because female parents of the F_1 hybrids are heterozygous at the S-locus (see Table 1), linkage of these markers to alleles S_1 or S_3 is determined by co-segregation analysis in F_1 populations. Markers linked to the S_1 allele of the female parent #3 are expected to segregate in a 1:1 ratio within F_1 seed populations #4 and #5, while markers linked to the S_3 allele of female parent #8 are expected to co-segregate in the F_1 populations #9 and #10. Preliminary work revealed only one marker in each of the female parents #3 (OPE-1) and #8 (OPN-13) that segregate in corresponding F_1 populations. The low number of segregating markers is mostly due to the fact that these parental lines are very closely related. The likelihood of the linkage with the S-locus, however, will be high.

Conclusions

We developed a RAPD-based diagnostic system for the certification of F_1 hybrids in canola. The protocol used for DNA extraction is short and yields uniform amounts of DNA that permit up to 100 RAPD reactions. Our study indicates that RAPD markers intended for routine diagnostic analysis must be major products of amplification, as opposed to minor products which were found to be unreliable unless great care is taken for their analysis. The simplicity of our system now provides breeders with tools that enable screening on a commercial scale of various F_1 hybrid populations and allows a quick measure of the relative efficiency of various S-alleles (here we tested two S-alleles) under different environmental conditions (field vs greenhouse) and genetic backgrounds. As an indirect result of our study, we were able to identify conditions that yield higher levels of hybridity. We were also able to confirm the suitability of our parental lines (highly homozygous) for the production of F_1 hybrids and identified RAPD markers potentially linked to the S-locus. In order to further simplify our system, we are currently cloning the additional diagnostic RAPD markers that were less stable in order to design longer oligonucleotides (Paran and Michelmore 1993) that would amplify specific DNA bands or SCARs (sequence characterized amplified regions) from the male parent. These SCAR markers will be much easier to score because unstability related to the use of these RAPD markers is greatly reduced.

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