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Development of a SCAR (sequence characterised amplified region) marker for molecular tagging of the dwarf BREIZH (*Bzh*) gene in *Brassica napus* L.

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Abstract A SCAR (sequence characterised amplified region) has been developed for optimal tagging of the dwarf *Bzh* gene in *Brassica napus*. A RAPD marker named OPMO7-730 was previously found closely linked to the dwarf locus at 0.8 ± 0.7 cM. The DNA band corresponding to this marker was cloned and sequenced, and specific PCR primers were designed. The PCR test allowed us to amplify the locus corresponding to OPM07-730. With a restriction endonuclease digest and optimal electrophoresis conditions, three allelic forms of this marker have been recovered on the 40 *B. napus* accessions tested. The usefullness of this marker in breeding dwarf rapeseed lines is discussed.

Key words: *Brassica napus* · Dwarfing gene · PCR markers · Marker-assisted selection

Introduction

Susceptibility to lodging is a worrying problem in oilseed rape (*Brassica napus* L.) crops (Islam and Evan 1994) whose yield might increase with the present development of hybrid varieties characterised with a height gain of more than 20 cm in size. The lodging resistance trait in rapeseed is under complex genetic control and is influenced by environmental conditions. A dwarf mutant was obtained at INRA, Rennes by Foisset et al. in 1995. Genetic analysis of this character revealed that it is under the control of a single mutated gene (*Bzh*) with an additive effect, the dwarf plant being *bzh*/*bzh*. As the first agronomic tests of the dwarf and semi-dwarf progeny were interesting with regard to the level of resistance to lodging and yield performance, breeding programs were initiated. In developing a back-cross pedigree, employing recurrent as well as haploid breeding schemes in field or greenhouse conditions, difficulties appeared in the accurate determination of homozygous (dwarf; *bzh*/*bzh*) and heterozygous (semidwarf; *Bzh*/*bzh*) plants in segregating progenies due to the effect of the genetic background and the environment on the expression of this character. In order to overcome these difficulties, molecular mapping of the dwarf locus was initiated. The population consisted of doubled-haploid lines derived from the cross 'Darmor.*bzh*' × 'Yudal'. A linkage group of 84.5 cM was constructed arround the *Bzh* gene. The nearest flanking marker, named OPM07-730, was linked at 0.8 ± 0.7 cM (Foisset et al. 1995).

In order to obtain a specific co-dominant PCR-based marker which would be easier to use widely in breeding schemes, we decided to transform OPM07–730 into a PCR-based SCAR marker. The transformation of RAPD (random amplified polymorphic DNA) markers into SCAR markers was described by Paran and Michelmore (1993). This method has been used to obtain markers linked to downy mildew resistance genes in lettuce (Maisonneuve et al. 1994), to anthracnose resistance gene in common bean (Adam-Blondon et al. 1994), to the leaf rust resistance gene in wheat (Dedryver et al. 1996) and for the genetic characterisation of *Fusarium* strains (Ouellet and Seifert 1993). In the present paper we describe the development of the M07 SCAR marker and its applications in dwarf *Bzh* gene selection.

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Materials and methods

Plant material

Initially, primers were tested on two control lines, 'Darmor.*bzh*' (French dwarf line) and 'Yudal' (Korean tall line), previously used to establish a genetic map (Foisset et al. 1996) and to tag the dwarf gene (Foisset et al. 1995). Four tall lines were included in the analysis: 'Stellar' (Canadian line), 'Drakkar', 'Darmor' and 'Samouraï' (French lines). Tests were also made on *B. oleracea* 'Rapid Cycling' and on *B. campestris* 'R500'. Then, selected markers were studied on 35 more *B. napus* accessions: two spring tall lines, 24 winter tall lines and nine near-isogenic dwarf lines selected through back crosses from 9 out of the 25 winter tall lines (see Table 1).

DNA extractions

DNA was extracted from young leaves according to the method of Doyle and Doyle (1990).

Cloning the M07-730 band

RAPD procedures using the primer OPM07 5′ CCG TGA CTC A 3′ were as described by Foisset et al. (1995). After electrophoresis, the gel was de-stained in distilled water for 1 h to eliminate the TAE buffer. The OPM07-730 band originating from 'Darmor-*bzh*' was cut out with a sterile cutter. DNA was recovered from agarose by Sephaglass band preparation (Pharmacia). Approximately 0.1 ng of DNA was subjected to re-amplification with the same protocol as for RAPD analysis with the exception that 30 cycles were employed instead of 45. A final extension time of 90 min was applied to maximize the formation of poly-A ends (Li and Guy 1996). The PCR product was cloned using the TA cloning kit (Invitrogen). Recombinant white colonies were grown at 37°C/300 rpm in 300 µl of Terrific Broth buffer (Sambrook et al. 1989) for 3 h and subjected to PCR amplification as described by Barret et al. (1998). After agarose electrophoresis, the PCR products were transfered to a Hybond N+ nylon membrane (Amersham) and hybridized with the OPM07-730 band recovered from the RAPD gel using the direct ECL kit (Amersham). The sequencing of one clone was performed by Genome Express (Grenoble, France).

PCR-specific amplification

PCR-specific primers were designed using Oligo 4 software. The sequences of the four oligonucleotides employed were: SCM07UP1 5′ CCG TGA CTC ACG TCA AAG AG 3′; SCM07LP1 5′ CCG TGA CTC ATT TAA AGA AAC 3′; SCMO7UP2 5′ ATG ACG TTC GAC TGA GAA TG 3′; and SCM07LP2 5′ CAC TGA AAA GGT TTG TGA CAA G 3′. The PCR reaction mixture (25 µl) contained 1 × concentrate *Taq* polymerase buffer (Eurobio, Les Ulis, france), 150 µM of each dNTP, 1.2 mM of $MgCl_2$ 20 pmole of each primer, 0.5 units of *Taq* polymerase (Eurobio) and 50 ng of plant genomic DNA. PCR was performed in Perkin Elmer 480 apparatus with 30 cycles of 94°C/30 s, 55°C/30 s, 72°C/30 s and a final extention step of 72°C/5 min. PCR products were resolved on a 2.5% agarose gel or on a 6% non-denaturing acrylamide gel in TAE buffer.

Southern-blot hybridization for RFLP

Southern-blot hybridization was previously described by Sharpe et al. (1995)

Genetic mapping of the SCAR marker

Linkage analysis was performed on Mapmaker/exp version 3.0b (Lincoln et al. 1992). A minimum LOD of 4.0 and a maximum recombination frequency of 0.4 were chosen.

Sequence analysis

Sequences homologies were analysed with the GCG package (University of Wisconsin, Madison) using the fasta subroutine (Pearson and Lipman 1988).

Results

Sequence of the OPM07-730 fragment

Seven positive clones of the approximately expected size which cross hybridized with the purified OPM07-730 band were recovered on 47 white colonies tested. Sequence analysis of one of the OPM07-730 cloned fragments revealed that it contained 803 bp. The presence of the OPM07 RAPD primer at the two extremities was checked (Fig. 1). No homology was found with other sequences in the databases.

Specific amplification of the OPM07-730 locus

PCR amplification with the selected SCM07UP1 and SCM07LP1 specific primers (Fig. 1) was performed using the DNA extracted from both parental lines,

Fig. 1 The OPM07–730 fragment sequence, together with the OPM07 RAPD primer sequence (in *bold type*), the restriction enzyme cutting sites (in *italics*) and the primers used for specific PCR amplification

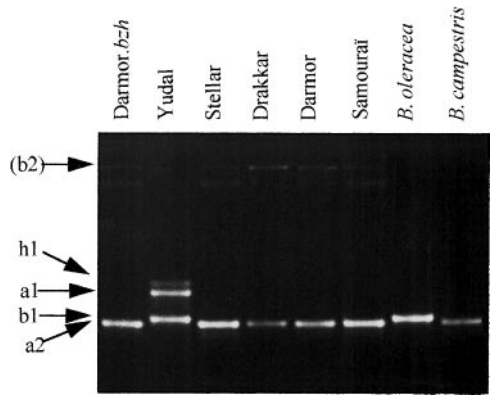

Fig. 2 2.5% agarose-gel electrophoresis of PCR amplification products with the SCM07UP1 and SCM07LP1 primers. The 803-bp fragment was recovered in Darmor.*bzh*. The two allelic forms (a1 and a2) corresponding to the OPM07-730 locus are indicated by an *arrow*

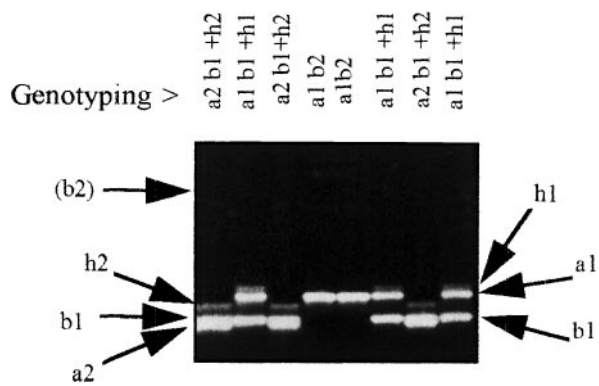

Fig. 3 Segregation of the SCM07UP1 + LP1 marker in a doubled-haploid population obtained from the cross Darmor.*bzh* × Yudal on a 2.5% agarose gel. Allelic forms a1 and a2 of the locus linked to the dwarf gene, b1 and b2 of the other locus, and heteroduplex bands h1 and h2 are indicated

'Darmor.*bzh*' and 'Yudal', as well as from the four other tall *B. napus* lines. The 803-bp fragment corresponding to the expected size (fragment a2, Fig. 2) was monomorphic except in 'Yudal' where two bands of higher molecular weight were amplified (Fig. 2). Segregation of this marker in a doubled-haploid population obtained from the cross 'Darmor.*bzh*' × 'Yudal' revealed that these profiles corresponded to two loci, named a and b. Bands a1 and a2 were allelic forms of the SCAR locus corresponding to the OPM07-730 locus on DY6 (Fig. 3). This latter locus was linked at 0.8 ± 0.7 cM to the dwarf locus, which corresponds to one recombinant plant among 96 tested. The second 'Yudal' band, named b1, segregated with a very faint band of higher molecular weight, named b2, and this b locus mapped on the linkage group DY12 of our genetic map (Foisset et al. 1996). The third faint band in 'Yudal', named h1 (Figs. 2 and 3), is a heteroduplex between a1 and b1. Another heteroduplex named h2, was observed when a2 and b1 allelic forms were simultaneously amplified (Fig. 3).

Amplification of a *B. oleracea* and a *B. campestris* accession with SCM07UP1 + LP1 primers indicated that the *B. campestris* band corresponds to the a2 allele of the a locus and that the *B. oleracea* band corresponds to the b1 allele of the b locus (Fig. 2). Moreover, although primers SCM07UP1 + LP1 amplified the a and b locus in rapeseed, amplification of a *B. juncea* accession (AABB genomes) affected only the a locus (data not shown).

Polymorphism detection among the 40 accessions tested

Thirty five additional *B. napus* accessions (Table 1) were genotyped with the SCAR marker. The allelic

Table 1 Allelic forms of the 40 *B. napus* accessions tested and the numbers used in Fig. 4 (Maxol, number 29, had been genotyped on another gel)

Accession	No. (Fig. 4)	Dwarf (D)/ tall (T)	Geographic origin	SCM07 allelic
Olymp ②	1	T	Germany	a2-2
Olymp ③	2	T	Germany	a2-2
Tapidor	3	T	France	a2-1
Doublol	4	T	Germany/France	a2-1
2405	5	T	France	a2-2
Darmor	6	T	France	a2-1
Gaspard	7	T	France	a2-2
Mohican	8	T	Great Britain	a2-1
Express	9	T	Germany	a2-1
Capitol	10	T	France	a2-1
Columbus	11	T	France	a2-1
Vivol	12	T	Germany/France	a2-1
Link	13	T	Great Britain	a2-2
Goéland	14	T	Germany	a2-1
Falcon 2	15	T	Germany	a2-2
Falcon 1	16	T	Germany	a2-2
Bristol	17	T	France	a2-1
Aligator	18	T	Germany	a2-2
Yudal	19	T	Korea	a1
Wesbrook	20	T	Australia	a2-2
Stellar	21	T	Canada	a2-2
Jaguar	22	T	Danemark	a2-2
Drakkar	23	T	France	a2-2
PB 1	24	T	Great Britain	a2-2
PB 2	25	T	Great Britain	a2-2
PB 3	26	T	Great Britain	a2-2
PB 4	27	T	Great Britain	a2-1
PB 5	28	T	Great Britain	a2-2
Maxol	29	T	Germany/France	a2-1
V 10	30	T	France	a2-1
Darmor.*bzh*	31	D	France	a2-1
2405.*bzh*	32	D	France	a2-1
Gaspard.*bzh*	33	D	France	a2-1
Doublol.*bzh*	34	D	Germany/France	a2-1
Tapidor.*bzh*	35	D	France	a2-1
Olymp.*bzh*	36	D	Germany	a2-1
PB2.*bzh*	37	D	Great Britain	a2-1
Bristol.*bzh*	38	D	France	a2-1
Maxol.*bzh*	39	D	Germany/France	a2-1
V 10.*bzh*	40	D	France	a2-1

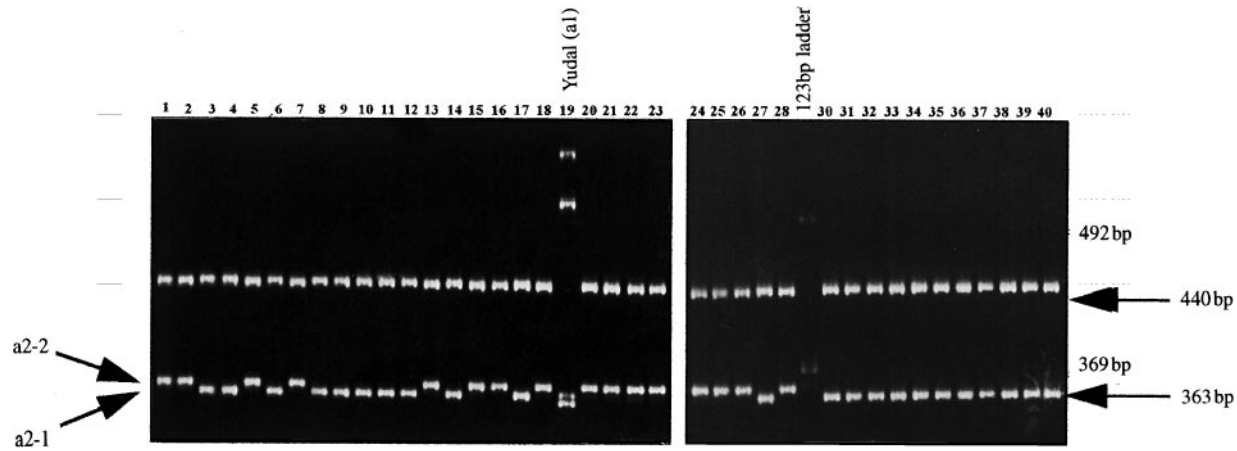

Fig. 4 Genotyping of the 40 rapeseed accessions listed in Table 1 with the SCM07UP1 + LP1 marker digested by *Eco*RV endonuclease. The restriction products were resolved by non denaturing 6% acrylamide gel electrophoresis

form a1 was only recovered in 'Yudal'; the 39 other tall or dwarf accessions presented the a2 allelic form. A search for other polymorphisms was then carried out. Restriction sites of the endonucleases *Dra*I, *Rsa*I, *Eco*RV and *Hha*I were detected on the DNA sequence. After PCR amplification, the 803-bp fragment was cut with the four restriction endonucleases. No restriction polymorphism was observed on agarose among the 39 accessions tested. To visualise the size, base composition and/or sequence polymorphism, non-denaturing acrylamide-gel electrophoresis of the DNA fragments could be performed (Sambrook et al. 1989). The 803-bp fragment was digested with the *Eco*RV endonuclease to obtain two bands of 440 and 363 bp compatible with acrylamide-gel electrophoresis (Fig. 1). These digestion products were then subjected to non-denaturing acrylamide-gel electrophoresis. Two new allelic forms, a2–1 and a2–2, corresponding to the previous a2 allele could be observed for the 363-bp band (Fig. 4). The distribution of the three allelic forms (a1, a2-1 and a2-2) among the 40 *B. napus* accessions tested is presented in Table 1 and in Fig. 4.

Optimisation of polymorphism detection

For optimal detection of polymorphism, primers SCM07UP2 and LP2 were defined (Fig. 1). SCM07 UP2 + LP2 amplified a 164-bp fragment which was not polymorphic. Then, a 528-bp fragment was amplified with SCM07UP1 + LP2 primers. The polymorphism between a1 and a2-1/a2-2 alleles was clearly detected with this PCR test, with no interference with the second locus (Fig. 5). However, the polymorphism between the a2-1 and a2-2 alleles is very faint on this 528-bp fragment and is difficult to detect, especially for heterozygous plants. A 439-bp fragment was amplified

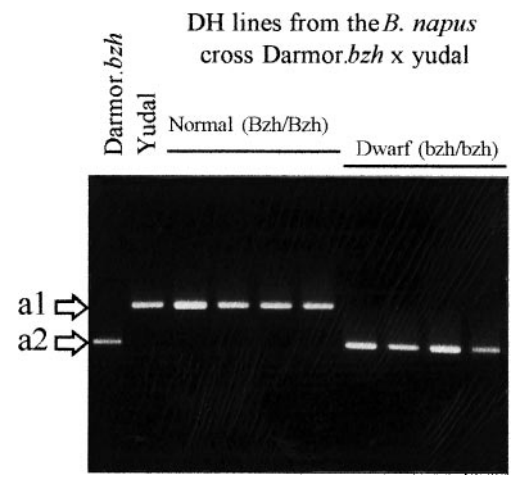

Fig. 5 Segregation of the SCM07UP1 + LP2 marker in a doubled-haploid population obtained from the cross Darmor.*bzh* × Yudal on a 2.5% agarose gel

with the SCM07UP2 + LP1 primers. Although the three allelic forms can be detected on acrylamide gels for the OPM07-730 locus with this test, the second locus, also amplified, gave a strong interference which did not allow us to genotype the locus linked to the dwarf gene (data not shown).

In conclusion, in comparison to a2-1 and a2-2, the a1 allelic form could be easily detected on agarose with the SCM07UP1 + LP2 test, while a2–1/a2–2 allelic forms could be differentiated on non-denaturing acrylamide with the SCM07UP1 + LP1/*Eco*RV-digest test.

Discussion

The OPM07-730 fragment was cloned and sequenced. Specific primers were defined to amplify the locus linked to the dwarf gene and three allelic forms were observed among the 40 *B. napus* accessions tested.

Cloning the OPM07-730 fragment

Seven positive clones of the approximately expected size which cross hybridized with the purified OPM07-730 band were recovered on 47 white colonies tested. The relatively low percentage of colonies containing the fragment of interest could be explained by the use for cloning of crude PCR re-amplification DNA. This PCR product contains the band of interest and many other bands because the original OPM07-730 fragment cut from the RAPD gel was not pure. We decided to use crude PCR DNA for cloning because manipulations for purification of the band under ultra-violet light could affect the quality of the poly-A ends, and hence the efficiency of the cloning procedure.

Sequence of the OPM07-730 clone

After sequencing, it appeared that the OPM07-730 fragment in fact contains 803 bp. The 730 bp of the RAPD band were initially estimated with a standard linear regression in comparison to the respective RF of the bands of the size marker (phage lambda DNA *Eco*RI and *Hin*dIII digest). The 9% error observed was probably due to the imprecision of the measurement of the RF of the ladder bands on a standard 8×10 cm photograph.

Polymorphism at the SCM07a locus

Three allelic forms were determined at the SCM07a locus. As a1 could be amplified with the SCM07UP1 + LP2 primers, and as this polymorphism was not revealed by the primers SCM07UP2 + LP2, we supposed that it was located 5′ of the EcoRV restriction site (Fig. 1). The a2-1 and a2-2 allelic forms were well distributed among the *B. napus* accessions tested (Fig. 4 and Table 1), and only a2-1 was recovered in the dwarf accessions. As a2-1 and a2-2 alleles were identified in the 363-bp band (Fig. 4) and were not revealed by the SCM07UP2 + LP2 primers, we concluded that the associated polymorphism was located 3′ of the *Eco*RV fragment. Due to the relatively low number of plants tested (96), while we could assess that these two polymorphic regions were allelic forms of the same locus we could not exclude the fact that a recombination event could occur between the two regions. If we consider that one centimorgan on the genetic map was approximately 500-kb pairs on the physical map [1200-Mb pairs for the *B. napus* genome (Arumuganathan and Earle 1991) and 2400 cM estimated from Foisset et al. 1996], the genetic size of the 100-bp estimated length between a1 and a2-1/a2-2 was 0.0002 cM. As the a1 and a2-1/a2-2 polymorphisms were not in the same site on the DNA, they could not have been created by the same molecular event. The allelic form a1 was only present in Yudal, a Korean accession relatively distant from the other accessions tested. This could be due to the large genetic distance between this accession and the others tested, but also because the molecular mechanism that had created a1 could be much less frequent than the one that had created a2-1/a2-2.

The SCM07 locus is duplicated in the *B. napus* genome

Two loci were amplified by primers SCM07UP1 and LP1. The a locus corresponds to the OPM07–730 locus located on the linkage group DY6 of the *B. napus* genetic map at 0.8 cM from the *Bzh* locus and at 8.1 cM from the RFLP 1NG3a locus (Foisset et al. 1995). The b locus, which is easily detectable in 'Yudal' (Fig. 2), is located on DY12 at 8.1 cM from the RFLP 1NG3b locus. As the two loci a and b were associated with *B. campestris* and *B. oleracea* genomes respectively (Fig. 2), we concluded that these two regions could be homoeologous. These results were confirmed by Southern bloting on genomic DNA, which detected two or three loci with the OPM07-730 probe (data not shown).

The SCM07 marker is more useful than the OPM07-730 RAPD marker

Although RAPD mapping with bulked segregant analysis (Michelmore et al. 1991) was the most efficient method to find markers linked to the dwarf locus (Foisset et al. 1995), these RAPD markers had been described to be *Taq* polymerase dependant (Schierwater and End 1993) and DNA extraction protocol dependant (Pandey et al. 1996); hence, problems could occur for the recovery of these markers in other laboratories. We tested SCM07UP1 + LP1 amplification with three different *Taq* polymerases (Eurobio, Promega and Perkin Elmer) and observed no difference in the amplification products, which was not the case for RAPD amplification of OPM07-730 (data not shown). Thus, the SCM07 marker is more reliable than the OPM07-730 RAPD marker.

Moreover, the SCAR marker was more rapidly amplified than the RAPD (2 and 5 h respectively in our conditions). The most important advantage of the SCM07 marker is its co-dominant status, which makes it possible to detect heterozygous plants in polymorphic segregating populations.

Applications in rapeseed breeding

According to the genetic background of the material selected, the breeding methodology applied (pedigree breeding, in vitro microspore culture) and the environmental (natural or artificial) conditions, it can be difficult to select accurately for the dwarf *Bzh* gene in

a segregating population. For instance, when regenerating doubled-haploid plants from microspores in a greenhouse, erroneous selection of homozygous H0 dwarf plants can be due to the effects of in vitro culture and colchicine treatment on the growth of the plants. It is the same when selecting semi-dwarf or dwarf plants in segregating F_2 (selfing) or BnF_1 (back cross) populations due to severe competition between plants. The impact of this is exacerbated by the fact that it is necessary not to select too short lines to be sure not to affect the yield potential of the selected varieties. Thus the SCM07 marker will be very useful to accurately genotype plants in such segregating populations at any stage when the two parental lines can be differentiated by the marker. For instance, we can now imagine the selection at an early developmental stage of homozygous *bzh*/*bzh* dwarf plants in BnF_2 progeny in order to individually increase seeds of each selected plant under a small cage before field testing. For optimal selection of the dwarf character, the gene has to be cloned. For this, a candidate-gene approach or a map-based cloning approach could be initiated. After cloning the gene, a PCR test to detect the EMS-created mutated allele could be choosen. This kind of diagnostic should allow us to detect the mutated allele of the dwarf gene in all genetic backgrounds.

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