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Inheritance of seed colour and identification of RAPD and AFLP markers linked to the seed colour gene in rapeseed (*Brassica napus* L.)

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Abstract In China Polima cytoplasmic male sterility (cms) is currently the most important hybrid system used for the breeding of hybrids. In an effort to develop yellow-seeded Polima cms restorer lines, we used yellow-seeded, doubled haploid (DH) line No.2127-17 as the gene source in crosses with two elite black-seeded Polima cms R lines, Hui5148-2 and 99Yu42, which originated from our breeding programme. The inheritance of seed colour was investigated in the F_2, BC_1 and F_1-derived DH progenies of the two crosses. Seed colour was found to be under the control of the maternal genotype and the yellow seed trait to be partially dominant over the black seed trait. Segregation analysis revealed a single gene locus for the partial dominance of yellow seed colour. Of 810 randomly amplified polymorphic DNA (RAPD) primers, 240 (29.6%) revealed polymorphisms between the parents. Of the 240 RAPD primers and 512 amplified fragment length polymorphism (AFLP) primer pairs, four RAPDs and 16 AFLP pairs showed polymorphisms between the bulks, with two RAPD and eight AFLP markers being identified in the vicinity of the seed-coat colour gene locus using a DH progeny population—derived from the cross Hui5148-2×No.2127-17—of 127 individuals in combination with the bulked segregant analysis strategy. Seven of these latter ten markers were linked to the allele for yellow seed, whereas the other three were linked to the allele for black seed. The seed-coat colour gene locus was bracketed by two tightly linked markers, EA02MG08 (2.4 cM) and S1129 (3.9 cM). The partial dominance and single gene control of the yellow seed-coat colour trait together with the available molecular markers will greatly facilitate the future breeding of yellow-seeded hybrid varieties.

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Introduction

The genus *Brassica* provides a few important oilseed crop species, of which *B. napus* is worldwide the most important economically. A primary objective in the breeding of oilseed *B. napus* is to increase the oil yield per unit area, which is determined by the seed yield potential and oil content in the seeds of a particular variety. A common strategy to enhance the yield potential is to develop hybrid varieties, i.e. by utilizing heterosis. The approach that has be adopted to enhance oil content in the seeds is to increase oil content in the embryos or reduce the hull proportion of the seeds. Compared with black seeds, yellow seeds of *Brassica* have a significantly thinner seed coat, thereby leading to a lower hull proportion in the seed and, consequently, a higher oil content (Stringam et al. 1974). Some other advantages of yellow seeds include a more transparent oil and a higher protein and lower fiber content of the meal. These latter two properties ensure a better feeding value for livestock (Tang et al. 1997; Meng et al. 1998).

Various groups of researchers have studied the inheritance of seed colour in *Brassica* species. In *B. rapa*, Mohammad et al. (1942) reported that three independent genes controlled the black seed-coat colour trait, but Stringam (1980) concluded that brown seed colour trait was determined by two independent dominant genes. A single gene was responsible for the dominant brown seed colour of the Indian *B. rapa Toria* lines over the yellow seed of Yellow Sarson lines (Ahmed and Zuberi 1971). Hawk (1982) also reported a single dominant gene for brown seed colour in *B. rapa*. Similarly, Chen and Heneen (1992) indicated that black seed colour was conditioned by a single dominant gene and was

mainly controlled by the maternal genotype but influenced by the interplay between the maternal and endosperm and/or embryonic genotypes in this species. Heneen and Brismar (2001) reported both maternal and embryonic control of seed colour by different *B. alboglabra* chromosomes. In *B. juncea*, Vera et al. (1979), Vera and Woods (1982) and Anand et al. (1985) concluded that black seed colour was dominant over yellow and controlled by two pairs of duplicated genes. In *B. carinata*, Gentinet and Rakow (1997) suggested that a dominant repressor gene (*Rp*) exits in yellow seed which inhibits the expression of the seed-coat pigment synthesis genes. In *B. napus*, Shirzadegan (1986), Liu (1992) and Van Deynze and Pauls (1994) reported that black seed colour was dominant over yellow and controlled by three independent duplicated genes and that yellow seeds were produced only when all three loci were in the recessive homozygous condition. Rahman et al. (2001) reported that three to four recessive genes were involved in the determination of yellow seed colour in *B. napus*. In addition to the effect of genotype on seed-coat colour expression, the latter has also been shown to be affected by environmental conditions during seed development, such as temperature (Van Deynze et al. 1993) and red/blue light (Lian et al. 2003).

The recent development of PCR techniques has made marker-assisted breeding more feasible. Breeding strategies for yellow-seeded *B. napus* varieties would greatly be facilitated by the identification of molecular markers linked to the seed colour gene. Randomly amplified polymorphic DNA (RAPD) marker analysis is a simple, quick and convenient procedure requiring only limited amounts of DNA (Halldēn et al. 1994; Charters et al. 1996). Amplified fragment length polymorphisms (AFLPs) have been used for DNA fingerprinting (Vos et al. 1995) and the mapping of genes for traits of interest (Negi et al. 2000; Sardesai et al. 2002; Lu et al. 2004). In order to identify markers linked to important genes in plants, bulked segregant analysis (BSA) constitutes a particularly efficient method (Michelmore et al. 1991). Using the BSA method, Negi et al. (2000) were able to identify three AFLP markers linked to seed-coat colour in *B. juncea* and further converted one into a sequence-characterized amplified region (SCAR) marker. Van Deynze et al. (1995) identified a restriction fragment length polymorphism (RFLP) marker linked to seed-coat colour in *B. napus*. Somers et al. (2001) reported that in *B. napus* a single major gene (*pigment 1*) and two additional genes controlled seed colour and that these genes were flanked by eight, one and two RAPD markers, respectively.

The main objectives of the study reported here were to determine the inheritance mode of seed colour and to identify RAPD and AFLP markers tightly linked to the seed colour gene that would be useful for marker-assisted selection (MAS) and would also serve as a starting point for the map-based cloning of the yellow seed allele. The ultimate goal is the development of yellow-seeded Polima cytoplasmic male-sterile (cms) restorer lines for use in the major hybrid system of our breeding programmes.

Materials and methods

Plant materials

The black-seeded *Brassica napus* parents were two Polima cms restorer lines, Hui5148-2 and 99Yu42, from Huazhong Agricultural University, while the yellow-seeded parental line, No.2127-17, was a doubled haploid (DH) from a resynthesized *B. napus* line derived from the interspecific cross between *B. alboglabra Baily* and *B. rapa* L. (Chen et al. 1988). To study seed colour inheritance, we crossed the two black-seeded parents reciprocally with the yellow-seeded parent. In each cross combination, the F_1 (black seed × yellow seed) and reciprocal F_1 (RF_1: yellow seed × black seed) plants were self-pollinated to produce the F_2 and RF_2 progenies. The F_1 plants were backcrossed with black-seeded and yellow-seeded parents to generate the BC_1 and BC_2 progenies. Meanwhile, the F_1 plants of the two crosses were used to develop DH lines by microspore culture using the method described by Shi and Liu (1993). The parents, F_1, RF_1, F_2 RF_2, BC_1 and BC_2 as well as DH progenies were grown on the research farm of Huazhong Agricultural University. The selfed seeds of individual plants were harvested and scored for seed colour to determine the inheritance mode of the trait. The 127 DH lines from the F_1 of the cross (Hui5148-2 × No.2127-17) were used as the mapping population to identify the RAPD and AFLP markers linked to the seed colour gene.

Seed colour classification

The seed colour of each individual plant was measured with a CR-300 colorimeter (Minolta, Japan). The light reflectance values for yellow to black seeds varied along a continuous gradiant from 50 to 17 (a.u.). Plants were classified into six groups on the basis of their seed colour (Fig. 1): group 1, with a value of greater than 40; group 2, with a value of 40–35; group 3, with a value 35–30; group 4, with a value 30–25; group 5, with a value 25–20; group 6, with a value of less than 20. To determine the inheritance mode of seed colour, we pooled groups 1–4 into the yellow-seeded type, whereas groups 5–6 were pooled into the black-seeded type. The χ^2 goodness-of-fit test was performed on the pooled segregation data.

DNA isolation

Total DNA from each of the 127 DH lines and the two parents Hui5148-2 and No.2127-17 was extracted using a modified sodium dodecyl sulphate (SDS) method (Li et al. 1994). For each sample, DNA concentration

Fig. 1 Seed colour of the six groups. The values *in parenthesisis* are the light reflectance values

and purity were measured by a Beckman spectrophotometer at a wavelength of 260 nm versus 280 nm. To identify molecular markers for the seed colour gene, we pooled equal quantities of DNA from ten yellow-seeded plants of group 1 and ten black-seeded plants of group 6, selected from the mapping DH population, to create the yellow-seeded bulk and black-seeded bulk, respectively. The concentration of template DNA of the two bulks and the two parents as well as of the DH lines was adjusted to 25 ng/µl with sterilized double-distilled water (ddH_2O).

RAPD and AFLP marker analysis

The two bulks along with the two parental lines were subjected to RAPD and AFLP analysis to identify putative markers linked to the seed colour gene. The RAPD reactions were carried out as follows: each reaction mixture contained 50 ng genomic DNA, 0.08 m*M* each dNTP, 0.45 μM decamer random primer (Sangon, Shanghai, China), 1.35 m*M* $MgCl_2$, 1 U *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania), 2 µl 10× PCR reaction buffer [750 m*M* Tris-HCl, pH 8.8, 200 m*M* $(NH_4)_2SO_4$, 0.1% Tween 20]. The final reaction was adjusted to 20 µl with sterilized ddH_2O. Reactions were performed in a PTC-225 Thermocycler (MJ Research, Waltham, Mass.) using the following cycling parameters: one cycle of 3 min at 94°C; 38 cycles of 30 s at 94°C, 45 s at 40°C,1 min at 72°C; one cycle of 10 min at 72°C. Amplification products were analysed following separation by gel electrophoresis on 1.2% agarose gels in 1× TAE buffer for 2.5 h at 3 V/cm. Gels were visualized by staining with ethidium bromide and photographed on a digital gel-documentation system. AFLP methodology was according to Vos et al. (1995) with minor modifications. Briefly, genomic DNA (250 ng) was restricted with 3 U each of the rare-cutting enzyme *Eco*RI and the frequent-cutting enzyme *Mse*I in a volume of 25 µl. The specific double-stranded *Eco*RI and *Mse*I adapters were subsequently ligated to the restriction fragment ends. The pre-amplification reaction was then carried out in a volume of 25 µl, including 75 ng each of primers EA and MC or MG, each having one selective nucleotide, and 5 ng of adaptor-ligated DNA. Following the amplification, 5 µl of the PCR products was analysed in a 1.0% agarose gel; the presence of a low-molecular-weight smear indicated successful amplification. The pre-amplified DNA was diluted (1:20) with sterilized ddH_2O, and an aliquot (1 µl) was used for selective amplification with M + 3 (*Mse*I-directed primer with three additional selective nucleotides at the 3′ ends; the other ends are similar) and E + 3 primers. The PCR products were then resolved on 6% denaturing polyacrylamide gels and visualized by the silver staining system (Promega, Madison, Wis.).

Data analysis

The putative markers were those RAPD and AFLP fragments that showed reproducible polymorphism between the two bulks as well as between the two parents. Each polymorphic band was treated as a separate character that was scored as a dominant marker and recorded as one for presence and zero for absence.

Linkage analysis was performed on the segregation data of all markers and seed colour in the 127 DH lines using the software package MAPMAKER/EXP 3.0 (Lander et al. 1987; Lincoln et al. 1992). The recombination frequencies between the seed colour gene and the molecular markers were calculated using the two-point analysis. The most likely map order was determined by three-point or multi-point analysis with a minimum LOD threshold of 4.0 and a maximum recombination fraction of 0.3. The recombination frequencies were converted into centiMorgans (cM) using the Kosambi function (Kosambi 1944).

Results

Inheritance of seed colour

The immediate F_1 seeds of the reciprocal crosses between black-seeded lines Hui5148-2 or 99Yu42 and the yellow-seeded line No.2127-17 were identical in colour to that of the self-pollinated seeds borne on the maternal parents of the particular cross, thereby indicating the control of seed colour by the maternal genotype. The self-pollinated seeds produced on both F_1 and RF_1 plants were of a yellow that was slightly darker than those produced by the yellow-seeded parent (Fig. 2). This revealed that seed colour was under the control of nuclear rather than cytoplasmic genes and that yellow seed was partially dominant over black. No black-seeded plants were observed in the two BC_2 (backcrosses to the yellow-seeded parent) progenies, further confirming the dominance of yellow seed over black. Table 1 shows the segregation ratios of the different progenies in the two cross combinations. A segregation ratio of 3:1 was observed for the yellow- versus black-seeded plants in the F_2 and RF_2 progenies, while a ratio of 1:1 was observed in the BC_1 and DH progenies. We therefore concluded that the seed colour segregation observed here is due to a single partially dominant gene "Y".

Identification of RAPD and AFLP markers linked to the seed colour gene

We initially used 810 random decamer primers (S1-S410 and S1000-S1400) to screen for RAPD polymorphisms between the two parents; of these, only 240 (29.6%) revealed polymorphic bands varying in size from 250 bp to 2,500 bp. One primer generated three to ten fragments. Of the 1,400 fragments observed, 540 (38.6%) were polymorphic. The 240 primers were subsequently used to detect polymorphisms between the two bulks as

Fig. 2 Seed colour of two parents and F_1

Table 1 Segregation of seed colour in the F_2, RF_2, BC_1 and DH progenies of the two crosses

Combination	Population	No. yellow seed					No. black seed			Expected ratio	χ^2 value[a]
		Group 1	Group 2	Group 3	Group 4	Total	Group 5	Group 6	Total		
Hui5148-2 × No.2127-17	F_2	7	50	70	79	206	15	63	78	3:1	0.92
	RF_2	4	40	54	71	169	11	55	66	3:1	1.19
	BC_1	0	3	26	29	58	7	60	67	1:1	0.65
	DH	21	39	2	0	62	5	60	65	1:1	0.07
99Yu42 × No.2127-17	F_2	5	29	34	47	115	7	23	30	3:1	1.44
	RF_2	7	25	36	53	121	11	33	44	3:1	0.24
	BC_1	0	1	12	29	42	4	30	34	1:1	0.84
	DH	3	13	1	0	17	2	17	19	1:1	0.11

[a] $\chi^2_{(0.05,\ 1)} = 3.84$

well as between the two parents; four of these revealed polymorphisms.

Since every AFLP assay can generally produce a large number of fragments with some polymorphisms, 512 pairs of AFLP primers (256 for E + 3/MC + 2 and 256 for E + 3/MG + 2) were used directly on both the two parents and the two bulks to identify putative markers linked to the seed colour gene. Of the 512 AFLP primer pairs, 16 were able to reveal polymorphisms between the two bulks. Typically, each primer pair could produce 50–120 distinct AFLP fragments (size range: 50 bp–1,000 bp), with an average of 87.2 fragments per primer pair.

On the basis of these results, four RAPD primers and 16 AFLP primer pairs were selected for further analysis of the ten DH lines forming the yellow-seeded bulk and the other ten DH lines of the black-seeded bulk along with the two parents (Fig. 3). Two RAPD and eight AFLP markers were determined to be closely associated with the seed colour gene based on recombination frequencies that were lower than 0.15 (Table 2). In addition, these two RAPD markers and five of the eight AFLP markers were revealed to be linked to the allele for yellow seed while the remaining three AFLP markers were linked to the allele for black seed.

Linkage analysis and map construction

To confirm the linkage relationship of the seed colour gene with the two RAPD and eight AFLP markers, we performed a co-segregation analysis of these ten markers and seed colour on the mapping population consisting of the 127 DH lines. The ten markers all segregated in accordance with the expected Mendelian ratio of 1:1 (Table 2). Tight linkage was indeed observed between the seed colour gene and the ten markers with recombination frequencies that varied from a minimum of 2.4% to a maximum of 9.4% (Table 2). The map order and genetic distances among the seed colour gene and the ten markers are presented in Fig. 4. The chromosomal region covered a map length of 53.8 cM with an average of 5.4 cM per marker. Five markers (S1129, S1130, EA05MC11, EA06MG13, EA11MG16) were located on one side of the seed colour gene, while the other five markers (EA02MG08, EA05MC12, EA07MC13, EA03MC03, EA10MG16) were found on the other side. The two flanking markers (EA02MG08 and S1129) closest to the gene had a genetic distance of 2.4 and 3.9 cM, respectively.

Discussion

In contrast to many previous reports of the yellow seed being a recessive trait in *Brassica* (see Stringam 1980 for turnip rape; Vera et al. 1979 for *B. juncea*; Shirzadegan 1986 for *B. napus*), we conclude, based on the results of the present study, that the yellow seed is partially dominant over the black seed in the plant material tested. Furthermore, the yellow seed is controlled by a single gene locus. To our knowledge, this is the first

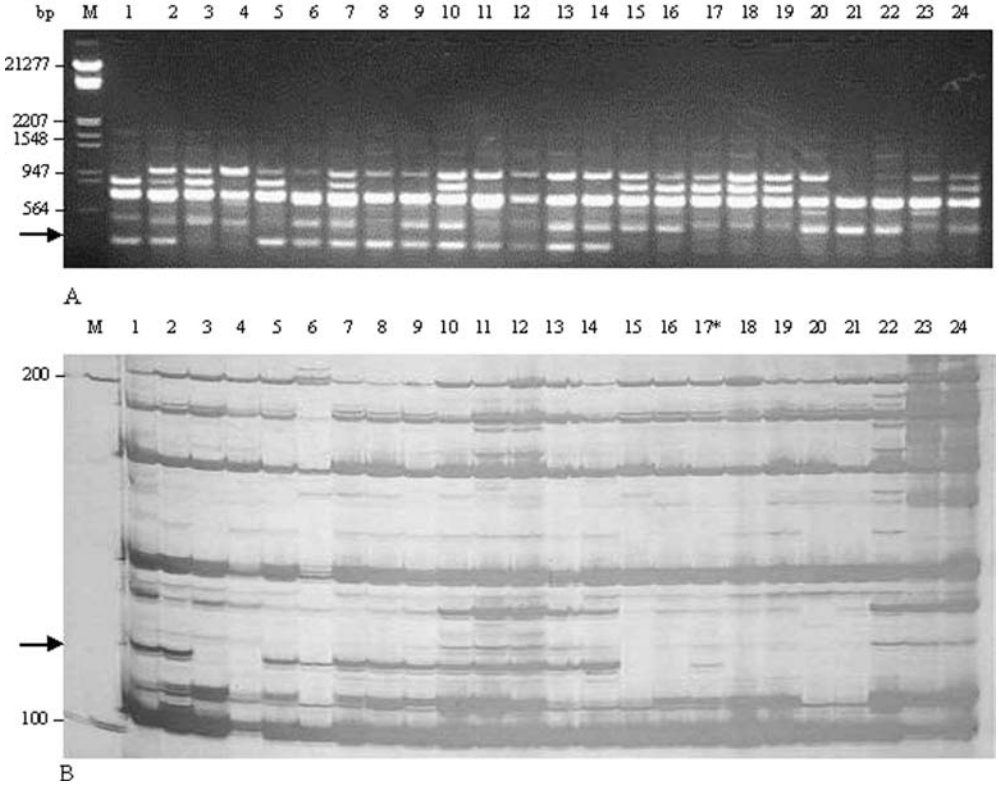

Fig. 3 Amplification patterns in the individuals of the bulks generated by S1129 (RAPD) (**A**) and EA07MC13 (AFLP) (**B**). *Lanes*: *1* No.2127-17, *2* yellow-seeded bulk, *3* black-seeded bulk, *4* Hui5148-2, *5–14* the yellow individuals forming the yellow-seeded bulk, *15–24* the black individuals forming the black-seeded bulk, *M* the size marker. *Arrow* indicates the marker, * indicates the recombinant

Table 2 Sequence, colour specificity, fragment sizes, χ^2 values, recombination values and map distances of RAPD and AFLP markers showing linkage with the yellow seed-coat gene

Marker names	Sequence[a] 5′ → 3′	Specific for:	Approximate size of marker (bp)	χ^2 value[b] (1:1)	Recombination value (%)	Map distance (cM)
S1129	GGGGGAGATG	Yellow	350	0.64	3.9	3.9
S1130	CTGTGTGCTC	Yellow	360	0.95	4.7	4.7
EA03MC03	E + AAC/M + CAC	Yellow	260	0.95	5.5	5.5
EA05MC11	E + ATA/M + CCC	Yellow	200	0.19	6.3	6.3
EA05MC12	E + ATA/M + CCG	Yellow	540	0.07	3.9	3.9
EA07MC13	E + ATC/M + CGA	Yellow	120	0.38	5.5	5.5
EA02MG08	E + AAT/M + GTG	Yellow	140	0.01	2.4	2.4
EA11MC16	E + ACC/M + CGG	Black	220	1.33	9.4	9.6
EA06MG13	E + ATT/M + GGA	Black	190	0.20	5.5	5.5
EA10MG16	E + ACT/M + GGG	Black	160	0.20	6.3	6.3

[a] *E*, *Eco*RI primer: 5′-GACTGCGTACCAATTC-3′; *M*, *Mse*I primer: 5′-GATGAGTCCTGAGTAA-3′
[b] $\chi^2_{(0.05,\ 1)} = 3.84$

report of the yellow seed trait being dominant over the black seed trait in *B. napus* . Tang et al. (1997) and Li et al. (1998) reported that different yellow-seeded genes exist in different plant materials. Consequently, it is probably not so surprising that both dominant yellow-seeded types (the present study) and recessive types (Shirzadegan 1986; Van Deynze and Pauls 1994) have been found in *B. napus*.

No natural yellow-seeded forms occur in *B. napus*. Many breeders have attempted to develop yellow-seeded *B. napus* due to the desirable quality characteristics associated with the trait (Chen et al. 1988; Liu 1992; Rashid et al. 1994; Tang et al. 1997; Meng et al. 1998; Rahman 2001; Somers et al. 2001), but only limited success has thus far been achieved, partly due to the complex and multigenic inheritance of the recessive yellow seed trait. The lack of elite and stable yellow-seeded germplasm in *Brassica napus* has been a major hindrance to success in earlier breeding efforts. The plant material that we used, No.2127-17, is a DH line that stably produces pure yellow seeds. From a breeding perspective, No.2127-17 is indeed an elite new gene source for yellow seed due to the monogenic control and partial dominance of the trait. Dominant yellow seed has particular advantages in combination with hybrid breeding. Although it is a desirable trait for quality, yellow seed is more prone to physical damage during harvesting and seed treatments due to its seed coat being thinner than that of black seed. Using a black-seeded male-sterile line and a dominant yellow-seeded restorer line, breeders will be able to produce certified black hybrid seeds for planting that will yield yellow commodity seeds for the crushing industry. We are currently performing marker-assisted backcross conversion of our elite black-seeded Polima cms restorer lines to the dominant yellow-seeded type. We anticipate that the monogenic dominant yellow seed of line No.2127-17 will be a new gene source that will greatly facilitate the future development of yellow-seeded varieties.

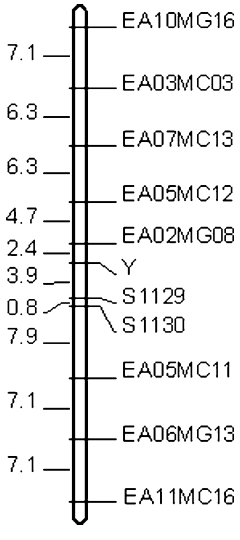

Fig. 4 Linkage map showing the RAPD and AFLP markers flanking the yellow seed-coat gene locus. Map distance (*left*) is reported in Kosambi units (centiMorgans, cM)

The tightly linked ten markers that we found will be useful for MAS in the breeding of yellow-seeded *B. napus*. Assuming no double crossover interference, application of the two closet flanking markers (S1129 and EA02MG08 at 3.9 cM and 2.4 cM, respectively; Fig. 4) will allow selection for yellow seed in a DH progeny at an accuracy of 99.91%. Moreover, the three AFLP marks linked to the allele for black seed can be used to determine if a yellow-seeded plant is heterozygous or not in pedigree selection.

In the present study, all of the RAPD and AFLP markers found to be linked to the seed colour gene followed a dominant inheritance mode, as indicated by the presence or absence of a specific fragment. Consequently, these markers cannot distinguish the homozygous from heterozygous genotypes. Moreover, RAPD primers usually anneal with multiple sites in different regions of the genome, which may thus amplify several genetic loci and be sensitive to reaction conditions, thereby resulting in poor reproducibility and reliability. The AFLP technique is generally expensive to employ. Unfortunately, these factors will likely limit the appli-

cation of these markers in the breeding for yellow-seeded *B. napus*. It is therefore highly desirable to convert these RAPD and AFLP markers (especially the S1129 and EA02MG08) markers into co-dominant SCAR markers that are fast, simple, reliable and readily applicable in breeding programmes. The two RAPD and EA05MC12 markers are larger than 350 bp, which makes them easy to convert into SCAR markers. However, the other AFLP markers, ranging between 120 bp and 260 bp, showed an inability to be directly converted into SCAR markers. We are currently examining the PCR-walking (Negi et al. 2000) or inverse-PCR (Bradeen and Simon 1998) approaches for isolating the flanking regions for conversion into SCAR markers.

One of the crucial steps in positional cloning is the discovery of molecular markers bracketing the gene of interest (Mammadov et al. 2003). Results from this study will provide the basis for physical mapping and eventual positional cloning of the dominant allele for yellow seed in the future.

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