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Development and characterization of microsatellite markers from chromosome 1-specific DNA libraries of *Vicia faba*

D. POŽÁRKOVÁ*, A. KOBLÍŽKOVÁ*, B. ROMÁN**, A. M. TORRES**, S. LUCRETTI***, M. LYSÁK+, J. DOLEŽEL+ and J. MACAS*[1]

*Institute of Plant Molecular Biology, ASCR, Branišovská 31, České Budějovice, CZ-37005, Czech Republic**
*C.I.F.A., Departamento de Mejora y Agronomía, Aptdo. 3092, E-14080 Córdoba, Spain***
ENEA C.R. Casaccia, Biotechnology and Agriculture Division,
*Via Anguillarese 301, S.M. di Galeria, I-00060 Rome, Italy****
Institute of Experimental Botany, ASCR, Sokolovská 6, CZ-77200 Olomouc, Czech Republic+.

Abstract

An integrated approach has been developed for targeted retrieval of microsatellite markers from selected regions of the field bean (*Vicia faba* L.) genome. The procedure is based on a combination of advanced physical and genetic mapping techniques and includes the following steps: *1*) flow-sorting of metaphase chromosomes, *2*) construction of microsatellite-enriched chromosome-specific DNA libraries, *3*) isolation of polymorphic microsatellite sequences from the libraries, *4*) testing chromosome specificity of the microsatellites using polymerase chain reaction with purified fractions of individual chromosome types, and *5*) integration of chromosome-specific markers into a genetic map. Several strategies for isolation of microsatellite clones were tested, including direct screening of non-enriched libraries with single or mixed probes and screening of the libraries after one or two rounds of enrichment. Finally, the usefulness of this approach was demonstrated by the retrieval of novel markers from a selected portion of the largest field bean chromosome (No. 1).

Additional key words: field bean, flow-sorting, genetic map, physical mapping, plant chromosomes.

Introduction

Microsatellites or simple sequence repeats (SSRs) are composed of tandemly arranged repetitions of short (1 - 5 bp) nucleotide motifs that form arrays generally less than 150 bp long (Tautz and Renz 1984, Litt and Luty 1989). They are widely distributed in all eukaryotic genomes (Hamada *et al.* 1982, Ellegren 1993, Cregan *et al.* 1994) although abundances of individual motifs vary among and within individual species (Beckmann and Weber 1992, Lagercrantz *et al.* 1993). Microsatellites frequently exhibit length polymorphisms, caused by variations in the number of repeats and by insertion-deletion events in adjacent regions (Kostia *et al.* 1995). This variability can be detected using a polymerase chain reaction (PCR) with primers complementary to microsatellite flanking sequences. Microsatellite loci that give polymorphic bands segregating in a Mendelian fashion can be used as powerful and informative genetic markers, as was shown in a wide variety of species including humans (Aydin 1999), animals (Grimm *et al.* 1997) and plants (Brondani *et al.* 1998, Ponce *et al.* 1999, Saal and Wricke 1999). Besides identification of microsatellites in known sequences deposited in DNA databases (Morgante and Olivieri 1993, Wang *et al.* 1994, Smulders *et al.* 1997), the microsatellite markers are usually developed

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Abbreviations: SSRs - simple sequence repeats; DOP-PCR - degenerate oligonucleotide-primed polymerase chain reaction.
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[1] Corresponding author; fax: (+420) 38 5300356, e-mail: macas@umbr.cas.cz

based on newly identified sequences retrieved from various kinds of genomic DNA libraries (Wu and Tanksley 1993, Powell *et al.* 1996, Pfeiffer *et al.* 1997). These libraries are often enriched for microsatellite sequences using hybridization-based subtraction prior to cloning, in order to increase the frequency of the desired clones (Armour *et al.* 1994, Kijas *et al.* 1994, Lench *et al.* 1996, Hamilton *et al.* 1999, Lunt *et al.* 1999, Paetkau 1999).

Development of genetic linkage maps, useable in marker-assisted breeding of crop plants, requires their saturation especially in the regions harboring agronomically important traits. However, most of the current approaches produce markers randomly distributed along all chromosomes. This obstacle could be circumvented by isolation of molecular markers from selected portions of a genome (*e.g.* individual chromosomes) known to contain genes of interest. Using DNA libraries prepared from individual chromosome types proved to be a powerful tool for isolation of chromosome-specific sequences and physical genome mapping in human and animals (Van Dilla and Deaven 1990, Vaiman *et al.* 1997). Recently, the methodology for obtaining highly purified fractions of metaphase chromosomes using flow-sorting has also been developed for several plant species, including field bean, garden pea, maize, barley, and wheat (Doležel *et al.* 2001). However, due to technical difficulties, the preparation of molecular markers from isolated chromosomes has only rarely been used for plants and remained limited to using RFLP markers (Wang *et al.* 1992, Arumuganathan *et al.* 1994). In our previous experiments (Macas *et al.* 1996) we successfully tested the possibility of isolating microsatellite clones from chromosome-specific libraries of *V. faba*. Therefore, in this study we also concentrated on developing the microsatellite markers, as they are generally more polymorphic and easier to score than RFLPs (Kijas *et al.* 1997, Barreneche *et al.* 1998, Hicks *et al.* 1998, Korzun *et al.* 1999).

Due to its large chromosomes and availability of lines with reconstructed karyotypes (Schubert *et al.* 1986), field bean (*Vicia faba* L., 2n = 12) has become a model species for plant flow cytogenetics and chromosome sorting (Lucretti *et al.* 1993, Doležel and Lucretti 1995). Flow-sorted field bean chromosomes were used for physical mapping of seed storage protein genes (Macas *et al.* 1993) and for preparation of a set of chromosome-specific libraries covering the whole genome of this species (Macas *et al.* 1996). Several physically localized sequences have also been integrated into a genetic linkage map (Vaz Patto *et al.* 1999). Although the field bean genetic map is being continually improved, new approaches allowing more efficient mapping of this large genome (1C = 12.780 Mbp, Bennett and Leitch 1995) are needed. These include both the development of new markers and the integration of genetic and physical maps. The latter aim can be achieved using trisomic lines at least for chromosomes 3, 4, 5 and 6 (Cabrera *et al.* 1989, Torres *et al.* 1995, 1998, Satovic *et al.* 1996, Vaz Patto *et al.* 1999), however, this approach does not allow targeted retrieval of the new markers from selected genomic regions.

In this work we focused on preparing microsatellite markers from a defined part of the field bean chromosome 1. This chromosome is the largest one in a wild-type field bean karyotype (Schubert *et al.* 1986) and the lines trisomic for this chromosome are not available. The chromosomes were purified using flow-sorting and used for preparation of DNA libraries that subsequently served for isolating fragments containing microsatellite sequences. The efficiency of microsatellite isolation from several libraries constructed with or without an enrichment step was compared and the isolated markers were localized using both physical and genetic mapping. The data presented in this paper demonstrate that the methodology for isolation of chromosome-specific microsatellite markers can be successfully applied to plants and provides a means for the targeted retrieval of markers from selected regions of the plant genome.

Materials and methods

Plants: Seeds of field bean (*Vicia faba* L.) translocation lines EF and JF were kindly provided by Dr. I. Schubert (IPK Gatersleben, Germany). Parental lines Vf 6 (*equina* type) and Vf 27 (*paucijuga* type) used for linkage analysis were obtained from the collection of genetic variants at the Escuela Técnica Superior de Ingenieros Agronomos de Montes in Cordoba, Spain. Total genomic DNA from the translocation lines was extracted from leaves as described by Dellaporta *et al.* (1983); DNA from the parental lines and F_2 individuals was extracted from young leaf tissue using the method proposed by Lassner *et al.* (1989) and modified by Torres *et al.* (1993).

Library construction: Purified fractions of individual chromosome types were flow-sorted and their DNA was cloned essentially as described by Macas *et al.* (1996). In brief, samples of 250 chromosomes No. 1 from the field bean translocation line JF (Fig. 1) were collected using the *FACStar Plus* flow cytometer and sorter (*Becton Dickinson*, San José, USA) and amplified in a modified degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR; Telenius *et al.* 1992, Macas *et al.* 1996) using the primer K6-MW (5′-CCG AGG TAC CNN NNN NAT GTG G-3′). Amplified fragments were either directly cloned into *pBluescript*^{R} *II SK*$^{(+)}$ plasmid vector (*Stratagene*, La Jolla, USA) using *Kpn*I restriction

site present in the primer, or subjected to an enrichment step prior to the cloning. The enrichment procedure consisted of hybridization of denatured DOP-PCR products to 5'-biotinylated $(GA)_{13}$ or $(GATA)_6$ oligonucleotides immobilized on streptavidine-coated magnetic beads (*Dynabeads® M-280 Streptavidin, Dynal AS,* Oslo, Norway) as described by Koblížková *et al.* (1998). After releasing retained DNA from the particles, the fragments were reamplified for 28 cycles in a DOP-PCR reaction using only a stringent primer annealing temperature (56 °C). The amplified DNA was cloned or subjected to a second round of subtraction prior to cloning.

Clones containing microsatellite sequences were identified by probing duplicate colony lifts (*Hybond-N+* membrane, *Amersham*, UK) with alkaline phosphatase-linked oligonucleotide probes using the *Quick-Light* Genome Mapping Probe and Hybridization Kit (*LIFECODES*, Stamford, USA). The screening was performed with corresponding microsatellite probes $(GA)_n$ or $(GATA)_n$, respectively and, in the case of the unsubtracted library, also with a mix of probes for $(CTTT)_n$, $(ACT)_n$, $(AAG)_n$, $(CAC)_n$, and $(AAC)_n$. Hybridization, washing and signal detection were performed according to the manufacturer's recommendations, using 55 °C as hybridization and washing temperature for $(GA)_n$, and 42 °C for remaining probes. Positive colonies were analyzed with respect to size of the inserts, presence of the microsatellite, and its location within the insert using PCR with combinations of microsatellite- and vector-derived primers (T3 and T7 primers).

Characterization of microsatellite clones: Selected clones were sequenced by the dideoxy-chain termination method (Sanger *et al.* 1997) and resulting sequences were subjected to computer analysis. Regions flanking microsatellite repeats were searched for homology to known sequences in the *GenBank* database (release 114) using *BLASTN*, *BLASTX 2.0.2* (Altschul *et al.* 1997) or *FASTA 3.2* (Pearson and Lipman 1988). Primers for PCR amplification of individual microsatellite loci were designed using the *PRIMER 0.5* program (Lincoln *et al.* 1991) and synthesized by *Generi Biotech* (Hradec Králové, Czech Republic). The amplification conditions were optimized for each primer pair using Vf6 and Vf27 genomic DNA as a template. The reactions were performed in 0.015 cm^3 of 1x PCR buffer, 1.5 mM $MgCl_2$ 0.2 mM dNTPs, 0.2 μM of each primer, 0.6 U of *Taq* DNA polymerase (*Promega,* Madison, USA), and 50 ng of template DNA. Reactions were overlaid with two drops of mineral oil to prevent evaporation and subjected to a touchdown PCR using *Techne Cyclogene* (Cambridge, UK) thermal cycler. The PCR profile consisted of initial denaturation (95 °C for 3 min) followed by cycles of denaturation at 95 °C for 50 s, primer annealing for 30 s, and primer extension at 72 °C for 1 min, and by a final extension step at 72 °C for 10 min. Initial cycles were performed using elevated annealing temperature that was decreased by 2 °C every two cycles until the annealing temperature based on the primer's T_m was reached (profiles used for individual primer pairs are listed in Table 2). The PCR products were separated on vertical non-denaturing 10 % polyacrylamide gel using *PROTEAN II xi Cell* electrophoresis apparatus (*Bio-Rad,* Hercules, USA) at 19 mA for 6 - 8 h (1x TBE was used as a running buffer). The resulting DNA bands were visualized by ethidium bromide staining (0.5 μg cm^{-3} in 1x TBE, 30 min at room temperature). Allele sizes were determined by comparison with a λ/*Pst*I ladder.

Physical and genetic mapping of microsatellite clones: In order to confirm their chromosomal specificity, selected microsatellite loci were physically localized as described by Macas *et al.* (1993). PCR reactions with 350 flow-sorted chromosomes were performed in 0.05 cm^3 of 1x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, and 2.5 U of *Taq* DNA polymerase using the following conditions: initial denaturation at 94 °C for 5 min, 35 or 40 cycles of 1 min at 94 °C, 50 s at respective annealing temperature (see Table 2), and 2 min at 72 °C, with final elongation step of 72 °C for 10 min. 0.025 cm^3 of the PCR products were analyzed on ethidium bromide-stained 2 % agarose gels.

Segregation and linkage analysis of the polymorphic microsatellite loci was performed on F_2 populations obtained by crossing parental lines Vf 6 and Vf 27. Fifty six to sixty individuals from three F_2 families (disomic, trisomic for chromosome 4, and trisomic for chromosome 6) were analyzed. Goodness-of-fit to the normal codominant 1:2:1 or the dominant 3:1 ratio was tested by χ^2-analysis. Linkage relationship were determined by the software *MAPMAKER V 2.0* (Lander *et al.* 1987) using a LOD score of 3.0 as the threshold for considering significant linkage. Recombinant fractions were converted to centiMorgans (cM) using the Kosambi mapping function (Kosambi 1944).

Results and discussion

Construction of chromosome-specific DNA libraries and retrieval of microsatellite clones: Field bean line JF with a reconstructed karyotype was used as a source of chromosomes for flow-sorting. In this line, the chromosome 1 is greatly reduced in size due to reciprocal translocation of its shorter arm with a small portion of the

short arm of chromosome 5 (Fig. 1*A*). It can be reliably discriminated on a flow karyotype (Fig. 1*B*) and

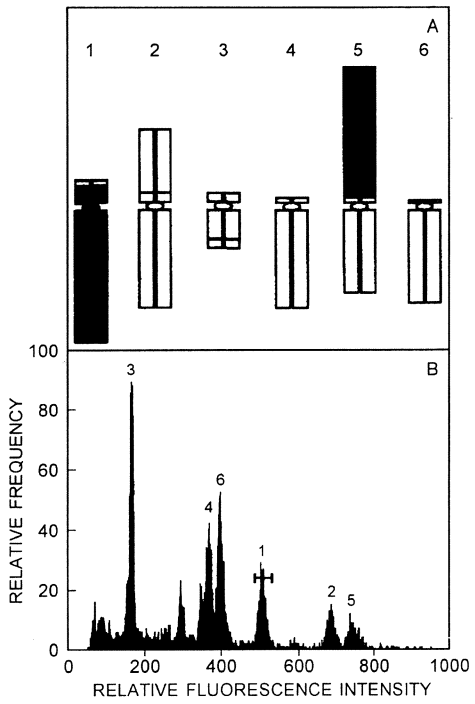

Fig. 1. Flow-cytometric analysis of *V. faba* with reconstructed karyotype JF. *A* - Scheme of the karyotype JF with segments of wildtype chromosome 1 displayed in black. In this karyotype, chromosome 1 is composed of the long and a small portion of the short arm of wildtype chromosome 1 fused with a part of the short arm of chromosome 5. *B* - A histogram of relative fluorescence intensity (flow karyotype) obtained after analysis of DAPI-stained chromosomes isolated from the line JF. Peaks representing individual chromosome types are labeled by numbers. A window used to sort chromosomes 1 is indicated by a *bar*.

subsequently sorted with high purity (the fractions used in this study generally exceeded 95 % purity). Samples of 250 flow-sorted chromosomes 1 were amplified using DOP-PCR, subjected to one or two rounds of hybridization-based enrichment for microsatellite $(GA)_n$ or $(GATA)_n$, and cloned into a plasmid vector.

Three libraries were constructed by cloning chromosomal DNA after one and two cycles of enrichment for $(GA)_n$, and after two cycles of enrichment for $(GATA)_n$, respectively (Table 1). The libraries were screened with probes for microsatellites used for the enrichment to identify clones containing corresponding sequences. Compared to the library VfJF01 constructed previously from the same chromosome but without the enrichment (Macas *et al.* 1996), the frequency of positive clones was 14.5-fold higher in the library after one, and 245-fold higher after two rounds of enrichment for $(GA)_n$, respectively (Table 1). The observed degree of enrichment was comparable with other studies using libraries prepared in a similar way. For example, Lyall *et al.* (1993) achieved 19-fold enrichment in $(CA)_n$ library constructed from flow-sorted human chromosomes. In higher plants, Huang *et al.* (1998) have reported 50-fold enrichment for (AC/GT) and (AG/CT) repeats in genomic libraries of kiwifruit (*Actinidia chinensis*).

Due to very low abundance of $(GATA)_n$ microsatellites in eukaryotic genomes (Beckmann and Weber 1992, Wang *et al.* 1994) the screening for these repeats was performed only in the enriched library that yielded 1.1 % of positive clones (Table 1). Twenty-six $(GA)_n$-positive clones and 12 $(GATA)_n$ clones were sequenced after having determined the presence and positions within inserts of the microsatellite regions using PCR. Two of the $(GATA)_n$ clones occurred more than once (GATA11, eight-times; GATA3, two-times); thus, only four different sequences were identified among the twelve clones. On the other hand, all $(GA)_n$ clones were unique and no duplication was found. Primers were designed for 17 of these sequences (Table 2).

As an alternative to the approach described above, a non-enriched library VfJF01 was screened with a mix of $(CTTT)_n$, $(ACT)_n$, $(AAG)_n$, $(CAC)_n$, and $(AAC)_n$ probes. Fourteen randomly selected positive clones were sequenced and four of these were used for primer design (clones SM4, SM9, SM11 and SM15; Table 2). The primers were also prepared for four loci containing $(GA)_n$ or $(AAC)_n$ repeats isolated from the same library in our previous study (Macas *et al.* 1996) (clones JF1-AG2, JF1-AG3, JF1-AAC2, JF1-AAC3; Table 2).

Table 1. Enrichment of chromosome 1-specific DNA libraries for clones with $(GA)_n$ and $(GATA)_n$ motifs

Microsatellite motif	Number of enrichment cycles	Number of clones screened	Number of positive clones	Proportion of positive clones [%]	Degree of enrichment
$(GA)_n$	0	8 682	2	0.02	-
	1	4 733	14	0.29	14.5 x
	2	7 305	359	4.90	245.0 x
$(GATA)_n$	2	1 440	16	1.11	-

Table 2. Characterization of selected microsatellite loci ([a] - the structure of the microsatellite region observed in the allele sequenced, [b] - the length of the allele sequenced, [c] - touchdown PCR: PCR amplification was started from reported annealing temperature that was decreased by 2 °C every two cycles until final annealing temperature was reached.

Locus	Repeat structure[a]	Product size [bp][b]	PCR profile[c] Touchdown from [°C]	Final annealing [°C]	No. cycles	Forward primer (5'- 3')	Reverse primer (5'- 3')
GA3	$(GA)_{14}$	192	67	55	25	TTGGGAGTTTAAGGGAAGAA	ATCATGACCTAGCGGATGAT
GA4	$(CT)_{16}$	232	68	62	23	GAACTAAGGTGTACACGCGGG	GGGGGGTAGATCTTGTTTTTTCC
GA6	$(GA)_2GT(GA)_8TA(GA)_6$	184	65	55	35	GAAAGGTTTTCTGATTCCAACAA	GACAGTGTACGTAGACCCACGC
GA11	$(GA)_{14}$	242	65	55	23	GTTGAGGCAGGTTAGGAGGG	GTTTGCCAGTTACTTTGTTCACG
GAII3	$(CT)_{17}$	300	63	51	26	GAAAAAAAAAAAAGAAGCCAGGG	GGGGTTTCCGGGTTAGATGTT
GAII5	$(CT)_{20}$	198	64	52	35	GGCATGTACTGGATCGCAAGA	GCAAAGAGAGTGGAGCGTCG
GAII8	$(CT)_{18}CC(CT)_3$	351	63	51	26	GTTATTATTATGTACGCGCGTGC	GAATAAGCAGAAACGCGACGT
GAII12	$(CT)_{22}TT(CT)_6$	341	63	51	26	GGTTTCGAAGGGGAAATGGAT	GTTGGAGAAGATGAAGTGATTTT
GAII30	$(CT)_{21}$	281	65	55	25	GGAAAATATGATGAAAAAGCCGC	GAGTCGATATCACGTCGGAGG
GAII54	$(CT)_2AT(CT)_{16}TT(CT)_6$	251	66	58	35	GGGTCACACATCAAGCATTGG	GCATGGCGGTTTTAGGGTG
GAII59	$(CT)_{28}$	250	66	58	35	GTAATGTGGCCCAATCCAATT	GTGAATTGTTGAAGATGGATGAA
GAII67	$(CT)_{18}$	248	66	58	22	GATTCAGAGAAAAGGTGGAAAGC	GTCACATGATGAAGCTAGATGGC
GAII68	$(CT)_2TT(CT)_{19}$	285	66	58	35	GATGTGAAGCGCAAGTGACTG	GCAATTTCCGGTCAGTGGC
GATA2	$(GA)_3(GATA)_4(GA)_4$	152	66	58	27	GACGCACCCTACTGCATCAC	GGTGTCCCACCAGGTAACTCG
GATA3	$(CA)_3(CT)_5(CTAT)_5$	205	66	58	27	GTGACCGGTCAACAATTTTCA	GTTGAAGAGAGTTGAGAAAACGG
GATA5	$(GATA)_2GGT(GATA)_6$	291	66	58	27	GAAACGTGCCTATGTAAAGGGA	GAATCTGATTCTGCTCAAAAGCA
GATA11	$(CT)_5(CTAT)_4$	233	66	58	27	GTTGCACTACATTTCGTCTTTGG	GTTGCAACAGAAGAGGCCAC
SM4	$(CTT)_7$	335	65	55	23	GTGTTGAAGGCACTTCAGCAG	GCCCATTGACCTTGCTTGG
SM9	$(CAC)_4$	184	68	62	18	GGCAGTGGAGGTTTCATTTCC	GGCATCCCCTGTTGATCAATC
SM11	$(GAA)_5$	211	68	62	18	GTGGTCGAAAACCTGACAAGA	GGAGCATCCTGAATCCAAGTACC
SM15	$(CTT)_4CCT(CTT)$	173	66	56	23	GCGATCATATAGGGCGAATGG	GGGTGAAGGAGAGGGAAAAGG
JF1-AG2	$(CT)_7AT(CT)_2T(CT)GA(CT)$	181	65	55	35	CAAAGCAGTTTTTGTTACATGG	TTGTTAAACTAAATAATGGCGTG
JF1-AG3	$(GA)_5TA(GA)_4TA(GA)_{11}$	350	67	55	35	ATGCTGAGGATGCAGGATCGA	TAATTTGTTGGTCTCAGTGC
JF1-AAC2	$(AAC)_4A(AAC)_3TC(AAC)$	136	65	55	35	GATGTATGCGACACAACATGTAT	GCTCACATCCAAAGTTGAATC
JF1-AAC3	$(AAC)_4TAATTC(AAC)$	183	65	55	35	GCCAACTTAACGAGCATCAGC	CTGAATTGAGAAATTGCAGG

Characterization of microsatellite clones: Altogether, total of 15 $(GA)_n$, 4 $(GATA)_n$, 3 $(AAG)_n$, 1 $(CAC)_n$ and 2 $(AAC)_n$ clones were used for further analysis (Table 2). They included 11 perfect tandem repeats, 11 imperfect (interrupted by several non-repeat nucleotides) and 3 compound repeats with the main tandem block of the $(GATA)_n$. The length of the longest uninterrupted microsatellite was 56 bp [$(GA)_{28}$ in the clone GAII59].

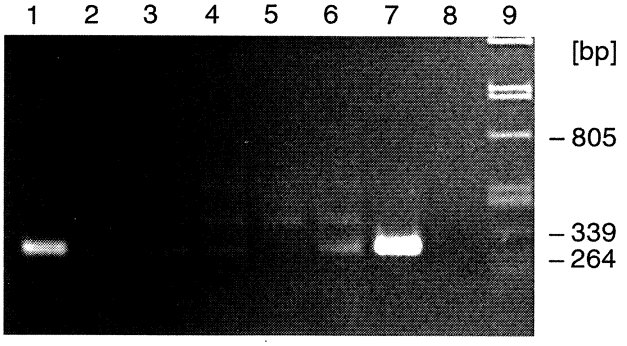

Fig. 2. Confirmation of chromosome-specificity of newly isolated markers. An example of agarose gel electrophoresis of PCR products obtained after amplification of DNA from flow sorted field bean chromosomes with the GAII68 primer pair. Chromosomes 1 to 6 of karyotype EF (*1* - *6*), genomic DNA (*7*), no DNA (*8*), size marker (*Pst*I-digested λ DNA - *9*).

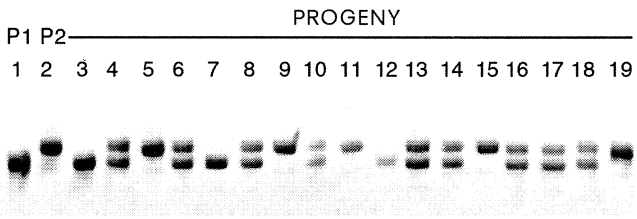

Fig. 3. Segregation of the marker GATA2 in F_2 populations. *Lanes P1* and *P2* represent parental lines Vf 6 and Vf 27, respectively. *Lanes 3* to *19* are individuals of F2 population derived from the cross Vf 6 × Vf 27.

Twenty five primer pairs were tested for amplification of the expected fragments using plasmid clones and for length polymorphisms using three sets of plants representing the cross Vf 6 × Vf 27. All primers successfully amplified the expected fragments when corresponding plasmids were used as a template. In order to obtain highly specific and background-free amplification with genomic DNA, the PCR conditions were individually optimized for each primer pair using a touchdown profile (Don *et al.* 1991) with the primer annealing temperature set above calculated T_m and gradually decreasing over initial PCR cycles (Table 2). Seven primer pairs (28 %) turned out to give a single-locus polymorphic patterns; the other primers amplified either a single monomorphic fragment of the expected size (9; 36 %), many fragments of various sizes (8; 32 %) or failed to generate amplification products (1; 4 %). The polymorphic microsatellite loci (GA4, GAII8, GAII59,

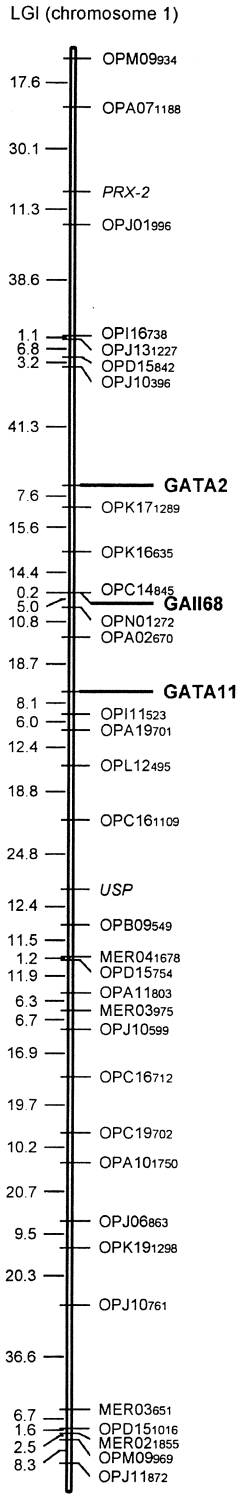

Fig. 4. Map positions of GATA2, GAII68 and GATA11 microsatellite markers on a field bean genetic linkage map of chromosome 1. Microsatellite loci are indicated in bold letters.

GAII68, GATA2, GATA11, and JF1-AG3) were also tested for their chromosome 1-specificity using PCR with fractions of all six chromosome types separated using flow-sorting. Six primer pairs amplified expected fragments only in the samples containing chromosome 1 and gave no or only weak products in reactions with other chromosomes (Fig. 2). Remaining primer pair (GAII8) produced bands in reactions with several chromosomes but not with the chromosome 1. This suggests that it amplified a moderately repetitive sequence that originated from other chromosomes contaminating sorted fractions used for the library construction. However, since all six single-copy loci were detected on the chromosome 1, it can be concluded that the process of targeted retrieval of markers from the selected chromosome proved to be highly efficient.

Genetic mapping of newly isolated microsatellite markers: In order to incorporate new microsatellite markers into the *V. faba* map, segregation and linkage analysis of polymorphic loci GA4, GAII59, GAII68, GATA2, GATA11, and JF1-AG3 were performed on an F_2 populations obtained by crossing parental lines Vf 6 × Vf 27. A total of 175 individuals from three F_2 families (disomic, trisomic for chromosome 4 and trisomic for chromosome 6) were analyzed. The GA4, GATA2 (Fig. 3) and JF1-AG3 microsatellites revealed a codominant segregation, whereas GAII59, GAII68 and GATA11 showed a dominant segregation. As the markers under study were not located on the chromosomes in trisomy (4 or 6), the normal segregation ratio was not modified due to the presence of the extra chromosome. The markers GATA2, GAII68 and GATA11 were mapped to the linkage group LG-I (Fig. 4) which was previously assigned to chromosome 1 based on the presence of physically localized *USP* genes (Macas *et al.* 1993, Vaz Patto *et al.* 1999). Therefore, the three new microsatellite markers confirmed the assignment of this linkage group and allowed its association with a defined chromosomal region. The microsatellites GA4, GAII59 and JF1-AG3 could not be assigned to any known linkage group since they remained unlinked. This result can be explained by the considerable size of the field bean genome and its relatively low saturation with markers. We expect that further saturation of the *V. faba* genome map will allow their inclusion in a specific linkage group and, in turn, it will also allow assignment of this group to chromosome 1.

Marker-assisted breeding of crop plants requires high-density maps in order to identify markers closely linked with the traits of interest. Especially in species with large genomes and less advanced genetic maps, the isolation of such markers could be highly accelerated using approaches based on the targeted retrieval of markers. As demonstrated in this paper, the methodology for such approaches is available, and due to the recent progress in chromosome flow-sorting (Doležel *et al.* 2001) it is also applicable to a growing number of crop plants.

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