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ORIGINAL PAPER

Molecular mapping of an apical branching gene of cultivated sunflower (*Helianthus annuus* L.)

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Abstract Commercial hybrids of cultivated sunflower (*Helianthus annuus* L.) are obtained by crossing a cytoplasmic male sterile line (A-line) with a restorer pollinator (R-line). The incorporation of a recessive branching trait to extend the pollination period of R-lines during hybrid seed production is laborious and time-consuming. By using target region polymorphism (TRAP) and bulked segregant analysis (BSA), we identified 15 TRAP markers linked to the b_1 (branching) locus in a population of 229 F_2 plants derived from a cross between nonbranched (HA 234) and branched (RHA 271) lines. TBr4-720 and TBr8-555 markers were linked to the b_1 gene in the coupling phase at 0.5 cM (0.004 recombination frequency). The Tbr20-297 and Tbr20-494 markers flanked the b_1 locus in the repulsion phase at genetic distances of 7.5 and 2.5 cM, respectively. Tbr19-395, also in the repulsion phase, mapped at 3.8 cM from the b_1 locus and on the opposite side of the marker Tbr20-297. The 8A1 and 15B3 restriction fragment length polymorphic (RFLP) markers of linkage group (LG) 16 of the RHA 271 × HA 234 cultivated sunflower map anchored the b_1 LG onto the RFLP map. Polymerase chain reaction (PCR)-based markers tightly linked to the recessive b_1 gene have been developed. Their identification and the incorporation of the LG containing the b_1 locus onto an RFLP map will be useful for marker-assisted selection (MAS) in breeding programs and provide the bases for map-based cloning of this gene.

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Introduction

Cultivated sunflower (*Helianthus annuus* L.) is one of the few major crops indigenous to the United States and one of the most important oilseed crops in the world. It was domesticated from wild sunflower by the Native Americans and is derived from one of the 51 wild *Helianthus* species of North American origin (Seiler and Gulya 2004). The domestication of the plants from their wild ancestors involved the increase of seed and inflorescence size, retention of the seeds until harvest and more determinate growth as compared to their wild progenitors. Determinate growth is characterized by an increase in apical dominance with the suppression of axillary branches. Wild sunflower is characterized by dominant branching with many branches along its stem, each branch with numerous small heads and small achenes (i.e. single-seeded fruits). In contrast, cultivated sunflower is characterized by a nonbranched stem terminating in a single large head and large achenes (Burke et al. 2002).

The branching trait in wild sunflower is often controlled by dominant genes. A single dominant gene (*Br*) was identified by Putt in 1940 (Miller and Fick 1997). Two decades later, Putt (1964) reported a single recessive gene b_1 at another locus controlling profuse branching. Hockett and

Knowles (1970) reported two additional duplicated genes (Br_2 and Br_3) with dominant effects as well as two complementary genes (b_2 and b_3), which produce a fully branched phenotype when both are homozygous recessive. More recently, Kovacik and Skaloud (1990) verified the existence of two genes that exhibit dominant control of branching, as well as two additional genes that produced branched plants when either gene was homozygous recessive. Gentzbittel et al. (1999) found that top branching and basal branching that are controlled by two different loci were located on the same linkage group. However, in spite of the evidence for the importance of major genes, Burke et al. (2002) suggested that control of branching is genetically complex based on the existence of several quantitative trait loci (QTL) with small effects scattered throughout the sunflower genome.

The recessive b_1 gene reported by Putt (1964) is responsible for apical branching, and has been mapped in cultivated sunflower by molecular markers developed with independent linkage group nomenclature (Gentzbittel et al. 1999; Tang et al. 2006). The gene has been located at the extreme distal end of linkage group (LG) 7 of the RFLP composite map developed by Gentzbittel et al. (1999), and at the center of LG 10 of the public simple sequence repeated (SSR) map developed by Tang et al. (2002, 2006). The closest RFLP marker identified is 3 cM from the gene in XQR × PSC8 (Bert et al. 2002), and the closest SSR marker is at 6 cM from the gene in RHA 280 × RHA 801 (Tang et al. 2006).

The genomic zone surrounding the b_1 locus seems to harbor multiple pleiotropic or linked QTLs for several important agronomic traits. It has been demonstrated that branching affects seed oil content, seed weight and other seed and capitulum characters. Branched inbred lines have higher seed oil concentration than single-head types mainly due to smaller heads, smaller achenes, and thinner hulls inherent among branched lines (Fick et al. 1974; Dedio 1980; Fick and Miller 1997). Different QTLs have been identified near or in the same zone of the recessive branching b_1 locus. Those included QTLs associated with *Sclerotinia* resistance (Mestries et al. 1998; Jouan et al. 2000; Bert et al. 2002), and QTLs explaining partially the phenotypic variance of seed oil concentration and other seed traits in populations segregating for apical branching derived from crosses of low- × high-oil inbred lines (Tang et al. 2006) and high- × high-oil inbred lines (Mestries et al. 1998; Jouan et al. 2000; Bert et al. 2003).

The discovery of cytoplasmic male sterility (CMS; Leclercq 1969) and the subsequent identification of genes for the restoration of male fertility by Kinman in 1970 (Fick and Miller 1997) led to the current high proportion of sunflower production based on hybrid seed. The commercial hybrids are produced by crossing a male-sterile inbred line (A-line) with a restorer male-fertile line (R-line). The sterility of the A-line is maintained by crossing it with its isogenic male-fertile line (B-line or M-line). Typically R- and B-lines coalesce into distinct genetic clades as a result of the necessity for maintaining heterotic groups and hybrid seed production traits such as fertility restoration and branching (Berry et al. 1994; Gentzbittel et al. 1994; Fick and Miller 1997; Mercy and Knapp 1998; Tang and Knapp 2003). Branching extends pollen shed period, facilitating synchronized flowering with female lines in hybrid seed production fields (Fick and Miller 1997). The recessive gene b_1 for apical branching reported by Putt (1964) has been widely used in the production of hybrid seed (Gentzbittel et al. 1995; Fick and Miller 1997).

The development of new R-lines requires the incorporation of the recessive branching trait, which is laborious and time-consuming. Molecular markers tightly linked to the recessive gene would identify heterozygous nonbranched plants and facilitate the development of R-lines by backcrossing. With high-resolution marker systems, markers tightly linked to a target gene can be identified in complex genomes (Bendahmane et al. 1997; Brigneti et al. 1997) by bulked segregant analysis (BSA; Michelmore et al. 1991).

The target region amplification polymorphism (TRAP) technique uses bioinformatics and EST database information to generate polymorphic markers around targeted putative candidate gene sequences. Compared with restriction fragment length polymorphism (RFLP) or other PCR-based marker systems, TRAP is quick, reproducible, and easy to set up (Hu and Vick 2003). It may be especially useful for high density mapping in regions containing genes of interest. Its utility in sunflower has been demonstrated by the identification of TRAP markers associated with disease resistance (Hu et al. 2004; Chen et al. 2007) and the nuclear male sterility gene, *ms9* (Chen et al. 2006).

Since the apical recessive branching gene (b_1) is usually incorporated in sunflower R-lines for hybrid seed production, the objectives of this study were to identify markers closely linked to the b_1 gene from F_2 progeny from an HA 234 × RHA 271 cross. This would allow easy detection of the recessive allele in backcross breeding programs using MAS, and for the incorporation of the linkage group containing the b_1 gene onto the RFLP map of cultivated sunflower developed by Jan et al. (1998). Our goal is to map simply inherited genes and PCR-based markers onto the current RFLP map to increase the efficiency of applied breeding efforts.

Materials and methods

Plant materials and phenotypic identification

A population of 229 F_2 plants derived from the cross between the USDA inbred lines HA 234 and RHA 271 was used in this study. Both parental line have $2n = 2x = 34$. HA

234 is an oilseed maintainer (B) line homozygous for the nonbranched dominant allele (B_1B_1). RHA 271 is a branched oilseed fertility restorer (R) line homozygous for the recessive b_1 allele. Jan et al. (1998) developed an RFLP linkage map from an F_2 population derived from the reciprocal cross RHA 271 × HA 234. The F_2 mapping population used by Jan et al. (1998) segregated for male sterility due to segregation of the fertility restoration gene *Rf1*, and was no longer adequate for further mapping because of the lack of F_3 families from those CMS plants when the extracted DNA was depleted.

The F_2 population was used to identify DNA markers tightly linked to the recessive gene b_1 for apical branching and a subgroup of 113 F_2 plants was used to integrate the LG containing the b_1 gene onto the RFLP map (Jan et al. 1998). Of the 229 F_2 plants, 116 plants were grown in a greenhouse at Fargo, ND and phenotyped for the apical branching trait during the winter of 1996. The F_3 families from selfed, nonbranched F_2 plants of this population were grown in the field in Fargo, ND, during 1997 to differentiate between heterozygous and homozygous nonbranched F_2 plants. The remaining 113 F_2 plants were grown in the greenhouse in Fargo and classified according to their branching phenotype during the winter of 2003. All F_2 plants and F_3 plants derived from nonbranched F_2 plants were classified as apically branched or nonbranched at the flowering stage.

Marker development and genotyping

DNA extraction

Genomic DNA was extracted from parents, F_1, and the F_2 plants following a modified version of the protocol described by Rogers and Bendich (1985). Three fully expanded leaves were cut from each of the plants grown in a greenhouse and frozen at −80°C. The leaf tissue was then lyophilized and ground to fine powder with a laboratory mill.

TRAP marker development

Two different approaches were followed for the design of fixed primers. The first approach consisted of designing PCR-primers based on the sequence of cloned genes for branching reported in the GeneBank of the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/GeneBank). The second approach was designing primers from expressed sequence tags (ESTs) of sunflower available at the Compositae Genomics Initiative database (http://cgpdb.ucdavis.edu/sitemap.html). Two fixed primers were built from the *Arabidopsis thaliana More Axillary Branching3* (*MAX3*) gene sequence (GenBank accession: At2g44990), two from the *teosinte branched1* (*tb1*) gene sequence (GenBank accession: ZMU94494), two from the rice (*Oryza sativa* L.) *Oryza sativa TB1* (*OsTB1*) gene sequence (GenBank accession: AB088343), and two were designed against the QH_CA_Contig5177 sequence that was an assembly of two ESTs. The contig sequence was aligned by BLAST searching for the proteins *MAX3* [28% (40/138) identity], *MAX4* [35% (46/131) identity] and *Ramosus Mutants1* (*RMS1*) of pea (*Pisum sativus* L.) [36% (48/131) identity] (Booker et al. 2004; Sorefan et al. 2003; Table 1). Fixed primers were designed with the program Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi; Rozen and Skaletsky 2000). Sequences of primers for markers mapped in the branching LG are listed in Tables 1 and 2.

Fifteen other fixed primers were selected from those that were previously used to isolate branching genes: *MONOCULM 1* (*MOC1*) in rice (Li et al. 2003), *OsTB1* in rice (Takeda et al. 2003), *RMS1* in pea (Sorefan et al. 2003), tb_1 in maize (*Zea mays* spp *mays*; Doebley et al. 1997), and *MAX3* in *Arabidopsis thaliana* (Booker et al. 2004).

TRAP markers were based upon 23 fixed and 14 arbitrary primers according to the protocol of Hu and Vick (2003). A single fixed primer was combined with two arbitrary primers in a PCR reaction. Arbitrary primers were 3′

Table 1 Fixed primers that amplified loci tightly linked to the b_1 locus for top branching in cultivated sunflower

Primer	Sequence (5′–3′)	Designed on base of sequence	Species
CTG5177L	TGGCACTTTCCCTAACAAAGAG	QH_CA_Contig5177[a]	Sunflower
Primer	**Sequence (5′–3′)**	**Used to isolate**	**Species and source**
M779	ACCAGGAGCAACCGAAGAAGATG	*RMS1* gene	Pea Sorefan et al. (2003)
MAX3BF	TCTTTGCCAACCGAGTCAA	*max3* alleles	*Arabidopsis* Booker et al. (2004)
MAX3BR	ACGTTATGAGCCCCATGAAGA	*max3* alleles	*Arabidopsis* Booker et al. (2004)
MOC1R3	CTAACTAGAGATCGAGTAGC	*MOC1* gene	Rice Li et al. (2003)

[a] Homologue by BLAST to *MAX3, MAX4, RMS1* proteins (GenBank accession: AAD32836, NP-195007, AAS66907)

Table 2 Arbitrary primers that amplified loci tightly linked to the b_1 locus for top branching in cultivated sunflower

Arbitrary primer	Sequence (5′–3′)	Labeled with
TRAP03	CGTAGCGCGTCAATTATG	700
TRAP04	CGTAGTGATCGAATTCTG	700
TRAP13	GCGCGATGATAAATTATC	800
Sa14	TTACCTTGGTCATACAACATT	700
Ga5	GGAACCAAACACATGAAGA	800
Odd8	CACAAGTCGCTGAGAAGG	800
Odd26	CTATCTCTCGGGACCAAAC	700

end-labeled with IR dye 700 or IR dye 800 for autodetection of the amplified fragment with a Global DNA Sequencer (Li-Cor Biosciences, Lincoln, NE). PCR was accomplished in a volume of 15 µl with the following composition: 2 µl of the 10–20 ng/µl DNA sample, 1.5 µl of 10× reaction buffer, 1 µl of 25 mM $MgCl_2$, 1 µl of 5 mM dNTPs, 0.3 pmol each of IR-800 and IR-700 dye labeled arbitrary primers, 1.0 pmol of the fixed primer, and 1.5 units of *Taq* DNA polymerase (QIAGEN, Valencia, CA). Thermal cycling consisted of an initial denaturing of template DNA at 94°C—3 min, then 5 cycles of 94°C—30 s, 37°C—45 s and 72°C—40 s, followed by 33 cycles of 94°C—30 s, 55°C—45 s and 72°C—40 s, then a final extension of 72°C for 5 min. Electrophoresis of PCR products was conducted on a 6.5% polyacrylamide gel at 1,500 V for 3.5 h in the Global DNA Sequencer. Gel images were collected with SAGA Genotyping software (Li-Cor Biosciences).

RFLP analysis

Ten micrograms of sunflower genomic DNA was digested with the enzymes *Eco*RI, *Eco*RV, and *Hind*III following the supplier's (Promega) instructions. The DNA fragments were separated by electrophoresis on an agarose gel and transferred onto a charged membrane (Hybond N+, Amersham Bioscience) by high salt alkaline conditions. The DNA was bound to the filters by UV cross-linking.

Twenty RFLP markers (5 probes × *Eco*RI, 6 probes × *Eco*RV, and 9 probes × *Hind*III) were selected from the linkage map developed by Jan et al. (1998). Plasmids with pGEM-11Zf vectors containing the above probes were picked from frozen stocks and cultured at 37°C overnight in Luria Agar (LA) containing 50 mg ampicillin/ml. A single colony picked from the plate was cultured in Luria Broth (LB) containing 50 mg ampicillin/ml overnight at 37°C with shaking at 200 rpm. Plasmid DNA was purified using a conventional method, and inserts from each of the probes were amplified using PCR with T7 and SP6 promoter primers. Probes were labeled with [^{32}P]dCTP using the Rediprime II Labeling Kit (GE Healthcare formerly Amersham Bioscience) and hybridized to membranes using conventional methods (final wash = 0.25× standard saline citrate at 60°C) to membranes containing DNA from a single line of parents, F_1, and F_2 plants. Autoradiograms were performed by exposing to X-ray films with two intensifying screens at −80°C, for 3–15 days as needed.

Screening for polymorphic markers

BSA was used to identify TRAP markers associated with the branching trait in the F_2 population. Bulks consisted of equal amounts of genomic DNA from 20 homozygous branched F_2 individuals and 20 homozygous nonbranched F_2 individuals. The parent lines and the two bulks were then subjected to TRAP analysis. TRAP fragments showing polymorphism between parents and between the two bulks were tested in the 20 individuals that made up the bulks. Primer combinations that generated reproducible polymorphic bands were assessed on the 229 F_2 plants. TRAP fragments were visually scored from a printed digital image and each one was regarded as a single dominant marker.

Map construction

RFLP markers were used as anchors for positioning TRAP markers and the b_1 locus on the RFLP map developed by Jan et al. (1998). Nineteen RFLP markers, one per LG of the RFLP RHA 271 × HA 234 map, were chosen for assaying with DNA from the parents, F_1 and the subgroup of 113 F_2 plants. The selected cDNA probes hybridized to a single restriction fragment with co-dominant pattern. Once the linkage between one RFLP locus and the LG for the b_1 gene was found, another cDNA probe was chosen from the same LG of the sunflower RFLP map and was hybridized to verify the linkage found and to obtain the orientation of the LG.

Chi-square analyses were performed on each locus to detect deviations from the expected Mendelian ratios for dominant (3:1) and codominant (1:2:1) markers in the F_2 generation. All the markers that did not fit to the expected ratio were excluded for the assignment of markers to linkage groups. The linkage map was constructed using MAPMAKER 3.0 (Lander et al. 1987). Markers were sorted in LGs using the two-point analysis "group" command with a recombination value of <0.30 and a threshold LOD score >3. An initial framework order of markers within the LG was established by selecting five loci based on their distances and LOD values, and the most likely order was determined by using the "compare" command. The framework map was retained when the difference of LOD value for the next best order was >3. Additional markers were placed into the framework at a LOD > 2.5 value using the

"try" command. The resulting marker order was tested with the "ripple command".

In the sub-F_2 population of 116 plants, nonbranched F_2 plants were scored as B_1B_1 and B_1b_1, whereas branched F_2 plants were scored as b_1b_1 so that gene b_1 was scored as a codominant marker. In the subgroup of 113 F_2 plants, the genotype for the apical branching trait was scored as B_1– for nonbranched plants and b_1b_1 for branched, because F_3 families from selfed nonbranched F_2 plants were not evaluated. Since RFLP markers were only analyzed on 113 plants, they were scored as missing data in the remaining plants of the F_2 population.

The LG nomenclature follows Jan et al. (1998). Map units (centimorgans, cM) between markers were calculated using the Kosambi's (1944) mapping function and the linkage group maps were drawn using the MapChart software (Voorrips 2002). The prefix "TBr" was used for the TRAP marker loci linked in the coupling phase and the prefix "Tbr" was used for marker loci at the repulsion phase. The prefix was followed by the number of the primer combinations and by its size in base pairs (bp; Table 3).

Results

The subgroup of 116 F_2 plants derived from the cross HA 234 × RHA 271 segregated 20 homozygous nonbranched, 66 heterozygous nonbranched and 30 homozygous branched plants, which fit to the expected ratio 1:2:1 ($\chi^2 = 3.93$, $P = 0.14$) for a major single gene. The group of 113 F_2 plants used to incorporate the LG containing the b_1 gene onto the RHA 271 × HA 234 RFLP map built by Jan et al. (1998) fit to the expected ratio 3:1 [$\chi^2 = 0.14$, $P = 0.71$] for a single gene, with 83 nonbranched plants to 30 branched plants. The segregation of the whole F_2 population was consistent with segregation of 3:1 [$\chi^2 = 0$, $P = 1$] expected for the single recessive gene b_1.

Twenty-three of the 333 primer combinations assayed to identify markers closely linked to the gene b_1 for apical branching in sunflower, using the BSA technique, showed polymorphisms associated with the branching trait. Altogether, the 23 primer combinations were used to genotype the whole F_2 population derived from the cross HA 234 × RHA 271. The 23 TRAP primer combinations involved 11 fixed primers and twelve IR-dye-labeled arbitrary primers. Among the 23 primer combinations, 21 amplified bands from both HA 234 and RHA 271 genomes (data not shown). These 23 primer combinations showed 1–14 polymorphisms, resulting in 117 markers in total, 57 dominant markers amplified from genomic DNA of HA 234 and 60 dominant markers amplified from genomic DNA of RHA 271. The 117 loci did not exhibit distorted segregation, so they fitted to the 3:1 ratio expected for a dominant marker.

The screening of the subgroup of 113 F_2 plants with the set of 19 cDNA probes, one from each LG of the RFLP RHA 271 × HA 234 map (Jan et al. 1998), showed that the b_1 locus was linked to the 15B3 marker of LG16 of the RFLP map at a distance of 44 cM. The linkage at LG16 was verified by assaying the RFLP marker 8A1 that was found linked at 15 cM from the b_1 locus in the subgroup of 113 F_2 plants.

The final map containing the branching gene was constructed with the segregating TRAP markers' data of the whole F_2 population (229 plants) and RFLPs of the subgroup 113 F_2 plants. The LG contained 18 markers, spanning a genetic distance of 45.7 cM on LG16 of the RFLP linkage map of the cultivated sunflower (Jan et al. 1998). The average distance between markers was 2.5 cM. The nearest marker linked to the b_1 locus was the cluster constituted by the TBr4-720 and TBr8-555 markers that mapped at 0.5 cM in the coupling phase (M/B_1) with the nonbranching allele B_1. The recombination frequency between the gene and TBr4-720/TBr8-555 cluster was only 0.004 (one recombinant was observed among 229 F_2 individuals); therefore, the b_1 locus and the cluster TBr4-720/TBr8-555 were tightly linked. The Tbr20-297 and Tbr20-494 markers were linked to the branching allele b_1 in the repulsion phase (M/b_1) flanking the gene at distances of 7.5 and 2.5 cM, respectively (Figs. 1, 2).

The TBr4-720 marker was a product of the fixed primer designed to clone the *RMS1* gene, and the TBr8-555 marker was generated using a primer to isolate the *MAX3* gene. The TBr10-366, TBr10-440 and TBr11-107 markers were obtained from primers previously used for cloning the *MOC1* gene (Table 4). Therefore, no special affinity was found between the branching genes from which one of the

Table 3 Combinations of primers that generated b_1-linked TRAP markers and the corresponding number of TRAP fragments amplified from genomic zones of the oilseed sunflower lines HA 234 and RHA 271

Code[a]	Primer combination	No. of TRAP markers mapped	
		HA 234 line	RHA 271 line
4	M779/Trap03-700	1	0
5	M779/Ga5-800	2	1
6	MAX3BF/Sa14-700	0	1
8	MAX3BR/Trap03-700	1	1
9	MAX3BR/Trap13-800	1	1
10	MOC1R3/Trap04-700	2	0
11	MOC1R3/Ga5-800	1	0
19	CTG5177L/Odd26-700	0	1
20	CTG5177L/Odd8-800	0	2

[a] Number corresponds to the extension of "TBr" or "Tbr" prefixes

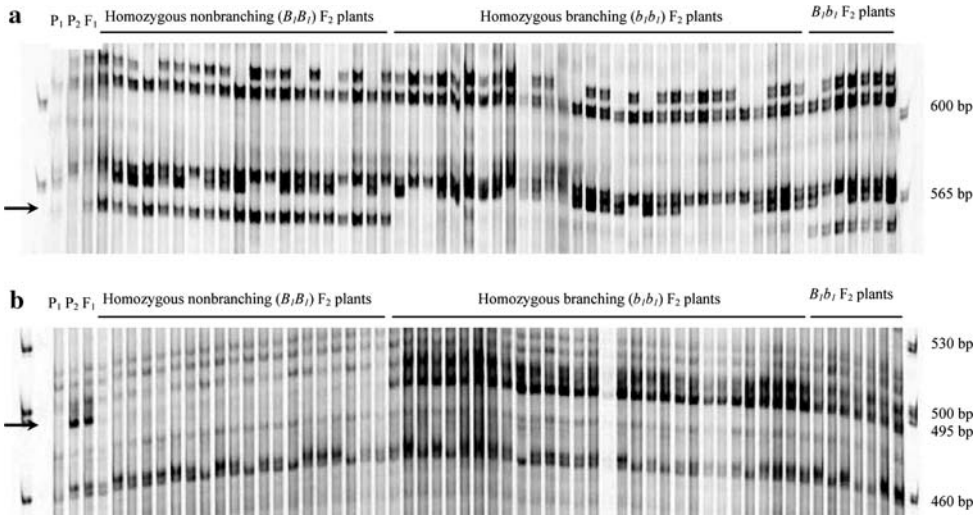

Fig. 1 TRAP profiles amplified by primer combinations TBr8-555 (**a**) linked in the coupling phase, and Tbr20-494 (**b**) linked in the repulsion phase showing polymorphism associated with the apical branching gene b_1 in F_2 plants, parents and F_1 derived from the cross between non-branched line HA 234 (P_1) and branched line RHA 271 (P_2)

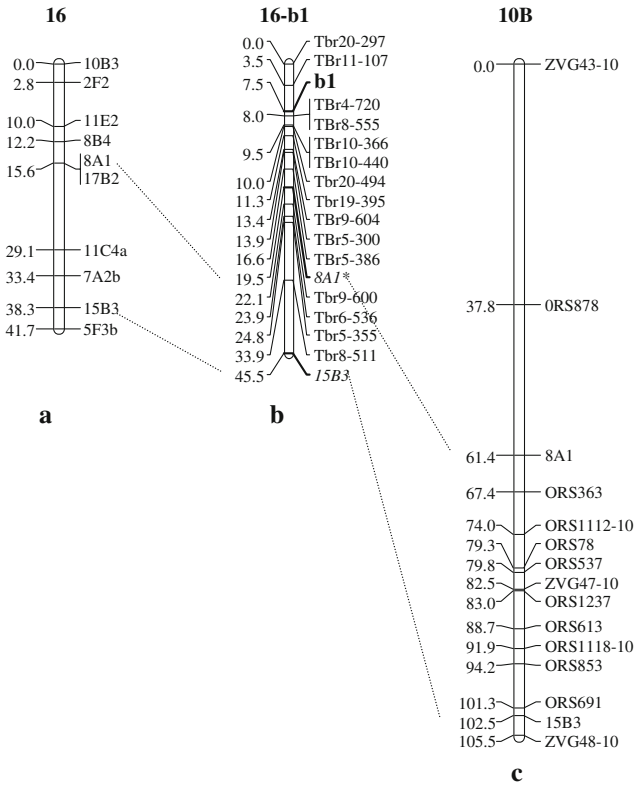

Fig. 2 **a** Map of linkage group (LG) 16 of a sunflower RFLP linkage map of RHA 271 × RHA 234 (Jan et al. 1998). **b** Molecular linkage map LG 16-*b1* of the cultivated sunflower map containing the b_1 gene for apical branching. The map was constructed using the F_2 population derived from the HA 234 × RHA 271 cross using TRAP marker segregation data. Anchor RFLP markers are identified in *italics*. The mapped marker identified with an *asterisk* was integrated with a LOD < 2.5. Prefixes *TBr* and *Tbr* were used for markers in the coupling and repulsion phases with apical branching alleles, respectively. **c** Linkage map of LG 10B of the HA370 × HA372 RFLP-SSR linkage map (Yu et al. 2003). Genetic distances on the *left* of the maps are given in centimorgans (cM) estimated with the Kosambi (1944) mapping function

fixed primers was designed and the proximity of the markers obtained to the locus b_1. On the other hand, 53, 47 and 20% of b_1-linked TRAP loci were the products of fixed primers related to *MAX3*/*MAX4*, *RMS1*, and *MOC1* genes, respectively (Table 4). It should be pointed out that only a small proportion of the markers linked to the b_1 locus is amplified from the target sequences showing high homology to the fixed primers because a low annealing temperature was used during the first five PCR cycles. This allowed for mismatch between the primers and templates (Hu et al. 2005). BSA also plays an important role in concentrating polymorphic markers around the target locus.

Discussion

Several genes involved in plant branching have been mapped and identified in maize (Doebley et al. 1997), rice (Li et al. 2003; Takeda et al. 2003), *Arabidopsis thaliana* (Otsuga et al. 2001; Tantikanjana et al. 2001; Stirnberg et al. 2002; Booker et al. 2004), pea (Sorefan et al. 2003), foxtail millet (*Setaria italica*; Doust et al. 2004), and tomato (*Lycopersicum esculentum* Mill.; Schumacher et al. 1999, Schmitz et al. 2002). In sunflower, the b_1 gene for apical branching has been previously mapped onto LG7 of a composite map by means of RFLP markers (Gentzbittel et al. 1999), and on LG10 of the public linkage group nomenclature (Tang et al. 2002, 2006; Yu et al. 2003).

In the present study, a molecular genetic map (Fig. 2) containing the gene b_1 was developed. The apical branching gene locus was positioned at the upper extremity of LG16 of the RFLP map developed by Jan et al. (1998) and was flanked by TRAP markers. LG16 of the RFLP map constructed by Jan et al. (1998) corresponds to LG10 of the public sunflower RFLP-SSR linkage map (Tang et al. 2002;

Table 4 Fixed primers that produced b_1-linked TRAP fragment linked in an F_2 population derived from the HA 234 × RHA 271 cross, the gene used to design the primers, and their distances from the branching b_1 gene

Fixed primer	Gene	TRAP marker	Distance from b_1 locus (cM)	Linkage phase to the b_1 allele
M779	*RMS1*	TBr4-720	0.5	Coupling
MAX3BR	*MAX3*	TBr8-555	0.5	Coupling
MOC1R3	*MOC1*	TBr10-366	2.0	Coupling
MOC1R3	*MOC1*	TBr10-440	2.0	Coupling
CTG5177L	*MAX3/MAX4/RMS1*	Tbr20-494	2.5	Repulsion
CTG5177L	*MAX3/MAX4/RMS1*	Tbr19-395	3.8	Repulsion
MOC1R3	*MOC1*	TBr11-107	4.0	Coupling
MAX3BR	*MAX3*	TBr9-604	5.9	Coupling
M779	*RMS1*	TBr5-300	6.4	Coupling
CTG5177L	*MAX3/MAX4/RMS1*	Tbr20-297	7.5	Repulsion
M779	*RMS1*	TBr5-386	9.1	Coupling
MAX3BR	*MAX3*	Tbr9-600	14.6	Repulsion
MAX3BF	*MAX3*	Tbr6-536	16.4	Repulsion
M779	*RMS1*	Tbr5-355	17.3	Repulsion
MAX3BR	*MAX3*	Tbr8-511	26.4	Repulsion

Yu et al. 2003), according to sunflower cross-reference maps (Gedil et al. 2001; Yu et al. 2003). So, our results agree with a previous report where the b_1 gene was mapped onto LG10 (Tang et al. 2006).

The public RFLP-SSR map (Tang et al. 2002; Yu et al. 2003) does not include RFLP markers for pairing LGs from the map developed by Gentzbittel et al. (1995, 1999). However, the cross-reference of the locus b_1 between LG16 and LG7 shows that LG16, LG10 and LG7 correspond to the same chromosome of the sunflower genome. The presence of the phenotypic marker b_1 locus in one extreme of LG7 of the RFLP map of Gentzbittel et al. (1999) and LG16 of the RFLP map of Jan et al. (1998) and in the center of LG10 of the public linkage group nomenclature (Yu et al. 2003; Tang et al. 2006) suggests that RFLP markers of LG7 could overlap with molecular markers of LG10 and LG16 and could extend the coverage of the chromosome.

The use of CMS for sunflower hybrid seed production led to the use of the male-fertility-restorer lines (R-line) which are profuse pollen producers during an extended time to cover the flowering period of the CMS female lines. This behavior in R-lines has been obtained by introducing recessive branching genes (Fick and Miller 1997). For this purpose, the b_1 gene governing apical branching has been used more widely than the *bbr* gene controlling basal branching (Gentzbittel et al. 1995). However, since a single recessive gene controls apical branching, the presence of the recessive b_1 allele in backcrossed lines can only be detected by progeny testing (Fehr 1987). Therefore, the use of MAS for the gene will be particularly powerful in eliminating the delay associated with progeny tests.

The present work has established the first set of markers tightly linked to the recessive allele b_1 for apical branching in sunflower employing the strategy of BSA. Although markers flanking the b_1 locus had been previously reported, they were not developed specifically for MAS. The b_1 locus had been mapped between the AFLP E32M49-215 and RFLP S012E1H3 markers in PSC8 × XQR at distances of 3.0 and 11 cM, respectively (Gentzbittel et al. 1999; Bert et al. 2002), and between the SSR markers ORS1088 and ORS930 in RHA 280 × RHA 801 at the distances of 10.6 and 5.8 cM, respectively (Tang et al. 2006). We developed two TRAP markers that linked in the repulsion phase with the recessive b_1 allele from HA 234 × RHA 271, Tbr20-494 and Tbr20-297, with 0.03 and 0.06 recombination units (2.9 and 6.7 cM), respectively, from the b_1 locus and also flanked the branching gene region. The next TRAP locus closest to the gene being in repulsion to the b_1 recessive allele was Tbr19-395 at 0.03 recombination units (3.9 cM). Linked in the coupling phase were TBr4-720 and TBr9-555 with 0.004 recombination units (0.5 cM) from b_1 locus (Fig. 1). We demonstrated that the TRAP technique as well as the two strategies followed for designing fixed primers were both effective and powerful approaches to generate polymorphic markers around a targeted putative candidate gene sequence.

The efficiency of MAS depends on the ability to select heterozygous genotypes during the introgression of the recessive trait into the recurrent parent background. Dominant markers linked in the repulsion phase allow the identification of heterozygous genotypes. MAS efficiency is increased by employing markers flanking the target locus compared to when a single marker is used (Chahal and Gosal 2002). The markers Tbr20-494, Tbr20-297 and Tbr19-395 TRAP are products of the primer combinations CTG5177L/Odd26-700 and CTG5177L//Odd8-800; there-

fore, they can be scored simultaneously running a single PCR reaction. In addition, they are linked in the repulsion phase and flank the gene, which makes them very useful for marker-assisted selection. Additional use of the TBr4-720 marker or the TBr9-555 marker during the selection, both of which are tightly linked in the coupling phase to the apical branching gene, will make the identification of heterozygosity even more accurate. This is due to the combination of the repulsion and coupling phase markers for MAS that can serve as quasi-codominant markers (Johnson et al. 1995; Kelly and Miklas 1998). However, the predictability of molecular markers for MAS is determined by its applicability in different genetic backgrounds; therefore, it would be recommended to screen these sets of markers in branched and nonbranched sunflower inbreds with differing genetic backgrounds.

QTLs affecting several important agronomic characters such as seed oil concentration, *Sclerotinia* resistance and seed weight have been mapped in the genome region flanking the b_1 locus (Jouan et al. 2000; Bert et al. 2002, 2003; Tang et al. 2006). QTLs overlapping on the same chromosome interval often reflect correlation between traits that may result from pleiotropic or linkage effects. Bert et al. (2003) concluded that the seed oil content was affected by QTLs linked to the b_1 locus, whereas Mestries et al. (1998) and Tang et al. (2006), studying the effect of b_1 in a different genetic background, concluded that the b_1 locus pleiotropically affected the levels of seed oil. Only pleiotropic effects were proposed for b_1-linked QTLs associated with *Sclerotinia* resistance (Mestries et al. 1998; Jouan et al. 2000; Bert et al. 2002). Pleiotropic effects of the apical branching gene on undesirable agronomic traits would be not very useful for breeders because commercial F_1 hybrids are nonbranched. Another frequent problem in backcross programs is the negative effect of the "linkage drag", i.e. undesirable genes will be introduced into the genetic background of a recipient elite line (Brinkman and Frey 1977; Tanksley et al. 1989). Linkage drag can be significantly reduced by inclusion of a marker tightly flanking the target gene in the selection process (Frisch et al. 1999; Frisch and Melchinger 2001).

The tight linkage between TRAP markers TBr4-720 and TBr9-555 with the b_1 locus will allow us to develop codominant markers cosegregating with the b_1 locus. This forms the basis for map-based cloning of the b_1 gene in sunflower and could be useful for molecular genetic and evolutionary studies. Also, it would be of interest to increase the density of this map with codominant markers to improve the coverage of this specific zone in the sunflower genome and increase the power of detection of QTL. This will help to distinguish between pleiotropic and linkage effects of b_1-linked QTL, and to develop markers for minimizing "linkage drag" effects by breaking undesirable linkages. A higher-resolution map of the b_1 locus zone could be achieved by screening the HA 234 × RHA 271 F_2 population with the SSR and RFLP loci previously mapped at LG10 of the cultivated sunflower (Berry et al. 1997; Tang et al. 2002; Yu et al. 2003) and incorporating them into LG16.

The molecular markers identified in this study will reduce or even replace progeny tests in breeding programs, and provide the bases for the positional (or map-based) cloning of the b_1 gene for apical branching of cultivated sunflower. The LG including the b_1 locus has been integrated into LG16 of the RFLP map developed by Jan et al. (1998), and corresponds to LG10 of the public map for cultivated sunflower (Tang et al. 2002; Yu et al. 2003).

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