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Combined mapping of DALP and AFLP markers in cultivated sunflower using F9 recombinant inbred lines

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Abstract A genetic map was constructed with specific PCRs, DALPs and AFLPs using F8-generation sunflower recombinant inbred lines. RI lines generated from a F2 population of one cross between the two cultivated inbred lines HA89 (maintainer for Pet1 CMS) and LR4 (restorer for Pet1 CMS) were used. A total of 305 markers were located using seven sPCR, 64 DALP and 301 AFLP loci. They were generated with one, seven and 14 primer pairs, respectively. The map construction consisted of a two-step strategy using 6 and 3.1 LOD scores revealed by a simulation file. Mapped markers were assembled into 18 linkage groups covering 2,168.6 cM with an average of 6.1 cM. The distribution of DALPs and AFLPs revealed that both markers tagged different regions to enable covering most of the sunflower genome. This leads to the longest map published so far for sunflower.

Keywords Cultivated sunflower · *Helianthus annuus* · Specific PCR · DALP · AFLP · Genetic map · Recombinant inbred lines

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Introduction

Construction of a genetic map using molecular markers is now routinely performed to assign traits and to locate genes. The genetic structure of the segregation populations is one of the main factors determining the quality of the result and the rapidity to obtain it. In plants, back-cross and one F2 population are usually preferred because they are fast and cheap to produce. On the other hand, recombinant inbred lines (RI Lines) require several years to achieve an acceptable level of fixation, but they represent a major advantage to estimate genetic and environmental effects on every trait in segregation (Burr et al. 1988).

Sunflower (*Helianthus annuus* L.) is the fourth world's leading oil crop, after oil palm, soybean and rape seed in the world, and the second in Europe after rapeseed. A major research effort was directed toward breeding improved cultivars, disease and insect controls, and components of seed quality. Considering the economic importance of the sunflower crop, there have been significant advances in the understanding of the genetic determinants of disease resistance genes (Gentzbittel et al. 1998; Brahm et al. 2000).

The increasing interest in the sunflower genome is becoming important. Several maps have been constructed until the development of large numbers of molecular markers (Isozymes, RFLPs, RAPDs, AFLPs and microsatellites) using different segregating populations (F2, F3, BC1 and RI Lines). Moreover, polymorphisms in elite lines are limited and thus some of the markers are mapped in one population but are not useful in others (Gedil et al. 2001). Consequently, anchor-marker points are still lacking to produce a consensus map with acceptable confidence. Recently, 18 expressed gene homologues have been assigned to the composite map of the sunflower genome (Gentzbittel et al. 1999) that enhances the chance to find a correlation between a gene and a trait.

Direct Amplification of Length Polymorphism (DALP), a new technique developed by Desmarais et al. (1998), combines the advantages of a high-resolution

fingerprint technique with that of providing the possibility to sequence each new marker locus. In addition, it is relatively simple and rapid to obtain in a laboratory with no special expertise. Their main advantage is that the fingerprints are obtained with the minimum number of primers: each experiment requires only two primers and between two different experiments only one is changed; all the fragments can be sequenced by the same two universal sequencing primers. Thus the cost of the study to obtain enough markers in a map is really low. Thus, the results are obtained very rapidly since one PCR followed by electrophoresis is sufficient for multi-locus analysis of population diversity (Hoarau and Borsa 2000) or to detect and define new monolocus codominant markers (Perrot-Minnot et al. 2000).

We therefore developed DALP, AFLP and specific primers on sunflower RI Lines to handle a significant sample of reliable markers for mapping. We obtained the longest map for sunflower. Several technical and scientific implications are therefore discussed.

Materials and methods

Plant materials

The parents of the cross are two inbred lines of cultivated sunflower (*H. annuus* L.) that shares the same chromosome constitution ($2n = 34$). LR4 was derived from the NSH45 commercial hybrids (Institute of Field and Crops, Novi Sad, Yugoslavia). The maintainer HA89 sunflower line was chemically emasculated with gibberellins and pollinated by the restorer LR4 line. Recombinant inbred lines were constructed according to the single-seed descent method (SSD). At the F9 generation, 232 recombinant inbred lines

were available of which only 171 were used in this study. Young leaves were harvested at the star bud stage in the field and stored at -70°C for DNA preparation.

Molecular methods

DNA preparation

DNA was extracted from 5 g of frozen leaves (without mid-veins) with CTAB using the procedure described by Gentzmittel et al. (1992). DNA concentration was adjusted to 10 ng/ μl .

AFLP analysis

AFLP markers (Vos et al. 1995) were generated according to Flores Berrios et al. (2000) with the combined set of *EcoRI* (E) and *MseI* (M) primers (Table 1). We used the Life Technologies AFLP Analysis System 1, AFLP Starter Primer Kit (Gibco BRL), according to the manufacturer's instructions.

Specific PCR (sPCR) and DALP analyses

Specific oligonucleotides were designed from cDNA library coding for the induced pathogenesis-related (IPR) proteins STH-2 and STH-21 expressed in potato during *Phytophthora infestans* (g169578) infection (Matton et al. 1993). We generated the reverse primer STH21A (19-mer) 5'-GCGTAGACAGAAGGAT-TGG-3' and the forward STH21B (19-mer) 5'-CACTTGA-GACCACAACACC-3'. Final products were not yet sequenced; thus they were considered as anonymous markers.

DALP protocol was adapted to sunflower (Desmarais et al. 1998). Amplifications were performed with seven forward selective primers and a single reverse primer (Table 2).

DALP and sPCR reverse primers were radiolabeled with 1 μl of ATP ^{32}P (converted to 3 μCi of Bq/nmol, 10 mCi idem of ^{32}P), 50 pmol of Oligo-reverse, (final concentration) 2 μl of T4 polynucleotide (PN) kinase buffer 10 \times , and 1 μl of T4 PN Kinase

Table 1 Comparison of AFLP, DALP and specific primer combinations for their capacity to generate polymorphic markers between the two sunflower inbred lines LR4 and HA89. The P%

Primer combination	Scored bands	Polymorphic bands		Mapped markers		P%	Clusters with N markers	
		LR4	HA89	LR4	HA89		N = 2	N \geq 3
E-AAC/M-CAT	136	8	10	7	9	13.6	1	0
E-AAC/M-CAG	106	6	10	5	8	15.1	0	0
E-AAC/M-CAA	126	9	12	8	10	16.7	2	0
E-AAC/M-CTT	113	13	10	9	10	20.4	1	0
E-AAC/M-CTC	103	8	10	6	8	17.5	0	0
E-ACA/M-CTG	98	10	14	9	11	24.5	2	0
E-ACA/M-CAG	117	13	15	13	14	23.9	0	0
E-ACA/M-CAC	127	16	10	14	9	20.5	7	3
E-ACC/M-CAT	99	13	6	10	6	19.2	2	0
E-ACC/M-CAA	115	14	13	10	11	23.5	2	0
E-ACC/M-CAG	98	7	12	7	12	19.5	3	0
E-ACC/M-CTA	111	15	11	8	14	23.4	3	0
E-ACC/M-CTC	103	11	10	6	7	20.4	1	0
E-ACC/M-CAC	95	9	6	4	4	15.8	0	0
DALP232	57	3	4	3	2	12.3	0	0
DALP233	67	7	4	5	4	16.4	1	0
DALP234	81	7	7	7	6	17.3	1	0
DALP235	89	5	6	5	6	12.4	1	0
DALP242	77	4	4	4	3	10.4	1	0
DALP243	83	4	5	3	4	10.8	0	0
DALP244	66	1	3	1	1	0.61	0	0
STH21	83	6	1	6	1	0.96	1	0

polymorphism rate, the number of mapped markers, and the clusters formed in linkage groups (N: the number of clustered mapped markers generated by the corresponding primer combination)

Table 2 Sequences of primers used for DALP analysis

Amplification and sequencing:USP ^a	DALPR	5'TTTCACACAGGAAACAGCTATGAC3'
Selective primers: DALP2X _i	DALP232	5'GTTTTCCAGTCACGACGAC3'
	DALP233	5'GTTTTCCAGTCACGACACG3'
	DALP234	5'GTTTTCCAGTCACGACCAG3'
	DALP235	5'GTTTTCCAGTCACGACCAC3'
	DALP242	5'GTTTTCCAGTCACGACCTAG3'
	DALP243	5'GTTTTCCAGTCACGACTACG3'
	DALP244	5'GTTTTCCAGTCACGACTGAC3'

^a USP, universal-sequencing primers. The bold group of nucleotides indicates the – 40 USP sequence

(10 U/50 pmol) in a final volume of 20 µl. The mix was incubated at 37 °C up to 1 h and reaction was stopped at 70 °C for 5 min. Amplifications were performed in a final volume of 20 µl with 1 µl of dNTP (2 µM) [final concentration 1.5 µl MgCl₂ (25 mM), 5 pmol of Oligo-forward primer, 2 µl of labeled Oligo-reverse primer, 2 U of *Taq* buffer 10×, 2 µl of DNA (10 ng/µl) and 0.5 U of *Taq* polymerase. The PCR protocol was optimized with the following program: 4 min pre-incubation at 94 °C followed by 40 cycles at 94 °C for 1 min, 49 °C for 2 min, 72 °C for 2 min with a final 6-min elongation at 72 °C. A negative control was used in each experiment to test for the presence of DNA contamination in reagents and reaction mixtures. Electrophoresis and characterization were performed according to the published protocol (Desmarais et al. 1998).

Data analyses

Two different persons visually scored AFLP and DALP autoradiograms and only markers consistent through the two readings were further taken into consideration for map construction. Segregation of alleles into genotypic classes at each locus was checked against the expected 1:1 ratio for a set of RI Lines by using a chi-square test of the MapDisto program (<http://www.mpl.ird.fr/~lorieux/mapdisto>) with a significance level of 5%.

Map construction was performed with MAPMAKER version 3.0 (Lander et al. 1987). The genomic constitution of each RI line was determined from the frequencies of each genotypic class across all loci. We first identified all pairs of linked markers using the 'two-point/group' command with LOD >6.0 and a maximum recombination fraction of 0.35. All artefacts causing wrong linkages were then avoided. The most-likely order of markers in each group was determined by multipoint analysis using the 'order', 'compare' and 'ripple' commands. Secondly, we decreased the LOD score step by step to 3.1 and assigned all linked small groups (two) and remaining markers using the 'try' command. Finally, all suitable and befitted markers were mapped and ordered without ambiguity using a LOD threshold higher than 2.0. The Kosambi mapping function was used to calculate distances (cM) between markers (Kosambi 1944). The total sunflower genome length was calculated by the formula described by Hulbert et al. (1988) with the Mathematica software (Wolfram 1988).

Results

Polymorphism rate

DNA of the two parents and the F1 hybrids were used to test the reproducibility in four replicate experiments for each primer combination. The sPCR did not produce the expected single band corresponding to the targeted *STH-2* gene but to a multilocus pattern. Nevertheless, it was further exploited as the result of arbitrarily primed PCR fingerprinting. All scored bands revealed by DALP, sPCR and AFLP pairing primers are correctly inherited and can be reproduced consistently. For each AFLP primer combination, the number of revealed bands

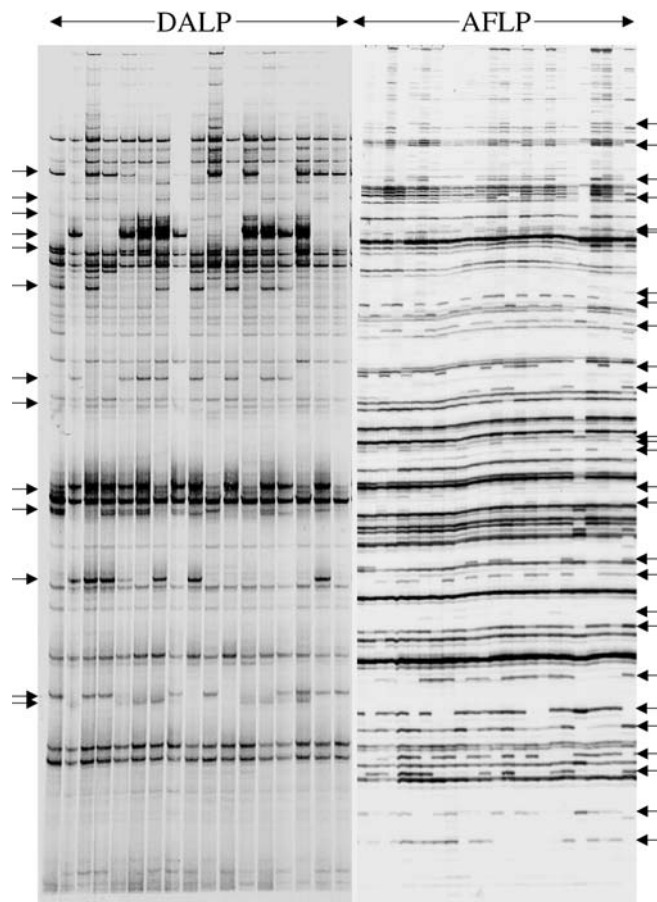


Fig. 1 Autoradiogram showing DALP and AFLP fingerprints of different RI lines generated with primer combinations DALP-Reverse/DALP235 and E-ACA/M-CAC, respectively

ranged between 95 and 136, with the mean of 110 and an average of 21.5% polymorphic bands (Table 1), whereas the number of visible bands for the DALP pairing primer was lower, with a mean of 74 and an average of 12.5% polymorphic bands. The total number of scored bands for different pairing primers was not correlated with the number of polymorphic bands ($R^2 = 0.30$). The percentage of polymorphic fragments was higher for AFLPs than for DALPs. Nevertheless, DALP markers were very easy to obtain and autoradiograms were faster and less difficult to read (Fig. 1). Their molecular weight ranged between 200 and 1,000 bases.

Seven polymorphic fragments for 83 scored bands were generated by the sPCR pairing primer (*STH21*). Six of them involved the parental LR4 markers.

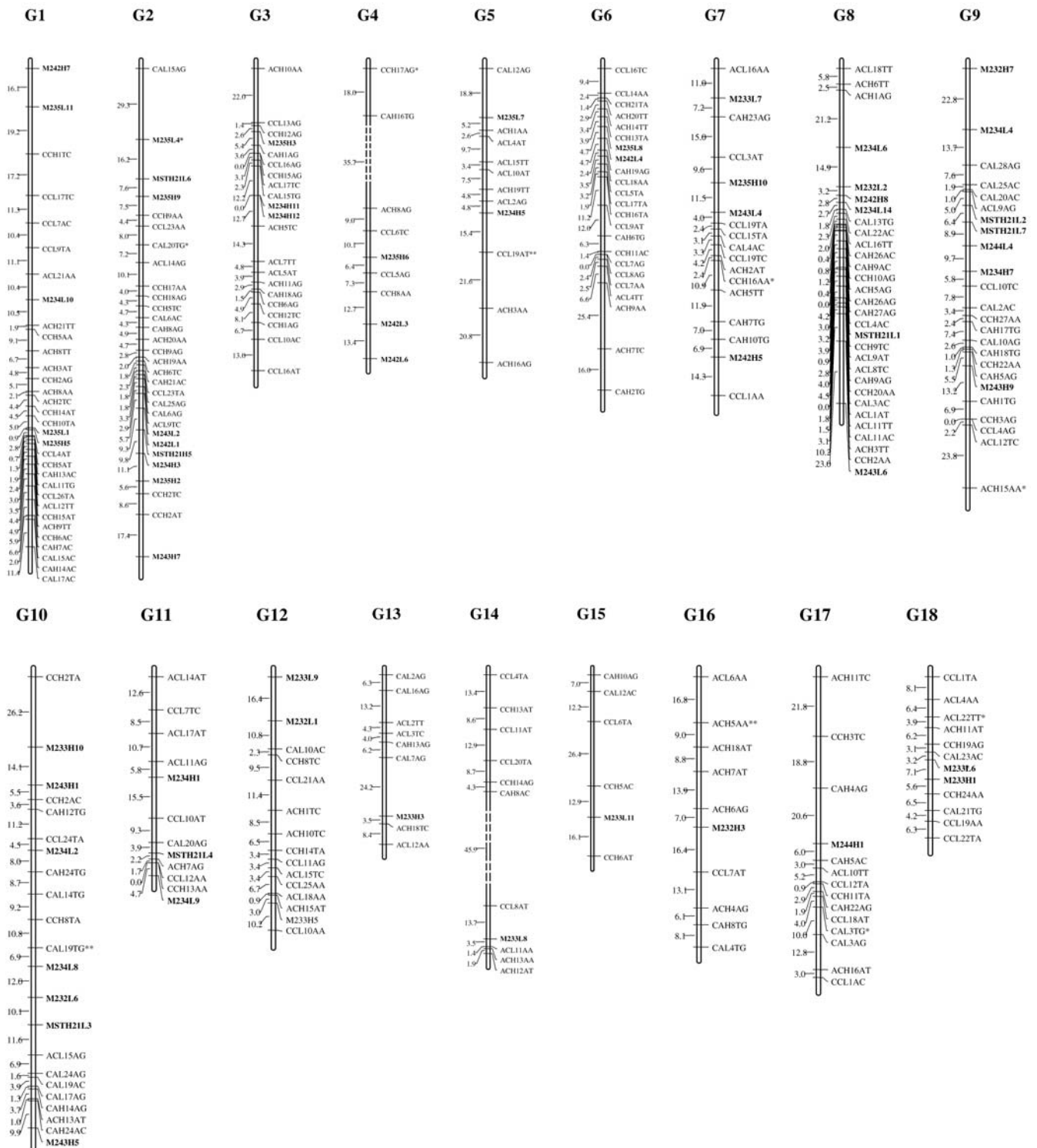


Fig. 2 The molecular linkage map of 171 (F8/F9) sunflower RI lines derived from a cross between the two cultivated inbred lines (>F6) HA89 and LR4 combining 245 AFLP, 57 DALP and seven specific PCR (sPCR) markers. DALP and sPCR markers are designated, respectively, by the letters M2Xi (Table 1) and STH21, followed by the letters L (LR4 fragment) or H (HA89) and the decreasing order of molecular weights. AFLP markers are designated by the two last letters of the *Eco*RI primer followed by the letters L or H, the decreasing order of molecular weight

and the two last letters of the *Mse*I primer (Table 1). The molecular weight of several mapped markers ranged between 200 and 1,000 bp to the nearest 10-bp value. One and two asterisks indicate markers with distorted segregation ratios at $P < 0.05$ and $P < 0.01$, respectively. All linked markers are indicated on the right of the framework. Map distances (left) were estimated by the Kosambi function (cM). Dotted lines indicate the map of two groups with a 3>LOD>2 in G4 and G14

Segregation and mapping

A total of 64 DALP, seven sPCR and 301 AFLP markers were held in segregation analysis. A majority of them (96.5%) fitted the expected 1:1 Mendelian ratio. Only 3.1% of the DALP markers and respectively 3.7% of the AFLPs deviated significantly from the expected ratio ($P < 0.01$) and then have been removed from linkage analysis. Two DALP (3%) and ten AFLP (3.3%) markers, which presented the same profiles, were coupled into six markers. Two AFLP bands (1.9%) appeared to be combined into one locus. Also, we noted the high level of mapped markers (see Table 1) originating from the parent HA89 (160/183 \cong 87.4%) compared to those derived from the male parent LR4 (150/189 \cong 79.3%).

The whole map contained 305 markers arranged in 18 linkage groups and 54 (17.7%) single markers (Fig. 2). The full-length map was 2,168.6 cM and the average interval distance between markers was 7.1 cM. The number of mapped loci for each linkage group varied between 6 and 32, with assessed genetic distances of 60.3 to 203.2 cM. Seventyone percent of genetic distances between pairwise-mapped markers were less than 10 cM and 94.7% were less than 20 cM (Fig. 3). Only 5% of our genetic map presented regions where the distance between adjacent markers was greater than 20 cM. This produced some gaps in the map, which are located at the end of several linkage groups where few AFLP but frequently DALP markers were dispersed. Nevertheless, 5–11 AFLP markers were clustered and formed a highly saturated linkage block in some linkage groups (Fig. 2), while mapped DALP markers were randomly distributed. Moreover, the fine distribution analysis of mapped primer pair bands indicated that the AFLP (E-ACA/M-CAC)-generated markers were clustered in short blocks of two and more than three markers. The majority of them were located in the highly saturated AFLP clusters (Table 1, Fig. 2). Finally, two pairs of STH21 markers were located in two linkage groups (G2, G9) and three markers were dispersed in three linkage groups (G8, G10, G11).

Discussion

The polymorphism rate revealed in this cross by AFLP primer pairs was higher (21.5) than those obtained by DALP (12.5). This corresponds to an average of two DALP markers per primer combination. Nevertheless, the percentage of AFLP bands that remained unlinked (14.9%) was equivalent ($P > 0.05$) to the unlinked DALP markers (12.5%). However, the level of LR4 mapped markers (79.3%) was lower than that of HA89 mapped markers (87.4%). This suggests that a substantial portion of the male-parent LR4 genome is genetically under-represented in our map. This difference could originate from the complex pedigree of LR4, incorporating wild sunflower genotypes of diverse origin (D. Skoric, personal communication). Indeed meiotic abnormalities

relating to the ancestral polyploid origin of *H. annuus* have also been reported (Sossey-Alaoui et al. 1998) and may explain the male recombination suppression on interspecific hybrids (Rieseberg et al. 1995).

The high levels of unlinked molecular markers have been previously reported in maps of sunflower and other species. Jan et al. (1998) found that 14.4% of RFLPs were unlinked to the map of cultivated sunflower, while Flores Berrios et al. (2000) reported a 17.5% level of un-mapped AFLP markers in sunflower inbred lines originating from a cross between PAC2 and RHA266. A spectacular level of unlinked AFLP markers (46%) was reported in a high-density map of maize (Vuylsteke et al. 1999). The low number of classical informative markers segregating in a single cross could explain these results; only limited information about the process of recombination in the whole genome could be obtained from each single cross. A composite map would be a good way to overcome this problem.

The total number (96.5%) of markers fitted the expected 1:1 Mendelian ratio. Only 3.5% (3.7% of AFLP markers and 3.1% of DALP markers) deviated significantly from the expected ratio. Similar proportions of markers (3%) showing distorted segregation were reported in *Brassica rapa* (Song et al. 1991). These rates are less than those reported by Gentzbittel et al. (1995) in a consensus linkage RFLP map of cultivated sunflower (8%).

AFLP markers are clustered on the map leading to the formation of highly saturated linkage blocks in seven groups. In contrast, there is an average distance between markers of over 6 cM in the total length of the group. Such unequal distributions of recombination along the chromosomes have been quoted in other maps of sunflower and other species by many authors (Tanksley et al. 1992; Van Deynze et al. 1995; Peerbolte and Peleman 1996; Wei et al. 1999; Gedil et al. 2001). They explained such phenomena by a more important suppression of recombination at centromeric and telomeric regions of chromosomes, so that AFLP markers were closely packed in these regions. However, our map revealed that some AFLP, sPCR and DALP markers were scattered between clusters (Fig. 2). Moreover, Lotti et al. (2000) did not observe such phenomena with the integration of AFLP markers to an RFLP-based map of durum wheat RI lines. The same observation was noted by Park et al. (2000) in a genetic map of cucumber composed of RAPDs, RFLPs and AFLP markers. This suggested that AFLP clusters were not always located in telomeric/centromeric regions. The use of telomeric probes or markers may be a good way to judge such a hypothesis. The centromeric, telomeric and some intermediate regions of chromosomes are extremely enriched in repeated sequences. If the basic DNA repeat unit contains a recognition site for the restriction enzyme used in AFLP, then a large number of restriction fragments will be produced from these special sequences. Moreover, the sequence of the repeat unit can be largely variable leading to inter-individual restriction polymorphisms. Conse-

quently a large number of AFLP markers may be located in centromeric, telomeric and intermediate regions leading to a biased distribution over the whole map. This is a well-described problem that was first encountered by the pioneers in polymorphism analysis using RFLP.

Gap areas on linkage groups are also sparsely populated. These results are consistent with the reports from barley, tomato and other crops (Tanksley et al. 1992; Becker et al. 1995). Compared to other sunflower maps, our map presented less gaps (5.3%) than those obtained (7.9%) by Flores-Berrios et al. (2000) in which more than three gaps were higher than 40 cM. DALP markers were more evenly distributed across the sunflower genome and reduced such regions. Furthermore, the addition of RAPD and simple sequence repeat (SSR) markers to RFLPs map have been proved to fill larger gaps (Giovannoni et al. 1991; Joobeur et al. 2000). This suggested that additional regions could be covered if we diversified the use of molecular-marker techniques.

PCR amplification with primers designed based on conserved resistance-gene sequences is an efficient approach in identifying and mapping resistance-gene analogs (Leister et al. 1996; Michelmore and Meyers 1998). Sequence comparisons between these genes for several plant and mammalian species revealed structural similarities of domains, suggesting putative functional roles for the encoded proteins. The use of generated primer pairs for homology cloning of known genes scored a good success. Our specific PCR strategy was to amplify conserved motifs in the STH-2 fungal resistance gene (Matton et al. 1993), and then all polymorphic generated bands were added in a dense map of the RI population. Seven polymorphic STH21 bands fitted the ratio 1:1 at $\alpha < 0.05$ and were added to the map. Six of them came from LR4.

The combination of DALP and AFLP markers appears to be an efficient method to map sunflower in a RI line population. Some DALP markers covered regions more than 20 cM long and appeared as a good complement to the AFLP data. Such results were highly visible in some linkage groups like G1(M242H7, M235L11), G2(M235L4, M235H9), G4(M242L3, M242L6), G7(M235H10, M243L4), G8(M234L6, M242H8, M234L14), G9(M232H7, M234L4), G10(M234L8, M232L6), G12(M233L9, M232L1), G15 and G17 (Fig. 2). The total length of the genome map was 2,168.6 cM, which is less than estimated by the Hulbert method (2,200 cM). This indicated that we were on the brink of a saturated map. Even eight linkage groups were stable at LOD >9 and probably represented real chromosomes. Such result have never been reported in other published sunflower maps. Nevertheless, in comparison of the combined AFLP/RFLP maps with the original CARTISOL RFLP maps, Peerbolte (1996) found some rearrangements in order and in the number of linkage groups at a LOD score of 5.5. The first trial performed with DALP here make up their suitable quality, besides their lower cost (9.9 Euro less than half the cost of AFLP 20.7 Euro), to randomly cover the

previous sunflower map. However, some gaps in this map still remain uncovered. The number of linkage groups (18) is superior to the number of chromosomes ($n = 17$). A continued effort to develop and map markers will be necessary to fill the gaps in the sunflower comparative map and to continue its refinement. This will provide an essential tool for detailed functional analyses of the sunflower genome, which has already began.

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