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High-throughput genetic mapping in *Arabidopsis thaliana*

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Abstract To facilitate rapid determination of the chromosomal location of novel mutations, we have improved current approaches to gene mapping using microsatellite length polymorphisms. The high-throughput linkage analysis method described here allows a novel gene to be tested for linkage against the whole genome of a multicellular eukaryote, *Arabidopsis thaliana*, in a single polyacrylamide gel. The procedure is based on the simultaneous co-amplification of 21 microsatellites in a single tube, using a multiplex PCR mix containing 21 primer pairs, each including one oligonucleotide labeled with one of three fluorescent dyes that have different emission wavelengths. The amplification products, which range in number from 21 to 42, depending on the genotype of the individual being tested, are electrophoresed in a single lane on a polyacrylamide gel. The use of an automated fragment analyzer makes it possible to perform linkage analysis on a one gel-one gene basis using DNA samples from 19 F_2 individuals obtained from an outcross involving a mutant and a wild-type that is genetically polymorphic with respect to the ecotype in which the mutant was generated. Discrimination of the amplification products is facilitated not only by labeling with different fluorochromes, but also by prior testing of different sequences for the ability to prime the amplification of each microsatellite, in order to ensure that multiplex PCR yields compatible amplification products of non-overlapping size. The method is particularly useful in large-scale mutagenesis projects, as well as for routine mapping of single mutants, since it reveals the map position of a gene less than 24 h after the F_2 individuals to be analyzed have become available. The concepts employed here can easily be extended to other biological systems.

Key words Simple sequence length polymorphism (SSLP) · Multiplex PCR · Fluorescence-based genetic mapping · *Arabidopsis*

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Introduction

Among the procedures currently available for gene mapping by linkage analysis, one of the most widely preferred is the simple sequence length polymorphism (SSLP) method, which is based on the presence of highly polymorphic microsatellites in most, if not all, eukaryotic genomes. SSLPs were first discovered (Litt and Luty 1989; Smeets et al. 1989; Tautz 1989; Weber and May 1989) and exploited for linkage analysis (Hearne et al. 1992) in mammals. More recently, mapping procedures based on SSLP have been introduced in plants, with the description of about 50 microsatellites in *Arabidopsis thaliana* (Bell and Ecker 1994; http://cbil.humgen.upenn.edu:80/~atgc/SSLP_info/nga_sequences.html).

Gene mapping by SSLP is performed in *Arabidopsis* on genomic DNA samples obtained from the F_2 progeny of an outcross between a mutant and a wild-type strain that is genetically polymorphic with respect to the ecotype in which the mutant was obtained. Linkage analysis is performed by amplification of the polymorphic DNA regions, followed by determination of the PCR product sizes. For recessive alleles of a gene whose map position is unknown, DNAs from at least twenty F_2 individuals displaying the mutant phenotype are used as a template on a one microsatellite (one primer pair) – one PCR amplification basis. The usual approach consists of choosing five microsatellites, one from each of the five *Arabidopsis* chromosomes, which are then sequentially tested for linkage. If linkage is observed at the first attempt, a minimum of 20 PCR amplifications is necessary, each amplification requiring one lane in a

polyacrylamide or high-resolution agarose gel for visualization of the amplification products (by radiolabeling or ethidium bromide staining). However, since the average genetic size of the five chromosomes of *Arabidopsis* is about 100 cM, it often happens that no obvious linkage is detected after testing five microsatellites, each from a different chromosome. In other words, it is not unusual to perform 100 PCR amplifications, visualized in 100 gel lanes, before having to conclude that additional microsatellites need to be tested.

We have tried to improve this mapping technique, with the dual aim of (a) maximizing the number of microsatellites co-amplified in the same PCR tube, which should reduce the number of amplifications required, and (b) minimizing the number of gel lanes required to visualize the PCR products. Such an approach would substantially reduce the time needed to find at least one microsatellite linked to the gene being mapped. The protocol presented here involves reaction conditions and a combination of primer pairs which can be used for multiplex PCR so that mapping can be achieved on a one gel-one gene basis.

Materials and methods

Plant materials and growth

Both sterile (grown in 150 mm Petri dishes filled with 100 ml of GM solid medium) and nonsterile (grown in pots containing a 1:1:1 mixture of perlite, vermiculite and sphagnum moss) cultures were performed at 20 ± 1° C and 60–70% relative humidity under continuous fluorescent light (7000 lx) as described in Ponce et al. (1998).

Some of the *A. thaliana* mutant lines used in this work were supplied by the Nottingham *Arabidopsis* Stock Centre (NASC). Plants homozygous for EMS- and fast neutron-induced mutations and displaying phenotypes mainly characterized by abnormal leaf morphologies were isolated in a L*er* background and used as the female parents in outcrosses to the Col ecotype. F_2 inbred individuals displaying the recessive mutant phenotype were collected 20 days after sowing.

DNA isolation

Single whole plants were homogenized in 1.5-ml Eppendorf tubes containing 200 µl of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM β-mercaptoethanol). Then 300 µl of extraction buffer and 35 µl of 20% SDS was added and the mixture was incubated for 10 min at 65° C; 130 µl of 5 M potassium acetate was added and the tube kept on ice for 5 min, before being centrifuged for 10 min at 13,000 rpm. The supernatant was transferred to a new tube and DNA was precipitated for 10 min at −20° C with isopropyl alcohol containing 0.3 M sodium acetate, pelleted by centrifugation, washed with 70% ethanol, resuspended in 50 µl of water and stored at 4° C. Since several mutants displayed some degree of dwarfism, the weight of plant material extracted varied from 4.5 to 35 mg. This procedure is based on that described at http://www.caltech.edu/~meyerowitz/protocols/quickdna.html

Multiplex PCR

Synthetic oligonucleotides were purchased from Perkin-Elmer Applied Biosystems. Each primer pair included one oligonucleotide labeled with 6-FAM, TET or HEX phosphoramidites, as indicated in Table 1. PCR amplifications were performed in a Perkin Elmer 2400 thermocycler, using 0.2-ml thin-walled tubes, in a 5-µl volume

Table 1 Primer sets used in multiplex PCR amplifications of *Arabidopsis* microsatellites

Microsatellite[a]	Chromosome	Oligonucleotide sequences (5′→3′)[b]		Fluorochrome	PCR product size (bp)			
		Labeled forward primer	Unlabeled reverse primer		L*er*	Col	En-2	Ws-2
AthACS[2]	1	AGAAGTTTAGACAGGTAC	AAATGTGCAATTGCCTTC	TET	276	281	282	285
AthZFPG[1]	1	TTGCGTTTCCACATTTGTTT	TGGGTCAATTCACATGTAGAGA	6-FAM	137	144	137	137
T27k12Sp6[3]	1	GGACAACGTCTCAAACGGTT	**TTGAACTTAGCAAACGATCC**	6-FAM	339	328	374	342
AthGENEA[2]	1	**TGCTACGCGTTGTCGTCGTG**	ACATAACCACAAATAGGGGTGC	TET	419	423	427	425
nga111[1]	1	CTCCAGTTGGAAGCTAAAGGG	TGTTTTTTAGGACAAATGGCG	6-FAM	162	128	192	146
nga1145[1]	2	CCTTCACATCCAAAACCCAC	GCACATACCCACAACCAGAA	HEX	220	214	202	194
nga1126[3]	2	CGCTACGCTTTTCGGTAAAG	**TCAGTGCTTGAGGAAGATAT**	TET	460	438	460	438
nga361[3]	2	AAAGAGATGAGAATTTGGAC	ACATATCAATATATTAAAGTAGC	TET	115	101	112	95
nga168[3]	2	**CTACTTTGCTCTTTCTTATACG**	**ACCACATATGTATACTGGCC**	6-FAM	374	390	374	374
AthCHIB[3]	3	**CTTCGGCCAGACTTCCCATGA**	**GGAATATTCGTAAGTTCCTTG**	HEX	392	400	400	399
nga162[2]	3	CATGCAATTTGCATCTGAGG	CTCTGTCACTCTTTTCCTCTGG	6-FAM	89	107	87	72
AthGAPAab[1]	3	**TCTGGTCACCATGGCTTCGG**	**GCCACCTTAAGCTTGGCCTC**	6-FAM	475	466	475	470
nga6[1]	3	GGATTTCTTCCTCTCTTCAC	TGGAGAAGCTTACACTGATC	HEX	120	142	121	150
nga12[2]	4	AATGTTGTCCTCCCCTCCTC	**CCTTGTAGATCTTCTGATGC**	HEX	252	262	262	262
nga1111[1]	4	GGGTTCGGTTACAATCGTGT	AGTTCCAGATTGAGCTTTGAGC	HEX	157	152	152	151
AM4[3]	4	**GTTACACTATTCAGATTCAATC**	**CAATGGCTGAATAACGACGA**	TET	300	322	301	318
nga1139[2]	4	**CTAGGCTCGGGTGAGTCAC**	TTTTTCCTTGTGTTGCATTCC	HEX	306	295	295	314
nga1107[1]	4	GCGAAAAAACAAAAAAATCCA	CGACGAATCGACAGAATTAGG	TET	132	154	136	133
AthCTR1[2]	5	TATCAACAGAAACGCACCGAG	CCACTTGTTTCTCTCTCTAG	TET	143	159	161	145
nga139[1]	5	AGAGCTACCAGATCCGATGG	GGTTTCGTTTCACTATCCAGG	TET	136	182	148	136
AthPHYC[2]	5	CTCAGAGAATTCCCAGAAAAA-TCT	AAACTCGAGAGTTTTGTCTAGATC	6-FAM	226	211	211	211
MBK5[1]	5	**CTGTCAGTTGTTGGTGAAAG**	TGAGCATTTCACAGAGACG	HEX	360	381	366	366

[a] The *superscripts* indicate concentrations used in the PCR mix for a given set of oligonucleotides: [1]15 nM, [2]30 nM and [3]45 nM

[b] Oligonucleotide sequences indicated in *bold* differ from those recommended by Bell and Ecker (1994). They were designed on the basis of the microsatellite sequences deposited at http://cbil.humgen.upenn.edu:80/~atgc/SSLP_info/nga_sequences.html

containing 200 μM of each deoxyribonucleotide, 0.2 units of Bio-*Taq* enzyme (Bioline), 0.5 μl of 10× reaction buffer (Bioline) and 2 mM $MgCl_2$. The reaction mix also included 1 μl of the 50-μl DNA solution obtained from a single 20-day-old plant. The final concentration of oligonucleotides in the reaction mix was 15, 30 or 45 nM, as detailed in the legend to Table 1. Such concentrations were attained by taking 0.75 μl of a master mix containing the 42 oligonucleotides at concentrations of 0.1, 0.2 and 0.3 μM. The thermocycle program started with an initial 1.5-min denaturation at 94° C, followed by 40 cycles of PCR (30 s at 94° C, 15 s at 50° C, 2 min at 70° C), and a final 7-min incubation at 70° C.

Electrophoresis and sizing of PCR amplification products

For gel analysis, 1.2 μl of loading buffer, comprising a 5:1:1 mixture of deionized formamide, 50 mM blue dextran and 4 nM GeneScan-500 (TAMRA) internal size standard (Perkin Elmer Applied Biosystems) was combined with 0.8 μl of PCR mix. The samples were heated at 94° C for 3 min and placed on ice immediately prior to gel loading. Electrophoresis was carried out in an ABI PRISM 377 DNA sequencer, using a 4.25% acrylamide-bisacrylamide (29:1) gel containing 6 M urea, cast between 36 cm well-to-read glass plates. Such an acrylamide-bisacrylamide ratio greatly improves peak definition. The samples were run in the GS 36C-2400 module for 3 h. DNA fragment analysis was performed using GeneScan 2.1 software (Applied Biosystems) as described in the manufacturer's manual. Peak sizes were calculated using the "Local Southern Method" sizing option.

Results

Selection of microsatellites for simultaneous testing

As previously mentioned, in SSLP mapping in *Arabidopsis* one normally uses at least 20 F_2 plants to test for linkage between the gene responsible for the mutant phenotype and a given microsatellite. Given the small number of individuals under study, only recombination frequencies of less than 30% should be accepted as unequivocal evidence for linkage. Since the whole *Arabidopsis* genome is about 500 cM long (Koornneef et al. 1983; Chang et al. 1988; Nam et al. 1989; Hauge et al. 1993) (Fig. 1), the probability of finding linkage between a given SSLP marker and a mutation causing a mutant phenotype is $(2 \times 30)/500$ or approximately 1/9. Based on this conservative assumption, a minimum of 500/30 or about 17 microsatellites need to be tested in order to obtain acceptable evidence of linkage between a gene under study and two flanking microsatellites. Furthermore, it should not be forgotten that only about 50 microsatellites are known in *Arabidopsis*, and that they are not homogeneously distributed in the genome.

With the aim of simultaneously testing a sufficient number of microsatellites to be certain of finding linkage to at least one of them, we divided the *Arabidopsis* genome into regions of approximately 30 cM, so that no mutation would be located more than 30 cM from the closest microsatellite being tested. In other words, we tried to choose the markers to be tested in such a way that the distance between adjacent ones would not exceed 60 cM (Fig. 1). Starting from such a premise, 22 microsatellites were chosen in the hope of amplifying them together in a single tube, as well as of discriminating the amplification products in a single gel lane.

Fluorescence-based discrimination of amplified microsatellites

Another factor to be considered in the selection of the microsatellites to be tested was the size of their PCR amplification products: the fragments obtained should be sufficiently dissimilar to make it possible to distinguish between them. We have taken into account the SSLP sizes described by Bell and Ecker (1994), who demonstrated that most microsatellites show polymorphism between the Landsberg *erecta* (L*er*) and Columbia (Col) ecotypes, which were used in this work together with Enkheim-2 (En-2) and Wassilewskija-2 (Ws-2). The amplification products obtained for *Arabidopsis* L*er* and Col microsatellites using the oligonucleotides described by Bell and Ecker (1994) vary in size from 70 to 280 bp, a very narrow range which would make it difficult to distinguish more than 40 different molecules in a single gel lane. In order to facilitate discrimination between molecules of identical or very similar sizes, we differentially labelled them with fluorochromes. Three types of fluorescent dyes can be simultaneously used for fragment analysis in a Perkin-Elmer ABI 377 sequencer, and these were used to label the 5′ end of one of the oligonucleotides comprising each primer pair (Table 1). Such differential fluorochrome staining allowed us to distinguish the amplification products of some microsatellites which overlapped in size, although the technique was not sufficiently accurate to discriminate between the members of some clusters that included more than three molecules of almost identical length. Faced with this problem, we decided to modify the size of the amplification products of some microsatellites by designing new oligonucleotides on the basis of the known sequence of the genomic regions flanking the microsatellite. Some of these new primers were shown to be effective for individual amplifications but not for multiplex PCR and these were replaced by more appropriate oligonucleotides. Since all the amplification products observed with the new oligonucleotides were bigger than the original ones, the spectrum of sizes of the PCR amplification products expanded from 70–280 to 89–475 bp.

The sizes of the molecules amplified from microsatellites calculated by the GeneScan software were almost identical to those obtained from the sequences which can be found in http://cbil.humgen.upenn.edu:80/~atgc/SSLP_info/nga_sequences.html. We ran only 19 samples per gel since our attempts to use all 36 wells available in a typical ABI 377 sequencing gel were unsuccessful: the intensity of the signal from some bands in a given lane was so high that it was also detected in the neighboring lanes.

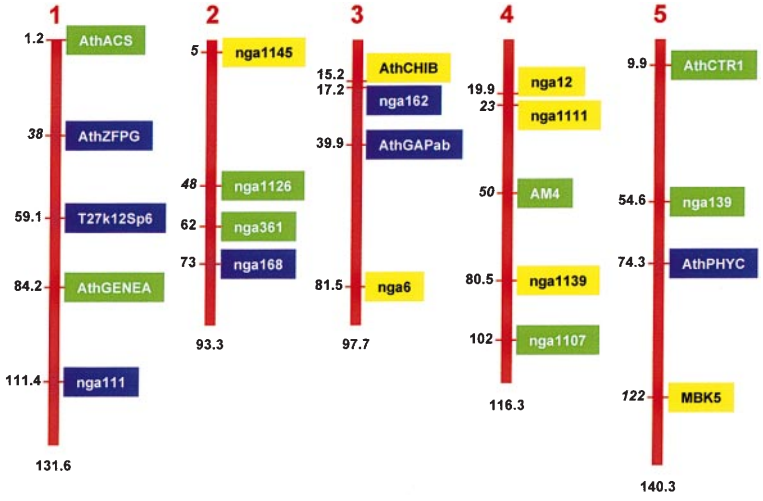

Fig. 1 *Arabidopsis thaliana* genetic map showing positions of microsatellite markers used in this work. *Green*, *blue* and *yellow boxes* indicate the fluorochrome label (TET, 6-FAM and HEX, respectively) used for one member of the primer pair used to amplify a given microsatellite. No mutation can lie further than 30 cM from a marker. Most map positions were obtained from the last available version of the Recombinant Inbred (RI) map (Lister and Dean 1993; http://nasc.nott.ac.uk/new_ri_map.html). Map positions indicated in *italics* were deduced from the information found at http://cbil.humgen.upenn.edu/~atgc/SSLP_info/coming-soon.html, as follows: nga1145 maps between NOR2 (0.04 cM in the RI map) and m246 (11.04 cM in the RI map); nga1126 is located between Gpa1 (47.1 cM in the RI map) and *ER* (49.5 cM in the RI map); nga1111 maps between *HY4* (15 cM in the integrated genetic/RFLP map of Hauge et al. 1993) and m518A (28.9 cM in the RI map); AthZFPG maps between m235 (31.9 cM in the RI map) and g17286 (47.8 cM in the map available at http://cbil.humgen.upenn.edu/~atgc/SSLP_info/SSLP_map.html); MBK5 maps 2 cM centromere distal to *LFY3* (118.4 cM in the RI map); AM4 (ESSA1 project contig fragment no. 3; accession number Z97338; P. Puigdomènech and A. Monfort, personal communication) is 0.5 Mb from SC5 CAPS [ESSA1 project contig fragment no. 5] between mi260 (51.7 cM in the RI map) and m226 (54.2 cM in the RI map)

Multiplex PCR amplification of microsatellites

The reaction conditions must be optimized if a balanced yield of the different amplification products expected from a multiplex PCR is to be achieved. Several compromises have to be made, since different oligonucleotide primers and their target sequences may require different amplification conditions (Edwards and Gibbs 1994; Henegariu et al. 1997). In addition, it is not easy to avoid the appearance of unexpected amplification products – artefacts generated by the presence of a complex mixture of oligonucleotide primers.

We attempted to optimize our multiplex PCR conditions by varying the following parameters individually and in combination: the amount of plant material used to purify genomic DNA (from a rosette leaf to a whole plantlet), the volume of the reaction mix (5–10 µl), the concentration of Mg^{+2} (2–3.5 mM), the amount of enzyme (0.05 to 1 units), the number of cycles in the thermocycling program (25–40), the concentration of primers (5–50 nM), the annealing (50–55° C) and polymerization (68–72° C) temperatures, the length of the polymerization step (30 s–3 min), and the concentration of dNTPs (200–400 µM). We found that the higher the amount of plant material used, the better the yield of amplification products. No differences were observed on changing the PCR volume from 5 to 10 µl, the concentration of dNTPs from 200 to 400 µM, or the amount of enzyme from 0.2 to 1 unit. Unexpected products appeared when $MgCl_2$ was used at concentrations exceeding 2 mM. Several microsatellite amplification products were not obtained when less than 0.2 units of enzyme or annealing temperatures below 50° C were used, whereas some of the largest products required a polymerization time of at least 2 min. On the basis of these optimization experiments, we ultimately defined reaction conditions which allowed us to use multiplex PCR on a total of 21 different microsatellites (Fig. 2). Not all of these markers were amplified to the same extent in the multiplex reaction, although yields also varied in some cases of individual amplification. Three of the microsatellites studied, T27K12Sp6, AthCHIB and AM4, all of which contain the dinucleotide repeat $(AT)_n$, consistently showed large differences in the yield of the amplification products of their alleles. This did not prevent the unequivocal genotyping of the former two, but AM4 was not scored in most mapping experiments because of its inconsistent behavior. Alleles of this microsatellite, however, are easily amplified in single, non-multiplex reactions.

One critical parameter that strongly influenced the reproducibility of the multiplex reaction was the quality of the DNA used as template. The rapid method for plant DNA extraction described by Edwards et al. (1991), was found to be applicable for the amplification of individual markers, but not for multiplex PCR, since only two-thirds of the multiplex reactions yielded sufficient product to be analyzed. The small-scale method chosen, which although slightly laborious was not excessively time consuming (see Methods), is a modification of that described by Dellaporta et al. (1983), and allowed reproducible multiplex PCR amplification from

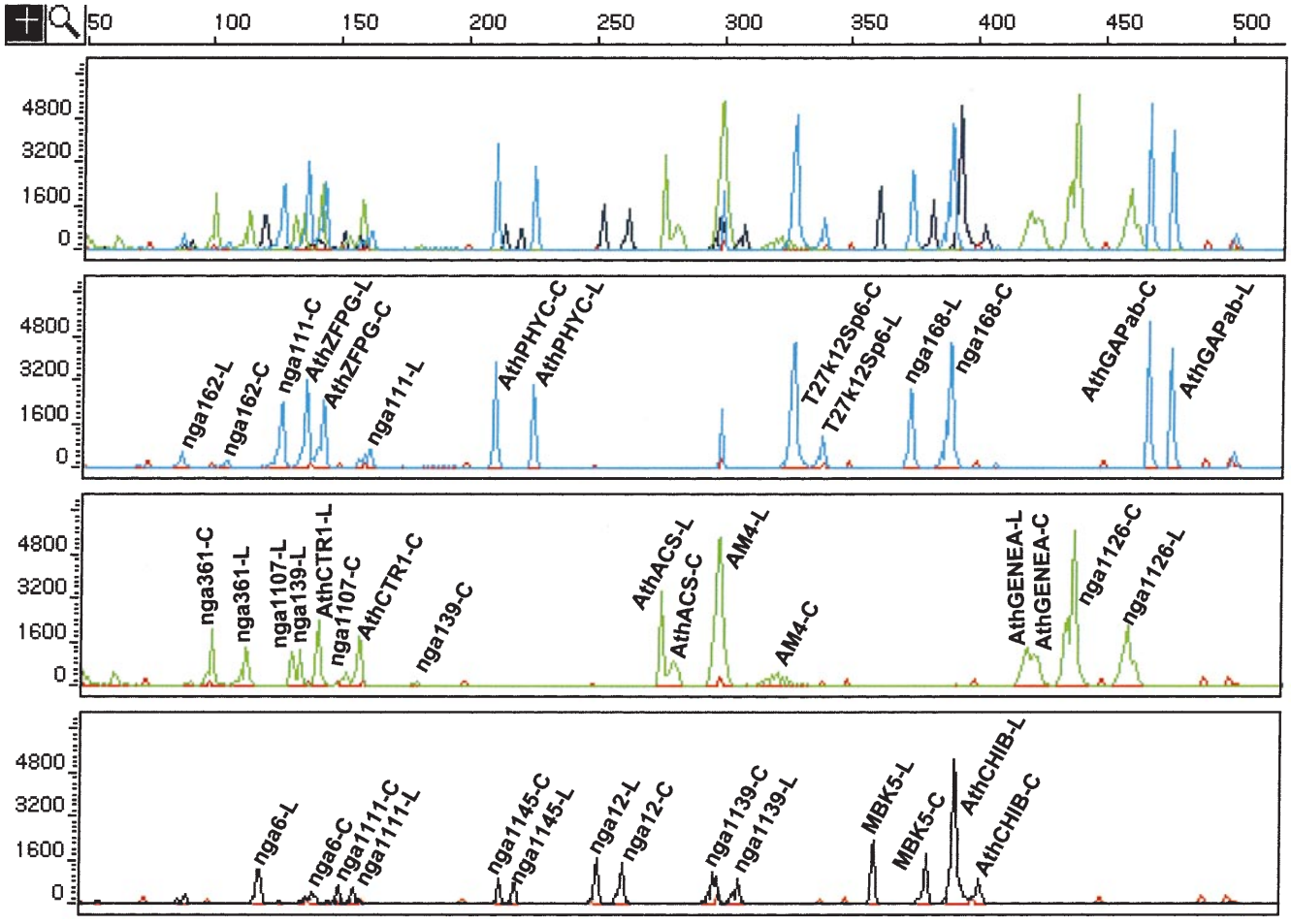

Fig. 2 Gel profile illustrating the results of a typical mapping experiment following the method presented in this paper. The *horizontal and vertical axes* indicate, respectively, the size of the fragments (in nucleotides) and the intensity of fluorophore emission (in arbitrary units). The profile in the *upper panel* corresponds to a single gel lane and contains peaks that represent the molecules obtained from the multiplex PCR amplification of 22 microsatellites. The profile can be decomposed by the GENESCAN 2.1 software into other three plots, as shown in the remaining panels, each of which represents peaks corresponding to only one of the fluorochromes used. *Green*, *blue* and *black* peaks indicate TET, 6-FAM and HEX fluorochromes, respectively. Each peak is denoted by the name of the corresponding microsatellite and that of the parental allele (L for L*er* and C for Col). Peaks corresponding to the internal molecular weight standard appear in *red*. The figure shows 44 peaks because it was obtained from genomic DNA of an F_1 individual from a cross between L*er* and Col, two ecotypes which are polymorphic for all the microsatellites analyzed in this work (see Table 1). Primers for the AM4 microsatellite, whose alleles are shown to be amplified in this profile, were not included in the optimized reaction mix, which included 21 primer pairs, yielding up to 42 amplification products

most, if not all, of the plant DNA samples tested under our experimental conditions.

Efficiency of the method

The effectiveness of the technique presented here has been demonstrated by the mapping of more than 90 *Arabidopsis* genes, most of which were identified in screens using ethyl methanesulfonate or fast neutrons as the mutagen (Berná, Robles and Micol, submitted; Quesada, Ponce and Micol, in preparation). Linkage was rapidly and unequivocally established in all cases, irrespective of the genetic background of the mutants; L*er*, Col, En-2 and Ws-2 were all used, depending on the mutation. Table 2 presents a few representative examples of the results that were obtained when the method presented in this paper was used for low-resolution mapping on a one gene-one gel basis. The *DEN1* (*DENTICULATA1*), *OND4* (*ONDULATA4*), *SCA4* (*SCABRA4*), *SEA2* (*SERRATA2*) and *VEN1* (*VENOSA1*) genes were identified in a large-scale screen for EMS-induced *A. thaliana* mutants that have abnormal leaf morphology (Berná, Robles and Micol, submitted).

Discussion

Our initial intention was to develop a genetic mapping procedure that could be applied rapidly to large groups of genes identified by mutations. Although several procedures for genetic mapping by linkage analysis are available in *Arabidopsis*, those based on the use of classical genetic markers (Koornneef et al. 1983; Hauge et al. 1993), restriction fragment length polymorphisms

Table 2 Recombination frequencies obtained in mapping experiments performed in single gels

Microsatellite	Chromosome	Locus[a]				
		DEN1	*OND4*	*SCA4*	*SEA2*	*VEN1*
AthACS	1	55.5 (36)	41.7 (36)	44.7 (38)	55.5 (38)	31.6 (38)
AthZFPG	1	36.8 (38)	55.3 (38)	57.9 (38)	55.3 (38)	39.5 (38)
T27k12-Sp6	1	62.5 (32)	56.7 (30)	57.9 (38)	63.2 (36)	61.8 (34)
AthGENEA	1	47.2 (36)	50.0 (36)	52.8 (36)	61.2 (34)	60.5 (38)
nga111	1	36.8 (38)	50.0 (38)	60.5 (38)	60.5 (38)	65.8 (38)
nga1145	2	38.9 (36)	34.2 (38)	50.0 (38)	47.4 (38)	44.7 (38)
nga1126	2	52.9 (34)	**02.8** (36)	55.3 (38)	27.8 (36)	44.1 (34)
nga361	2	52.8 (36)	**10.5** (38)	52.6 (38)	**18.4** (38)	42.1 (38)
nga168	2	52.8 (36)	40.6 (32)	44.7 (38)	**05.3** (38)	72.2 (18)
AthCHIB	3	35.3 (34)	30.5 (36)	**08.3** (36)	20.6 (34)	33.3 (30)
nga162	3	47.2 (36)	50.0 (38)	**15.8** (38)	41.7 (36)	23.7 (38)
AthGAPab	3	44.4 (36)	44.1 (34)	23.7 (38)	39.5 (38)	26.3 (38)
nga6	3	70.0 (30)	67.6 (34)	47.4 (38)	62.5 (32)	39.5 (38)
nga12	4	47.2 (36)	47.4 (38)	50.0 (38)	50.0 (38)	42.1 (38)
nga1111	4	50.0 (36)	57.9 (38)	50.0 (38)	47.4 (38)	39.5 (36)
nga1139	4	58.3 (36)	60.5 (38)	50.0 (38)	44.7 (38)	47.4 (38)
nga1107	4	44.4 (36)	47.4 (38)	47.2 (36)	23.7 (38)	50.0 (36)
AthCTR1	5	47.2 (36)	47.4 (38)	65.8 (38)	47.4 (36)	**07.9** (38)
nga139	5	38.9 (38)	52.6 (38)	60.5 (38)	55.3 (38)	**15.6** (38)
AthPHYC	5	27.8 (36)	44.7 (38)	57.9 (38)	50.0 (38)	21.0 (38)
MBK5	5	**08.3** (36)	76.3 (38)	44.7 (38)	50.0 (38)	44.7 (38)

[a] The values indicate raw recombination frequencies. The numbers *in parentheses* indicate the absolute number of chromosomes analyzed. Values highlighted in *bold* are considered as unequivocal evidence of linkage

(RFLPs) (Chang et al. 1988; Nam et al. 1989), random amplified polymorphic DNAs (RAPDs) (Reiter et al. 1992) or cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel 1993), were discarded because they were too time consuming. Based on its ease of use rather than on any other consideration, the SSLP (Bell and Ecker 1994) method was considered the best choice, although improvements were necessary: conventional PCR was replaced by multiplex PCR, and ethidium bromide staining or isotope labeling by fluorescence-based differential labeling.

The procedure presented here is partly based on advances made by other authors in analyzing the human genome, such as Reed et al. (1994), who first reported linkage analysis and genotyping by means of a combination of multiplex PCR and fluorescence-based semi-automated allele sizing. Given the large size of the human genome, these authors designed several primer sets, most of which included 20 oligonucleotides, able to amplify up to 20 products from 10 microsatellites. More recently, Mitchell et al. (1997) reported multiplex PCR and fluorescent detection of 11 microsatellite amplification products using 22 primers for genotyping of *Brassica napus* cultivars, showing coamplification of four polymorphic loci from hybrids. By contrast, we have obtained and detected 42 amplification products using 42 primers in *Arabidopsis thaliana*. It should be noted that the number of available human polymorphic microsatellites runs to several thousands, and while the physical size of the *Arabidopsis* genome is about thirty times smaller, the number of known *Arabidopsis* microsatellites is also much smaller, making it difficult to substitute a microsatellite that fails to be efficiently amplified in a multiplex PCR by another. Furthermore, we have found no reference in the literature to successful multiplex amplification of more than 13 targets in one step and in a single PCR tube (Edwards and Gibbs 1994), regardless of the genome under study.

The mapping procedure described here has several clear advantages over previous approaches. First, the number of PCR amplifications is reduced to a minimum, since only one reaction per F_2 individual is required in order simultaneously to test linkage with respect to 21 markers covering the whole *Arabidopsis* genome. Second, the number of electrophoresis runs necessary to determine linkage is reduced to one. Furthermore, data acquisition by a computer-controlled device saves time and allows unequivocal genotyping (Carrano et al. 1989; Ziegle et al. 1992). Other advantages of our method include the fact that no gel lane need be sacrificed for size standards, since they are internal, and neither ethidium bromide staining nor radiolabeling is required for visualization of the PCR-amplified microsatellites, as is usual for mobility measurements on agarose or polyacrylamide gels, respectively. It should be emphasized, however, that one of the most important contributions of our method is the intentional modification of the positions of the microsatellite regions used for PCR priming (based on their already known sequence) in order to obtain multiplex PCR-compatible amplification products of non-overlapping sizes.

One of our objectives was to cut to a minimum the cost of the mapping procedure. For this we reduced the volume of the PCR mix to 5 μl, the total amount of each labeled primer (the most expensive component of the PCR mix) to 0.075–0.225 pmoles per reaction, and the

amount of enzyme to 0.2 units, an extremely low level of enzyme, taking into account the fact that 21–42 different molecules have to be amplified. We tested several polymerases and found that the relatively inexpensive Bio-*Taq* (Bioline) produced the best balance between the different microsatellite amplification products. Other enzymes dramatically improved the yield of the amplification products of specific microsatellites, but impaired the amplification of others and/or caused the appearance of unexpected bands, the best quality/price ratio being obtained with Bio*Taq*. The cost of mapping a gene by the procedure presented here is significantly lower than with any known alternative for determining linkage using SSLP.

Last, but not least, we wish to stress that it is with respect to the time required that our procedure excels, since only one PCR amplification and only one gel lane are required per F_2 individual. Since 19 different individuals can be analyzed in a 36-cm polyacrylamide gel run in a Perkin-Elmer ABI 377, a total of 38 chromosomes can be tested for linkage to every marker. In other words, the equivalent to $21 \times 19 = 399$ single PCR amplifications are visualized in a single gel. Even in those rare cases in which some of the 19 individuals show no amplification, or where some of the primers behave in an unusual and irregular way, a single gel is sufficient to detect linkage to one or more of the microsatellites tested. When the F_2 individuals displaying the mutant phenotype under study are ready for processing, only 1 day is required to obtain an approximate map position for the mutated gene. This includes DNA isolation, multiplex PCR amplification, gel electrophoresis and fragment sizing. The protocol described here makes it possible routinely to run three gels per day per sequencing machine. It is therefore possible, when fine-scale mapping is required, to test a total of 91 F_2 inbred individuals in less than 24 h. In such cases, the first gel is run with samples obtained from multiplex PCR amplifications involving all 42 primers, and the other two using only the primers specific for the linked microsatellite(s). A total of 36 individual samples can be analyzed in each of the latter two gels, provided that odd and even lanes are loaded after a reasonable interval. Since recent modifications made to both the software and hardware of ABI 377 sequencers allow the simultaneous sequencing of 96 samples, it will not be difficult to apply such improvements to increase the number of *Arabidopsis* F_2 individuals simultaneously tested in a single gel after multiplex PCR.

For studies involving *Arabidopsis* mutants obtained in genetic backgrounds with previously undetermined microsatellite allele sizes, our method allows their straightforward determination, using only one plant DNA sample and only one gel lane per group of three primer sets marked with different fluorophores.

Large-scale mutant screens are well established approaches for the identification of genes controlling developmental processes in multicellular systems (Nüsslein-Volhard and Wieschaus 1980; Jürgens et al. 1991; Haffter et al. 1996). Such screens usually provide a large number of new mutants, which have to be subjected to complementation and linkage analyses before the number and map positions of the novel genes found can be established. These are universal rules that all geneticists are expected to observe irrespective of the type of biological system under study. In a large-scale mutant screen, the availability of a rapid mapping procedure is extremely convenient for determining how many of the new mutants need be subjected to specific allelism tests. Once the map position of the new mutation is known, the new mutant can be crossed to previously described mutants, in order to ascertain whether they are mutations in as yet undescribed genes or new alleles of already known genes. This can only be done for a large group of new mutants if a high-throughput procedure such as the one described in this paper is used.

The results presented here clearly indicate that multiplex PCR amplification of a large number of genomic targets can be achieved without greatly modifying the reaction conditions and without the need for the use of sophisticated additives in the reaction mix. The upper limit to the number of genomic targets that can be co-amplified in a single reaction tube by a conventional thermostable polymerase has probably not yet been reached.

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