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Low-Resolution Mapping of Untagged Mutations

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Summary

The mapping method detailed here is based on the multiplex polymerase chain reaction (PCR) coamplification of 32 molecular markers, using fluorescently labeled oligonucleotides as primers. For the genotyping of a single plant from a mapping population, only two simultaneous amplifications are required, the products of which are finally electrophoresed in an automated DNA sequencer controlled by fragment analysis software. An analysis of the genotypes of 50 plants allows mapping of the mutation of interest within a candidate genomic interval of about 15 cM (3 Mb, corresponding to about 40 BAC clones).

Key Words: Positional cloning; linkage analysis; SSLP; In/Del; multiplex PCR.

1. Introduction

A typical departure point for genetic approaches to identify which genes are involved in a given biological process is to isolate mutants. Once a mutant phenotype has proved to be unequivocally distinguishable from the wild type and stable through several generations, it is relatively easy to approach the gene damaged by mutation.

Mutations tagged by insertional mutagenesis have been, and continue to be, instrumental for the rapid identification of the genomic sequences flanking the insertion. However, there are a number of drawbacks in the use of T-DNA, which in many cases generates multiple unlinked insertions or internally reorganized and large concatamers, and in some instances causes untagged mutations as a result of abortive insertions. Such behavior of T-DNA-based constructs sometimes complicates the cloning of the genes damaged by their insertions. In addition, most T-DNA-induced mutations are null and some cause recessive lethality.

The use of physical or chemical mutagens represents an alternative to insertional mutagenesis. A major objection raised against the use of these mutagens is that positional approaches must be followed to clone the corresponding genes. However, although map-based cloning has until recently been considered time-consuming and expensive, the available techniques and the information provided by the *Arabidopsis* Genome Initiative make it possible to complete positional cloning projects in a short period of time ([1](#)). In addition, physical and chemical mutagens have mutation rates higher than those of insertional mutagens, and they induce null and lethal alleles as well as hypomorphic and viable alleles.

To map an untagged mutation, a mapping population should first be obtained by crossing the mutant of interest to a wild-type line. The genetic background of the latter must be different and as polymorphic as possible compared with that of the mutant under study. With this regard, the most usual crosses in *Arabidopsis thaliana* are those involving Landsberg *erecta* (*Ler*) and a mutant with a Columbia-0 (*Col-0*) genetic background, or vice versa, whose F1 progeny is allowed to self in order to obtain F2 plants. Although a single cross is usually sufficient for the

low-resolution mapping of a mutation, minimal genotyping of its F1 progeny is convenient in order to confirm heterozygosis.

Phenotypically recessive F2 plants are collected to individually extract DNA, which is then used to assess the linkage between the mutation under study and molecular markers more or less evenly spaced along the genome of *Arabidopsis thaliana*. Low-resolution mapping of mutations is facilitated by the availability of a plethora of polymorphic microsatellites, also named simple sequence length polymorphisms (SSLPs; [2](#)), and small-insertion and deletions (In/Dels; [3](#)), all of which are easy to score codominant markers, given that their alleles yield polymerase chain reaction (PCR) amplification products of distinguishable sizes. Differences between the widely used *Ler* and *Col-0* accessions at the DNA sequence level mean that it is possible to find at least one polymorphism per BAC clone, many of which can be used to develop molecular markers ([3](#)).

The method detailed here is based on multiplex PCR coamplification of a large set of molecular markers using fluorescently labeled oligonucleotides as primers ([4](#)). Only two simultaneous PCR amplifications are required for the genotyping of a single F2 plant for the 32 markers, which include 20 SSLPs and 12 In/Dels. An analysis of the genotypes of 50 plants allows mapping of the mutation of interest within a candidate genomic interval of about 15 cM (3 Mb, corresponding to about 40 BAC clones).

Once a mutation has been low-resolution mapped, there are several procedures that can be used to further refine the candidate genomic interval ([3,5](#)), some of which are covered in other chapters of this book. Needless to say, a prudent practice is to survey the literature and congress proceedings for already-described phenotypes associated to mutations in the candidate interval, as recloning a gene already cloned by others is becoming increasingly common in the postgenomics era.

2. Materials

Use autoclaved distilled water to prepare all solutions. All plastic materials should be sterile.

2.1. Equipment and Supplies

1. 1.5-mL Eppendorf tubes.
2. 0.2-mL thin-walled PCR tubes.
3. 3-mm tungsten carbide beads (Qiagen, cat. no. 69997).
4. Vortex.
5. Microfuge.
6. Heat block.
7. MM 300 Mixer Mill (Qiagen, 85120).
8. Autoclavable plastic pestles (Sigma, Z35,994-7).
9. Cordless motor grinder (Sigma, Z35,997-1).
10. 96-well optical reaction plate (Applied Biosystems, part no. 4306737).
11. 3100 Capillary Array 36 cm (Applied Biosystems, 4315931).
12. ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).
13. GeneAmp PCR System 2700 (Applied Biosystems).
14. Genescan 3.7 software (Applied Biosystems).
15. Mapmaker 3.0b program ([6](#)).

2.2. Reagents and Buffers

1. Hi Di formamide (Applied Biosystems, 4311320).
2. 20% SDS.
3. 5 M KOAc.
4. Isopropanol.
5. 3 M NaOAc.
6. 70% ethanol.
7. *Taq* DNA polymerase.

8. 10X PCR buffer.
9. Oligonucleotide mixes (*see Table 1*).
10. 50 mM MgCl₂.
11. DNA extraction buffer: 100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl.
12. Deoxyribonucleotide mix: 2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, and 2.5 mM dTTP.
13. GeneScan-500 LIZ Size Standard (Applied Biosystems, 4322682).
14. 3100 POP-6 polymer (Applied Biosystems, 4316357).

3. Methods

3.1. DNA Extraction

Select 50 homozygous plants (*see Note 1*) from the F₂ mapping population derived from an outcross of the mutant of interest to a wild-type plant. This should allow the detection of at least one marker linked to the mutation under study (*see Note 2*).

1. Collect 50 to 80 mg of plant material from each individual to be genotyped in 1.5 mL-Eppendorf tubes.
2. Add to each tube one tungsten carbide bead and 500 μ L of DNA extraction buffer.
3. Grind the samples twice for 1.5 min at 30 Hz in the Mixer Mill (*see Note 3*).
4. Add 35 μ L 20% SDS, mix gently, and incubate for 5 min in a heat block at 65°C.
5. Add 130 μ L 5 M KOAc, mix gently, and incubate on ice for 8 min.
6. Centrifuge at 13,000 rpm for 15 min in a microfuge.
7. Transfer the supernatant to properly labelled 1.5 mL-Eppendorf tubes. Repeat **steps 6 and 7** if the supernatant is not completely free of cellular debris.
8. Add 640 μ L isopropanol and 60 μ L 3 M NaOAc and mix gently.
9. Precipitate DNA at –20°C for 20 min.
10. Centrifuge at 13,000 rpm for 10 min in a microfuge.
11. Remove supernatant.
12. Wash the DNA precipitate with 300 μ L 70% ethanol.
13. Centrifuge at 13,000 rpm for 5 min in a microfuge.
14. Remove supernatant and let the pellet air dry (usually 1 h).
15. Resuspend the pellet in 100 μ L of water (or 50 μ L if the starting plant material was less than 50 mg).
16. Store the DNA samples at 4°C or –20°C.

3.2. Multiplex PCR Amplifications

3.2.1. Preparation of Oligonucleotide Mixes

The method described here allows the simultaneous genotyping of a total of 32 markers (**Fig. 1**) by simply performing two PCR amplifications per plant. Two oligonucleotide mixes are required, which include 17 and 15 primer pairs, respectively (**Table 1**; *see Note 4*). Each primer pair includes an oligonucleotide that has been labeled with one of four fluorescent dyes (NED, 6-FAM, VIC, or PET), with nonoverlapping emission wavelengths. Both oligonucleotide mixes are prepared in the same way (*see Notes 5 and 6*), as follows:

1. Prepare 10 μ M oligonucleotide stock solutions (*see Note 7*).
2. Prepare primer mixes 1 and 2, both of which contain some oligonucleotides at a 250 nM concentration and some others at 375 nM. In this way, their final concentrations in the PCR mix will be 50 or 75 nM, respectively (*see Note 8*).
To prepare primer mix 1, add the following to an Eppendorf tube:
 - a. 2.5 μ L of each 10 μ M oligonucleotide stock solution of the primers, which should be at a 50 nM final concentration in the PCR mix ($11 \times 2 = 22$ primers, $22 \times 2.5 \mu\text{L} = 55 \mu\text{L}$) (*see Table 1*).
 - b. 3.75 μ L of each 10 μ M oligonucleotide stock solution of the primers that should be at a 75 nM final concentration in the PCR mix ($6 \times 2 = 12$ primers, $12 \times 3.75 \mu\text{L} = 45 \mu\text{L}$) (*see Table 1*).

Table 1
Oligonucleotide Sets for Low-Resolution Mapping

Marker	Type of marker	Oligonucleotides					Amplification	
		Sequences (5'→3')		Label	Final concentration	Mix	product size (bp)	
		Unlabelled reverse primer	Labelled forward primer				Ler	Col-0
nga59	SSLP	GCCTAACAAATTTAAAGTTAAGACT	ATCTGTGTTCACTCGCCGCC	NED	50 nM	1	173	169
JV18/19	SSLP	AATTCAGTATCGAGATACCCCT	TGTCGTATATCAATCGAAAAAGAG	6-FAM	75 nM	1	284	290
SGCSNP10026	In/Del	ACTATTGACTTCGTGTTAACAAAG	CAAGCCCTCTTATGCTTTAGAAA	6-FAM	50 nM	1	238	231
F1I21	In/Del	ACCTGACTAGCACTTGCATCTT	GTTGGTAGTGGTTGCAAAACAG	VIC	50 nM	1	453	468
nga1145	SSLP	GAGCACATACCCACAACCAGA	CTTCACATCCAAAACCCACCT	VIC	50 nM	1	220	214
SGCSNP922	In/Del	AACTCGAACCCGAAATACCCA	GTCAAATACTAGTTACAATCTCAG	VIC	50 nM	1	316	302
SGCSNP8895	In/Del	ACTTTGGGAGAGCGATTTC	GGAGTTAGTCAGTTAGCTAACAA	6-FAM	50 nM	1	339	327
nga6	SSLP	CTTCACCGATTTCCTAGATCTTC	AATCCGAAAATAATGGAGAAGCTT	NED	75 nM	1	117	137
nga1111	SSLP	GTTGGTTCGGTTACAATCGTGT	GTTACCAGATTGAGCTTTGAGC	VIC	50 nM	1	158	151
AthF28J12	SSLP	GCCTTTATCAATGGATGAGGAA	AAATCTCAGATCCGTCATTTCCA	VIC	50 nM	1	257	265
AthCTR1	SSLP	ATCACCAAAATATGAAATACCAAAAT	CCACTGTTTCTCTCTCTAGTTT	6-FAM	50 nM	1	359	377
nga76	SSLP	TCCACCGCCATCACCGTCG	TGGATCTTTTGACAAATCAGGAT	6-FAM	75 nM	1	248	177
SO191	SSLP	TAATGCTCTCTTAATTGTGGTTAC	CTCCACCAATCATGCAAAATGTTT	NED	75 nM	1	492	482
MNF13	In/Del	AATCGTTTACGTCGGAGAAAAC	GGATTTAATTCCTCAGCCATCGC	PET	75 nM	1	434	446
MCL19	In/Del	GGTAATGAGTAAAGTTTCAATATCA	GATGTTGATTTCTCATAAAGTCATA	VIC	50 nM	1	422	411
MNB8	In/Del	TCCTGCACATTCATAGCGGAA	CTCTACACGTGTATATATCAACA	NED	75 nM	1	390	402
K8K14	SSLP	CACAAATCCTTGCGATGGAAC	CCCAGGATTTGTCTCAATCGA	VIC	50 nM	1	345	351
AthZFPG	SSLP	AGAAATGGGTCAATTACATGTA	TTGCGTTTCCACATTTGTTAAAC	PET	75 nM	2	143	149
nga128	SSLP	TAAATCTTGAAACCTTAGGGAG	GGTCTGTTGATGTCGTAAGTC	PET	75 nM	2	193	183
SGCSNP10490	In/Del	GGGAAAGAAAAGACTTAATTAGCT	GAGTCGAACGGCTATTTTCGT	NED	50 nM	2	283	271
nga111	SSLP	TGTTTTTTAGGACAAATGGCGAAA	CTCCAGTTGGAAGCTAAAGGG	6-FAM	50 nM	2	160	124
nga1126	SSLP	GGTTAATGTTTCTTCAGTGCTTG	ATCTTCTCCTCTACGCTTTTC	NED	50 nM	2	479	459
nga168	SSLP	GCCAACATTGTGTATCATCAATC	CTACAGAGCTGTCAATCGCTGA	VIC	50 nM	2	381	397
nga126	SSLP	CGTAATGATTGTGACTTTTTTGC	GAAAAACGCTACTTTCTGTGGTA	PET	75 nM	2	408	382
MYF24	In/Del	CGCCAGCGGCCTCAAAACATA	GGTAAATAGCCTGAAATCACAC	NED	50 nM	2	308	340
AthGAPab	SSLP	GCCACCTTAAGCTTGGCCTC	TCTGGTCACCATGGCTTCGG	PET	75 nM	2	476	467
T32N15	In/Del	TATCGGAAATAGGATTGACTACG	TACTTTTCATGGGAGTAGGCACT	6-FAM	50 nM	2	243	250
F28P10	In/Del	GTTATTGTTGACAACTTTTAGCCT	ATCCACCAATTCAGGAGCCA	VIC	75 nM	2	353	361
nga1139	SSLP	GGCTCGGGTGAGTCACCAT	TTTTTCCTTGTTGCATTCCG	PET	75 nM	2	309	297
nga151	SSLP	TCGTTGTAGTAGCAGTTTACTATA	TGACCCATATGTTCCAATGTGTT	6-FAM	50 nM	2	420	448
AthPHYC	SSLP	GAAACTCGAGAGTTTTGTCTAGA	CTCAGAGAATCCCAGAAAAATC	NED	75 nM	2	225	209
MUA2	In/Del	CCGTTATTTCTCAATGATCTTTC	GCTATGATGAAGAAGAAAGAAGG	NED	75 nM	2	319	368

To prepare primer mix 2, add the following to an Eppendorf tube:

- 2.5 μ L of each 10 μ M oligonucleotide stock solution of the primers, which should be at a 50 nM final concentration in the PCR mix ($7 \times 2 = 14$ primers, $14 \times 2.5 \mu\text{L} = 35 \mu\text{L}$) (*see Table 1*).

- b. 3.75 μL of each 10 μM oligonucleotide stock solution of the primers, which should be at a 75 nM final concentration in the PCR mix ($8 \times 2 = 16$ primers, $16 \times 3.75 \mu\text{L} = 60 \mu\text{L}$) (see **Table 1**).
- c. 5 μL of water.

3.2.2. PCR Amplifications

PCR amplifications are performed in a 5- μL volume, thus minimizing the amount of labeled primers required. In order to avoid pipetting errors, a master mix for at least 10 reactions should be prepared.

1. Prepare PCR master mixes 1 and 2, both of which should include the following components per plant to be genotyped: 1.86 μL of water, 0.5 μL 10X PCR buffer, 0.4 μL dNTP mix (2.5 mM each), 1.0 μL primer mix 1 (or primer mix 2), 0.2 μL 50 mM MgCl_2 , and 0.04 μL 5 U/ μL Taq polymerase (see **Note 9**).
2. Dispense 4- μL aliquots of the PCR master mixes into 100 0.2-mL thin-walled tubes (50 tubes per PCR master mix). Add to each tube 1 μL of plant DNA. Two amplifications per plant are required to genotype the whole set of markers.
3. Place the tubes in a thermocycler, and run the following program: 3 min at 94°C, followed by 40 cycles (30 s at 94°C, 15 s at 50°C, and 2 min at 70°C), and a final 7-min incubation at 70°C (see **Note 10**).

3.3. Electrophoresis and Sizing of PCR Amplification Products

1. Dilute each PCR mix 1:4 by adding 15 μL of water in order to avoid saturation of the genetic analyzer.
2. Prepare a loading buffer by mixing 1 mL of Hi Di formamide and 25 μL of GeneScan-500 LIZ size standard.
3. Add 1 μL of the diluted PCR mix and 10 μL of loading buffer per well of a 96-well optical reaction plate.
4. Immediately before electrophoresis in an ABI PRISM 3100 Genetic Analyzer, heat the 96-well optical reaction plate containing the samples at 94°C for 2 min in a thermocycler or a water bath, and place it on ice for at least 5 min (see **Note 11**).
5. Perform the electrophoresis of the amplification products using a capillary array of 36 cm, the POP-6 polymer and the Data Collection 3.1 program with the GeneScan36_POP6Default Module.
6. After the end of the electrophoresis, use the Genescan 3.7 software to size the electrophoresed molecules (**Fig. 2**).

3.4. Genotyping

1. Genotype all the F2 plants under study for all the 32 markers (see **Note 12**). Although the Genotyper 3.6 NT software can be used for this purpose, manual genotyping is usually possible (see **Note 13**).
2. Represent the genotypes in a data matrix as shown in **Table 2**.

3.5. Testing Linkage and Estimating Map Distances

1. Calculate recombination frequencies for each marker and all the F2 plants studied as the recombinant chromosomes/total chromosomes ratio.
2. Map distances between a mutation and its neighboring markers, as well as their relative positions (their maximum-likelihood map), can be determined using the Mapmaker 3.0b program (**6**).

4. Notes

1. If the mutation to be mapped is recessive, choose phenotypically mutant F2 plants to extract their DNA; if the mutation is completely dominant, select the wild-type plants; if the mutation is semidominant, select those with the most extreme phenotypes, either the unequivocally wild type or the most severely mutant ones, in order to avoid selecting unnoticed heterozygotes.

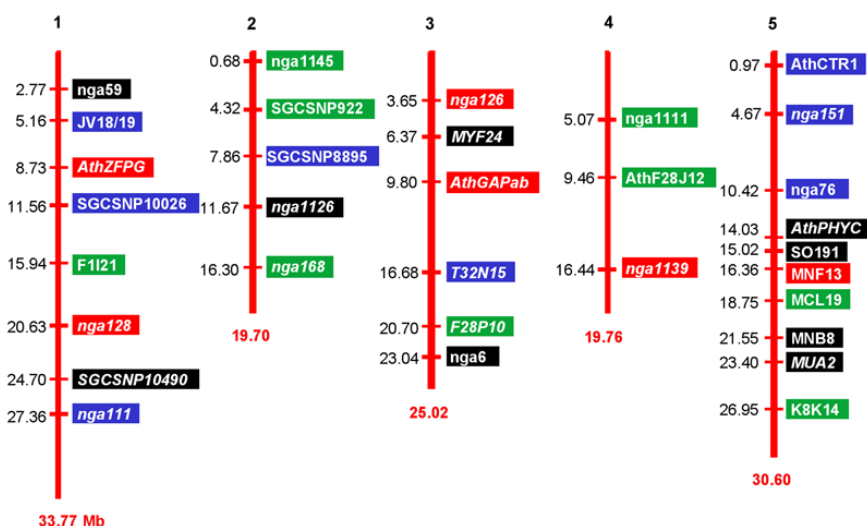


Fig. 1. *Arabidopsis thaliana* physical map with indication of the positions of the markers used in the linkage mapping method described here. All figures indicate Mb, the only exceptions being chromosome numbers. Markers are colored to indicate the fluorophore used to label one of their corresponding oligonucleotide PCR primers (VIC, green; 6-FAM, blue; NED, black; PET, red). Markers indicated in italics are genotyped using primer mix 2 and the remaining ones with primer mix 1 (see Table 1).

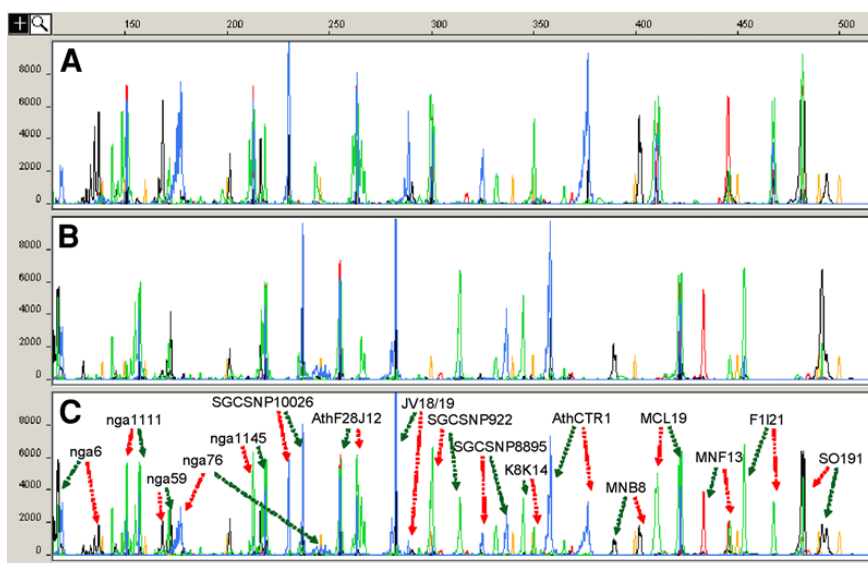


Fig. 2. Electrophoregram illustrating the results of a mapping experiment following the method described in this chapter. The horizontal and vertical axes indicate, respectively, the size of the amplification products (in nucleotides) and the intensity of fluorophore emission (in arbitrary units). The profiles represent the molecules obtained from the multiplex PCR coamplification of 17 markers (primer mix 1), using as a template genomic DNA from single plants of the Col-0 (panel A) and Ler (panel B) accessions, and their F1 progeny (panel C). Green, blue, black, and red peaks indicate VIC, 6-FAM, NED, and PET fluorochromes, respectively. Peaks corresponding to the internal molecular weight standard (GeneScan-500 LIZ Size Standard) appear in orange. In panel C, peaks are denoted by the name of their corresponding marker and the arrows point to the Ler (green arrows) or Col-0 (red arrows) alleles.

Table 2
Example of Results of Low-Resolution Mapping Experiment

Marker	Chromosome	Physical map position (bp)	F ₂ plants										r (%)	Sr	D (cM)	Sd	n
			1	2	3	4	5	6	7	8	9	10					
nga59	1	2.768.000	C	C	L	H	C	L	C	L	H	C	50.00	5.05			98
JV18/19	1	5.160.594	C	C	L	L	C	L	C	L	-	C	67.07	5.19			82
AthZFPG	1	8.727.056	C	C	L	H	C	L	H	L	C	C	55.10	5.02			98
SGCSNP10026	1	11.562.061	C	C	L	H	C	L	H	L	H	C	53.06	5.04			98
F1121	1	15.937.110	C	C	L	H	C	L	H	L	H	C	53.26	5.20			92
nga128	1	20.633.251	H	C	L	L	C	L	L	L	H	H	59.18	4.96			98
SGCSNP10490	1	24.696.198	H	C	H	H	C	L	L	H	L	H	57.14	5.00			98
nga111	1	27.356.874	L	C	H	H	C	L	L	H	L	L	53.06	5.04			98
nga1145	2	683.625	H	H	L	L	C	C	L	H	L	C	59.18	4.96			98
SGCSNP922	2	4.321.924	H	H	L	L	C	C	L	H	L	H	54.26	5.14			94
SGCSNP8895	2	7.860.251	H	C	L	L	C	L	L	H	L	L	57.14	5.00			98
nga1126	2	11.670.000	H	H	C	H	H	L	L	H	L	L	53.06	5.04			98
nga168	2	16.298.919	H	H	C	H	H	L	L	H	L	H	52.04	5.05			98
nga126	3	3.649.000	C	C	H	H	H	L	H	H	L	C	39.80	4.94	54.37	13.49	98
MYF24	3	6.370.809	C	C	H	H	H	L	H	H	L	C	36.73	4.87	46.94	10.58	98
AthGAPab	3	9.796.456	C	C	C	C	H	L	H	C	H	C	23.47	4.28	25.46	5.49	98
T32N15	3	16.676.053	C	C	C	C	C	C	H	C	H	C	7.14	2.60	7.19	2.66	98
F28P10	3	20.702.928	C	H	C	H	H	C	C	H	C	C	15.31	3.64	15.81	4.01	98
nga6	3	23.042.025	C	H	C	H	H	C	C	H	H	H	27.55	4.51	30.99	6.48	98
nga1111	4	5.074.681	H	H	H	H	L	H	C	H	H	C	62.24	4.90			98
AthF28J12	4	9.456.646	H	H	H	H	L	H	C	H	H	C	55.10	5.02			98
nga1139	4	16.444.155	H	C	C	H	C	H	H	H	H	C	44.90	5.02	73.08	25.94	98
AthCTR1	5	974.507	C	L	H	C	L	C	H	L	H	C	45.92	5.03			98
nga151	5	4.669.932	H	L	H	C	L	C	H	L	H	C	48.98	5.05	114.37	124.99	98
nga76	5	10.418.614	H	L	H	C	H	C	C	H	C	H	31.63	4.70	37.29	7.83	98
AthPHYC	5	14.025.127	H	L	C	C	H	C	C	H	H	H	34.69	4.81	42.77	9.27	98
SO191	5	15.021.915	H	L	H	C	H	C	C	H	C	H	31.91	4.81	37.76	8.11	94
MNF13	5	16.359.785	H	L	H	C	H	C	C	H	H	H	33.70	4.93	40.89	9.03	92
MCL19	5	18.753.210	H	L	H	C	H	C	C	H	H	H	36.96	5.03	47.43	11.09	92
MNB8	5	21.549.766	H	L	H	H	H	H	H	H	H	H	48.91	5.21			92
MUA2	5	23.396.513	H	H	H	C	L	C	H	H	H	H	43.88	5.01	68.25	21.80	98
K8K14	5	26.947.209	H	H	H	C	L	C	H	H	H	H	51.02	5.05			98

Genotyping results obtained for a mapping population of F₂ plants derived from a cross involving the wild-type *Ler* and a mutant with a Col-0 genetic background. For the sake of clarity, the genotypes of only 10 of the 50 F₂ plants analyzed are shown (columns headed as F₂ plants 1–10). Values in all the remaining columns correspond to calculations made for the whole mapping population of 50 plants. C: homozygote for the Col-0 allele (to be scored as 0 recombinant chromosomes). H: heterozygote (1 recombinant chromosome), L: homozygote for the *Ler* allele (2 recombinant chromosomes), –: no amplification obtained for a given marker. r: recombination frequency, obtained as 100-recombinant chromosomes/total chromosomes studied. Sr: Standard error of recombination frequency calculated as $[r(100 - r)/n]^{1/2}$. D: map distance in cM, calculated using the mapping function of Kosambi (8) as $25 \ln[(100 + 2r) / (100 - 2r)]$. Sd: Standard error of map distance, calculated as $(2500Sr) / (2500 - r^2)$. n: total number of chromosomes genotyped. Physical map positions of the markers were obtained from the Munich Information Center for Protein Sequences (MIPS) *Arabidopsis thaliana* database.

Data corresponding to recombination frequencies smaller than 30% are highlighted in bold and were considered unequivocal evidence of linkage. Analysis of the table indicates that the mutation under study is flanked by the T32N15 and F28P10 markers. In addition, recombination frequencies indicate that the mutation is linked to AthGAPab, T32N15, F28P10 and nga6. More specific information can be obtained from the genotypes of plants, which suggest that the mutation is located above F28P10 (plants 2, 4, 5, and 8) and below T32N15 (plants 7 and 9).

2. A very rough, but in many cases good enough, map position can be obtained by genotyping only 18 to 20 F2 plants (4,7).
3. As an alternative to using a Mixer Mill, plants can be individually ground using autoclavable plastic pestles attached to a cordless motor grinder. In this case, add 250 μ L of DNA extraction buffer to each tube, completely grind the tissue using one pestle per sample. Add another 250 μ L of extraction buffer and mix by vortexing.
4. Many of the primer sequences used here are different from those described in some databases for a given marker, and have been designed to obtain PCR amplification products of nonoverlapping sizes.
5. A 0.020–0.025 μ mol oligonucleotide synthesis scale should be sufficient to perform a very large number of PCR amplifications. We purchase labeled and nonlabeled oligonucleotides from Applied Biosystems and Sigma, respectively.
6. Less complex mixes than those used here can be obtained by using only a subset of the primer pairs shown in Table 1. Some markers of chromosome 5, for instance, can be omitted.
7. We recommend using lyophilized primers, which can be dissolved in water to reach the required concentration. To avoid contamination, we use filtered micropipet tips for the preparation and manipulation of oligonucleotide stock solutions.
8. Some oligonucleotides should be used at a 75 nM final concentration in PCR mixes, as we have found that 50 nM is not sufficient to ensure a good yield of some amplification products.
9. Although we use a polymerase provided by a local supplier (Ecoaq; Ecogen cat. no. ETAQ500), any other *Taq* polymerase should provide efficient amplification.
10. Although we routinely use ABI thermocyclers, many others can be used to perform the amplifications described here. Nevertheless, some optimization of the thermocycling program may be necessary.
11. DNA sequencers other than those from ABI can be used with the appropriate software for data collection and analysis. Check the list of fluorophores compatible with your sequencer before ordering labeled oligonucleotides.
12. In a multiplex PCR amplification, it is common to obtain different yields of different amplification products. In addition, the intensity of the signal of the amplification products labeled with PET is lower than that of the remaining fluorophores at similar concentrations. Although the yield of amplification products for a few markers may be below the detection threshold in some reactions, the data collected after genotyping 50 plants will be sufficient to find linkage to one or more markers.
13. To map a large number of mutations, we recommend using the Genotyper program rather than manual genotyping.

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