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Molecular mapping of some Arabidopsis thaliana genes determining leaf shape and chlorophyll defects

Jana Řepková*, Sylvie Hlaváčová, Pavel Lízal, Zdeňka Kyjovská, & Jiřina Relichová

Masaryk University Brno, Faculty of Sciences, Department of Genetics and Molecular Biology, Kotlářská 2, CZ–61137 Brno, Czech Republic; tel.: +42549496895, fax: +42549492570, e-mail: repkova@sci.muni.cz

Abstract: Six Arabidopsis thaliana mutations were developed in our laboratory and are held at the Nottingham Arabidopsis Stock Centre. Morphological mutations were called cupuliformis, rotundata and involuta. The second group of mutants with chlorophyll defects were chlorominuta, lucida and lucida(S). The aim of our work was to localise the six mutant alleles on the genetic map of A. thaliana. The level of DNA polymorphism among ecotypes of A. thaliana, S96 and Gijon-G (genetic backgrounds of mutations) vs. Columbia and Landsberg erecta, had to be evaluated to determine suitable parental plants for the crosses. DNA markers, 16 microsatellites and 6 cleaved amplified polymorphic sequences, were used for recombination analysis in F_2 populations. The location of the cupuliformis mutation was on the short arm of chromosome 1, the rotundata mutation on the long arm of chromosome 4 and the involuta mutation on the long arm of chromosome 2. The chlorominuta mutation with a chlorophyll defect was located on chromosome 3 and the positions of both lucida and lucida(S) mutations were on chromosome 4. This mapping study simplified gene identification and evaluation of potential allelism with mutants already isolated by previous authors.

Key words: *Arabidopsis thaliana*, CAPS, DNA markers, genetic mapping, SSR

Introduction

During the last fifteen years, the efforts of geneticists and molecular biologists have been concentrated on the study and understanding of the molecular basis of plant development and the identification and characterisation of new genes. In this field, *Arabidopsis thaliana* (L.) Heynh. has played an important role as the major plant model system. It was the first plant species for which the entire genome was sequenced (The *Arabidopsis* genome initiative, 2000), but our knowledge of gene function is still limited. The goal of the new project, *Arabidopsis* 2010 Program, is to establish the function of as many *Arabidopsis* genes as possible by the year 2010 (Ausubel, 2002).

Mutagenesis and molecular-genetic strategies have been developed to facilitate the isolation of new genes and have become an attractive method for functional analysis (Meinke et al., 1998). There are basically two ways to link the sequence and function of a specific gene: forward and reverse genetics (Peters et al., 2003b). Reverse approaches start with specific sequences (genomic, expressed sequence tags) and try to identify the underlying functions by selecting mutations that disrupt the sequence and its function. Reverse approaches largely include widely applicable strategies such as the use of transposable elements or T-DNA constructs. The recently developed, targeting induced local lesions in genomes (McCallum et al., 2000), applied after chemical mutagenesis and capable of targeting single-nucleotide changes in protein coding genes, has the potential to also be useful in nonmodel plant species. The forward-genetic approach, where mutagenesis is followed by phenotypic screens, requires a map-based cloning strategy in which markers linked to the mutated gene are used to delimit the region containing the gene of interest. The availability of saturating marker systems and the progress made in methods to detect DNA polymorphisms make fast map-based cloning of a gene in a model species, such as *Arabidopsis*, feasible (Chandler & Webb, 2003).

Most supposed genes have not yet been located on the genetic map. Recently, mapping strategy has been aimed at DNA markers. Single sequence repeats (SSR; Bell & Ecker, 1994), cleaved amplified polymorphic sequences (CAPS; Konieczny & Ausubel, 1993) and amplified fragment length polymorphisms (AFLP; Alonso-Blanco et al., 1998) have been utilised to locate genes. The most useful are SSR and CAPS markers, because they are codominant. In The *Arabidopsis* Information Resource (TAIR) database (http://www.arabidopsis.org/), 335 CAPS

* Corresponding author

Table 1. Characteristics of mutations tested.

Mutation	Symbol	Phenotype	Genetic background	Mutagen	Catalogue number[1]
cupuliformis	*cp*	leaves asymmetric, roundish, wrinkled, edges rolled up	S96	MNU	N242
rotundata	*rd*	leaves broad, roundish, petioles short, vigorous, late flowering	S96	MNU	N254
involuta	*iv*	cotyledons deformed, leaves lapped, very diminutive, very early flowering	S96	EMS	N247
chlorominuta	*chm*	cotyledons light green, leaves light yellow green,	S96	EMS	N246
lucida	*lc*	cotyledons, leaves and siliques light yellow green	Di-G	MNU	N251
lucida(S)	*lc(S)*	cotyledons and leaves light yellow green	S96	X	not included

EMS – ethyl methanesulfonate, MNU – methyl nitrosourea, X – X-rays
[1]NASC catalogue number (http://nasc.nott.ac.uk/)

and 288 SSR markers are available for *Arabidopsis*, but only 81 and 53, respectively, have known positions on the genetic map derived from recombinant inbred lines (Lister & Dean, 1993). Single nucleotide polymorphisms (SNP) and insertion/deletion polymorphisms (I_ND_{EL}) represent a virtually inexhaustible source of polymorphic markers in plants (Peters et al., 2003b). In addition to SNP and I_ND_{EL}, the AFLP technique is particularly suitable for map-based cloning projects. It is especially suitable for genome-wide mapping, because it detects many markers per reaction (Peters et al., 2003a).

The identification of genes controlling leaf development was previously enabled by means of the study of many mutants of *Arabidopsis* (Berná et al., 1999; Serrano-Cartagena et al., 1999), *Nicotiana* (McHale, 1993), *Pisum* (Hofer et al., 1997; Hofer et al., 2001) or *Antirrhinum* (Waites & Hudson, 1995). Berná et al. (1999) performed a large-scale screening for mutants with abnormal leaves in *Arabidopsis*. They analysed 255 mutant lines belonging to 94 complementation groups. Serrano-Cartagena et al. (1999) subjected 57 leaf morphogenesis mutants to genetic analysis.

The aim of our work was to localise on the genetic map of *A. thaliana* six mutant alleles determining altered leaf shape and chlorophyll defects. These mutants originated in our laboratory and are stored at the Nottingham *Arabidopsis* Stock Centre (NASC, http://nasc.nott.ac.uk/). DNA markers were used for recombination analysis and mapping. The level of DNA polymorphism among available ecotypes of *A. thaliana* was evaluated to determine suitable parental plants for the crosses. Localising the genes on the genetic map will facilitate determining if they are alleles of mutants already isolated by previous authors. The knowledge of map positions of new mutant loci of *Arabidopsis* is also important as a starting point for isolation of the affected genes by map-based cloning.

Material and methods

Plant material and cultivation conditions

The tested mutant lines were previously developed in our laboratory mostly by means of classical chemomutagens (Relichová, 1976) and are held at the Nottingham *Arabidopsis* Stock Centre (NASC). Morphological mutations were called *cupuliformis* (*cp*, Fig. 1A), *rotundata* (*rd*, Fig. 1B) and *involuta* (*iv*, Fig. 1C). The second group of mutants were those with chlorophyll defects, *chlorominuta* (*chm*, Fig. 1D) and *lucida* (*lc*, *lc(S)*, Fig. 1E). For all these mutations, the genetic background was S96 (Fig. 1E), except for *lc*, which had Dijon-G (Di-G). The exception is a mutation with a chlorophyll defect, *lc(S)*, which was obtained from ecotype S96 by X-raying. Morphological and other characters of tested mutants are given in Table 1. Standard genotypes Columbia (Col), Landsberg *erecta* (L*er*), S96 and Di-G were used for polymorphism testing.

Plants were grown to maturity in a controlled climate cultivation chamber at 20–22 °C with irradiation of 70 μmol m^{-2} s^{-1}, in 16 h light/8 h dark cycles.

DNA markers

SSR and CAPS markers have been chosen from http://www.arabidopsis.org/, which includes data concerning oligonucleotide sequences in SSR and CAPS markers and PCR conditions for SSR markers. Altogether, 16 SSR and 6 CAPS markers were taken for molecular analyses. Their positions on the genetic map of *Arabidopsis* based on the Lister and Dean RIL population (Lister & Dean, 1993) are given in Table 2.

The amplification reactions were carried out on a Genius thermal cycler (Techne) in 20 μL, containing 2 μL of DNA (5 ng), 200 μmoL of each dNTP (Sigma), 5 pmoL of each of the primers, 1.5 unit of *Taq* DNA polymerase (Sigma) and 1× reaction buffer (100 mM TRIS-HCl, pH

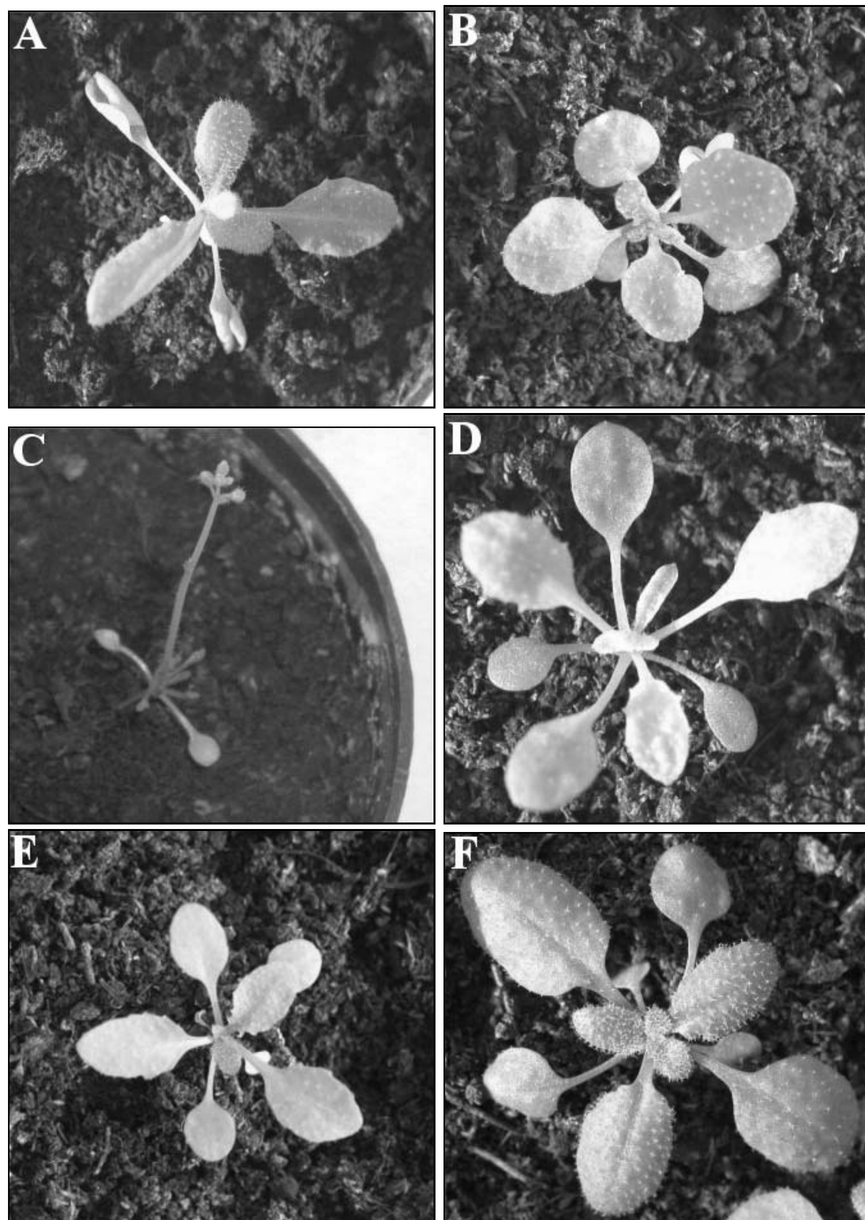

Fig. 1. Morphological mutants. A – *cp* with leaves asymmetric, roundish, wrinkled, edges rolled up, B – *rd* with leaves broad, roundish, petioles short, C – *iv* with cotyledons deformed, leaves lapped, very diminutive, very early flowering, D – *chm* with cotyledons light green, leaves light yellow green, E – *lc* with cotyledons leaves and siliques light yellow green, F – standard plant of S96 ecotype.

8.3, 500 mM KCl, 11 mM $MgCl_2$, 1% gelatine). PCR conditions were as follows for SSR markers: 94°C 2 min, 40× (94°C 15 s, 55°C 15 s, 72°C 30 s), 72°C 5 min. For CAPS markers, they had to be individually optimised.

PCR reactions were usually evaluated on 3% agarose gels containing 0.5 μg/mL ethidium bromide. Microsatellites nga 249, nga 1111 and nga 1145 were visualised on 4% agarose gels. CAPS markers were evaluated on 1.7% agarose gels.

Polymorphism testing

For polymorphism evaluation, genetic backgrounds of the tested mutations, i.e., S96 and Di-G, were compared with those of available standard genotypes, Col and L*er*. All 22 DNA markers mentioned above were used in this analysis. Length polymorphism in all microsatellite sequences was tested after PCR, and, in the case of CAPS markers, length polymorphism after restriction enzyme cleavage of DNA fragments arising from amplification reactions was analysed. On the basis of these analyses, the genetic background of each mutation tested was assigned to that of standard genotype with resembling polymorphism.

Molecular mapping

Mapping of the mutations was performed in F_2 populations. The two parents used in the mapping crosses were the mutant plant as a female (recessive homozygote *mm*) and a wild-type plant of a polymorphic ecotype ++ as a male. F_1 progeny from this cross was self-fertilised to produce F_2 individuals. Only mutant plants of the F_2 population were scored with molecular markers. Single mutant plant DNA was extracted from leaf tissues using the CTAB miniprep method described by Rogers & Bendich (1988). The DNA was dissolved in 40 μL of distilled sterile water. SSR and CAPS markers analyses were documented with the Gel Detection System EDAS 290 and processed with 1D Image Analysis Software 3.5. The number of recombinants for each cross was determined, and linkage was evaluated by recombination frequency (r, %) between the gene of interest

Table 2. SSR and CAPS markers used in mapping analyses and polymorphism Col versus L*er* testing.

Marker	Polymorphism fragment length (bp)[1]		Map position (cM)
	Col	L*er*	
nga 63	111	89	1; 9.7
G2395*	XbaI; 183, 154	XbaI; 336	1; 28.1
UFO*	TaqI; 983, 316	TaqI; 600, 383, 316	1; 49.6
AthATPASE	85	69	1; 115.8
nga 1145	213	217	2; 9.6
nga 1126	191	199	2; 50.7
nga 168	151	135	2; 73.8
GAPC*	EcoRV; 735, 713	EcoRV; 713, 390, 340	3; 8.4
nga 162	107	89	3; 20.6
AthGAPAb	142	150	3; 43.8
nga 6	143	1	3; 86.4
GA1.1*	BsaBI; 707, 527	1196	4; 17.7
nga 1111	150	140	4; 29.6
G4539*	HindIII; 600	HindIII; 480, 120	4; 57.6
nga 1139	114	118	4; 83.4
nga 1107	150	140	4; 104,7
nga 225	119	189	5; 14.3
nga 249	125	115	5; 23.7
R89998*	RSAI; 350, 50, 30	RSAI; 440, 10	5; 38.7
nga 139	174	132	5; 50.5
nga 76	231	> 250	5; 68.4
AthSO191	148	156	5; 79.9

*CAPS marker, [1]fragment length after restriction cleavage

and the DNA marker (number of recombinant chromosomes / total number of tested chromosomes). The estimate of map distance (D, cM) was given by Kosambis mapping function $D = 25\ln(100+2r/100-2r)$ (Kosambi, 1944). The relative positions of the genes on the chromosomes were assigned with DrawMap 1.1 (Van Ooijen, 1994).

Results

DNA markers

For genetic mapping, only codominant molecular markers were screened, 16 SSR and 6 CAPS markers altogether. Cycling conditions were suitable for all markers tested except nga 249 and nga 76. For nga 249, annealing temperature was 60℃ and the cycles were repeated 35 times. For nga 76, annealing temperature was 65℃. For CAPS markers, PCR conditions were for UFO: 98℃ 30 s, 45× (94℃ 20 s, 70℃ 60 s, 72℃ 1 min 20 s), 72℃ 10 min; for G2395 and G4539: 94℃ 4 min, 30× (94℃ 30 s, 55℃ 60 s, 72℃ 45 s), 72℃ 5 min; for GAPC: 98℃ 30 s, 45× (94℃ 20 s, 60℃ 60 s, 72℃ 1 min 20 s), 72℃ 10 min; for GA1.1: 98℃ 30 s, 40× (94℃ 20 s, 60℃ 60 s, 72℃ 1 min 20 s), 72℃ 10 min; for R89998: 94℃ 4 min, 30× (94℃ 30 s, 50℃ 60 s, 72℃ 45 s), 72℃ 5 min.

Polymorphism testing

The S96 and Di-G genetic backgrounds have not been frequently used for mapping; therefore, polymorphism testing was necessary. S96 showed polymorphism with Col, but not in all markers tested. The non-polymorphic markers were CAPS markers G2395 and UFO on chromosome 1, GAPC on chromosome 3, G4539 on chromosome 4, R89998 on chromosome 5 and SSR markers nga 1139 on chromosome 4 and nga 139 and AthSO191 on chromosome 5 (Tab. 3). They showed polymorphism with L*er*. Di-G showed polymorphism with Col; the only exception was nga 1139 on chromosome 4. This microsatellite showed polymorphism with L*er* (Tab. 3).

Molecular mapping

In our experiments, 20 F_2 mutant plants were subjected to initial molecular analysis and assignment of a gene to a linkage group. To determine the exact position of the gene in the linkage group, the number of plants tested was increased to 30 or 40, according to the tightness of the linkage. The *cp* mutation was linked with nga 63, and 39 plants were analysed. The location of this mutation is on the short arm of chromosome 1 at position 31.2 ± 5.5 cM. Linkage with nga 1107 and nga 1139 was determined for the *rd* mutation, and 40 plants for each DNA marker were analysed. Microsatellite nga 1139 was tightly linked with it (1.3 cM). One recombination event occurred in 80 chromosomes. The mutation was mapped to chromosome 4 in position 84.7 ± 1.2 cM. Linkage with nga 168 and nga 1126 was deter-

Table 3. SSR and CAPS markers and their polymorphism in the genetic backgrounds S96 and Di-G versus Col and L*er*.

Chromosome	Polymorphism			
	S-96		Di-G	
	Col	L*er*	Col	L*er*
1	nga 63		nga 63	
		G2395*	G2395*	
		UFO*	UFO*	
	AthATPASE		AthATPASE	
2	nga 1145	nga	nga 1145	
	1126	nga	nga 1126	
	168	nga 168		
3		GAPC*	GAPC*	
	nga 162		nga 162	
	AthGAPAb		AthGAPAb	
	nga 6		nga 6	
4	GA1.1*		GA1.1*	
	nga 1111		nga 1111	
		G4539*	G4539*	
		nga 1139		nga 1139
	nga 1107		nga 1107	
5	nga 225		nga 225	
	nga 249		nga 249	
		R89998*	R89998*	
		nga 139	nga 139	
	nga 76		nga 76	
		AthSO191	AthSO191	

*CAPS marker

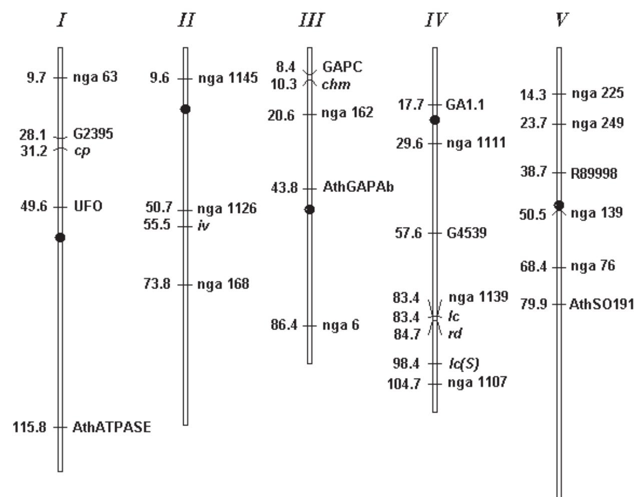

Fig. 2. Genetic map of *Arabidopsis thaliana* with DNA markers and new mutant loci indicated.

mined in the *iv* mutation; 37 and 13 plants, respectively, were analysed. This mutation was located at position 55.5 ± 5.0 cM on the long arm of chromosome 2, flanked by the two DNA markers tested. The *chm* mutation with a chlorophyll defect was located on chromosome 3 after linkage detection with nga 162 and GAPC. The mutation was located at the top of this chromosome at position 10.3 ± 1.4 cM. Molecular analysis of the *lc(S)* mutation revealed linkage with markers nga 1139 and nga 1107 on chromosome 4 at position 98.4 ± 4.1 cM. The *lc* mutation was linked with the same markers as the *lc(S)* mutation. Linkage with nga 1139 was very tight, and no recombination event was detected among the 76 chromosomes tested. This indicated that the position of *lc* on chromosome 4 is the same as that of nga 1139, i.e. 83.4 cM. Mapping efforts are summarised in Table 4 and Figure 3.

Discussion

One of the possible approaches to a plant gene function study in a model plant, such as *Arabidopsis*, involves the determination of the position of a targeted gene on the genetic map, the choice of a candidate gene and the isolation of DNA sequence underlying the phenotype of interest. Chemical mutagenesis generates a greater diversity of mutations and thus allows identification of

Table 4. Results of recombination analysis and map positions of mutations tested.

Mutant	Marker	n^1	No. of S96 (Di-G) chrom.	No. of Col chrom.	$r \pm s_r^2$ (%)	$D \pm s_D^3$ (cM)	Map[4] location (cM)
Morphological defect							
cp	nga 63	39	62	16	20.5 ± 4.6	21.8 ± 5.5	1; 31.2 ± 5.5
rd	nga 1139	40	79	1	1.3 ± 1.2	1.3 ± 1.2	4; 84.7 ± 1.2
iv	nga 168	37	61	13	17.6 ± 4.4	18.4 ± 5.0	2; 55.5 ± 5.0
Chlorophyll defect							
chm	GAPC	26	51	1	1.9 ± 1.4	1.9 ± 1.4	3; 10.3 ± 1.4
lc	nga 1139	38	76	0	0.0 ± 0.0	0.0 ± 0.0	4; 83.4 ± 0.0
lc(S)	nga 1139	45	77	13	14.4 ± 3.7	14.9 ± 4.1	4; 98.4 ± 4.1

[1] number of plants tested, [2] recombination frequency and standard error, [3] estimate of map distance according Kosambi's function, [4] chromosome and map position

genes that are likely to not be identified by insertion mutagenesis (Peters et al., 2003b).

A large number of gene products participate in leaf morphogenesis. Despite the availability of a large number of mutations affecting the development of the *Arabidopsis* leaf, very few genes have been characterised. Candidate genes for leaf shape are discussed by Tsukaya (2002) on the basis of cell theory, genetic regulation of cell sizes, cell shapes and orientations of cell division.

The morphological mutants of *A. thaliana* with altered growth potential in their leaves and overall growth habit described in this work are *rd* and *cp*. They have aberrantly shaped (rounded) leaves. The *rd* mutation was mapped on chromosome 4 at 84.7 ± 1.2 cM. The interval of 2.4 cM represents approximately 490 kb in *Arabidopsis* (1 cM is equivalent, on average, to 205 kb; Peters et al., 2003b). Similar mutants to our *rd* have been identified before and were named *rotundifolia* (*rot*) (Tsuge et al., 1996; Kim et al., 1998) and *rotunda* (*ron*) (Berná et al. 1999; Robles & Micol, 2001). *RON2* and *RON3* proved to be two different alleles of one gene. Their chromosomal location on the linkage map (Robles & Micol, 2001) nearly agrees with the location of our mutation *rotundata* on chromosome 4. Recently, Peters et al. (2003a) determined the chromosome regions of three leaf-form mutants, *ron1*, *ron2* (chromosome 4) and *ron3* (chromosome 5). A complementation test of *rd* with *ron* will determine if they are allelic or not.

The mutation c*upuliformis* was located on chromosome 1 at position 31.2 ± 5.5 cM. The relatively large chromosome interval makes candidate gene determination entirely impossible. A similar gene, *CP2* (*COMPACTA2*), is known on chromosome 2 in position 38.0 cM (TAIR database). Mutation in this gene is characterised by a compact, semi-dwarf phenotype.

Involuta is a typical developmental mutant with both vegetative structures and inflorescence architecture affected. Even though various morphological traits are altered, monogenic determination has been confirmed. The extremely dwarfed phenotype may be connected with the function of phytohormones, such as auxin, which has been implicated in a broad variety of developmental processes (Berleth & Sachs, 2001).

Processes including photosynthesis and exchange of gases with the environment are important for proper leaf and plant body structure (Berná et al., 1999). It could be expected that mutations in these genes would be connected with pigment chlorophyll defects. Three mutations with chlorophyll defects, *chm*, *lc* and *lc(S)*, were analysed and mapped. The pale green leaf phenotype of mutants indicates that some step of the process of normal plastid development and chloroplast production is blocked.

The *chlorominuta* mutation was located on chromosome 3 at 10.3 ± 1.4 cM, 600 kb. The publicly available *Arabidopsis* database (TAIR) was examined for candidate genes on this chromosome in the identified 600 kb region. For this region, a large number of AGI-annotated genes are available, and therefore we were unable to select a prospective candidate gene. Therefore, fine-mapping will continue. Gene with a similar name, *CHM1* (*CHLOROPLAST MUTATOR*), is known on chromosome 3 at position 32.0 cM (TAIR database). Mutation in this gene affects mitochondrial gene expression and impairs mitochondrial function. Owing to their positions on different chromosomes, the loci are unquestionably distinct from each other.

Surprisingly, *lc* and *lc(S)* mutations were assigned to different positions on chromosome 4, even though complementation tests confirmed the allelism of both mutant alleles (three repetitions, data not shown). This discrepancy could be explained by polymorphism between Di-G and S96 in the region of the nga 1139 and nga 1107 markers. This result would indicate chromosome aberration of the deletion/insertion type in this region. The tight linkage of *lc* with nga 1139 enabled the identification of two possible candidate genes from *Arabidopsis* chloroplast and mitochondrial gene families (genomic loci At4g25570 and At4g25700; http://www.arabidopsisorg/info/-genefamily/Chloroplast.html).

CAPS markers are SNP markers and are a very suitable type of DNA marker because of their even dispersion across the entire genome, not only in *A. thaliana* but also in other plant species. PCR cycles for all CAPS markers used in our work had to bee separately optimised. Also, methods for a few SSR markers available in the public database http://www.arabidopsis.org/ had to be optimised.

To be able to map the mutations on the S96 and Di-G backgrounds, DNA markers specific for S96 and Di-G vs. Col or L*er* ecotypes had to be identified. Polymorphism testing revealed the necessity of crossing mutants with an S96 background with standard plants of both Col and L*er* ecotypes.

The use of DNA markers permits a great reduction in the number of plants required for mapping in the F_2 generation when compared to classical morphological markers. The number of plants to be analysed is equivalent to twice the number of chromosomes to be analysed. Konieczny & Ausubel (1993) considered 28 plants sufficient for molecular mapping by CAPS markers. Between 20 to 30 of the appropriate F_2 individuals (in most cases F_2 mutants) are sufficient to establish a linkage in genome-wide mapping (6 Mb region). About 120 appropriate F_2 individuals (mutants) are sufficient to identify a 200–800 kb region containing the gene of interest (Peters et al., 2003b). In our mapping work, 40 plants were sufficient for linkage determination within 1.3 cM for the *rd* mutation. Between *lc* mutation and marker nga 1139, no recombinant event was detected among 76 chromosomes. No more plants were available for molecular analysis, but the F_2 population is being expanded for further fine-mapping.

This mapping study simplified gene identification and evaluation of potential allelism with mutants already isolated by previous authors. Saturation of the *A. thaliana* genetic map with genes of known phenotypic manifestation will aid in further detailed analysis of its genome. All six mutations were located on individual *Arabidopsis* chromosomes. Five of them seem to be new genes, the functions of which have not been determined. Determination of the chromosomal locations of phenotypic mutants is only the first step on the road to positional cloning, and, therefore, the fine-mapping procedure with SNP markers will continue.

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