

TECHNICAL ADVANCE

Development of an AFLP based linkage map of *Ler*, *Col* and *Cvi Arabidopsis thaliana* ecotypes and construction of a *Ler/Cvi* recombinant inbred line population

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Summary

An amplified fragment polymorphism (AFLP) based linkage map has been generated for a new Landsberg *erecta*/Cape Verde Islands (*Ler/Cvi*) recombinant inbred line (RIL) population. A total of 321 molecular PCR based markers and the *erecta* mutation were mapped. AFLP markers were also analysed in the Landsberg *erecta*/Columbia (*Ler/Col*) RIL population (Lister and Dean, 1993) and 395 AFLP markers have been integrated into the previous *Arabidopsis* molecular map of 122 RFLPs, CAPSs and SSLPs. This enabled the evaluation of the efficiency and robustness of AFLP technology for linkage analyses in *Arabidopsis*. AFLP markers were found throughout the linkage map. The two RIL maps could be integrated through 49 common markers which all mapped at similar positions. Comparison of both maps led to the conclusion that segregating bands from a common parent can be compared between different populations, and that AFLP bands of similar molecular size, amplified with the same primer combination in two different ecotypes, are likely to correspond to the same locus. AFLPs were found clustering around the centromeric regions, and the authors have established the map position of the centromere of chromosome 3 by a quantitative analysis of AFLP bands using trisomic plants. AFLP markers were also used to

estimate the polymorphism rate among the three ecotypes. The larger polymorphism rate found between *Ler* and *Cvi* compared to *Ler* and *Col* will mean that the new RIL population will provide a useful material to map DNA polymorphisms and quantitative trait loci.

Introduction

During the last decade, *Arabidopsis thaliana* has been accepted as the model plant organism for molecular genetic studies (Meyerowitz, 1989) and therefore a large international effort has been devoted to developing the tools to dissect its genome (Goodman *et al.*, 1995). One of these basic tools is the availability of reliable molecular markers, a necessary key step for map-based cloning. The efficient use of genetic markers requires knowledge of their accurate map positions. The first *Arabidopsis* genetic maps were constructed using different F₂/F₃ mapping populations (Chang *et al.*, 1988; Koornneef *et al.*, 1983; Nam *et al.*, 1989), but due to the statistical errors associated with the recombination estimates and to differences in recombination frequency among crosses, markers could only be integrated in a joint map of limited accuracy through a statistical approach (Hauge *et al.*, 1993). In order to provide a better mapping alternative, especially with respect to the marker order, it is important that markers are mapped in the same population. For this, Reiter *et al.* (1992) and Lister and Dean (1993) constructed recombinant inbred lines (RILs) which constitute permanent mapping populations because they are practically homozygous genotypes and can therefore be multiplied indefinitely enabling any laboratory to use them. New markers can be added to the same database containing the markers previously mapped and thus the *Ler/Col* RIL population (Lister and Dean, 1993) became the canonical mapping population of *Arabidopsis* (*AtDB*).

The molecular markers available in *Arabidopsis* are mostly RFLPs (restriction fragment length polymorphisms), which are co-dominant and very reliable but laborious (Chang *et al.*, 1988; Liu *et al.*, 1996; Nam *et al.*, 1989). Polymerase chain reaction (PCR) based markers can be analysed more rapidly and require smaller amounts of DNA. However, in *Arabidopsis*, PCR markers are either dominant and not consistently reproducible (RAPDs -random amplified polymorphic DNAs-, Reiter *et al.*, 1992)

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Table 1. Nomenclature of primers and primer combinations used in the AFLP analysis

Primer	Selective nucleotides	Nomenclature	M47	M48	M49	M50	M59	M60	M61	M62
			CAA A	CAC B	CAG C	CAT D	CTA E	CTC F	CTG G	CTT H
E11	AA	A	AA	AB	AC	AD	AE	AF	AG	AH
E12	AC	B	BA	BB	BC	BD	BE	BF	BG	BH
E13	AG	C	CA	CB	CC	CD	CE	CF	CG	CH
E14	AT	D	DA	DB	DC	DD	DE	DF	DG	DH
E23	TA	E	EA	EB	EC	ED	EE	EF	EG	EH
E24	TC	F	FA	FB	FC	FD	FE	FF	FG	FH
E25	TG	G	GA	GB	GC	GD	GE	GF	GG	GH
E26	TT	H	HA	HB	HC	HD	HE	HF	HG	HH

E, *EcoRI* primer; M, *MseI* primer.

or highly polymorphic and co-dominant, and therefore very useful (microsatellites or SSLPs – simple sequence length polymorphism; Bell and Ecker, 1994), but with only a limited number available. Consequently, different strategies have been developed to accelerate the analysis of restriction polymorphisms such as the conversion of RFLP markers into PCR markers called CAPS (cleaved amplified polymorphic sequences; Konieczny and Ausubel, 1993), and the establishment of multilocus RFLP sets like the *Arabidopsis* RFLP mapping set (ARMS, Fabri and Schöffner, 1994).

Recently, a new marker technology has been developed, called AFLP (amplified fragment polymorphism) which combines the advantages of being a multi-locus (fingerprinting) technique and PCR based (Vos *et al.*, 1995; Zabeau and Vos, 1993). The AFLP technique detects restriction fragments by PCR amplification and its versatility arises from the fact that it does not require prior knowledge of sequence. A virtually unlimited number of restriction fragments can be detected in complex genomes.

In the present work we have evaluated the efficiency and robustness of AFLP technology for linkage analyses in *Arabidopsis*. For that, two different recombinant inbred line populations have been studied: (i) the Landsberg *erecta*/Columbia population (Lister and Dean, 1993) and (ii) a new RIL population developed from crosses between the laboratory strain Landsberg *erecta* and the ecotype Cape Verde Islands. AFLPs were used to estimate polymorphism rates and to construct AFLP based linkage maps of both populations. The two maps were compared and integrated through the common markers. The quantitative nature of the AFLP technology was used to map the centromere of chromosome 3 by the measurement of allele dosage in trisomic plants. The distribution of AFLP markers is discussed in relation to the centromere positions.

Results

Polymorphism rates among Ler, Col and Cvi

The *Arabidopsis* ecotypes Landsberg *erecta* (*Ler*), Columbia (*Col*) and Cape Verde Islands (*Cvi*), the three parental lines of the two RIL populations used in the present work, were analysed with the AFLP technique using 64 different primer combinations (Table 1). The 64 fingerprinting patterns were compared among the three genotypes. The patterns obtained with five primer combinations (AA, AE, DA, FA and HA from Table 1) were not analysed further because they contained high intensity bands, probably due to repetitive DNA, and therefore the rest of the pattern was too weak to be scored reliably. For the remaining 59 fingerprinting patterns only clearly detectable bands were taken into account. The number of well-amplified bands per primer combination and genotype varied between 23 and 78, with an average of 41 bands. For each fingerprinting pattern the approximate molecular sizes of the DNA fragments amplified were compared, and when a band of similar size was observed in two lines this was taken as a shared band. When comparing the three ecotypes two by two, the number of polymorphic bands per primer combination, i.e. bands present in only one of the two parental lines, varied between 6 and 31 with an average of 16. Table 2 shows, for each pair of ecotypes, the total number of shared bands and the total number of ecotype specific bands. *Ler* and *Col* differed in 30.4% of the bands, with higher polymorphism being observed between *Cvi* and either *Ler* or *Col* (34.4% and 33.7%, respectively). Comparing the three ecotypes simultaneously, they shared 1770 out of the 3204 bands and *Ler*, *Col* and *Cvi* showed 293, 236 and 356 ecotype specific bands, respectively.

Table 2. Total number of shared bands (indicated with the '=' symbol) and total number of ecotype specific bands obtained with 59 primer combinations for each pair of ecotypes

Ler/Col			Ler/Cvi			Cvi/Col		
Ler=Col	Ler	Col	Ler=Cvi	Ler	Cvi	Cvi=Col	Cvi	Col
1983	469	396	1946	506	516	1930	532	449

Construction of a Ler/Cvi AFLP based linkage map

A set of 162 recombinant inbred lines between Ler and Cvi has been generated by single-seed descent procedure. Fourteen AFLP primer combinations were selected to make a linkage map of this population in such a way that (i) most of the different primers were used; (ii) half of the primer combinations should include an E-A+1 primer and half of them should be E-T + 1 (Table 1 and Materials and Methods); and (iii) the largest number of ecotype specific (polymorphic) bands between Ler and Cvi were obtained. The 14 fingerprinting patterns generated a total of 302 polymorphic bands which were individually scored as A (Ler), B (Cvi) or H (heterozygous, when the corresponding AFLP band showed approximately half intensity). Segregating bands showing variation in intensity among the RILs were not included to avoid bands originating from multiple loci. Approximately the same number of polymorphic bands was amplified with either the E-A+1 or E-T+1 primers (157 and 145, respectively). Figure 1 shows an example of an AFLP image containing both parental lines and several RILs.

In order to identify the linkage groups of Arabidopsis and their orientations (top and bottom), and to ensure a complete genetic coverage of its genome, 17 CAPS markers, the microsatellites nga139 and nga158, and the *erecta* mutation (which is segregating in the population), all with well-established map positions, were also analysed. The segregation data of these markers were used to obtain the linkage maps shown in Figure 2. The 322 markers were assigned to five linkage groups with a total genetic length of 475 cM. The genetic length of each linkage group was comparable to the lengths reported for other mapping populations and all markers other than the AFLP markers were located at similar positions as on previous Arabidopsis maps (Chang *et al.*, 1988; Koornneef *et al.*, 1983; Lister and Dean, 1993; Nam *et al.*, 1989; Reiter *et al.*, 1992). All genetic distances between consecutive pairs of markers were smaller than 10 cM, except for the top of chromosome 4. The distribution of AFLP markers among the five linkage groups was not proportional to the genetic lengths, chromosome 4 containing fewer markers than expected and chromosome 3 more ($\chi^2 = 18.49$, d.f. = 4, $P < 0.01$). Although AFLP markers seem to have a similar distribution independent of whether they were generated with the E-A+1 or E-T+1 primers, the overall distribution within the

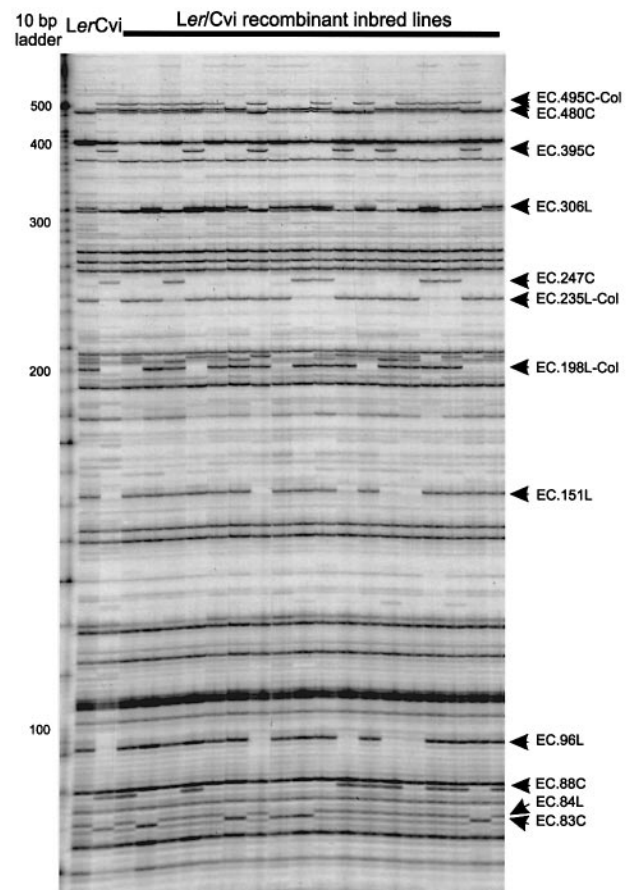


Figure 1. An AFLP image obtained with the primer combination EC, including the Ler and Cvi parental lines and a set of recombinant inbred lines. Segregating scored bands are indicated with arrows.

linkage groups was not uniform; at least one large cluster of AFLP markers is observed in each linkage group (Figure 2).

RILs are expected to be essentially homozygous by the F_8 generation, the theoretical chance of a heterozygous RIL for a given marker being 0.78%. AFLP markers are dominant markers when only the presence or absence of bands is recorded, but heterozygous genomic regions can be identified when the band intensities are taken into account (see bands marked with * in Figure 4). In the first step of the linkage analysis the putative heterozygous genotypes were included as missing data, and in most cases these markers appeared linked. Thereafter, each RIL was carefully checked in the markers surrounding the putative hetero-

zygous region which might have been missed previously. In general, consistency was obtained for the heterozygous regions which, when possible, were confirmed with CAPS markers; the average frequency of heterozygous lines per marker was 0.71% with no marker exceeding the expected maximum value of four heterozygotes ($P < 0.01$). Therefore, selection for maintaining heterozygous plants in any genomic region has not occurred.

The segregation ratio of the two homozygous classes at each marker was tested for the 1:1 expected proportion in case of no selection for any of the parental alleles. Figure 3 shows a simplified version of the *Ler/Cvi* genetic map indicating the genomic regions deviating from this ratio ($P < 0.05$). Although the selection of plants used to obtain the next generation was random, it is clear that segregation distortion was obtained for a large portion of the genome, mainly involving chromosomes 1, 3 and 5. The largest distortions were of a 1:2.3 magnitude for top chromosome 5 with an excess of *Ler* alleles, the rest of the distorted segregation genomic regions showing about a 1:2 ratio. Comparable magnitude and extension of distortions were

observed in the *Ler/Col* RIL population (Lister and Dean, 1993) in some cases involving the same genomic areas, such as the bottom of chromosome 1 and the top of chromosome 5 where *Ler* alleles behaved in a similar manner. Possible sources and the origin of this segregation distortion in the *Ler/Cvi* RIL population are under study and will be reported elsewhere.

AFLP markers can be considered as allele markers, since some of the bands scored as different markers might be allelic. To identify possible allelic band pairs that could be used as co-dominant markers, the obtained map was analysed, taking into account the following genetic criteria: (i) two AFLP bands might be allelic when they are derived from different parents, with the same primer combination; (ii) the two AFLP markers segregate complementary. Twenty-nine pairs of bands (19.2% of the bands) satisfied these criteria, and in all cases their molecular sizes differed in a few base pairs (between 1 and 5 bases, with the exception of three pairs differing in 25, 17 and 12). In 27 out of these 29 putative co-dominant locus markers, RILs with both bands were found and in these putative hetero-

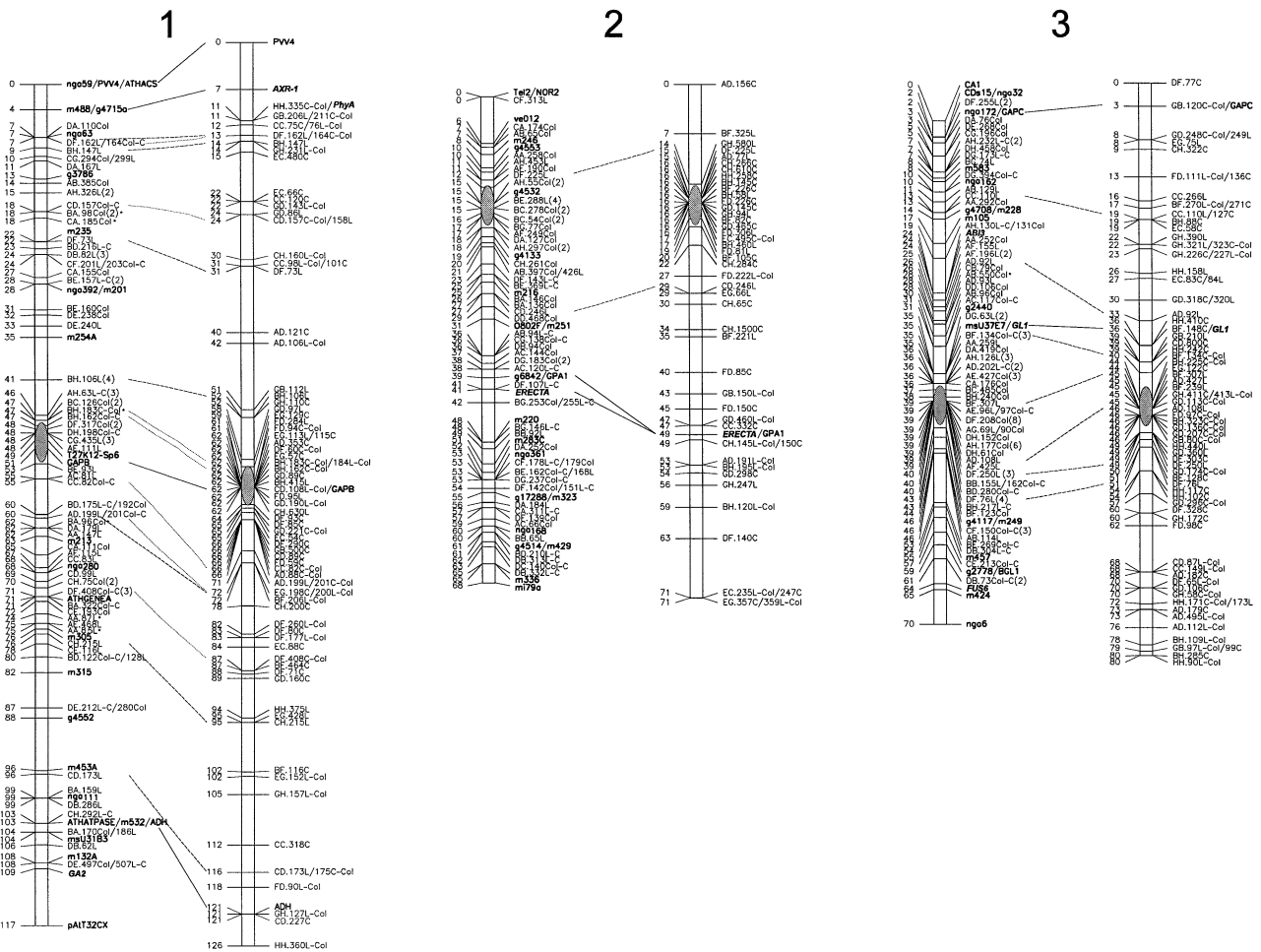


Figure 2.

zygotes both bands showed consistently weaker intensity than in the lines containing only one band, strongly suggesting true allelism. These pairs of AFLP markers are kept as single locus markers in Figure 2, their names being composed of both allele names, although true allelism should be confirmed by sequence analysis. Assuming these pairs of bands as allelic, the maximum total number of AFLP loci that have been mapped might be estimated as $302 - 29 = 273$. In addition, a few other pairs of bands were observed that might be allelic, although each band is amplified with a different primer combination, and pairs of bands from the same parent might be co-segregating thus representing a single polymorphism. Taking into account the complete linkage among some of the AFLP markers, 107 AFLP bands appeared genetically linked to 35 positions, which would represent a minimum number of 35 loci, giving an underestimation of 201 loci. Therefore,

the total number of loci mapped with the 302 AFLP allele markers is somewhere between 201 and 273.

Construction of a Ler/Col AFLP based linkage map

To generate an AFLP linkage map of the Ler/Col RIL population (Lister and Dean, 1993) the 32 primer combinations involving the E-A+1 primers (Table 1) were used. A total of 395 segregating AFLP bands were scored individually in 88 RILs, and detailed information on these markers has been given by Kuiper (1998). Heterozygotes were replaced by missing data, since RFLPs and AFLPs were scored at different laboratories from different sources of DNA and therefore heterozygosity of the lines might be different.

To integrate the AFLP markers with the molecular markers previously mapped in this population a set of 122 RFLPs, CAPSs, SSLPs (AtDB) were included in the linkage

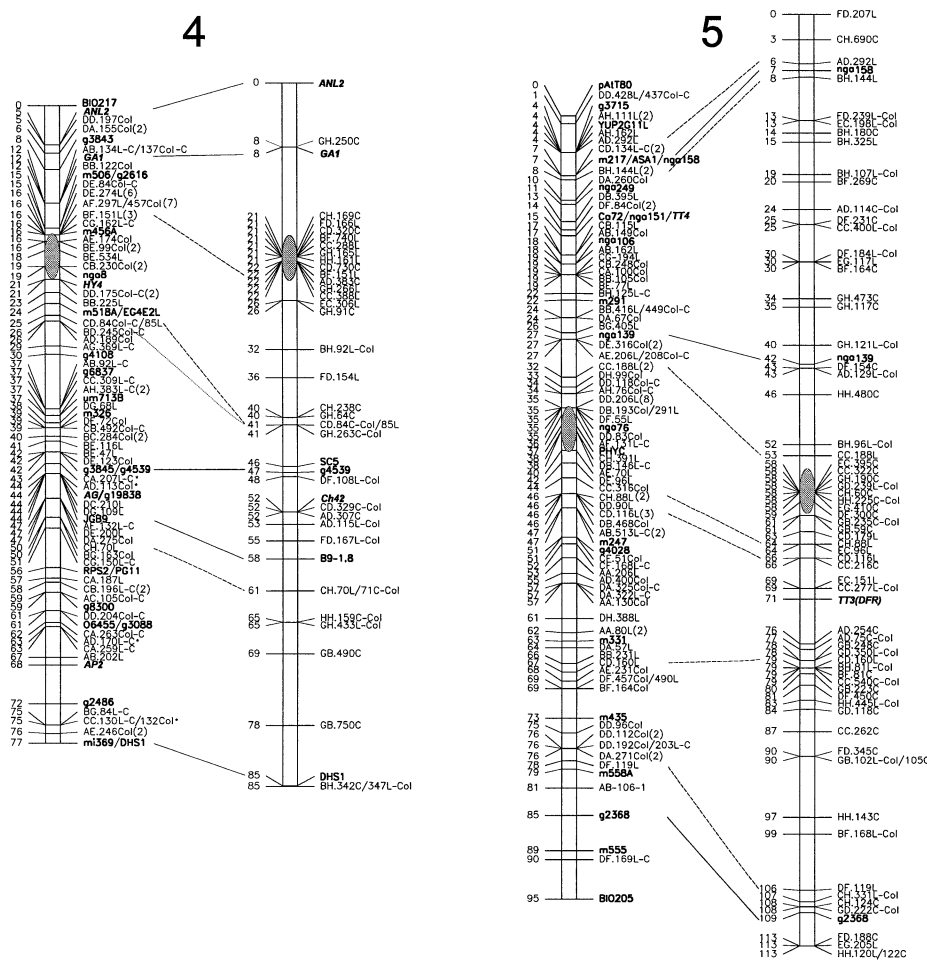


Figure 2. The Ler/Col (left chromosome) and Ler/Cvi (right chromosome) AFLP based linkage maps. Markers other than AFLP markers are shown in bold, and markers of mutant genes are indicated in bold italic. Putative pairs of allelic AFLP markers are shown as single co-dominant markers, their names being composed of both allele names separated by a slash. Centromere positions are shown as an ellipse. Common markers integrating both maps are connected by lines; continuous lines correspond to CAPS and microsatellite markers and the *erecta* mutation, dashed lines to Ler (L) specific AFLP markers, and dotted lines to AFLP bands present in both Cvi (C) and Col. In the Ler/Col map, numbers in parentheses close to some of the AFLP markers indicate the number of AFLPs completely linked to that position and which have been removed from the figure to simplify (these markers can be found in Appendix 1). Asterisks close to some of the Ler/Col AFLP markers indicate molecular size estimated by eye.

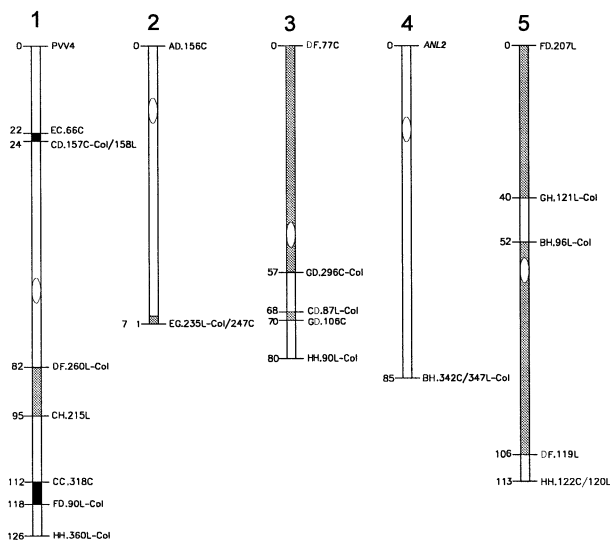


Figure 3. Simplified *Ler/Cvi* genetic map showing the genomic regions deviating from the 1:1 ratio ($P < 0.05$).

Grey boxes indicate genomic regions with significantly higher proportion of RILs with *Ler* alleles. Black boxes indicate genomic regions with significantly higher proportion of lines with *Cvi* alleles.

analysis. Markers were selected in such a way that they were evenly distributed over the complete genome, and relatively complete data sets for the 88 RILs were available. Special attention was given to include PCR-based markers such as CAPSs and microsatellites, and markers of mutant genes, since these are more often used in Arabidopsis research. The 517 markers were assigned to five linkage groups with a total genetic length of 427 cM (Figure 2). Given the large number of markers analysed in this population, 96 AFLPs completely linked to another AFLP are not included in Figure 2 but are shown in Appendix 1. Most of the genetic distances between consecutive pairs of AFLP markers were smaller than 5 cM, the largest distance being 8 cM. As for the *Ler/Cvi* RIL map, the distribution of AFLP markers among the five linkage groups was not proportional to the genetic lengths, chromosome 3 again containing more markers than expected ($\chi^2 = 17.19$, d.f. = 4, $P < 0.01$). The distribution of AFLP markers within each linkage group was not uniform, and at least one cluster is observed in each group (Figure 2 and Appendix 1). The order of markers within the clusters could not be established, and particular difficulties were observed for chromosome 3 in the region between the RFLPs g2440 and g4117 where markers could not be ordered unambiguously. The few recombinants available between markers in this region could not be interpreted to give a preferential order.

In order to estimate the number of loci corresponding with the 395 AFLP allele markers, we proceeded as in the *Ler/Cvi* population (see above). Twenty-eight pairs of AFLP bands (14.2%) behaved as allelic, both bands from the two parents being amplified with the same primer combination, and in many cases differing in size just in a few base pairs

(fewer than 10). These putative allelic bands are kept as single co-dominant markers, their names composed of both allele names in Figure 2. Taking into account the clustering of AFLP markers in certain regions, the number of loci mapped with the 395 AFLP bands might be somewhere between 270 and 367.

Integration of the *Ler/Col* and *Ler/Cvi* molecular genetic maps

Figure 2 shows the *Ler/Col* and *Ler/Cvi* molecular maps linked through the anchoring markers scored in both populations. A total of 49 markers evenly distributed over the five linkage groups allows integration of the two maps. The segregation of 13 CAPS markers, two microsatellites and the *erecta* mutation was scored in both populations (joined by continuous lines in Figure 2). *AXR-1* and m488 have been considered as comparable genetic markers because they are physically located very close to one another (AtDB). In addition, seven common primer combinations were used to generate AFLP markers in both populations. Since the two populations share *Ler* as a common parent, it was expected that the *Ler*-specific DNA fragments, i.e. the AFLP markers obtained as present only in *Ler*, should map genetically at similar positions. Twenty-five AFLP *Ler*-specific markers were scored in both populations and they all mapped at similar positions (markers linked by dashed lines in Figure 2). On the other hand, some bands of similar molecular size were amplified with the same primer combination in *Col* and *Cvi* but not in *Ler*, and these AFLP markers might correspond to either the same allele shared by both ecotypes or to two different loci. Eight AFLP markers of this type were recorded and in all cases they were assigned to the same linkage group and to similar map positions in both populations, suggesting that they are the same locus (indicated as dotted lines in Figure 2). Nevertheless, the exploitation of this type of marker requires a very careful comparison of the molecular sizes and true allelism should be confirmed by sequence analysis. The genetic order of all the anchoring markers within each linkage group was the same in both maps.

The relative lengths of each linkage group are similar in both crosses. However, the absolute genetic lengths of the *Ler/Col* linkage groups are slightly smaller than the *Ler/Cvi* groups. We do not think these differences represent a lower recombination rate in the first population, but more probably are due to a compression effect obtained when inconsistencies are found while trying to build a map order. Taking into account the fact that many of the RFLPs and CAPS markers included in the *Ler/Col* population were analysed in different laboratories, and therefore not subject to cross-checking, it is expected that this gave rise to a higher frequency of genotype misclassifications than in

the *Ler/Cvi* map where all markers were recorded from the same single plant DNAs.

Co-location of AFLP marker clusters and the Arabidopsis centromeres

The location of the AFLP clusters obtained in certain genomic regions of the five linkage groups and in the two RIL mapping populations (Figure 2) suggested that some of them might correspond to the centromeres of the *Arabidopsis* chromosomes (Koornneef *et al.*, 1983). The centromere of chromosome 1 has been genetically mapped with RFLPs (Richards *et al.*, 1991) and is closely linked to the marker GAPB. This marker was included in both RIL maps and colocalises with the largest cluster of AFLP markers of this linkage group. The centromeres of chromosome 2 and 4 have been located on the recently developed physical YAC maps, from the positions of centromere associated repeated sequences (Schmidt *et al.*, 1995, 1996; Zachgo *et al.*, 1996). The centromere of chromosome 2 is physically very close to the RFLP marker g4532 and the chromosome 4 one lies between the RFLP marker m506 and *HY4*. These markers were included in our analysis and map to the largest AFLP clusters of these linkage groups. The centromere of chromosome 5 has been physically mapped by *in situ* hybridisation of YAC clones (P. Fransz and M. Stammers, personal communication) and is located north and close to the molecular marker *PHYC*, at a similar position to one of the AFLP clusters.

In contrast, little information is available about the chromosome 3 centromere, so far only assigned by telotrismic analysis between the morphological markers *GL1* and *TT5* (Koornneef *et al.*, 1983). To locate this centromere more accurately, a quantitative analysis of AFLP markers of trisomic plants was performed. Nine different genotypes were used: diploid *Ler* plants, *Ler* primary trisomic Tr3 plants which carry an extra chromosome 3, and *Ler* telotrismic Tr3A plants carrying an extra 3A arm; diploid F_1 *Ler* × *Col* plants; telo-trisomic F_1 *Ler* × *Col* plants with an extra *Ler* 3A arm, and diploid *Col* plants; diploid F_1 *Ler* × *Cvi* plants; telo-trisomic F_1 *Ler* × *Cvi* plants with an extra *Ler* 3A arm, and diploid *Cvi* plants. The intensity of 22 *Ler* AFLP bands which had been assigned to chromosome 3 by the linkage analyses was quantified in each genotype using a densitometer, and each value was divided by either the *Ler* intensity band value (in the three *Ler* background genotypes), or the F_1 *Ler* × *Col* value (in the three *Col* background genotypes) or the F_1 *Ler* × *Cvi* (in the three *Cvi* background genotypes) (Table 3 and Material and methods). Thus, the three different genetic backgrounds provide independent tests for the assignment of the AFLP bands to either the telocentric chromosome arm or the upper arm of chromosome 3, by comparing the trisomic and normal diploid plants. From Table 3 it can be deduced

that our AFLP protocol allowed a good distinction between 1 and 2 doses of a band, the difference in intensity of an AFLP band between genotypes carrying 2 and 3 doses being less precise and probably requiring a reduction in the amount of starting template DNA. As it is shown in Figure 4, this analysis located the centromere of chromosome 3 between two completely linked AFLP markers, BF.307 L mapping in the upper arm and BF.239 L in the lower one. The centromere of chromosome 3 therefore also maps in a cluster of AFLP markers.

Figure 2 shows the approximate positions of the centromeres in the different linkage groups. Because marker order could not be established unambiguously within the AFLP clusters and the surrounding markers, a rather broad location of 5 cM has been assigned, and further mapping would be required to refine the accurate location.

Discussion

Efficiency and robustness of AFLP markers for linkage analysis

An AFLP based linkage map has been generated for a *Ler/Cvi* RIL population, including a total of 321 molecular PCR based markers and the *erecta* mutation. AFLP markers were also analysed in the *Ler/Col* RIL population (Lister and Dean, 1993) and 395 new markers have thus been integrated in the *Arabidopsis* molecular map through their linkage with 122 previously mapped RFLP, CAPS and SSLP markers. The analysis of these maps show that the AFLP markers generated with the *EcoRI/MseI* restriction enzymes are not distributed among the five linkage groups in proportion to the genetic lengths of the chromosomes; chromosome 3 contains more markers than expected, and this is mainly due to an enrichment of markers around its centromere. It has been shown by Fransz *et al.* (1998) that a chromosomal rearrangement may be involved around the chromosome 3 centromere of *Ler* in comparison to *Col* and *Cvi*, which might have led to certain local suppression of recombination. Nevertheless, AFLP markers were found along almost the entire length of the *Arabidopsis* genome, and most genetic distances between consecutive pairs of AFLP markers were smaller than 10 cM. Therefore, AFLP markers provided a good coverage of the majority of the *Arabidopsis* genome, showing the efficiency of this technology to generate dense molecular linkage maps in a relatively short period of time compared with RFLPs or other PCR based markers. The mapping populations were AFLP genotyped at different laboratories using different systems of separation and visualisation of the amplified DNA fragments, and data were analysed independently. Thus, the robustness of the AFLP markers could be tested. Twenty-five AFLP markers segregating from the common parent (*Ler*) and eight AFLP markers corresponding to

Table 3. Relative band intensities of chromosome 3 AFLP bands in the nine different genotypes analysed to map the centromere of chromosome 3. The values correspond to densitometer intensity ratios between the corresponding genotype value and the value of either *Ler*, or F_1 *Ler* × *Col*, or F_1 *Ler* × *Cvi*, for each of the three genetic backgrounds, respectively (see text)

Marker name	Map position*	<i>Ler</i>	<i>Ler</i> Tr3	<i>Ler</i> Tr3A	F_1 <i>Ler</i> × <i>Col</i>	F_1 <i>Ler</i> × <i>Col</i> Tr3A	<i>Col</i>	F_1 <i>Ler</i> × <i>Cvi</i>	F_1 <i>Ler</i> × <i>Cvi</i> Tr3A	<i>Cvi</i>	Location on T3-A
FD.111L-Col	12.9	1	1.22	0.88	1	1.06	1.01	1	0.85	0	-
BF.270L-Col	17.2	1	1.22	0.96	1	1.08	1.11	1	0.82	0	-
GH.390L	22.3	1	1.33	0.91	1	0.96	0	1	0.97	0	-
GH.321L	22.3	1	0.99	0.77	1	0.81	0	1	0.88	0	-
GH.227L-Col	22.9	1	1.24	0.89	1	1.07	0.97	1	0.92	0	-
HH.158L	26.3	1	1.43	1.07	1	0.89	0	1	0.76	0	-
GD.320L	30.0	1	1.09	0.90	1	0.97	0	1	1.10	0	-
AD.92L	32.9	1	1.09	0.87	1	1.05	0	1	0.81	0	-
BF.307L	45.2	1	1.31	1.17	1	0.95	0	1	0.97	0	-
AD.427L	45.2	1	1.52	1.57	1	1.69	0	1	1.96	0	+
BF.239L	45.2	1	1.45	1.29	1	2.16	0	1	2.15	0	+
GH.413L-Col	45.2	1	1.28	1.17	1	1.22	1.00	1	2.04	0	+
AD.108L	46.1	1	1.17	1.12	1	1.35	0.82	1	1.81	0	+
HH.440L	48.5	1	1.68	1.83	1	1.69	0	1	1.43	0	+
GD.360L	48.5	1	1.63	1.76	1	2.04	0	1	1.81	0	+
DF.250L	48.5	1	1.41	1.72	1	1.50	0	1	1.86	0	+
DF.76L	50.5	1	1.30	1.45	1	2.16	0	1	1.75	0	+
DF.65L-Col	70.2	1	1.30	1.56	1	1.28	0.97	1	2.61	0	+
HH.173L	72.4	1	1.35	1.34	1	1.67	0	1	1.93	0	+
AD.495L-Col	73.2	1	1.31	1.48	1	1.27	1.09	1	2.21	0	+
AD.112L-Col	75.7	1	1.14	1.15	1	1.28	1.07	1	1.58	0	+
HH.90L-Col	79.6	1	1.41	1.47	1	1.07	0.97	1	1.81	0	+

*Map position in *Ler*/*Cvi* map, since all these markers segregated in that population.

+The AFLP band is concluded to locate on the trisomic chromosome 3 arm.

-The AFLP band is concluded to locate on the non trisomic chromosome 3 arm.

similar size DNA fragments amplified with the same primers in *Col* and *Cvi* but not in *Ler* were compared between the two maps. All these markers were assigned to similar positions. This shows that segregating bands from a common parent can be compared between different mapping populations, and that AFLP bands of similar molecular size amplified with the same primer combination in two different ecotypes are likely to correspond to the same locus. The correspondence in map position of AFLP bands of common size has also been observed between potato mapping populations (Roupe van der Voort *et al.*, 1997) where it was confirmed by sequence analysis, showing the usefulness of AFLP markers for comparative mapping. Nevertheless, this utility might be restricted in genomes containing a higher degree of duplication, or across taxa.

AFLP markers are allele markers, i.e. marker bands are amplified from one parental line but not from the other, and the maps that we have constructed can be understood as three ecotype specific maps for *Ler*, *Col* and *Cvi*. It has been shown previously that AFLP markers can be applied efficiently for high resolution mapping and chromosome landing (Cnops *et al.*, 1996; Thomas *et al.*, 1995). Therefore, these maps can be used to identify sets of primer combinations that will provide markers covering most of the

genome or primers for a specific target genomic area, in crosses involving one of these three ecotypes.

AFLP clustering around the centromeres

We have shown that AFLP markers cluster around the centromeric regions of all five linkage groups. Since AFLPs are allele markers, part of the clustering might be explained by the allelism between some AFLP bands. Around 14–19% of the AFLP markers analysed are likely to be allelic pairs, as strongly suggested by the weaker band intensity present in both bands in the putative heterozygous RILs, both bands being amplified with the same primers and showing in most cases small differences in molecular sizes. Nevertheless, this allelism does not account for the complete clustering (Figure 2). The five Arabidopsis pachytene chromosome pairs, as described recently by Fransz *et al.* (1998), show prominent pericentromeric heterochromatin representing a considerable portion of about 6% of the length of each chromosome. It is likely that the Arabidopsis pericentromeric AFLP clusters lie within this pericentromeric heterochromatin or nearby euchromatic areas. However, without information on the precise physical location of the AFLP markers it is impossible to know whether clustering is due to: (i) a reduced

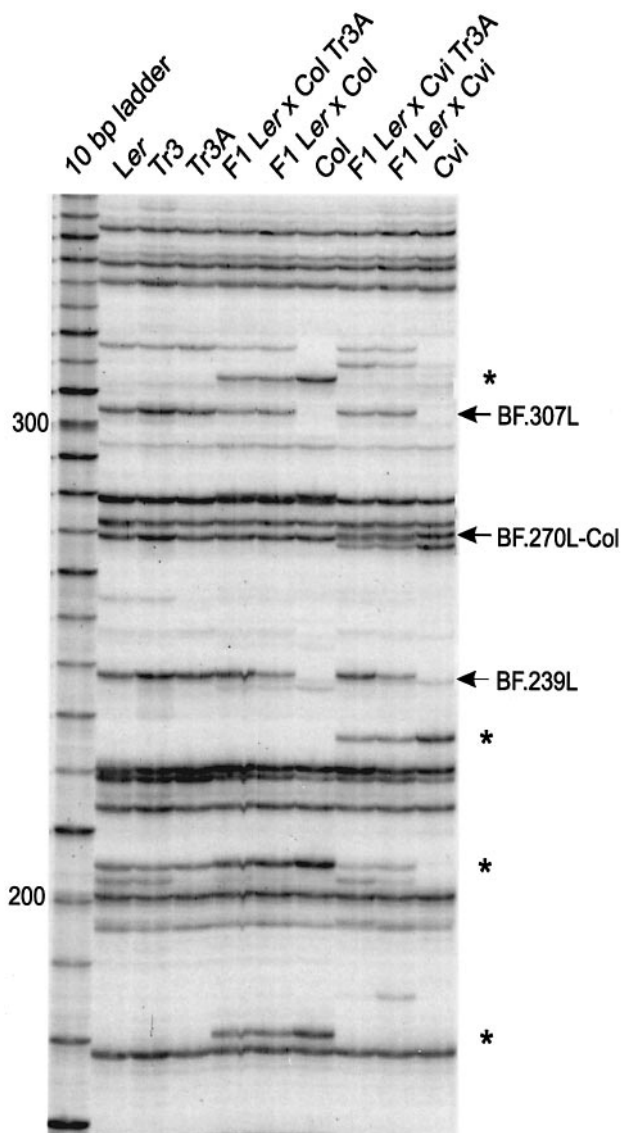


Figure 4. An AFLP image obtained with the primer combination BF and including the nine genotypes analysed to map the centromere of chromosome 3.

Markers located on chromosome 3 are indicated with arrows. Asterisks (*) indicate AFLP markers that illustrate the band intensity difference between homozygous and heterozygous genotypes.

recombination rate around the Arabidopsis centromeres; (ii) an enrichment of AFLP markers in these regions; or (iii) a combination of both previous effects. It is unlikely that lower recombination rates are the only cause of the AFLP clustering because such a phenomenon has not been observed previously in Arabidopsis when using morphological markers (Hauge *et al.*, 1993; Koornneef *et al.*, 1983), RFLPs (Chang *et al.*, 1988; Lister and Dean, 1993; Nam *et al.*, 1989), RAPDs (Reiter *et al.*, 1992) or microsatellites (Bell and Ecker, 1994). AFLP cluster regions correspond to areas for which a limited number of other types of markers are present in the current maps (AtDB). This might reflect

the low content of single copy sequences present in the pericentromeric regions of the Arabidopsis chromosomes. Pericentromeric regions contain mainly repeated sequences of unknown function, such as the 180 bp repeat (Fransz *et al.*, 1998; Maluszynska and Heslop-Harrison, 1991), and it is likely that markers in these regions have been missed in the Arabidopsis RFLP maps because when screening for RFLPs, probes showing repeated DNA patterns are often discarded. With the AFLP technique, polymorphisms in these regions will probably be less discriminated against, and single restriction polymorphisms would be identified and mapped even when the amplified band corresponds to a repeated DNA sequence. An extra enrichment of AFLP markers could probably be due to the combined effect of (i) using *MseI* as enzyme to generate most of the restriction polymorphisms. *MseI* recognises the target sequence 5'-TTAA-3' and will therefore cut more frequently in A+T rich regions, such as the pericentromeric heterochromatin areas since they show a bright colour when stained with the fluorochrome DAPI (Ross *et al.*, 1996) and this fluorochrome is known to show preference for A+T rich DNA (Sumner, 1990); (ii) a larger polymorphism frequency might be associated with non-coding genomic regions (either repeated DNA or not) than coding single copy regions. It is therefore suggested that both low recombination rate and enrichment for AFLP markers in the pericentromeric areas are involved in producing this clustering.

The clustering of AFLP markers obtained in Arabidopsis with the *EcoRI/MseI* restriction enzymes also appears in other plant AFLP linkage maps such as potato (van Eck *et al.*, 1995), barley (Becker *et al.*, 1995; Powell *et al.*, 1997), soybean (Keim *et al.*, 1997) and maize (M. Vuylsteke *et al.*, in preparation). This suggests that some clusters might also represent the centromeres in other plant species since the presence of pericentromeric heterochromatin is a general feature of plant chromosomes. On the other hand, this reduces the number of useful markers in genomic regions other than the centromeres. Therefore, in order to determine whether the clustering is specific to this enzyme, and to increase the efficiency of the generation of AFLP markers useful for linkage analyses, it will be necessary to investigate the genetic location of AFLP markers obtained with different restriction enzymes.

The use of Ler/Cvi RIL population

We have generated and characterised a set of 162 RILs between the laboratory strain *Ler*, originating from Northern Europe (Rédei, 1992), and an exotic ecotype, *Cvi*, from the tropical Cape Verde Islands (Lobin, 1983). Using the AFLP technique it is shown that *Ler* and *Cvi* differ in about 34% of the amplified DNA bands, while *Ler* and *Col* differ in 30%. The higher polymorphism rate found between

Ler and *Cvi* compared to *Ler* and *Col* will mean that the new RIL population will provide a useful material to map molecular markers when polymorphisms are not found in the *Ler/Col* population.

In addition, the combination of recombinant inbred lines as a permanent mapping population with a dense molecular genetic map also allows the mapping of quantitative trait loci (QTLs) (Burr and Burr, 1991; Lister and Dean, 1993; McCouch and Doerge, 1995). This usefulness has already been shown in *Arabidopsis* with the *Ler/Col* RIL population in which several quantitative traits such as flowering time, trichome number, seed dormancy and resistance to *Xanthomonas campestris* have been analysed, and some of these traits have been studied in multiple environments enabling the detection of genotype-by-environment interactions (Buell and Somerville, 1997; Jansen *et al.*, 1995; Larkin *et al.*, 1996; Mitchel-Olds, 1996; van der Schaar *et al.*, 1997). It is expected that the *Ler/Cvi* RIL population will also provide a useful genetic material to identify loci involved in important developmental and physiological traits, for which a large variation is found (C. Alonso-Blanco *et al.*, unpublished observations). Thus, the natural genetic variation affecting ecological traits can be analysed and exploited in *Arabidopsis*.

Experimental procedures

Plant material

Two recombinant inbred line (RIL) populations involving three different ecotypes of *Arabidopsis thaliana* were used. One population consisted of 88 RILs derived from a cross between the ecotypes *Landsberg erecta* (*Ler*) and *Columbia* (*Col*). These RILs were developed by Lister and Dean (1993) and have previously been genotyped for many molecular markers including RFLPs (Lister and Dean, 1993; Liu *et al.*, 1996; *AtDB*), CAPS markers (Jarvis *et al.*, 1994; Konieczny and Ausubel, 1993), and microsatellites (Bell and Ecker, 1994). The *Ler/Col* lines used correspond to the standard set of 100 RILs (*AtDB*) except lines 19B, 53, 182, 188, 193, 194, 237, 259, 263, 394, 397, 400. The other population consisted of 162 RILs derived from crosses between the ecotypes *Ler* and *Cape Verde Islands* (*Cvi*) (Lobin, 1983), which was generated at the Laboratory of Genetics, Wageningen Agricultural University (see below). These RILs will be available through the stock centers and the AFLP data through the *AtDB*. Seeds of *Cvi* were obtained from G. Coupland (John Innes Centre, Norwich, UK) who obtained it from A.R. Kranz and selfed it by single seed descent during three generations. Current *Cvi* lines at the *Arabidopsis* stock centres also come from Kranz's collection (Accession numbers N902 and N1096) (M. Anderson, personal communication) and are probably very similar to the line used in the present work.

To locate the centromere of chromosome 3, *Ler* telo-trisomic plants *Tr3A* (Koornneef, 1983) were crossed with *Cvi* and *Col* plants. *F*₁ plants carrying an extra *Ler* arm 3A were distinguished from diploid plants on the base of morphological characteristics. *Ler* primary trisomic plants for chromosome 3, *Tr3* (Koornneef and van der Veen, 1983) were also analysed.

Generation of *Ler/Cvi* recombinant inbred lines

The ecotype *Cvi* was reciprocally crossed to *Ler*. Seeds from three *F*₁ plants of a single cross in which *Cvi* was used as the female (*Cvi* × *Ler*), and seed from one *F*₁ plant of a cross using *Cvi* as male (*Ler* × *Cvi*) were grown and self-fertilised. One hundred and eighteen *F*₂ plants from the *Cvi* × *Ler* cross, and 45 from the *Ler* × *Cvi* were self-fertilised and advanced to the *F*₃ generation by single seed descent. To avoid bias in the selection of plants taken to the next generation six seeds per line were sown and grown in a row, and plants at positions 1, 3 and 5 were harvested. Plant 3 was used to obtain the next generation, but when this did not grow or was not fertile plant 5 was used, or plant 1 when 5 was not available. Although certain segregation for sterility was observed during the generation of the lines, only one line disappeared because of fertility problems.

In all generations seeds were sown in plastic Petri dishes on a water-soaked filter paper and incubated in a cold room for at least 5 days to overcome remaining dormancy. Thereafter, seeds were transferred to a climate room (25°C, 16 h light per day) and incubated for 2 days. The germinated seeds were subsequently planted on potting compost in individual clay pots and grown in a greenhouse with long-day light conditions (at least 14 h day-length).

AFLP analysis

DNA was prepared from leaf material of the 88 *Ler/Col* RIL plants grown in the greenhouse, basically following the protocol of Bernatzky and Tanksley (1986), but with mercaptoethanol eliminated. For the 162 *Ler/Cvi* RILs, genomic DNA was prepared in the same way but the protocol was scaled down for use with leaf material from single *F*₃ plants. DNA was purified with the Prep-A-Gene purification system (BioRad Laboratories Inc., Hercules, CA, USA) or BIO-101 glass milk (BIO 101 Inc. La Jolla, CA, USA) prior to use, following the manufacturers' recommendations. Fifty nanograms of DNA per sample were used as the starting amount for all AFLP reactions.

The AFLP analysis was performed according to Vos *et al.* (1995) using the 'tetra cutter' restriction enzyme *Mse*I and the 'hexa cutter' *Eco*RI. AFLP reactions were carried out using a primer specific to the *Eco*RI adapter containing two selective nucleotides (E+2), and a primer to the *Mse*I adapter containing three selective nucleotides (M+3). Eight different E+2 primers and 8 M+3 primers were used (see Table 1). All the amplification reactions were performed in a Perkin Elmer 9600 thermocycler (Perkin Elmer Corp., Norwalk, CT, USA).

AFLP analyses were carried out in two different laboratories, using different systems of separation and visualisation of the amplified DNA fragments. The *Ler/Col* RIL population was genotyped for 32 different primer combinations (combinations of the 4 E-A+1 primers with the 8 M+3 primers) at Keygene N.V. (The Netherlands) using [γ -³²P]ATP (specific activity 37–110 TBq mmol⁻¹, Amersham Int. plc, Little Chalfont, UK) labelled E+2 primers in the AFLP reactions, and the amplified DNA fragments were separated in a SequiGen 38 × 50 cm gel apparatus (BioRad Laboratories Inc., Hercules, CA, USA). After electrophoresis, gels were fixed for 30 min in 10% acetic acid, dried on glass plates, and exposed to Fuji phosphorimage screens for 16 h. Fingerprinting patterns were visualised using a Fujix scanner (Fuji Photo Film Company Ltd, Japan). The sizes of the amplification products were estimated on the bases of the SequaMark 10 base-ladder (Research Genetics, Huntsville, AL, USA). The size of 22 of the AFLP markers were estimated by eye. The rest of the molecular

size estimates in this population and the scoring of the gels was performed using the special AFLP image analysis software developed by Keygene N.V. for internal use.

The *Ler/Cvi* RIL population was genotyped for 14 primer combinations (AD, BF, BH, CC, CD, CH, DF, EC, EG, FD, GB, GD, GH and HH; Table 1) at the Laboratory of Genetics, Wageningen Agricultural University (The Netherlands) using [γ - 32 P]ATP (specific activity 37–110 TBq mmol $^{-1}$, Amersham) labelled E+2 primers, and separating the DNA fragments in a S2 sequencing gel electrophoresis apparatus (Gibco BRL, Life Technologies USA, Maryland, USA). After electrophoresis, gels were fixed for 30 min in 10% acetic acid, dried on glass plates and exposed to Konica AX X-ray films (Konica Corporation, Tokyo, Japan) for 2–5 days. The approximate size of the amplification products were estimated by eye on the bases of the SequaMark 10 base-ladder (Research Genetics, Huntsville, AL, USA). Fingerprinting patterns were analysed and scored by eye.

The AFLP markers were named with the code of the corresponding primer combination (Table 1) followed by the molecular size of the DNA fragment, and followed by codes indicating in which ecotypes the DNA fragment was amplified (L = *Ler*; Col = Col, C = *Cvi*). This nomenclature allowed the identification of AFLP markers scored in both mapping populations.

To locate the centromere of chromosome 3, DNA was isolated from single plants of the parental lines, F $_1$ *Ler* \times Col and *Ler* \times *Cvi* diploid and telo-trisomic Tr3A, *Ler*Tr3 and *Ler*Tr3A (Koorneef, 1983; Koorneef and van der Veen, 1983). Fingerprinting patterns were generated by AFLP technique with seven different primer combinations (AD, BF, DF, FD, GD, GH and HH of Table 1) at the Laboratory of Genetics, Wageningen Agricultural University (The Netherlands) and analysed at Keygene N.V. (The Netherlands) using the special AFLP analysis software mentioned above. Using a densitometer, band intensity was measured in each genotype for 22 AFLP bands located in chromosome 3, and for at least 10 more bands per primer combination not located on chromosome 3 and present in all genotypes. For each chromosome 3 AFLP band and each genotype, its intensity value was divided by the corresponding mean intensity value of the control non-chromosome 3 bands, thus compensating for lane differences due to different amounts of loaded DNA. These corrected values were used to obtain ratios of band intensities between the different genotypes.

CAPS and microsatellite analysis

Thirty-six CAPS markers previously described for *Ler* and Col (AtfDB) were analysed in *Cvi* according to Konieczny and Ausubel (1993) and polymorphisms between *Cvi* and *Ler* or Col have been submitted to the AtfDB. From those CAPS markers, PVV4, PhyA, GAPB, ADH, GPA1, GAPC, GL1, GA1, SC5, g4539, CH42, B9-1.8, DHS1, g2368 and DFR were scored in at least 50 *Ler/Cvi* RILs. A new CAPS marker was developed and scored in the *Ler/Cvi* population, from the *AXR1* gene sequence (Leyser *et al.*, 1993), using the primers 5'-TGGTGGTGGTGGTAGCATC-3' and 5'-GTGATTGACCCGTGACAGA-3'. The 1478 bp amplified DNA fragment includes a *Xba*I restriction site in *Cvi* ecotype, at position 765 approximately, which is absent in *Ler* and Col. Another CAPS marker was generated for the *ANL2* gene sequence (Kubo *et al.*, in preparation) and scored in the *Ler/Cvi* population. The same marker has been scored as an RFLP in the *Ler/Col* population (Kubo *et al.*, in preparation).

The microsatellite markers nga139 and nga158 were analysed in the *Ler/Cvi* population according to Bell and Ecker (1994). The *Cvi* allele at nga139 locus has an approximately similar size to the

Col allele (Bell and Ecker, 1994); the *Cvi* allele at locus nga158 has an approximate size of 98 bp.

Linkage analysis

Linkage analyses were performed with the software package JOINMAP version 2 (Stam, 1993; Stam and van Ooijen, 1995) using the mapping population type option for RILs. Mapping was carried out with the following thresholds: REC of 0.5, LOD of 0.001, JUMP of 4–5, and triplet of 7 (Stam and van Ooijen, 1995). No order was forced during the linkage analyses and a ripple was performed after the addition of every 1–5 markers. The recombination frequencies were converted to map distances in cM with the Kosambi function (Kosambi, 1944). Maps were drawn using the computer program Drawmap (van Ooijen, 1994).

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Appendix 1. The AFLP markers appearing on the *Ler/Col* map are indicated with the map positions. All AFLP markers listed below are completely linked to the markers shown on the *Ler/Col* map (Figure 2). Asterisks close to some of the markers indicate molecular size estimated by eye.

Chromosome 1		Chromosome 3		Chromosome 4		Chromosome 5	
AH.326L(2) DE.118L	14.9	DF.255L(2) DH.254Col	2.2	DA.155Col(2) BA.139Col-C	5.7	AH.111L(2) BD.99L-C	4.4
BA.98Col(2)* BA.97Col*	17.5	AH.232L-C(2) BA.80L*	7	DE.274L(6) AB.276Col AE.249Col	15.6	CD.134L-C(2) CD.135Col*	7.2
DB.82L(3) BA.127Col-C AE.126L	23.5	AF.196L(2) AE.196Col-C	24.8	AG.210Col-C DA.130L DC.259L		BH.144L(2) DD.110L-C	7.7
BE.157L-C(2) AA.200L*	27.6	DG.63L(2) DF.45L*	35	AF.297L/457Col(7) AB.146L-C	15.6	DF.84Col(2) CH.238Col	13.8
BH.106L(4) AH.133Col DA.219L DE.159L	40.9	BF.134Col-C(3) DA.464L DD.264L-C	35	AH.116L-C DA.100L* DB.275Col-C DH.92Col		DE.316Col(2) CE.245Col	27
AH.63L-C(3) DB.184L DF.275L	45.9	AH.126L(3) BG.529L AA.112L*	36.4	BF.151L(3) AA.158Col-C BH.156Col	15.6	CC.188L(2) CD.189Col	31.6
BC.126Col(2) DH.315Col	46.6	AD.202L-C(2) BA.75L*	36.4	BE.99Col(2) AE.377L-C	16.4	DD.206L(8) DH.146L-C AB.376Col AE.117Col-C AE.459Col AA.150L	35.3
DF.317Col(2) DF.290Col*	47.8	AE.427Col(3) BA.208Col-C DE.86Col	36.4	CB.230Col(2) DB.86Col	19.4	BB.159Col-C BE.495L	
CG.435L(3) AG.120Col-C BD.91Col	48.4	DF.208Col(8) AB.155L AH.378L BB.191L CF.116L	38.8	DD.175Col-C(2) AE.211L	21	CD.116L(3) DG.52Col BB.55L	45.6
CH.75Col(2) AC.48Col	70	CG.311Col DD.358L DG.409Col		AH.383L-C(2) BA.177L	37.4	CH.88L(2) AE.161Col-C	45.6
DF.408Col-C(3) DD.128L AB.73Col-C	71.2			BC.284Col(2) DH.333Col	39.9	AB.513L-C(2) DF.95L	47.3
		AH.177Col(6) AH.188Col-C BB.57Col-C CG.519Col-C CH.328Col DG.329Col	38.8	CB.196L-C(2) BH.124L-C	57.6	AA.80L(2) BH.119L-C	62.4
Chromosome 2				AE.246Col(2) DG.60L	75.8	DD.112Col(2) CC.126Col*	76.4
AH.55Col(2) AG.247Col-C	14.7	DF.250L(3) BG.91Col DD.337Col	39.5			DA.271Col(2) BC.55L	76.4
BE.288L(4) BC.317Col CF.48L DC.56L	15.3	DF.76L(4) BH.165Col AE.95Col CH.52L	42.6				
BC.278Col(2) CF.83Col	15.3						
BC.54Col(2) BG.53L	16	CF.150Col-C(3) BB.257Col-C DH.293Col	45.5				
AH.297Col(2) BG.215L	18	DB.73Col-C(2) BF.434Col-C	60.9				
DG.183Col(2) DE.183L-C	37.7						