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J. L. Peters · G. Cnops · P. Neyt · J. Zethof · K. Cornelis · M. Van Lijsebettens · T. Gerats

An AFLP-based genome-wide mapping strategy

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Abstract To efficiently determine the chromosomal location of phenotypic mutants, we designed a genome-wide mapping strategy that can be used in any crop for which a dense AFLP (Amplified Fragment Length Polymorphism) map is available or can be made. The AFLP technique is particularly suitable to initiate map-based cloning projects because it detects many markers per reaction. First a standard set of AFLP primer combinations that results in a framework of AFLP markers well dispersed over the genome is selected. These primer combinations are applied to a limited number of mutant individuals from a segregating population to register linkage and non-linkage of the AFLP markers to the gene-of-interest. Further delineation of the area of interest is accomplished by analyzing the remaining recombinants and additional mutant individuals with AFLP markers that lie within the identified region. We illustrate the usefulness of the method by mapping three *rotunda* (*ron*) leaf-form mutant loci of *Arabidopsis thaliana* and show that in the initial phase of map-based cloning projects a 400–600 kb interval can be identified for the average mutant locus within a few weeks. Once such an area is identified and before initiating the more time-consuming fine-mapping procedure, it is essential to examine publicly available databases for candidate genes and known mutants in the identified region. The 390-kb interval on chromosome 4 that harbors the *ron2* mutation, also carries a known flower mutant, *leunig* (*lug*); upon crossing, the two mutants appeared to be allelic. When no such candidates are found, the mapping procedure should be continued. We present a strategy to efficiently select recombinants that can be used for fine mapping.

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J. L. Peters (✉) · T. Gerats
Department of Experimental Botany, Plant Genetics,
University of Nijmegen,
Toernooiveld 1, 6525ED Nijmegen, The Netherlands
e-mail: japet@sci.kun.nl
Tel.: +31-24-3652757
Fax: +31-24-3652787

G. Cnops · P. Neyt · J. Zethof · K. Cornelis · M. Van Lijsebettens
Department of Plant Systems Biology,
Flanders Interuniversity Institute for Biotechnology,
Ghent University,
K.L. Ledeganckstraat 35, 9000 Gent, Belgium

Introduction

In this era of genomics, plant biologists aim to understand the function of all plant genes. The function of a particular gene can be determined when the gene of interest is mutated and the resulting mutant is analyzed for differences compared to its wild-type. There are basically two ways to mutate a gene: reverse and forward genetics (Weigel and Glazebrook 2002). In reverse-genetic approaches, one starts with a (sequenced) gene of interest, selects a mutation in that gene, and then tries to identify a phenotypic change. In forward-genetic approaches, one predicts the specific effect of a mutation for a process under research, and then tries to isolate mutants with the predicted phenotype or one tries to isolate the genetic sequence that underlies any phenotype of interest. Thus, contrary to reverse genetics, forward genetics starts with a phenotypic mutant and tries to identify the gene responsible for the altered phenotype. Both approaches are valuable and complementary. The advantage of forward genetics is that it is a process that does not need any prior knowledge about the gene and that it is in principle applicable to any species. Both insertional and chemical mutagenesis can be used to perform mutant screens. When the gene that causes the mutation is tagged by a T-DNA or transposon insertion, a rapid identification of the gene of interest is possible, whereas identification of a gene affected by a chemically induced mutation requires a more time-consuming posi-

tional cloning approach. However, there are good reasons to keep screening for mutants in chemically mutagenized populations, implying that an efficient positional cloning procedure represents a very important research tool. For example, chemical mutagenesis generates a greater diversity of mutations and thus allows identification of genes unlikely to be identified by insertional mutagenesis. It also results in more mutations per plant, which reduces the number of plants to be screened to find the phenotype of interest. Moreover, many interesting second-site mutations, also called suppressors because they suppress the original phenotype, have been induced by the chemical mutagen EMS (ethyl methane sulphonate). Finally, positional cloning techniques are essential for the isolation of genes underlying natural genetic variants such as those of QTLs (Alonso-Blanco and Koornneef 2000; Yano 2001). In conclusion, the positional cloning approach is a very important tool to facilitate the identification of gene functions. However, it is a relatively time-consuming method. We therefore searched for a method to accelerate the map-based cloning process.

The efficiency of positional cloning directly depends on the availability of markers and sequence information. Recent progress in genome sequencing projects has provided plant biologists with two nearly complete sequences, that of *Arabidopsis thaliana* (ecotype: Columbia) (The Arabidopsis Sequence Initiative 2000) and rice, *Oryza sativa* L. (ssp. *japonica* and ssp. *indica*) (Goff et al. 2002; Yu et al. 2002). We made use of the *Arabidopsis* sequence to develop a procedure that considerably accelerates the normally time-consuming procedure for building genetic maps (Peters et al. 2001). In essence, we combined *in silico* AFLP analysis of the *Arabidopsis* sequence with gel-based AFLP analysis on several *Arabidopsis* ecotypes, which resulted in the placement of more than 1,250 Col/L*er* AFLP markers on the genome sequence. These markers are described at the TAIR web site and can be used as individual markers during map-based cloning procedures. However, the strength of the AFLP technique (Vos et al. 1995), the analysis of multiple markers per primer combination, is not exploited in this way and it is exactly this characteristic that makes AFLP markers very valuable for the first steps of map-based cloning projects. Hence, we developed a strategy that uses AFLP markers in a genome-wide mapping strategy. We have successfully used this strategy to map several genes and will present data on the mapping of three leaf-form mutants, *rotunda1* (*ron1*), *ron2* and *ron3* (Berná et al. 1999), as examples to demonstrate the rapid identification of relatively small areas containing the gene of interest.

Materials and methods

Plant material and growth conditions

A. thaliana (L.) Heynh. ecotypes Columbia (Col-0, NASC stock number N1092) and Landsberg *erecta* (L*er*-0, NASC stock number NW20), the leaf-form mutants *rotunda1* (*ron1*), *ron3* (Robles and Micol 2001), *ron2-1* and *ron2-2* (Berná et al. 1999; Robles and Micol 2001), and two allelic flower mutants *lug-1* and *lug-16* (Liu and Meyerowitz 1995; Conner and Liu 2000) were used in this study. F2 populations derived from a cross of *ron1*, *ron2-1* and *ron3* to Col were kindly provided by J.L. Micol (Universidad Miguel Hernández, Alicante, Spain), whereas *lug-1* and *lug-16* (L*er* ecotype) were provided by Dr. Z. Liu (University of Maryland, College Park, Md., USA). Plants were grown in a soil vermiculite (3:1) mixture in a 16-h light, 8-h dark regime at 22°C, 100 μm m^{-2} s^{-1} light intensity, and 70% relative humidity.

DNA preparation

The method for DNA preparation of Edwards et al. (1991) was modified as described below. Plants were grown in 2×96 grid-trays (one grid represents 1 cm^2) (n.v. August de Clercq, Zwijnaarde, Belgium). One leaf of 2 to 3 week-old seedlings was sampled and put in 96 well-plates (Qiagen, Hilden, Germany) with one grinding ball (4-mm diameter) per collection of the microtube. The laboratory vibration mill MM300 (Retsch GmbH and Co, Haan, Germany) was used to disrupt the samples (30 s at 30 Hz). After adding 400 μl of extraction buffer (200 mM of Tris HCl pH 7.5, 250 mM of NaCl, 25 mM of EDTA and 0.5% SDS), the samples were mixed well and left at 55–65°C for 15 min to extract. The extracts were centrifuged for 10–20 min at 4,000 rpm in a Sorvall RC-5B centrifuge using the SH 3000 rotor (DuPont Sorvall Products, Newton, Conn., USA); 300 μl of the supernatant was transferred to fresh collection tubes and mixed with 300 μl of isopropanol. After centrifugation at 4,000 rpm for 10–20 min, the supernatant was poured off and the pellet was washed with 70% ethanol. The ethanol was poured off after 10 min centrifugation at 4,000 rpm and allowed to dry. The pellet was dissolved in 500 μl of water or TE and left in the fridge overnight.

Experimental AFLP analysis

The AFLP analysis was performed as described in Peters et al. (2001). For the initial step in map-based cloning, eight standard AFLP primer combinations (Table 1) were performed on the parents and at a maximum of 22 F2 mutant individuals. These eight

Table 1 Standard set of eight AFLP primer combinations used to detect linkage between 85 Col/L*er* AFLP markers and any locus of interest

Marker code[a]	Selective nucleotides				Number of AFLP markers
	*Sac*I+1	*Sac*I+2	*Mse*I+1	*Mse*I+2	
SM8	A	A	C	T	9
SM57	A	T	G	A	13
SM61	A	T	T	A	9
SM205	T	A	T	A	12
SM229	T	G	C	A	14
SM233	T	G	G	A	10
SM236	T	G	G	T	10
SM240	T	G	T	T	8

[a] according to Peters et al. 2001

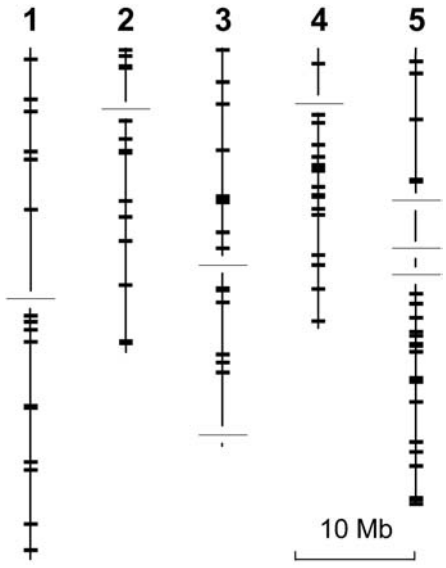

Fig. 1 Distribution of 85 Col/L*er* AFLP markers resulting from eight primer AFLP combinations over the five chromosomes of *Arabidopsis*. Each *bold horizontal line* represents a marker, whereas a *thin horizontal line* disrupting the chromosome, stands for a sequence gap

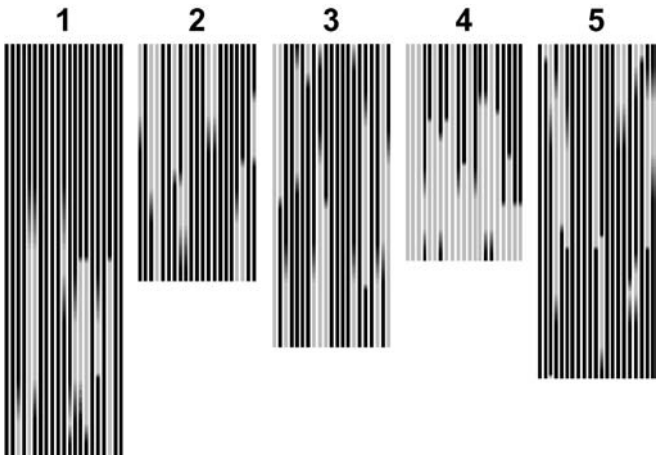

Fig. 2 Linkage of the *RON2* locus with Col/L*er* AFLP markers at the bottom of chromosome 4 and non-linkage with Col/L*er* AFLP markers on chromosomes 1, 2, 3, top of 4 and 5. Twenty one F2 mutant individuals resulting from a cross between the mutant (L*er*) and wild-type (Col) were tested for the 85 Col/L*er* AFLP markers described in Fig. 1. *Black* represents Col (homozygous and heterozygous), whereas *grey* represents L*er* (homozygous). Because the exact location of recombination breakpoints is unknown, each cross-over event between two markers is shown as a gradient between *black* and *grey*

primer combinations lead to 85 Col/L*er* AFLP markers distributed over the *Arabidopsis* genome (see Fig. 1, Table A). Subsequently, AFLP markers that were within the region identified were used to further delineate the region of interest. Details on the position of the AFLP markers in the *Arabidopsis* genome can be found on the TAIR website (http://www.arabidopsis.org/) (Peters et al. 2001). AFLP is a registered trademark of Keygene N.V.

Results and discussion

Genome-wide mapping strategy using AFLP markers

We propose a genome-wide mapping procedure that uses AFLP markers to efficiently determine the chromosomal location of a specific mutation. The availability of a dense AFLP map is a pre-requisite to establish this strategy. First, a standard set of AFLP primer-combinations, resulting in AFLP markers well dispersed over the genome, needs to be selected. In our example organism, *Arabidopsis*, the physical AFLP map as published by Peters et al. (2001) was used to accomplish this. Initially we identified AFLP primer combinations that produce the largest number of AFLP markers. Subsequently additional primer combinations were chosen to deliver markers to fill the remaining gaps. The result is a set of eight primer combinations that amplifies 85 clearly discernable AFLP markers well distributed over the five chromosomes of *Arabidopsis* (Fig. 1, Table 1). For *Arabidopsis* we experienced that these eight primer combinations produce an adequate number of markers, but organisms with larger genomes or more linkage groups might require coverage of the genome with more markers. Information on the precise location of the 85 Col/L*er* AFLP markers as well as their degree of polymorphism in other *Arabidopsis* ecotypes (C24, Ws and Cvi) is available as supplemental data (Table A).

Second, relevant individuals need to be selected from a mapping population. In case of a monogenic recessive trait, F2 mutants are the individuals of choice in *Arabidopsis*. When constructing a mapping population and selecting AFLP markers for the genome-wide mapping strategy, the fact that AFLP is mainly used as a dominant marker technique should be taken into account. This implies that linkage of AFLP markers to the mutant locus must be detected by scoring for absence of the marker in (most of) the F2 mutant individuals. In general, analyzing between 20 to 30 F2 mutants is sufficient to detect linkage to a distinct part of the genome. The exact number of F2 mutants under analysis can be made dependent on the system used to run AFLP gels. For example, running eight AFLP primer combinations on 22 F2 mutants (plus the two parental lines) will restrict the workload to the analysis of two 96-well AFLP gels.

Figure 1 and Table A show that on average one Col/L*er* AFLP marker is present every 1.3 Mb (i.e. on average about 6 cM). Only 10% of the markers is separated by more than 3.5 Mb, whereas the maximum gap is about 5.6 Mb. The first step of our genome-wide mapping procedure is therefore expected to determine linkage of the mutant locus to a specific chromosomal region of less than 5.6 Mb, and non-linkage to the rest of the genome as exemplified for the *RON2* locus in Fig. 2. Once a segregating mapping population is available, this first step can be performed within 3 days. For the second step, available AFLP markers positioned in between the flanking AFLP markers are used for further mapping.

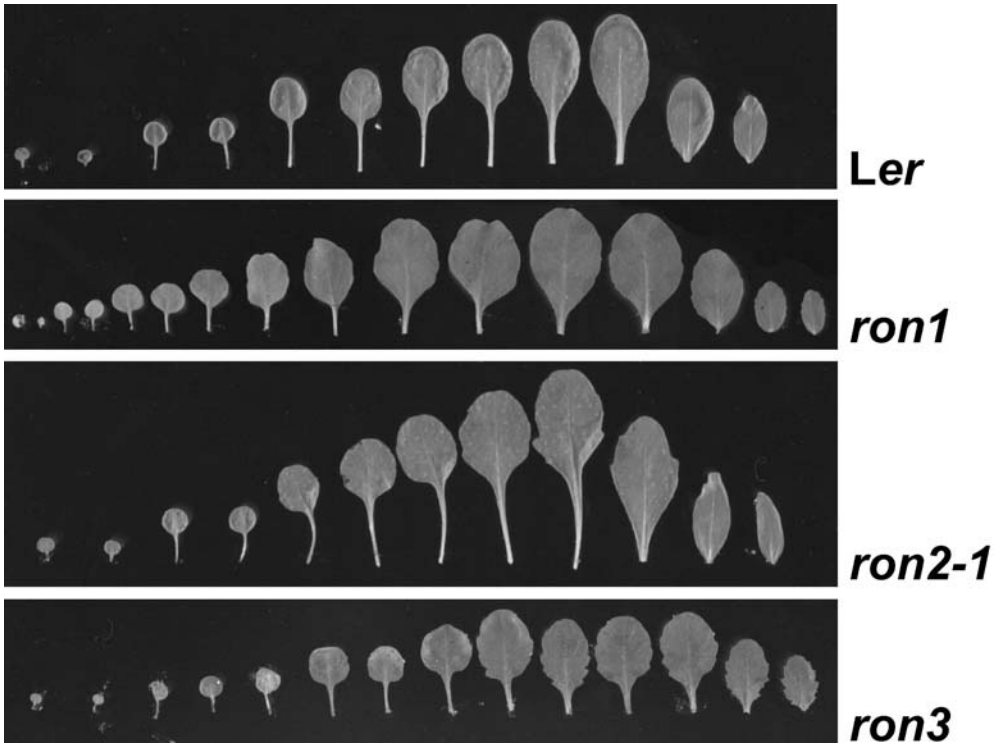

Fig. 3 Leaf morphology of *rotunda1* (*ron1*), *ron2* and *ron3* mutants. Series of fully expanded leaves from wild-type (L*er*) are compared with leaves of *ron1, ron2* and *ron3* homozygous plants. The leaves in each row are from left to right: two cotyledons, rosette leaves 1 to 7, and two to three cauline leaves

At this stage the benefits of a detailed mapping position must be balanced against the work involved in the AFLP-characterization of a larger number of F2 mutant individuals. In the following section we will show that in the case of *Arabidopsis*, analyzing about 100–150 F2 mutant individuals on average, exhausts the presently available set of more than 1,250 AFLP markers.

Mapping of three leaf-form mutations using the genome-wide mapping strategy

To demonstrate the genome-wide mapping strategy, we present the mapping results of three leaf-form mutants, *rotunda1* (*ron1*), *ron2* and *ron3* (Fig. 3). The investigation of these mutants is part of a research program on the study of the genetic control of leaf growth in *Arabidopsis*. The mutants were obtained from the collection of 255 mutant lines induced by EMS mutagenesis (Berná et al. 1999). The *rotunda* class of leaf-form mutants is defective in growth along the width and length axes. The L*er* mutants were crossed with the Col wild-type and the resulting F1s were allowed to self, in order to produce F2 mapping-populations (Robles and Micol 2001). Twenty one F2 *ron2-1* mutants, and 22 F2 *ron3* mutants, together with their L*er* and Col parents, were analyzed using our standard set of eight AFLP primer combinations. After scoring the resulting 85 AFLP markers, linkage to one chromosome and non-linkage to all other chromosomes was observed. For the *ron2-1* mutant this is visualized in Fig. 2. Each vertical line symbolizes one F2 mutant individual. Presence of the AFLP marker signifies that the marker behaves as the Col parent and is represented in black. For an F2 individual this means that the marker is either homozygous or heterozygous. Absence of the AFLP marker indicates that the marker is homozygous L*er* (represented in gray). Recombination breakpoints are shown as a gradient between black and gray to indicate that the exact place of recombination between the two AFLP markers involved is unknown. Since the mutant locus is in a L*er* background, it is straightforward to recognize that the AFLP markers at the bottom of chromosome 4 are linked to the *RON2* locus. Due to the distances between the AFLP markers resulting from the eight selected primer combinations (Fig. 1, Table A), the genome-wide mapping procedure is expected to determine linkage of the mutant-locus to a specific chromosomal region of less than 5.6 Mb, provided the required recombinants are present. This is indeed the case for the regions containing the *ron2* and *ron3* mutations, which were delimited to 4.4 and 3.3 Mb, respectively (Table 2). Additional AFLP marker analysis of 120 F2 mutant individuals delimited the *RON1* interval to 455 kb on chromosome 5, and the *RON2* and *RON3* intervals to 390 kb and 600 kb both on chromosome 4 (Table 2). Only for the *ron2* mutant, more AFLP markers are available within the identified area, meaning that a larger number of F2 mutants could have furthered the mapping to a smaller region containing the *RON2* gene (Table 2).

In summary, for *Arabidopsis* mapping projects we grow an F2 population of approximately 500 individuals. Twenty two mutants are analyzed for the first step of the genome-wide mapping procedure, whereas the remaining mutants are used to delineate a 400 to 600 kb area

Table 2 Intervals (kb) to which three leaf-form loci can be delineated, using AFLP markers. The standard set of AFLP markers signifies 85 AFLP markers resulting from the application of eight primer combinations (see Fig. 1). The AFLP interval and the number of AFLP markers that is still available after performing AFLP with 120 mutant individuals vary per locus

Locus	Chromosome	Standard set of AFLP marker interval (kb)[a]	AFLP markers interval (kb)[b]	AFLP markers available[c]
RON1	5	n.a.[d]	455	0
RON2	4	4,405	390	3
RON3	4	3,337	606	0

[a] Data obtained using 21 *ron2-1* and 22 *ron3* F2 mutants
[b] Data obtained using 120 F2 mutants
[c] From the set of 1,267 AFLP markers (Peters et al. 2001)
[d] Data not available

containing the locus of interest. This procedure takes 2–3 weeks to perform, provided the F2 material is available.

Candidate genes

Once the mutant locus is mapped to a 400–600-kb area and before initiating the more time-consuming fine-mapping procedure, it is essential to examine publicly available databases for candidate genes in the identified region. Especially for model organisms like *Arabidopsis*, an increasing number of genes is being characterized, implying that the chance a gene is already cloned is increasing. Besides the TAIR web site (http://www.arabidopsis.org/), the recently published sequence-based map with 620 mutant phenotypes (Meinke et al. 2003) is a great help to identify candidate genes that might represent the same locus as the mutation under investigation. Performing such a study for the three leaf-form mutants shows that 139, 86 and 170 AGI-annotated genes can be identified for the regions containing the *RON1*, *RON2* and *RON3* loci, respectively (data not shown). Although this still is a rather large number of genes, it is advisable to carefully consider whether any of these could be a favored candidate gene. As a strategy, one can start to check whether mutants are known for any of the genes involved that resemble the mutant under investigation. In the *RON2* region, three mutants could be identified, *YUC* (At4g32540), *LUG* (At4g32551) and *SQD1* (At4g33030) (Meinke et al. 2003). *LEUNIG* (*LUG*) was a very likely candidate to represent *RON2* since the two available *ron2* alleles, *ron2-1* and *ron2-2* (Berná et al. 1999), display a flower and silique phenotype comparable to the one described for the flower developmental mutant, *lug* (Liu and Meyerowitz 1995; Conner and Liu 2000). Allelism tests between *ron2-1*/+ heterozygotes and *lug-1* or *lug-16* homozygotes, together with sequence analysis, revealed that the *RON2* and *LUG* loci indeed correspond to the same gene (Gerda Cnops, personal communication). For *RON1* and *RON3* no such potential candidates have been identified, meaning the mapping project should be continued.

Strategy to select recombinants for fine-mapping

For fine-mapping, a large population of 1,500 to 2,000 F2 plants should be screened with markers flanking the region containing the gene of interest. Despite the large numbers, this is workable when the plants are grown in 96-grid trays; DNA is isolated from young plantlets using a corresponding 96-well high-throughput method, and only the recombinants are selected and grown to maturity. All F2 individuals have to be subjected to a screen with two flanking markers. Since the advantage of the AFLP technique (i.e. many physically dispersed markers per primer combination) is lost at this point, we advise using other PCR-based markers. For *Arabidopsis*, the Cereon collection of InDel (insertion, deletion) polymorphisms and SNPs is a very valuable collection of polymorphisms that can be used for this purpose (Cereon, Cambridge, Mass., USA). InDel polymorphisms flanking the region of interest are identified, and primers developed to score the flanking markers in a co-dominant way (Fig. 4A). In Fig. 4B, a schedule of all expected recombination events is presented. The two markers X and Y represent InDel markers flanking the region of interest from above and below, respectively. In the example, both InDel markers resulted in a larger fragment for the Col parent compared to the L*er* parent. The F2 individuals that harbor a recombination event in the region flanked by X and Y can easily be recognized, because they produce three PCR fragments instead of two (homozygous F2 non-recombinants) or four (heterozygous F2 non-recombinants). In case of a recessive mutation, homozygous and heterozygous wild-type plants cannot be distinguished in an F2 population. Therefore, relevant recombinants have to be selfed and grown to determine the genotype of wild-type F2 recombinants. InDel markers are developed in such a way that they can be analyzed simultaneously on a polyacrylamide gel (Fig. 4C). The recombinants can subsequently be used to further delimit the area containing the gene of interest. Markers needed for this can be developed from the available Cereon InDel and SNP collection. The principles of mapping have recently been described in reviews (Lukowitz et al. 2000; Jander et al. 2002).

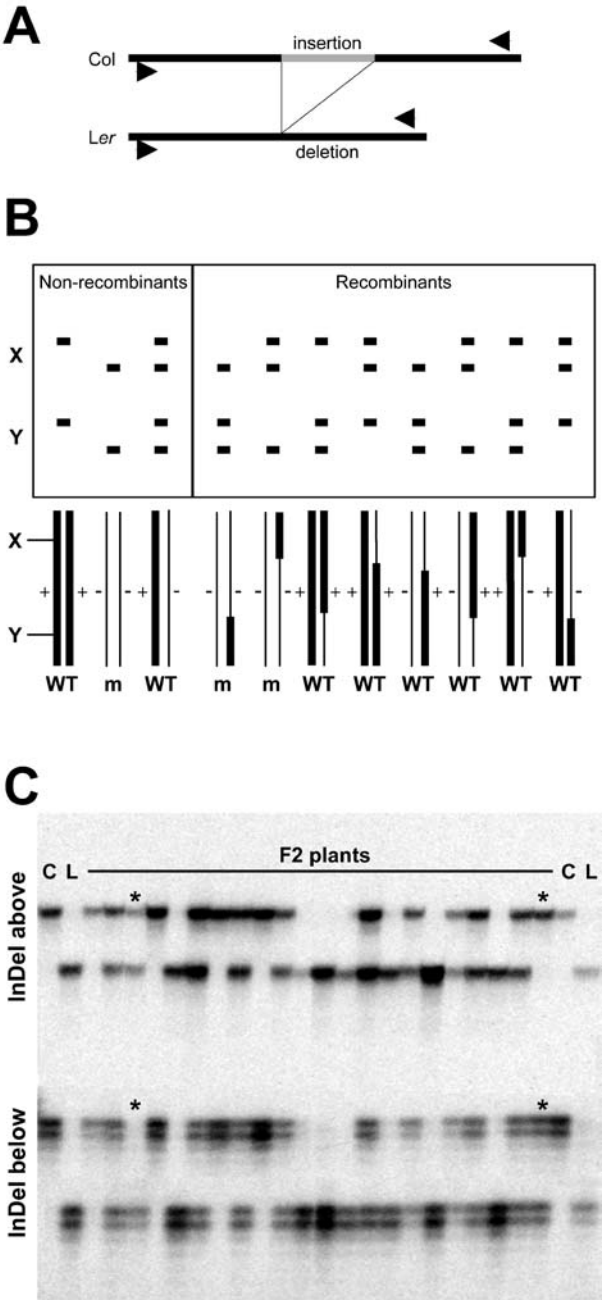

Fig. 4A–C Screening of a large F2 population with two flanking InDel markers. **A** Schematic example of an InDel marker. Amplification with the primer pair shown will result in a larger fragment for the *Col* parent compared to the *Ler* parent. **B** Two markers (*X* and *Y*) represent InDel markers flanking the region of interest from above and below, respectively. Both InDel markers result in a larger fragment for the Col parent compared to the L*er* parent. F2 individuals are subjected to amplification with the primer pair belonging to markers *X* and *Y* and the two reactions are run simultaneously on a polyacrylamide gel. F2 individuals that show a recombination event in the region flanked by *X* and *Y* can easily be distinguished from non-recombinants because they produce three PCR fragments instead of two (homozygous F2 non-recombinants) or four (heterozygous F2 non-recombinants). Further delineation of the region of interest is an iterative process, using markers localized more inward. The F2 genotype and phenotype belonging to non-recombinants and recombinants are represented in the bottom panel. The mutant allele is represented by –, whereas the wild-type allele is represented by +. **C** Example gel with 22 F2 individuals resulting from the [*ron2-1* mutant (L*er*)×WT (Col)] cross. The parentals, wild-type Col (*C*) and mutant L*er* (*L*), are loaded on both sides of the gel, flanking the 22 F2 individuals. Note that for the marker below, double bands were observed in parents as well as recombinants. *Asterisks* indicate two recombinants

Concluding remarks

The proposed genome-wide mapping procedure can be used for map-based cloning projects in any organism for which a dense AFLP map is available, e.g. barley (Qi et al. 1998), maize (Vuylsteke et al. 1999), tomato (Haanstra et al. 1999), lettuce (Jeuken et al. 2001) and Petunia (Strommer et al. 2002). Since no sequence information is needed to construct an AFLP map, the procedure can be applied to any organism. In addition to the identification of loci responsible for induced mutations, genome-wide genotyping with AFLP markers is very helpful in the search for QTLs underlying natural variation. We have shown that the first step of the genome-wide mapping approach results in linkage to one specific chromosomal area as well as non-linkage to all others within 3 days. Gross genomic rearrangements, such as translocations, will be visualized at this phase because apparent linkage to more than one chromosomal area is predicted to occur under these circumstances. We have indeed observed linkage to markers, normally located on two different chromosomes in an irradiation-induced mutant (data not shown). Recognition at this early stage prevents spending time on unproductive projects. Furthermore, cases of repetitive cloning can theoretically be identified at this early stage. Especially in model organisms an increasing number of genes is being characterized. Since the occurrence of mutants that exhibit a visual phenotype is rather restricted, an increase of repetitive cloning cases can be anticipated which makes the recognition of such events at an early stage, the more important. Likewise, many EMS-induced mutants have pleiotropic phenotypes, which can lead to situations where two groups interested in different processes are in fact investigating the same mutant. In this paper we have shown that the flower mutant, *lug*, is caused by a mutation in the same gene as the leaf-form mutant, *ron2-1*.

Other recently published procedures for genome-wide mapping use simple sequence length polymorphisms (SSLPs) (Ponce et al. 1999; Lukowitz et al. 2000) or SNPs (Cho et al. 1999). Like the AFLP genome-wide mapping system, these two systems analyze a limited number of F2 individuals (19 and 28, respectively) to provide a first localization on the *Arabidopsis* genome. The SSLP system works with co-dominant markers for which other than solely Col/L*er* polymorphisms are known. However, it uses only 22 markers and thus provides a rather rough localization. The SNP system has a resolution of greater than 3.5 cM, with a maximum of 15 cM and detects Col/L*er* polymorphisms. However, it is

array-based (Affymetrix, Santa Clara, Calif., USA) and thus relatively costly.

In conclusion, we are confident that our genome-wide mapping strategy will be a valuable addition to the systems already available. Genome-wide mapping strategies merely facilitate the identification of an area containing the locus of interest. In certain breeding-research programs this result might be satisfactory (e.g. marker-assisted breeding), but when the purpose is to actually identify the gene(s) under investigation the efficiency depends on the availability of sequence information. Therefore, the recent progress of genome sequencing projects, the availability of polymorphism collections and advances in the methods used for DNA marker detection are of great importance for facilitating positional cloning (Jander et al. 2002). Hence a model system like *Arabidopsis* has the great advantage that fine mapping and the final cloning of the gene of interest can be carried out rapidly.

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