EMBO COURSE

Practical Course on Genetic and Molecular Analysis of Arabidopsis

Module 2

MAPPING MUTATIONS USING MOLECULAR MARKERS

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The objective of this module is to learn how to map a mutation using SSLP and CAPS molecular markers.

1-INTRODUCTION

Mapping a novel mutation to a well-defined chromosomal region is an essential step in the genetic analysis of this mutant, and is also (unless the mutant is tagged) a prerequisite for molecular cloning of the corresponding gene. Determining the map position of a gene (as identified by its mutant phenotype) consists in testing linkage with a number of previously mapped markers. Once linkage with a specific marker is detected, a refined mapping can be achieved by analysing linkage relations to more markers in that region.

Historically, mapping in *Arabidopsis* primarily utilised morphological markers such as mutants with an easily scorable phenotype and a defined map position. Typically, the mutant of interest is crossed to another mutant used as phenotypic marker, the resulting F₁ double heterozygote is allowed to self, and the segregation of the two phenotypes is analysed in the F₂ population. The mutation used as marker should of course not interfere with the phenotype of the mutant to be mapped. The genetic distance is the number of meiotic recombination events that occur between the two loci in 100 chromosomes. The genetic distance is expressed in centiMorgans (cM), and can range from 0 cM (absolute linkage) to 50 cM (non-linked loci). To facilitate mapping, tester lines that are recessive for several morphological markers have been constructed (Koornneef and Stam, 1992) and can be ordered from the Nottingham *Arabidopsis* Stock Centre (NASC; http://nasc.nott.ac.uk/ ). It remains however difficult to score many different phenotypes in a single population. Hence, detailed mapping using morphological markers is tedious because it requires numerous crosses.

In contrast, a single cross can be used to analyse linkage with an essentially unlimited number of molecular markers. DNA markers were incorporated into mapping strategies once it was recognised that distantly related individuals differ in DNA sequence throughout their genome (Botstein et al., 1980). In *Arabidopsis*, molecular markers exploit the natural differences between distinct ecotypes. For instance, it has been estimated that the widely used Landsberg *erecta* and Columbia ecotypes differ by approximately 0.5 to 1% at the DNA sequence level (Chang et al., 1988; Hauser et al., 1998) These local differences or *polymorphisms* of the DNA sequence are due to point mutations, insertions or deletions that randomly occurred in one ecotype and not in the other. As described below, these DNA polymorphisms can be conveniently visualised by several methods.

To map a novel mutation that was generated in ecotype A, this mutant is crossed with a wild-type plant of a polymorphic ecotype B, and the F₁ progeny is allowed to self. The resulting F₂ population can then be used to analyse the linkage between the mutation of interest and any DNA marker that distinguishes ecotypes A and B. As compared to morphological markers, an additional advantage of molecular markers is that in most cases homozygous and heterozygous individuals can be readily distinguished (see below).
2-DIFFERENT TYPES OF DNA MARKERS

2-1-Restriction Fragment Length Polymorphisms (RFLPs)

The first type of DNA markers that were used for mapping in *Arabidopsis* were RFLPs. The DNA sequence differences between polymorphic *Arabidopsis* ecotypes may create differences in the length of restriction fragments derived from genomic DNA. For instance, a given restriction site may be present in one ecotype and not in the other. As illustrated in Figure 1, this polymorphism can be revealed by genomic DNA blot hybridisation (Southern) using as probe a DNA fragment corresponding to that region. The polymorphic bands can then be used as genetic markers to distinguish the two ecotypes. Multiple RFLP markers have been identified in *Arabidopsis* and assembled into genetic maps (Chang et al., 1988; Nam et al., 1989; Liu et al., 1996). Details on the polymorphisms detected by these RFLP probes, as well as ordering information, can be found on the web page of the *Arabidopsis* Biological Resource Center (ABRC) at the Ohio State University, USA: http://aims.cps.msu.edu/aims/. (see also AtDB Database http://genome-www.stanford.edu/Arabidopsis/ , look for RFLP).

An advantage of the RFLP mapping approach is that RFLP markers are co-dominant. Distinct patterns are indeed obtained for plants that are homozygous or heterozygous for the parental alleles (Figure 1). Hence, all the chromosomes of a given F2 population can be scored. In contrast, a main disadvantage is that RFLP mapping necessitates relatively large amounts of DNA because distinct RFLP markers may require digestion of genomic DNA samples with different diagnostic restriction enzymes. To circumvent this problem, a set of 12 RFLP markers that all detect EcoRI polymorphisms and cover the five *Arabidopsis* chromosomes has been developed, and can be used for a rough mapping of a mutation to a chromosomal region (Fabri and Schäffner, 1994). Nevertheless, a more detailed RFLP mapping usually necessitates the pooling of F3 individuals because a sufficient amount of DNA could not be isolated from individual F2 plants for analysis with multiple RFLP probes.

In the past recent years PCR-based DNA markers have become the most widely used because they require only small samples of DNA and eliminate the need for the time-consuming DNA blotting and hybridisation steps.

2-2-Cleaved Amplified Polymorphic Sequences (CAPS)

The principle of CAPS markers is very similar to that of RFLP markers. The main difference is that PCR is used instead of DNA blot hybridisation to detect a restriction site polymorphism. As illustrated in Figure 2, a genomic DNA region is amplified by PCR using specific primers and those amplified fragments are then digested with a diagnostic restriction enzyme to reveal the polymorphism. Hence, whereas RFLP probes can be anonymous clones, CAPS markers require sequence information to design the specific PCR primers.

Like RFLPs, CAPS markers are co-dominant (Figure 2). CAPS markers are based on PCR for detection, and thus require only small quantities of genomic DNA. Typically, a single leaf will provide enough DNA for analysis with multiple CAPS markers (see section 4-2 below). Finally, CAPS markers can be easily assayed using standard agarose gel electrophoresis.

The feasibility of using CAPS markers to map *Arabidopsis* mutations was initially demonstrated by Konieczny and Ausubel (1993). Since then, CAPS markers continue to be
developed by members of the *Arabidopsis* research community. A list of currently available CAPS markers is regularly updated by Eliana Drenkard and Fred Ausubel, and provides primer sequences, restriction enzyme and number of cuts for each of the examined ecotypes. This list can be accessed via the AtDB Database http://genome-www.stanford.edu/Arabidopsis/ (look for molecular markers, CAPS).
Figure 1. Principle of RFLP markers. This figure illustrates an RFLP marker which utilises a site for the restriction enzyme (E) which is present in ecotype A and not in ecotype B.
Figure 2. Principle of CAPS markers. This figure illustrates a CAPS marker which utilises a restriction enzyme (E) that cleaves the amplified fragment at one site in ecotype A and not at all in ecotype B.
2-3-Simple Sequence Length Polymorphisms (SSLPs)

Like other eukaryotic genomes, the *Arabidopsis* genome contains tandem repeats of one-, two-, or three-nucleotide motifs. These microsatellite repeat sequences are usually polymorphic in different ecotypes because of variations in the number of repeat units. These polymorphisms are called SSLPs, and can be conveniently used as co-dominant genetic markers. As illustrated in Figure 3, specific primers are used to PCR amplify a small genomic region (150 to 250 bp) that contains a polymorphic microsatellite sequence. The size of the amplified fragment will vary depending on the number of repeats present in a given ecotype. These polymorphic fragments can be separated and visualised by electrophoresis in agarose or polyacrylamide gels. As compared to CAPS markers, SSLPs offer the additional advantage that they do not involve the use of restriction endonucleases and thus avoid the problems associated with partial digestions.

Bell and Ecker (1994) described an initial set of *Arabidopsis* SSLP markers that were largely based on polymorphisms of (GA)_n repeats (the text of this article is also available at http://genome.bio.upenn.edu/SSLP_info/SSLP.html). All these markers were positioned on the *Arabidopsis* genetic map. Since then, the number of mapped *Arabidopsis* SSLP markers has steadily increased, and a regularly updated list is provided by the group of Joe Ecker at the *Arabidopsis thaliana* Genome Center (ATGC), University of Pennsylvania, USA: http://genome.bio.upenn.edu/SSLP_info/SSLP.html (see also AtDB Database http://genome-www.stanford.edu/Arabidopsis/; look for molecular markers, SSLP). These web sites provide detailed informations on primer sequences, conditions for PCR reaction, and size of the polymorphic fragment amplified for each of the examined ecotypes.

2-4-Random Amplified Polymorphic DNA (RAPD)

RAPD markers are another type of PCR-based markers that have been used for mapping *Arabidopsis* mutations (Williams et al., 1993). This approach is based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence. The oligonucleotide (around 10-bp long) is used for PCR at low annealing temperatures. When the oligonucleotide hybridises to both DNA strands at sites within an appropriate distance from each other, the DNA region delimited by these two sites will be amplified. Small nucleotide changes (polymorphism) at one of the two sites may prevent hybridisation of the oligonucleotide and hence also prevent DNA amplification (Williams et al., 1990). Typically a RAPD primer will amplify a given fragment from ecotype A and not from ecotype B. It will thus be impossible to distinguish an homozygous individual AA from an heterozygous individual AB. In other words, RAPDs are dominant markers and are thus less efficient than co-dominant markers in extracting information from a given F_2 population. Another limitation of RAPD markers is that because of the low annealing temperatures used, the amplification of a given polymorphic band seems to be highly sensitive to PCR conditions and hence less consistently reproducible in different laboratories.

2-5-Amplified Restriction Fragment Length Polymorphism (AFLP)

AFLP™ is a patented technology developed by KeyGene, Wageningen, The Netherlands (Vos et al., 1995). In this procedure, the genomic DNA is digested by two different restriction enzymes, a rare cutter and a frequent cutter. Double-stranded adapters are then ligated to the ends of the restriction fragments. The fragments are then amplified by PCR using primers that correspond to the adapter and restriction site sequences. These primers have additional
nucleotides at the 3’ ends extending into the restriction fragments, in order to limit the number of fragments that will be amplified. The AFLP products are detected by labelling one of the two primers, and the labelled DNA fragments are separated by electrophoresis in denaturing polyacrylamide gels (similar to sequencing gels). Typically, 50 to 100 amplification products are detected in a single lane. Polymorphic bands can be identified by comparing the amplification products derived from two ecotypes. AFLP protocols adapted to the *Arabidopsis* genome have been described (Alonso-Blanco et al., 1998; Vos, 1998), and are being used to construct a high density genetic map (Alonso-Blanco et al., 1998; Kuiper, 1998). Like RAPDs, AFLPs are typically dominant markers.
Figure 3. Principle of SSLP markers. This figure illustrates an SSLP marker which utilises the fact that the number of (GA) repeat units is higher in ecotype B than in ecotype A.
Figure 4. Principle of dCAPS marker (from Michaels and Amasino, 1998). The region of DNA containing a point mutation is amplified using a primer that contains a mismatch. The introduction of this mismatch into the PCR product generates an MboI site in product 2 only.
2-6-Single Nucleotide Polymorphisms (SNPs)

The most common class of DNA polymorphisms present both in natural ecotypes and after induced mutagenesis is single nucleotide polymorphisms (SNPs). As described above, the RFLP and CAPS methods can detect only the SNPs which alter a recognition site for a restriction enzyme. The RAPD and AFLP methodologies can in principle detect any type of SNPs, however these two techniques are not very convenient to target a selected genomic region. In contrast, the Arabidopsis genome project is generating a wealth of sequence information which provides a starting point for the development of PCR-based markers. In other words, once the sequence of a region is known, primers can be synthesised to amplify alleles (or ecotypes) of interest which can then be analysed to find allele-specific polymorphisms in that region.

Although they have not been extensively used in Arabidopsis thus far, a large number of techniques have been developed to scan a defined region of DNA for SNPs (reviewed by Cotton, 1997). In particular, single-strand conformation polymorphism (SSCP) is based on the fact that a strand of single-stranded DNA folds differently from another if it differs by a single base, which leads to different mobilities of these two strands in non-denaturing gel electrophoresis. Heteroduplex analysis is based on the different mobilities of homo- and hetero-duplexes in non-denaturing gel electrophoresis, or in slightly denaturing high-performance liquid chromatography. A recent report describes the first use of heteroduplex analysis in Arabidopsis (Hauser et al., 1998). It should be noted that methods to detect SNPs can also be extremely useful in the last step of a positional cloning of a mutant locus, namely to locate the mutation within the DNA region that has been delimited by mapping.

Finally, an efficient method has been described that allows to create a PCR-based marker for any known point mutation. This technique is called derived cleaved amplified polymorphic sequence (dCAPS), and has been recently applied to Arabidopsis (Michaels and Amasino, 1998; Neff et al., 1998). The dCAPS method is primarily used when the point mutation of interest does not alter an existing restriction site. In this case, the dCAPS technique consists in designing a primer with one or two mismatches which, together with the mutation, will create a unique restriction site in one only of the two alleles (Figure 4). A second primer (usually without mismatch) is used to PCR amplify the region, and the amplification products are digested with the appropriate restriction enzyme, exactly as for CAPS markers. dCAPS are useful for genetic mapping, and to follow known mutations in segregating populations. A computer program that facilitates the design of dCAPS primers is available (Neff et al., 1998).

3-GENETIC MAPS

When selecting DNA markers on genetic maps, or later in interpreting linkage data to these markers, it is important to bear in mind how these reference genetic maps were constructed. A genetic map only displays the relative genetic distances between the particular set of markers that were analysed in a given mapping population. Hence, a given marker will typically have different map positions in genetic maps that were constructed independently, or in successive versions of the same map as new markers are incorporated.

The first molecular genetic maps of Arabidopsis were constructed using different F2/F3 mapping populations (Chang et al., 1988; Nam et al., 1989). Several common markers were included as tentative contact points. Nevertheless, because of statistical errors associated with
the recombination estimates and of differences in recombination frequency among crosses, attempts to integrate distinct individual maps resulted in a joint map of only limited accuracy (Hauge et al., 1993). In order to construct a better reference map, in particular with respect to the relative order of markers, it is essential that all markers are mapped in the same population. Even with the development of PCR-based markers which require a smaller amount of genomic DNA than RFLP markers, the small size of Arabidopsis plants limits the number of markers than can be mapped in a given F₂ population. This provided the impetus for the generation of populations of recombinant inbred (RI) lines for mapping.
To construct an RI population, individual F2 plants are selfed, and for each F3 family a single F3 plant is selected at random and allowed to self. This process, called single-seed descent, is repeated to the F8 (Figure 5). At each generation, the average level of heterozygocity is reduced by 50%. Hence, F8 lines are over 99% homozygous. These RI lines thus constitute permanent mapping populations because they are near-homozygous and can therefore be multiplied indefinitely enabling multiple laboratories to use the same mapping population. Populations of Arabidopsis RI lines have been generated from crosses between the Landsberg erecta (Ler) and Wassileskija (WS) ecotypes (Reiter et al., 1992), between Ler and Columbia (Col) (Lister and Dean, 1993), and more recently between Ler and Cape Verde Islands (Cvi) (Alonso-Blanco et al., 1998).

The collection of Ler / Col RI lines developed by Lister and Dean is currently the canonical mapping population of Arabidopsis. New markers are added to the same database containing the previously mapped markers. These segregation data are used to regularly update the RI genetic map which is accessible via NASC (http://nasc.nott.ac.uk/) or AtDB (http://genome-www.stanford.edu/Arabidopsis/), and is presently the densest and most reliable molecular genetic map of Arabidopsis. Furthermore, the physical maps and sequence data generated by the Arabidopsis genome project can be very useful to ascertain the relative order of tightly linked molecular markers.
Figure 5. Development of RI lines by single-seed descent from individual F2 plants. At each generation, the expected level of homozygocity is indicated on the right.
4-EXPERIMENTAL PROCEDURES

A newly-arisen mutation will be mapped using SSLP and CAPS markers. One of the easily scorable phenotypes of the mutant used here is the reduced root length of plantlets. The mutation was generated in the ecotype Landsberg erecta. For mapping, the homozygous mutant was crossed to the (wild-type) polymorphic ecotype Columbia. The F₁ was allowed to self and the F₂ segregating population will be used for mapping.

4-1-Select F₂ individuals to be used for DNA extraction

Select F₂ individuals that are homozygous for the recessive allele of the locus of interest. In the case of a recessive mutation, select F₂ individuals showing the mutant phenotype. In the case of a dominant mutation, select F₂ individuals showing the wild-type phenotype.

The mutation analysed here is dominant. Hence, you will select F₂ individuals showing the wild-type phenotype (long root). All these individuals will thus be homozygous for the Columbia allele at the locus that we want to map.

The resolution of the mapping is directly linked to the number of F₂ individuals analysed. For an initial mapping, 30 to 40 F₂ individuals are sufficient. Always include the two parental lines of the cross and the heterozygote F₁ as controls.

4-2-Rapid DNA preparation

A crude and rapid extraction from a small leaf disk is sufficient to isolate genomic DNA suitable for PCR amplification. The present protocol is based on the method described by Edwards et al. (1991). All steps are performed at room temperature.

1. Use the lid of a 1.5 ml Eppendorf tube to pinch out a disc from a young leaf into the tube. (Note A)
2. Use a small pestle to grind the leaf material in the tube without buffer for approx. 15 seconds.
3. Add 400 µl of extraction buffer (Note B), and vortex for 5 seconds (Note C).
4. Spin 1 minute at full speed in a microfuge to pellet the debris.
5. Transfer 300 µl of the supernatant into a fresh 1.5 ml Eppendorf tube (avoid taking debris from the pellet).
6. Add 300 µl isopropanol, mix and leave at room temperature for approx. 2 minutes.
7. Spin 5 minutes at full speed in a microfuge to pellet the DNA.
8. Remove all the supernatant and dry the pellet gently (for instance by incubating the open tubes at 37°C for a few minutes). Do not let the pellet get too dry, otherwise it will be very difficult to redisolve the genomic DNA.
9. Add 100 µl T₁₀E₁, and dissolve the pellet by gentle shaking (do not vortex).

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A. It is important to avoid using too much starting plant material. In contrast, a smaller piece of leaf or a single cotyledon can be used, in which case all volumes should be reduced by 50% in the subsequent steps of the extraction procedure.

B. Extraction buffer: 200 mM Tris-HCl pH 7.5 (pH 8.0 is OK), 250 mM NaCl, 25 mM EDTA, 0.5% SDS

C. At this stage, the samples can be kept at room temperature for up to one hour until all the samples have been extracted

4-3-Analysis with SSLP markers

The conditions for PCR amplification and gel electrophoresis will vary from one marker to the other. Refer to the informations provided for instance in the web pages mentioned above, and run tests on DNA from the two parental lines and the F<sub>1</sub> before analysing F<sub>2</sub> individuals.

The conditions given below are appropriate for the SSLP markers used during the course.

1. Set up a 20 µl reaction mix containing:

   - 1 µl Genomic DNA from the miniprep method described above
   - 1 µl Forward primer (150-250 ng/µl)
   - 1 µl Reverse primer (150-250 ng/µl)
   - 2 µl 10xBuffer
   - 0.6 µl 50 mM MgCl<sub>2</sub>
   - 8 µl 0.5 mM dNTPs
   - 0.2 µl Taq DNA Polymerase (5 U/µl)
   - 6.2 µl H₂O

2. Run PCR program:

   - 1x 94°C 1 minute
   - 40 x 94°C 15 seconds
   - 55°C 15 seconds
   - 72°C 30 seconds

3. Prepare a 3% agarose gel (1.5% regular agarose, 1.5% low melting agarose) in 1xTAE, and containing ethidium bromide (1ng/ml - wear gloves!) for DNA staining. To allow a good resolution of the polymorphic SSLP bands, thin (0.7 mm) combs are used.

4. Take 5 µl of the PCR reaction, add 2 µl of loading dye, and run the electrophoresis.

5. Photograph the gel under UV light.

6. Score the genotype of each F<sub>2</sub> individual by the polymorphic bands. Use as standards control samples corresponding to DNA from each of the two parental lines, and DNA from the heterozygote (or an equimolar mixture of DNA from both parents).

4-4-Analysis with CAPS markers
The conditions for PCR amplification and gel electrophoresis will vary from one marker to the other. Refer to the informations provided for instance in the web pages mentioned above, and run tests on DNA from the two parental lines and the F₁ before analysing F₂ individuals.

The conditions given below are appropriate for the CAPS markers used during the course.

1. Set up a 20 µl reaction mix containing:

   - 1 µl Genomic DNA from the miniprep method described above
   - 1 µl Forward primer (150-250 ng/µl)
   - 1 µl Reverse primer (150-250 ng/µl)
   - 2 µl 10x Buffer
   - 0.6 µl 50 mM MgCl₂
   - 8 µl 0.5 mM dNTPs
   - 0.2 µl Taq DNA Polymerase (5 U/µl)
   - 6.2 µl H₂O

2. Run PCR program:

   - 1x 94°C 4 minutes
   - 30 x 94°C 30 seconds
   - 30 x 55°C 60 seconds
   - 30 x 72°C 45 seconds

3. Set up a 10 µl restriction-digest mix containing:

   - 5 µl PCR reaction
   - 1 µl 10x Restriction Buffer
   - [5 units] Restriction Enzyme
   - qsp 5 µl H₂O

4. Incubate for at least 1.5 hours at the optimal temperature for the restriction enzyme.

5. Prepare a 1.7% agarose gel (regular agarose) in 1xTAE, and containing ethidium bromide (1ng/ml - wear gloves!) for DNA staining.

6. Add 2 µl of loading dye to the restriction digest, and run the electrophoresis.

7. Photograph the gel under UV light.

8. Score the genotype of each F₂ individual by the polymorphic bands. Use as standards control samples corresponding to DNA from each of the two parental lines, and DNA from the heterozygote (or an equimolar mixture of DNA from both parents).

4-5-Analysis of the CAPS and SSLP mapping data

In large-scale mapping projects, analysis of the segregation data and generation of the genetic map cannot be achieved without a computer-implemented procedure (Koornneef et al., 1998). MAPMAKER (Lander et al., 1987) and JOINMAP (Stam, 1993) are two mapping software packages which are widely used. In contrast, for the purpose described here (detecting linkage
between a new mutation and a few mapped markers, and evaluating the genetic distances between the mutation and these linked markers), the use of a mapping computer program is not required because the amount of segregation data to analyse is very limited.

In the example used in the course, all the F2 plants that we selected for mapping are homozygous for the Columbia allele at the locus that we want to map (see section 4-1 above). CAPS and SSLPs are co-dominant markers. Hence, for a given DNA marker, each F2 individual can be scored as homozygous for the Landsberg erecta allele (Ler/Ler), or homozygous for the Columbia allele (Col/Col), or heterozygous (Ler/Col). In our example, the recombination frequency between the DNA marker and the locus of interest is thus the number of chromosomes scored Ler divided by the total number of chromosomes analysed. Linkage is detected when the recombination frequency is significantly lower than 50%.

Once linkage is detected, it is necessary to convert the recombination frequency to map distance (Koornneef and Stam, 1992). This conversion is needed to account for two facts: i) chromosomes in which two recombination events occurred between the marker and the locus of interest are counted as having no recombination event; and ii) recombination events can influence the probabilities of a second recombination event occurring in the vicinity, a phenomenon called interference. In Arabidopsis, a reasonable estimate of map distance is given by the Kosambili function: 
\[ D = 25 \times \ln \left( \frac{100 + 2r}{100 - 2r} \right) \]
where r is the recombination frequency expressed as a percentage, and D is the map distance in centiMorgans (cM) (Koornneef and Stam, 1992). Map distances over adjacent intervals are additive whereas recombination frequencies are not.

Once linkage to a particular DNA marker is detected, it is very informative to make a table summarising the results obtained for each F2 plant (rows) with all the DNA markers (columns) tested on that same chromosome. Because of the small number of F2 plants analysed, there is a very low probability that one of these F2 plants has multiple recombination events in a small interval. If the experimental results predict such multiple recombination events in a F2 plant, it is very likely that one or several DNA markers were scored wrong for this F2 plant, and/or that the relative order of the DNA markers on the genetic map is wrong. This analysis is particularly important for fine mapping which ultimately relies on very few plants with recombination events in the vicinity of the locus of interest.

5-REFERENCES


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