P. Robles · J.L. Micol

Genome-wide linkage analysis of *Arabidopsis* genes required for leaf development

Received: 5 March 2001 / Accepted: 15 May 2001 / Published online: 11 July 2001
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**Abstract** In most crop species, primary productivity depends mainly on the leaf. However, the genes that contribute to the making of a leaf remain to be unraveled (reviewed in Hake and Sinha 1991; Smith and Hake 1992; Sinha et al. 1993; Teller and Poethig 1994; Tsukaya 1995; Hall and Langdale 1996; Poethig 1997; Brutnell and Langdale 1998; Tsiantis and Langdale 1998; Van Lijssebetens and Clarke 1998; Pozzi et al. 1999; Sinha 1999).

In an attempt to identify genes involved in leaf morphogenesis in *Arabidopsis thaliana*, we have screened for new mutants that form abnormal leaves (Berná et al. 1999) and conducted genetic analyses of mutants that were already available (Serrano-Cartagena et al. 1999; 2000). Our large-scale mutant search, which came close to, but did not quite reach, saturation of the genome, showed that the lines obtained fell into 94 complementation groups (Berná et al. 1999). We present here the results of our linkage analysis of most of these genes which were identified by mutation. We have taken advantage of the availability of a high-throughput mapping procedure previously developed for this purpose in our laboratory, based on the simultaneous coamplification by PCR of 21 polymorphic microsatellites and the semiautomated fluorescent detection of the products. The map positions and 

**Keywords** Plant leaf morphogenesis · *Arabidopsis* · Genetic map

**Introduction**

The primary pathway for carbon and energy uptake by plants is the leaf, an organ that is of the utmost importance to agriculture. However, little is known about the genetic controls that underlie leaf development, in spite of the fact that its biotechnological manipulation offers great potential. At the present time, the nature, action and interactions of the genes which drive the sequence of developmental events that contribute to the making of a leaf remain to be unraveled (reviewed in Hake and Sinha 1991; Smith and Hake 1992; Sinha et al. 1993; Teller and Poethig 1994; Tsukaya 1995; Hall and Langdale 1996; Poethig 1997; Brutnell and Langdale 1998; Tsiantis and Langdale 1998; Van Lijssebetens and Clarke 1998; Pozzi et al. 1999; Sinha 1999).

In an attempt to identify genes involved in leaf morphogenesis in *Arabidopsis thaliana*, we have screened for new mutants that form abnormal leaves (Berná et al. 1999) and conducted genetic analyses of mutants that were already available (Serrano-Cartagena et al. 1999; 2000). Our large-scale mutant search, which came close to, but did not quite reach, saturation of the genome, showed that the lines obtained fell into 94 complementation groups (Berná et al. 1999). We present here the results of our linkage analysis of most of these genes which were identified by mutation. We have taken advantage of the availability of a high-throughput mapping procedure previously developed for this purpose in our laboratory, based on the simultaneous coamplification by PCR of 21 polymorphic microsatellites and the fluorescent semiautomated detection and sizing of the products (Ponce et al. 1999). The molecular markers used are spaced evenly over the entire genome at intervals of 6.7 to 57.7 cM.

**Materials and methods**

Plant materials and growth

The *Arabidopsis thaliana* mutant lines studied in this work had already been isolated and subjected to complementation analysis as described in Berná et al. (1999). All the mutations studied here are recessive, with only two exceptions (ven3-2 and ven6, both semidominant), and were obtained in the genetic background of the ecotype Ler after ethyl methanesulfonate (EMS) treatment. Plants were grown at 20 ± 1°C and 60–70% relative humidity under continuous illumination of 7,000 lx, under both sterile (in 150 mm petri dishes containing agar medium, each plate sown with 100 regularly spaced seeds) and non-sterile (in pots containing a 2:2:1 mixture of perlite, vermiculite and sphagnum moss) conditions, as described in Ponce et al. (1998).
Linkage analysis

To assess linkage to polymorphic microsatellite markers, also called Simple Sequence Length Polymorphisms (SSLP; Bell and Ecker 1994), at least one mutant line from each of the complementation groups described in Bernà et al. (1999) was outcrossed to the Columbia-0 (Col-0) ecotype; mutant plants were always used as female parents, allowing us easily to recognize any self-pollination event. The seeds of two or three F1 plants were harvested and stored. For each complementation group, about 300 seeds from a single F1 plant were sown on petri dishes. Inheritance patterns, although already studied in F2 populations obtained from backcrosses (Bernà et al. 1999), were tested again in the F2 families derived from the outcrosses.

F2 plants displaying the recessive phenotype were harvested 3–4 weeks after sowing, and stored at −20°C. DNA isolation, multiplex PCR, electrophoresis and sizing of amplification products were performed as described in Ponce et al. (1999). In brief, DNA from each F2 plant was amplified in a single-tube multiplex PCR mix containing 21 primer sets, each set including an oligonucleotide labeled with one of three different fluorescent dyes. The amplification products were electrophoresed in a single lane of a polyacrylamide gel, in an ABI PRISM 377 DNA sequencer, and sized with the help of the GeneScan 2.1 fragment analysis software (Applied Biosystems). Most map positions were determined from DNA samples extracted from 19 F2 plants, the maximum number of samples per gel, since our attempts to use all the 36 wells available in our ABI 377 sequencing gels were unsuccessful: the intensity of the signal from some bands in a given lane was so high that it was also detected in the neighboring lanes (Ponce et al. 1999).

Estimation of map distances

The microsatellite markers used in this work were the following, their corresponding chromosome and map positions in cM are indicated in parentheses: AthACS (1; 3.31), AthZFP8 (1; 37.36), T27k12Sp6 (1; 61.22), AthGENEA (1; 88.90), nga111 (1; 115.55), nga1145 (2; 9.60), nga1126 (2; 50.65), nga361 (2; 63.02), nga168 (2; 73.77), AthCHIB (3; 19.10), nga162 (3; 20.56), AthGAPab (3; 43.77), nga6 (3; 86.41), nga12 (4; 22.92), nga111 (4; 29.64), nga1139 (4; 83.41), nga1107 (4; 104.73), AthCTR1 (5; 9.32), nga139 (5; 50.48), AthPHYC (5; 71.13), MBK5 (5; 119). These map positions were taken from the latest version of the recombinant inbred (RI) map of Lister and Dean (http://nasc.nott.ac.uk/new_ril_map.html), the only exception being MBK5, which is known to be 2 cM distal to LFY3, which in turn is at 116.88 cM in the RI map.

Recombination percentages (r) and their standard errors (S_r) were obtained as described in Koornneef and Stam (1992), and transformed into map distances in centimorgans (D), using the mapping function of Kosambi (1944), as follows:

\[
D = \frac{n}{25 \ln \left( \frac{100 + 2r}{100 - 2r} \right)}
\]

where \( n \) is the number of chromosomes studied. In those cases where linkage between a mutation and a marker was complete, the standard deviation of map distances (S_D) was calculated as:

\[
S_D = \frac{1}{\sqrt{n}}
\]

Map distances between a mutation and its neighboring markers, as well as their relative positions (their maximum-likelihood map), were determined using the “lod table” and “compare” commands, respectively, in the Mapmaker 3.0b program (Lander et al. 1987).

Results

In this work, we have mapped 76 of the 94 genes that we previously identified by complementation analysis of EMS-induced Arabidopsis mutants with aberrantly shaped leaves. Their phenotypes are named and described in Table 1 and illustrated in Fig. 1. All the mutant lines of four of the 94 loci were lost (api2, den14, ...
Fig. 1a–t Leaf form in the wild type (ecotype Ler; a) and representatives of the pheno-
typic classes defined during the complementation analysis of
EMS-induced mutants with
abnormal leaves mapped in this
work (b–t). See Table 1 for the
full name and a brief descrip-
tion of each phenotypic class.
Photographs were taken
21 days after sowing. The scale
bars indicate 5 mm

den16, and sca2). Mutants belonging to four other
complementation groups had already been found to be
alleles of genes mapped by other authors (Berná et al.
1999; ASYMMETRIC LEAVES1 (ASI; Rédei 1965a,
1965b; Barabas and Rédei 1971), which maps on chro-
mosome 2, AS2 (Fabri and Schaffner 1994), on chro-
mosome 1, and CURLY LEAF (CLF; Goodrich et al.
1997) and RETICULATA (RE; Rédei and Hirono
1964), two genes that we initially called INCURVATA1
(ICU1) and VENOSA2 (VEN2), respectively, both of
which map on chromosome 2. Other genes not mapped
in this work were ICU3 (Serrano-Cartagena et al. 2000),
which has been named HASTY (HST) by Telfer and
Poethig (1998), and ICU6, ICU7, ICU8, ICU9 and
UCU1, the genetic analyses of which will be published
elsewhere.
Outcrosses were performed between Col-0 and 81 mutant strains in the Ler background, each representative of a different complementation group. The resulting F₁ populations were wild type in all cases, the only exceptions being those involving the semi-dominant mutations ven3-2 and ven6. Even though monogenic inheritance for all such mutants, as well as recessiveness for 79 of them, had previously been demonstrated in the progeny of backcrosses to the Ler ecotype (Berná et al. 1999), we wanted to test their inheritance patterns in the F₂ populations of the outcrosses to Col-0. More mutant F₂ plants than expected for a 3:1 segregation were found for five of the outcrosses – those involving anu5-1b, anu9-1b, den13, exi7, and ero3-1a. In contrast, fewer phenotypically mutant individuals than expected for 3:1 segregation were found in the F₂ progenies of 32 mutant strains (ang2, ang3, ang4, anu1-1, anu2, anu3, anu4, anu6, anu8-1b, anu10-1a, anu11, anu12, api3, api5, api7-1a, den1, den2, den8, den10, den17, elo1-1a, elo4, ero1, exi2-1a, ond1, ond2, ond3, ron1, ron3, tcu1-1a, tcu2-1b and tcu3-1a). A similar observation was made by Koornneef and Hanhart (1988), who proposed the existence of certation – a reduced rate of transmission of mutant alleles through the gametophyte – as the most likely explanation for the reduced proportion of phenotypically mutant individuals that they found in nine of the F₂ progenies of the outcrosses to the Niederzenz (Nd) ecotype of 18 mutants obtained in a Ler background. Alternatively, this finding may indicate the influence of the genetic background on the phenotype associated with these recessive mutations, whose penetrance and/or expressivity in the F₂ of the outcrosses was lower than that of the corresponding backcrosses. This could also be the reason why the mutations ven3-2 and ven6 mutations, which are semi-dominant in the Ler background, behaved as recessives in the F₂ populations of their outcrosses to Col-0.

Some mutant strains (ron3, sca4, sca5 and tcu3) were found to be carriers of additional mutations, other than the mutation responsible for the phenotype of interest, since we found unexpected phenotypes in the F₂, apparently caused by recessive mutations. The den3 strain was not subjected to linkage analysis since some Exiguia individuals but no Denticulata plants were found among its F₂ progeny. Neither exi8-1, deal 1 nor exi4 was mapped, since the F₂ seeds of exi8-1 did not germinate and only wild-type individuals were found among the progeny of the others.

Table 2 presents a few representative examples from the entire set of raw recombination data that are obtained when the method used in this paper is applied to mapping on a one gene-one gel basis. For each mutation, only those recombination frequencies considered as unequivocal evidence of linkage are listed in Table 3, where only distances to the microsatellites flanking each gene are given. Most of the map positions presented here were determined from DNA samples extracted from 19 F₂ plants (see Materials and methods). However, it was not possible to detect linkage on one gel one-gene basis for some mutations. We studied linkage on more than one gel (i.e., genotyping more than 19 F₂ plants) in those cases where fewer than 30 chromosomes could be genotyped for the two nearest microsatellites, due to their poor amplification (ANG2, ANG4, ANU4, ANU6, ANG1).
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<th>Flanking markers</th>
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Table 3 (Contd.)

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<td>AthGAPab (38), 0.00 ± 0.16; nga162 (38), 10.69 ± 5.21</td>
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</table>

The figures in parentheses indicate the number of chromosomes analyzed to test for linkage to a given microsatellite marker.

API4, DEN2, DEN7, OND1, ORB2 and VEN3, or when the map distance to the nearest microsatellite was greater than 20 cM (DEN6, DEN8, DEN9, DEN13, EXII, EXI3, OND2 and SEA3). In this latter group, although a second gel helped to refine map positions, five of the eight genes studied still fell more than 20 cM away from their closest neighboring microsatellites (DEN6, DEN8, DEN9, DEN13 and EXII). The distances between the markers flanking four of these genes (DEN6, DEN9, DEN13 and EXII) are longer than 34 cM (nga1111 and nga1139, AthACS and AthZFPG, AthGAPab and nga6, separated in the last RI map by 53.77, 34.05 and 42.64 cM, respectively). The remaining one (DEN8) was distal to the nga168 marker, which lays 23.68 cM from the telomere.

A map is shown in Fig. 2 indicating the positions of the microsatellites used as markers and those of the 76 genes subjected to linkage analysis in this work. To test the robustness of our method, we pooled all the mapping data to calculate map distances between the markers themselves. The distances obtained in this way between adjacent microsatellites were very close to those given in the last version of the Lister and Dean RI map (see...
Materials and methods), with an average deviation of 4.76 cM. The greatest differences found were the distance between AthGAPAb and nga6, and that between AthCTR1 and nga139, which diverged 15.1 and 15.6 cM respectively, with respect to the RI map. Such deviations are likely to be due to the different types of mapping populations used, since the map of Lister and Dean was obtained from recombinant inbred lines and ours from the F2 progeny of outcrosses.

Allelism tests performed after linkage analysis

We performed some allelism tests between mutant strains that share some phenotypic traits and carry mutations that map to similar positions, such as some of the *ero*, *anu* and *den* mutants that have toothed leaves. Only two of the mutants that were crossed were shown to carry allelic mutations: *ero2* and *anu1*, both mapping on chromosome 1, at 27.00 and 20.57 cM respectively. Complementation was found in the progeny of the remaining crosses, which were the following (indicated as female × male, each mutation shown with its chromosome and approximate map position, in cM): *ero2-1a* (1; 27.00) × *anu10-1a* (1; 40.59), *ero2-1a* (1; 27.00) × *anu12* (1; 74.06), *ang1-1* (2; 43.25) × *den5-1* (2; 0.00), *api3* (4; 42.39) × *den6-1* (4; 27.29), *den17* (4; 74.84) × *ang2* (4; 59.81), *den17* (4; 74.84) × *api7-1a* (4; 81.08), *api7-1a* (4; 81.08) × *ang2* (4; 59.81), *den11* (5; 18.52) × *ven1* (5; 18.43), *sca5* (5; 35.64) × *anu3* (5; 30.55), *sca5* (5; 35.64) × *anu2* (5; 27.75) and *api6* (5; 109.02) × *den10* (5; 101.94).

Discussion

Much information can be gained from large-scale mutant screens aimed at identifying the genes controlling developmental processes (Nüsslein-Volhard and Wieschaus 1980; Jürgens et al. 1991; Haftler et al. 1996; Berná et al. 1999). Such screens usually yield a large number of mutants, which must then be subjected to complementation and linkage analyses in order to ascertain whether they carry mutations in as yet undescribed genes or new alleles of already known genes. This can only be done for a large group of new mutants in a short period of time if a high-throughput procedure such as the one used in this paper is available.

Almost twenty years have passed since the first large *Arabidopsis* genetic map, which included 76 loci, was obtained (Koornneef et al. 1983). Such pioneering work was based on the use of classical genetic markers, which were later used to map more than two hundred *EMBRYO DEFECTIVE* (*EMB*) genes (Patton et al. 1991; Castle et al. 1993; Franzmann et al. 1995), obtained in the laboratory of D. Meinke in an attempt to saturate the genome for mutations affecting embryonic development. Allelism tests involving members of such a large collection of *emb* mutants were limited to those with neighboring map positions (Franzmann et al. 1995). In this case, proximity between genetic map positions was considered to be a better criterion than an apparent resemblance between phenotypes on which to base the choice of candidates for allelism tests. More recently, molecular markers have been used to map nine genes that cause organ fusion (Lolle et al. 1998), nine *SENSITIVE TO FREEZING* (*SFR*) genes (Thorby et al. 1999), and nine *RESPONSIVE TO DEHYDRATION* (*RD*) and 16 *EARLY RESPONSIVE TO DEHYDRATION* (*ERD*) genes (Taiji et al. 1999).

We contribute here to enriching the genetic map of *A. thaliana* by determining the positions of 76 genes identified by mutation, whose activity is required for the leaf to acquire its wild-type architecture. The mapping of this large collection of mutations that perturb leaf development establishes the basis for future advances that will shed light on the processes involved in the making of a leaf. On the other hand, our collection of mutants, together with their map positions presented here, will facilitate studies of leaf mutants isolated by others.

Although until recently map-based cloning has been considered time-consuming and expensive in *A. thaliana*, techniques developed in recent years, and the information provided by the *Arabidopsis* Genome Initiative, now make it possible to complete positional cloning projects in a short period of time (Lukowitz et al. 2000). The mapping data, as well as the F2 mapping populations obtained in this work, lay the foundations for attempts to positionally clone the mutated genes, which will be identified by narrowing down the genetic intervals presented in this paper. In fact, efforts to clone some of the genes mapped in this work are already underway, in collaboration with other groups.

Acknowledgements Thanks are due to H. Candela, A. Martínez-Laborda, J. M. Pérez-Pérez, M. R. Ponce, P. Piñeras and V. Quesada for comments on the manuscript, and S. Gerber and J. M. Serrano for their expert technical help. This research was supported by a grant from the Ministerio de Educación y Cultura of Spain (PB98-1389). P.R. was a Fellow of the Ministerio de Educación y Cultura of Spain.

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