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Use of recombinant inbred lines (RILs) to identify, locate and map major genes and quantitative trait loci involved with in vitro regeneration ability in *Arabidopsis thaliana*

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Abstract The Landsberg *erecta* × Columbia recombinant inbred lines (RILs) of *Arabidopsis* have been used in order to identify and localize chromosome regions involved in the genetic control of the in vitro regeneration ability. Callus morphology (CM) and shoot regeneration (SR) traits have been considered for both leaf and root explants. The MAPMAKER analysis of leaf culture data has revealed at least one chromosome region involved with CM and several with SR, the 29–30 region of chromosome 1 being common for the two traits. Root explants did not segregate for CM but several QTLs have been detected for SR. The chromosome regions involved with leaf culture regeneration seem to be different from those of root cultures, although the regeneration of abnormal shoots in leaf explants share two chromosome regions with the regeneration of normal shoots in root cultures.

Keywords *Arabidopsis thaliana* · In vitro regeneration · QTL · Gene mapping · RILs

Introduction

Several papers have been published with the aim of clarifying the heritance of in vitro regeneration in higher plants (Naldoska-Orezyk and Malepszy 1989; Birhman et al. 1994; Yasutani et al. 1994; Fennell et al. 1996; Sarrafi et al. 1996; Taguchi-Shiobara et al. 1997a; Sugiyama 1999). Genetic markers have been used to locate the genes involved in regeneration ability (Armstrong et al. 1992; Koornneef et al. 1987, 1993; Ben Amer et al. 1997; Komatsuda et al. 1993; Mano et al. 1996; Taguchi-Shiobara et al. 1997b; He et al. 1998). In *Arabidopsis thaliana* three mutants, exhibiting temperature sensitivity at different stages of organogenesis, of three genes located on chromosome 1 have been isolated (Ozawa et al. 1998). These authors also propose an hypothetical scheme for *Arabidopsis* in vitro organogenesis. We have described (Candela et al., submitted) genotype differences related with the in vitro regeneration response between two *A. thaliana* parental lines for which recombinant inbred lines (RILs) are available, Columbia N933 and Landsberg *erecta* NW20 (Lister and Dean 1993). RILs became a useful tool for genetic because they avoid the number of marker limits due to DNA requirements occurring in small-size plant species for RFLP analysis and minimize the environmental variation in QTL analysis (Kearsey and Farquhar 1998). RILs can be reproduced indefinitely providing that the data obtained by different researchers come from the same gamete sample and contribute to the same database (Alonso-Blanco et al. 1998). We have performed an experiment with leaf and root cultures of *Arabidopsis* using RILs for analyzing the character callus morphology, which is indicative of further regeneration ability, and the number of normal and abnormal regenerated shoots in order to identify chromosome regions involved in the genetic control of in vitro plant regeneration.

Materials and methods

We have analyzed 30 RILs from those obtained by Lister and Dean (1993) and distributed by the Nottingham *Arabidopsis* Stock Center (NASC). The chosen lines were those recommended by the NASC to begin the mapping approach for the genes or chromosome regions responsible for the phenotype differences observed in the parental lines. They have the highest frequency of recombination over the five *Arabidopsis* chromosomes and should therefore be the most informative for mapping purposes.

Two experimental non-simultaneous replicas have been carried out either with leaf or root explants. A third replica was made with leaf explants to analyze only the callus-morphology character. In each replica several plants per line were used and the parental lines Columbia N933 (Col) and Landsberg *erecta* NW20 (Ler) were always included as a control.

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Culture procedures

Seeds were surface-sterilized by a 1-min immersion in 70% ethanol and 5 min in 2.5% (w/v) sodium hypochlorite, followed by several washes in sterile water. They were then germinated in growth medium (GM) and vernalized for 4 days at 4 ºC. Afterwards the plantlets were grown at 22 ± 2 ºC with 16 h of light per day. We follow the Feldman and Marks (1986) protocol which yields the best regeneration results but introduced some modifications into the callus inducing medium (CIM) and in the shoot inducing medium (SIM) as described (Candela et al., submitted), CIH and SIH being the media used for leaf cultures. In the case of root explants we used the CIR and SIR media based on those described by Valvekens et al. (1988). In each experiment five to eight plants, 3-weeks old, were used per line. Four leaves per plant cut in two halves were placed in CIH medium for callus induction, resulting in eight calluses per plant. From the same plants the roots were cut into 8–10-mm sections and grouped into a bunch which was set in CIR medium for callus induction, resulting in a single callus per plant. After 5 days both leaf and root explants were transferred to shoot-inducing media (SIM) and 10–12 days afterwards the callus morphology (CM) was observed. After $3^1/_2$ weeks of culture the number of normal and abnormal regenerated shoots per plant were scored and the average mean per line was calculated in each case.

Mapping analysis

The MAPMAKER/EXP 3.0 and MAPMAKER/QTL 1.1 programs, distributed by the Whitehead Institute for Biomedical Research, Cambridge Mass. (USA) (Lander et al. 1987; Lincoln et al. 1992 a,b), were used to analyze the experimental data. A 163 molecular-marker matrix was elaborated from the more than 810 molecular-marker data available at the NASC web site. The 163 chosen markers cover the five *Arabidopsis* chromosomes in such a way that the maximum map distance between two neighbor markers was 5 cM, with very few exceptions to this rule. When MAPMAKER/QTL is used to analyze RILs data the log-likelihood (LOD) score threshold is doubled; thus, the resulting LOD threshold needs to be 4 (NASC web site advise). MAPMAKER/EXP is already prepared to work with RILs data having a recommended LOD threshold of 3**.**

Results

Root explants were able to develop callus in almost all the cases, while the response of leaf explants were lower but always higher than 70%. No correlation has been found between the percentage of callus formation and the regeneration ability.

Callus morphology

Leaf explants

After 10 to 12 days of culture in SIH medium the Col and Ler calluses present a very characteristic morphology. Col explants grow slowly and become small pale green calluses with a soft and aqueous-like texture. Those of Ler grow faster (with a bigger callus) have a darker green color and their texture is compact and granular. Ler calluses show wide dark areas probably due to the presence of phenolic metabolites (Fig. 1). In some case Ler calluses only became dark in those quite large areas through which regeneration occurs. In contrast, Col calluses very seldom show a small dark point just in the area from which a shoot is regenerating. These callus characteristics are indicative of the further regeneration ability observed after $3^1/_2$ weeks of culture. Thus, Ler calluses have a higher shoot-formation frequency while this is null or very low in Col calluses. Each of the 30 Ler × Col analyzed RILs show a characteristic callus phenotype which can be like one parent, the other, or else intermediate. In the second replica, RILs were classified only as Col or Ler depending on the major similarities of their callus appearance, while in replicas 1 and 3 various intermediate phenotypes were also considered: IC = intermediate near Col**,** IL = intermediate near Ler, and I = intermediate; however, the qualitative analysis was made with these same data grouped into only two phenotypic classes (C or L) (Table 1, in brackets). This analysis was carried out using the MAPMAKER/EXP program (Table 2). In the three experimental replicas the

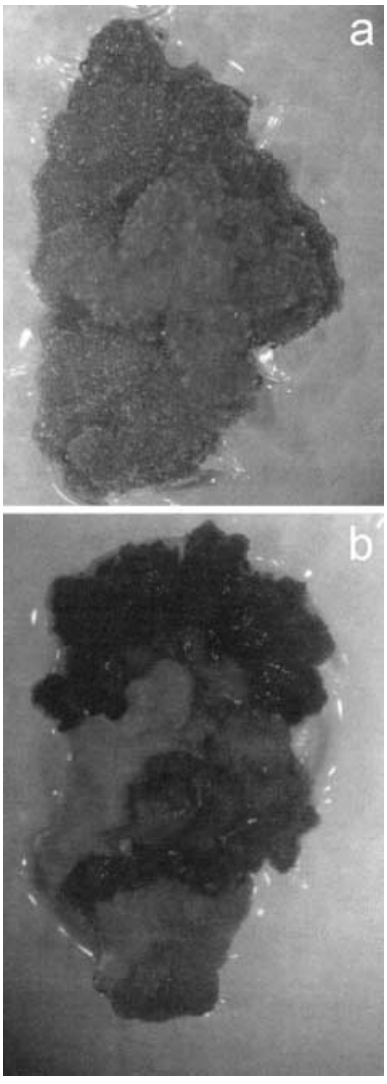

Fig. 1a, b Leaf explant calluses after 12 days of culture on SIH medium. **a** Col N933 callus. **b** Ler NW20 callus showing the characteristic dark areas

Table 1 Callus morphology in leaf explant-derived cultures from the 30 analyzed RILs and their parental lines. In brackets the assignation for the qualitative analysis.L = Landsberg erecta (Ler)-type callus, C= Columbia (Col)-type callus, IL = intermediate callus with major Ler characteristics, I = intermediate callus, IC = intermediate callus with major Col characteristics

Line	Replica 1	Replica 2	Replica 3
N933 (Col)	C	C	C
NW29 (Ler)	L	L	L
4	C	C	C
5	C	C	C
13	I (L)	L	L
33	IC (C)	C	IC (C)
35	C	C	IC (C)
37	C	C	C
115	IL (L)	L	L
190	IL (L)	L	IL (L)
191	IC (C)	L	IC (C)
194	IL (L)	L	IL (L)
217	L	L	L
231	C	C	C
232	C	C	C
238	C	C	C
242	I (C)	C	IC (C)
245	C	C	IC (C)
263	C	C	C
267	C	C	IC (C)
283	C	C	IC (C)
284	I (L)	L	IL (L)
288	I (C)	C	C
295	IL (L)	L	L
302	C	C	IC (C)
303	L	L	L
332	C	C	C
356	I (C)	C	IL (L)
358	C	C	IC (C)
367	IL(C)	C	IC (C)
370	L	C	C
377	IC (C)	C	IL (L)

Table 2 Map marker nomenclature (NASC data base)

Map marke number	Map marker name	Map position (in cM)	Chromosome
3	GT451	6.42	1
4	nga63	9.36	1
23	mi72	61.46	1
24	mi291a	69.84	1
28	mi303	81.67	1
29	g4026	84.90	1
30	mi353	88.60	1
45	m497 A	13.32	2
56	SEP5B	60.93	2
59	m323	67.97	2
103	GT148	12.65	4
104	Gsl_ohp	16.76	4
107	m456 A	23.98	4
108	nga8	26.56	4
109	H2761	31.65	4
124	m214	91.58	4
125	AP2	95.90	4
126	g2486	101.67	4
135	ca72	29.60	5
136	C1	36.43	5
137	mi138	39.60	5
142	Tn139	59.69	5
145	mi137	72.26	5

CM trait was assigned to chromosome 1 with LOD values of 3.2, 3.9 and 3.9, respectively; although the probable map location shows some differences, the regions between the markers 23 to 25 and 27 to 30 were principally involved.

The described morphological classes were assigned to numerical values in order to analyze the data quantitatively. To the first replica data the value 0 was given to Col, 1 to near Col, 2 to intermediate, 3 to near Ler, and 4 to Ler. The analysis using the MAPMAKER/QTL program shows the possible presence of a peak on chromosome 1 between the map markers 29 and 30, although its LOD value (2.5) does not reach the minimum level of significance required (4.00). When the same data were grouped giving the value 0 to those lines assigned to Col callus morphology and 1 to those with Ler characteristics, two peaks, one at position 29–30 (LOD = 2.9) and the other at position 24–25 (LOD = 2.8) (both below the significance level), were found.

The second replica data were also analyzed using the MAPMAKER/QTL program assigning the values 0 or 1 to the Col- or Ler-type calluses, respectively. Again a peak in the location 29–30 was found, now with a LOD = 33.84 surrounded by a wider area also over the threshold. In the case of the third replica data, a first analysis was made giving the value 0 to Col-type lines, 1 or 2 to those with intermediate characteristics, and 3 to the Ler-type lines. In this case four peaks in positions 23–24, 24–25, 28–29 and 29–30 appeared on chromosome 1 with LOD values of 2.9, 3.00, 3.5 and 3.2, respectively. When the same data were grouped giving the values 0 and 1 to the Col- and Ler-like lines, respectively, two peaks on chromosome 1 between the map markers 24 and 25 (LOD = 3.00) and between markers 29–30 (LOD = 2.95) were found (Fig. 2).

All these results suggest that in leaf explant cultures the chromosome-1 regions 23–25 and 27–30 could be responsible for those callus morphology features related to their further regeneration ability.

Root explants

Calluses from root explants do not show appreciable morphological characteristics distinctive for the parental lines. The calluses are compact, large in size, showing a white color which evolves into green and, occasionally, with dark regenerating areas. These calluses develop secondary white roots. This morphological homogeneity, probably as a result of no differences between the parental lines for those genes controlling early regeneration from root explants, makes it impossible to classify the RILs and, consequently, no statistical analysis can be made.

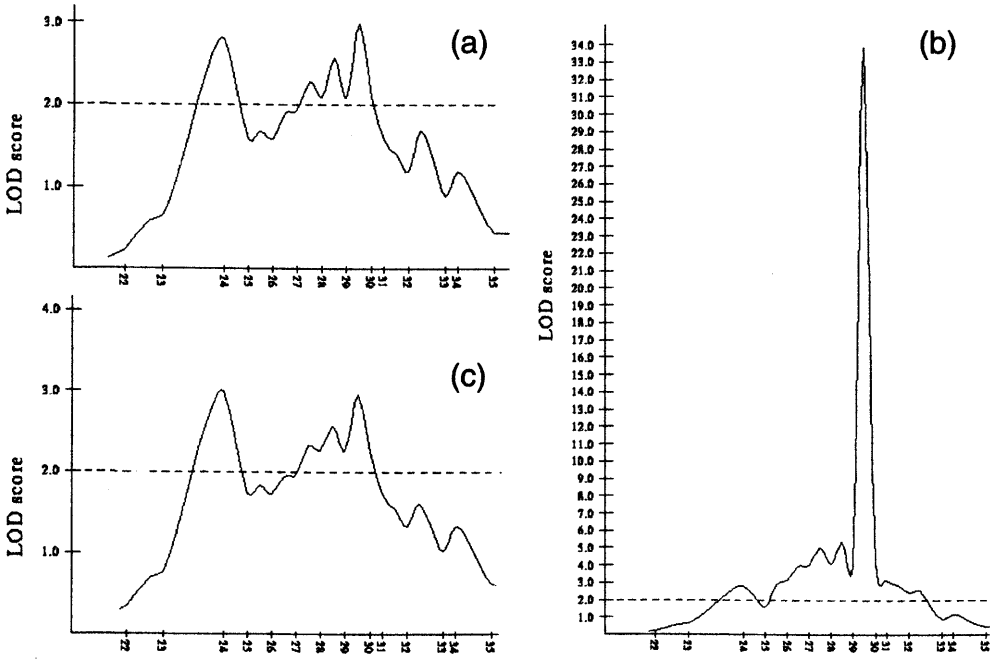

Fig. 2a–c MAPMAKER/QTL analysis of the callus morphology trait. The values 0 and 1 have be assigned to C- and L-callus phenotypes, respectively. (**a**) Replica-1 data. (**b**) Replica-2 data. (**c**) Replica-3 data

Regeneration ability

Leaf explants

Three parameters have been used to evaluate the regeneration ability of the leaf explants from the 30 analyzed RILs together with their parental lines in the two experimental replicas: namely, the average mean of the total regenerated shoots per plant, the average mean of normal regenerated shoots per plant, and the average mean per plant of those shoots with abnormal morphology. The parental line Ler NW20 regenerates considerably (15.37 ± 1.26 as a mean average of total regenerated shoots per plant, 3.50 ± 0.62 being the mean average of normal regenerated shoots per plant; these data have been calculated averaging the data from both replica), Col N933 does not regenerate at all or does so very poorly (0.53 ± 0.33 as the average mean of total regenerated shoots per plant, 0.35 ± 0.25 being the value considering only normal regenerated shoots). The different RILs behave repetitively like one parental line, like the other parental line, as intermediates, or even show regeneration values more extreme than the parental lines (the average mean of total regenerated shoots per plant varyed from 0 to 21.00 ± 2.27, 5.75 ± 1.92 being the value when only normal regenerated shoots are considered).

The MAPMAKER/QTL analysis of replica-1 data of normal and abnormal (total) regenerated shoots only detected the 29–30 region on chromosome 1 as being involved with the regeneration response, although its LOD value does not reach the threshold level. When only either normal regenerated shoots or abnormal regenerated shoots were considered, the same peak appeared with a LOD value slightly lower. The MAPMAKER/QTL analysis of the total number of replica-2 regenerated shoots data showed three peaks over (or very close to) the threshold on chromosome 1, together with two peaks over the level of significance on chromosome 4, and a peak close to the threshold around positions 136–137 on chromosome 5. Considering only normal regenerated shoots, only the 29–30 peak on chromosome 1 appeared above the level of significance. When the data of abnormal regenerated shoots were considered several peaks appeared on different chromosomes: three on chromosome 1, three on chromosome 4, and one on chromosome 5 (Table 3).

Root explants

The parental line Ler NW20 shows a mean average of total regenerated shoots per plant of 2.85 ± 0.56, 1.64 ± 0.49 being the average mean of normal regenerated shoots per plant. Contrary to the leaf explants, root explants from Col regenerate better than those from Ler (11.57 ± 1.39 for total regenerated shoots per plant with 5.05 ± 0.77 considering only normal regenerated shoots). The RIL values vary from 0.81 ± 0.3 to 17.28 ± 3.94 as an average mean of the total regenerated shoots per plant, 0.44 ± 0.22 and 10.63 ± 2.63 being the average means of normal regenerated shoots, respectively. When the replica-1 data, collected from 30 RILs, were analyzed using the MAPMAKER/QTL program no peak over the level of significance was found considering either the total number of regenerated shoots or the number of normal regenerated shoots. However, in the second replica, in which 85 RILs were analyzed, one peak over the significance level was obtained on chromosome 4, and two peaks were evident for chromosome 5 when taking into account only the normal regenerated shoots.

Table 3 Regeneration ability results from leaf explant-derived cultures

Item	Replica 1		
	Chromosome	Map markers	LOD
Total regenerated shoots	1	29–30	3.61
Normal regenerated shoots	1	29–30	3.32
Abnormal regenerated shoots	1	29–30	3.30

Item	Replica 2		
	Chromosome	Map markers	LOD
Total regenerated shoots	1	3–4	3.92
	1	23–24	4.74*
	1	29–30	6.62*
	4	103–104	4.30*
	4	125–126	4.63*
	5	136–137	3.79
Normal regenerated shoots	1	29–30	4.46*
Abnormal regenerated shoots	1	3–4	4.05*
	1	23–24	4.90*
	1	29–30	6.20*
	4	103–104	4.32*
	4	108–109	5.00*
	4	125–126	4.62*
	5	136–137	4.20*

* = Significant values

Table 4 Regeneration ability results from root derived cultures

Item	Replica 2		
	Chromosome	Map markers	LOD
Total regenerated shoots	5	135–136	3.80
Normal regenerated shoots	4	107–109	4.29*
	5	131–133	3.9
	5	136–137	4.80*
Abnormal regenerated shoots	2	45–46	5.10*
	2	56–59	5.20*
	5	135–136	3.50
	5	138–139	5.00*
	5	142–143	3.86
	5	144–145	3.80

* = Significant values

Analyzing only abnormal regenerated structures, several peaks, over or very near the threshold, appeared on chromosomes 2 and 5. When the total number of regenerated shoots (normal plus abnormal) was considered, a peak close to the threshold was evident in chromosome-5 region 135–136 (Table 4).

Shoot regeneration showed different results in leaf and root cultures. In the case of root explants the peak on chromosome 1, region 29–30, did not appear whereas it was very clear in leaf explants. However, there are some peaks common to both kinds of explant, this is the case for peak 107–109 (chromosome 4) and peak 136–137 (chromosome 5) which appeared in the abnormal regenerated-shoot analysis from leaf explants as well as in that of normal regenerated shoots from root explants.

Discussion

The very distinctive callus morphology shown by the leaf explant calluses of the parental lines, together with the differences in their regeneration ability from both leaf and root explant calluses, generates variability for these two traits among the derived RILs as a consequence of the segregation of those genes involved in the in vitro regeneration response. This variability includes descendant RILs with phenotypes more extreme than those shown by the parental lines (transgressive segregation). This fact has already been described by several authors in relation to the genetic control of in vitro plant regeneration (Naldoska-Orezyk and Malepszy 1989; Komatsuda et al. 1993; Taguchi-Shiobara et al. 1997b) and is an indication of polygenic inheritance. The occurrence of transgresive segregation indicates the existence of some alleles which promote, and others which inhibit,

in vitro regeneration, and not all the alleles with positive effects occurred in the same parent. The analysis of such variation led us to identify chromosome regions responsible, in a major or a minor degree, for the *Arabidopsis* in vitro regeneration process.

When various parameters are used as a measure of the observed phenotypic variation related to the in vitro regeneration capability, it is possible to dissect the process. In our experiment we have detected several chromosome regions involved with *in vitro* regeneration, which seem to act at different times. Thus, when the parameter callus morphology is analyzed (both as a qualitative and a quantitative character) in those cultures derived from leaf explants, one area (or maybe two) on chromosome 1 around map-markers 23 to 30 appears to be involved with the inheritance of such a trait. With the data of shoot regeneration the results are again consistent with the same area of chromosome 1 playing a role in the genetics of this character but, in this case, new regions on other chromosomes also appear to be involved. The differences in regeneration ability shown by the parental lines Ler and Col could be the consequence of various genes acting at different steps of the cell-differentiation process which occurs from the de-differentiated callus cells to shoot morphogenesis. A similar situation has been described by Ben Amer et al. (1997) who detected three QTLs for tissue culture response (*Tcr*) which mapped on chromosome 2B in barley; two of them (*Tcr-1B* and *Tcr-2B*) affect both green-spot initiation and shoot regeneration but the third QTL (*Tcr-B3*) only influences regeneration. Likewise, Taguchi-Shiobara et al. (1997b) detected five QTLs in rice on chromosomes 1, 2 and 4 using the number of regenerating shoots per callus, while only four of them were detected when analyzing the regeneration-rate data. Koornneef et al. (1987) found two major genes involved with the regeneration response of leaf-disk cultures in tomato and also suggested the possible occurrence of minor genes playing a role in the process.

In contrast to what happens with leaf explants, root explant-derived cultures do not present callus morphology differences either between the parental lines or among the analyzed RILs. This, together with the appearance of different chromosome regions involved with shoot regeneration in cultures derived from leaf or from root explants when the regeneration data are analyzed, could be an indication of different genetic requirements of the de-differentiated cells coming from one or the other tissue in order to follow a re-differentiation process which, at the end, will originate shoots. However, we must not forget that the culture media which allow shoot regeneration in leaf and root explants have a different hormone composition and so would introduce a source of variability other than just the differences in the involved genes. Several authors have reported differences in the in vitro regeneration genetic control depending on the explant type. Thus, Taguchi-Shiobara et al. (1997b) detected several QTLs controlling the regeneration ability of rice seed callus. These QTLs were located on different chomosome areas from those reported by Yamagishi et al. (1996) using rice anther cultures. Koornneef et al. (1993) also found differences between primary and established callus cultures from tomato root explants; thus, in young cultures the presence of the dominant allele *Rg-1* seems to be sufficient to regenerate shoots while in established cultures the additional presence of a second dominant allele (*Rg-2)* seems to be required. Ozawa et al. (1998) analyzing the organogenic responses in the tissue culture of three temperature-sensitive mutants of *Arabidopsis* found that the requirements for root or hypocotyl cultures to reach the competence acquisition phase are different. Thus, hypocotyl explants required the gene *SRD2* in addition to *SRD3*, while root explants required only *SRD3*. All the *SRD* genes are located on chromosome 1 and one of them (*SRD1)* maps close to the marker m315 which is located quite near to the 29–30 area. The *SRD-1* product seems to play a role in a later stage of the re-differentiation of shoots when the hypocotyl cultures are transplanted into SIM medium.

In our experiment, different QTLs seem to be involved with the regeneration of either normal or abnormal shoots in cultures derived from both leaf and root explants. When normal shoot regeneration is analyzed, two QTLs (chromosome 4 position 106–109 and chromosome 5 around position 136) appeared to be involved in this trait; these QTLs are coincident with two of the regions found in the leaf explant analysis when only abnormal shoots were considered. It is possible that the allelic combination which controls the different regeneration steps giving rise a normal shoot would be different in the two kinds of explant. In this respect, it is of interest to note the existence of a mutation *(stm1)*, which has been reported in *Arabidopsis*, acting in such a way that root explants are unable to regenerate normal shoots but are able to form abnormal shoots and leaves in culture (Barton and Poethig 1993).

We are now trying to determine the precise location of the different QTLs here described on the *Arabidopsis* genetic map, as well as their molecular identification, isolation and cloning. This effort will require the analysis of more RILs together with a molecular approach based on a knowledge of the heterologous sequences of similar genes.

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