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ORIGINAL PAPER

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Quantitative trait loci controlling root growth and architecture in *Arabidopsis thaliana* confirmed by heterogeneous inbred family

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Abstract *Arabidopsis thaliana* provides a scientifically attractive and simple model for studying root growth and architecture and, subsequently, for discovering new genes involved in the control of these characters in plants. We have used the natural variation available in Arabidopsis accessions and mapped quantitative trait loci (QTLs) for primary root length (PRL), lateral root number (LRN) and density (LRD) and for total length of the lateral root system (LRL) in the Bay-0 × Shahdara population. Total phenotypic variation was very large, and despite the importance of the environmental component we were able to map 13 QTLs and one epistatic interaction between QTLs. Our results highlight the biological relevance and genetic control of lateral root density in this material. We were also able to show that variation in the extent of the lateral root system depends mainly on the growth of the existing lateral roots rather than in a change in LRN. Factors controlling lateral root growth seemed to have no major effect on primary root growth. Moreover, Shahdara QTL alleles always increased the length of the lateral roots, which may be taken as an adaptation to its very dry natural environment in Tadjikistan. A QTL for PRL was confirmed using a type of near-isogenic line called a heterogeneous inbred family (HIF), and this QTL is a candidate for further fine-mapping and cloning.

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Introduction

The root system provides, among others, anchoring and water/nutrient absorption to the plant. As such, its extension, a phenomenal post-embryonic transformation (Malamy and Ryan 2001), is extremely important as it ensures the stability of the plant and its adaptability to the surrounding environment. Despite its essential role in plant growth and adaptation, the root system in general has been studied far less intensively than the shoot because it often remains the 'hidden-half'.

In addition to its status as a model plant system for the study of plant genetics, *Arabidopsis thaliana* is also a very attractive tool for studying root architecture and growth as a model for plant organ development due to the simple structure of its root system and the relative easiness in which the latter can be visualized in vitro on vertical agar plates (Benfey and Schiefelbein 1994). The mostly vertically extending primary root consists of single layers of epidermal, cortical and endodermal cells surrounding the vascular tissues. Some of the pericycle cells along the primary root undergo a series of transverse and periclinal divisions, thereby giving rise to lateral root primordia (initiation and emergence; Casimiro et al. 2003). The number of primordia is not predetermined and is a response to different growth control points and environmental cues (Smith and Fedoroff 1995; Dubrovsky et al. 2000). The lateral root subsequently extends at a certain angle from the vertical (elongation) in a manner exactly similar to that of the primary root itself—by cell division and elongation—and becomes a secondary root. Mutant screens have already identified a number of genes involved in the pathway regulating lateral root development in Arabidopsis, and a recent review by Casimiro et al. (2003) reports 28 of these genes. Many of these show direct or indirect effects linked to the hormone auxin, a signal derived from the shoot that globally promotes root growth (Fu and Harberd 2003). Genes potentially associated with auxin synthesis, transport or sensitivity

(Xie et al. 2000; Casimiro et al. 2001) are certainly over-represented due to the original indirect screens used to detect the mutants, such as screens for the presence of auxin or auxin-transport inhibitor in the environment. Despite these numerous mutants, the exact mechanisms explaining the regulation of, for example, lateral root spacing by auxin remain to be established (Casimiro et al. 2003). To date, very few genes have been reported to specifically affect lateral root initiation (Malamy and Ryan 2001), and most of the mutants summarized by Casimiro et al. (2003) have a quantitative phenotype based on the number of lateral roots.

Root development is also under strong environmental control, especially through its response to nutrient (nitrate, phosphate, sulfate, etc.) and water availability, which possibly involves both specific and general mechanisms (Forde and Lorenzo 2001). For example, nitrate and phosphate are known to have contrasting effects on primary root length and lateral root density, but similar effects on lateral root length (Linkohr et al. 2002). Of all the areas of root development that have been investigated, the complex effects of nitrate on lateral root development (and its signaling) is the most studied of these interactions with the environment, and several different regulatory mechanisms have been revealed(Casimiro et al. 2003). The nitrate-induced localized stimulatory effect seems to act on the elongation rate of those lateral roots directly in contact with a localized nitrate supply in a generally low-nitrate environment (Zhang et al. 1999); the high-nitrate-induced inhibitory effect acts specifically on immature, emerging lateral roots, and the hormone abscisic acid plays an important role in its mediation (Malamy and Ryan 2001; Signora et al. 2001).

The quantitative and environmentally responsive nature of root growth and architecture make traditional genetics screens difficult. Another source of allelic variability, which has only recently become widely used, lies in the natural variation of Arabidopsis accessions (Alonso-Blanco and Koornneef 2000). Previous screens of accessions have shown extended variation for the growth of the primary root and lateral roots in different environments (Narang et al. 2000; Beemster et al. 2002; Chevalier et al. 2003). Several different groups have also noticed that traditional laboratory ecotypes (i.e., Col, L *er* and WS) show differences with respect to root architecture. Moreover, it seems that the root development is adaptive and determines fitness in Arabidopsis, particularly with respect to phosphate (Narang et al. 2000; Fitter et al. 2002) and nitrate acquisition (Linkohr et al. 2002).

Quantitative trait locus (QTL) mapping using recombinant inbred line (RIL) populations derived from crosses between these accessions is one way to identify the genes underlying root development. With respect to such investigations, QTL mapping to date has mostly been used to study root architecture in rice in response to environmental constraints such as drought (Price and Courtois 1999; Ali et al. 2000; Kamoshita et al. 2002). However, it could be successfully applied to clone new genes in Arabidopsis, and different laboratories have started to exploit this approach. Publications available on QTL analysis of root traits in Arabidopsis report only on root mass or maximum root length (Kobayashi and Koyama 2002; Rauh et al. 2002), both of which are known to have little effect in determining anchoring and/or absorption (Forde and Lorenzo 2001). QTL were mapped in the L *er*/Col population for primary root length under aluminum stress, but Hoekenga et al. (2003) were unable to identify a QTL in their control (non-stressed) environment. Recently, Mouchel et al. (2004) have cloned a major gene (*BREVIS RADIX*) explaining most of the phenotypic variation for primary root elongation in a cross between accessions Uk-1 and Sav-0. They have shown that Uk-1 has a premature stop codon in this member of a novel, plant-specific gene family.

We describe here a full QTL analysis of root architecture characters measured in original material (the Bay-0 x Shahdara population; Loudet et al. 2002) showing extensive variation for all of the traits studied. A total of thirteen QTL were detected, representing the intrinsic developmental variation of root architecture in this material under standard in vitro conditions. Taking advantage of the residual heterozygosity and the large size of the RIL population, we were able to confirm one of our QTLs in near-isogenic lines (NIL) using the 'heterogeneous inbred family' (HIF) strategy.

Materials and methods

Plant material

The material used in this study was developed in our laboratory and has been deposited in public Arabidopsis stock centers. The Bay-0 × Shahdara RIL population has been fully described in another publication (Loudet et al. 2002) and is available at http://www.inra.fr/qtlat. F_7 seeds obtained from the last generation of single-seed descent were used. These seeds were harvested from plants grown once for all lines, thus minimizing the maternal environment effect. A sample of 150 RILs were arbitrarily chosen for this analysis, mainly lines between nrs. RIL145 and RIL300. The exact list of lines can be found at http://www.inra.fr/qtlat.

NILs were developed as an HIF following the idea published by Tuinstra et al. (1997). At the F_6 stage, lines RIL196 and RIL090 (from the Bay-0 × Shahdara population) are homozygous everywhere on their genome except for a small region around marker MSAT4.9 (RIL196) and a small region around marker MSAT4.18 (RIL090). For each of these lines, we planted 20 F_7 seeds and genotyped the plants individually, selecting two plants fixed with the Bay-0 allele and two other plants fixed with the Shahdara allele at the respective segregating marker (seeds from these plants are then used for further phenotyping). HIF196C and HIF196M were the plants with the Shahdara allele at marker MSAT4.9 and differed from HIF196G and HIF196L (Bay-0 at

MSAT4.9) only at this region. At marker MSAT4.18, the lines compared were HIF090C/HIF090G (Shahdara allele) and HIF090J/HIF090H (Bay-0 allele).

Phenotyping display

Seeds were sterilized using Bayrochlore in 95% (v/v) ethanol for 10 min and then washed with absolute ethanol once. They were then stratified for 48 h at 4°C in the dark in a 0.1% (w/v) sterile agar solution. On day 0, eight seeds from each line were distributed individually at regular intervals (using a pipetor) 2 cm from the top of a 12×12 cm square plate containing 65 ml of solid medium [basic Arabidopsis media (Estelle and Somerville 1987) specifically supplemented with 10 m *M* nitrate (= 10 m *M* N), 1% (w/v) sucrose and 0.6% (w/v) agarose (LSM 5000 protein grade)]. The rather low carbon to nitrogen (C:N) ratio was intended as a manner to avoid the inhibition of lateral root development under our conditions. The plates were sealed with air-pore tape to ensure air renewal within the plate volume. A uniform area with respect to temperature, light and air flow was determined in the growth room to receive the 160 plates (150 RIL and the parental genotypes Bay-0 and Shahdara, repeated throughout the growth chamber). Since no plate-to-plate variation was detected during the preliminary experiments, we decided to place all replications (eight plants per line) on the same plate. The plates lay horizontally for 3 days (germination occurred on day 2) to ensure that the emerging root would penetrate into the media and not stay on its surface. On day 3, possible late-germinating seeds were recorded, and the plates were placed quasi-vertically on a rack maintaining them regularly spaced at an angle of 20° with respect to the vertical axis (see Fig. 1). For the QTL experiment, all lines and controls were studied at one time in a growth room. The same growth room and conditions were also used as for NIL (HIF) phenotyping.

Growth conditions

During the complete culture period the plates were maintained at a constant temperature of 21°C under long-day conditions (16/8 h; light/dark) with light supplied by three cool-white 36-W fluorescent bulbs providing a photosynthetic photon flux density of approximately 120 µmol m^{-2} s^{-1}. Humidity was maintained at 70% in the growth room.

Traits measured

After 9 days of 'vertical' growth, all of the plates were harvested at the same time (day 12), the lids were removed and the plates were scanned on a Vista scanner (Umax Systems, Willich, Germany) using the 'transmissive' mode at 300 dpi. The pictures were then analyzed using a semi-automatic graphical interface that enabled the root extremities to be specified and their precise length to be measured. Plants from seeds that did not germinate by day 2 or those with a root system at the surface of the media were not considered in the analysis. The length of the primary root was measured (PRL), and the number and length of each discernible (≥1 mm) lateral root emerging from the primary root [lateral root number (LRN)] was recorded. Eventual adventitious roots (lateral root emerging from the hypocotyl) were not taken into account. The sum of the lengths of all of the lateral roots provided the LRL. The lateral root density (LRD) was calculated from the ratio LRN/PRL. Table 1 summarizes the traits studied.

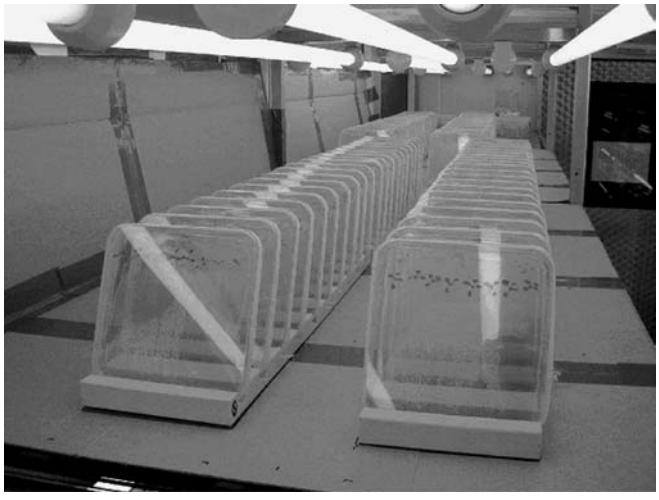

Fig. 1 The phenotyping display during the experiment, 2 days before harvest (day 10)

Statistical analysis and QTL mapping

The complete set of data was included in an analysis of variance (ANOVA) model to determine the specific effect of the 'genotype' factor. This ANOVA enabled us to quantify the broad-sense heritability (genetic variance/total phenotypic variance). Again, as specific analysis detected no plate-to-plate variation across the replicated parents, subsequent analyses involved mean values for each line. Phenotypic correlations were calculated for all combinations of traits. ANOVA and correlation estimations were performed using *aov*() and *lm*() functions of the S-PLUS ver. 3.4 statistical package (Statistical Sciences, Seattle, Wash.).

The original set of markers (38 microsatellite markers) and the genetic map obtained with MAPMAKER 3.0, as previously described (Loudet et al. 2002; http://www.inra.fr/qtlat), were used in this study. All QTL analyses were performed using the Unix version of QTL CARTOGRAPHER 1.14 (Basten et al. 1994; 2000). We used standard methods as previously described (Loudet et al. 2002), interval mapping (IM) and composite interval mapping (CIM). IM (Lander and Botstein 1989) was first used to determine putative QTLs involved in the variation of the trait. CIM model 6 of QTL CARTOGRAPHER 1.14 (Basten

Table 1 Traits, 'genotype' effect and heritability (*RIL* recombinant inbred line)

Abbreviation	Trait (units)	RIL mean	RIL range (minimum–maximum)	Genotype effect[a]	Heritability
PRL	Primary root length (in millimeters)	79.7	60.2–94.8	47,748*	0.63
LRN	Lateral root number (in number of roots)	21.2	9.8–33.8	16,068*	0.56
LRD	Lateral root density, LRN/PRL (in roots mm^{-1})	0.264	0.147–0.408	1.81*	0.55
LRL	Total lateral root length (in millimeters)	216.6	45.5–393.9	4,035,871*	0.59

*Significant at the 0.1% level
[a]The sum of squares of the 'genotype' factor (one-way ANOVA) and its significance

et al. 2000) was then performed on the same data: the closest marker to each local LOD score peak (putative QTL) was used as a cofactor to control the genetic background while testing at another position of the genome. When a cofactor was also a flanking marker of the region tested, it was excluded from the model. The number of cofactors involved in our models did not exceed four and the window size used was 3 cM. The walking speed chosen for all QTL analyses was 0.1 cM. The LOD significance threshold (2.4 LOD) was estimated from permutation test analyses, as suggested by Churchill and Doerge (1994). One thousand permutations of phenotypic data were analyzed using the CIM model with the specific conditions described above for each trait, and the maximum 'experiment-wise threshold' obtained (overall error level:5%) was used for all traits.

Additive effects (Table 3; '2a') of the QTLs detected were estimated from the CIM results, with 2a representing the mean effect of the replacement of both Shahdara alleles by Bay-0 alleles at the locus investigated. The contribution of each identified QTL to the total phenotypic variance R^2) was estimated by variance component analysis. For each trait, the model involved the genotype at the closest marker to the corresponding detected QTL as random factors in ANOVA. Only homozygous genotypes were included in the ANOVA analysis. Significant QTL × QTL interactions were also added to the linear model via the corresponding marker × marker interactions, and their contribution to the total variance was also estimated. Complete Model R^2 (Table 3) was estimated for each trait as the sum of individual R^2 (QTL and epistatic interactions). We used two types of support interval estimations—an anti-conservative one-LOD support interval based on real QTL LOD Score profile analyses and conservative bootstrap simulated confidence interval with ten series of 1,000 resampling data sets as proposed by Visscher et al. (1996). Estimations were calculated for different R^2 classes.

Results

Approximately 900 root systems were studied in detail, representing on average 5.5 plants per RIL (following elimination of the late-germinated seedlings). No major source of heterogeneity was detected within the growth room (results not presented; see Fig. 1), and the main source of variation is the genotype. As shown in Table 1, genotype effect was highly significant for all traits. Figure 2 illustrates this genotypic variation on six lines chosen from the Bay-0 × Shahdara population; heritabilities (estimated on RIL variation) are high—in the 0.55–0.65 range—for the four traits studied.

Phenotypic variation and correlations among traits

Figure 3 shows the distribution of the phenotypic values for each trait among the 150 lines. Note that the distributions are relatively normal, with the population mean at or between the parental values. The four traits showed strongly significant transgression; this is especially true for LRN and LRD for which we could not distinguish between the parental phenotypes and still observe a large variability in the population. LRN varied from fewer than ten roots per plant up to more than 33 roots per plant, and this variation was partly correlated with the length of the primary root, PRL (+ 0.63; see Table 2 for correlation coefficient between traits). However, as illustrated on Fig. 4, a large panel of LRN phenotypes could be found in the population for any given PRL level (except the extreme ones). This degree of freedom of the total number of lateral roots with respect to the length of the organ that holds them is represented through LRD (=LRN/PRL) variation, which varied from 0.15 root mm^{-1} to 0.40 root mm^{-1}. LRD was therefore linked to LRN variation more than to PRL variation (correlations are +0.91 and +0.25, respectively; Table 2). The total length of the lateral root system was highly variable in our material, from extremely poorly developed systems (less than 50 mm of lateral roots) to dense and highly extended systems (400 mm of lateral roots). LRL was mainly correlated with LRN (+0.85; Table 2), with the extreme phenotypes of both traits being exclusively linked, even though plants with the same average number of lateral roots (i.e. approx. 20 roots) covered a wide range of LRL phenotypes (from 150 mm to 300 mm of lateral roots; results not presented).

QTL mapping

Thirteen QTLs and one QTL × QTL interaction were detected in this analysis of the four root architecture parameters in the Bay-0 × Shahdara population. Table 3 describes the three QTLs found for PRL, LRN

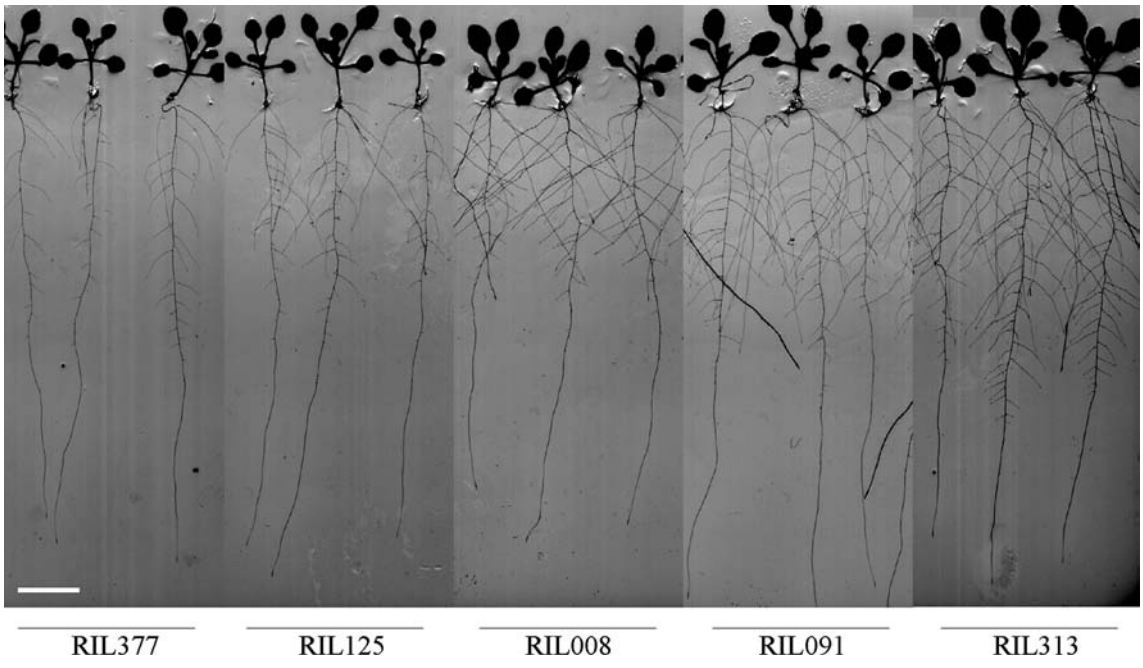

Fig. 2 Variability for root architecture in the Bay-0 × Shahdara population. Six lines (each line is represented by three plants) are pictured at the same age (10 days post-germination, day 12). *White bar*: 10 mm

and LRL and the four QTLs plus one epistatic interaction found for the LRD trait. Individual QTL contributions R^2) ranged from 3% to 16%, with most being in the 6–10% range. Three QTLs had high contributions (around 15% of the total phenotypic variance), two of which were detected for the same trait—the density of branching (LRD). Figure 5 presents a summary of the position and effects of the QTLs found in this study.

Primary root length analysis revealed three loci contributing to 5%, 7% and 14% of the trait variance. PRL2 on the bottom of chromosome 3 was the only one with a positive allelic effect (the Bay-0 allele at this locus increased the length of the root) and partly explains the transgression above the Shahdara phenotypic value and below the Bay-0 phenotypic value. At the PRL3 locus on chromosome 4, the Bay-0 alleles were responsible for a 7-mm decrease in root length with respect to the Shahdara alleles.

The number of elongated lateral roots was significantly controlled by three medium-effect loci in our population, among which LRN2 on chromosome 4 was the only one with a negative allelic effect. The initiation and/or elongation of more than three lateral roots on average was dependent on the allele present at this locus; the LRN3 allelic effect was almost as strong but in the opposite direction.

The analysis of the density of branching along the primary root revealed four QTLs: LRD1 and LRD3 had moderate phenotypic contributions and negative allelic effects (the Shahdara alleles increased the value of the trait), while LRD2 and LRD4 were two of the main-effect QTLs found in this study and showed positive allelic effects. Maximum individual allelic effects were 0.04 root mm^{-1}, which is one additional lateral root every 2–3 cm of primary root. The small-effect QTLs (LRD1 and LRD3) together were involved in an epistatic interaction explaining 4% of the total variance.

The three QTLs detected for total length of the lateral roots acted in the same direction, with the Shahdara alleles always contributing positively to the length of the system. This explains why only very few lines significantly exceeded the Shahdara phenotypic value. As much as 54 mm of LRL variation can be explained by loci LRL1 and LRL2.

Confirmation of a QTL in near-isogenic lines

To build a HIF type of NIL, a RIL is chosen that is still heterozygous around the QTL and homozygous elsewhere, This RIL is then selfed and genotyped (F_7 plants) so that each homozygous genotype at the region of interest can be identified and studied in detail. HIFs are not to be compared with the reference parental genotype but with one another within the descendants (family) of the chosen RIL. In contrast with 'conventional' NILs, the genetic background is not homogeneous, but a mix of both parental genomes, since these lines originate from one RIL of the population. In a relatively large population of F_6 plants such as the Bay-0 × Shahdara population (420 RILs genotyped) used in this investigation, we observed that for each possible QTL of interest (represented by a 5- to 10-cM-long candidate region), we were usually able to find two to three candidate RILs for the construction of the HIF (for the numerous loci that we tried, the number of candidate

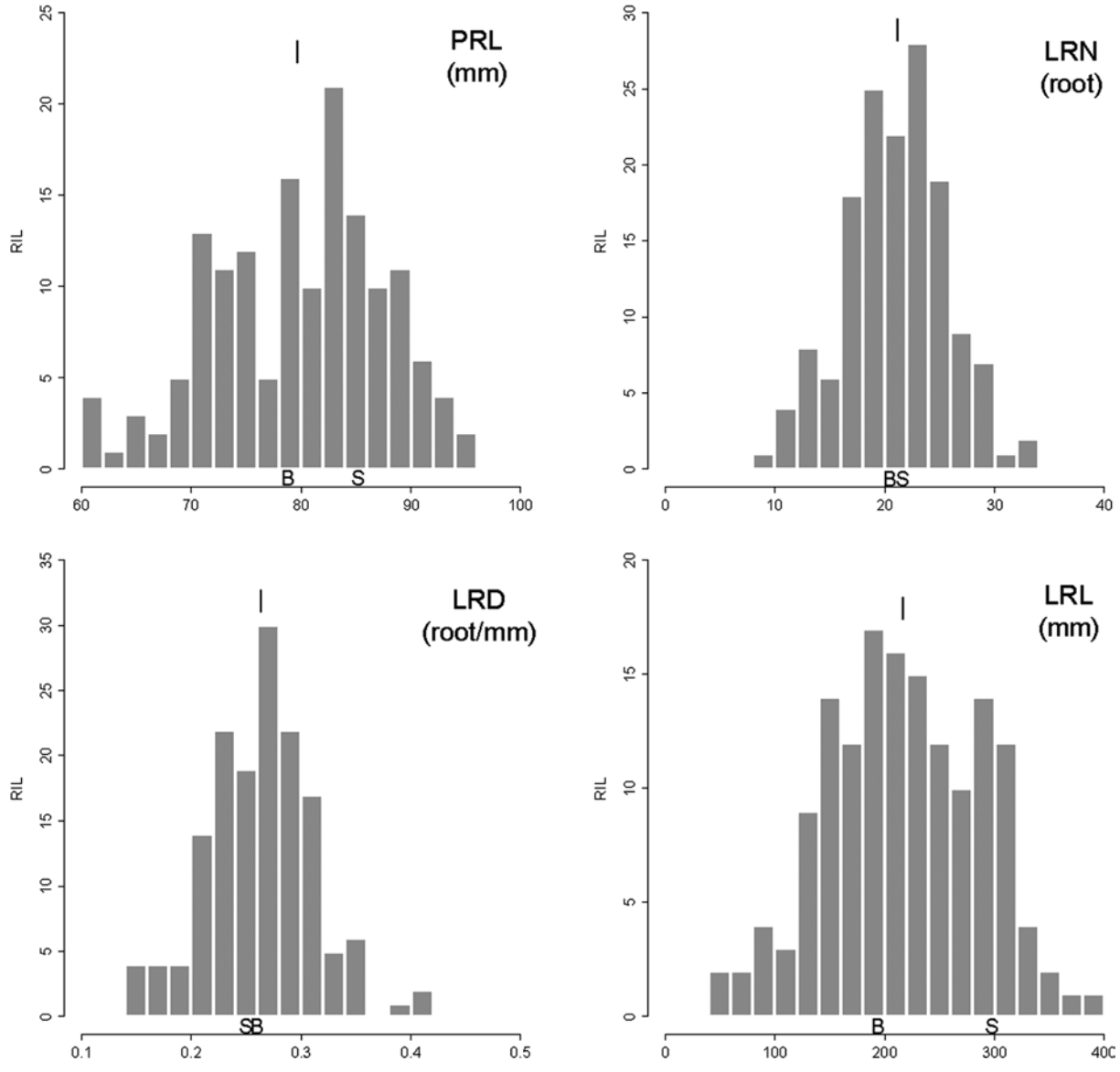

Fig. 3 Histograms of distribution of the phenotypic values for root traits in the Bay-0 × Shahdara population. For definition of the traits, refer to Table 1. *B*, *S* indicate the values obtained for parental accessions, Bay-0 and Shahdara, respectively, *vertical line above bars* indicates the population mean value

RILs varied between one and six). Under these circumstances, almost any QTL was then potentially workable into several independent HIFs that represented different genetic backgrounds, which is particularly useful, for example when the QTL is involved in epistatic interaction(s) with another locus. By making use of recombination within the population, the HIF technique allows finely mapped NILs to be constituted in only one generation (F_7/F_8 seeds already available for all RILs) with fewer than a hundred PCR reactions.

Table 2 Phenotypic correlations among traits (see Table 1 for definitions of traits). Explained variables are in rows

	LRN	LRD	LRL
PRL	+0.63**	+0.25*	+0.67**
LRN		+0.91**	+0.85**
LRD			+0.71**

*, **Significantly different from 0 at the 1% and 0.1% level, respectively

The QTL PRL3 was mapped in between marker MSAT4.18 in the north (10.9 Mb) and MSAT4.9 in the south (14.8 Mb). Two sets of NILs were developed, one showing the difference between the Shahdara and Bay-0 alleles in the region surrounding marker MSAT4.9 (HIF196) and the other showing the difference between the Shahdara and Bay-0 alleles in the region surrounding MSAT4.18 (HIF090). Figure 6 illustrates the genotypes and phenotypes of these lines, showing that PRL3 did not segregate in the interval that was heterozygous in RIL196. On the other hand, the difference between the

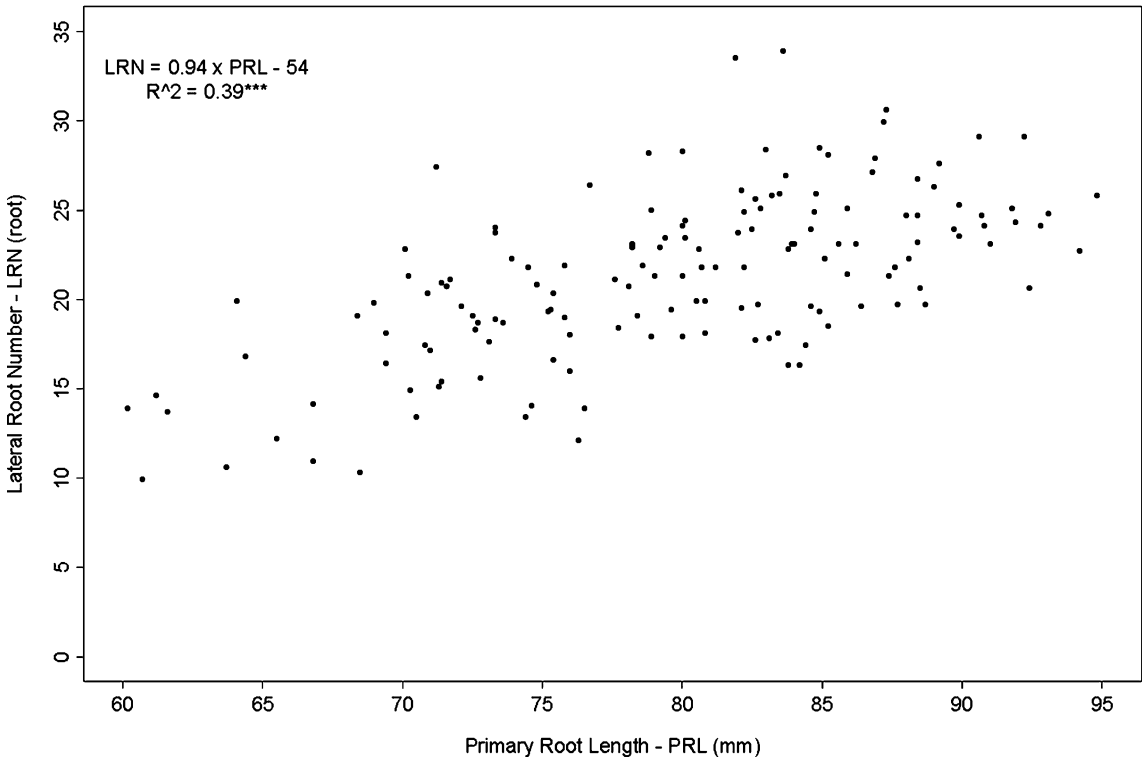

Fig. 4 Relation between the number of lateral roots (LRN) and the length of the primary root (PRL) in the Bay-0 × Shahdara population. Each one of the 150 lines studied is represented by a *dot*. The highly significant linear regression equation is indicated

Shahdara and Bay-0 alleles in the region that differentiated HIF090C/HIF090G from HIF090J/HIF090H (−6.2 mm) was very close to the estimated allelic effect of PRL3 (− 7.1 mm). Consequently, the molecular origin of PRL3 had to be in the interval segregating in HIF090. A new microsatellite marker located between MSAT4.18 and MSAT4.9 showed that HIF090 was not segregating at and south of physical position 12.7 Mb.

Table 3 Results of QTL analyses for root architecture traits in the Bay-0 × Shahdara population

QTL[a]	Chromosome—marker[b]	Position[c]	LOD Score	R^{2d}	2a[e]
PRL1	Chrom 2—MSAT2.38	16.7	2.4	5	−4.0
PRL2	Chrom 3—MSAT3.18	61.5	2.5	7	+4.5
PRL3	Chrom 4—MSAT4.18	52.1	6.4	14	−7.1
PRL complete model				26%	
LRN1	Chrom 1—MSAT1.13	73.4	2.7	6	+2.6
LRN2	Chrom 4—MSAT4.18	49.8	3.7	9	−3.3
LRN3	Chrom 5—NGA139	25.3	3.2	8	+3.0
LRN complete model				23%	
LRD1	Chrom 1—MSAT1.10	13.0	2.7	3	−0.026
LRD2	Chrom 1—MSAT1.13	70.4	7.0	16	+0.040
LRD3	Chrom 4—NGA8	16.4	2.4	4	−0.022
LRD4	Chrom 5—NGA139	28.4	5.5	13	+0.040
LRD1 × LRD3				4	
LRD complete model				40%	
LRL1	Chrom 2—MSAT2.36	28.2	3.3	8	−54
LRL2	Chrom 3—ATHCHIB2	3.1	4.7	9	−54
LRL3	Chrom 4—MSAT4.18	40.8	2.5	5	−44
LRL complete model				22%	

[a]The name given to a local LOD score peak contains the trait name suffixed with an order number

[b]The corresponding marker is the one used in CIM model 6 as well as in ANOVA analysis

[c]The position of the QTL is expressed in centiMorgans from the first marker of the chromosome

[d]Percentage of variance explained by the QTL or by QTL × QTL interaction, when significant

[e]2a represents the mean effect (in trait units; see Table 1) of the replacement of both Shahdara alleles by Bay-0 alleles at the QTL

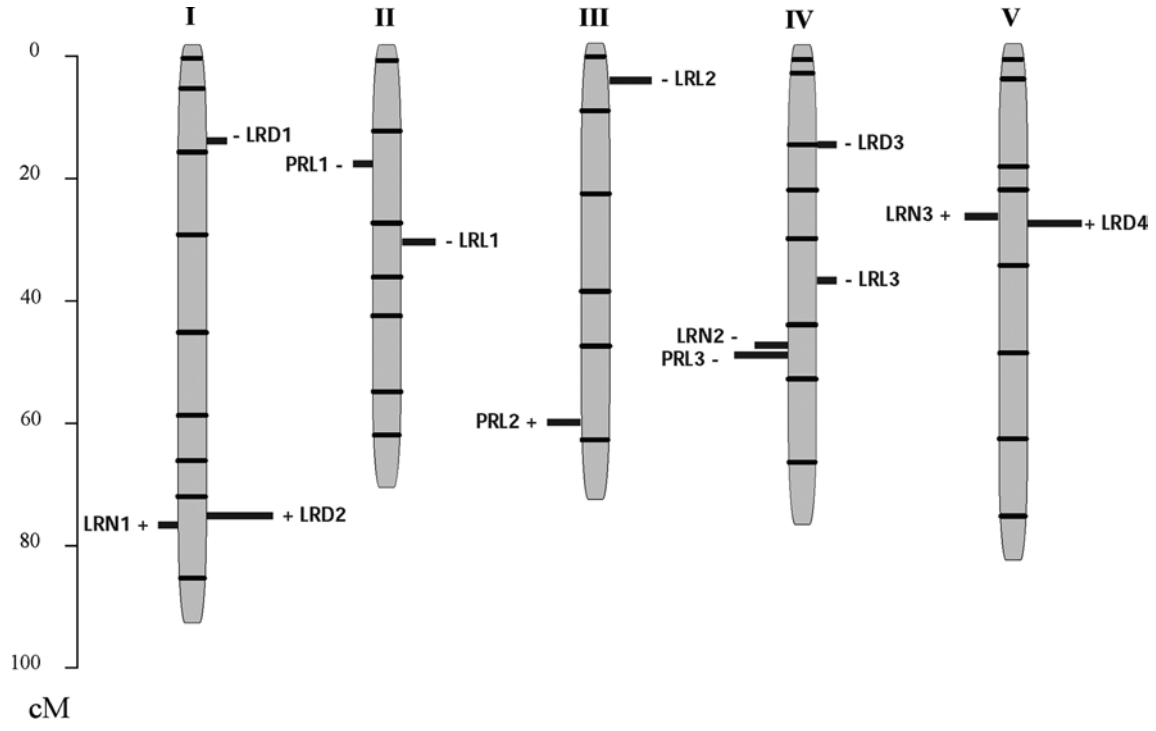

Fig. 5 QTLs detected for root traits in the Bay-0 × Shahdara population. Each QTL is represented by a *bar* located at its most probable position. The *length of the bar* is proportional to the QTL contribution R^2). The sign of the allelic effect (2a) is indicated for each QTL. The framework genetic map (indicating positions of the markers) is from Loudet et al. 2002

Together with the calculated interval from QTL mapping, PRL3 was then predicted to be somewhere between 10.9 Mb and 12.7 Mb.

Discussion

In the past the study of root architecture on a large number of genotypes was mostly carried out by unearthing root systems from plants grown in the field or pots—a radically destructive means. Other systems, such as 'rhizotrons', enable direct observations of the roots to be made but not necessarily on a scale involving hundreds of plants in which the results are reproducible. The small physical size of the Arabidopsis root system allows thousands of root systems to be observed under controlled conditions in vitro on vertical agar plates for at least the first 2 weeks of their development. By comparing the architecture of contrasting genotypes in sand culture and on vertical plates, we were able to check the relevancy of the observations made in vitro (O. Loudet, unpublished results). In a previous investigation, Fitter et al. (2002) also observed similar effects and reactions when growing Arabidopsis plants (wild-type and mutants) on agar plates and in thin layers of soil placed between two glass plates.

We chose to examine root systems as they grew in medium, essentially to avoid the wave-like growth of the primary and lateral roots when these are grown on the surface of a medium. As such, it was important to limit the thickness of the medium to ensure that the root did not experience any anoxia. We combined our specific light and temperature conditions with a low C:N ratio in the medium in order to create conditions allowing our genetic material to express maximum phenotypic diversity (especially in terms of lateral root development). In actual fact, the variation in these traits in the Bay-0 × Shahdara population measured in a single environment was very large (Figs. 2, 3) and equivalent to what is usually observed with mutants or in different environments.

When we compared the heritability of the traits (approx. 60%) with the total percentage of variance explained by our QTL models, we found that either we under-estimated the individual R^2 or that a large number of very small-effect QTLs remained undetected. These loci could account for the part of the transgression not explained by our models (LRN). The smaller number of RILs studied could be responsible for the difference in power with previous studies on the same population (Loudet et al. 2003a, 2003b). Another possible explanation is that we over-estimated the heritabilities due to a degree of confounding environmental variation with the genotype effect as a result of our display; however, this did not affect QTL mapping as the RILs themselves constituted a replication of the different genotypic combinations across the growth room. Hence, some QTLs displayed quantitatively interesting allelic effects, such as LRL2 on total lateral root length or PRL3 on primary root length, lateral root density

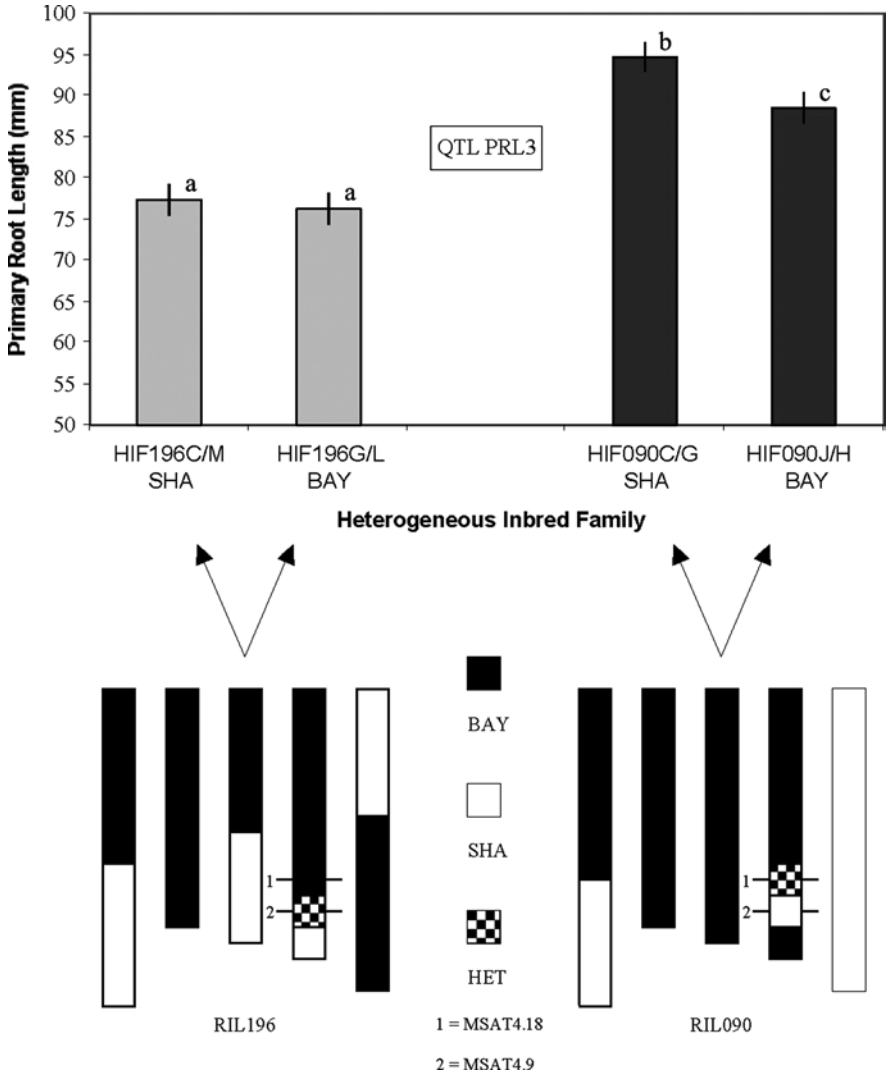

Fig. 6 Confirmation of QTL PRL3 for primary root length through the NIL. Heterogeneous inbred family HIF196 segregates for a region around marker MSAT4.9 (and is homozygous elsewhere), while HIF090 segregates for a region around MSAT4.18 (and is homozygous elsewhere). Each value is the average of at least 70 different PRLs from two independent plants with the same genotype. *Bars denoted with the same letters* are not significantly different at the 5% level. *Vertical bars* represent the standard deviation for each genotype

seemed to be more precisely dissected than the other traits (especially those from which it was calculated), which is probably an indication of the biological relevance of this derived variable.

Our analysis of the length of the primary root after 10 days of growth (PRL) identified QTLs that seem to be primarily specific to this trait (Fig. 5). However, the most important of these, PRL3, co-localizes with a QTL explaining 9% of the variation for the number of lateral roots (LRN2). If these QTLs underlie the same gene, then it is very likely that the change in the number of laterals we observed is a consequence of the change in the length of the primary root (more initiated and elongated laterals, in total, but the density remains the same). This relationship is expected if, for example, lateral root spacing is fixed. However, other lateral root number QTLs showed that this is not necessarily the case: in fact, LRN1 and LRN3 identified loci whose effect is to change the degree of branching with no detectable effect on PRL. This means a change in the density of branching, as confirmed by the associated LRD loci, LRD2 and LRD4, very likely corresponds to the same gene(s) as LRN1 and LRN3, respectively. It is interesting to note that the effect (2a, R^2) of these loci appeared to be stronger on LRD despite a heritability similar to LRN, which indicates that taking into account the length of the primary root significantly changes our understanding of branching variation. However, LRD depended mainly on lateral root number: the loci affecting only the length of the primary root (PRL2, for example) do not co-localize with LRD QTL. In fact, smaller-effect LRD loci appeared to be specific to this trait as they were not shared by LRN (although this could be explained by a lack of power in the analysis of the other traits). Our analysis of lateral roots could not distinguish between more initiated and more elongated roots because we measured and counted all elongated laterals (≥1 mm) together.

Total LRL is the first trait studied in this population for which we can only map QTLs acting in a single direction (Shahdara alleles always contribute to the extension of the root system relative to the Bay-0 alleles). LRL1 and LRL2 showed that (in our material and conditions) the total length of the lateral root system

varied mainly through the specific elongation of existing laterals and less through the number of laterals initiated. Also, the mechanisms explaining the variation in lateral root growth seemed to be different from those explaining the variation in primary root growth in this material. However, due to the uncertainty associated with QTL mapping, we cannot totally exclude that LRL3 and PRL3/LRN2 correspond to the same locus. Whatever the story behind these loci (either linkage or pleiotropy), this is the only genetic evidence that we detected which explains the strong correlation between LRL and LRN. A more detailed analysis of root anatomy would allow the estimation of the respective contribution of cell division and cell elongation into these process. Using a wide variation in Arabidopsis accessions, Beemster et al. (2002) showed that these processes contributed equally to the variation in primary root growth.

Among the other QTLs mapped using this population, those for shoot water content and anion contents are particularly interesting (Loudet et al. 2003a). LRL1 and LRL2 each co-localized with a QTL for water content (HU10.4 and HU10.5, respectively) with the same allelic effect sign. Nitrate content variation in the same direction was also associated with each of these loci (NO10.5 and NO10.6, respectively, mapped in non-limiting nitrogen conditions). Although these results were obtained on soil-grown plants and at a different stage, they are interesting in the context of the regulation of root development by environmental signals (like nitrate), which involves both local and long-range signaling pathways (Zhang and Forde 2000; Forde 2002). In our particular case, it is difficult to identify the causal relationship without cloning the gene(s) responsible for the QTL. Root development could modify nitrate and water uptake; otherwise, differing root lengths could be a consequence of a difference in water use efficiency or nitrogen metabolism. Recently, Malamy and colleagues found that a mutant (*lin1*; Malamy and Ryan 2001) showing a highly branched root system under conditions that usually repress the development of lateral roots (high C:N ratio in the medium) was affected at *NRT2.1*, which codes for a high-affinity nitrate transporter (Filleur et al. 2001; Rao et al. 2003).

Hypocotyl and root growth are known to be affected by shared/linked processes, such as the auxin pathway (Bhalerao et al. 2002), and genes have been cloned that elucidate a molecular link between shoot and root development regulation (Nakazawa et al. 2001). However, in our material, it seems that the polymorphic (variable) steps are not part of this inter-connection since the hypocotyl length QTL (O. Loudet, unpublished results) did not co-localize with the root QTL published here.

It is particularly interesting to compare the localization of QTLs for different traits in the same material. If a co-localization of two QTLs has to be interpreted with care because it could be either pleiotropy or linkage, the absence of co-localization (at least for medium- and large-effect QTLs) is very informative. In accordance with other authors (El-Assal et al. 2004; Koornneef et al. 2004) we believe that, with the availability of more and more QTL maps and NILs to confirm them, some specific populations will become extremely powerful tools in the drawing of a complete picture of the phenotypically expressed polymorphisms between some accessions. At this stage of our knowledge, it seems that the most studied populations in Arabidopsis are L *er*/Cvi, L *er*/Col and Bay-0 × Shahdara (see the Natural website at http://www.natural-eu.org and the QTLAT website at http://www.inra.fr/qtlat).

As a first step in the path leading to the cloning of the gene(s) responsible for complex trait variation, fine-mapping usually requires the construction of NILs that differ only at a small region around the QTL of interest (Glazier et al. 2002). NILs are usually created by introgressing one parent allele at the QTL region in the other parent background, and this process requires several backcrosses. In the investigation reported here we used another type of NIL, in which we took advantage of the residual heterozygosity left at the F_6 generation (approx. 3%; Loudet et al. 2002) to find lines that still segregated only at the region around the QTL of interest. These types of NILs were proposed by Tuinstra et al. (1997) and called HIFs.

Using HIFs, we were able to confirm medium-effect QTL PRL3, our most important QTL for PRL (chromosome 4), and also confirm that the variation observed in the unique phenotyping experiment on the RILs is relevant and repeatable. Two independent HIFs were used (illustrated on Fig. 6) to test for the presence of the QTL in the northern or southern half of the confidence interval of PRL3. The QTL was shown to segregate only in HIF090. A new marker was then added to delimit this region to a 1,750-kb candidate region for PRL3. The strategy to be adopted for further fine-mapping is to accumulate recombinations in the interval by screening descendants of heterozygous plants and to use these, once fixed, as new HIF with a smaller candidate region.

LRD2 (/LRN1) and LRD4 (/LRN3) are also obvious candidate loci to be investigated in this way. Despite these tools, it might be challenging to fine-map the QTLs explaining LRL itself, since their allelic effects seem to be slight compared to the environmental variance. The difficulty in estimating the phenotype will at least make it labor-intensive, requiring probably more than 100 analyzed root systems per genotype. However the locus position of LRL2 will be determined by testing the HIF made to fine-map different QTLs co-localizing at a locus called 'L3' (Loudet et al. 2003b).

Another manner to analyze these loci is to use the candidate gene approach (from both positional and functional information). Among the genes known to control root architecture, *ABI1* (coding a protein phosphatase involved in abscisic acid sensitivity; Vartanian 1996) and *SUR2* (coding a cytochrome P450 involved in auxin homeostasis; Delarue et al. 1998) are in the original LRN2/PRL3 confidence interval. However, *SUR2* can now be excluded from the PRL3 candidate interval

as confirmed by HIF analysis. *IAA28* codes for a putative transcription factor and maps very close to LRD4/LRN3. A gain-of-function mutation was found in this gene that induces a drastically reduced number of lateral roots through resistance to auxin (Rogg et al. 2001). Consistent with our finding no PRL (this paper) or hypocotyl-length QTL (O. Loudet, unpublished results) at that locus, the *iaa28* mutant has no other marked seedling phenotype than root branching.

Our investigation of the material described in this report has revealed the differing genetic control of root growth and architecture in the Bay-0 and Shahdara accessions despite a strong convergence towards similar phenotypes for traits such as lateral root number or density. On the other hand, the growth of these lateral roots in Shahdara seems to be highly constrained (if not selected) toward high values, as shown by the LRL QTL with allelic effects only in one direction (and few positively transgressing lines above the level of the Shahdara phenotype in the population). Shahdara originally comes from a mountainous environment that is particularly dry in late spring and summer (Khurmatov 1982; see climatic data gathered per accession on the VNAT website: http://dbsgap.versailles.inra.fr/vnat). It is then easy to imagine an adaptive value of this pattern of variation for drought tolerance. However, this remains to be confirmed in terms of whole-plant growth and, ultimately, fitness, as Fitter et al. (2002) did recently for nutrient uptake. Continuous variation for root development (the RIL population) as well as discrete variation in different backgrounds (the HIF lines) is available to test these hypothesis. The next step is to use this intriguing genetic material to dissect developmental and physiological features in different environments, which will help distinguish the intrinsic variations (developmental) from the reaction to a specific factor of the environment. Root growth and development is particularly interesting when studied in interaction with nitrogen availability but also under drought or osmotic stress, soil acidity, salt stress and phosphate availability.

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