Quantitative Trait Loci Analysis of Water and Anion Contents in Interaction With Nitrogen Availability in *Arabidopsis thaliana*

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ABSTRACT

In plants, water and anion parameters are linked, for example through the integration of nutritional signaling and the response to diverse stress. In this work, *Arabidopsis thaliana* is used as a model system to dissect the genetic variation of these parameters by quantitative trait loci (QTL) mapping in the 415 recombinant inbred lines of the Bay-0 × Shahdara population. Water, nitrate, chloride, and phosphate contents were measured at the vegetative stage in the shoots of plants grown in controlled conditions. Two contrasting nitrogen (N) conditions were studied, one leading to the complete depletion of the nitrate pool in the plants. Most of the observed genetic variation was identified as QTL, with medium but also large phenotypic contributions. QTL colocalization provides a genetic basis for the correlation between water and nitrate contents in nonlimiting N conditions and water and chloride contents in limiting N conditions. The 34 new QTL described here represent at least 19 loci polymorphic between Bay-0 and Shahdara; some may correspond to known genes from water/anion transport systems, while others clearly identify new genes controlling or interacting with water/anion absorption and accumulation. Interestingly, flowering-time genes probably play a role in the regulation of water content in our conditions.

NITROGEN (N) is certainly the most important nutrient that plants have to capture from the soil solution, mainly in the form of nitrate. Nitrate itself, apart from this role as a nutritional compound, is also known as a signaling molecule involved in the integration of N metabolism at the whole-plant level (Stitt 1999) and as an osmoticum involved in the regulation of plant turgor (McIntyre 1997). Therefore, nitrate limitation in the plant environment has direct consequences on growth and indirect consequences, for example, through modifications of nutritional status (as indicated by nitrate availability in plant cells and tissues) or osmotic pressure (Fricke and Flowers 1998). Developmental processes such as root architecture are known to be strongly constrained by nitrate availability, for example, through the initiation and elongation of lateral root primordia (Zhang *et al.* 1999). Moreover, other osmotically active compounds are involved in the regulation of plant response to the fluctuations of nitrate availability; they replace nitrate as an osmoticum, especially when N is limiting. In these conditions, chloride has been shown to play a major role in maintaining osmotic pressure (White and Broadley 2001). To a lesser extent, phosphate is known to accumulate in N-starved plants (Lamaze *et al.* 1984). A common feature of these compounds is their accumulation in the vacuole (Leigh 1997) and their link with water tissue in which they are dissolved (Cardenas-Navarro *et al.* 1999; Karley *et al.* 2000). Generally, solutes and water contents are correlated; homeostasis for endogenous nitrate (described as the apparent stability of the nitrate concentration in tissues) has been reported in a large range of external N conditions (Cardenas-Navarro *et al.* 1999).

The precise description of ion accumulation process and control still has to be achieved, particularly in the context of environmental constraints such as N or water stress. The genetic regulations involved in the control of solutes and water contents are still poorly known because of their quantitative nature and strong interactions with environment. We need to assign precise regulatory function to known genes involved in these pathways and to find new genes responsible for physiological variation. This could be particularly informative for biologists and geneticists who try to adapt plants to, for example, limited supplies of micro- and macronutrients or potentially toxic loads of ions.

Quantitative trait loci (QTL) mapping is a means to identify the individual genetic factors influencing the value of a quantitative trait. This approach is then particularly interesting when multiple sources of variation prevent us from understanding the individual mechanisms leading to a phenotype. QTL studies in the model plant species, like *Arabidopsis thaliana*, began only recently (Alonso-Blanco and Koornneef 2000) and are particularly amenable to map-based cloning of QTL (Remington *et al.* 2001). The completion of Arabidopsis sequence (Arabidopsis Genome Initiative 2000) provides the ultimate physical map, a decisive advantage for

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map-based cloning (Lukowitz *et al.* 2000; Yano 2001). Many Arabidopsis QTL mapping studies concern flowering time, but recently complex physiological and developmental processes have also been dissected (Mitchell-Olds and Pedersen 1998; Bentsink *et al.* 2000; Kliebenstein *et al.* 2001; Borevitz *et al.* 2002).

Most of these studies have been performed using two recombinant inbred line (RIL) populations, namely Ler/Col and Ler/Cvi populations. We recently described a new RIL population that is derived from the cross between Bay-0 and Shahdara ecotypes (Loudet *et al.* 2002; the Bay-0 × Shahdara tool is described on the web site http://www.inra.fr/qtlat). This cross between a Central-Asian accession and a European accession should maximize interesting variation reflecting the genetic distance between them (Loridon *et al.* 1998; Breyne *et al.* 1999; Sharbel *et al.* 2000) and ecological contrast between their habitats (Khurmatov 1982). Moreover, this population has high power for QTL mapping, due to the large population size (Loudet *et al.* 2002).

In this article, we describe the genetic analysis of water and anion content variation in the Bay-0 × Shahdara population in response to nitrate availability. The traits were measured at a vegetative stage in two contrasting N conditions, one leading to the complete depletion of the nitrate pool in plants. We identify and discuss several loci explaining the variation of water, nitrate, chloride, and phosphate contents. This represents, to our knowledge, the first QTL analysis of these traits and their dependence on the N status of the plant.

MATERIALS AND METHODS

Plant material: The material used in this study has been developed in our laboratory and deposited in public Arabidopsis stock centers; the Bay-0 × Shahdara RIL population has been fully described in a recent publication (Loudet *et al.* 2002) and on http://www.inra.fr/qtlat. F_7 seeds obtained from the last generation of single seed descent for 415 lines were used. These seeds were harvested from plants grown at the same time for all lines, thus minimizing the maternal environment effect.

Phenotyping display: The production of homogeneous vegetative plant material for the 415 lines was performed in controlled conditions (growth chamber). Two N environments were compared at the same time and in the same growth chamber in each experiment (cultivation repetition) using the whole set of RIL. The experimental unit was a small pot (L = 60 mm, l = 65 mm, h = 60 mm) containing six plants positioned on a circle. With only one repetition per RIL (one pot, *i.e.*, six plants) and 17 connecting controls (Bay-0 and Shahdara repetitions), the whole population studied in one N environment represented 432 experimental units, organized in 18 blocks of 24 pots. The RIL were completely and independently randomized in each cultivation repetition (performed successively in the same growth chamber). The blocks were rotated every other day, following a scheme that allowed each block to move all around the growth chamber. Two N environments were compared in this study: the first one (N+) did not limit plant growth at any stage during our experiment and the second one (N−) strongly limited growth (for details, see the watering solutions described below). The data from three cultivation repetitions of N+ environment and two repetitions of N− environment have been collected and analyzed in detail.

Growth conditions: Pots were carefully filled with a homogeneous nonenriched compost composed of blond and brown peats (1/1) sifted at 2–3 mm (Basis substrat II, Stender GmbH, Schermbeck, Germany). The pH of this compost was stabilized between 5.5 and 5.9 and it contained only very small amounts of nitrate (<0.5 mm in the soil solution). Every other day the pots were watered (by immersion of the base of the pots) in a solution containing either 10 mm (N+) or 3 mm (N−) nitrate. Phosphate and sulfate were present in both solutions at the same concentration (0.25 mm), as well as magnesium (0.25 mm) and sodium ions (0.20 mm). The difference between N+ and N− solutions concerned only potassium (5.25 mm and 2.75 mm, respectively, in N+ and N− solutions), calcium (2.50 mm and 0.50 mm, respectively), and chloride ions (0.20 mm and 0.70 mm, respectively), but all these concentrations were supraoptimal for plant growth. N+ and N− solutions contained desirable amounts of Fe and other micronutrients. The pH of the watering solutions remained between 5.1 and 5.5.

The seeds were stratified for 48 hr in 0.1% agar solution (in water) at 4° in the dark. Then six positions on a circle were determined in each pot and received approximately seven seeds per position, from the same RIL (with a pipetor, the distribution of a small volume of the stratification solution ensured the sowing of a steady number of seeds at each position). Homogeneous germination occurred 2 days after sowing. Six days after sowing, only one seedling per position was retained while the others were removed, resulting in six homogeneous seedlings per pot. The plants were maintained in short days with a photoperiod of 8 hr during all the culture. The day and night temperatures were regulated at 21° and 17°, respectively. The hygrometry fluctuated between 65% during the day and 90% during the night. Light was provided by 20 mercury-vapor bulbs, ensuring a photosynthetic photon flux density of ~160 μmol/m^2/sec. The last watering occurred 33 days after sowing and plants (shoot) were harvested 35 days after sowing.

Measured traits: The six plants harvested for each RIL were pooled for one cultivation repetition and one N environment. Shoot fresh weight was measured before the plants were freeze-dried for 72 hr. Shoot dry weight was then measured and the water content (humidity, HU) was estimated as % (fresh weight − dry weight)/fresh weight. The dry material was finely ground in a vibrator using steel beads. An aliquot of the powder (between 8 and 10 mg) was weighed and extracted with a two-step ethanol-water procedure conducted in a 96-deep well plate. The first step consisted of a 25-min extraction at 80° using 500 μl of 80% ethanol, while the second step completed the extraction by using 500 μl of water at 80° for 20 min. Extracts from both steps were pooled and diluted before analyzing anion concentration by HPLC on a DX-120 (Dionex, Jouy en Josas, France). Nitrate content (NO), chloride content (CL), and phosphate content (PO) were expressed in nanomoles per milligram of dry matter. Table 1 summarizes the traits measured.

Statistical analysis and QTL mapping: The complete set of data from each environment was included in an analysis of variance model (ANOVA) to determine the specific effects of "genotype" (*i.e.*, the RIL) and "repetition" (*i.e.*, the cultivation repetition) factors. This ANOVA allowed the quantification of the broad-sense heritability (genetic variance/total phenotypic variance). The genotype × repetition interaction could be tested only by using grouped N+ and N− data (corresponding to common cultivation repetitions) in the

same analysis. Using the same set of data, we performed a two-factor ANOVA to determine the significance of the N environment effect and the genotype × N interaction. Subsequent analyses involved unadjusted mean values from the different repetitions in each N environment. Phenotypic correlations were calculated for all combinations of traits in each N environment and across N environments for each trait. ANOVA and correlation estimations were performed using *aov*() and *lm*() functions of S-PLUS 3.4 statistical package (Statistical Sciences).

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). First, IM (Lander and Botstein 1989) was used to determine putative QTL involved in the variation of the trait. CIM Model 6 of QTL Cartographer 1.14 (Basten *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 a position of the genome. When a cofactor was also a flanking marker of the tested region, it was excluded from the model. The number of cofactors involved in our models did not exceed seven and the window size used was 3 cM. The walking speed chosen for all QTL analysis was 0.1 cM. The LOD significance threshold (2.3 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 "experimentwise threshold" obtained (overall error level, 5%) was used for all traits.

Additive effects ($2a$ in Table 3) of detected QTL were estimated from CIM results; $2a$ represents the mean effect of the replacement of both Shahdara alleles by Bay-0 alleles at the studied locus. 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). When QTL for the same trait appeared to be shared in both N environments, QTL × N environment interaction was assessed by a two-factor ANOVA, with the corresponding marker genotype and N environment as classifying factors. We used two types of support interval estimations, anticonservative one-LOD support interval based on 50 real QTL LOD score profile analyses and conservative bootstrap simulated confidence interval with 10 series of 1000 resampling data sets as proposed by Visscher *et al.* (1996). Estimations were calculated for different R^2 classes.

RESULTS

The names of the traits obtained in N+ environment (10 mm nitrate) are suffixed with 10 (for example, HU10), while the names of the traits obtained in N− environment (3 mm nitrate) are suffixed with 3 (for example, HU3). Nitrate content in plants cultivated in N− environment, as well as phosphate content in plants cultivated in N+ environment, were extremely low (very close to zero) and could not be correctly estimated by our analysis. Therefore, these traits were not studied here.

Decomposition of variance and heritability: Table 1 indicates that the genotype effect is highly significant for all traits [$P(f) < 0.001$]. The magnitude of this effect is particularly strong on chloride and phosphate contents in N− environment (CL3 and PO3). Cultivation repetition also significantly influences all traits [$P(f) < 0.01$; data not shown]: some uncontrolled environmental factor(s) is responsible for a variation between repetitions, although these were performed in the same growth chamber. We detect a significant genotype × repetition interaction only for water content [$P(f) < 0.001$; using pooled N+, N− data], whereas chloride content genotype × repetition interaction is not significant [CL $P(f) > 0.22$; data not shown]. We chose to perform all subsequent QTL analyses on unadjusted mean values across the different repetitions, which should represent a good estimation of the average behavior of a genotype in a specific N environment. Heritabilities of the different traits are presented in Table 1, indicating for most of the traits that about half of the total phenotypic variation is explained by the genetic differences between lines. Remarkably, chloride content in N− environment (CL3) is much more heritable than the other traits ($h^2 = 0.70$). The effect of the N environment is highly significant for all traits, as well as the genotype × N environment interaction [$P(f) < 0.001$ for HU and CL; data not shown], leading globally to a decrease in plant water content and an increase in chloride content concomitant with the limitation of plant growth due to restricted N availability. Nitrate content was depleted to zero in almost all lines in N− environment, as well as phosphate content in N+ environment. We have verified that the difference in chloride concentration in N+ and N− watering solutions was not responsible for the difference between CL10 and CL3, which is then imputed to the lower nitrate concentration in N− watering solution (data not shown).

Phenotypic variation and correlations among traits: The variability of each trait is illustrated in Figure 1. Phenotypic values in the whole population show a relatively normal distribution, around a population mean that lies between the parental values (except for CL3, for which the population mean is superior to both parents). Strong transgressive variation is the general rule, especially for CL3, CL10, and HU3 for which most of the lines have phenotypic values exceeding the parental values (in one or the other direction). Water content and nitrate content in N+ environment (HU10 and NO10) show an unbalanced transgression, with only a few lines exceeding Shahdara value when many lines have phenotypes below Bay-0 value. CL10 distribution is also deviated

TABLE 1

Traits, genotype effect, and heritability

Name	Trait	Unit[a]	RIL mean	RIL range (min-max)	Genotype effect[b]	Heritability
HU10	Water content (N+)	% FM	92.5	90.4–93.5	0.44***	0.55
HU3	Water content (N−)	% FM	86.2	83.0–89.6	2.37***	0.40
NO10	Nitrate content (N+)	nmol/mg DM	2015	1326–2712	137,695***	0.40
CL10	Chloride content (N+)	nmol/mg DM	37.8	29.0–50.4	49.4***	0.45
CL3	Chloride content (N−)	nmol/mg DM	396.9	250.6–587.0	8,448***	0.70
PO3	Phosphate content (N−)	nmol/mg DM	15.2	3.1–33.4	49.2***	0.50

[a] FM, fresh matter; DM, dry matter.
[b] Variance associated with the genotype effect and significance. ***Significant at the 0.1% level.

toward low values with significantly more RIL below Shahdara phenotypic value than above Bay-0 value. Bay-0 and Shahdara phenotypes for chloride content in limiting N conditions (CL3) are almost identical (374.0 and 363.5 nmol of chloride per milligram of dry matter, respectively), whereas line phenotypes vary from 250 to 590 nmol/mg. Phenotypic correlations among the traits and across N environments are presented in Table 2. The two traits measured under both N conditions (water and chloride contents) are positively correlated across N environments (+0.53 and +0.34, respectively), despite the strong differences between CL10 and CL3 phenotypic values (on the average, CL3 = 10 × CL10). Water content is highly correlated with nitrate content in N+ environment (HU10 and NO10, +0.60) and with chloride content in N− environment (HU3 and CL3, +0.50). Phosphate content (PO3) is not significantly correlated with any other traits measured in N− environment. The correlation between HU10 and HU3 is illustrated in Figure 2. This representation also shows the strong interaction with N environment: for example, some lines, despite high HU10 phenotypes, strongly reduce their water content in reaction to N stress almost down to the lowest HU3 values found in the population. As illustrated in Figure 3, the correlations between HU10 and NO10 and between HU3 and CL3 are not exclusive and a large part of plant water content variation seems to be controlled by factors not linked to anion contents (nitrate, chloride): for example, average-HU10 lines (92.5% of water) contain from 1600 to 2500 nmol of nitrate per milligram of dry matter; similarly, average-HU3 lines (86% of water) contain from 250 to 500 nmol of chloride per milligram of dry matter.

QTL mapping: QTL mapping results are presented in Table 3, where the name of the QTL contains the trait name suffixed with an ordering number from the first chromosome. Excluding NO10 QTL (from Loudet *et al.* 2003), we identified a total of 34 new QTL for the whole set of traits. Each trait is significantly controlled by 6–8 QTL. We found epistatic interactions between detected QTL for chloride content in N+ and N− environments (2 and 1 QTL × QTL interactions, respectively). Individual phenotypic contributions of QTL (R^2) range from 2 to 21%, with 15 QTL explaining <5% of the phenotypic variation and 6 QTL explaining 10% or more of the phenotypic variation. For the different R^2 values, one-LOD support interval size is estimated to be approximately half the size of the intervals obtained using bootstrap simulation (data not shown). This difference is even more pronounced for small-effect QTL ($R^2 < 5\%$). When analyzing QTL colocalizations, only one-LOD support interval overlaps are used to avoid false-positive colocalizations as much as possible.

Water content in nonlimiting N conditions (HU10) revealed eight significant QTL distributed on all chromosomes, most of them being medium-effect QTL (R^2 from 6 to 10%). Individual QTL are responsible for a gain or loss of at the most 0.32 point in water content (estimated allelic effect 2*a* in Table 3). Only one QTL for HU10 (HU10.6) has a positive 2*a* allelic effect (for the others, Bay-0 always carries the allele with a negative effect on HU10 with respect to the Shahdara allele). In limiting N conditions (HU3), six QTL were detected with relatively homogeneous contributions (R^2 from 4 to 7%). Individual allelic effect amplitudes are larger than those for HU10, rising at ±0.70 point in water content. Two QTL hold a Bay-0 allele with positive effect on HU3 (HU3.4 and HU3.5 on chromosomes 2 and 4, respectively). When compared between N environments, QTL mapping reveals four loci where QTL are found in both N environments: HU10.3/HU3.2 on chromosome 1, HU10.4/HU3.3 on chromosome 2, HU10.6/HU3.5 on chromosome 4, and HU10.7/HU3.6 on chromosome 5. The direction of the allelic effects is also consistent with each of these being a single locus. Among them, HU10.6/HU3.5 represent the uncommon positive allelic loci. Moreover, HU10.6/HU3.5 and HU10.7/HU3.6 do not interact with N environment (data not shown; tested by ANOVA through neighboring markers MSAT4.8 and NGA249, respectively). On the contrary, HU10.3/HU3.2 and HU10.4/HU3.3 effects are significantly modified by N availability (data not shown; tested by ANOVA through neighboring

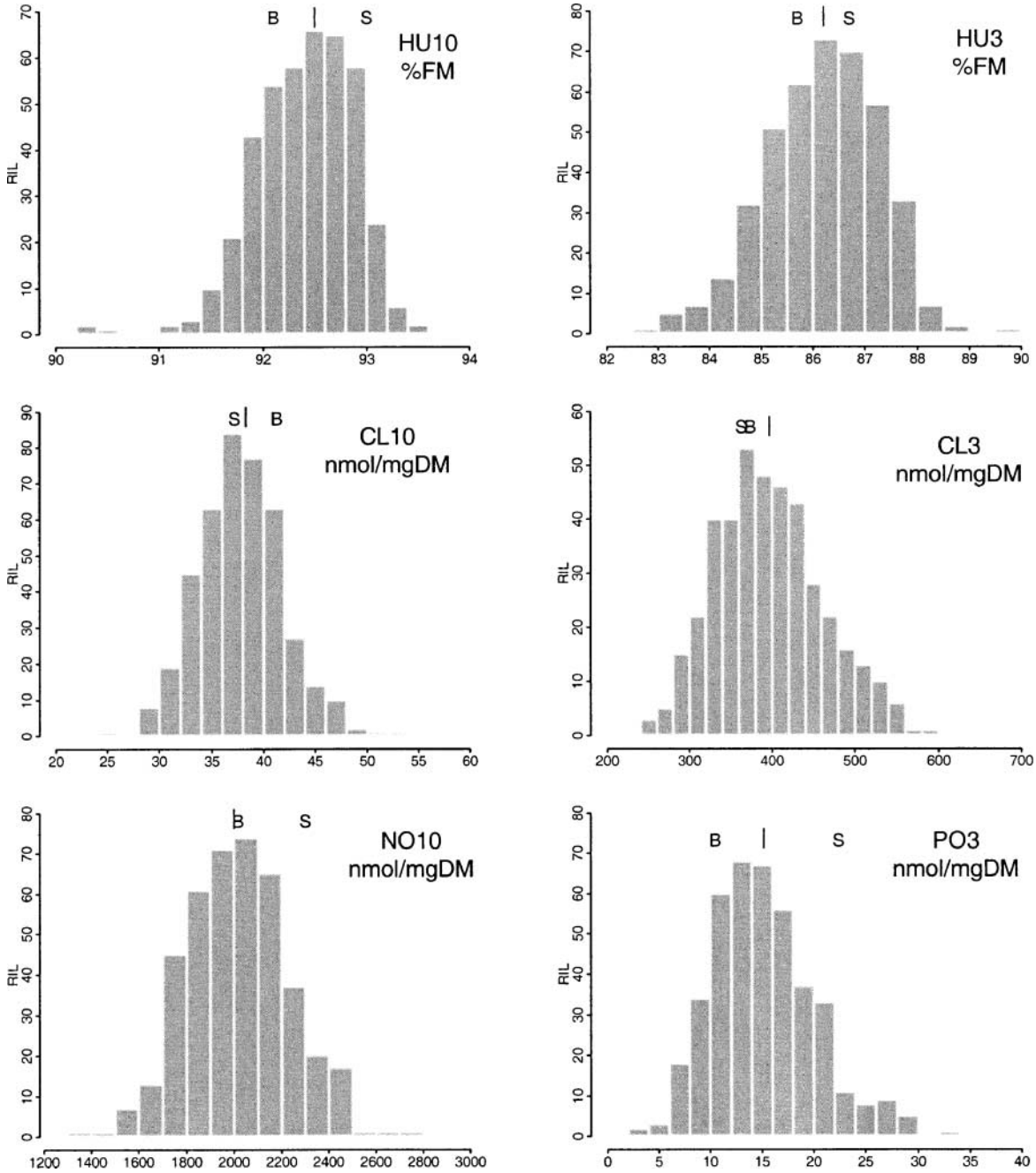

FIGURE 1.—Histograms of repartition of the phenotypic values in the Bay-0 × Shahdara population. For meaning of traits refer to Table 1. B and S positions indicate the values obtained for parental accessions, Bay-0 and Shahdara, respectively. The position of the vertical line above bars indicates the population mean value.

markers MSAT1.5 and MSAT2.41, respectively). All other QTL appear to be specific to one N environment or the other.

Chloride content genetic dissection in N+ environment (CL10) reveals eight significant QTL (R^2 from 2 to 13%) and two significant (but small-effect) epistatic interactions between QTL. An equal number of positive and negative allelic effect QTL are detected, but the R^2 distribution is unbalanced, the larger-effect QTL (CL10.7 and CL10.8) carrying a Bay-0 allele with positive effect on CL10 with respect to the Shahdara allele. Both epistatic interactions involve CL10.1, together with the main-effect QTL CL10.7 or CL10.8. In N− environment, six QTL and a QTL × QTL interaction were detected (R^2 from 2 to 21%). Main-effect QTL CL3.2 (chromosome 1) and CL3.3 (chromosome 2) have opposite sign allelic effects, individually responsible for a variation of 60 nmol chloride per milligram of dry matter (Table 3). The epistatic interaction involves CL3.2 and a minor negative-effect QTL (CL3.5). Three pairs

TABLE 2

Phenotypic correlations among traits

	N−			
N+	HU	NO	CL	PO
HU	+**0.53*****	NA	+0.50***	NS
NO	+0.60***	**NA**	NA	NA
CL	NS	+0.09*	+**0.34*****	NS
PO	NA	NA	NA	**NA**

***Significant at the 0.1% level; *significant at the 5% level. NS, not significant; NA, data not available. Values below the boldface diagonal correspond to N+ environment correlations; values above the diagonal correspond to N− environment correlations; values on the diagonal correspond to across-N environment correlations. Explained variables for N+ and N− correlations are in column and line, respectively.

of QTL could potentially represent genetic factors common to both N environments: CL10.2/CL3.2 (chromosome 1, positive allelic effect; highly significant QTL × N interaction when tested through marker MSAT1.13, data not shown), CL10.4/CL3.4 (chromosome 2, positive allelic effect; not significant QTL × N interaction when tested through marker MSAT2.22), and CL10.5/CL3.5 (chromosome 3, negative allelic effect; highly significant QTL × N interaction tested through marker MSAT3.32). All other QTL and, remarkably, CL3.3, CL10.7, and CL10.8 appear specific to one N environment.

Phosphate content study in limiting N conditions (PO3) identified six loci, four of which have negative allelic effects. QTL PO3.1 and PO3.4 each explain >10% of the total phenotypic variation (Table 3), with opposite-sign allelic effects. As much as 4.3 nmol phosphate per milligram of dry matter variation can be caused by the replacement of both Shahdara alleles by Bay-0 alleles at a single QTL, the average phosphate content in the population being 15 nmol/mg dry matter.

DISCUSSION

This work is part of an extensive analysis of the Bay-0 × Shahdara RIL population (Loudet *et al.* 2002) to perform the genetic dissection of N metabolism variability and its relations with whole-plant physiology. The experimental display that we designed allowed us to measure novel traits and their interaction with N availability, on 415 RIL grown in controlled conditions. The genetic variation described here is very large for each of the four traits in both environments. The N stress (N− in comparison with N+) resulted in a complete depletion of shoot nitrate (NO close to zero in N− environment). Together with this drastic change in N metabolism, water content (HU, %) was strongly reduced (on the average, six points less in N− than in N+), chloride accumulated in the shoot (10 times more in N− than in N+), as well as PO. Moreover, a strong genotype × N environment interaction is observed, indicating that the lines' reactions to N stress are not equivalent.

Global QTL features: A summary of the QTL found for all traits is presented in Figure 4. Taking into consideration the possible colocalizations, these QTL identify at least 19 loci polymorphic between Bay-0 and Shahdara. At least six and up to eight QTL were detected per

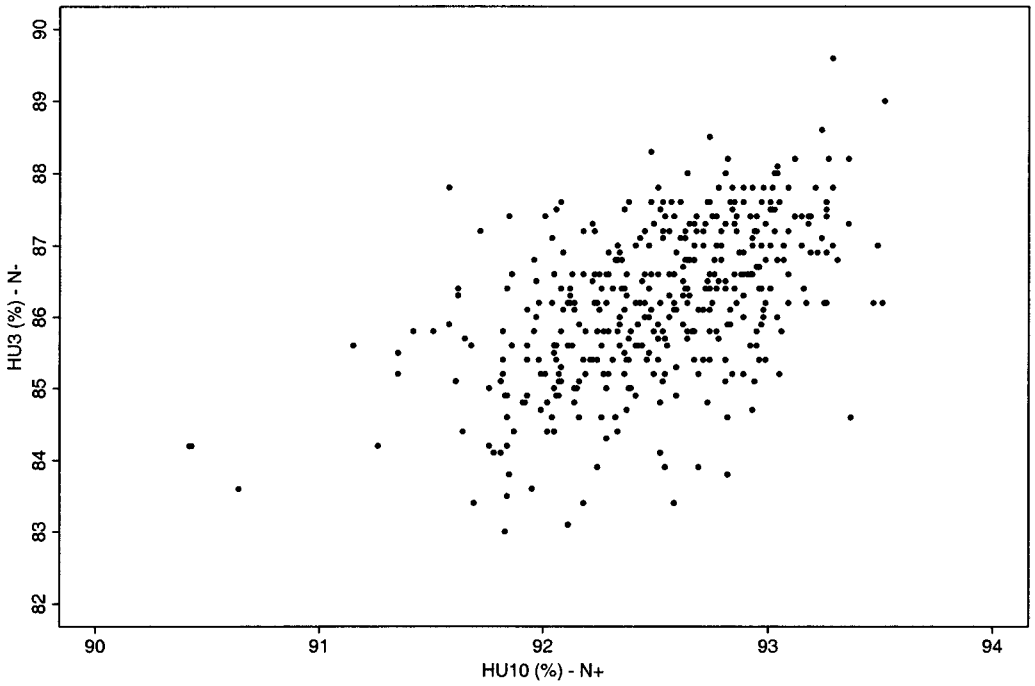

Figure 2.—Variation for shoot water content in both N environments in the Bay-0 × Shahdara population. Each one of the 415 RIL is represented by a dot.

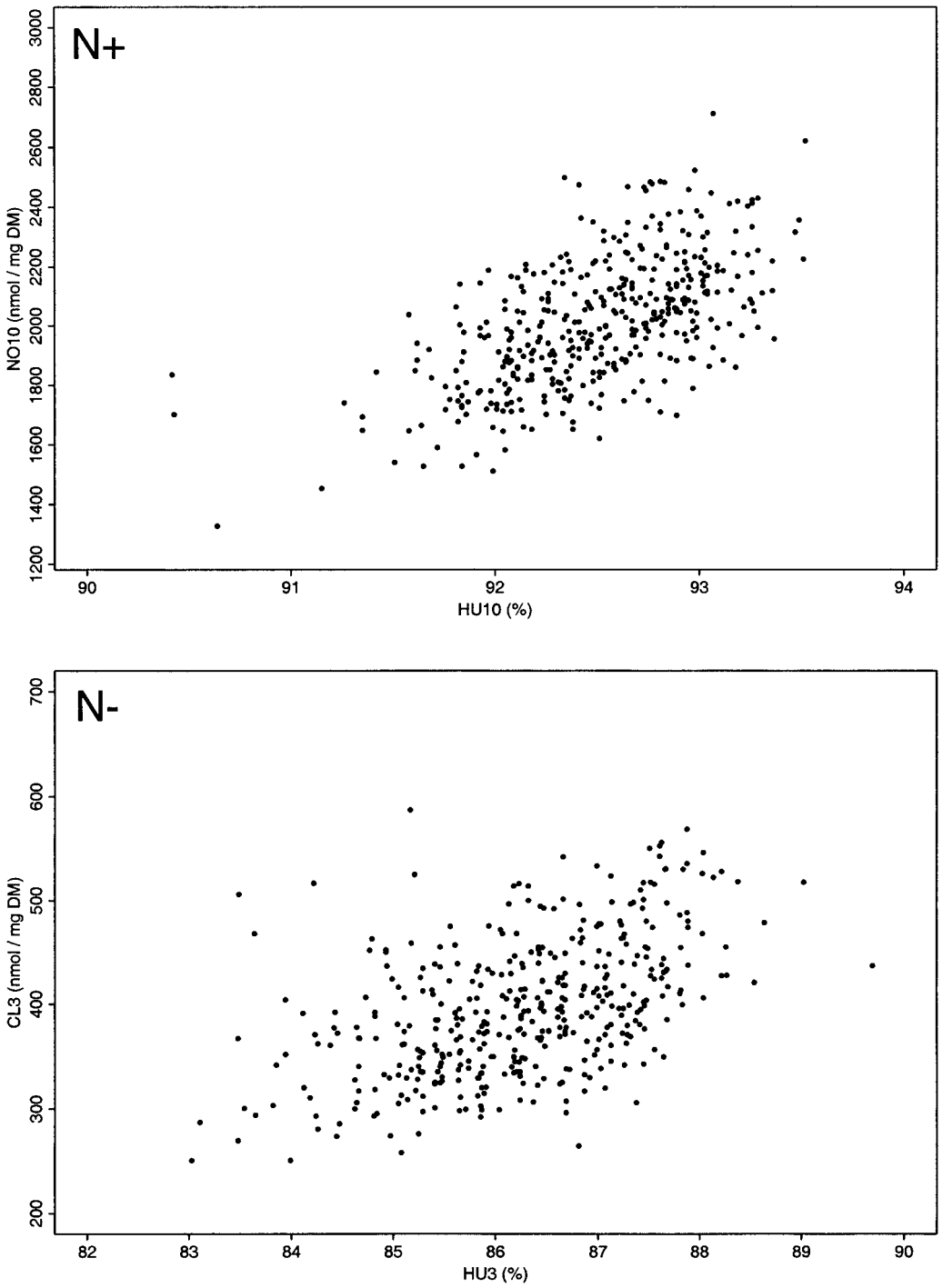

FIGURE 3.—Relationship between shoot water content and anion content in the Bay-0 × Shahdara population. Top graph represents water (HU10) and nitrate (NO10) content relationship in N+ environment; bottom graph represents water (HU3) and chloride (CL3) content relationship in N− environment. Each one of the 415 RIL is represented by a dot.

trait, which is large, especially for moderately heritable traits (ALONSO-BLANCO and KOORNNEEF 2000; JUENGER *et al.* 2000). Epistatic interactions between detected QTL were found to provide only a small contribution to the total phenotypic variation, which is in contrast to other traits studied in the same population (LOUDET *et al.* 2002, 2003). For all traits studied here, the mapping of both positive- and negative-effect QTL provides a genetic basis for the transgression illustrated in Figure 1. In the absence of significant epistatic interaction, HU10 unbalanced transgression is more likely due to a physiological upper limit of the water content in the shoot than to genetic variation. For each trait, the percentage of genetic variance revealed through QTL and QTL × QTL interactions (calculated as: complete model R^2 sum from Table 3/heritability from Table 1) is high, between 75 and 95% (results not presented). Traits are globally less deeply dissected in N− environment than

TABLE 3

Results of QTL analyses for water and anion traits in the Bay-0 × Shahdara population

QTL[a]	Chromosome-marker[b]	Position[c]	LOD score	$R^{2\,d}$	$2a^{e}$
HU10.1	Chrom 1-NGA248	34.0	9.5	10	−0.32
HU10.2	Chrom 1-NGA128	50.8	9.6	6	−0.30
HU10.3	Chrom 1-MSAT1.5	83.9	3.4	3	−0.16
HU10.4	Chrom 2-MSAT2.41	30.8	4.8	6	−0.18
HU10.5	Chrom 3-NGA172	0.1	4.4	2	−0.16
HU10.6	Chrom 4-MSAT4.8	1.5	12.5	9	+0.30
HU10.7	Chrom 5-NGA249	6.0	12.7	9	−0.32
HU10.8	Chrom 5-MSAT5.19	73.9	6.7	6	−0.22
HU10 complete model				51%	
HU3.1	Chrom 1-MSAT1.10	19.4	6.7	4	−0.56
HU3.2	Chrom 1-MSAT1.5	83.9	7.4	7	−0.54
HU3.3	Chrom 2-MSAT2.41	34.5	9.9	5	−0.70
HU3.4	Chrom 2-MSAT2.10	55.7	8.2	6	+0.64
HU3.5	Chrom 4-MSAT4.8	1.7	6.3	4	+0.52
HU3.6	Chrom 5-NGA249	7.7	6.2	5	−0.58
HU3 complete model				31%	
NO10.1	Chrom 1-NGA248	38.0	7.5	6	−130
NO10.2	Chrom 1-NGA128	49.1	9.4	7	−166
NO10.3	Chrom 1-MSAT1.13	73.6	5.9	4	+102
NO10.4	Chrom 2-MSAT2.38	22.6	4.5	6	−112
NO10.5	Chrom 2-MSAT2.41	29.5	6.7	5	−116
NO10.6	Chrom 3-NGA172	1.9	9.9	5	−124
NO10.7	Chrom 4-MSAT4.15	38.2	3.7	3	−72
NO10.8	Chrom 5-NGA249	3.1	4.6	2	−60
NO10 complete model				38%	
CL10.1	Chrom 1-NGA248	35.5	5.0	2	−2.1
CL10.2	Chrom 1-F5I14	63.1	6.9	3	+2.0
CL10.3	Chrom 2-MSAT2.38	13.1	3.1	3	−1.2
CL10.4	Chrom 2-MSAT2.22	62.5	3.2	4	+1.3
CL10.5	Chrom 3-MSAT3.32	34.4	5.1	2	−1.8
CL10.6	Chrom 4-MSAT4.9	55.8	3.7	2	−1.4
CL10.7	Chrom 5-NGA139	25.4	8.8	9	+2.4
CL10.8	Chrom 5-MSAT5.12	58.3	16.9	13	+3.4
CL10.1 × CL10.7				2	
CL10.1 × CL10.8				3	
CL10 complete model				43%	
CL3.1	Chrom 1-MSAT1.10	22.0	16.6	6	−47
CL3.2	Chrom 1-MSAT1.13	70.0	30.9	21	+60
CL3.3	Chrom 2-MSAT2.41	34.1	24.6	11	−58
CL3.4	Chrom 2-MSAT2.10	57.3	10.0	5	+36
CL3.5	Chrom 3-MSAT3.32	34.9	6.7	3	−30
CL3.6	Chrom 5-MSAT5.12	69.5	9.8	4	+35
CL3.2 × CL3.5				2	
CL3 complete model				52%	
PO3.1	Chrom 1-NGA248	24.9	21.2	16	−4.3
PO3.2	Chrom 1-MSAT1.13	70.4	4.8	3	−1.9
PO3.3	Chrom 3-MSAT3.19	17.4	5.6	3	−2.4
PO3.4	Chrom 4-MSAT4.8	4.8	14.4	11	+3.6
PO3.5	Chrom 4-MSAT4.18	48.8	3.2	3	+1.6
PO3.6	Chrom 5-MSAT5.9	43.0	10.4	6	−3.1
PO3 complete model				42%	

[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 cM from the first marker of the chromosome.
[d] Percentage of variance explained by the QTL or by QTL × QTL interaction, when significant.
[e] The mean effect (in trait unit, see Table 1) of the replacement of both Shahdara alleles by Bay-0 alleles at the QTL.

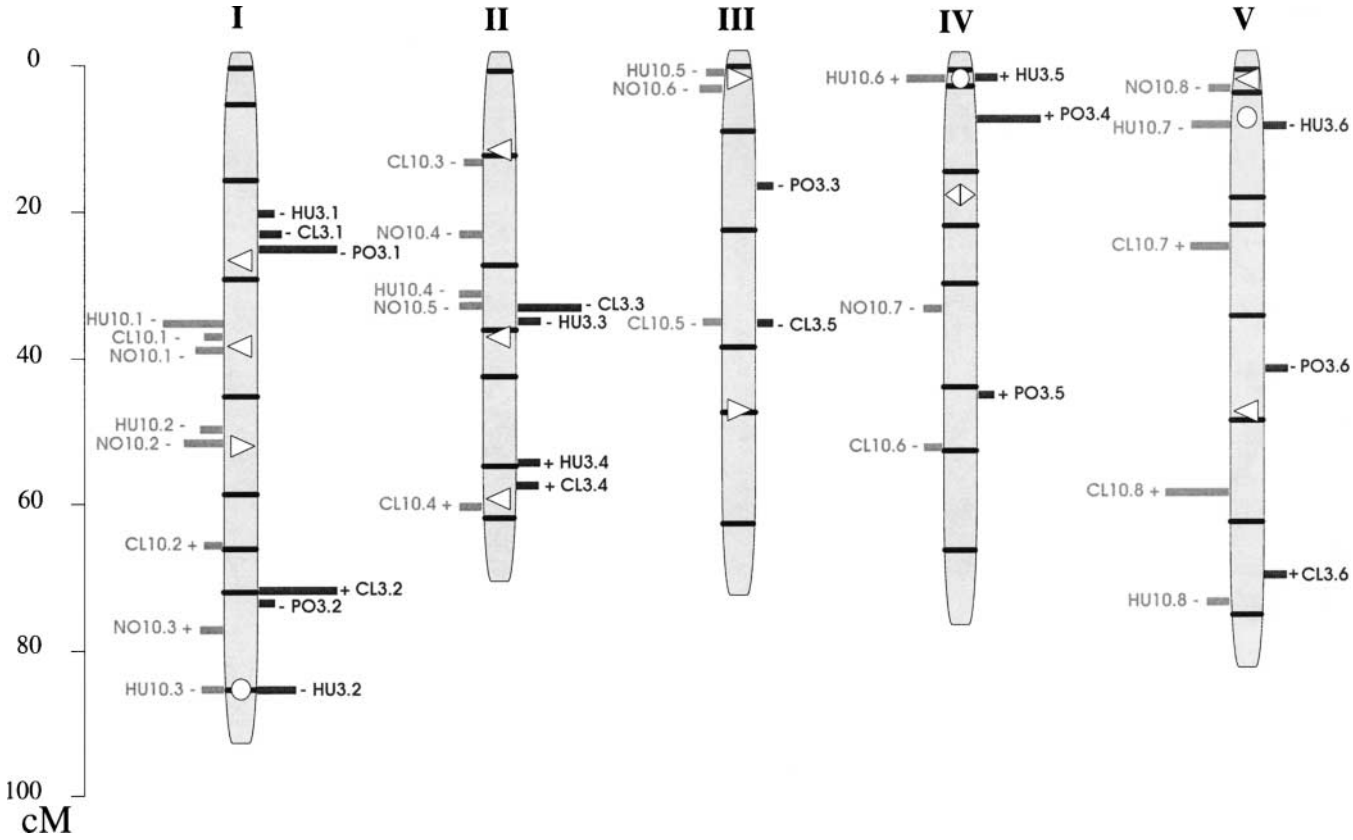

FIGURE 4.—QTL detected for water and anion traits in the Bay-0 × Shahdara population. Each QTL is represented by a bar located at its most probable position (or nearby). QTL on the left side of the chromosomes are those detected in N+ environment; QTL on the right side of the chromosomes are those detected in N− environment. The length of the bar is proportional to the QTL contribution (R^2). The sign of the allelic effect is indicated for each QTL. The framework genetic map (indicating marker position) is from LOUDET *et al.* (2002). Flowering-time QTL positions (from LOUDET *et al.* 2002) are indicated with open circles on the chromosomes. Shoot dry matter QTL positions (from DM trait in LOUDET *et al.* 2003) are indicated with open triangles on the chromosomes; left-pointing triangles indicate QTL obtained in N+ environment (DM10), while right-pointing triangles indicate QTL obtained in N− environment (DM3).

in N+ environment, perhaps because a number of small-effect QTL were not significantly detected. Nevertheless, this work gives access to an interesting part of the genetic variation of water and anion contents in the Bay-0 × Shahdara population.

QTL stability with N limitation: In plants, the role of nitrate in the regulation of osmotic pressure and turgor is well known (CARDENAS-NAVARRO *et al.* 1999). We present a quantitative genetics study detailing nitrate's interaction with water parameters and other osmoticum. Here, we analyze the genetic effect of nitrate deprivation by the identification of QTL that are specific to a particular N environment or subject to QTL × N interaction. Most of the water content variation and, even more, the chloride content variation is controlled differently in N+ and N− environments. This can be a direct consequence of the depletion of the nitrate pool or an indirect consequence of N metabolism modification, by its effect on growth or through other osmotically active N compounds such as malate (NIEDZIELA *et al.* 1993; FRICKE *et al.* 1997; CLARKSON *et al.* 2000). These N interacting QTL are candidate factors influencing the way N nutritional information is transduced into a hydraulic response (CLARKSON *et al.* 2000). As expected, chloride content seems to be more genetically constrained in N limiting conditions (see CL3 heritability in Table 1 and complete model R^2 in Table 3), where its role as an osmoticum is reinforced (WHITE and BROADLEY 2001). Concerning water content, it is noteworthy that two important loci, HU10.6/HU3.5 on chromosome 4 and HU10.7/HU3.6 on chromosome 5, remain stable despite N deprivation.

Loci involved in different traits: One of the major issues of our study comes from the interpretation of the colocalization of QTL from different traits, because it allows us to isolate individual genetic factors explaining the correlations between these traits. However, it is difficult to distinguish between linkage, pleiotropy, and causality solely on the basis of QTL results.

Water content and flowering time: Among the four N-stable loci controlling water content variation, three share a very interesting feature: HU10.3/HU3.2 on chromosome 1, HU10.6/HU3.5 on chromosome 4, and HU10.7/HU3.6 on chromosome 5 strongly colocalize

with previously published flowering-time QTL (obtained in the same short-day photoperiod), SD3, SD1, and SD2, respectively (from Loudet *et al.* 2002; SD QTL are indicated with open circles in Figure 4). Additive effects are compatible with this triple colocalization, since HU and SD QTL always show opposite allelic effects. At these loci, flowering-delaying effect is accompanied by a reduction in shoot water content in 35-day-old plants in both N environments. The significant correlation between flowering time and water content reflects this situation (-0.42 in N+ environment and -0.32 in N− environment). Unexpectedly, no significant HU10.6 × HU10.7 or HU3.5 × HU3.6 interactions parallel the SD1 × SD2 epistatic interaction, which is, however, much less important in short days than in long days (Loudet *et al.* 2002). Such a triple colocalization appearing fortuitously is highly improbable (<1 chance in 42,000, considering an average 10-cM confidence interval overlap) and likely reveals a newly discovered pleiotropic relationship between flowering time and water content. According to Loudet *et al.* (2002), SD1 and SD2 QTL very probably correspond to *FRIGIDA* and *FLOWERING LOCUS C* (*FLC*) genes, which are direct components of the flowering pathway (Sheldon *et al.* 2000). Recently, QTL mapping possibly identified *FLC* as a locus regulating the circadian rhythm of leaf movements in the Ler/Col population (Swarup *et al.* 1999). Because, in addition to governing leaf movement and hypocotyl elongation, circadian rhythms also govern the rhythmic opening of stomata (Somers *et al.* 1998), *FLC* could participate in general water content regulation through the control of stomatal transpiration. The implication of *FLC* in water content regulation could be interestingly tested using *flc* null mutants (Michaels and Amasino 1999); unfortunately, these mutants are available in the Col background, making it difficult to extend the comparison to our material. Floral evocation, indeed, was shown to be associated with increases in cellular, cytoplasmic, and nucleolar volume in the apical meristem and these histological changes are thought to play an essential role in the transition to flowering (Havelange 1980). Water regulation may be central in these interactions. It is then possible that a modulation of plant water content proceeds from the regulation of flowering precocity, even if the former is observed well before flowering transition. McIntyre (1997) also reported evidence of water parameter regulation controlled by precocity that appeared prior to flowering; according to the author, this was linked to solute accumulation. Our QTL colocalizations (Figure 4) show that the control of water content linked to flowering-time regulation seems to be independent of anion content regulation (although we cannot totally exclude that NO10.8 corresponds to the same locus as HU10.7/HU3.6).

This relationship is novel since other studies on the same genetic material and in the same conditions did not reveal any obvious relationship between flowering time and growth or N-related traits (total N content, free-amino-acid content) through QTL colocalization (Loudet *et al.* 2003).

Water content and osmoticum: All other HU10 QTL except one (HU10.8) colocalize with NO10 QTL with the same allelic effect sign. Interestingly, at these QTL Bay-0 always carries the allele that decreases the trait value. This association is not surprising and reflects the control for nitrate homeostasis since nitrate concentration in the water reservoir is not modified by these loci (Cardenas-Navarro *et al.* 1999; Clarkson *et al.* 2000). This result could be explained by the hypothesis that nitrate acts osmotically to increase water uptake (McIntyre 1997). This would be consistent with the data published by Wang *et al.* (2001) showing an upregulation of some aquaporin genes expression after exposure to nitrate. Some of the NO10 QTL involved in this relation have already been hypothesized to be directly implicated in N metabolism regulation (like NO10.2), while others (like NO10.5) could represent a pleiotropic consequence of, for example, a developmental factor, revealed through shoot dry matter linked variation (Loudet *et al.* 2003).

All the HU3 QTL that are not linked to flowering-time QTL strongly colocalize with CL3 QTL showing the same allelic effect sign (positive or negative). Chloride has already been shown to replace nitrate as an osmoticum when the latter is not available (Fricke and Flowers 1998; White and Broadley 2001). Furthermore, the positive locus in the bottom of chromosome 2 (HU3.4/CL3.4 together with CL10.4) reveals that an N-independent regulation of chloride content has consequences on shoot water content only when the role of chloride as an osmoticum is reinforced by nitrate deprivation. One remarkable genetic situation is identified through the locus in the middle of chromosome 2 (HU10.4/NO10.5-HU3.3/CL3.3 QTL): in this case it seems more likely that turgor or osmotic pressure itself is the overriding feature then controlling anion (nitrate or chloride) content in the shoot, as some authors have already proposed (Leigh 1997; White and Broadley 2001).

Chloride content regulation: Apart from the CL10.4-CL3.4 colocalization described above, most chloride content QTL map to different positions in different N environments. Some major-effect QTL specific to N+ (CL10.8) or even N− (CL3.2) environment do not contribute to the well-known strict correlation between osmoticum and water contents (Cardenas-Navarro *et al.* 1999). Lack of statistical power in small-effect QTL detection could explain this situation when chloride concentration in the shoot tissue is low (N+); but in N− environment, we conclude that some genetic factors clearly modify chloride concentration in the water reservoir. In nonlimiting N conditions, an interesting colocalization of CL10 and NO10 QTL is found on chromo-

some 1 (CL10.1-NO10.1-HU10.1). If this regulation is exerted first on water parameters, then several osmotic compounds together (nitrate, chloride . . .) follow this variation, certainly through accumulation in the vacuole (Karley *et al.* 2000). Geelen *et al.* (2000) already brought molecular evidence of a putative genetic link between nitrate and chloride: their study of the putative chloride channel *AtCLCa* gene revealed that it was involved in regulating the nitrate status of the plant.

Phosphate content regulation: Phosphate content variation decomposition is very interesting because it reveals loci relatively distinct from those analyzed above. They could therefore represent specific elements of the phosphate acquisition pathway, like phosphate transporters. Some of these loci, however, could correspond to genes controlling root architecture, as this parameter is known to be one of the most important factors affecting or interacting with phosphate acquisition (Narang *et al.* 2000). Whatever they may be, their specific interaction with nitrate availability is worth elucidating. Nevertheless, major-effect QTL PO3.1 explains one-third of the total genetic variation and colocalizes with previously detailed QTL HU3.1 and CL3.1 (all three with negative allelic effects). If identical, this locus could then participate in osmotic regulation through unspecific osmoticum (chloride, phosphate) variations. Moreover, we cannot exclude the possibility that PO3.4 QTL reveals the same locus as HU3.5 and HU10.6, but if *FRIGIDA* is confirmed as the origin of the water content variation through flowering-time change, then it is highly improbable that PO3 would not be affected also by other flowering-time loci.

Conclusions: Our approach proved to be very efficient for dissection of the genetic relationships between different physiological traits. This is especially the case because the variation of the observed traits can be interpreted with respect to variation in whole-plant physiology. The number of lines involved in the experiment ensures the quality and power of QTL detection, revealed for example by the ability to detect even small-effect QTL for most traits. Some characteristics, like HU3 or NO10, seem to be controlled mostly by homogeneous medium-effect loci. This is different from the more classically described situation in which most of the variation originates from a small number of large-effect QTL, together with small-effect loci (Ungerer *et al.* 2002).

Our results shed a new light on the relationship either between plant development and water parameters or between water and anion contents. Two flowering-time loci, *FRI* and *FLC*, are likely to be involved in the control of water content variation in our conditions. Moreover, this effect is essentially stable with N availability change. Developmental variation (as revealed with shoot dry matter QTL analysis) could also explain other loci through osmotic adjustment leading to unspecific anion content regulation in the shoot (HU10.4/HU3.3). It is also noteworthy that the only QTL previously found for shoot dry matter that is stable across N environments (on chromosome 4) does not colocalize with any of the QTL published here (see Figure 4; DM10.6/DM3.4 from Loudet *et al.* 2003). Globally, other water content variation seems to induce or result from anion variations, essentially nitrate in N+ conditions and chloride in N− conditions; phosphate provides only a poor contribution to water content variability in our conditions, despite its large genetic variation. However, other specific genetic factors are involved in the regulation of the water status of the plant without any change in anion contents. Each anion content is also specifically and independently controlled, leading to a variation of anion concentration in the water reservoir. This information is particularly interesting because it reveals the integration of nitrate variation at the whole-plant level (through its multiple roles: nitrate as a nutritional compound, a signaling molecule, or an osmoticum) in interaction with nitrate availability in the soil. Chloride accumulation in different N conditions also reveals interesting variability that could be useful for the study of salt (NaCl) stress tolerance in Arabidopsis.

Candidate genes for these QTL could comprise, for example, the numerous structural genes encoding aquaporin and chloride or phosphate transporter/channel. The locus in the middle of chromosome 2 (HU10.4/NO10.5-HU3.3/CL3.3 QTL) colocalizes with a gene coding for a putative aquaporin (accession no. AT2G25810), which is not in contradiction with our previous hypothesis that anion content variation probably results from water variation in this case. Another water channel-like protein is encoded by locus AT4G-23400, very closely linked to PO3.5 QTL. Finally, both main-effect CL10 QTL on chromosome 5 colocalize with chloride channel genes: CL10.7 maps very close to *AtCLC-d* and CL10.8 maps very close to the AT5G49890 locus.

The ultimate goal of such a work is the cloning of genes explaining the QTL. In Arabidopsis, this is now technically feasible using the physical map provided by the available complete genome sequence and near-isogenic lines (NIL). Taking advantage of the residual heterozygosity in F_6 plants, NIL can be rapidly constructed particularly in the Bay-0 × Shahdara population, following the method of heterogeneous inbred family (Tuinstra *et al.* 1997). If the locus proves to behave as a single Mendelian locus, then the cloning of the gene itself uses conventional positional cloning techniques (Lukowitz *et al.* 2000; Yano 2001). QTL such as CL3.2 or PO3.1 are interesting candidates for such an approach, because they represent new large-effect loci involved in the control of these traits.

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