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ORIGINAL PAPER

Using a physiological framework for improving the detection of quantitative trait loci related to nitrogen nutrition in *Medicago truncatula*

Delphine Moreau · Judith Burstin · Grégoire Aubert · Thierry Huguet · Cécile Ben · Jean-Marie Prosperi · Christophe Salon · Nathalie Munier-Jolain

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Abstract *Medicago truncatula* is used as a model plant for exploring the genetic and molecular determinants of nitrogen (N) nutrition in legumes. In this study, our aim was to detect quantitative trait loci (QTL) controlling plant N nutrition using a simple framework of carbon/N plant functioning stemming from crop physiology. This framework was based on efficiency variables which delineated the plant's efficiency to take up and process carbon and N resources. A recombinant inbred line population (LR4) was grown in a glasshouse experiment under two contrasting nitrate concentrations. At low nitrate, symbiotic N_2 fixation was the main N source for plant growth and a QTL with a large effect located on linkage group (LG) 8 affected all the traits. Significantly, efficiency variables were necessary both to precisely localize a second QTL on LG5 and to detect a third QTL involved in epistatic interactions on LG2. At high nitrate, nitrate assimilation was the main N source and a larger number of QTL with weaker effects were identified compared to low nitrate. Only two QTL were common to both nitrate treatments: a QTL of belowground biomass located at the bottom of LG3 and another one on LG6 related to three different variables (leaf area, specific N uptake and aboveground:belowground biomass ratio). Possible functions of several candidate genes underlying QTL of efficiency variables could be proposed. Altogether, our results provided new insights into the genetic control of N nutrition in *M. truncatula*. For instance, a novel result for *M. truncatula* was identification of two epistatic interactions in controlling plant N_2 fixation. As such this study showed the value of a simple conceptual framework based on efficiency variables for studying genetic determinants of complex traits and particularly epistatic interactions.

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D. Moreau (✉) · J. Burstin · G. Aubert · C. Salon · N. Munier-Jolain
INRA, UMR 102 Génétique et Ecophysiologie des Légumineuses, 17 rue Sully, BP 86510, 21065 Dijon cedex, France
e-mail: delphine.moreau@dijon.inra.fr

D. Moreau
INRA, UMR 1210 Biologie et Gestion des Adventices, 17 rue Sully, BP 86510, 21065 Dijon cedex, France

T. Huguet
INP-ENSAT, Laboratoire Symbioses et Pathologies des Plantes, 18 chemin de Borde rouge, 31326 Castanet Tolosan, France

C. Ben
Université de Toulouse, INP, UPS, UMR 5245 EcoLab (Laboratoire Ecologie Fonctionnelle et Environnement), ENSAT, 18 chemin de Borde rouge, 31326 Castanet Tolosan, France

C. Ben
CNRS, EcoLab, 31326 Castanet Tolosan, France

J.-M. Prosperi
INRA, IRD, Montpellier SupAgro, Université de Montpellier 2, UMR 1097 Diversité et Adaptation des Plantes Cultivées, 34130 Mauguio, France

Abbreviations

ABR	Aboveground:belowground biomass ratio
LG	Linkage group
N	Nitrogen
NLA	N into leaf area conversion efficiency
QTL	Quantitative trait loci
RIL	Recombinant inbred line

RUE Radiation use efficiency
SNU Specific N uptake

Introduction

The ability of legumes to establish a symbiosis with *Rhizobium* bacteria in root nodules confers on them the capacity to fix atmospheric nitrogen (N_2). Nitrogen (N) nutrition can then derive from both root assimilation of mineral N and symbiotic fixation of N_2. In the context of reducing the environmental impact of agriculture, the possibility of cultivating legume crops without N fertilizers constitutes an asset when compared to other crops such as cereals or rapeseed. The instability of both the grain yield and the grain protein content is, however, a major limitation to legume production. To unravel this issue, *M. truncatula* has been widely used as a model plant in order to explore the genetic and molecular determinants of N-fixing symbiosis of legumes (Barker et al. 1990; Sagan et al. 1995; Cook 1999; Colebatch et al. 2002; Stacey et al. 2006). This species appears as a model of choice for identifying genes of interest in legume crops because of its relatively small genome (Blondon et al. 1994), its tractable genetic properties, its high level of synteny with several legumes of interest (for review, see Zhu et al. 2005) and its forthcoming genomic sequence. The study of either natural or induced genetic variants in *M. truncatula* has provided new clues into the processes involved in the establishment of symbiosis and in the regulation of nodulation at a molecular level (Penmetsa and Cook 1997; Wais et al. 2000; Penmetsa et al. 2003; Mitra et al. 2004; Schnabel et al. 2005; Baier et al. 2007; Laporte et al. 2010). Recently, studies on plant N nutrition have been carried out at a whole plant level bringing new insight on how the two pathways (i.e. symbiotic N_2 fixation and root nitrate assimilation) complemented each other to acquire N (Moreau et al. 2008; Terpolilli et al. 2008; Jeudy et al. 2009; Salon et al. 2009; Sulieman and Schulze, 2010). Searching for quantitative trait loci (QTL) is a useful complementary approach to clarify the complexity of N nutrition. Using available recombinant inbred lines (RIL) mapping populations and genetic maps, QTL have been recently detected in *M. truncatula* for: aerial morphogenesis (Julier et al. 2007); flowering time (Pierre et al. 2008); seed mineral concentrations and content (Sankaran et al. 2009); seed germination and pre-emergence growth (Menna Baretto Dias et al. 2011); seed vigor (Vandecasteele et al. 2011); and resistances to (i) *Ralstonia solanacearum* (Vailleau et al. 2007), (ii) anthracnose and powdery mildew (Ameline-Torregrosa et al. 2008), (iii) *Aphanomyces euteiches* (Djebali et al. 2009; Pilet-Nayel et al. 2009) and (iv) spring black stem and leaf spot (Kamphuis et al. 2008). However, QTL have never been identified for plant N nutrition traits. Developing such an approach could represent a great opportunity to identify genome regions involved in N nutrition. This would potentially impact on genetic improvement of legume crops, considering the high level of synteny between *M. truncatula* and crop legume species such as pea (*Pisum sativum*) and alfalfa (*Medicago sativa*) (Zhu et al. 2005; Bordat et al. 2011).

One difficulty with QTL mapping is that the plant traits under study are frequently complex: they result from the integration of a large number of physiological processes under the control of genetic and environmental factors. As a consequence, plant traits are often controlled by numerous genes with weak effects and are therefore imprecisely detected by QTL analyses. Thus, identifying the roles of the genes underlying the QTL remains difficult. An innovative approach initiated a few years ago combines genetic and crop physiology approaches. Crop physiology models enable the dissection of complex traits into simpler components for which QTL can be identified. Applied to the study of the foliar elongation of maize (Reymond et al. 2003), growth of peach fruit (Quilot et al. 2004); flowering time of barley (Yin et al. 2005) and rice (Nakagawa et al. 2005); and to the N nutrition of wheat (Laperche et al. 2006), this synergy between genetic and crop physiology has improved our understanding of the genetic bases of plant functioning (Tardieu 2003).

Here, our objective was to analyze genetic determinants of the N nutrition of *M. truncatula* combining a simple conceptual framework stemming from crop physiology and QTL detection. This study was based on the population LR4 (175 RIL from the cross Jemalong A17 × DZA315.16) which was shown to display variation useful for studying N nutrition and especially N_2 symbiotic fixation (Moreau et al. 2009).

Materials and methods

Plant material and culture conditions

A population of 175 RIL (LR4) in F8 generation was obtained from the cross Jemalong A17 × DZA315.16 (Huguet et al. 2004). The line Jemalong A17 was derived from one plant of the Australian cultivar Jemalong (registered in 1955, http://www.pi.csiro.au/ahpc/legumes/legumes.htm) selfed twice. The line DZA315.16 was derived from one plant of a wild Algerian ecotype also selfed twice. Jemalong A17 is known to be poorly efficient for N_2 fixation with *Rhizobium meliloti* strains 2011 and 1021 (Moreau et al. 2008; Terpolilli et al. 2008). No data

had to our knowledge been published on the efficiency of nodulation for DZA315.16. A preliminary experiment conducted at low nitrate (data not shown) indicated that the growth of DZA315.16 was higher than that of Jemalong suggesting a better efficiency of nodulation for DZA315.16.

The experimental design was previously reported in Moreau et al. (2009): the population LR4 and the two parental lines (Jemalong A17 and DZA315.16) were all grown in a glasshouse in the presence of *R. meliloti* strain 2011. Two nutrient solutions were used: a low (0.625 mM) and a high (10.5 mM) nitrate concentration where plants relied either on N_2 fixation or root nitrate assimilation, respectively (Moreau et al. 2008). Seed preparation, soil substrate, *Rhizobium* inoculation and watering of pots were identical to Moreau et al. (2008). The experimental design was made of 14 complete blocks per N treatment. Each RIL was represented by one 1.0-L pot containing one plant in each block. As such 14 replications per N treatment were used in this experiment. The photoperiod was 14-h, air temperature was 17.86 ± 0.84°C and the mean incident photosynthetically active radiations (PAR) was 14.23 ± 1.55 mol m^{-2} day^{-1}.

Plant measurements

Plant leaf area was measured non-destructively once a week from 294 to 638 degree-days after sowing (base 5°C; see Bonhomme 2000 for information on degree-days and Moreau et al. 2006 for base temperature) using a setup presented in Moreau et al. (2009) and Mathey et al. (2011). Three plants were analyzed for each RIL × N treatment combination. In addition, two destructive measurements were done at 463 and 730 degree-days after sowing (base 5°C). At each of the two dates, three plants were analyzed for each RIL × N treatment combination: the aboveground and the belowground plant biomass were measured after oven drying at 80°C during 48 h. The N content of the whole plant (aboveground and below ground) was estimated using the Kjeldahl method (AOAC 1965).

Physiological framework

The framework of analysis was derived from Laperche et al. (2006) (Fig. 1). It was based on four main meaningful "state variables" characterizing plant growth: plant leaf area, total biomass, root and nodule biomass (belowground biomass), the amount of plant N. These variables were linked by "efficiency variables" representing the capacity of plants to extract and utilize resources, i.e. carbon and N: (1) Radiation use efficiency (RUE, g of total biomass MJ^{-1} of intercepted PAR); (2) Aboveground:belowground biomass ratio (ABR, g of aboveground biomass g^{-1} of belowground biomass); (3) Specific N Uptake (SNU, g N g^{-1} of belowground biomass); and (4) N to leaf area conversion efficiency (NLA, cm^2 of leaf g^{-1} of N). These efficiency variables were calculated as the slope of a linear regression with an intercept at (0; 0). SNU was calculated by plotting total amount of N and belowground biomass; NLA was calculated by plotting plant leaf area and total amount of N; RUE was calculated by plotting total plant biomass and intercepted PAR; (4) ABR was calculated by plotting aboveground biomass and belowground biomass. For each RIL × N treatment combination, the values used in the QTL analyses were: (1) the mean values calculated on the three plants at the second sampling date for the state

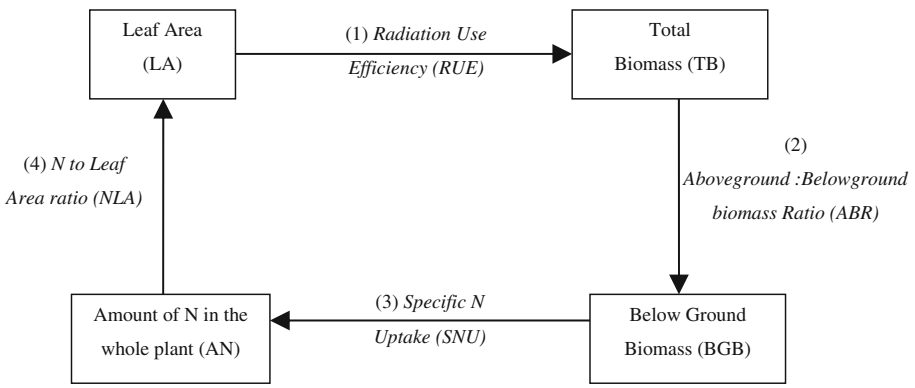

Fig. 1 Conceptual framework of plant functioning in which plant carbon metabolism and plant N metabolism are closely linked (Gastal and Lemaire 2002). State integrative variables are in *boxes* whereas efficiency variables are in *italic*. The relationships between carbon and N fluxes at the plant level can be conceptualized as follows: (1) Due to its role of radiation sensor, plant leaf area defines strongly the amount of carbon assimilated through photosynthesis (Monteith, 1977); (2) One part of the carbon assimilated is allocated to root and nodules (belowground biomass) for their morphogenesis, their activity and their maintenance (Minchin et al. 1981); (3) In return, roots and nodules assimilate N; (4) The amount of N taken up is then allocated to aboveground parts; the amount of N which is assimilated influences directly carbon assimilation, principally by modulating leaf area expansion (Moreau et al. 2008)

variables; (2) the mean slope of the regressions calculated on the two sampling dates for the efficiency variables.

Statistical analysis

The normality of the distribution of the variables was tested: it was considered as normal when the kurtosis value (k) ranged within the interval [−0.74; 0.74] and when skewness value (s) ranged within the interval [−0.37; 0.37]. Both intervals were assessed as two times the kurtosis and skewness standard error. ANOVA were performed to test genotype and block effects using the SAS GLM procedure (SAS Institute 2000). Broad sense heritability (h^2_b) was estimated for each trait as a ratio between genotypic (σ^2_g) and residual (σ^2_e) variances: $h^2_b = \sigma^2_g/(\sigma^2_g + \sigma^2_e)$. Due to the method of calculation of efficiency variables (see above), h^2_b could not be calculated for these variables. ANOVA was also performed to test for the genotype, N treatment and genotype × N treatment effects. Genotype mean values were obtained using the lsmeans command of the SAS GLM procedure.

QTL detection

The genetic map was based on 62 frame markers. The frame markers were well distributed on the 8 chromosomes and the framework map span 576 cM with an average distance between markers of 10.7 cM (with minimum and maximum distances at 1.1 and 29.1 cM, respectively). Primers and map conditions are presented in Table S1.

Quantitative trait loci detections were performed using MCQTL software (Jourjon et al. 2005) by the iterative QTL mapping method (iQTLm). Cofactor selection and QTL detection were performed using F tests. F thresholds were determined for traits showing various h^2_b by 1,000 permutation tests for a global genome-wide I risk of 10% for cofactors and 5% for QTL detection. Cofactors were searched by forward regression, using a threshold of $F = 10.1$ (equivalent to LOD = 2.2). Then QTL were searched by iQTLm using a threshold of $F = 11.6$ (equivalent to LOD = 2.5). Two-LOD support CI corresponding to a conservative 95% CI were determined. For each trait, a global R^2, individual R^2 (percentage of variance explained by each detected QTL), global R^2 (percentage of variance explained by the sum of the detected QTL) and allelic effect at each QTL were estimated. Epitasis was tested by two-way ANOVA between MTE55 and all the other markers on the 8 linkage groups. It was tested for all the variables under study (state and efficiency variables) for the two nitrate conditions. Finally, the *M. truncatula* candidate gene reference map was projected onto the LR4 QTL map to search for co-localizations. The QTL map was drawn using MapChart, version 2.2. (Voorips 2002).

Fig. 2 Histograms of distribution of the RIL for different variables in both nitrate treatments. *White and black columns* indicate low and high nitrate treatments, respectively. Data were obtained at 730 degree-days (base 5) after sowing. Leaf area was not measured at this date: it was extrapolated by modeling (data not shown). **a** Corresponded to state integrative variables whereas **b** corresponded to efficiency variables. The position of the two parental lines Jemalong A17 (*J*) and DZA315.16 (*D*) are indicated. *SNU* specific N uptake, *RUE* radiation use efficiency, *NLA* N into leaf area conversion efficiency and *ABR* aboveground:belowground biomass ratio

Results

Our analysis was based on two types of plant growth variables at two conditions of N nutrition: (1) "State variables" such as the amount of N in the plant, plant leaf area, total, aboveground and belowground biomass; and (2) "Efficiency variables" computed from "state variables" which delineated the plant's efficiency to take up and process carbon and N resources: RUE, ABR, SNU and NLA.

Phenotypic variations among RIL

The ANOVA revealed a significant genotypic effect ($P < 0.0001$) for all measured variables, an N treatment effect ($P < 0.0001$ for all variables except $P < 0.05$ for belowground biomass) and a genotype × N treatment interaction effect ($P < 0.0001$). Increasing nitrate concentration tended to stimulate plant growth for all the state variables except belowground biomass (Fig. 2a). It tended to simulate plant growth for two efficiency variables: ABR and SNU (Fig. 2b). Broad sense heritability (h^2_b) was higher at low nitrate (on average 0.69) than at high nitrate (on average 0.34) (Table 1). h^2_b varied among traits (Table 1): for both N treatments, it was lowest for belowground biomass and highest for the amount of N in the plant.

For each state variable and each N treatment, a wide range of variation was observed within the RIL population (Fig. 2). The belowground biomass varied by a fourfold factor in both N treatments. The aboveground biomass and the amount of N at low nitrate varied sevenfold and fourfold among RIL, respectively. Both variables varied threefold at high nitrate.

For most of the variables, the parental line DZA315.16 generally showed higher values than Jemalong A17. Transgressive segregants were observed for all state variables in both nitrate treatments. At low nitrate, an average of 48% of the RIL lied outside the range of variation of their parents. At high nitrate, only 17% of the RIL transgressed the parental values. At low nitrate, RIL did not follow a normal distribution for belowground biomass, leaf area, SNU, NLA and ABR (Table 1). At high nitrate, RIL did not follow a normal distribution for leaf area and efficiency variables.

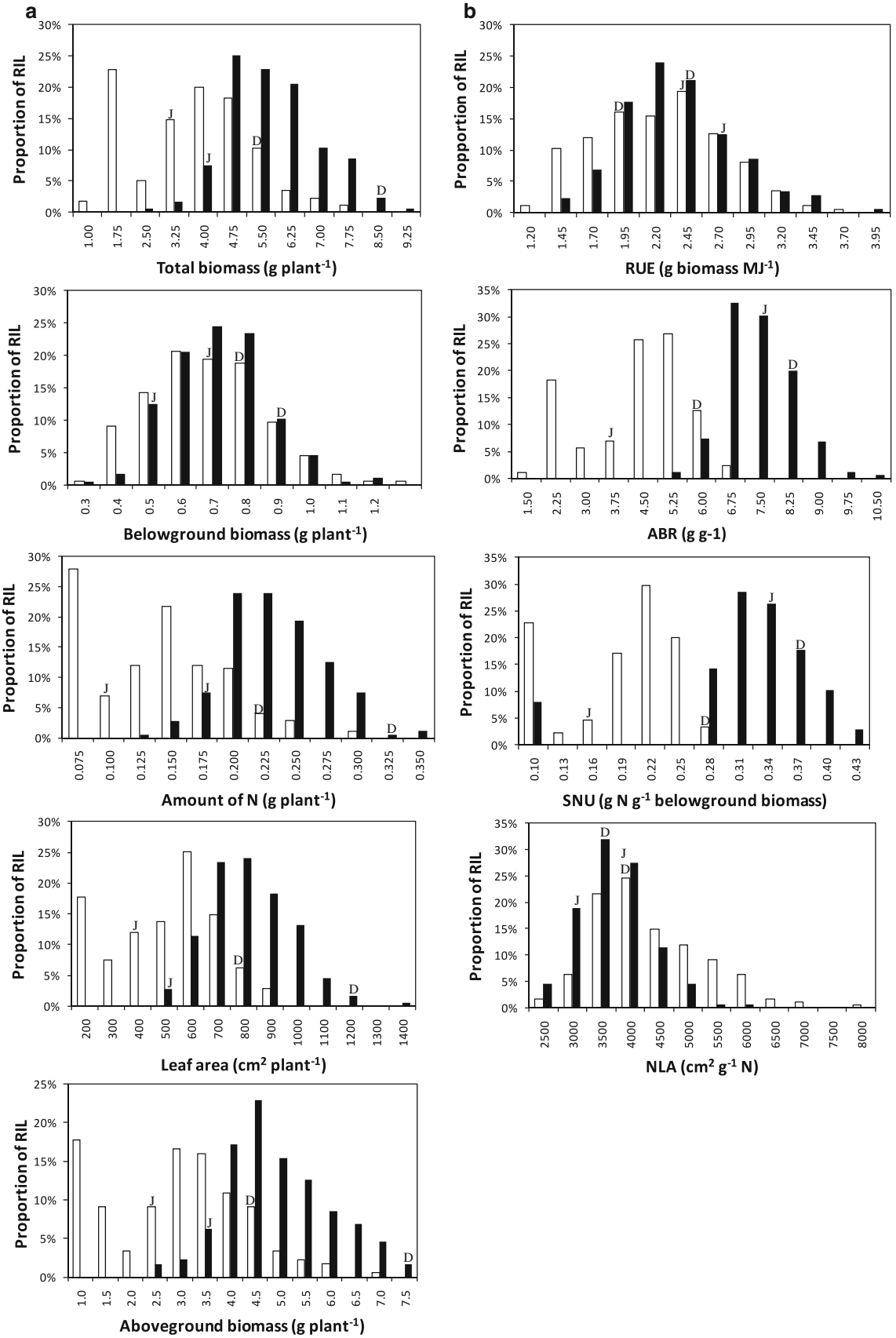
a
b
Proportion of RIL
Total biomass (g plant-1)
RUE (g biomass MJ-1)
Belowground biomass (g plant-1)
ABR (g g-1)
Amount of N (g plant-1)
SNU (g N g-1 belowground biomass)
Leaf area (cm2 plant-1)
NLA (cm2 g-1 N)
Aboveground biomass (g plant-1)
J
D

Table 1 Broad sense heritability (h^2_b), kurtosis (k) and skewness (s) values for each variable

	Low nitrate			High nitrate		
	h^2_b	k	s	h^2_b	k	s
Total biomass	0.70	−0.56	0.21	0.31	−0.26	0.31
Aboveground biomass	0.72	−0.59	0.18	0.31	−0.29	0.33
Belowground biomass	0.46	0.31	0.56	0.25	0.14	0.33
Amount of N	0.85	−0.73	0.00	0.51	0.05	0.15
Leaf area	0.70	−1.02	−0.16	0.30	0.17	0.43
RUE		−0.30	0.28		0.27	0.52
ABR		−0.86	−0.48		0.11	0.46
SNU		−0.89	−0.66		−0.57	0.39
NLA		0.52	0.79		0.83	0.62

Due to its mode of calculation, h^2_b could be estimated only for state variables. Distributions were considered as normal when k value ranged within the interval [−0.74; 0.74] and when s value ranged within the interval [−0.37; 0.37]

SNU specific N uptake, *NLA* N into leaf area conversion efficiency, *RUE* radiation use efficiency, *ABR* aboveground:belowground biomass ratio

QTL detection

Quantitative trait loci mapping results are shown in Table 2 and Fig. 3. QTL were named according to the variable names. A total of 34 QTL was detected (16 and 18 at low and high nitrate treatments, respectively) for 9 variables (5 state variables and 4 efficiency variables). They were found on all linkage groups (LG) except LG7. Variables were controlled by 0–4 QTL. The part of phenotypic variance explained by individual QTL (R^2) varied from 0.06 to 0.52 with, on average, higher values of R^2 in the low than in the high nitrate treatment. Only two QTL were common to both nitrate treatments: a QTL of belowground biomass located at the bottom of LG3 and another one on LG6 related to three different variables (leaf area, SNU and ABR). Only four QTL out of the 34 identified were associated with positive effects of the parental line Jemalong A17.

State variables

Low nitrate revealed ten QTL on LG3, LG5, LG6 and LG8 with R^2 ranging from 0.07 to 0.46 (Table 2a; Fig. 3a). One locus on LG8 harbored QTL for all the state variables whereas LG5 harbored two QTL (leaf area and amount of N) and LG3 and LG6 harbored each one QTL (belowground biomass and leaf area, respectively). At high nitrate, thirteen QTL were detected on LG1, LG2, LG3, LG4 with R^2 varying from 0.06 to 0.16 (Table 2a; Fig. 3b). A locus on LG4 harbored QTL for all the state variables. Another locus on LG3 harbored QTL for all the state variables except leaf area (Table 2).

Efficiency variables

At low nitrate, six QTL for efficiency variables were identified at two main loci (LG5 and LG8) (Table 2b; Fig. 3a). On LG5, QTL of SNU and ABR were identified in the same region as that detected for state variables. On LG8, QTL for efficiency variables were located in the same genomic region as the one detected for state variables with particularly high R^2 (0.52) (Table 2a; Fig. 3a). Epistasis was tested for all the variables under study and found only for SNU (Fig. 4). Two significant epistatic interactions were found ($P < 0.0005$). Both involved MTE55 on LG8 in interaction with MTE122 on LG5 and MT64 on LG2 (Fig. 4: Jemalong A17 and DZA315.16 alleles corresponded to J and D). The highest SNU occurred when the allele for MTE55 came from DZA315.16 regardless of the allele for MTE64 and MTE122. The lowest SNU values corresponded to a combination of alleles of Jemalong A17 (for MTE55 and MTE122) and DZA315.16 (for MTE64).

At high nitrate, five QTL for efficiency variables were detected (Table 2b; Fig. 3b): three different QTL were detected on LG3 (ABR and SNU in the middle of LG3 and RUE at the bottom of LG3) and two QTL were mapped on LG6 (SNU and ABR); no QTL for state variables had been previously located in this LG6 genomic region.

A positive effect of Jemalong A17 alleles was identified for only one state variable (leaf area) and three efficiency variables (NLA, SNU, ABR; Table 2). The positive effect of Jemalong A17 allele for NLA was associated to a relatively high R^2 (0.22).

Identification of candidate genes

Mtsym6 (Tirichine et al. 2000) is a mutation associated with non-fixing nodules that was mapped on LG8 (Thoquet et al. 2002). In our study, it appeared as a good candidate for the QTL cluster on LG8 detected at low nitrate for two main reasons. The phenotypes in our study revealed strongly phenotypes similar to those obtained for a *MtSym6* mutation

Table 2 QTL related to N nutrition for *M. truncatula* RIL of LR4 population

Nitrate concentration (mM)	State variable	Linkage group	QTL position (cM)	Confidence interval (cM)	Closest marker	Peak LOD score	R^2	Effect of Jemalong allele
(a)								
0.625	Amount of N	8	51.4	48.12–55.39	MTE55	32.2	0.46	−0.0256
		5	65.5	39.02–79.54	MTE122	3.4	0.08	−0.0075
		Total					0.48	
	Leaf area	8	57.8	40.62–61.4	MTE55	17.2	0.32	−53.18
		8	36.5	18.24–57.4	MTE54	3.3	0.08	−25.06
		6	53.9	36.26–63.8	MTE41	2.6	0.07	+20.82
		5	63.5	27.12–85.4	MTE122	2.6	0.07	−18.94
		Total					0.51	
	Total biomass	8	51.4	48.46–56.91	MTE55	28.8	0.43	−0.6139
	Aboveground biomass	8	51.4	48.53–56.48	MTE55	30.6	0.45	−0.5566
	Belowground biomass	8	53.4	47.07–63.49	MTE55	13.1	0.23	−0.0512
		3	49.6	39.26–59.9	MTE20	3.3	0.08	−0.0261
		Total					0.33	
10.5	Amount of N	4	76.7	69.6–77.2	MTE93	5.1	0.12	−0.0068
		3	54.6	47.88–59.9	MTE133	4.2	0.10	−0.0079
		1	41	0–70	MTE78	2.4	0.06	−0.006
		Total					0.26	
	Leaf area	4	77.2	69.35–77.2	MTE93	4.4	0.11	−25.32
		2	64.4	51.88–70	MTE80	4.3	0.10	−27.13
		Total					0.18	
	Total biomass	4	76.7	70.27–77.2	MTE93	6.9	0.16	−0.2313
		3	54.6	48.27–59.83	MTE133	4.6	0.11	−0.248
		2	60.4	0–70	MTE80	2.7	0.07	−0.14
		Total					0.28	
	Aboveground biomass	4	76.7	70.45–77.2	MTE93	7.1	0.16	−0.2081
		3	54.6	48.5–59.9	MTE133	4.4	0.11	−0.2156
		2	60.4	0–70	MTE80	2.9	0.07	−0.1347
		Total					0.29	
	Belowground biomass	3	51.6	45.73–57.81	MTE20	5.1	0.12	−0.0314
		4	74.7	67.01–77.2	MTE95	3.8	0.09	−0.0226
		Total					0.20	

Nitrate concentration (mM)	Efficiency variable	Linkage group	QTL position (cM)	Confidence interval (cM)	Closest marker	Peak LOD score	R^2	Effect of Jemalong allele
(b)								
0.625	SNU	8	49.4	47.12–52.70	MTE55	40.4	0.52	−0.0277
		5	63.5	52.53–71.24	MTE122	5.3	0.12	−0.0082
		Total					0.54	
	NLA	8	49.4	42–62	MTE55	10.8	0.22	+283.735
	RUE	8	53.4	47.2–63.7	MTE55	14.3	0.28	−0.1543
	ABR	8	51.4	47.91–54.81	MTE55	33.7	0.47	−0.547
		5	65.5	50.42–84.66	MTE122	3.2	0.08	−0.1532
		Total					0.49	

Table 2 continued

Nitrate concentration (mM)	Efficiency variable	Linkage group	QTL position (cM)	Confidence interval (cM)	Closest marker	Peak LOD score	R^2	Effect of Jemalong allele
10.5	SNU	3	36	25.87–53.18	MTE84	4.0	0.10	+0.0068
		6	57.9	41.36–63.8	MTE42	3.4	0.08	−0.0625
		Total					0.18	
	RUE	3	56.6	44.11–59.9	MTE22	3.0	0.07	−0.0883
	ABR	3	37.8	28.78–43.32	MTE84	3.4	0.08	+0.1791
		6	57.9	44.05–63.8	MTE42	3.0	0.07	−0.1801
		Total					0.17	

They were identified by the iterative QTL mapping for (a) state variables and (b) efficiency variables under two nitrate concentrations. The QTL were represented in Fig. 3

SNU specific N uptake, *NLA* N into leaf area conversion efficiency, *RUE* radiation use efficiency, *ABR* aboveground:belowground biomass ratio

(Tirichine et al. 2000) and these phenotypes were co-segregating with marker MTE55. Moreover, phenotypic data obtained on 124 F2 RIL (Thoquet et al. 2002) allowed us positioning *MtSym6* in the QTL confidence interval (data not shown). Another QTL involved in the efficiency of N uptake (SNU) at low nitrate was located on LG5 at 63.5 cM in the vicinity of *MtLb1*. *MtLb1* is a leghaemoglobin gene expressed specifically in the nitrogen-fixing root nodule of *M. truncatula* (Thoquet et al. 2002). At high nitrate, a QTL of RUE (which accounts for photosynthesis) located on LG3 at 57 cM, in the region harboring a gene encoding a putative rubisco-activase (RCA, corresponding to EST contig TC189863) mapped in this region (Huguet, pers. comm.). This gene could be involved in rubisco activity, a key enzyme involved in photosynthesis. Furthermore at high nitrate, a gene encoding for a glutamine synthetase (GS, Genbank accession number Y10267, EC = 6.3.1.2) was placed on LG6 in the genomic region corresponding to QTL of SNU and ABR, using the 3.0 version of the *M. truncatula* genome.

Co-localizations with QTL previously identified in the literature

Co-localizations between our 34 QTL and QTL influencing aboveground morphogenesis that were previously identified by Julier et al. (2007) for *M. truncatula* were analyzed (data not shown). At high nitrate, some co-locations were identified: QTL for branch length, biomass and leaf to stem ratio were localized on LG3 at the same location that the two clusters of QTL that we identified on LG3 for biomass, amount of N in the plant, ABR, SNU and RUE. Moreover, branch diameter, branch length, biomass, leaf to stem ratio and the number of internodes were localized on LG4 at the same location that QTL of biomass, leaf area and amount of N in the plant.

Co-localizations between the QTL of our study and QTL influencing root, nodule and aboveground variability in pea (Bourion et al. 2010) were analyzed using recent syntenic information between pea and *M. truncatula* (Bordat et al. 2011). The pea plants analyzed by Bourion et al. (2010) were grown in the presence of nitrate. At high nitrate, QTL detected in the present study on LG3 for total, aboveground and belowground biomass, amount of N, ABR, SNU and RUE were localized in syntenic region of pea LGIII QTL related to root development and growth, leaf appearance rate and beginning of flowering.

Discussion

While numerous genes involved in N symbiosis had been identified in *M. truncatula*, no study had so far reported QTL involved in N nutrition efficiency in this species. Little was known about how symbiotic genes interact and control N acquisition and the whole plant phenotype. In the present study, a *M. truncatula* RIL population was cultivated under two conditions of N nutrition (low vs. high nitrate). Both the genomic regions controlling plant N nutrition and the underlying physiological processes controlling plant growth integrative traits were revealed.

The level of nitrate treatment strongly affected QTL location

All the variables were strongly influenced by the nitrate concentration in the nutrient solution (0.625 or 10.5 mM) (Fig. 2). Such a result was not necessarily expected for a legume species because the complementarity between the two N nutrition sources (atmospheric N_2 and nitrate in the nutrient solution) is generally sufficient to reach an optimal N nutrition whatever the N source (in pea: Sagan et al. 1993; Voisin et al. 2002). But the two nitrate treatments generated a wide range of N nutrition levels in the RIL progeny. A N-deficient phenotype was found when N_2 fixation was the main N source (Moreau et al. 2009). This finding may be

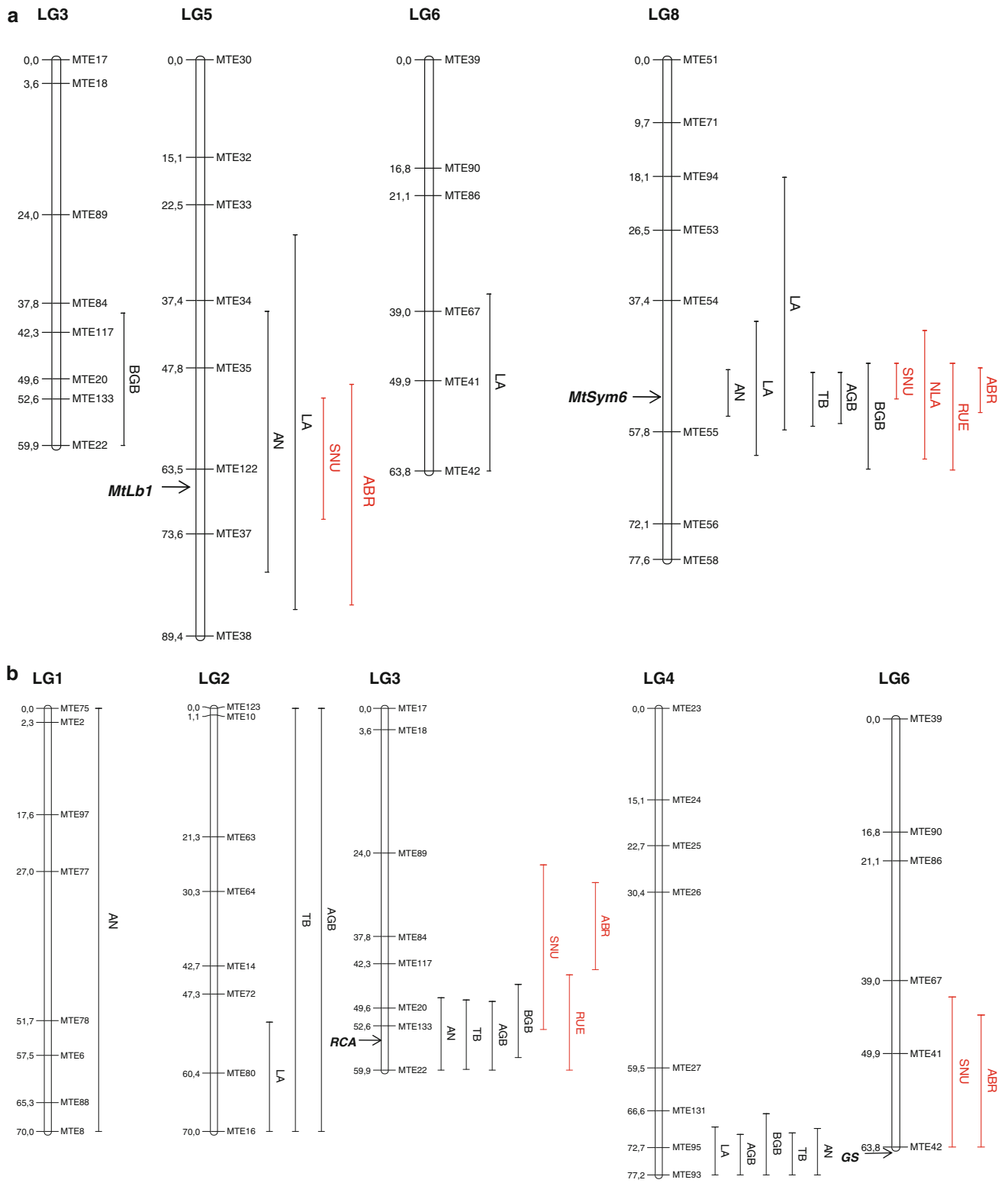

Fig. 3 LR4 linkage map and QTL position (*AGB* aboveground biomass, *BGB* belowground biomass, *TB* total biomass, *LA* leaf area, *AN* amount of N, *NLA* N to leaf area conversion efficiency, *RUE* radiation use efficiency, *ABR* aboveground:belowground biomass ratio, *SNU* specific N uptake). **a** Corresponded to low nitrate concentration in the nutrient solution. **b** Corresponded to high nitrate concentration in the nutrient solution. State variables and efficiency variables are represented, respectively, in *black and in red*. On *the left side* of the LG, the marker positions are indicated. On *the right side*, the marker names are indicated. The *vertical bars* indicate the position of the QTL with their interval confidence. Candidate genes are shown: *RCA* (rubisco-activase; Huguet, pers. comm.) implied in photosynthesis, *MtLb1* (Thoquet et al. 2002) and *MtSym6* (Tirichine et al. 2000) implied in symbiotic N_2 fixation; *GS* (glutamine synthetase)

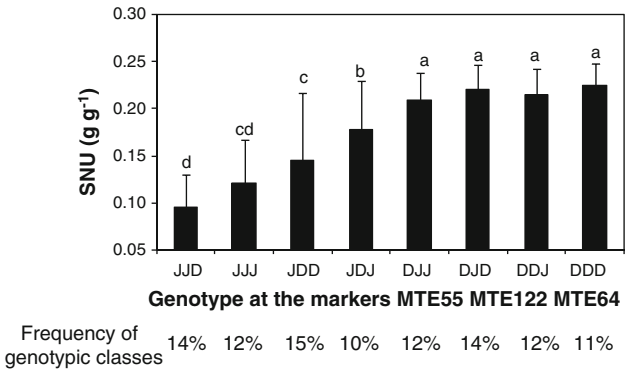

Fig. 4 Relationship between the genotype of the recombinant inbred line (RIL) at the different markers and the response of specific N uptake (SNU) at low nitrate concentration. Markers are, respectively, MTE55 on LG8, MTE122 on LG5 and MTE64 on LG2. The Jemalong A17 and the DZA315.16 alleles correspond, respectively, to J and D. *Error bars* are standard deviations. Mean values were classified using a Student–Newman–Keuls test ($P < 0.05$): *identical letters* indicate no significant differences between group of RIL. The *numbers* below the graph indicate the proportion of RIL for each combination of markers. Only 136 RIL were genotyped at the three markers

linked to the genetic constitution of the LR4 population. The main effect observed was that of a deleterious mutation preventing the plant from fixing N_2. It was difficult to compare our findings with the literature as only few papers to date have dealt with QTL in legumes in contrasting conditions of mineral N supply: Souza et al. (2000) and Tsai et al. (1998) in *Phaseolus vulgaris*. In both papers, the number of nodules was the only growth variable (related to N nutrition) that was measured. No information was given about both shoot growth and the amount of N in the plant. In accordance with our findings, a *Lotus japonicus* mutant, *Ljsym75*, which forms ineffective symbiotic nodules, has been characterized (Suganuma et al. 2003): no N_2-fixing activity was detectable in its nodules at any stage during plant development and, as in our study, plant growth was markedly affected.

Low and high nitrate treatments generated two different modes of N nutrition: mainly N_2 fixation and nitrate assimilation, respectively (Moreau et al. 2008). At low nitrate, most of the RIL experienced a strong N-shortage whereas it was not the case at high nitrate. This was suggested in this study by the higher values of total amount of N in the plant at high than at low nitrate treatment. It was confirmed by calculating a N nutrition index on the same dataset (Moreau et al. 2009). We assumed that this strong N-shortage at low nitrate impacted plant growth with dramatic consequences on all the variables used for phenotyping (both state and efficiency variables) due to the close correlation between plant N nutrition and plant growth traits demonstrated for many species (Gastal and Lemaire 2002). That was probably the reason why such differences were observed between the QTL of both nitrate treatments.

At low nitrate, one region on LG8 harbored QTL for all the studied variables. These were either state variables (e.g. leaf area, biomass, amount of N) or variables representing efficiency for acquiring resources (carbon and N). The possibility that a single gene on LG8 would regulate all variables was supported by the fact that allelic effects were in the same direction whatever the variable except NLA. However, NLA represents N dilution in the plant and is therefore negatively correlated with other growth variables.

A gene involved in the primary steps of N nutrition efficiency at low nitrate could display large and varied effects on plant phenotype. Indeed, a wide range of variability was observed among RIL in their capacity to acquire N at low nitrate (Moreau et al. 2009). As N_2 fixation is the main N source when nitrate concentration was low, this indicated a wide range of capacity to fix N_2 among the RIL. Due to the strong link between the amount of N and aboveground growth (Moreau et al. 2008, 2009), this wide range of capacity to fix N_2 among the RIL resulted in a wide range of aboveground biomass. As a consequence, data obtained at low nitrate did not allow the study of the genetic determinants of processes other than plant N_2 fixation. Heritability was lower at high nitrate than at low nitrate. Therefore, QTL detection at high nitrate was less effective. This result was in accordance with the weaker effects of QTL at high nitrate. According to Calenge et al. (2006), loci common to both nitrate treatments should encode key enzymes or control elements of carbon metabolism which are essential independently of the N status. Conversely, loci that are not stable across nitrate treatments reflect adaptation of metabolism due to the constraint. One QTL for belowground biomass located on LG3 was common to both nitrate treatments. Interestingly, the distribution of the RIL was poorly influenced by the nitrate treatment for this variable (Fig. 2a). It can be assumed that the belowground growth at low nitrate was relatively high (compared to shoot growth) in order to compensate the weakness of N uptake (mainly N_2 fixation).

For QTL on LG1, LG2 and LG4, other physiological functions (such as carbon assimilation via light interception) may have been determining phenotypes at high nitrate when N nutrition was not a limiting factor.

The use of efficiency variables increased the accuracy of QTL detection

At high nitrate, QTL for state variables were detected on four LG (Fig. 3b). The positioning of the QTL related to efficiency variables allowed the formulation of hypotheses concerning the function of the genes underlying detected chromosomal regions. For example the region at the bottom of LG3 contained QTL of RUE, aboveground, belowground and total biomass and amount of N. RUE

represents the efficiency of conversion of intercepted light to biomass and is consequently linked closely to photosynthesis. A gene involved in photosynthesis and/or energetic metabolism may underlie this cluster of QTL with consequences on biomass accumulation. Interestingly, a gene encoding a rubisco-activase (RCA), which modulates the activity of rubisco, a key photosynthetic enzyme which represents about 50% of the total protein content in leaves, is located near this cluster and so appears to be a good candidate gene. Similar conclusions could be drawn from co-localization of the QTL SNU on LG6 with a gene of glutamine synthetase (GS), an enzyme that plays an essential role in N metabolism.

When symbiotic N_2 fixation was the main N source (at low nitrate) (Fig. 3a), two main chromosomal regions were detected on LG5 and LG8. Interestingly on LG8, the confidence interval of the QTL for the efficiency variable SNU was smaller than that of the QTL for state variables. Moreover, the QTL of SNU on LG8 displayed the highest R^2. The use of efficiency variables (especially SNU) was also essential for positioning more precisely another QTL on LG5: the QTL for SNU was of higher effect than that of the QTL controlling leaf area and amount of N. This QTL seems to interfere with N_2 fixation but to a lesser extent than that on LG8. Moreover, the use of the efficiency variable SNU revealed a third QTL on LG2. This QTL was not found by simple QTL detection but required epistatic interaction detection, indicating that using the efficiency variable SNU allowed a more precise QTL detection than state variables. It has to be mentioned that detection of epistatic interactions was improved in this study using efficiency variables but this is not always the case when ratios are used for QTL mapping. Revealing epistatic interactions is generally regarded as depending on the size of the population under study and statistical power, whereas we have shown here that adapted phenotyping can also improve their detection.

N_2 fixation was controlled by two epistatic interactions

QTL in epistatic interaction were shown to control N_2 fixation at low nitrate: MTE55 locus on LG8 × MTE122 locus on LG5 on the one hand, and MTE55 locus on LG8 × MTE64 locus on LG2. The study of candidate genes strongly suggested that MTE55 and MTE122 corresponded to *MtSym6* and *MtLb1*, respectively (Tirichine et al. 2000; Thoquet et al. 2002). MTE55 showed a dominant epistatic effect: if a genotype carried the DZA315.16 allele, N_2 fixation was at its maximum value (high SNU), whatever the genotype on the two other loci (Fig. 4: Jemalong A17 and DZA315.16 alleles corresponded to J and D, respectively). However, if a genotype had the allele of Jemalong A17 at MTE55, it was MTE122 that determined the phenotype. *MtSym6* is known to influence the capacity for *M. truncatula* plants to create a symbiosis with a *R. meliloti* bacteria and to form efficient nodules (Tirichine et al. 2000). We used a different strain to the one described by these authors. However, if *MtSym6* corresponded to the QTL at MTE55, our results showed that the symbiotic response was similar with the *Rhizobium* strain 2011. When a genotype carried the Jemalong allele at MTE55 and the DZA315.16 allele at MTE122, SNU was decreased slightly (Fig. 4). *MtLb1* encodes a leghaemoglobin; a nodule-specific protein with a very high affinity for oxygen which supports the oxidative phosphorylation carried out by the N-fixing *Rhizobium* (Gallusci et al. 1991). The interaction between MTE55 and MTE122 and their possible correspondence with *MtSym6* and *MtLb1*, respectively, was interesting: it could indicate that when nodules have formed, the ability of the plant to supply enough oxygen might become limiting. In this context, a variation in the *MtLb1* gene could strongly influence N nutrition. In our study, if a genotype carried the DZA315.16 allele neither at MTE55 nor at MTE122, then a gene on LG2 (MTE64) determined the expressed phenotype. In this case, SNU was higher when the genotypes carried the Jemalong allele. The allele combination resulting in the lowest SNU consisted of the alleles of Jemalong at both MTE55 and MTE122 and the allele of DZA315.16 at MTE64. Additional experiments (for example association genetics studies or functional validation) will be necessary to show that these candidate genes are responsible for the different QTL.

48% of the RIL lied outside the range of variation of their parents for SNU. Transgressive segregation of parental characters can generally be explained by the fact that none of the parent cumulated the most favorable allele combination for all genes and/or by epistatic interaction between these genes. Two QTL with the strongest effects were detected for SNU and the most favorable alleles originated from the same parent (DZA315.16). This suggested that other genes with minor undetected effects could be involved in the control of this trait.

QTL detection was in accordance with QTL previously identified in the literature

The variables measured by Julier et al. (2007) are components of the more integrative variables measured in our study. For instance, the co-locations at high nitrate on LG4 suggested that the differences of plant leaf area among RIL in our study could be due to both growth (branch length) and development (internode number) processes. Co-localizations of QTL of belowground growth in *M. truncatula* with QTL related to roots in pea (Bourion et al. 2010) could be expected on the basis of their close phylogenetic similarity.

Conclusion

A wide phenotypic variation for plant growth was observed among the RIL of the LR4 population of *M. truncatula* (DZA315.16 × Jemalong A17). The use of a conceptual framework stemming from crop physiology allowed us to decipher the functional processes involved in the phenotypic variations. In particular, at low nitrate, the ability of RIL to fix N_2 determined their growth characteristics. The genomic regions underlying this variation were identified by QTL analysis. QTL are classically localized only on the basis of integrative traits. Through the measurement of traits that are easily accessible and compatible with large scale phenotyping, we showed that using efficiency variables that represented elementary physiological processes (efficiency for acquiring resources: carbon and N) enabled a more precise localization of QTL and the detection of supplementary loci. From a methodological point of view, a key result was that coupling a simple physiological framework and QTL detection enabled (i) enhancing the sensitivity of QTL detection, (ii) interpreting the role of the underlying loci and thus (iii) identifying the genetic determinants of plant environmental response. A novel result for *M. truncatula* was identification of two epistatic interactions in controlling plant N_2 fixation.

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