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ORIGINAL PAPER

Genetic dissection of nitrogen nutrition in pea through a QTL approach of root, nodule, and shoot variability

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Abstract Pea (*Pisum sativum* L.) is the third most important grain legume worldwide, and the increasing demand for protein-rich raw material has led to a great interest in this crop as a protein source. Seed yield and protein content in crops are strongly determined by nitrogen (N) nutrition, which in legumes relies on two complementary pathways: absorption by roots of soil mineral nitrogen, and fixation in nodules of atmospheric dinitrogen through the plant–*Rhizobium* symbiosis. This study assessed the potential of naturally occurring genetic variability of nodulated root structure and functioning traits to improve N nutrition in pea. Glasshouse and field experiments were performed on seven pea genotypes and on the 'Cameor' × 'Ballet' population of recombinant inbred lines selected on the basis of parental contrast for root and nodule traits. Significant variation was observed for most traits, which were obtained from non-destructive kinetic measurements of nodulated root and shoot in pouches, root and shoot image analysis, ^{15}N quantification, or seed yield and protein content determination. A significant positive relationship was found between nodule establishment and root system growth, both among the seven genotypes and the RIL population. Moreover, several quantitative trait loci for root or nodule traits and seed N accumulation were mapped in similar locations, highlighting the possibility of breeding new pea cultivars with increased root system size, sustained nodule number, and improved N nutrition. The impact on both root or nodule traits and N nutrition of the genomic regions of the major developmental genes *Le* and *Af* was also underlined.

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Introduction

Pea (*Pisum sativum* L.) is the third most important grain legume worldwide, after soybean (*Glycine max* L.) and common bean (*Phaseolus vulgaris* L.) (FAOSTAT 2007). The increasing demand for protein-rich raw material for animal feed or human nutrition has led to a greater interest in this crop as a protein source (Santalla et al. 2001). Selection for high yield, high seed protein concentration and early maturity has been undertaken by pea breeders to develop cultivars with superior performance. However, seed yield and protein content are very variable, mostly because of biotic and environmental stresses. In all crops, nitrogen nutrition is one of the key processes involved in determining yield. In legumes, N acquisition relies on two complementary pathways: absorption of soil mineral nitrogen and fixation of atmospheric dinitrogen through the plant–*Rhizobium* symbiosis. N_2 fixation supplies the major part of plant nitrogen but is complemented by N absorption at critical stages, especially when N_2 fixation decreases during seed filling. Ecophysiological studies on legumes have established a relationship between respiration costs, which are mainly associated with energy demand for N_2 fixation in nodules, and C allocated to nodule and root structure (Voisin et al. 2003a). Nitrogen flux through symbiotic activity has been modelled as a function of C flux towards nodulated roots, which is independent of nitrate availability but depends on nodule biomass. From the vegetative phase

up to flowering, the developing nodules represent the largest carbon sink for the plant and N_2 fixation contributes to the largest part of N acquisition (Voisin et al. 2003b). Conversely, at the end of growth cycle when filling seeds become the largest carbon sink, N-fixing activity decreases. At this stage, exogenous N supply relies on N assimilation by roots and should be limiting if the root system is too small (Bourion et al. 2007).

We hypothesize that the genetic improvement of symbiotic efficiency and of nutrient acquisition by root systems can contribute to the improvement of legume crop performances and their stability. Due to the technical difficulty of studying the phenotype of root systems, the molecular determinants of root or nodule development are less understood than those for aerial plant parts. However, the molecular basis of the early nodule development has made significant progress since the discovery of plant responses to bacterial signals, known as Nod factors (Dénarié and Cullimore 1993). Three genes controlling early steps of Nod factor signal transduction have been identified both in *Medicago truncatula* (Catoira et al. 2000; Ané et al. 2004; Endre et al. 2002; Lévy et al. 2004) and in pea (Edwards et al. 2007; Endre et al. 2002; Lévy et al. 2004). Nodulation is then regulated through principally a major systemic regulation pathway known as autoregulation of nodulation (AON), and mutants impaired in AON display a hypernodulating phenotype (for review see Oka-Kira and Kawaguchi 2006; Magori and Kawaguchi 2009). In pea, the hypernodulation phenotype is under shoot control for *sym28* and *sym29* mutants (Sagan and Duc 1996) but under root control for *nod3* mutant lines (Postma et al. 1988). Most of these hypernodulating mutants have a reduced shoot and root growth (Salon et al. 2001; Bourion et al. 2007; Novak et al. 2009), which may result from the competition for C between these different structures (Voisin et al. 2007), and/or from complex meristematic response to hormonal signals (Krusell et al. 2002).

A few experiments have investigated the spontaneous pea genetic variability of root development in young seedlings under controlled conditions (Ali-Khan and Snoad 1977; McPhee 2005) or in field experiments (Thorup-Kristensen 1998; Kraft and Boge 2001; Bourion et al. 2007). Up to now, this variability in pea has not been associated with any plant genes. However, several regulators of root development have been identified in *Medicago truncatula*, and most of them monitor a fine and complex tuning of root versus nodule development. They include (1) genes involved notably in auxin, cytokinins and ABA transduction pathway: *MtLax* gene is involved in local auxin transport and controls lateral root and nodule development (de Billy et al. 2001; Mathesius 2008), cytokinin signalling mediated by *MtCre1* gene regulates nodule and lateral root organogenesis in an opposite manner (Gonzalez-Rizzo et al. 2006; Frugier et al. 2008), whereas ABA can rescue the root but not nodule meristem defect observed in *latd* mutants of *Mt* (Bright et al. 2005; Liang et al. 2007), (2) genes involved in nitrate sensing and response: low mineral N condition is a prerequisite to allow nodule formation and function, whereas high levels of nitrate or ammonium inhibit nodule formation (Caroll and Mathews 1990; Barbulova et al. 2007). This inhibition of nodulation is primarily a localised response (Caroll and Mathews 1990), but also includes AON, as exemplified by the nodule maintenance in hypernodulating mutants grown under high NO_3^- (Caroll and Mathews 1990; Sagan and Duc 1996; Wopereis et al. 2000; Schnabel et al. 2005). Recently, the *Cle* gene in *Lotus japonicus* was proposed to produce a root derived signal, which drives *Har1* mediated autoregulation and nitrate inhibition of nodulation (Okamoto et al. 2009).

Quantitative trait loci (QTL) mapping has become a widespread approach to dissect the genetic determinism of many economically important complex traits in plant breeding. In pea, several genetic maps have been constructed using different types of markers, and genes of known function have been integrated into consensus maps (Weeden et al. 1999; Aubert et al. 2006). QTL for important traits in pea have been localised, including QTL for resistance to root diseases (Pilet-Nayel et al. 2002), QTL for frost tolerance (Lejeune-Hénaut et al. 2008; Dumont et al. 2009) or QTL of seed yield and protein content (Timmerman-Vaughan et al. 1996, 2005; Tar'an et al. 2004; Burstin et al. 2007). Most QTL for seed traits were shown to coincide with genes or QTL for aerial developmental traits, indicating either that the genomics regions associated with these QTL carry group of linked genes, or that single developmental genes underlying the QTL have pleiotropic effects on plant morphology, nitrogen source capacity and seed protein content and yield (Burstin et al. 2007). Identifying the genetics determinants of nitrogen source capacity may unravel the molecular basis of the seed traits QTL. A few QTL for nitrogen source capacity were mapped in cereals, and showed overlaps between QTL of plant nitrogen use efficiency or root architecture and QTL for seed yield (Tuberosa et al. 2002; Coque and Gallais 2006; Laperche et al. 2006).

In this study, we questioned the feasibility of improving nitrogen nutrition in legumes: is there a significant genetic variability for root and nodule development traits in pea ecotypes? Is there an antagonistic relationship between nodule and root developments? Can we identify root and/or nodule characteristics that are associated with seed nitrogen and biomass accumulation? To answer these questions, we characterised the genetic variability of the nodulated root compartment in pea and its relationship with nitrogen accumulation in the plant, in seven pea genotypes. Then, we identified QTL for these traits in a recombinant inbred line

(RIL) population derived from the cross between two genotypes with contrasted nodulated root development.

Materials and methods

Plant material

Seven genotypes, parents of RIL populations ('Ballet', 'Cameor', China, VavD265, K586, 'Sommette', 'Terese') were assessed in glasshouse to evaluate root and nodule traits describing their nitrogen acquisition structure. These genotypes were described by Baranger et al. (2004), and their seed protein content and weight determined by Burstin et al. (2007). The population RIL4 comprised 207 RILs deriving from a cross between 'Cameor' and 'Ballet'. 'Ballet' differs from 'Cameor' by lower seed protein content and by its semi-leafless type, due to the effect of A*f* gene.

Field trials

The population RIL4 was sown in two field experiments, on 3 March 2004 (Exp04f; 180 F6:8 RIL) and on 21 March 2006 (Exp06f; 153 F6:9 RIL harvested from the F6:8 RIL sown in 2004), at INRA-Dijon, Domaine d'Epoisses, Bretenière, France. At the sowing date, the ploughed layer (0–30 cm) of soil contained about 60 and 5 kg of N ha^{-1} in 2004 and 2006, respectively. Three weeks after sowing, 1 kg N ha^{-1} of ^{15}N labelled ammonium nitrate was applied, providing 1% atom excess. P and K fertilisation was performed during the preceding autumn. Irrigation was provided at the beginning and end of flowering to avoid any drought stress. In these field trials, with two replicates in 2006 and one in 2004, each plot consisted in a 30-pea plant row grown on trellises and samples of ten pea plants per plot were harvested when seeds had ripened.

Glasshouse experiments

Four glasshouse experiments were carried out successively in February 2005 (Exp05a), December 2005 (Exp05b), February 2006 (Exp06) and February 2007 (Exp07). Plants were grown in controlled temperature (20°C/15°C) in a 16-h day-night cycle and under a mean photosynthetically active radiation (PAR) of 170 µmol photons m^{-2} s^{-1} guaranteed by high-pressure sodium lamps when daylight was declining. These experiments were performed in pots or in pouches, with surface sterilized seeds, in sterilized substrates inoculated with a cell suspension of a *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) (ca. 10^8 per seed; Smith and Wollum 1989; Lira Junior et al. 2005) and supplied with a low nitrate content (2.5 mM) nutrient solution for optimal root and nodule development. When grown in pots, plants were grown in 7-l pots filled with a 1:1 (v/v) mixture of sterilized atapulgite and clay balls (2–6 mm diameter). Inoculation was applied just after sowing. The sowing density was of four seeds per pot, but only the three most homogeneous seedlings over the four were kept after emergence. When grown in pouches, 15 seeds by genotype were firstly left to germinate in plastic boxes filled with one litre of 4% (w/v) Kalys agar HP 696 gel, during 4 days, and then the three most homogeneous seedlings, with taproot length of about 3 cm long, were transferred into sterilized growth pouches and inoculated the day after. Each pouch consisted on a transparent plastic bag of 19 cm in width and 21 cm in length, containing a wick paper forming a trough in which the seed was placed (Fig. 1). All pouches were covered with opaque paper. They received 50 ml of the low nitrate content nutrient solution, which was replaced twice a week. The solution had an initial pH of 6.5 (Novak et al. 2002), and whatever the pouch, a final pH between 6.3 and 6.5 when renewed after 3 or 4 days in contact with the root.

Exp05a was set up to assess the genetic variability of the nodulated root development in seven genotypes ('Ballet', 'Cameor', China, VavD265, K586, 'Sommette', 'Terese'). Seeds were sown in pots and inoculated with *Rlv* strain 1007 (Sagan and Duc 1996; Laguerre et al. 2007) in a three-block randomised design, with four pots per genotype in each block. Plants were harvested for measurements at four successive stages: the 4-leaf, 9-leaf, beginning of flowering (BF) and beginning of seed filling (BSF) stages. In order to assess the effect of pots and pouches growing conditions as well as of the *Rlv* strain, Exp5b was performed with genotypes 'Ballet', 'Cameor', and VavD265 grown both in pots and pouches, and inoculated using either *Rlv* strain 1007 or P221 (Tricot et al. 1997; Laguerre et al. 2007). Plants were harvested for measurements at BF. The two following experiments were carried out in order to map QTL of nodulated root development in the mapping population RIL4. In Exp06, seeds of 153 F6:9 RIL and of their two parents were sown in a two-block randomized design, with one pot per RIL and six pots per parent in each block. In Exp07, after measurement of their tap root length (TRootLd0), seedlings of 117 F6:9 RIL and of their two parents were grown in sterilized growth pouches, in a three-block randomized design, with one pouch per line and two per parent in each block. Seeds or seedlings were inoculated with *Rlv* strain P221 and plants watered throughout the experiment, with a nutrient solution supplemented with 2.5 mM ^{15}N labelled nitrate (1% ^{15}N). Plants were harvested for measurements at BF.

Root measurements

When plants were harvested, their root system was carefully spread onto a transparent sheet to minimise root

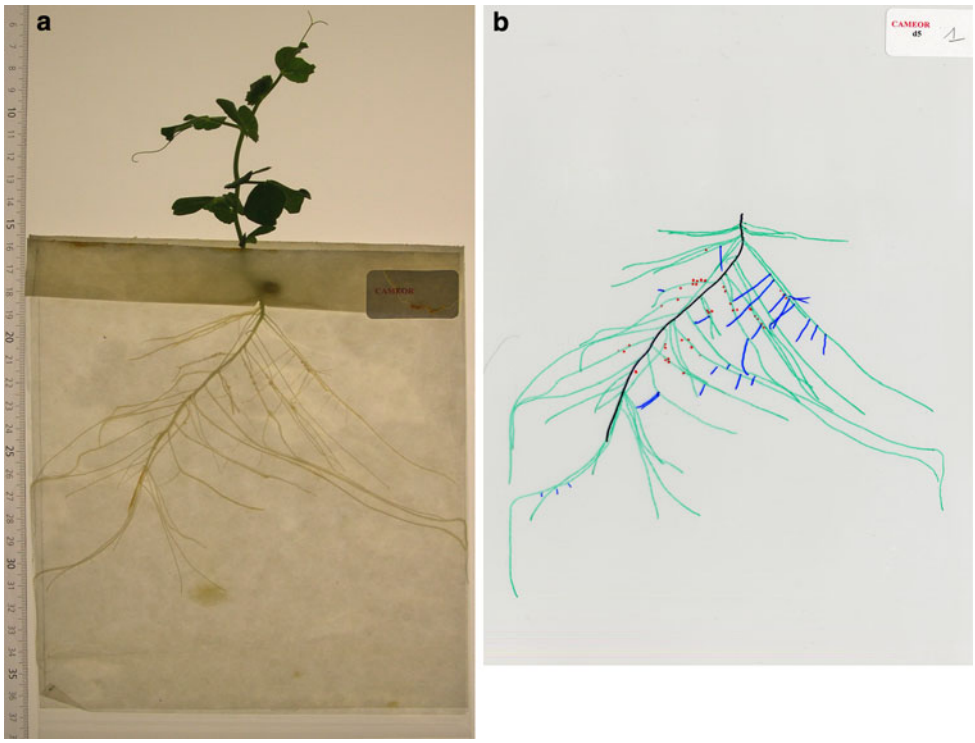

Fig. 1 Observation of pouch 15 days after seedling transfer into pouch (d5) **a** the pouch consists on a transparent plastic bag of 18 cm in width and 20 cm in length, containing a wick paper forming a trough in which the seedling was placed, **b** roots and nodules are drawn on a transparent sheet covering the pouch

overlapping and scanned as digital images with an A3 colour scanner (Epson; Tokyo, Japan). After image scanning, first order lateral roots and nodules were counted. Roots and nodules were then oven-dried at 80°C for 48 h, weighed separately, and their dry matter (RootB and NodB, respectively) and relative part of nodule upon belowground dry matter (NB:BGB) were determined. Total root length (TRootL) and nodule projected area (TNodPA) were further determined by image analysis using WinRhizo® Software (Regent Instruments, Quebec, Canada). Growth pouches also allowed for non-destructive observation throughout the Exp07. Twice a week, from date 1 until date 7 corresponding respectively to 2 and 27 days after the seedlings transfer in pouches, number of first order lateral roots (NLatRoot) and number of nodules (NNod) were counted. The increase of first lateral root number was linear between date 1 and date 4 (12 days after transfer), allowing the calculation of first order lateral root appearance rate (LatApR) within this period. First nodules appeared only at date 4, and the nodule appearance rate (NodApR) was calculated within the linear increase period between date 4 and date 6 (20 days after transfer). Roots and nodules were also drawn on a transparent sheet covering the pouch, in different colours according to the root order, and red indicated nodules (Fig. 1). After transparent sheets scanning, total root elongation rate (TRootER) and nodule projected area increase rate (TNodPAR) were calculated within the periods of linear increase. All these calculated traits were expressed as a function of cumulative °C/days from sowing, using a 0°C base temperature (Ney and Turc 1993).

Aerial part measurements

The date of beginning of flowering (BegFlo), Shoot length (ShootL), and number of basal branches (NBranch) were measured at harvest in all experiments. In all the glasshouse experiments, the main stem leaf number was measured throughout the vegetative period, allowing the calculation of leaf appearance rate (leafApR). Shoots were harvested and oven-dried for dry matter measurement (ShootB) and calculation of the relative part of belowground upon total dry matter (BGB:TB). Shoot nitrogen content (ShootNC) was estimated according to the Dumas method for glasshouse Exp05a, or by mass spectrometry (SOCHROM) for glasshouse Exp06 and Exp07, and field Exp04f and Exp06f. Shoot nitrogen accumulation (ShootQN) was then calculated. For glasshouse Exp06 and Exp07, and field Exp04f and Exp06f, the part of nitrogen accumulation derived from symbiotic fixation (NDFA) was calculated using the isotope dilution technique (Duc et al. 1988). In the glasshouse Exp06 and Exp07, specific nitrogen uptake (SNU) (Moreau 2007; Larigauderie et al. 1994) was estimated as the amount of total shoot nitrogen uptake per total belowground dry matter; root efficiency and respectively nodule efficiency (RootE and NodE) were calculated as the amount of shoot NO_3^- (respectively N_2) uptake per unit of

root (respectively nodule) dry matter (Voisin et al. 2007). In glasshouse Exp07, SPAD chlorophyll measurements (Minolta, Japan) were made on the three last expanded leaves of each harvested plant. Then, shoots were carefully spread onto a transparent sheet, the transparent sheets were scanned and analysed to determine total leaf area (LeafA), and thus estimate specific leaf nitrogen (SLN; Sinclair and Horie 1989) as total shoot nitrogen uptake per unit of leaf area. The leaf area was also estimated at successive dates, allowing the calculation of leaf area increase rate (LeafAR) and of radiation use efficiency (RUE) (Sinclair and Horie 1989; Kiniry et al. 1989).

Finally, in field Exp04f and Exp06f, the number of seed (SeedN), the straw, seed and total shoot dry matter (StrawB, SeedB and ShootB) and thousand seed weight (TSW) were measured at maturity. Straw and seed nitrogen content (StrawNC, SeedNC) were estimated by near-infrared spectroscopy, allowing the calculation of straw and seed nitrogen accumulation (StrawQN, SeedQN) and of total shoot nitrogen content and accumulation (ShootNC, ShootQN).

RIL4 map construction

The 207 F6:8 RIL were genotyped using microsatellite and gene framework markers chosen to cover the pea genome from Loridon et al. (2005) and Aubert et al. (2006). The genetic map was build from using the *near*, *try*, *ripple* and *map* commands of MAPMAKER/EXP version 2.0. The RIL4 map comprises 152 markers and covers 1140 cM (Fig. 5).

Statistical analyses

For each experiment, ANOVA were performed using the SAS GLM procedure (SAS Institute, 2000) to determine the significance level for genotype and block effects. The statistical model was: $Y_{ij} = \mu + g_i + b_j + e_{ij}$; where Y_{ij} is the value of the trait for genotype i in replicate j, μ the general mean, g_i the genotypic effect, b_j the replicate effect and e_{ij} the residual. Broad-sense heritability (h^2) was then calculated as $h^2 = \sigma_G^2 / [\sigma_G^2 + \sigma_R^2 / b]$, where σ_G^2 was the genotypic variance, σ_R^2 the error variance and b the number of blocks.

In glasshouse Exp05b, we tested the effect of the container, the *Rlv* strain, the genotype, as well as container × genotype and *rlv* strain × genotype effects. The analysis of variance did not show any significant *Rlv* strain effect or *Rlv* strain × genotype effect. Conversely, the effect of container (pot vs. pouch) was significant for all traits, and the effect of container × genotype interaction was significant for all traits except NLatRoot, RootB, TRootL and NodB (data not shown).

In glasshouse Exp06 and Exp07, and field Exp04f and Exp06f, ANOVA were performed to test for year, RIL, and RIL × year interaction effects. For QTL analysis, adjusted genotype mean values were obtained for each experiment, using the lsmeans command of the SAS GLM procedure. For QTL analysis for traits measured at flowering in the two glasshouse experiments, corrected genotype mean values were the residuals of the following model: $Y_{ij} = \mu + \mathrm{BegFlo}_{ij} + b_j + e_{ij}$; where BegFlo_{ij} is the beginning of flowering date for genotype i in replicate j. QTL were located using the composite interval mapping and iterative QTL mapping method (iQTLm) performed in MCQTL software (Jourjon et al. 2005). Cofactors were selected by forward regression and QTL were searched, both using F tests. F thresholds were determined for all traits by 1,000 permutations test, for a global genome-wide type I risk of 10% for cofactor selection and of 5% for QTL detection. Mean F values over the traits of 11.3 (equivalent to LOD = 2.4) and of 12.9 (equivalent to LOD = 2.8) were used for cofactor selection and for QTL detection, respectively. Allelic effect at each QTL and individual R^2, which represents the percentage of phenotypic variance explained by each QTL, were determined for all traits using MCQTL software. A global R^2 was also calculated for each trait by the multiple QTL model developed in iQTLm; its represents the percentage of phenotypic variance explained by all detected QTL. The global R^2 value was used to estimate p, the proportion of genotypic variance (σ_G^2) explained by all detected QTL, as p = global R^2/h^2 (Charcosset and Gallais 1996; Melchinger et al. 2000). Pearson genetic correlation coefficients between traits were calculated using XLSTAT software (version 2006.4, http://www.xlstat.com).

Results

Variability of root and nodule structure among seven pea genotypes and its relation with nitrogen accumulation

Root and nodule traits describing the nitrogen acquisition structure were measured for seven pea genotypes at four successive stages. A significant genotype effect was observed whatever the stage, on the number of nodules (NNod), nodule biomass (NodB), total nodule projected area (TNodPA), number of lateral root (NlatRoot), total root length (TRootL) and root biomass (RootB). Genotypes showed contrasted kinetics of root and nodule development (Fig. 2): in 'Cameor', NNod, NodB and RootB displayed a particularly fast increase at the beginning of the growth cycle, but rapidly reached their maximum at the 9-leaf stage; in 'Sommette', NNod, NodB, TNodPA and RootB were slow and remained limited until the end of the experiment; conversely, genotype K586

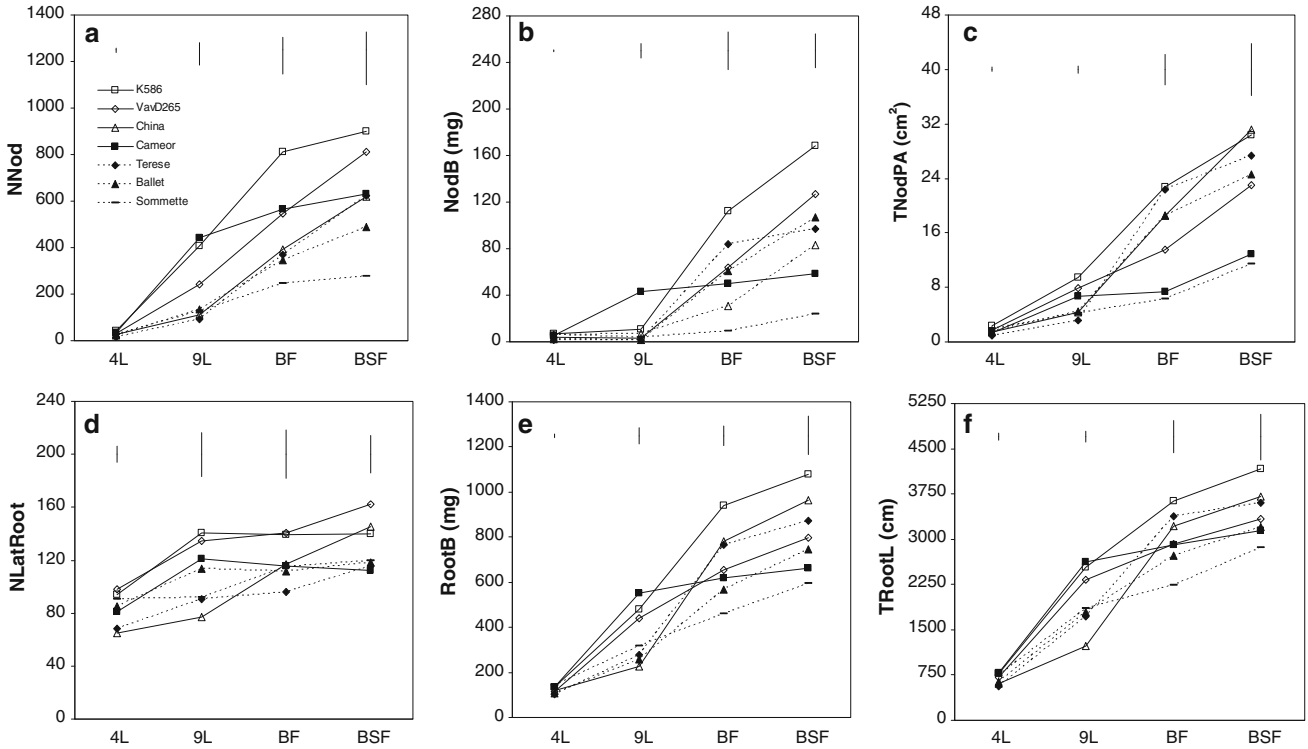

Fig. 2 Nodule number (**a**), nodule biomass (**b**), total nodule projected area (**c**), number of first order lateral roots (**d**), root biomass (**e**) and total root length (**f**) per plant, for seven pea genotypes, from the 4-leaf stage until the beginning of seed filling. 4L, 9L, BF and BSF indicate the developmental stage and mean 4-leaf stage, 9-leaf stage, beginning of flowering and beginning of seed filling, respectively. Each point is the mean value of three replicates. *Vertical bars* represent LSD ($P < 0.05$). *Open* and *solid symbols* indicate *Le* and *le* genotypes, respectively. *Full* and *dotted lines* indicate *Af* and *af* genotypes, respectively

showed a fast and prolonged increase in NNod, TNodPA as well as in RootB and TRootL. The *Le* and *Af* genes, which respectively control the internode length and the semi-leafless trait, are segregating among the seven genotypes (Fig. 2). Both *Le* and *Af* genes had a significant effect on all root and nodule traits, at one stage or another, with higher values for *Le* or *Af* genotypes than for *le* or *af* ones (Supplemental Table 1). Among the *le* genotypes, 'Cameor' and 'Ballet' presented contrasted phenotypes both on rate of NNod appearance and on NodB, RootB and TRootL increase (Fig. 2).

Shoot nitrogen accumulation (ShootQN) was significantly correlated with RootB from the 4-leaf stage until the beginning of flowering (BF), but neither with NLatRoot nor TRootL (Table 1). RootB was highly correlated with TRootL and to NLatRoot only at the 9-leaf stage. ShootQN was highly correlated with NNod and NodB at the 9-leaf stage, and with TNodPA from BF to the BSF. NodB was significantly correlated with NNod whatever the stage, and with TNodPA at BSF. Lastly, RootB and NodB were significantly correlated with each other at all stages except the 9-leaf stage, and NNod was significantly correlated with the TRoot at all stages except BF, and with NLatRoot only at the 9-leaf stage.

Variability of root and nodule structure in the 'Cameor' × 'Ballet' recombinant inbred population

Similar nitrogen acquisition structure traits were recorded in the 'Cameor' × 'Ballet' recombinant inbred line population (RIL4) during two glasshouse experiments: in 2006, plants were grown in pots and in 2007, plants were grown in pouches. Except for the number of lateral roots at BF, a highly significant effect of the genotype was detected for all root and nodule traits, included the number of lateral roots and the NNod as soon as their appearance date, which was respectively about 2 days (d1) and 12 days (d4) after the seedlings transfer in pouches (Supplemental Table 2). Heritabilities were moderate to high, up to 0.88 and 0.77, for root and nodule traits, respectively. 'Ballet' had slightly higher values than 'Cameor' for most of the root traits, whereas nodule number and biomass were slightly lower for 'Ballet' than for 'Cameor'. Compared with the parental values, transgressive segregants were observed for all traits in the two experiments (Fig. 3, 4; Supplemental Fig. 1). The effects of the experiment and of genotype x experiment interaction were also highly significant for all traits analysed ($P < 0.0001$) except NLatRoot. For all traits but NLatRoot, mean and range values differed markedly

Table 1 Pearson correlation coefficients between root and nodule structure traits and shoot nitrogen accumulation recorded on seven pea genotypes at four successive stages

Stage	Trait	TRootL	RootB	NNod	TNodPA	NodB	ShootQN
4-leaf	NLatRoot	0.62	0.45	0.57	0.75*	0.25	0.09
	TRootL		0.87**	0.77*	0.51	0.62	0.66
	RootB			0.69	0.60	0.86**	0.75*
	NNod				0.74	0.75*	0.16
	TNodPA					0.70	0.05
	NodB						0.42
9-leaf	NLatRoot	0.92**	0.79*	0.77*	0.89**	0.27	0.60
	TRootL		0.90**	0.82*	0.80*	0.43	0.71
	RootB			0.95**	0.80*	0.72	0.92**
	NNod				0.83*	0.77*	0.88**
	TNodPA					0.31	0.70
	NodB						0.81*
Beginning of flowering	NLatRoot	0.00	0.30	0.71	−0.02	0.28	−0.21
	TRootL		0.81*	0.59	0.70	0.82*	0.72
	RootB			0.72	0.78*	0.76*	0.76*
	NNod				0.30	0.74	0.25
	TNodPA					0.73	0.96**
	NodB						0.72
Beginning of seed filling	NLatRoot	0.52	0.49	0.60	0.47	0.53	−0.01
	TRootL		0.70	0.78*	0.48	0.46	−0.04
	RootB			0.74	0.92**	0.79*	0.57
	NNod				0.59	0.86**	0.26
	TNodPA					0.75*	0.79*
	NodB						0.59

*, ** Significant correlation at the 0.05 and 0.01 probability level, respectively

between the two experiments, with about threefold higher mean values in pots than in pouches. Nevertheless, TRootL, RootB, NNod, TNodPA and NodB measured in 2006 were highly significantly correlated with the same traits measured in 2007 (Supplemental Table 3). Whatever the experiment, NodB was significantly correlated with both NNod and TNodPA, whereas TRootL and RootB were highly correlated together ($r^2 > 0.6$), and both significantly correlated with all the nodule traits. Conversely, NLatRoot was neither highly correlated with RootB nor with nodule traits.

Variability of traits related to N acquisition efficiency, N and C accumulation in the plant, and plant development in the 'Cameor' × 'Ballet' recombinant inbred population

Different measurements related to C (ShootB, StrawB, SeedB, TSW) and N accumulation in aerial parts (ShootNC, ShootQN, StrawNC, StrawQN, SeedNC, SeedQN), and traits related to nitrogen acquisition efficiency (NDFA, SNU, RootE, NodE) were determined. Some developmental traits (ShootL, Nbranch, BegFlo, LeafApR) were also recorded.

The effect of the genotype was significant for most traits, with generally higher broad-sense heritabilities (h^2) in glasshouse experiments than in field experiments (Supplemental Table 4). In all experiments, 'Ballet' had higher BegFlo, LeafApR than 'Cameor'. Concerning traits related to C accumulation, 'Ballet' had higher ShootB, StrawB, TSW, RUE and ShootL than 'Cameor', and lower SeedN, LeafA and LeafAR (Supplemental Table 4). Concerning N acquisition efficiency and accumulation, 'Cameor' had higher NDFA, SPAD, ShootNC, SeedNC and SeedQN, and lower StrawQN and NodE.

The effects of year and genotype × year interaction were highly significant for most traits. Higher mean values were observed in pots than in pouches, with about a fourfold increase of ShootB or ShootQN, and a twofold increase of SNU, RootE and NodE (Supplemental Table 5). In field experiments, higher StrawB and SeedB were observed in 2004 than in 2006, associated with higher StrawQN and SeedQN in spite of lower SeedNC. NDFA was noticeably lower in 2004 (Supplemental Table 5), which is consistent with higher soil nitrate content at sowing (Sagan et al. 1993; Voisin et al. 2002). ShootQN and SeedQN were highly significantly correlated with RootB and TRootL measured at BF in pot or pouch experiment, and only in some cases with NLatRoot (Table 2). ShootQN and SeedQN were also significantly correlated with all the nodule traits and NDFA, and with ShootB, StrawB or SeedB.

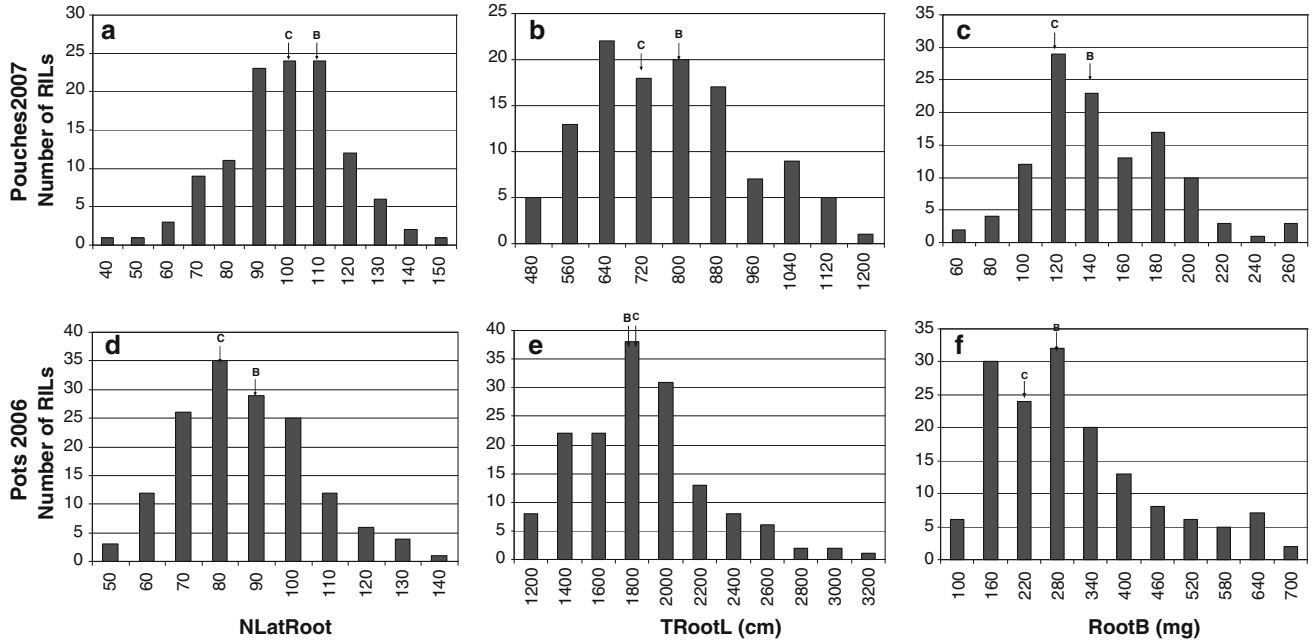

Fig. 3 Frequency distribution of RIL4 population for number of first order lateral roots (**a, d**), root biomass (**b, e**) and total root length (**c, f**) per plant, observed at the beginning of flowering in Pouches 2007 and Pots 2006 glasshouse experiments. *Arrows* indicate the mean value of the parental lines: *B* 'Ballet', *C* 'Cameor'

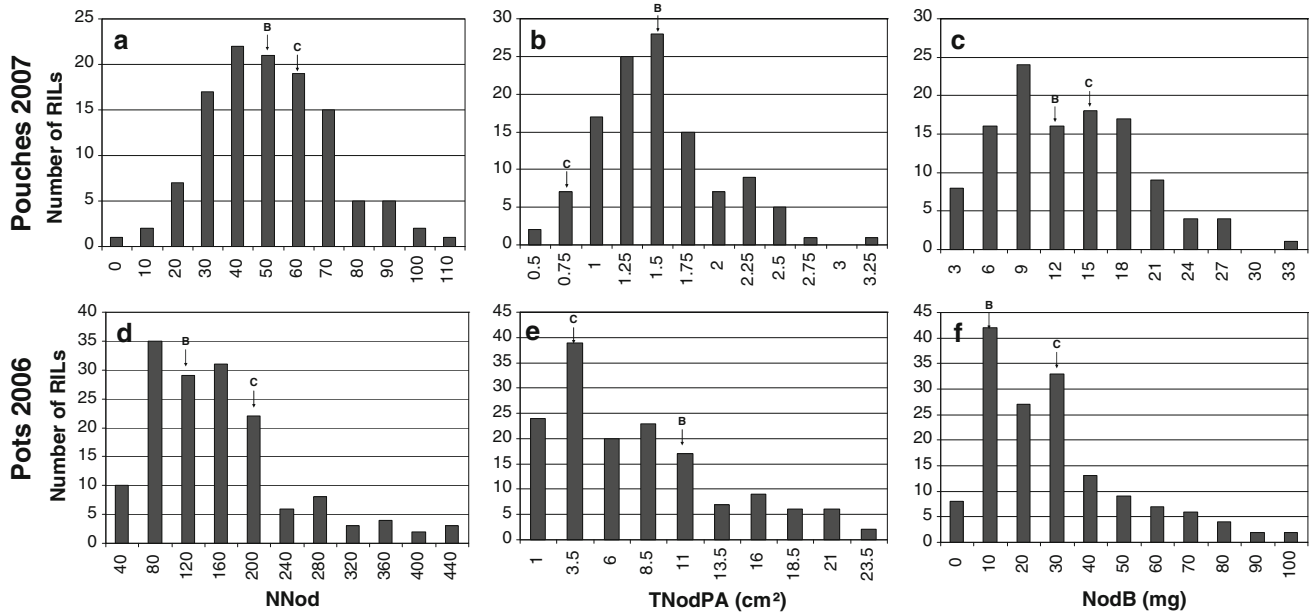

Fig. 4 Frequency distribution of RIL4 population for nodule number (**a, d**), nodule biomass (**b, e**), total nodule projected area (**c, f**) per plant, observed at the beginning of flowering in Pouches 2007 and Pots 2006 glasshouse experiments. *Arrows* indicate the mean value of the parental lines: *B* 'Ballet', *C* 'Cameor'

Conversely, ShootQN and SeedQN were hardly ever correlated with ShootNC, SeedNC, RootE and NodE. Lastly, ShootQN and SeedQN were significantly negatively correlated with SLN and RUE, whereas they were significantly positively correlated with leafA and LeafAR.

Mapping QTL for root and nodule structure

A total of 32 QTL was detected for root traits on six linkage groups (LG): 8 were related to number of lateral roots, 21 to root length, and 3 to root dry matter (Supplemental

Table 2 Pearson correlation coefficients between shoot or seed nitrogen accumulation and nitrogen acquisition functioning or carbon accumulation traits recorded in RIL4 population in four experiments

Trait	ShootQN-07	ShootQN-06	SeedQN-06f	SeedQN-04f
NLatRoot-07	0.33***	0.12	0.13	0.24*
NLatRoot-06	0.21*	0.04	0.21*	0.15
TRootL-07	0.77***	0.70***	0.49***	0.32**
TRootL-06	0.59***	0.72***	0.40***	0.25*
RootB-07	0.73***	0.72***	0.41***	0.29**
RootB-06	0.65***	0.88***	0.43***	0.27**
NNod-07	0.47***	0.34***	0.13	0.24*
NNod-06	0.52***	0.75***	0.39***	0.27**
TNodPA-07	0.67***	0.62***	0.35***	0.19
TNodPA-06	0.65***	0.91***	0.39***	0.31**
NodB-07	0.60***	0.38***	0.18	0.03
NodB-06	0.50***	0.82***	0.40***	0.33***
ShootB-07	0.83***	0.75***	0.35***	0.22*
ShootB-06	0.64***	0.97***	0.44***	0.30**
StrawB-06f	0.32**	0.43***	0.81***	0.40***
StrawB-04f	0.20*	0.38***	0.35***	0.62***
SeedB-06f	0.31**	0.47***	0.98***	0.44***
SeedB-04f	0.11	0.27**	0.46***	0.98***
NDFA-07	0.61***	0.40***	0.02	−0.03
NDFA-06	0.09	0.29**	0.26**	0.28**
NDFA-06f	0.21*	0.41***	0.64***	0.35***
NDFA-04f	0.18	0.38***	0.28**	0.52***
ShootNC-07	−0.07	−0.40***	−0.21*	−0.20*
ShootNC-06	−0.13	−0.13	−0.07	−0.07
StrawNC-06f	0.09	−0.10	−0.40***	−0.37***
StrawNC-04f	0.21*	0.10	−0.13	−0.53***
SeedNC-06f	−0.12	−0.14	0.21*	0.08
SeedNC-04f	0.13	0.18	0.07	0.36***
RootE-07	−0.21*	−0.42***	−0.15	−0.19
RootE-06	0.05	0.07	−0.12	−0.15
NodE-07	−0.12	−0.02	−0.03	−0.06
NodE-06	−0.08	−0.15	−0.27**	−0.14
LeafA-07	0.79***	0.72***	0.42***	0.28**
LeafAR-07	0.48***	0.48***	0.37***	0.36***
RUE-07	−0.60***	−0.57***	−0.33**	−0.13
SLN-07	−0.15	−0.41***	−0.35***	−0.28**

*, ** and *** Significant correlation at the 0.05, 0.01 and 0.001 probability level, respectively

Table 6). Concerning the nodule traits, a total of 26 QTL were detected on five linkage groups; 9 were related to nodule number, 8 to nodule area, 4 to nodule dry matter, and 3 to the relative part of the nodule dry matter. Five QTL for the relative part of belowground upon total dry matter (BGB:TB) were also detected. Seven of the 32 root QTL and 11 of the 26 nodule QTL were detected in region of LGI close to the *Af* gene (LGI-*Af*). All showed a positive additive effect of the 'Cameor' allele with parts of the phenotypic variation (R^2) explained by the QTL ranging from 9 to 49%.

The other QTL for root or nodule traits were located on fourteen other genomic regions, with R^2 ranging from 10 to 20%. Some clusters were observed (Fig. 5): nodule and root traits QTL were detected in LGII close to the marker Dioxase, with a negative additive effect of 'Cameor'; nodule traits QTL were found in LGIII near the marker AAP1; six of the eight QTL for lateral root number and three QTL for root length were detected in the same region of LGIII, near the marker AA374, with a negative additive effect of 'Cameor'; several QTL for nodule traits were found at LGIII near marker AB139, with a negative effect of

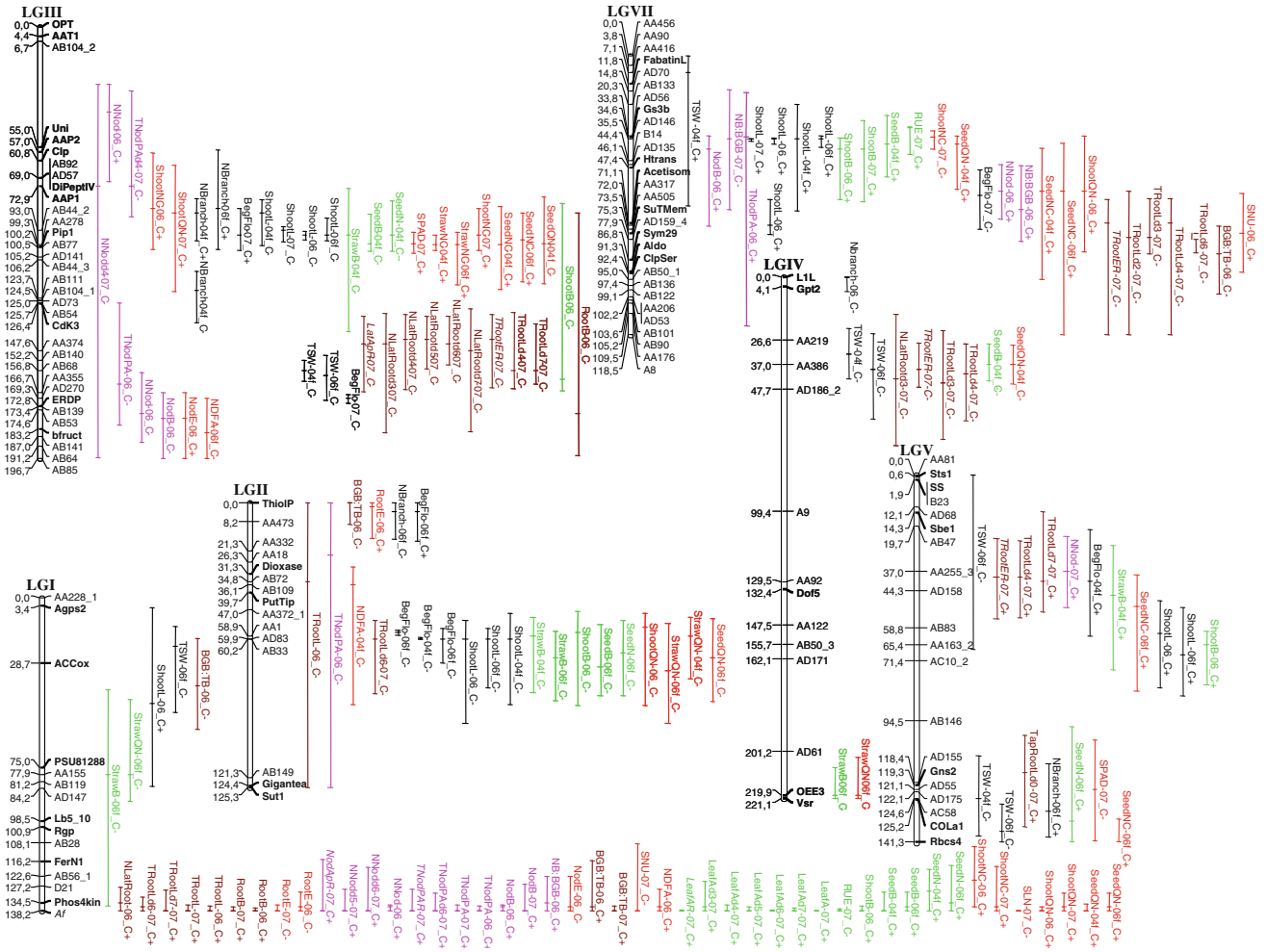

Fig. 5 Map position of QTL associated with nitrogen acquisition structure or functioning, carbon accumulation in aerial part or seed yield components, and nitrogen accumulation or content; in the pea population RIL4, in two glasshouse (07, 06) and two field experiments (06f, 04f). For each QTL, the name of the trait, the number of the experiment, and the sign of the additive effect of the parental 'Cameor' allele are indicated

'Cameor' allele; root traits QTL were found on LGIV near marker AA386, with a negative effect of 'Cameor' allele; root and nodule traits QTL were found on LGV near AD158, with a positive effect of 'Cameor' allele; on LGVII, three QTL for nodule traits were found near Gs3b, and two other QTL for nodule traits were found between Htrans and Acetisom, with a positive effect of 'Cameor' allele, whereas five different QTL for root length with a negative effect of 'Cameor' allele were detected between AD159_4 and Sym29.

Co-location of QTL for root and nodule structure with QTL for N acquisition efficiency, N and C accumulation in plant, and plant development

Ten QTL for N efficiency traits were detected (Supplemental Table 6): seven QTL related to the N acquisition efficiency (SNU, RootE, NodE), and three QTL for the percentage of N derived from fixation (NDFA). All of them mapped to genomic regions involved in root or nodule variation (Fig. 5). Five of them were detected in the genomic region LGI-*Af*, and their R^2 ranged from 9 to 21%, with a positive additive effect of the 'Cameor' allele for NDFA, and negative additive effect for all other N acquisition efficiency traits. Co-locations of QTL of N efficiency with root and nodule traits were also observed in four other genomic regions, with negative effect of the 'Cameor' allele for all the NDFA QTL and positive effect for all the efficiency QTL. Each of these QTL accounted for 8–12% of the phenotypic variation.

A total of 26 QTL related to C accumulation in aerial parts (LeafA, LeafAR, ShootB, StrawB, SeedB), 15 QTL for seed yield components (SeedN, TSW) and 3 QTL for RUE were detected (Supplemental Table 7). Sixteen QTL for N accumulation in the plant (ShootQN, StrawQN, SeedQN, SLN) and 15 QTL for N content (ShootNC,

StrawNC, SeedNC) and SPAD were detected (Supplemental Table 8). Twenty of these QTL were detected in the LGI-*Af* region (Fig. 5), with positive additive effect of the 'Cameor' allele for all traits, except RUE. QTL for RUE and for ShootB-06 accounted for, respectively, 76 and 42% of the phenotypic variation of these traits measured at BF. For StrawB, SeedB and SeedN, which were measured at harvest, R^2 ranged from 10 to 17%. Co-locations of N and C accumulation with root and/or nodule QTL were also observed on LGII-AB33, LGIII-AAP1, LGIV-AA386, LGV-AD158, LGVII-Gs3b and LGVII-Htrans (Fig. 5). A close inspection of LOG curves revealed a secondary peak for root and nodule traits near LGIII-AB44_2 (100 cM), which co-located with shoot and seed N accumulation traits (Supplemental Fig. 2). Interestingly, LGIII-AA44_2 was the only region in which QTL for SeedQN co-located with QTL for SeedNC, and they displayed antagonistic effects. Otherwise, whatever the genomic region, QTL for SeedQN always co-located with SeedB, with never antagonistic effects.

Most of the QTL related to aerial part or to seed N and biomass accumulation were consistent across experiments, and many correspond to genomic regions that we also identified in the pea mapping population RIL1 (Burstin et al. 2007). Among them, LGI-*Af* was contributing in both RIL4 and RIL1 to almost all the traits evaluated. Three other genomic regions in RIL4 involved in C and N accumulation in aerial parts and seeds (LGII-AB33 for ShootQN and flowering; LGIII-AB44_2 for StrawB, StrawNC, SeedN and flowering; LGVII-Gs3b for ShootB, ShootNC and SeedB) also correspond to genomic regions in RIL1. Moreover, four regions involved in the variation of TSW and/or SeedNC were located in similar regions in RIL1 and RIL4 (LGIII-AA374, LGIV-AA386, LGV-AD158, LGV-Rbcs4). Moreover, the root length QTL near LGVII-AD159_4 may correspond to the region of marker Amy associated with RootB reported by Weeden and Moffet (2002).

Discussion

This study was carried out to identify the genetic determinants of the nitrogen nutrition in pea, which could be involved in the determinism of seed yield and protein content. Some studies identified major gene effects for root biomass in pea (Weeden and Moffet 2002; Kof et al. 2006), and some searched for genomic regions involved in nodule number or biomass on common bean and soybean (Nodari et al. 1993; Souza et al. 2000; Nicolas et al. 2006). To our knowledge, our study is the first integrated approach of the genetic basis of nitrogen nutrition in legumes investigating not only QTL involved in nodulated root structure and functioning variability, but also those related with C accumulation variability, which is known to be closely linked to nitrogen acquisition capacity (Voisin et al. 2003a). We used a set of different methods, ranging from root washing at specific stages, kinetic measurements of roots and nodules in pouches, to ^{15}N quantification experiments in the field, in order to approach the different facets of the nodulated root development as related to N nutrition. Our experiments in artificial conditions used *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) strains previously demonstrated to be efficient on a large range of pea genotypes (Laguerre et al. 2007), and our field experiments were conducted on a soil previously shown to contain *Rlv* populations efficient on a majority of pea accessions in our collection. Our study in pouches allowed an establishment of nodules on all plant studied, confirming that the conditions of high inoculation level and 50 ml of nutrient solution with pH at 6.5 were suitable for nodulation, in agreement with various experiments on legumes (Smith and Wollum 1989; Novak et al. 2002; Lira Junior et al. 2005). Both pouch and pot experiments in artificial conditions provided a range of measurements of good quality, as revealed by the high heritabilities obtained for root and nodule traits (Supplemental Tables 4, 9), in comparison with those obtained in a field experiment within a soybean RIL population (Kuang et al. 2005). Our glasshouse experiments allowed detection of QTL for most of root and nodule traits, explaining up to 68% of the genetic variance observed for these traits (Supplemental Table 9). High heritabilities were also obtained for C and N accumulation traits in such conditions, and QTL were detected, explaining similar part of genetic variance existing within the RIL population than those detected in field conditions (Supplemental Table 10).

Towards the improvement of N nutrition through root and nodule structure traits

As for other legumes, N nutrition in pea relies both on atmospheric N_2 fixation by nodules and on soil mineral N uptake by roots, and it is considered a major limiting factor of plant growth (Voisin et al. 2007). In pea, N-fixing activity increases during the vegetative phase up to flowering, concomitantly with the development of nodules which are then the largest carbon sink for the plant (Voisin et al. 2003b). Then, both symbiotic fixation and mineral N root absorption decline at the end of the growth cycle when filling seeds become the largest carbon sink (Jeuffroy and Warembourg 1991; Voisin et al. 2003a). One strategy for enhancing exogenous nitrogen supply late during the growth cycle could be to select pea lines with a prolonged period of nodule development, which would maintain their symbiotic N_2 fixation activity during seed filling. Another complementary strategy is to increase root development

before the BSF, in order to enhance exogenous nitrogen supply at later stages (Bourion et al. 2007). This would also contribute through deeper roots to water stress tolerance, and probably interact with root rot disease tolerance.

In this study, we assessed the potential of naturally occurring genetic variability for root and nodule traits to improve nitrogen nutrition in pea. A significant variability was observed for both root (RootB, TRootL, NLatRoot) and nodule traits (NodB, NNod, TNodPA), among seven contrasted pea genotypes and among a pea RIL population. Nodule traits exhibited tenfold variations or more (Fig. 2), depending on the stage at which the nodulated root system was observed. Root traits, with a twofold average variation, were less variable (Fig. 2). Then, we assessed the relationship among desirable traits, to identify possible antagonistic relationship between root and nodule development, and between root or nodule and shoot development. A complex interaction between hormonal and trophic factors determines the root-nodule-shoot development. Indeed, hypernodulating mutants were considered potential candidates for enhancing N_2 fixation through an increase in nodule number (Caroll and Mathews 1990). However, various studies have indicated that hypernodulating mutants did not accumulate more nitrogen than the wild line (Sagan et al. 1993; Salon et al. 2001; Bourion et al. 2007), and often displayed depressed shoot and root growth, probably due to high C costs for nodulation and N_2 fixation (Voisin et al. 2007). Gonzalez-Rizzo et al. (2006) also demonstrated that the cytokinin receptor *MtCre1* regulates nodule and lateral root organogenesis in an opposite manner. In the present study, root and nodule traits did not show antagonistic relationship both among the seven genotypes and the RIL4 population (Table 1; Supplemental Table 3). Conversely, nodule traits were highly positively correlated with TRootL and RootB, and to a lesser extent with NLatRoot. Consistently, the four common QTL controlling root and nodule traits showed additive allele effects of the same sign (LGI-*Af*, LGII-Dioxase, LGIII-AB139, LGV-AD158; Fig. 5). On the other hand, some QTL regions were specific of roots traits (LGIII-AA374, LGIV-AA386). This makes it possible to select simultaneously or separately for root and nodule traits.

We further identified that NodB was determined both by NNod (3 QTL in common; LGI-*Af*, LGIII-AB139, LGVII-Htrans; Supplemental Fig. 2) and by TNodPA (3 QTL in common; LGI-*Af*, LGIII-AB139, LGVII-Gs3b), which reflects nodule number and nodule size, respectively. Conversely, RootB was hardly ever correlated to NLatRoot (Table 1; Supplemental Table 3) and highly correlated to TRootL (with 2 QTL in common; LGI-*Af*, LGIII-AA374). TRootL was correlated both with TRootER (4 QTL in common; LGIII, LGIV, LGV, LGVII) and little with LatApR (1 QTL in common; LGIII-AA374). Interestingly, QTL for TRootER were co-located either with NNod (LGV-AD158) or with NLatRoot (LGIII-AA374, LGIV-AA386). As nodules as well as lateral root primordia form on elongating parts of the root (Tricot et al. 1997), this result suggests that some genomic regions may control either nodule or root initiation, but not both.

Root and nodule structures contribute to N acquisition and accumulation in the plant

All QTL for NDFA, which described N acquisition by fixation, corresponded to QTL controlling both root and nodule traits (LGI-*Af*, LGII-Dioxase, LGIII-AB139; Fig. 5, Supplemental Fig. 2). They displayed additive effects of same sign, indicating that an increase of NDFA is tightly linked to an increase of NodB and RootB. This result seemed different from what was observed in pea hypernodulating mutants (Sagan et al. 1993; Salon et al. 2001), for which increased NodB was associated with decreased RootB and NDFA (Bourion et al. 2007). However, all these results suggest that the N accumulation through fixation relies on a good development of both roots and nodules. Interestingly, QTL for NDFA were not always co-located with QTL for SeedQN, which is in agreement with previous observations of no significant effect of N symbiotic fixation level in seed N accumulation (Sagan et al. 1993; Voisin et al. 2002).

In most cases, shoot and seed N accumulation were correlated with root and nodule traits. As such, among the seven pea accessions, ShootQN was significantly correlated with RootB at early stages (from 4-leaf stage to BF), NNod at the 9-leaf stage, and TNodPA at later stages (from BF to BSF); Table 1). Among the RIL4 genotypes, ShootQN and SeedQN were significantly correlated with RootB, TRootL, NNod, and TNodPA and NodB (Table 2). Consistently, common QTL displaying effects of the same sign were found between root and/or nodule traits and ShootQN, SeedQN or SeedNC (LGI-*Af*, LGII-AB33, LGIV-AA386, LGV-AA158, LGVII-Htrans; Fig. 5). These results may suggest that genes controlling nitrogen nutrition structure traits are significant determinants of shoot and/or seed N accumulation. Conversely, these results may equally suggest that ShootQN accumulation, which is assumed to control the elaboration of leaf area and thus the C supply (Laperche et al. 2006; Moreau et al. 2009), promotes root and nodule establishment and growth.

In other cases, shoot or seed N accumulation did not appear to be directly correlated with root or nodule traits. As such, the genomic regions near LGIII-AB44_2 and near LGVII-Gs3b displayed QTL with opposite effects for ShootNC or SeedNC on one hand, and SeedB, SeedN and SeedQN on the other hand, but no strong effect QTL for root and nodule traits. As these genomic regions appeared to also control ShootL and BegFlo, they may correspond to

a QTL controlling N partitioning between seeds and aerial parts through plant development. Lastly, the QTL clusters for root elongation rate, root length at early stages, and thousand seed weight that were found at LGIII-AA374 and LGIV-AA386 may illustrate the link between seed cotyledon reserves and root growth during the heterotrophic phase. Indeed, Tricot et al. (1997) observed a rough decline of root elongation rate and of roots number, between the 4- and 6-leaf stages in conjunction with the exhaustion of seed reserves. Other experiments have confirmed the impact of seed size on root elongation rate, root length or root dry matter during the early growth of the plant (Thorup-Kristensen 1998; McPhee 2005). Thus, the QTL controlling thousand seed weight in these regions may have a pleiotropic effect on the root elongation rate. However, a higher root elongation rate and root length could conversely enhance seed storage compound accumulation at the end of the plant life cycle by a sustained water and nutrient supply, and hence increase thousand seed weight.

Root and nodule traits are impacted by major developmental genes such as *Le* and *Af*

In pea, *Le*, which encodes gibberellin 3b-hydroxylase, controls inter-node length whereas *Af* controls the switch between leaflets and tendrils. In a previous work, we showed that in pea, the genomic regions encompassing the developmental genes *Le* and *Af* have pleiotropic effects on plant morphology, source capacity, and seed protein content and yield (Burstin et al. 2007). Consistently, Weeden and Moffet (2002) showed a significant association between *Le* and root biomass, in 42 RIL derived from a cross between a *Pisum elatius* line and a pea cultivar, and Kof et al. (2006) observed a significant effect of *Af* on root biomass. The results obtained herein confirm and specify these findings. In seven pea accessions, we found a significant effect of the genes *Le* and *Af* on root and nodule development, at different stages of plant development (Supplemental Table 1). The effect of *Le* on root and nodule traits increased from the earlier stage (at 4-leaf stage, only nodule traits showed a significant effect of *Le*) towards the latest stage analysed (at BSF, all root and nodule traits showed a significant effect of *Le*). Conversely, the effect of *Af* on root and nodule traits decreased from the earlier stage (at 4-leaf stage, all traits showed a significant effect of *Af*) towards the latest stages analysed (at BSF, only NLatRoot and NNod showed a significant effect). In the RIL4 population, *Le* is not segregating, and neither QTL for seed N content and yield nor QTL for root and nodule development were detected in the corresponding genomic region, at the bottom end of LGIII. *Af* is segregating in the RIL4 population. A major QTL cluster was identified in the LGI-*Af* region, where *Af* is the best candidate gene for having pleiotropic effects on leaf area, nitrogen acquisition structure traits, and seed or shoot N accumulation traits. This region controlled 16% of the variation of leaf area 8 days after germination when one leaf was fully expanded, and accounted for more than 60% 20 days after germination when five leaves were fully expanded. It also controlled 75% of the variation of RUE, indicating a reduction of C accumulation in *af* genotypes. This could be the cause of the reduction of root and nodule growth. Indeed, QTL for root and nodule dry matter and for the relative part of nodule upon belowground dry matter were consistently detected at beginning of flowering, with a negative effect of the *af* allele for all these traits including the relative part of nodule upon belowground dry matter. This may indicate a greater impact of low carbon availability on nodules than on roots, which, according to Voisin et al. (2003b) represent the largest carbon sink for the plant during the vegetative stage up to flowering. Consistently, no QTL for TRootER was detected at the *Af* locus, whereas QTL for NodApR and TNodPAR were. This reinforces the hypothesis of a trophic control of this locus on nodule appearance and growth rather than on root elongation. The potentiality of this region for N nutrition improvement depends on the nature of the gene involved: if the gene *Af* is responsible for the N nutrition variation, then the usefulness of this locus will be limited since the *afila* trait is extremely desirable for lodging resistance. If the gene responsible for the variation is a gene close to *Af*, then the linkage may be broken. This question could be checked when the *Af* gene will be identified.

Conclusion

This study showed the usefulness of experiment in pouches combined with image analysis for investigating the nodulated root development and growth in a large number of plants. This methodology provided consistent results with those acquired in pots and facilitated the selection of contrasted accessions for root and nodule features. This information can be used as a valuable baseline for breeding programs. Using this methodology, we investigated the variability and relationship of nitrogen acquisition structure together and with C and N accumulation in the plant. We have found a significant positive relationship between nodule establishment and root system growth, which should allow building a pea nitrogen nutrition ‘ideotype’, with increased root system size and no decreased nodule number. We also specified the significant contribution of N acquisition structure to seed N content and yield. Our results point to regions of interest for root and nodule development. Because QTL associations described herein may be caused either by pleiotropic effects of one gene or by linkage between different genes, these regions will need

to be refined through fine-mapping and/or the use of association genetics, and through the comparison with another large-seed legume such as soybean or with the model species *Medicago truncatula*.

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