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ORIGINAL PAPER

Multi-population QTL detection for aerial morphogenetic traits in the model legume *Medicago truncatula*

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Abstract *Medicago truncatula*, as a model species, is useful to study the genetic control of traits of agronomic interest in legumes species. Aerial morphogenesis is a key component of forage and seed yield. It was measured in four mapping populations originating from five parental lines. Single and multi-population quantitative trait locus (QTL) detections were carried out. A large variation was observed within populations and transgressive segregation was noted. Most traits showed high heritabilities in all seasons. Length of primary branches (LPB, cm) was positively correlated to branch elongation rate (BER, cm day^{-1}) and aerial dry matter (ADM, g). Flowering time (FT, °C day^{-1}) showed negative correlations with length of main stem (LMS, cm) and BER. One hundred and forty-one QTLs for BER, LMS, FT, LPB, diameter of primary branches (DPB), number of primary branches (NPB), number of nodes (NI) and ADM were identified and localized over all eight chromosomes. Single and multi-population analyses showed that the most important regions for aerial morphogenetic traits were chromosomes 1, 2, 7 and 8. Multi-population analysis revealed three regions of major QTLs affecting aerial morphogenetic traits (LPB, LMS, NPB, BER and FT). A region involved in flowering time variation was revealed on chromosome 6 on a single population. These results were used to identify candidate genes that could control variation for aerial morphogenesis traits in this species and in related crop legume species.

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Introduction

Medicago truncatula is a Mediterranean legume which has an autogamous mode of reproduction, with a small genome (500–550 Mbp) (Young et al. 2005) and a short growth cycle. It belongs to the galegoid clade, as do most cultivated legumes. The synteny among species within this clade was shown to be high (Choi et al. 2004). Currently, large collections of diverse ecotypes of *M. truncatula* and a large amount of sequence data are available (http://www.medicago.org). These resources and other genomic tools such as large mutant populations, molecular markers, genetic maps, expressed sequence tag (ESTs) and TILLING (Targeting Induced Local Lesions in Genomes) populations for reverse genetics (Le Signor et al. 2009) are useful to implement translational genomics in related forage and grain legume crops (Young and Udvardi 2009).

The broad lines of research addressed to *M. truncatula* as a model legume initially concerned the symbiotic and

mycorrhizal symbioses (Ané et al. 2004), but extended to disease resistance (Ameline-Torregrosa et al. 2008) and abiotic stress tolerance (Narasimhamoorthy et al. 2007). A large genetic variation for aerial morphogenesis, a set of traits that is responsible for plant architecture and phenology, was recently observed in natural populations and in a mapping population (Julier et al. 2007). These traits contribute to biomass production of forage legumes and to seed production of grain legumes.

Aerial morphogenesis integrates a wide range of biological processes that involve multiple quantitative traits. Genetic variation for these traits is controlled by several genes located in genomic regions known as quantitative trait loci (QTLs) (Maloof 2003). Mapping QTL in a legume model plant for aerial morphogenetic traits can help to understand the genetic basis of variation in plant growth and to select genomic regions and candidate genes (with a fine mapping strategy) to improve biomass and seed yield in legume crop plants.

Regions that contain QTLs have been identified for vegetative and reproductive traits in several legume species: *Lotus japonicus* (Gondo et al. 2007), soybean (Zhang et al. 2004), white clover (Cogan et al. 2006), pea (Burstin et al. 2007) and alfalfa (Brummer 2004; Robins et al. 2007a, b). In *M. truncatula*, Julier et al. (2007) detected QTLs for vegetative and reproductive traits in one recombinant inbred line (RIL) population. A major QTL for flowering date and branch growth was found in chromosome 7. In this region, six candidate genes involved in flowering were proposed by Pierre et al. (2008), and among them, an homologue of Constans-like gene and a Flowering locus T gene involved in the regulation of flowering by day length (Michaels 2009). In addition, minor year-specific QTLs were found for vegetative traits. Photoperiod and temperature are the main environmental factors that regulate several aspects of plant growth and development. Responses of plant to these factors affect flowering date, bud set and branching pattern (Beveridge et al. 2003). *M. truncatula* flowering is promoted by long photoperiod and vernalization (Clarkson and Russell 1975). These responses are genetically regulated and can be modified by other parameters of the surrounding environment (Leyser 2003).

Most of the QTL studies were performed considering one inbred population. But using connected multi-parental crosses of maize increased the number of QTLs detected, the accuracy of QTL position estimates and allowed the identification of the parental origin(s) of favourable allele(s) at each QTL (Blanc et al. 2006). Such an approach provides more effective detection and evaluation of the effects of the QTLs and their stability. Billotte et al. (2010) showed the efficiency of the multi-parent mapping design in full-sib families with a small number of individuals for detection of QTLs related to vegetative growth and yield in oil palm. Using the multi-parent approach, a reduction of the support interval and a better location of a major QTL for flowering time were obtained in three connected populations of *M. truncatula* (Pierre et al. 2008).

In this study, we first present a description of the effects of photoperiod duration on aerial morphogenesis in eight genotypes of *M. truncatula* to better understand the phenotypic variations observed in different environments. The second objective was to identify QTLs from four connected RIL populations of *M. truncatula*. Data for aerial morphogenetic traits from these populations grown in different seasons and years were used to (1) evaluate the genetic variation among RILs, (2) identify genomic regions involved in trait variation for each population, (3) apply a multi-population analysis using the four connected populations to identify consistent genetic effects and (4) detect candidate genes located in the QTL regions.

Materials and methods

Effect of photoperiod

To describe the effect of photoperiod on aerial morphogenetic traits, eight lines were used. Five of them are parents of RIL populations: DZA45.5 and DZA315.16 from Algeria, F83005.5 from France, Jemalong6 and A20, two lines selected in natural populations (Penmetsa and Cook 2000, Pierre et al. 2008) from Australia. The three other lines originated from Tunisia (TN6.18) and Israel (Meiron and Levahim-B). They were grown in growth chambers, under two photoperiods, 12 and 18 h, applied without significant change in total energy supply, in a 6-block randomised design (see Pierre et al. 2008). The length of the first two emerging primary branches was measured twice a week, the flowering date (transformed in degree-days with a temperature basis of 0°C) was scored, and when all the plants had flowered, the number of primary branches (NPB) was counted, the diameter of the first two emerging primary branches and the length of the main stem (LMS) were measured. The dynamics of primary branch elongation over time was modelled by a beta function (Verdenal et al. 2008), and the estimated parameters were: the final length, the elongation rate and the duration of elongation. Analysis of variance was performed on the recorded traits and model parameters to test the effects of photoperiod, genotype and the interaction photoperiod × genotype, using PROC GLM of SAS software package version 8.1 (SAS Institute Inc. 2000).

QTL detection

Four RIL populations of *M. truncatula* involving a total of five parental lines were studied: LR4 (Julier et al. 2007),

LR5 (Ameline-Torregrosa et al. 2008), LR1 (Pierre et al. 2008) and LR6 (Table 1). Each population was composed of 173–233 lines. The populations were analysed for aerial morphogenetic traits in experiments conducted in greenhouse at Lusignan (France) in 2002, 2003, 2004, 2005, 2007 and 2008. The five parental lines involved in the four RIL populations showed differences in aerial morphogenetic traits. Jemalong6 had fewer but longer primary branches, a higher branch elongation rate (BER) and flowered earlier than F83005.5, DZA315.16 and DZA45.5 (Julier et al. 2007). A20 was the genotype that had the earliest flowering date (Pierre et al. 2008). The description of experiments during 2002, 2003, 2004 (for LR4) and 2005 (for LR1 and LR5) is specified in Julier et al. (2007) and Pierre et al. (2008). These experiments were similar to that used for LR6 and described below. Seeds of RILs of LR6 population and the five parental lines described above were scarified and sown into Petri dishes on 05 March 2007 and 07 March 2008, respectively, imbibed for 24 h at room temperature and vernalized at 4°C for 48 h. Germinated seeds were transplanted in individual pots on 04 April 2007 and 10 March 2008, respectively, and grown in a greenhouse at INRA Lusignan (France). All lines of LR4 in 2002 and 2003 were repeated three times but, in order to maximise the number of RILs under study, there was no repetition for LR4 in 2004, LR1, LR5, LR6, except for 15 RILs randomly taken and five parental lines. These 20 lines were repeated three times to check if the experiment was accurate and to calculate a rough estimation of heritability. Each repetition was composed of one plant. The plants were grown under natural day-length and received two applications of 20 ml of a 2% NPK solution, 1 month and 2 months after transplantation. The flowering time (FT) was recorded when a plant had one open flower on a primary branch, and transformed in degree-days with a temperature basis of 0°C. The length of the first two emerging primary branches (LPB) was measured twice a week during the growth period. When all the plants of the trial had flowered, the experiment was harvested (13 June in spring 2005 and 20 December in autumn 2005 for LR1 and LR5, 30 May 2007 and 20 May 2008 for LR6) and the following data was collected: LMS, NPB, aerial dry matter (ADM) weight. In addition, on the first two emerging primary branches, the diameter of primary branches (DPB) and the number of internodes (NI) were determined. The curve of branch elongation as a function of sums of degree-days showed a linear phase. For each RIL, BER was calculated as the slope of this linear phase. For LR4, NPB and LMS were not recorded in all years. In addition, ADM was measured as the weight of the two primary branches that were sampled.

Correlations between mean values of traits within each season were calculated over all the RILS of each population × season using the procedure CORR of SAS (SAS Institute Inc. 2000). The data sets of 15 RILS from LR1, LR5 and LR6 populations replicated three times were subjected to independent analyses of variance by season to test the effect of lines on the traits. Variances of lines (σ^2_L), considered as a random effect and error (σ^2_R) were estimated using PROC VARCOMP of SAS and broad sense heritability (h^2) for each trait was calculated as $h^2 = \frac{\sigma^2_L}{\sigma^2_L + \sigma^2_R/b}$, where b is the number of repetitions.

Framework maps made with SSR markers were available for populations LR4, LR5 (Ameline-Torregrosa et al. 2008), LR1 and LR6 (T. Huguet, unpublished). They included 62, 62, 60, and 61 markers and covered 576.1, 597.7, 575.2 and 614.7 cM, respectively. QTL mapping was performed using QTL Cartographer (Basten et al. 1994, 2002) with the composite interval mapping (CIM) procedure (ZmapQTL model 6) with a maximum of five background parameters (determined from SRmapQTL with the default F test at $P = 0.1$) and a window size of 10 cM. The threshold for adding a QTL, determined at 5% risk by a permutation test method (1,000 replications), was set to 11.33 (LOD $\geq$ 2.46). A QTL position was estimated where the LOD score reached its maximum in the region under consideration. The limits of the confidence interval of QTL position were estimated at the positions where the LOD value drop-off was equal to 1 (Lander and Botstein 1989). The software BioMercator (Arcade et al. 2004) was used to draw the QTLs on the map of each population.

To better estimate the position of the QTLs that were common to different populations and their effects, a multi-population QTL analysis was carried out with the MCQTL software package (Jourjon et al. 2005). First a consensus genetic map was built from the genetic maps of LR1, LR4, LR5 and LR6 populations by iterative projection of loci using BioMercator software. The LR4 map was used as a reference. The LR5 map was first projected on this reference LR4 map to produce a second map. Then the LR6 map was projected on this second map to produce a third map. Finally, the LR1 map was projected on this latter map to produce a consensus map. The most likely position of each QTL and its support interval on LR6, LR5 and LR1 maps were projected on the LR4 map on the basis of their relative distance to flanking common markers with a homothetic function.

In a second step, adjusted means of aerial morphogenetic traits per RIL (LR1, LR4, LR5 and LR6 populations) were calculated over all seasons using the procedure GLM of the SAS. The model included the effects of season (year or spring vs. autumn), genotype and replication nested within season. The parental lines that were present in all years or seasons were used to connect the designs.

Table 1 Characteristics of the RIL populations of *M. truncatula*

RIL population	Cross	Number of RILs	Generation	Season/year of evaluation
LR1	DZA315.26 × DZA45.5	233	F7	Spring 2005
				Autumn 2005
LR4	Jemalong6 × DZA315.16	199	F6	Spring 2002
				Spring 2003
				Autumn 2004
LR5	Jemalong6 × F83005.5	173	F7	Spring 2005
				Autumn 2005
LR6	Jemalong6 × A20	179	F5	Spring 2007
				Spring 2008

DZA315.16 and DZA315.26 are assumed to be identical, since they were obtained from the same population and appeared phenotypically identical (see Pierre et al. 2008)

Adjusted means (Blanc et al. 2006) and the consensus map were used to launch the multi-population QTL analysis with the "connected" option. Two groups of analyses were conducted, one for the traits that were recorded in the four RILs populations and one for traits that were recorded in three of them. For QTL detection, an additive connected model was chosen, with the iterative QTL mapping method (iQTLm) using genetic cofactors, and a windows size of 5.5 cM. Cofactor selection and test of QTL effects were performed with *F* test. *F* thresholds were determined with 1,000 permutations to correspond to a global type I risk of 1% (across all populations and the total genome).

Medicago genome sequencing website (http://www.medicagohapmap.org/?genome) indicates the BACs included in the Version 3.5 of genome assembly and the gene annotations. A search of BACs that belong to the support interval of the major QTLs was made from the position of the upper and lower bound markers, The IMGAG gene models (Medtrxgxxxxxx.x) of each BAC were compared to TAIR and Swissprot protein databases on the website http://www.legoo.org. Predictions of candidate genes on these BACs were analysed to identify those involved in plant growth in other species. Then, the putative genes involved in plant growth, specifically in shoot formation and flowering (Michaels 2009; Anastasiou and Lenhard 2007; Beveridge 2006; Aida and Tasaka 2006; Matsubayashi 2003) were considered.

Results

Effect of photoperiod on aerial morphogenetic traits

For all traits, photoperiod and genotype effects were highly significant in analysis of variance but the effect of photoperiod was the highest (Table 2). At flowering, plants had shorter main stems, shorter branches, more branches and a higher BER at a long (18 h) than at a short (12 h) photoperiod, except DZA315.16 that had longer branches at a long than at a short photoperiod (Table 3). Duration of branch elongation was lower at a long than at a short photoperiod. Flowering of Jemalong6 and F83005.5 was more hastened by long photoperiod than that of the other genotypes (see Pierre et al. 2008). Main stem length was very short at a long photoperiod (below 15 cm) but reached about 50 cm under a photoperiod of 12 h, except for TN6.18 whose main stem was only 3.9-cm long. Branch length was shorter at a long than at a short photoperiod for six genotypes (Jemalong6, DZA45.5, F83005.5, A20, Meiron and Levahim-B), longer for one genotype (DZA315.16) and little affected for one genotype (TN6.18). Even if the effect of photoperiod induced an effect in the same direction for most genotypes, the interaction between genotype and photoperiod was always significant except for duration of primary branch elongation.

Genetic variation of aerial morphogenetic traits of RIL populations

A large variation for aerial morphogenetic traits was observed in all populations (Table 4). Means and ranges of variation were different among seasons for all traits. On average, earlier FT and lower BER, shorter main stem and primary branches were observed in spring than in autumn on LR1 and LR5 populations. In LR4, the plants had lower LPB, BER, DPB and ADM and later FT in autumn 2004 than in the spring harvests of 2002 and 2003. In LR6 population, the lines had an earlier FT, higher BER, LMS and NPB in 2008 than in 2007, but lower LPB. A large variation was observed among lines for all traits in each population. Figure 1 shows phenotypic distribution of the RILs for LPB based on line means, revealing a transgression towards lower and higher values than those of parents. Highly significant effects of lines were observed for most of the traits in the three populations studied, but low genetic variation was observed for DPB, NI and NPB traits (Supplementary Materials 1, 2 and 3). The effect of line × season interaction was analysed in LR1 and LR5 populations (data not shown). In LR1 population, this interaction was only significant for BER, LMS and NPB. In

Table 2 Mean squares in analysis of variance for aerial morphogenetic traits recorded on eight genotypes grown at photoperiods of 12 and 18 h

Trait	Photoperiod	Block (photoperiod)	Genotype	Photoperiod × genotype interaction	Error
Length of primary branches (LPB), cm	900**	61 NS	491***	182**	41
Branch elongation rate (BER), cm/°C day^{-1}	0.0381***	0.0002**	0.0023***	0.0004**	0.00007
Duration of primary branches elongation, E + 03°C day^{-1}	8744***	11 NS	457***	38 NS	12
Length of main stem (LMS), cm	43541***	55 NS	1312***	873***	42
No of primary branches (NPB)	1,067***	5 NS	59***	7*	3
Diameter of primary branches (DPB), mm	4.24***	0.04 NS	1.03***	0.27*	0.07
Flowering time (FT), E + 03°C°day^{-1}	19.543***	0.009 NS	2.118***	0.498***	0.024
Degree of freedom	1	10	7	7	70

* Significant ($P < 0.05$), ** significant ($P < 0.01$), *** significant ($P < 0.001$), *NS* non significant

Table 3 Means of aerial morphogenetic traits for eight genotypes of *M. truncatula* grown at two photoperiods

Trait	Photoperiod (h)	Genotypes								SED
		Jemalong6	DZA315.16	DZA45.5	F83005.5	A20	TN6.18	Meiron	Levahim-B	
Length of primary branches (LPB), cm	12	64.3	42.9	66.8	69.3	66	56.8	62.7	57.9	6.5
	18	60.3	50.8	54.9	64.5	49.5	55.8	57.7	44.2	
Branch elongation rate (BER), 10^{-2} cm/°C day	12	7.4	4.7	5.7	6.3	7.9	5.4	7.8	7.8	0.69
	18	13.6	8.2	9.3	10.4	10.1	9.8	12.2	11.3	
Duration of primary branches elongation, 10^3°C day^{-1}	12	1.45	1.43	1.74	1.7	1.29	1.61	1.27	1.15	0.11
	18	0.68	1.00	1.03	0.98	0.72	1.09	0.69	0.59	
Length of main stem (LMS), cm	12	58.8	47.8	57.2	58.2	50	3.9	58	51.2	6.5
	18	1	0.17	5	4.5	8.7	0.83	14.5	10.2	
No of primary branches (NPB)	12	6.8	6.3	3	4.8	5.3	11.8	4	3.5	1.7
	18	12.8	14.7	10.7	12.3	11.7	15.2	10.5	11.2	
Diameter of primary branches (DPB), mm	12	2.5	2.7	2.6	2.9	2.4	2.2	2.7	2.3	0.3
	18	3.1	2.9	3.4	3.7	3.0	2.5	2.7	2.6	
Flowering time, °C day^{-1} (FT)	12	1454	1685	1583	1995	1064	1359	1186	903	94
	18	633	1096	937	1049	716	979	741	671	

SED Standard error deviation

contrast, for LR5 population, the effect of interaction line × season was highly significant for all traits. The analysis of variance for morphological traits of LR4 population was previously performed by Julier et al. (2007) and showed a large effect of the line for all traits.

High broad sense heritabilities (Table 5) were detected for FT, LMS and BER in all environments and populations and to a lesser degree for LPB and ADM. DPB and NI had the lowest heritabilities.

Correlations between traits are presented in Table 6. Positive and significant correlations were observed between BER, NI and LPB in all seasons. A significant negative correlation between LMS and NPB except for LR5 in autumn was observed. Negative and significant correlations were observed between FT and LMS and between FT and LPB except for LR5 in autumn 2005. Finally, a positive and significant correlations were observed between LPB, LMS, BER and ADM in most cases. These correlations indicate that on average, genotypes with long primary branches, high number of internodes and a high BER showed an early flowering date, a long main stem and less primary branches. In addition, these genotypes showed a higher ADM.

QTLs for aerial morphogenetic traits

A total of 141 QTLs were identified for aerial morphogenetic traits in the four RIL populations (Fig. 2). The positions of QTLs and their confidence intervals for the traits on LR1, LR4, LR5 and LR6 genetic maps and the consensus map can be compared in Supplementary Material 4. In total, 31 QTLs were mapped for LPB, 16 for BER, 20

Table 4 Means and ranges of variation for aerial morphological traits in three populations of *M. truncatula* recombinant inbred lines

Trait	LR1		LR4			LR5		LR6	
	Spring 2005	Autumn 2005	2002	2003	2004	Spring 2005	Autumn 2005	2007	2008
Length of primary branches (LPB), cm									
Mean	52.1	101.8	81.6	77.9	42.3	62.0	103.3	87.0	80.5
Range	39.0–64.9	67.0–126.7	57.7–100.2	35.0–97.7	7.7–73.3	41.4–82.9	71.2–123.0	75.4–102.6	72.0–93.0
Branch elongation rate (BER), 10^{-2} cm/°C day^{-1}									
Mean	6.1	10.3	14.9	10.9	4.1	8.0	11.0	11.0	12.4
Range	3.4–7.3	5.7–13.3	11.2–19.0	6.5–13.8	0.8–7.6	6.7–9.6	7.7–14.6	8.9–13.6	11.1–13.9
Length of main stem (LMS), cm									
Mean	30.7	66.2	–	–	14.0	35.8	63.9	32.7	42.3
Range	10.8–45.8	23.5–89.7	–	–	0.1–57.5	15.2–59.5	33.3–89.5	2.5–59.8	19.6–62.6
Number of primary branches (NPB)									
Mean	4.6	5.5	–	9.5	5.1	6.4	5.9	5.6	7.4
Range	3.0–5.6	4.0–6.7	–	7.0–14.7	3.0–8.0	4.7–9.3	5.3–6.3	4.3–7.0	6.0–9.6
Diameter of primary branches (DPB), mm									
Mean	2.7	2.3	2.3	2.4	1.8	2.4	2.0	2.2	2.0
Range	2.4–2.9	1.9–2.6	1.9–2.8	1.7–2.8	1.4–2.4	1.9–3.0	1.8–2.4	2.0–2.6	1.7–2.1
Number of internodes (NI)									
Mean	–	18.1	15.7	16.5	14.8	–	17.9	18.5	14.8
Range	–	14.2–19.7	13.0–17.2	10.5–18.2	10.0–17.5	–	16.3–19.7	16.6–19.8	12.6–16.6
Aerial dry matter (ADM), g									
Mean	7.0	8.0	1.5	1.7	2.3	6.9	7.7	13.9	5.7
Range	3.1–13.0	3.8–10.4	0.8–2.2	0.7–2.4	0.2–6.5	4.6–10.9	5.3–9.8	9.1–19.2	2.5–7.2
Flowering time (FT), °C day^{-1}									
Mean	1,254	1,369	1,023	1,147	1,503	972	1,274	1,302	1,079
Range	994–1,390	1179–1,521	878–1,282	899–1,500	867–2,120	836–1,278	1,129–1,498	1,077–1,507	942–1,255

for LMS, 18 for NPB, 12 for DPB, 24 for FT, 6 for NI and 14 for ADM. The R^2 was above 20% for 28 QTLs and between 15 and 20% for 17 QTLs. QTLs were distributed on all chromosomes of *M. truncatula*, with a greater concentration on chromosomes 1, 2, 7 and 8.

The bottom of chromosome 7 carried the QTLs with the highest R^2 values. QTLs for FT, BER, LMS, NPB, LPB and NPB were identified on populations LR1, LR4 and LR5. Most of these QTLs were located between 44.9 and 61.5 cM. This region includes the QTLs described by Julier et al. (2007) on LR4. No QTL was found in this region in LR6, except for NI in 2008. Jemalong6, female parent of the LR4 and LR5 populations, induced an increase in LPB, BER, LMS and a decrease in FT, NPB (LR4 population) and NI (LR6 population). In contrast, DZA315.26 parent induced a positive effect on FT in LR1 population.

The strongest QTLs for FT in LR6 were located on chromosome 6 at the positions 15.4–21.1 cM, explaining 15–19.4% of the variation. Jemalong6 allele induced positive effects in this population and so a late flowering time. No QTLs for the other traits in LR6 or other populations were found in this region.

At the bottom of chromosome 1, 20 QTLs for LPB, BER, LMS, NPB, FT and ADM co-located between 62.8 and 69.7 cM in the four populations. Nine of these QTLs explained more than 15% of phenotypic variation.

The bottom of chromosome 2 carried 10 QTLs between 60.0 and 71.2 cM for BER and LMS (LR4, LR5, LR6), NPB (LR1), LPB (LR4, LR5), ADM (LR6). Four of these QTLs had R^2 above 10%.

Twenty-four QTLs were detected on chromosome 8 for LPB, BER, LMS, NPB, DPB, ADM and FT, mostly in LR1, LR4 and LR5 populations. They were located all along the chromosome. The QTLs that explained the highest part of the variation were: a QTL for FT detected at 1.4 cM in LR5 population and explaining 21.0% of total variation, QTLs for LMS in all populations and explaining from 9.0 to 32.3% of variation, QTLs for NPB in LR4 and LR5 populations and a QTL for DPB in LR5 population.

On chromosomes 3, 4 and 5, the QTLs tended to be gathered in specific regions but explained more limited part on variation. However, QTLs for DPB in LR4 population at the top of chromosome 5 explained between 21.8 and 32.4% of the variation.

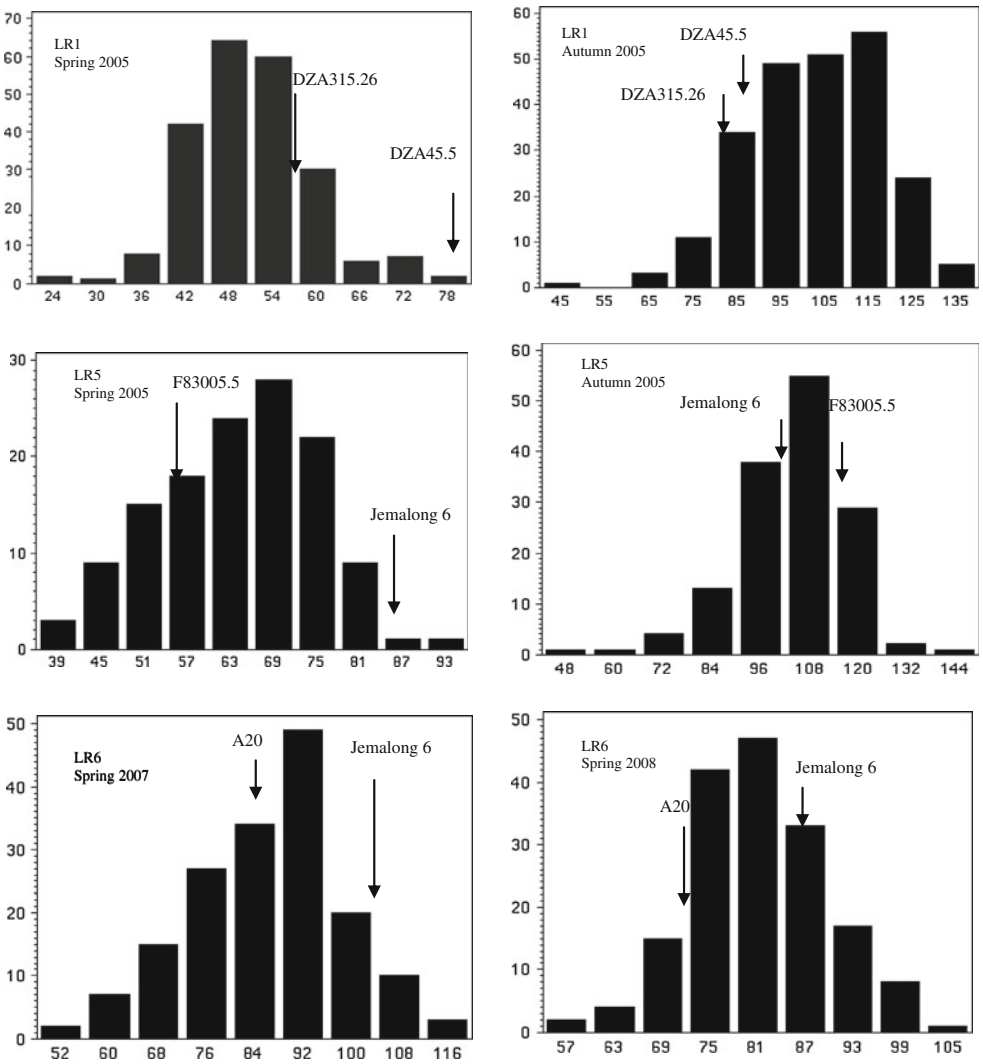

Fig. 1 Histogram for length of primary branch in three RIL populations of *M. truncatula*. The *arrows* indicate the mean value of the parental lines

Table 5 Broad sense heritabilities for aerial morphogenetic traits evaluated in three RIL populations of *M. truncatula*

Trait	LR1		LR4			LR5		LR6	
	Spring	Autumn	2002	2003	2004	Spring	Autumn	2007	2008
Length of primary branches (LPB), cm	0.33	0.91	0.55	0.61	0.51	0.84	0.86	0.41	0.65
Branch elongation rate (BER), cm.day^{-1}	0.58	0.87	0.50	0.48	0.93	0.50	0.75	0.69	0.66
Length of main stem (LMS), cm	0.73	0.85	–	–	0.84	0.89	0.85	0.86	0.95
Number of primary branches (NPB)	0.49	0.58	0.34	0.35	0.65	0.78	0.21	0.51	0.41
Diameter of primary branches (DPB), mm	0.0	0.66	0.29	0.60	0.69	0.86	0.45	0.71	0.35
Number of internodes (NI)	–	0.79	0.34	0.30	0.43	–	0.15	0.57	0.87
Aerial dry matter (ADM), g	0.59	0.76	0.47	0.56	0.02	0.53	0.95	0.40	0.65
Flowering time (FT), °C day^{-1}	0.68	0.82	0.57	0.71	0.36	0.93	0.79	0.92	0.93

Multi-parental QTL analysis

A total of 19 QTL (herein called mcQTL) were identified with the multi-population QTL analysis using MCQTL software for the six traits under study (Table 7). For FT, two mcQTLs were obtained, one on chromosome 7 (at 51.5 cM) where QTLs were detected on LR1, LR4 and LR5 populations and one on chromosome 8 (at 5.0 cM) where QTLs were detected on LR4 and LR5 populations, explaining 22.9 and 3.9% of total variation, respectively.

Table 6 Phenotypic correlations between aerial morphological traits in RILs populations LR1, LR5 and LR6

Trait[a]	Population/ season	Branch elongation rate (BER)	Length of main stem (LMS)	Number of primary branches (NPB)	Diameter of primary branches (DPB)	Number of internodes (NI)	Aerial dry matter (ADM)	Flowering time (FT)
LPB	LR6/2007	0.84***	0.38***	NS	NS	0.69***	0.55***	−0.50***
	LR6/2008	0.82**	0.38***	NS	NS	0.55***	0.43***	−0.45***
	LR1/Spring	0.48***	0.67***	NS	−0.21**	–	NS	−0.32***
	LR1/Autumn	0.82***	0.66***	0.18***	0.45***	0.42***	0.73***	−0.48***
	LR4/2002	0.74***	–	0.27**	0.28**	0.62***	0.60***	−0.59***
	LR4/2003	0.85***	–	NS	0.48***	0.69***	0.61***	−0.82***
	LR4/2004	0.81***	0.54***	−0.28***	NS	0.57***	0.40***	−0.48***
	LR5/Spring	0.68***	0.60***	NS	−0.25**	–	0.39***	−0.50***
	LR5/Autumn	0.76***	0.32***	NS	0.34***	0.73***	0.53***	NS
BER	LR6/2007		0.42***	0.84***	NS	0.47***	0.43***	−0.66***
	LR6/2008		NS	−0.19**	NS	0.44***	NS	NS
	LR1/Spring		0.45***	0.19**	0.27***	–	0.36***	0.29***
	LR1/Autumn		0.53***	0.15*	0.41***	0.46***	0.67***	−0.40***
	LR4/2002		–	0.21*	0.21*	0.29**	0.49***	−0.20*
	LR4/2003		–	NS	0.34***	0.62***	0.48***	−0.42***
	LR4/2004		0.39***	−0.20**	NS	0.28***	0.48***	−0.30***
	LR5/Spring		0.22*	NS	NS	–	0.32***	NS
	LR5/Autumn		0.20*	NS	0.32***	0.62***	0.50***	NS
LMS	LR6/2007			−0.59***	NS	NS	0.42***	−0.53***
	LR6/2008			−0.38***	−0.16	NS	0.18*	−0.65***
	LR1/Spring			−0.36***	NS	–	NS	−0.40***
	LR1/Autumn			−0.20**	−0.29***	NS	0.44***	−0.36***
	LR4/2002			–	–	–	–	–
	LR4/2003			–	–	–	–	–
	LR4/2004			−0.20**	NS	0.28***	0.48***	−0.30***
	LR5/Spring			−0.58***	−0.57***	–	0.23**	−0.75***
	LR5/Autumn			NS	NS	NS	NS	NS

Table 6 continued

Trait[a]	Population/ season	Branch elongation rate (BER)	Length of main stem (LMS)	Number of primary branches (NPB)	Diameter of primary branches (DPB)	Number of internodes (NI)	Aerial dry matter (ADM)	Flowering time (FT)
NPB	LR6/2007				NS	NS	NS	NS
	LR6/2008				NS	NS	NS	NS
	LR1/Spring				0.29***	–	0.32***	NS
	LR1/Autumn				0.16**	0.16**	0.31***	NS
	LR4/2002				NS	0.64***	0.43***	NS
	LR4/2003				NS	NS	NS	NS
	LR4/2004				0.43***	NS	NS	0.36***
	LR5/Spring				0.49***	–	NS	0.46***
	LR5/Autumn				NS	NS	NS	NS
DPB	LR6/2007					0.31***	NS	NS
	LR6/2008					0.19*	NS	NS
	LR1/Spring					–	0.32***	−0.52*
	LR1/Autumn					0.19**	0.31***	−0.66**
	LR4/2002					0.20*	0.69***	NS
	LR4/2003					0.58***	0.85***	−0.23*
	LR4/2004					0.21**	0.25**	0.29***
	LR5/Spring					–	NS	0.84***
	LR5/Autumn					0.41***	NS	NS
NI	LR6/2007						0.57***	−0.22**
	LR6/2008						0.60* **	NS
	LR1/Spring						–	–
	LR1/Autumn						0.41***	−0.25***
	LR4/2002						0.52***	−0.26**
	LR4/2003						NS	−0.30***
	LR4/2004						NS	−0.18*
	LR5/Spring						–	–
	LR5/Autumn						0.54***	NS

Table 6 continued

Trait[a]	Population/season	Branch elongation rate (BER)	Length of main stem (LMS)	Number of primary branches (NPB)	Diameter of primary branches (DPB)	Number of internodes (NI)	Aerial dry matter (ADM)	Flowering time (FT)
ADM	LR6/2007							−0.44***
	LR6/2008							−0.17*
	LR1/Spring							−0.13*
	LR1/Autumn							−0.44***
	LR4/2002							NS
	LR4/2003							NS
	LR4/2004							NS
	LR5/Spring							−0.23**
	LR5/Autumn							NS

NS Not significant ($P > 0.05$), *significant ($P < 0.05$), **significant ($P < 0.01$), ***significant ($P < 0.001$), – not available

[a] *LPB* Length of primary branches

These two mcQTLs correspond to those described by Pierre et al. (2008). The confidence interval for the first mcQTL was 5.5 cM. The effects of Jemalong6, A20 and F83005.5 were negative whereas those of DZA315.16 and DZA45.5 were positive. For the mcQTL on chromosome 8, negative effects were induced by Jemalong6 and A20; positive effects by F83005.5, DZA315.16 and DZA45.26. QTLs detected on chromosome 6 for FT was not revealed in the multi-population analysis although they were present in the LR6 population.

Four mcQTLs for LPB were located on chromosomes 1, 2, 3 and 7. For the mcQTL of chromosome 1, QTLs were detected for LBP in LR1, LR4 and LR5 populations. The mcQTL of chromosome 2 corresponded to the QTLs found in LR4 and LR5 populations. For mcQTL of chromosome 3, LR4, LR5 and LR6 populations showed QTLs. Finally for chromosome 7, LR1, LR4 and LR5 populations had QTLs for LPB. Total variation explained by the mcQTLs for this trait varied from 4.3 to 10.2%. The effects of parents differed according to the region of the chromosome involved. Jemalong6 alleles except one induced long branches, and DZA315.16 alleles induced short branches. For the other parents, the alleles had either positive or negative effects.

Two mcQTLs were detected for BER, one at position 52.3 cM on chromosome 2 where QTLs were detected for LR4 and LR6 populations and one at the bottom of chromosome 4 where a QTL was detected in LR1 population. Jemalong6 alleles had positive effects while F83005.5 alleles had negative effects. For DZA45.5 and A20, additive effects were positive when considering the alleles on chromosome 2 but negative for alleles on chromosome 4. On the contrary, DZA315.16 allele of chromosome 2 had a negative effect while allele of chromosome 4 had a positive effect.

No mcQTLs were revealed for DPB or NI in this multi-population analysis. For ADM, three mcQTLs were observed at the bottom of chromosomes 1 (QTLs in LR1 and LR5 populations) and 2 (a QTL in LR6 population) and one at the top of chromosome 3 (a QTL in LR1 population). Additive effects were negative for alleles of Jemalong6, DZA315.16 and F83005.5. A20 allele on chromosome 3 and DZA45.5 allele on chromosome 2 induced a decrease in ADM.

For NPB, two QTLs were observed at 65.3 cM on chromosome 1 (QTLs in LR1, LR4 and LR6 populations) and at the top of chromosome 8 (QTLs in LR4 and LR5 populations). Jemalong6 and DZA45.5 alleles induced negative effects whereas those of A20 had positive effects. Six QTLs were detected for LMS, on chromosomes 1, 2, 7 and 8. Most alleles of F83005.5 and DZA45.5 induced a short main stem but A20 alleles induced a long main stem. Jemalong6 and DZA315.16 had three alleles with positive effects and three alleles with negative effects.

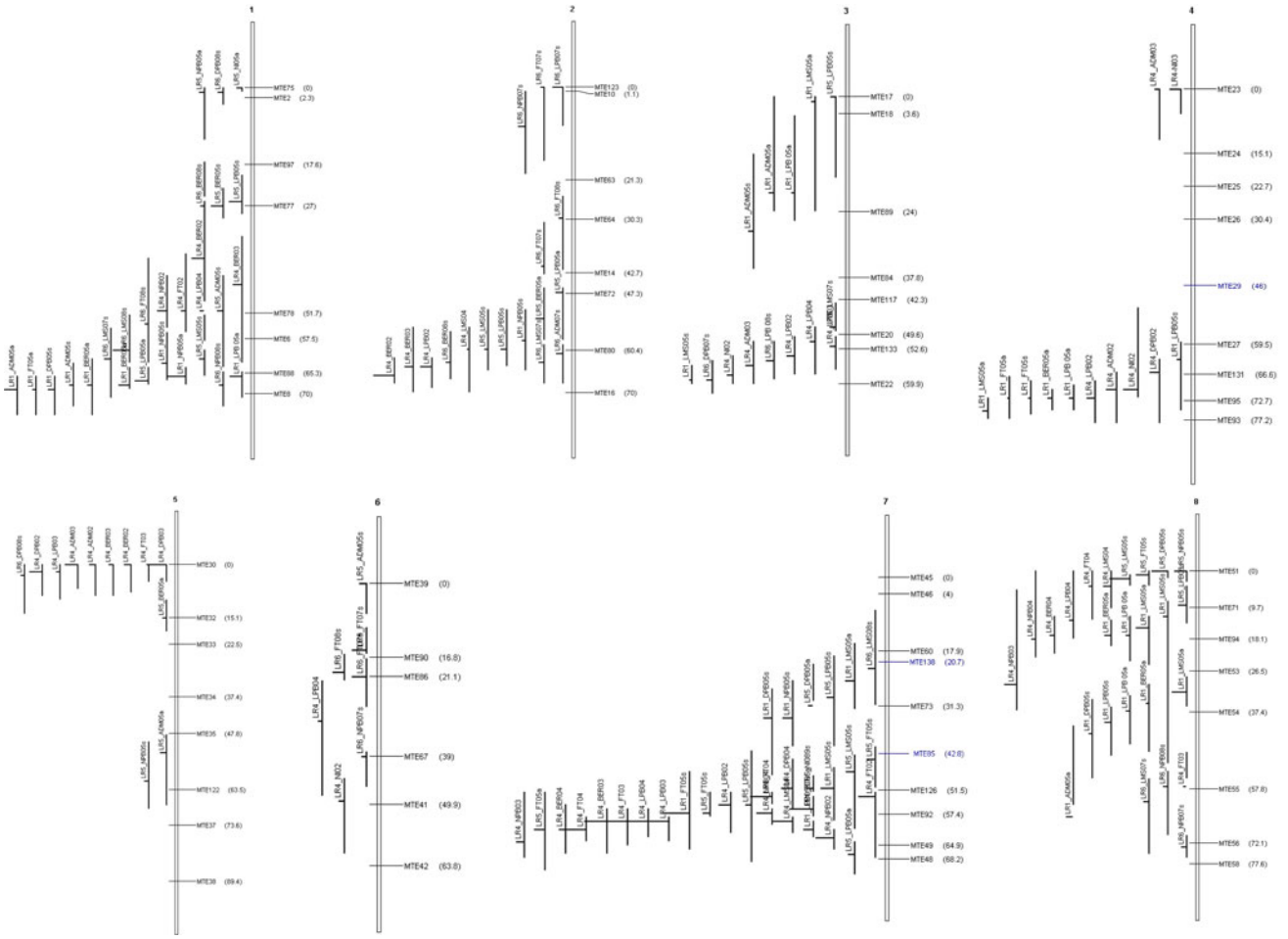

Fig. 2 Position and confidence interval of QTLs for aerial morphogenesis traits in LR1, LR4, LR5 and LR6 RILs populations of *M. truncatula* on the consensus map. On the right side of chromosomes, the marker names are followed by their position indicated within brackets. *Vertical bars* on the left side of chromosomes indicate the confidence interval of the QTLs. *Horizontal bars* represent the position of the QTLs and the bar length is proportional to R^2 value. See trait abbreviations in Table 6

Finally, at least one QTL was present in the region where the mcQTLs were detected. Conversely, some QTLs that seemed to co-localise in several populations (FT on chromosome 1, BER on chromosome 1, LPB on chromosomes 4 and 8, NPB on chromosome 7) were not recovered as mcQTLs.

The same positions for the mcQTLs were observed for LPB, ADM, NPB and LMS on chromosome 1, for LPB, LMS and BER on chromosome 2, for FT, LPB and LMS on chromosome 7, and for FT and LMS on chromosome 8.

Using a bio-analysis of data available in the TAIRpep and SWISSprot databank and existing knowledge of genes involved in the flowering pathways, stem and branching control, putative candidate genes were investigated in the confidence interval of major mcQTLs for FT on chromosome 7 and LMS on chromosome 1 (Table 8), in which a total of 919 and 1,961 genes were present, respectively. Three and ten candidate genes were found in each QTL region. In the confidence interval of the mcQTL for FT on chromosome 7, genes related to floral induction were identified: zinc finger protein CONSTANS-like, FLOWERING LOCUS T and PEBP genes (Michaels 2009). At the bottom of chromosome 1 where a mcQTL for LMS was identified close to QTLs for LPB, eight genes were detected (COP1, CLAVATA1, SBP, ARF/SAR, Auxin-binding protein, EMBRYOGENIC FLOWER 2, Aux/IAA protein and NAM) related to shoot and branching development through hormone response and signalling (Zhao et al. 2001; Beveridge 2006; Aida and Tasaka 2006; Anastasiou and Lenhard 2007). In the genomic region revealed on chromosome 6 for FT (7–18 cM), a gene FAR1 (AT4G38180.1, AT3G59470.1) involved in the far-red responses controlled by phytochrome A (Lin and Wang 2004), and a member of SKP1 gene family, homologue of ASK1 (AT3G61415.2) in *Arabidopsis* (Zhao et al. 2003), is annotated in the *M. truncatula* database and Swissprot and TAIRpep database. This ASK1 gene belongs to family called SCF complex (SKP1, cullin/CDC53, F-box protein)

Table 7 Location of QTLs for aerial morphogenesis, proportion of explained variation (R^2) and effects of parents from a multi-population QTL analysis

Trait	Chromosome	Position	Confidence interval	R^2	Effect of parents				
					Jemalong6	DZA315.26	DZA45.5	A20	F83005.5
Length of primary branches (LPB), cm*	1	65.3	64–70	5.5	−1.72	−0.32	3.19	−1.67	0.51
	2	52.3	49–58	6.0	2.40	−0.61	−1.31	2.16	−2.65
	3	52.6	51–55	4.3	2.06	−1.27	−1.69	−1.36	2.27
	7	56.5	54–60	10.2	3.29	−2.60	−4.42	2.17	1.56
Branch elongation rate (BER), 10^{-3} cm day^{-1}*	2	52.3	46–59	3.0	0.8	−1.9	2.6	0.2	−5.7
	4	72.7	68–77	3.5	0.8	2.8	−1.2	−0.6	−1.8
Length of main stem (LMS), cm**	1	62.5	55–64	11.7	−3.18	−0.49	0.49	3.68	−0.50
	2	57.3	48–58	5.0	−0.59	−0.55	0.55	3.59	−3.00
	7	22.9	17–26	8.5	−2.55	1.17	−1.17	4.25	−1.69
	7	50	41–54	4.9	2.13	1.19	−1.19	−1.49	−0.64
	8	5	1–9	5.2	1.16	1.09	−1.09	2.89	−4.06
	8	31.5	28–37	7.8	2.37	−2.51	−1.50	−0.87	2.51
Number of primary branches (NPB)**	1	65.3	61–70	8.2	−0.040	−0.103	−0.178	0.178	0.143
	8	0	0–7	3.6	−0.079	0.226	−0.015	0.015	−0.147
Aerial dry matter (ADM), g*	1	70	64–70	7.0	−0.295	−0.253	0.547	0.055	−0.0544
	2	65.4	61–70	4.7	−0.204	−0.207	−0.053	0.697	−0.233
	3	29	18–32	4.5	−0.190	−0.153	0.622	−0.030	−0.249
Flowering time (FT), °C day^{-1}*	7	51.5	50–55	22.9	−76.6	57.7	149.7	−68.8	−62.0
	8	5	0–9	3.9	−31.5	20.9	33.1	−36.2	13.7

* Analysis conducted on four populations, ** analysis conducted on three populations (DZA315.26 × DZA45.5, Jemalong6 × F83005.5 and Jemalong6 × A20)

Table 8 Putative candidate genes in the support interval of major QTLs for FT, LMS and LPB

Trait	Chromosome	Position and CI	Accession	Genecall	Locus TAIR9	Putative candidate gene
Length of main stem (LMS), cm	1	62.5 (55–64)	AC158374	Medtr1g090940.1	AT5G43310.1	COP1 (Constitutive photomorphogenic 1)
				Medtr1g094070.1	AT1G08590.1	CLAVATA1 receptor kinase (CLV1)
				Medtr1g105980.1	AT2G47070.1	SBP (Squamosa promoter binding protein, SBL in *Arabidopsis*))
				Medtr1106180.1.2	AT1G10630.1, AT3G62290.1	ARF/SAR superfamily
				Medtr1g111460.1	AT4G02980.1	Auxin-binding protein
				Medtr1g111940.1	AT5G51230.3	EMBRYONIC FLOWER 2
				Medtr1g117980.1	AT5G51230.3	
				Medtr1g117600.1	AT4G14550.1	Aux/IAA protein
				Medtr1g117720.1	AT1G04240.1	
				Medtr1g118000.1	AT1G25580.1	NAM (No apical meristem protein)
Flowering time (FT), °C day^{-1}	7	51.5 (50–55)	AC133580	Medtr7g096570.1	AT2G33500.1	Zinc finger protein CONSTANS-like
			AC123593	Medtr7g099820.1	AT1G65480.1	FT (Flowering Locus T)
				Medtr7g099890.1	AT1G65480.1	PEBP (Phosphatidylethanolamine-binding proteins), FT (Flowering Locus T)

whose main role is to select substrates for proteolysis by facilitating the ligation of ubiquitin to specific proteins. In plants, the SKP1 gene is involved in auxin responses and jasmonate signalling in vegetative and reproductive processes as flower development, circadian clock, gibberellin signalling and leaf senescence (Mizoguchi and Coupland

2000; Zhao et al. 2001; Sasaki et al. 2003; Beveridge 2006).

Discussion

Effect of photoperiod on aerial morphogenetic traits

A large genetic variation was reported for aerial morphogenetic traits in *M. truncatula* (Julier et al. 2007). It was confirmed in the present study on another set of genotypes studied in different environmental conditions. These traits are influenced by environmental factors, among which temperature and photoperiod are the most important in controlling the rate of plant development. The effect of temperature on plant development was described and modelled in *M. truncatula* (Moreau et al. 2007). We observed that aerial morphogenetic traits were strongly affected by day-length treatments. Except for DZA315.16 that had longer branches at long than at short photoperiods, the effect of photoperiod was the same for all genotypes. However, the intensity of the photoperiod effect was different for the genotypes. Some were little affected, such as Jemalong6 for branch length, Levahim-B for flowering date and TN6.18 for main stem length. Others were dramatically affected, such as Jemalong6 for flowering time. Effects of photoperiod have already been described in common bean (Wallace et al. 1993) and in pea (Arumingtyas et al. 1992; Beveridge et al. 2003). Genetic variation for the response to photoperiod was proved in several species such as maize, soybean, wheat or others (Blondon and Gallais 1976; Cregan and Hartwig 1984; Board and Settimi 1986; Slafer and Rawson 1994; Yan and Wallace 1998; Giauffret et al. 2000) including *Arabidopsis* and rice in which the molecular mechanisms were analyzed (Hayama and Coupland 2004). Detection and use of photoperiod-insensitive genotypes is an objective in breeding programs for adaptation to a wide range of latitudes.

Variation for aerial morphogenetic traits among RILs

The variation among RILs of each population was large and high heritabilities were calculated for most traits. Transgressive lines were observed for most traits in the populations. They suggest that parental lines carry alleles with positive and negative effects at several loci involved in trait variation, the recombination between loci thereby generating the transgressions. Data collected in this study were added to previously published ones (Julier et al. 2007; Pierre et al. 2008). The seasons had an effect on the average values and ranges of variation of each population (not shown). Photoperiod and light quality that vary with the seasons have an influence on plant morphogenesis (Górski 1980). However, as both temperature and photoperiod varied along each experiment, it is difficult to conclude on the effect of specific environmental factors on traits in this study. Generally, LMS, LPB and BER were positively correlated. LMS was negatively correlated to NPB, as if there was a trade-off between the investment in elongation of the main stem and in elaboration of branches. Early flowering genotypes had shorter branches and a shorter main stem. Negative correlations between flowering date and vegetative traits have also been observed in a set of *Lotus japonicus* recombinant inbred lines (Gondo et al. 2007).

QTL analyses

QTL mapping of aerial morphogenesis traits, using four genetic maps (LR1, LR4, LR5 and LR6) that shared most of the markers, revealed 141 loci involved in variation for these traits. Most of the QTLs were detected for five traits (LPB, FT, LMS, NPB and BER). Most of these traits showed high heritabilities in all seasons and populations. No more than 12 significant QTLs were detected for one trait in a season. Considerable variation was observed in the number of QTLs detected per chromosome. The 141 QTLs detected in this study were distributed over all eight chromosomes of the *M. truncatula* genome. Three chromosomes carried many QTLs: chromosomes 8, 1 and 7 had 24, 27 and 31 QTLs for aerial morphogenetic traits, respectively.

Co-localisation of QTLs for a trait measured in different populations and seasons was often observed. Multi-population analysis was applied to reveal major QTLs for LPB, LMS, NPB, BER, ADM and FT. The larger population size created by this approach provides a greater detection power for the QTLs shared by several crosses (Billotte et al. 2010). For each trait, all the QTL detected with the multi-population analysis were located in regions that carried at least one QTL. The part of the variation explained by these QTLs was often lower than that of the initial QTLs because not all populations × seasons produced these QTLs. However, the QTL detected over the global design for LPB on chromosome 1 had a high R^2 even if a single QTL (LR5 in autumn 2005) was identified. An hypothesis could be that the allelic effects in the other populations × seasons were individually too low to show a QTL but the multi-population analysis was able to reveal it. QTLs that were specific to one single cross such as the QTL for FT on chromosome 6 in LR6 were not revealed with this multi-population analysis, as if its effect was "diluted" in the whole design. In other cases, QTLs detected in some populations × seasons were not recovered in the multi-population analysis. Either the allelic effects were too low to detect a multi-population QTL or again, there was a

dilution of the effect of the QTL. The low level risk chosen in the multi-population analysis also contribute to reduce the number of detected QTLs. Similar results were observed using this multi-parental design in oil palm (Billotte et al. 2010). The multi-population analysis was useful to compare the allelic effects of the parents. For example, for the QTL for FT on chromosome 7, the effect of the allele of DZA45.5 was much higher than that of the other parents. For all traits, it was possible to identify parents that carried both positive and negative alleles, explaining the presence of transgressive RILs. For FT, the confidence interval was of 5.2 cM on chromosome 7. Using this multi-parental approach with a previous version of MCQTL and another LR4 genetic map that contained more markers, a QTL was detected in the same position on this chromosome by Pierre et al. (2008) for FT trait on LR1, LR4 and LR5 populations, although the confidence interval was only 0.9 cM. This small confidence interval was related to the additional markers but was of little interest because the position of each marker on LR4 map, calculated on a population with 199 RILS only, had a low accuracy.

Several QTLs of different traits were mapped in close position. QTLs for FT, LMS, BER, and LPB were located in the same region of chromosomes 7 (between 42.8 and 68.2 cM) and 8 (between 0 and 26.5 cM). Correlation between these traits and co-location could indicate a common genetic regulation, these regions of chromosomes 7 and 8 being involved in branching development, branch elongation and flowering. On chromosome 1, multi-population QTLs were detected around 65 cM for LBP, LMS, NBP and ADM, four traits that showed correlations. A single gene could be responsible for these traits. In soybean, QTLs for developmental and morphological traits were also distributed on all different linkage groups (Josie et al. 2007; Panthee et al. 2007), but tended to be clustered (Mansur et al. 1993); specifically the QTLs that condition plant height and number of nodes on the main stem were identified in the same region on linkage groups B1 and C2 (Mian et al. 1998; Zhang et al. 2004).

Multi-population QTLs for FT and LMS co-located in the same region on chromosome 7 at the positions 51.5 and 50.0 cM, respectively. As these traits are highly correlated, a gene with pleiotropic effects could be involved. But the correlation was negative and the allelic effects of parents Jemalong6, DZA315.26, DZA45.5, A20 and F83005.5 do not always act in the opposite direction. In addition, the confidence interval of the two QTLs only partly overlapped. Two different genes are thus likely to be involved to explain these QTLs on chromosome 7.

The QTL positions are useful to look for candidate genes, using the annotation given on *M. truncatula* genomic sequences. The knowledge available on the effect of the genes on the phenotypes in other species was used to sort out the most promising genes, as proposed in the positional candidate gene approach (Pflieger et al. 2001). This approach was previously adopted to analyse gene sequence and expression variation of the CONSTANS-like gene of chromosome 7 (Pierre et al. 2008, 2011).

Two major regions of QTLs affecting aerial morphogenetic traits on the *M. truncatula* genome were revealed by the multi-parental approach that was used. Because of the large confidence intervals obtained for the identified QTLs, it cannot be excluded that different but linked genes may control pleiotropic variation. This co-location of QTLs for vegetative morphogenesis traits has already been shown in different legumes (El-Lithy et al. 2004; Cogan et al. 2006; Gondo et al. 2007; Burstin et al. 2007). To reduce the confidence intervals and to confirm the exact position of the QTLs for aerial morphogenetic traits, especially for LMS, LPB, and NPB on chromosomes 1 and 7, it is necessary to carry out a fine-scale mapping, using adapted genotypes in which no other QTL segregate, such as near-isogenic lines or large F2 populations established from heterozygous lines in the regions of major QTLs. A similar approach must be performed for FT on chromosome 6, on which a new QTL region was revealed in LR6 population for this trait. The genomics tools developed on this model plant should help to identify the likely underlying candidate gene(s) that controls the phenotypic variation for aerial morphogenesis in the studied populations. These genes could be candidates to explain the genetic variation in crop legume species, as was shown on alfalfa (Herrmann et al. 2010), based on a candidate gene identified from a fine mapping strategy on *M. truncatula*.

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