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Identification of QTLs for shoot and root growth under ionic–osmotic stress in *Lotus*, using a RIL population

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Abstract. The genus *Lotus* includes a group of forage legume species including genotypes of agronomic interest and model species. In this work, an experimental hydroponic growth system allowed the discrimination of growth responses to ionic–osmotic stress in a population of recombinant inbred lines (RILs) developed from *L. japonicus* × *L. burttii* and the identification of the associated quantitative trait loci (QTLs). The analyses led to the identification of eight QTLs: three for shoot growth localised on chromosome 3, 5 and 6; one for root growth on chromosome 1; three for total growth on chromosome 1, 4 and 5; and one associated with shoot/root ratio on chromosome 3. An interaction of QTL × stress condition was established and the effect of the environment quantified. In summary, it was established that the allele from *L. burttii* explained most responses to osmotic stress, while the alleles of *L. japonicus* explained the responses related to ionic stress conditions. Of 49 markers linked to all QTLs identified, 41 expressed superiority of the *L. burttii* parental allele in the osmotic stress condition, but when an iso-osmotic concentration of NaCl was applied, *L. burttii* lost superiority in 21 of these markers. This shows the superiority of the *L. japonicus* parental allele in ionic stress conditions. This study is the first report in which a RIL population of lotus is analysed with the aim of providing molecular markers associated with plant responses to ionic or osmotic stress.

Additional keywords: hydroponic growth, *L. japonicus*, *L. burttii*, PEG stress, salt stress.

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Introduction

More than 30% of the world's potential crop productivity is lost annually due to abiotic stress, such as drought and salinity, on plants. Furthermore, more than 10% of the area under agricultural production is affected by abiotic stresses, and this area is expected to reach 50% by 2050 (Flowers 2004; Munns and Tester 2008; Athar and Ashraf 2009). Drought and salinity stress are often interconnected, and may induce similar cellular damage resulting in the disruption of cell homeostasis and distribution of solutes and ions in the cell (Wang *et al*. 2003). The response to salt stress is clearly separated in two phases. The first is similar to the effect induced by drought, and is associated with the osmotic unbalance caused by the decrease in water potential in the growth medium (Borsani *et al*. 2001; Wang *et al*. 2003; Munns and Tester 2008). During this phase, similar to the occurrence in drought, salt stress tolerance is initially determined by osmotic compensation at the cellular level, which is followed by a response directed to maintain ionic/osmotic homeostasis (Borsani *et al*. 2001). The second phase is associated with ionic stress, a consequence of the unbalance in the K^+/Na^+ relation and the increase in Na^+ concentration to lethal levels (Munns 2002; Borsani *et al*. 2003; Díaz *et al*. 2005; Munns and Tester 2008). Under conditions of ionic stress, the tolerance is linked with the capacity of tissues to exclude and transport Na^+. In this way, a higher stem/root ratio and a higher growth rate are necessary to control the high levels of Na^+ in leaves (Munns *et al*. 2002; Yamaguchi and Blumwald 2005). Also in this last phase, plants accumulate ions in the older leaves, inducing a reduction in the photosynthetic capacity and

death of plant organs, with an impact on growth rate (Munns 2009). On the other hand, drought stress also involves mechanisms related to water conservation, such as stomata closing and active water transport, among others (Khan *et al*. 2010). At the plant level, tolerance of both stresses would involve morphological, physiological, and biochemical responses to maintain active respiration, photosynthesis and water-nutrient transport (Wang *et al*. 2003; Díaz *et al*. 2005; Munns and Tester 2008).

Ionic–osmotic stress tolerance in plants is determined by several complex traits, explained by loci with quantitative effect (QTLs, quantitative trait loci) (Flowers 2004; Cuartero *et al*. 2006). QTL mapping has been successful in identifying genomic regions associated with complex quantitative traits in general (Mackay *et al*. 2009), and stress-related traits in particular (Tuberosa *et al*. 2002; Arbaoui *et al*. 2008; Collins *et al*. 2008). However, there are two main limitations to successful detection of a QTL for abiotic stress. First, experimental systems are lacking that allow efficient phenotyping of a large number of individuals in similar conditions; hence, phenotyping of stress responses is a challenge (Agbicodo *et al*. 2009; Salekdeh *et al*. 2009). Second, genotype × environment interactions (GEI) and consequently QTL × environment interactions (QEI) (Malosetti *et al*. 2004) make it difficult to appropriately compare results from different experimental systems and stress conditions (Ashraf 2010). QTLs have been reported for shoot and root growth and development (Cogan *et al*. 2006; Bouteillé *et al*. 2012). Such studies have pointed to QTLs either involved in constitutive traits related to root growth (Loudet *et al*. 2005) or associated with plant growth responses to different environments including stressing conditions (Collins *et al*. 2008). Also, quantitative genetic variation for vegetative morphogenesis traits such as leaf dimensions and plant height have been identified by a multi-environment analysis in forages legumes such as *Medicago truncatula* (Bonnin *et al*. 1996, 1997) and *Trifolium repens* (Cogan *et al*. 2006).

Efforts in genomics and genetics studies have significantly increased the information available in this area in a wide range of plant species, including important crops (Miflin 2000). However, the focus on genomics has not been followed by similar efforts to understand the expressed phenotype. This has contributed to what is known as the 'phenotype gap' (Miflin 2000;Verslues *et al*. 2006). In this context, plant models and phenotyping methods acquire great relevance as a way to assist plant breeding programs (Botella *et al*. 2005).

The species *Lotus japonicus* has been selected and investigated as model plant for genetic and genomic studies (Handberg and Stougaard 1992; Hayashi *et al*. 2001; Pedrosa *et al*. 2002; Young *et al*. 2005; Sandal *et al*. 2006; Sato *et al*. 2008; Sandal *et al*. 2012). *Lotus japonicus* is a perennial species closely related to birdsfoot trefoil (*L. corniculatus*), an important forage legume. The studies performed in *L. japonicus* include whole-genome sequencing, molecular-marker development, and the construction of high-density linkage maps (Sato *et al*. 2008; Ohmido *et al*. 2010). Three linkage maps have been reported for the species, including one map based on the intraspecific cross of *L. japonicus* Gifu × *L. japonicus* MG20 (Hayashi *et al*. 2001; Sato and Tabata 2006), another based on the interspecific cross of *L. filicaulis* × *L. japonicus* Gifu (Sandal *et al*. 2002), and a third map under construction from the interspecific cross of *L. japonicus* Gifu × *L. burttii* (Kawaguchi *et al*. 2005; Sandal *et al*. 2012). Therefore, model plants are expected to play a significant role as platform to identify QTLs associated with agronomics traits (Gondo *et al*. 2007).

In this study, we aimed to identify genomic regions linked to traits associated with osmotic–ionic stress tolerance by using a recombinant inbred line (RIL) population of *L. japonicus* × *L. burttii* and a novel experimental system designed for extensive phenotyping of lines combined with powerful statistical tools to map QTLs.

Materials and methods

Propagation and genotyping of RILs

In total, of 163 RILs from the cross between *L. japonicus* Gifu B-129 (Stougaard and Beuselinck 1996) and *L. burttii* B-303 (Kawaguchi *et al*. 2005) were obtained through single-seed descent of F_2-derived F_8 lines. The genetic characterisation of the RIL population is described in detail by Sandal *et al*. (2012). Briefly, 97 microsatellite markers covering all six linkage groups of the species were used for mapping.

Linkage map

A linkage map for the RIL population characterised with 97 markers was constructed using the R/onemap 2.0 package (Margarido *et al*. 2007) of R software (R Project for Statistical Computing, www.r-project.org/). The recombination fraction of all marker-pairs was estimated using a two-point analysis. To assign markers to linkage groups, a significance threshold was set at a logarithm of odds (LOD) score of 4.0 and a maximal recombination fraction of 0.5. After markers were assigned to linkage groups, markers were re-ordered using a LOD score of 3.0 and the Kosambi mapping function to estimate genetic distances. The genome similarity matrix from each line was obtained by a simple matching with the parental genotype using the free software Flapjack (Milne *et al*. 2010).

Experimental conditions

Parental lines and a subset of 100 RILs from the original RIL population were phenotyped. Two factors were evaluated simultaneously in the experimental system: the 100 genotypes, and three stress-conditions. The stress conditions were created under hydroponic conditions in Hornum nutrient solution (Handberg and Stougaard 1992): (*i*) control, (*ii*) NaCl (ionic and osmotic stress), and (*iii*) *polyethylene glycol* (PEG) (osmotic stress). The control treatment consisted of Hornum nutrient solution throughout the experiment. Ionic–osmotic stress was induced with 150 mM NaCl in Hornum nutrient solution during 15 days after an acclimation period of 7 days. Osmotic stress was induced by PEG 800 at 15% (w/v) in Hornum nutrient solution during 15 days after an acclimation period of 7 days. Both stress treatments generate the same osmotic pressure (–0.85 MPa).

Genotypes were evaluated in an incomplete block design with two or three replications arranged in a split-plot design, where the main plot consisted of a tray with a specific stress treatment and 30 genotypes. Seed germination was conducted in Petri dishes with 0.8% (w/v) agar/water under continuous

Fig. 1. Hydroponic growth system designed for evaluating plant stress responses. Detail of seedling growth measurements is shown in the upper right corner.

light at 28°C. Germinated seeds (radicles 2–3 mm length) were transferred to trays.

Plants were grown in 400-mL trays in a hydroponic system continuously subjected to an air flux with 0.5 cm diameter tubes connected to a Champion® CX-0098 air pump (Fig. 1). In each tray, six drilled acrylic rulers supported the seedlings.

Growth and development parameters

In order to quantify plant growth and development, the length of shoots and roots, and the total length, were recorded in plants 17 and 21 days old (7 and 14 days after treatment started, respectively). All plant evaluations were conducted in a non-destructive manner by image analysis of individual plants using the free software ImageJ (Abràmoff *et al*. 2004).

Shoot, root and total relative growth rates (RGR_{shoot}, RGR_{root} and RGR_{total}) were calculated as:

$$RGR = (\ln L_f - \ln L_i)/(t_f - t_i)$$

where L_f and L_i are final and initial length, respectively, and t_f and t_i are final and initial time. Shoot, root and total initial and final lengths were used to calculate RGR_{shoot}, RGR_{root} and RGR_{total}, respectively. These are used as growth estimates.

Shoot/root ratio (SRR) was calculated as:

$$SRR = L_{Sf}/L_{Rf}$$

where L_{Sf} is the final shoot length and L_{Rf} is the final root length.

Statistical analyses

A two-step analysis was used for QTL mapping. First, a table of GEI phenotypic means was created for each variable (i.e. RGR_{shoot}, RGR_{root}, RGR_{total}, SRR), and then a QEI mapping

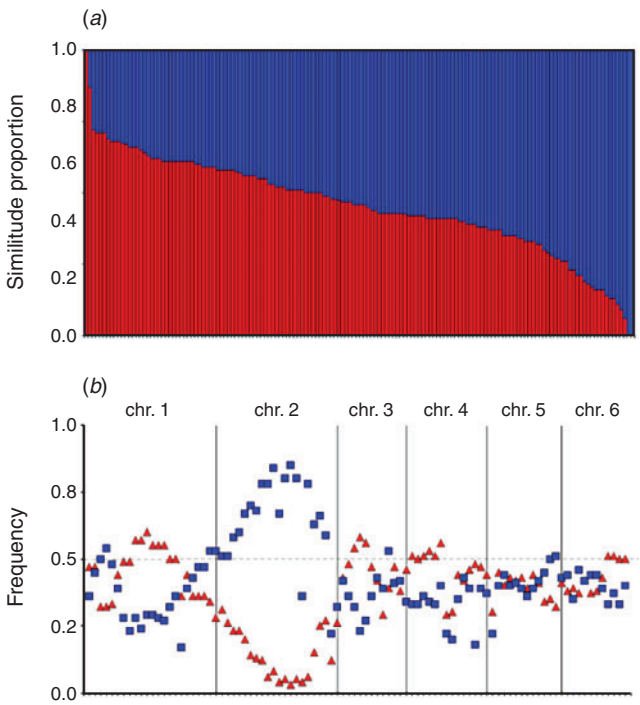

Fig. 2. Similarity proportion and allelic frequency of molecular markers in the *Lotus japonicus* × *L. burttii* RIL population. (*a*) Similarity proportion: each bar represents a unique genotype. Blue (upper) bars indicate the percentage of *L. burttii* genome, red (lower) bars the percentage of *L. japonicus* genome. First and last bars correspond to the parents *L. japonicus* and *L. burttii*, respectively. (*b*) Allelic frequency of *L. burttii* (blue squares) and *L. japonicus* (red triangles).

procedure was used where stress-specific and stress-general marker associations were detected. A table of phenotypic means was created by using the following linear model:

$$y_{ijk} = S_i + G_j + SG_{ij} + T_{k(i)} + e_{ijk}$$

where y_{ijk} is the observed variable, S_i is the ith stress treatment, G_j is the jth RIL genotype, and SG_{ij} is the stress × treatment interaction; $T_{k(i)}$ is a random variable associated with the kth tray in the ith stress treatment, with $T_{k(i)}$ ~N (0, σ^2_T); and e_{ijk} are random variables with e_{ijk} ~N (0, σ^2). When residuals from the model did not follow normal distributions, data transformations were used.

The genotype × stress interaction adjusted means were used in a GEI model to identify the variance–covariance structure (VCOV) that explained the correlation across environments:

$$y_{ij} = S_i + G_j + SG_{ij}$$

where G_j is the genotype main effect, S_i is the stress main effect, and SG_{ij} is the genotype × stress interaction. Different VCOV structures (diagonal heterogeneous variance compound symmetry, and unstructured) for SG_{ij} where compared and the best model was further used for QTL mapping.

A multi-QEI was performed in R using mixed models following Boer *et al*. (2007). In brief, the model for the genotypic effect is partitioned into QTL main effect and residual genotypic effect, while the genotype × stress interaction is partitioned into QTL × stress effect and residual genotype × stress effect as follows:

$$y_{ij} = S_i + X_j\alpha + G_j^* + X_j\alpha_i^* + SG_{ij}^*$$

where X_j is the marker allelic state, α is the QTL main effect, and α_i is the QTL × stress interaction effect; G_j* and SG_{ij}* are residual G_j and SG_{ij} effects.

The R/qtl package (Broman *et al*. 2003) was used to obtain genetic predictors for the RIL population at marker positions and at an additional grid of points with a maximum spacing of 10 cM. These genetic predictors were used as explanatory variables (X_j) in the mixed model described above. A composite-interval mapping approach was then followed where cofactors were chosen with a forward selection; markers with a significant marker–trait association in a single-interval mapping were used in subsequent rounds of the analysis as marker-cofactors to control for background genetic loci. A window of 10 cM was used for marker cofactors; within that

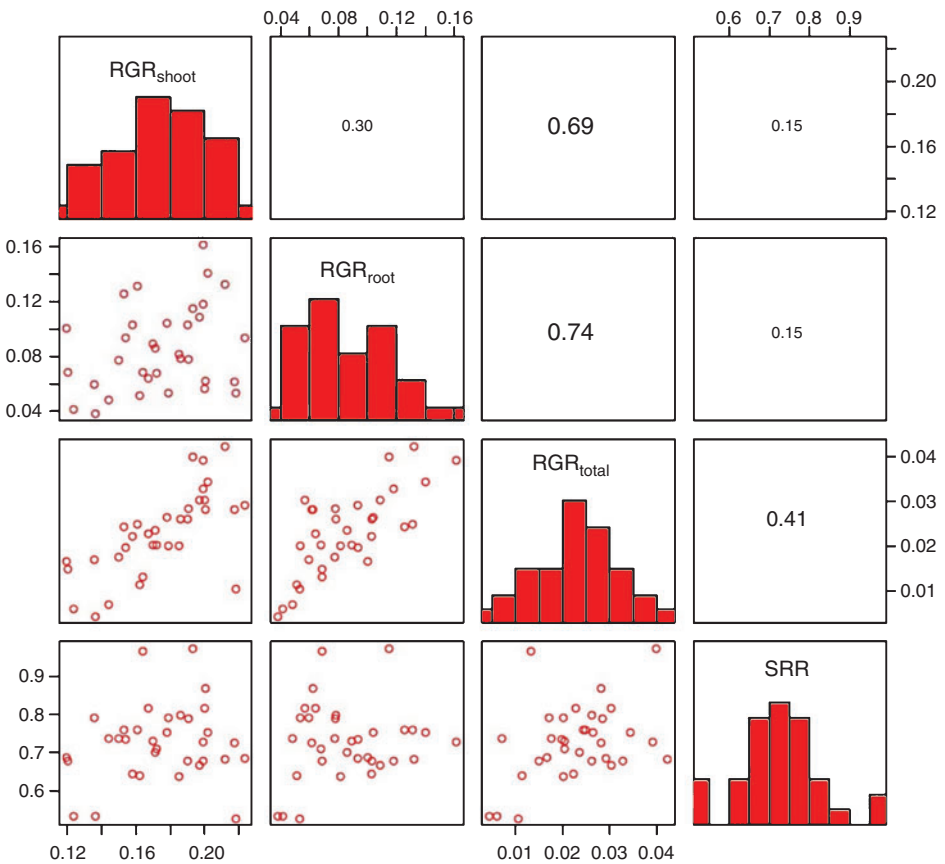

Fig. 3. Frequency distribution and correlation among growth and development parameters of the *Lotus japonicus* × *L. burttii* RIL population under the control treatment (Hornum growth medium). Histogram of frequency is shown in the diagonal. Bottom left corner: diagram of correlation between parameters. Upper right corner: correlation coefficient. RGR_{shoot}, RGR_{root}, RGR_{total}: Relative growth rates of shoot, root and total plant; SRR, shoot/root ratio. Numbers on the axes indicate the values of the parameters.

window, a cofactor was removed to avoid colinearity. The R/lme4 package (Bates and Maechler 2010) was used to fit the linear mixed model to detect environment-specific QTLs with a compound symmetry structure for the VCOV across environments (stress levels), and using markers × stress (genetic predictors × stress) as explanatory variables. A liberal *P*-value of 0.05 was used to detect QTLs because it is an exploratory study. As convention, a positive effect was assigned if the superior allele comes from the *L. burttii* parent, while a QTL negative effect indicates that the superior allele comes from *L. japonicus*.

Results

RIL genome analysis

The proportions of shared alleles showed that the RIL population combined appropriately the genomes of both parents *L. japonicus* and *L. burttii* (Fig. 2*a*). The allelic frequency along the genome of the RILs should approximate 0.5. In this case, the frequency was as expected in all chromosomes with the exception of markers on chromosome 2, which showed a higher proportion of the genome belonging to *L. burttii* (Fig. 2*b*). After removal of the markers or genotypes with a high percentage of missing data, >30 and 50%, respectively, a random pattern of the distribution of missing values in the genotype by marker matrix was observed (data not shown). Another step in the genotype data validation was the verification of the marker position in each linkage group. After examination of the missing data pattern and correction to the matrix of original genotypes, the recombinant fraction (r) for each marker in each linkage group was estimated and the probability for $r = 0.5$ was calculated. Recombination fraction analysis of markers with a linkage group indicated that the map obtained was consistent with a good marker alignment (see Supplementary data available at journal's website).

Phenotypic response of the RIL population under ionic–osmotic stress conditions

Analysis of frequency distribution and correlation among the growth and development parameters of the *L. japonicus* × *L. burttii* RIL population under control and stress treatments was performed.

The phenotypic evaluation of RILs showed that the growth parameters analysed were affected by the treatments applied. In the control treatment, all growth parameters showed a unimodal distribution (Fig. 3). The NaCl treatment did not modify any of the parameters with the exception of RGR_{shoot},

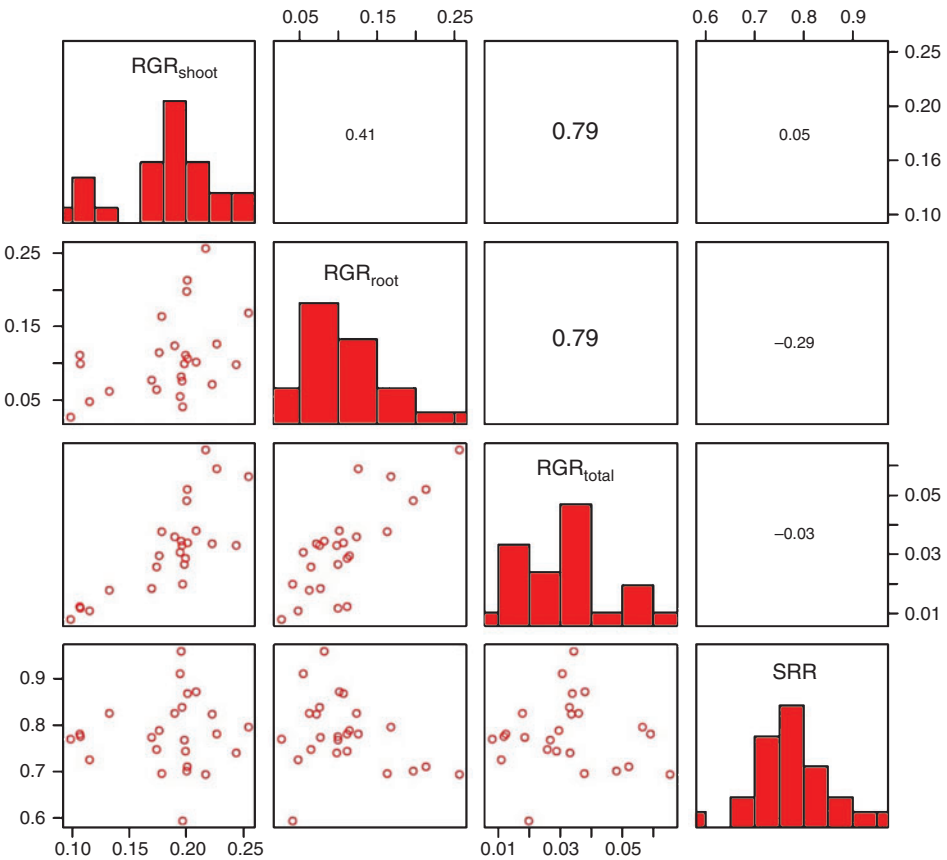

Fig. 4. Frequency distribution and correlation among growth and development parameters of *Lotus japonicus* × *L. burttii* RIL population under the NaCl treatment (Hornum growth medium supplemented with 150 mM NaCl). Histogram of frequency is shown in the diagonal. Bottom left corner: diagram of correlation between parameters. Upper right corner: correlation coefficient. RGR_{shoot}, RGR_{root}, RGR_{total}: Relative growth rates of shoot, root and total plant; SRR, shoot/root ratio. Numbers on the axes indicate the values of the parameters.

which was modified to a bimodal distribution (Fig. 4). In the PEG treatment, the parameters that changed to a bimodal distribution were the highly correlated ones, RGR_{root} and RGR_{total} (Fig. 5). The correlation coefficient among the variables was similar in all conditions evaluated, except in the case of the RGR_{total} and SRR correlation, where a significant correlation was observed in the control but not in the stressed condition (Figs 3–5).

Markers associated with the identified QTLs had different effects according to experimental conditions

We identified eight QTLs. Three QTLs for RGR_{shoot}, designated qS3, qS5 and qS6, were identified on chromosomes 3, 5 and 6, respectively. One QTL for RGR_{root} (qR1) was identified on chromosome 1.Three QTLs for RGR_{total} (qT1, qT4, qT5) were identified on chromosomes 1, 4, and 5. Finally, one QTL for SRR qSR3 was detected on chromosome 3 (Fig. 6).

A linear mixed model to detect environment-specific QTLs, and markers × stress as explanatory variables, were used, with a compound symmetry VCOV structure. A positive effect was assigned if the superior allele came from the *L. burttii* parent and a QTL negative effect if the superior allele came from *L. japonicus*.

From the 17 markers associated with QTLs for the parameter RGR_{shoot}, eight were assigned to chromosome 3 (qS3), seven to chromosome 5 (qS5), and two to chromosome 6 (qS6) (Fig. 6*a*). In case of RGR_{root}, all markers associated with QTLs were assigned to chromosome 1 (qR1) (Fig. 6*b*). For markers associated with QTLs for RGR_{total}, two were assigned to chromosome 1 (qT1), two to chromosome 4 (qT4), and two to chromosome 5 (qT5) (Fig. 6*c*). When the development parameter SRR was analysed, all markers associated with the identified QTL were assigned to chromosome 3 (qSR3) (Fig. 6*d*).

The *L. burttii* allele was superior in all markers linked to qS5 in all conditions, and qS3 and qS6 in PEG and control treatments (Fig. 6*a*). Results for RGR_{root} were similar to those for RGR_{shoot}. The *L. burttii* allele was superior in all markers linked to qR1 found in control and PEG treatment (Fig. 6*b*). However, the *L. japonicus* allele was superior in all markers associated with the QTL found in the NaCl treatment (Fig. 6*b*). The *L. burttii* allele was superior in the markers associated with qT1 and qT5 in control and NaCl treatments, respectively. However, the markers associated with qT4 showed superiority of the *L. japonicus* allele in the same treatments. The parental allele of *L. burttii* was superior in all markers linked to all QTLs found in the PEG treatment (Fig. 6*c*). The *L. burttii* allele was superior in most of

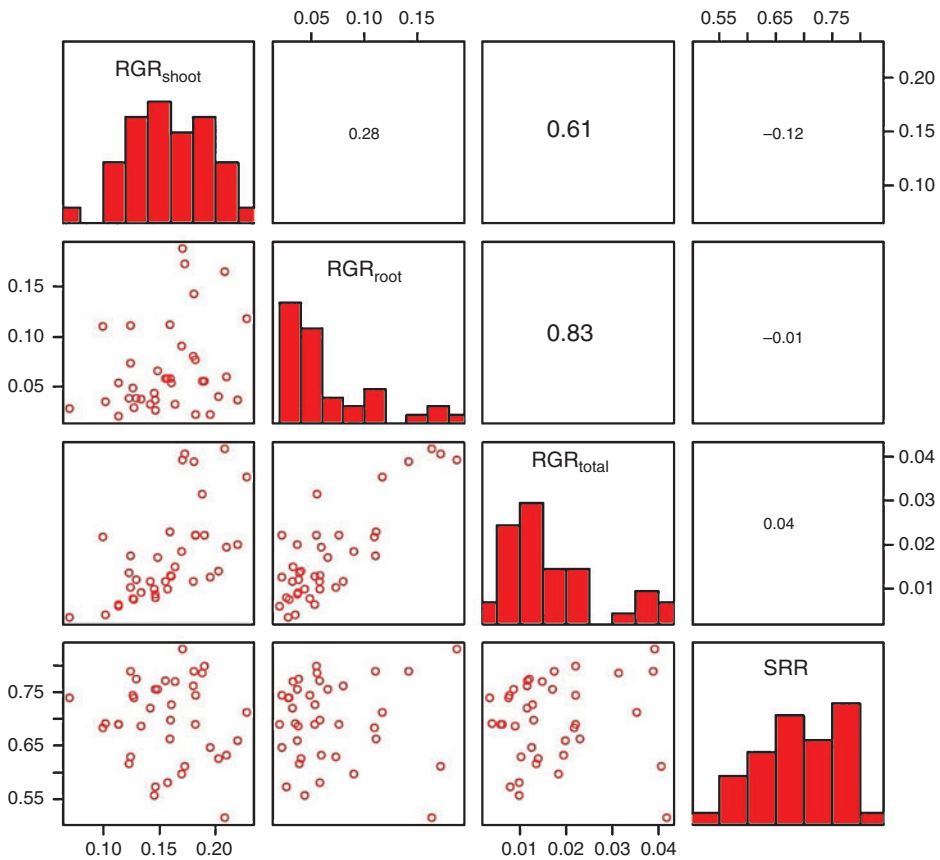

Fig. 5. Frequency distribution and correlation among growth and development parameters of *Lotus japonicus* × *L. burttii* RIL population under the PEG treatment (Hornum growth medium supplemented with 15% PEG). Histogram of frequency is shown in the diagonal. Bottom left corner: diagram of correlation between parameters. Upper right corner: correlation coefficient. RGR_{shoot}, RGR_{root}, RGR_{total}: Relative growth rates of shoot, root and total plant; SRR, shoot/root ratio. Numbers on the axes indicate the values of the parameters.

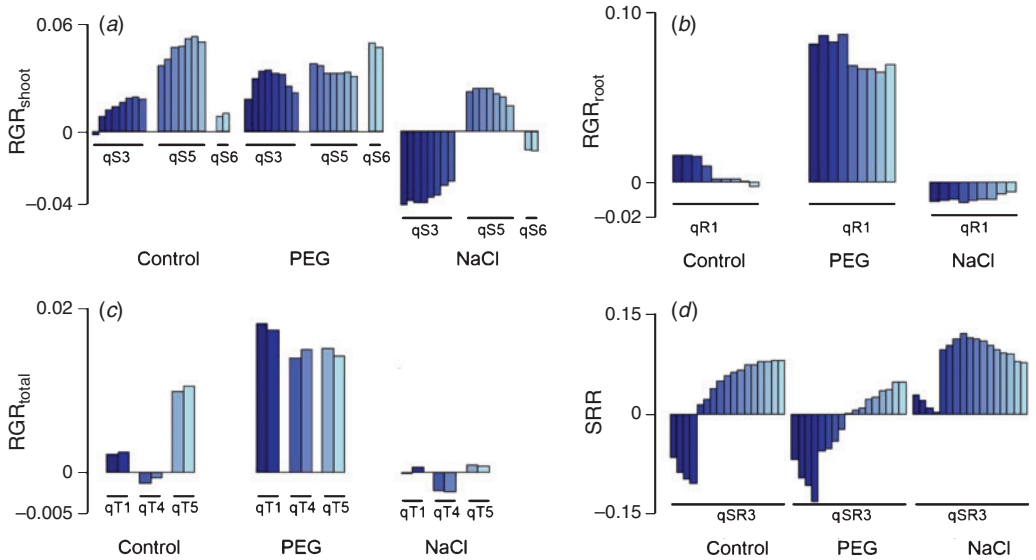

Fig. 6. QTL analysis for growth and development parameters of the *Lotus japonicus* × *L. burttii* RIL population; magnitude of QTL effect in each environment evaluated. Relative growth rates (RGR) of: (*a*) shoot, (*b*) root, and (*c*) total plant; and (*d*) shoot/root ratio (SRR). Each one of the significant markers and pseudo-makers within a QTL region (qS3, qS5 and qS6 for RGR_{shoot}; qR1 for RGR_{root}; qT1, qT4 and qT5 for RGR_{total}; qSR3for SRR) is represented as a vertical bar where the length of the bar on the *y*-axis indicates the magnitude of the effect. As convention, a positive effect is assigned if the superior allele comes from the *L. burttii* parent and a negative effect if the superior allele comes from the *L. japonicus* parent. The complete list of the markers and pseudo-marker associated is shown in supplementary Table S1.

the markers detected for qS3 found in control treatment. In case of PEG treatment, the parental allele of *L. burttii* was superior in half of the markers associated with the same QTL; however, in NaCl treatment the parental allele of *L. burttii* was superior in all of the markers associated (Fig. 6*d*).

Finally, analysis of QTL localisation along the *Lotus* genome showed that QTLs for growth parameters co-localised; the QTL for RGR_{total} co-localised with the QTL for RGR_{root} and the QTL for RGR_{shoot} on chromosome 1 and chromosome 5, respectively. Also, the QTL for RGR_{shoot} co-localised with the QTL for SRR on chromosome 3. On the other hand, chromosomes 4 and 6 showed QTLs for RGR_{total} and RGR_{shoot}, respectively (Fig. 7).

Discussion

The phenotype expressed during growth and development of plants is a result of genome interaction with different environmental conditions. Phenotype analysis in crop species is critical since it can reflect the plant productivity. However, efforts in genomic analysis have not been followed by a good understanding of phenotype, i.e. the phenotype gap (Miflin 2000; Verslues *et al.* 2006). Thus, a robust and trustworthy plant phenotyping system could improve the analysis of morphological and physiological–biochemical traits under different environmental stress conditions. In order to improve knowledge about the phenotypes expressed by *L. japonicus* and *L. burttii* under ionic and osmotic stress conditions, we set up and adjusted an assay in hydroponic conditions. The plant hydroponic system developed in this study worked well enough to grow the plants and quantify the ionic–osmotic stress effects on thousands of individual plants at the same time. Moreover, non-destructive analysis allows measurement of behaviour of different genotypes under particular stress conditions during a precise period. Parameters included the characterisation of plant growth and development responses to stress in genotypes of *L. japonicus* (Gifu) and *Lotus burttii* and a RIL population developed from interspecific crossing between these lotus species. The data demonstrated that RILs respond differentially to the ionic and osmotic stress applied and support the hypothesis that the genetic background selected could be influencing this response.

In summary, it was established that the allele from *L. burttii* explained most responses to osmotic stress, while the alleles of *L. japonicus* explained the responses related with ionic stress conditions. Data showed that from 49 markers linked to the QTLs identified, 41 expressed superiority of the *L. burttii* parental allele under the osmotic stress condition, but when an iso-osmotic concentration of NaCl was applied, *L. burttii* lost superiority in 21 of them.

It is important to take advantage of the genetic and genomics tools available (Melchiorre *et al.* 2009) to contribute to the establishment of a correlation between phenotypic and genotypic variables, with the concomitant identification of the genetic determinants of plant stress responses. The missing data pattern analysis allows the identification of problems in QTL detection (Broman and Sen 2009). Results of the quality-checking process indicated that the genotypic data are useful for performing an exploratory QTL mapping study.

Analysis of phenotypes of the RIL population under controlled conditions showed a unimodal frequency distribution. However, when plants were subjected to ionic stress, the RGR_{shoot} showed a frequency distribution of bimodal type, and this same behaviour

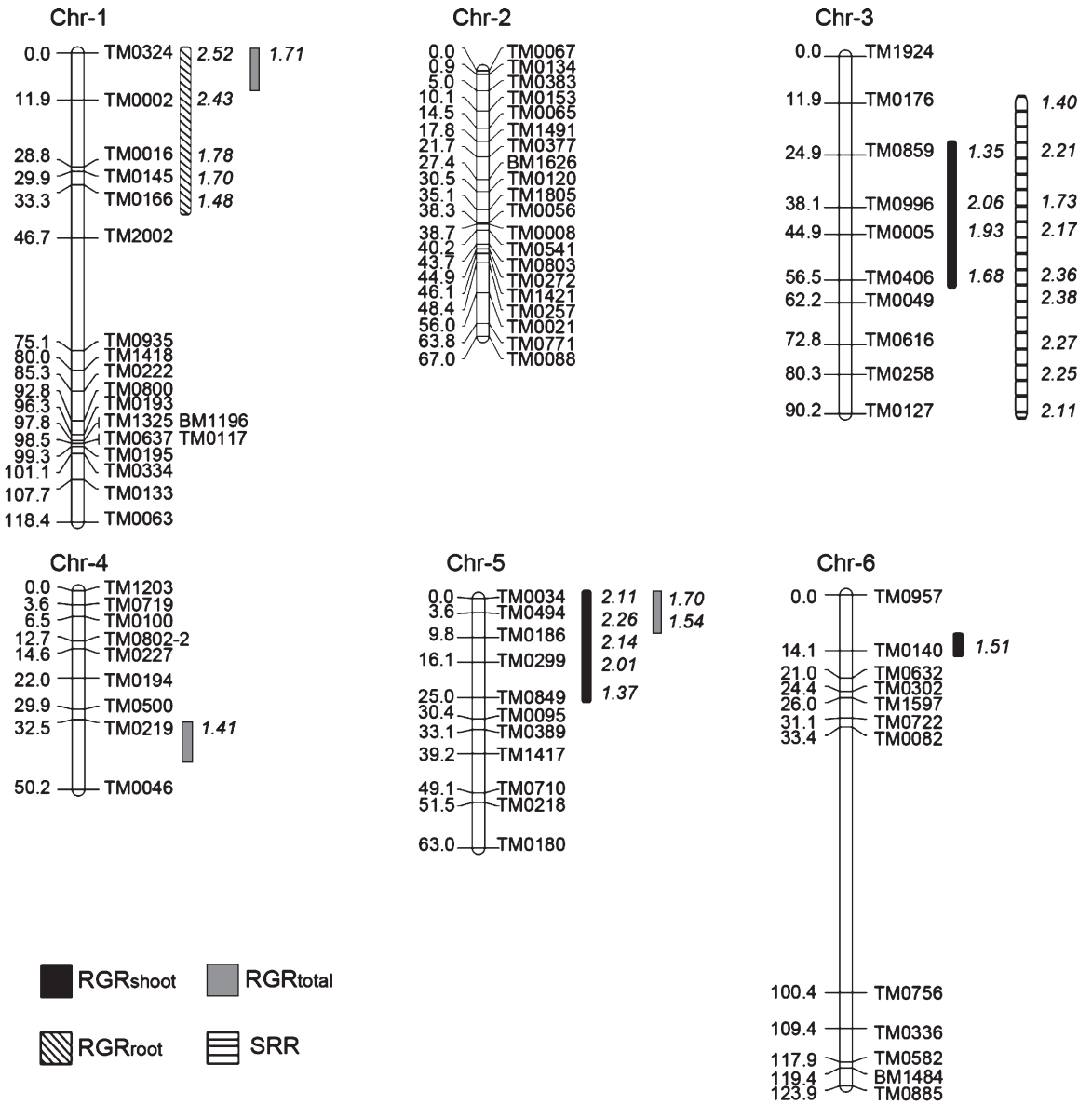

Fig. 7. Position on the genetic map and significance of QTLs detected in the *Lotus japonicus* × *L. burttii* RIL population. Vertical lines indicate the chromosome region containing the QTLs identified for each growth and development parameter evaluated. Results are given as *P*-values on a –log10 scale. RGRshoot: relative growth rate of shoot. RGR_{shoot}, RGR_{root}, RGR_{total}: Relative growth rates of shoot, root and total plant; SRR, shoot/root ratio. The complete list of the markers and pseudo-marker associated is shown in supplementary Table S1.

was observed for RGR_{root} when the plants were under osmotic stress conditions. These results are interesting, since each RIL is a unique genotype, and similarity proportion analysis showed that the RILs represent the combination of the genome of both parents. Therefore, the response in specific stress conditions could be associated with a particular genetic component (Tanksley 1993; Tuberosa *et al*. 2002; Collard *et al*. 2005).

If a distinct allele from a QTL differs in the magnitude or effect (+/−) depending on the original genetic background, then the QTL effect is dependent on the genetic and environment context (Mackay *et al*. 2009). Despite this, few efforts have been dedicated to incorporate GEI into QTL detection methods (Malosetti *et al*. 2004). Among the models used for QTL detection, the mixed models are especially adequate for modelling complex scenarios as generated by GEI (Boer *et al*. 2007; Malosetti *et al*. 2011). In this work, we used mixed models in order to identify a specific effect of each QTL in each experimental condition evaluated. Analysis showed that in specific conditions (stress or no stress) the parental allele of *L. japonicus* conferred an advantage over the parental allele of *L. burttii*, but under other conditions the *L. burttii* allele was superior to the *L. japonicus* allele, indicating that superiority of genotype is condition-dependent. An example of this is the QTLs associated with the growth parameters under PEG treatment, where the parental allele of *L. burttii* was always superior; however, under NaCl treatment the parental allele of

L. japonicus was superior for all QTLs associated with the RGR_{root} and for some of the QTLs associated with RGR_{shoot} and RGR_{total}.

Gondo *et al*. (2007), working with a population *L. japonicus* Gifu × *L. japonicus* MG-20 RIL, found a QTL for plant height in a region close to TM0996 (chromosome 3), in the same region where we found QTLs related to shoot growth, indicating that the region linked to this marker is highly associated with plant growth. Moreover, both Gondo *et al*. (2007) and our study find a negative effect of the Gifu ecotype allele on the QTLs associated with aerial plant growth; however, we show that in the NaCl treatment, the Gifu ecotype allele reverts to having a positive effect on RGR_{shoot}. In contrast with the results of Gondo *et al*. (2007), we were unable to detect QTLs on chromosome 2. Most of the QTLs detected by Gondo *et al*. (2007) are associated with reproductive traits; because our work was focussed on the vegetative stage, this could explain the absence of QTLs on this chromosome. However, detection of no QTLs on chromosome 2 due the strong distortion in the expected segregation in this chromosome should not be excluded.

Because of the presence of GEI, it is important that the analysis of allele contribution associated with a trait of interest in different lotus genotypes be performed in the context of multiple environments. This is especially important when plant stress-response analysis is required, and analysis through a hydroponic-growth phenotyping system appears useful to identify genomic regions associated with a specific stress response.

The importance of this study is that it suggests that particular QTLs display alleles with positive effects under osmotic stress but negative effects under ionic stress. Identification of the underlying gene/s would allow the understanding of this significant result and help in the selection of appropriate genetic backgrounds (accessions) depending on environmental conditions.

This study shows the superiority of the parental allele of *L. japonicus* in ionic stress conditions. Interestingly, the differential contribution of each allele of *L. burttii* and *L. japonicus* in the QTLs for the responses to osmotic and ionic stress, respectively, is concordant with the results obtained from the parental phenotypic characterisation in both stresses. A previous study in these species has demonstrated that *L. japonicus* is more tolerant to NaCl (Melchiorre *et al*. 2009). Therefore, a study directed at whether the QTLs associated with the salt tolerance from the *L. japonicus* allele would be related to osmoregulation and/or ionic homeostasis should be considered.

The complete sequencing of the *L. japonicus* genome opens the possibility of fine mapping of the QTLs. In this perspective, gene identification for ionic–osmotic stress tolerance in legumes using genetic map information and genome data is an achievable goal. Moreover, synteny existing with other *Lotus* species of agricultural importance (N Sandal, pers. obsv.) suggests the possibility of transfer of this information to forage-legume breeding programs.

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