Special Issue

Phenomic networks reveal largely independent root and shoot adjustment in waterlogged plants of *Lotus japonicus*

Gustavo G. Striker[1], Cecilia Casas[2], Milena E. Manzur[1], Rocío A. Ploschuk[1] & Jorge J. Casal[1]

[1]*IFEVA-CONICET, Cátedra de Fisiología Vegetal and* [2]*Cátedra de Edafología, Facultad de Agronomía, Universidad de Buenos Aires, Avenida San Martín 4453, Buenos Aires, CPA 1417 DSE, Argentina*

ABSTRACT

Waterlogging imposes severe stress to the plant, and the interplay between root and aerial organs in the adjustment to this stress is poorly understood. A set of recombinant inbred lines (RILs) of *Lotus japonicus* (Gifu B-129 × Miyakojima MG-20) was subjected to control and waterlogging conditions for 21 d, and 12 traits related to leaf physiological functioning, root aerenchyma formation, shoot and root morphology, and dry mass accumulation were assessed to generate phenomic networks. The phenomic network became more complex under waterlogging as a result of the incorporation of root aerenchyma and dark-adapted Fv/Fm. Significant waterlogging-induced variation was found for stomatal conductance, dark-adapted Fv/Fm and aerenchyma. The RILs with stronger induction of aerenchyma in response to waterlogging tended to show reduced negative impact of this stress on root growth but suffered average impact on shoot growth. The RILs that retained higher stomatal conductance under waterlogging tended to retain higher dark-adapted Fv/Fm and shoot growth under waterlogging conditions but showed average impact on root traits. We propose a model where, although the stress experienced by the roots during waterlogging is transmitted to the shoot, shoots and roots deal with waterlogging in a less interdependent manner than often assumed.

Key-words: inducible responses; legumes; phenotypic variation; QTL; recombinant inbred lines; waterlogging.

INTRODUCTION

Waterlogging is one of the most important constraints affecting crop and forage production worldwide due to the detrimental effects that it provokes on most terrestrial plants (Bailey-Serres & Voesenek 2008; Colmer & Voesenek 2009; Voesenek & Bailey-Serres 2013). Predictions by the Intergovernmental Panel on Climate Change (http://www.ipcc.ch/) are that global warming will result in more erratic weather patterns with increasing frequency of precipitations. Thus, the occurrence of soil water excess in flooded plains and cultivated lands is expected to be higher in the near future

(Arnell & Liu 2001). In addition, the increasing world population will enhance the pressure to incorporate marginal lands, including areas that experience frequent soil waterlogging, into production to cope with the rising food demand.

Legumes are the most important components of pastures after grasses. The *Lotus* genus includes more than 180 species distributed worldwide (Pajuelo & Stougaard 2005), and it is currently used to improve pastures in stressful environments, where other traditional forage legume species such as alfalfa or red clover do not succeed (Dear *et al*. 2003). Legumes such as *Lotus corniculatus*, *L. uliginosus* and *L. tenuis* have been domesticated and improved through selection and plant breeding. These three species have some degree of tolerance to soil waterlogging, but *L. tenuis* is the most tolerant to oxygen deficiency at root zone (James & Crawford 1998; Striker *et al*. 2005, 2012; Teakle *et al*. 2006). *L. japonicus*, which is taxonomically closely related to these forage species (Young *et al*. 2003), has arisen as a model for legumes because it is a small plant with short generation time, easy cultivation and is amenable to transformation (Pajuelo & Stougaard 2005). A set of genetic resources and tools is rapidly developing, including ecotypes, mutant lines, genetic maps, recombinant inbred lines (RILs), and a whole genome-sequencing project is underway (Sanchez *et al*. 2012). *L. japonicus* shows tolerance to waterlogging and is able to survive at least 12 d of submergence at seedling stage (Striker *et al*. 2012) and 30 d of waterlogging in adult plants (Rocha *et al*. 2010).

Plants develop a suite of anatomical, morphological and physiological responses to cope with anaerobic stress imposed by soil waterlogging (Visser & Voesenek 2004; Colmer & Voesenek 2009). Roots are affected directly by soil anaerobic conditions, and for this reason waterlogging normally has larger effects on root than shoot growth (Colmer & Voesenek 2009). At the anatomical level, the development of root aerenchyma, usually triggered by ethylene accumulation, is one of the most typical responses to anaerobiosis (Justin & Armstrong 1987; Visser *et al*. 2000; Striker *et al*. 2007; Abiko *et al*. 2012). This tissue provides a path of low resistance for the diffusive movement of oxygen from shoots to roots, thus enabling plant internal aeration (Justin & Armstrong 1987; Colmer 2003). At the morphological level, both shoot and root architecture can be altered by the lack of oxygen in the soil. In *L. tenuis*, shoots can become taller as a result of promotion of stem and/or leaf elongation induced

by waterlogging in tolerant genotypes (Manzur *et al.* 2009; Striker *et al.* 2012). The number of shoots can also be modified by waterlogging, depending on the balance between mortality and appearance of shoots (Malik *et al.* 2002). At the physiological level, waterlogging can induce early leaf senescence, plant yellowing, closing of stomata and photoinhibition under high irradiance conditions (Else *et al.* 1996; Smethurst & Shabala 2003; Striker *et al.* 2005; Colmer & Voesenek 2009).

In soybean (Cornelious *et al.* 2005), maize (Qiu *et al.* 2007) and wheat (Bai *et al.* 2013), waterlogging tolerance is controlled by several quantitative trait loci (QTL), each one with a relatively small contribution. On the contrary, tolerance to complete submergence in rice *via* the quiescence strategy involves a major locus, encoding the *SUB1* gene (Xu *et al.* 2006).

'Comprehensive whole-plant phenotyping, including both shoots and roots, is only occasionally practiced' (Dhondt *et al.* 2013). Here, we present the phenotypic analysis of *L. japonicus* plants subjected to control and waterlogging conditions for 21 d, by which the use of a population of 94 RILs and the measurement of different traits related to root and shoot growth, anatomy and physiology provide dimensionality to the study (Dhondt *et al.* 2013). The aim of the work was to investigate the links between traits by generating phenomic networks under both conditions to get insight into the coordination among the various plant responses to the stress imposed by waterlogging.

MATERIALS AND METHODS

Plant material

A total of 94 *L. japonicus* RILs derived from a cross between the lines Gifu B-129 (Gifu) and Miyakojima MG-20 (Miyakojima) self-pollinated to the F_8 generation (Kawaguchi *et al.* 2001) were used in this study. The seeds were obtained from the National BioResource Project, Miyazaki University (http://www.legumebase.brc.miyazaki-u.ac.jp/).

Seeds of each RIL were scarified, surface-sterilized with 0.04% (w/v) sodium hypochlorite, rinsed thoroughly in deionized water and germinated in an incubator (25 °C) in polystyrene boxes containing absorbent water-saturated white paper. After 2 to 3 d, germinated seeds were transplanted to 0.5 L plastic pots (two or three seedlings per pot) filled with sand and soil (1:1 v/v). The soil used corresponded to the non-saline A horizon (0–10 cm) of a Typic Natraquoll of loam texture and 3.3% of organic carbon extracted from a lowland grassland of the Flooding Pampa in Argentina (36°30′S, 58°30′W; Soriano 1991). After seeding, pots were transferred to a glasshouse at the Faculty of Agronomy at the University of Buenos Aires (Fig. 1a), and during the first week the seedlings were thinned to one per pot. In order to avoid nutrient limitation for plant growth, pots were additionally irrigated weekly before the treatments with 100 mL of a half-strength modified Hoagland solution, consisting of: 2 mM $Ca(NO_3)_2\cdot4H_2O$, 2 mM KNO_3, 0.5 mM NH_4NO_3, 0.5 mM $MgSO_4\cdot7H_2O$, 0.35 mM KH_2PO_4, 40 μM ethylenediaminetetraacetic acid iron III

sodium salt (FeEDTA), 30 μM H_3BO_3, 2 μM $MnCl_2\cdot4H_2O$, 2 μM $ZnSO_4\cdot7H_2O$, 0.5 μM $CuSO_4\cdot5H_2O$, 50 μM KCl, 0.075 μM $(NH_4)_6Mo_7O_{24}\cdot4H_2O$, 0.15 μM $CoCl_2\cdot6H_2O$. The solution was adjusted to pH 6.5 by adding KOH.

Experimental design

After a growth period of 60 d, when plants started flowering, pots were randomized into two groups with four replicates (94 RILs × 2 conditions × 4 replicates: 752 plants). In the control, pots were watered daily to field capacity and allowed to drain freely. In the waterlogging condition, pots were sealed with plastic bags to prevent water drainage, and irrigated to maintain the water level 5–10 mm above the soil surface. Soil hypoxia by waterlogging was characterized by measuring the oxygen diffusion rate (ODR) at a soil depth of 5 cm with platinum microelectrodes (Letey & Stolzy 1964). The ODR decreased quickly during waterlogging from 64 ± 1 to $5 \pm 1 \times 10^{-8}$ g cm^{-2} min^{-1} in the first 3 d, and to $0.6 \pm 0.3 \times 10^{-8}$ g cm^{-2} min^{-1} at the end of the experiment. Temperature at the root zone (4–5 cm depth) ranged between *ca.* 14 and 18 °C, and it was checked twice a week to keep it similar between treatments in 30 pots of each treatment randomly selected ($P > 0.60$). During the experiment, which lasted 21 d, the glasshouse temperature was controlled to remain between 18.5 and 25.3 °C.

Dry mass

Plants were harvested at the end of the experiment (day 21), and aerial parts were separated from roots. Aerial parts were dissected into shoots (i.e. leaves plus stems) and pods. Dry mass of each plant component was obtained after drying at 80 °C for 72 h (i.e. until constant weight). Roots were processed as described in subsequent sections, prior to oven-drying. Four true replicates per RIL and condition were harvested to assess dry mass responses.

Shoot morphology

The number of primary shoots and the number of secondary branches per plant were counted in all the replicate plants (four per RIL and condition) 21 d after the beginning of treatments. The length of the primary shoots was measured and then added to obtain the total primary shoot length.

Root morphology

The root systems of two plants per RIL and condition were harvested after 21 d of treatment. The roots were carefully washed and arranged in a 20-cm-wide and 30-cm-long acrylic container with approximately 1 cm height of deionized water to minimize overlapping among roots. Digital images of root systems were obtained by using an Epson Perfection V700 Photo scanner with a resolution of 400 dpi (Epson America Inc, Long Beach, CA, USA). Scanned root systems (*ca.* 400 images) were processed using the software WinRhizo Pro V
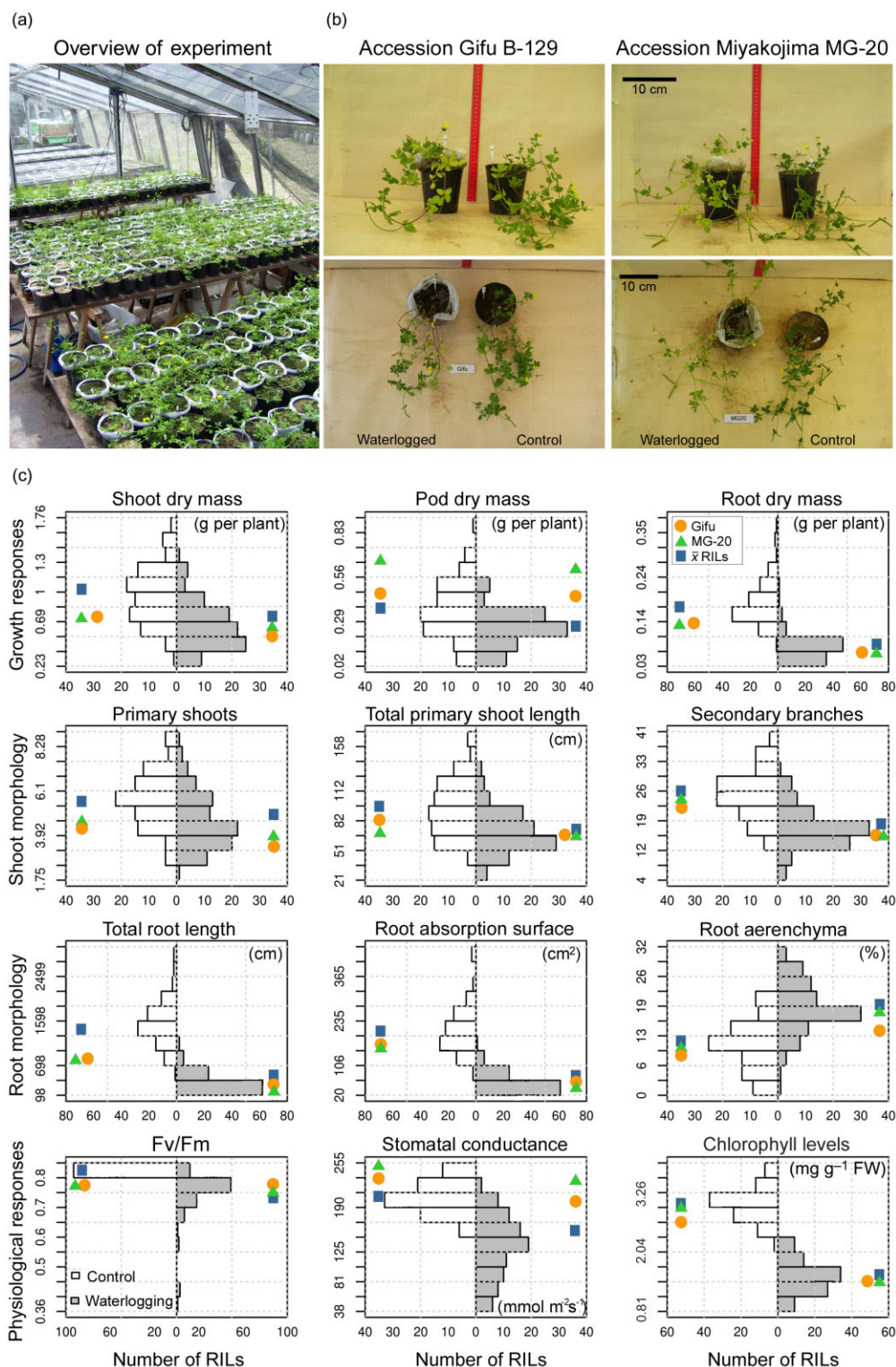
Figure 1. Variability in waterlogging impact on root and shoot growth and function in recombinant inbred lines (RILs) of *Lotus japonicus* derived from a cross between Miyakojima MG-20 and Gifu B-129. (a) Overview of the experiment. (b) Representative plants of the parental accessions plants after 2 weeks of treatment. Black bars represent the scale (10 cm). (c) Effects of waterlogging on root and shoot traits at the end of experiment (3 weeks of treatment). White bars show the histograms for control conditions and grey bars show histograms for the waterlogging condition. Circles represent average values of parental Gifu B-129, triangles represent average values of parental Miyakojima MG-20, and squares represent average values of RILs. Four replicates for dry mass responses, total primary shoot length, primary shoots and secondary branches. Two replicates for total root length, root absorption area and root aerenchyma, chlorophyll levels and dark-adapted Fv/Fm. Stomatal conductance had one replicate per RIL and condition.

2008 (Regent Instruments Inc, Quebec, Canada) to obtain the total root system length (lineal cm per plant), root absorption surface (cm^2) and a classification of root diameters in four categories: 0–0.2, 0.2–0.4, 0.4–0.8 and >0.8 mm of diameter. Distribution of root diameters among these categories was calculated according to the length of roots within each diametric range with respect to the total root length.

Root aerenchyma

Adventitious roots of two plants per RIL and condition were taken after 21 d treatment, washed and immediately fixed in ethanol 70%. Root samples of approximately 0.5 cm length between 2.2 and 2.7 cm above apex were dehydrated in an ethanol series and embedded in paraffin. Cross-sections of 7–8 μM thick were cut with a rotatory microtome and double-stained with Safranin–Fast Green. Representative images of root sections were taken using an optical microscope (Zeiss Axioplan; Zeiss, Oberkochen, Germany) connected to an image analyser (Imagenation Px; Imagenation Corp, Beaverton, OR, USA). Root aerenchyma, as the proportion of root cortex occupied by aerial lacunae, was measured in all cross-sections by using the public domain UTHSCSA ImageTool programme (University of Texas Health Science Centre at San Antonio, TX, USA) on digitalized photographs of the cross-sections as in Striker *et al.* (2007). We used the cross-section analysis of aerenchyma to infer on the potential of root aeration instead of measuring root porosity by pycnometric techniques. The large quantity of root tissue required for the latter method prevented the possibility of also measuring root porosity. Visser & Bögemann (2003) showed that for ranges of porosities between 10 and 25%, values matching with our measurements of aerenchyma, the correlation between these two methods is high.

Chlorophyll levels

Total chlorophyll was measured in fully expanded leaves located in the upper third of the plant (usually the third leaf from the newest leaf of a primary shoot), 19–21 d after the beginning of treatments using a portable chlorophyll meter (SPAD-502; Minolta, Ramsey, NJ, USA). Three measurements per individual plant were taken and averaged to obtain one replicate and two plant replicates were measured per RIL and condition. The calibration curve was y = 0.0705x–0.011 [$R^2 = 0.87$, $n = 24$, $P < 0.001$, where y = chlorophyll (mg g^{-1} fresh weight) and x = SPAD units]. Chlorophyll concentrations were determined spectrophotometrically (Ultrospec 2100 pro UV/Visible Spectrophotometer; Amersham Biosciences, Piscataway, NJ, USA) on leaf samples of known weight extracted in 1 mL of N,N-dimethylformamide and incubated for 3 d in darkness at 4 °C according to Moran & Porath (1980).

Dark-adapted Fv/Fm

Dark-adapted Fv/Fm was measured at night in young fully expanded leaves dark-adapted for at least 30 min, 19–21 d after the beginning of treatments using a portable modulated fluorometer (FMS-2; Hansatech Instruments Ltd, Kings Lynn, UK). Two plant replicates were measured per RIL and condition.

Stomatal conductance

Stomatal conductance was measured from late-morning to mid-afternoon (1100 to 1500 h, local time) between days 19 and 21, after the beginning of treatments in young fully expanded leaves using a portable Decagon SC-1 leaf porometer (Decagon Devices, Inc, Pullman, WA, USA). The short window-time to perform reliable measurements of stomatal conductance (at comparable conditions of atmospheric evaporative demand, i.e. air vapour pressure deficit that ranged from 2.5 to 2.8 kPa) for the large number of plants screened, allowed assessing one plant per RIL and condition.

QTL mapping

QTL mapping used the current marker positions and genotype data available at http://www.kazusa.or.jp/lotus/RIline/RI_map.html (verified on July 31, 2013). We used a total of 96 microsatellite markers, which cover >90% (420.7 cM) of the linkage map with an average genetic distance between markers of 4.4 cM, and the longest distance between two markers of 8.6 cM (Gondo *et al.* 2007). Firstly, we calculated the single-QTL genotype probabilities to determine the putative QTLs involved in the variation of the considered traits for the ratio between values of waterlogging and control (W/C) as well as for each condition separately (see Qiu *et al.* 2007). Single-QTLs genotype probabilities were performed with a step-size of 1.0 cM and significance at 0.05 via the function calc.genoprob [R/qtl package from R-cran (Broman *et al.* 2003)]. Single-QTL genome scan was performed with the function scanone which performed standard interval mapping considering normal model, maximum likelihood via the EM algorithm [R/qtl package from R-cran (Broman *et al.* 2003)]. A threshold LOD value of 2.1 was used to justify the presence of a QTL (van Ooijen 1999). After the single-QTL genome scans, multiple-QTL analysis was performed to bring the identified loci together into a joint model. A new QTL object, including single-QTL for each phenotype [makeqtl function, R/qtl package from R-cran (Broman *et al.* 2003)] were refined by multiple-QTL models using Haley-Knott regression method [fitqtl function R/qtl package from R-cran (Broman *et al.* 2003)]. QTL positions were assigned to maximum local LOD scores, and confidence intervals for the QTLs identified were indicated by 1.5 LOD support intervals (Mangin *et al.* 1994). When confidence intervals of two or more QTLs overlapped along 95% of their length, or one confidence interval fell within another, the QTLs were assumed to be co-located. The contribution of each detected QTL to the phenotypic explained variance (PEV) was estimated by variance component analysis. Data analysis was performed by R software environment (R Development Core Team 2011).

Phenomic networks and correlation analyses

Pearson's correlation coefficients among all possible combinations of measured traits were calculated separately under each condition (control and waterlogging) and for the ratio between waterlogged and control values (W/C) using a nominal 5% level of significance [cor function, basic package from R-cran (R Development Core Team 2011)]. Significant correlations were used to draw the phenomic networks showing the connections among all measured traits under each scenario (control, waterlogging and W/C). The online version of Circos software package (Krzywinski *et al.* 2009) was used to build circular layouts to visualize such networks of correlations (http://mkweb.bcgsc.ca/tableviewer/verified on 31 July 2013).

Linear regression analyses were used to determine the relationship between waterlogging and control condition across RILs for each trait [lm function, basic package from R-cran (R Development Core Team 2011)]. In addition, for specific functional traits, namely root aerenchyma, stomatal conductance, Fv/Fm and chlorophyll levels, quartile regression make it possible to group RILs in terms of minimum and maximum response to waterlogging (i.e. the highest and lowest responsive genotypes to hypoxia, which points above 0.75 and below 0.25 of the probability distribution, by using the rq function from quantreg package). In this way, we were able to analyse 'responses to waterlogging' with the advantage of avoiding the standardization of values at waterlogging proportionally to the values under control conditions (i.e. where all the responses are corrected proportionally to the magnitude observed for the variable under control conditions). With this approach, the 'responses' can be seen as a deviation from the 1:1 line (which represents the case where plants under control and waterlogging conditions have the same values). Therefore, we defined 'responsive genotypes' more or less according to their strong or weak deviation from the 1:1 line (Supporting Information Fig. S1) for root aerenchyma, stomatal conductance, Fv/Fm and chlorophyll levels. Hence, genotypes (i.e. RILs) of both groups, the upper and lower quartiles of the general regression from these specific traits were analysed along the other morphological and growth-related traits by adjusting lineal regressions (different symbols with respect to the other morphological and growth-related traits in Figs 5–7). Parameters of the equations between quartiles were also compared [slopes by slope.test function from smatr package and, when there were no differences between slopes, intercepts were compared by elev.test function from smatr package; ordinary least square method was used in all cases]. By doing this, potentially induced plastic responses to waterlogging were examined.

RESULTS

Overall responses and ranges of phenotypic variation

Two-month-old plants of 94 RILs of *L. japonicus* and their parent lines (Gifu B-129 and Miyakojima MG-20) were subjected to control and waterlogging treatments by 21 d

(Fig. 1a,b). All RILs survived the waterlogging period but, compared with controls, waterlogging reduced aerial (shoot plus pod) dry mass (31% in average across RILs) and root dry mass (61%; Fig. 1c). Waterlogging also reduced average total primary shoot length (26%), number of secondary branches (33%), total root length (72%), and root absorption surface (76%) (Fig. 1c), and shifted root diameter distribution towards a high proportion of thicker as well as thinner roots (Supporting Information Fig. S2). Conversely, waterlogging increased average root aerenchyma from 11 to 19%. Waterlogging severely compressed the range of variation explored by root size traits (dry mass, length and absorption surface) by lowering the maximum values for these parameters approximately to the minimal values observed under control conditions, but shoot size trends did not exhibit a comparable trend (Fig. 1c).

Waterlogging reduced dark-adapted Fv/Fm (11%), which is a measure of maximum quantum efficiency of photosystem II photochemistry, indicating the occurrence of photoinhibition (Maxwell & Johnson 2000), stomatal conductance (35%) and chlorophyll levels (48%; Fig. 1c). Waterlogging did not change the range of variation for chlorophyll levels but it increased by *ca.* nine and twofold the range of variation of Fv/Fm and stomatal conductance, respectively.

QTL detection

All the variables were analysed but some showed no statistically significant QTLs. We mapped four shared QTLs (same position in the chromosome) and 10 co-located QTLs (overlapping confidence intervals but different position of QTL peak) between waterlogging and control conditions (Fig. 2). Six QTLs were detected only under control conditions and four QTL were identified only under waterlogging conditions (Fig. 3). In general terms, QTLs accounted for 5.2–31.2% of the phenotypic variation (Supporting Information Table S1).

The 10 QTLs not shared by control and waterlogging conditions are relevant to the aims of this work (Fig. 3). We identified QTLs for shoot dry mass (chromosome 5), root dry mass (chromosome 4), number of secondary branches (chromosome 5), root absorption surface (chromosome 5) and chlorophyll levels (chromosomes 2 and 5) in control plants. We detected QTLs for shoot dry mass (chromosome 5), pod dry mass under (chromosome 1), root dry mass (chromosome 5) and primary shoot number (chromosome 4) in waterlogged plants (Fig. 3).

In order to focus on the response to waterlogging rather than on the absolute values of each trait (which are influenced by the values under control conditions), we also calculated the QTLs for the ratio between waterlogging and control conditions (W/C). The analysis allowed identifying a total of nine QTLs (Supporting Information Table S2). Five QTLs were related to root traits: total root length and root absorption surface (chromosomes 1 and 3), and root dry mass (chromosome 1). Four QTLs were associated to shoot traits: secondary branches (chromosomes 2 and 5), pod dry mass (chromosome 5) and chlorophyll levels (chromosome 6). It should be noticed that there was no overlapping between the
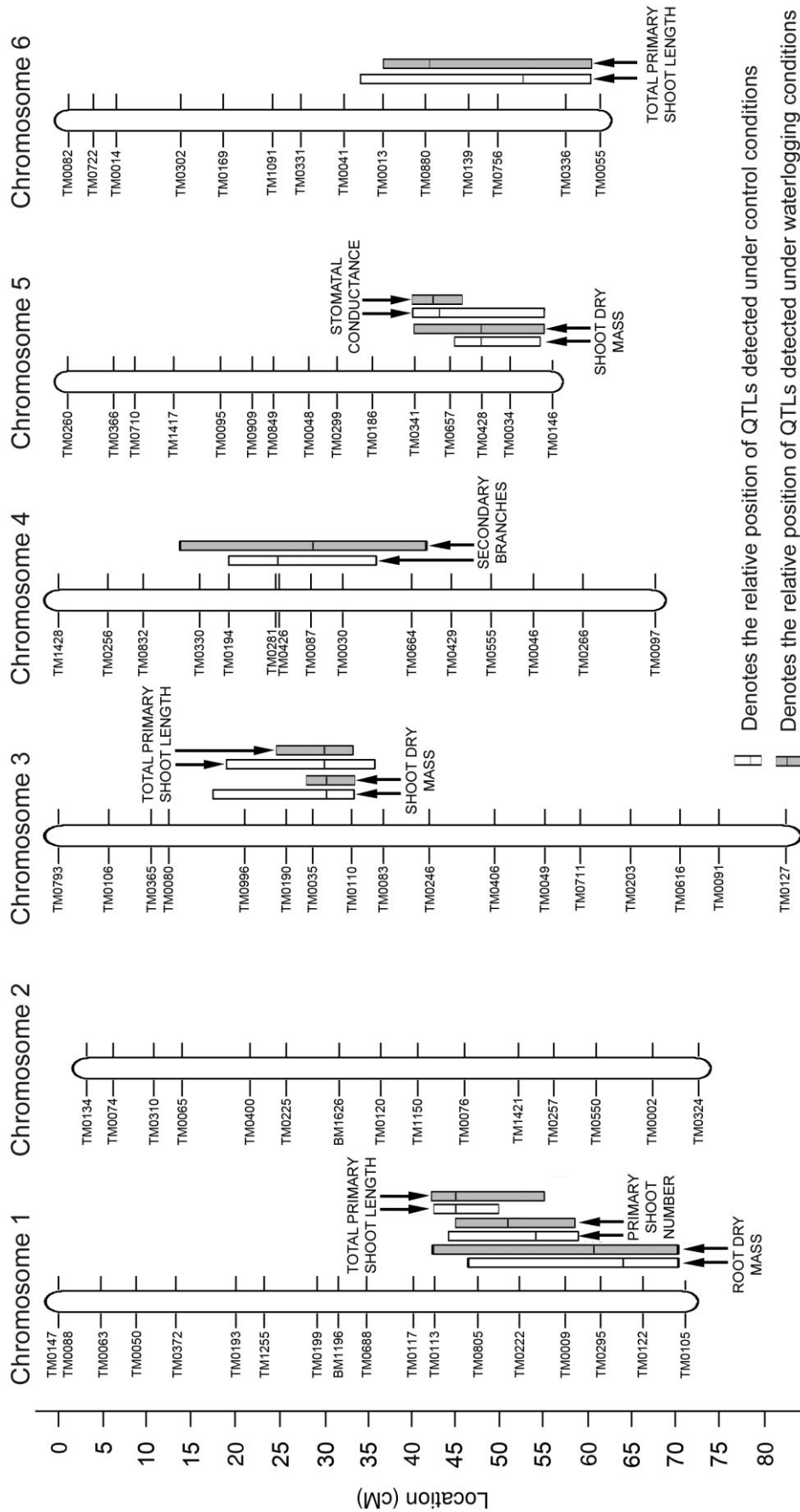
Figure 2. Molecular linkage map of *Lotus japonicus* showing quantitative trait loci (QTLs) shared and co-located between control and waterlogging conditions. Bars indicate the confidence interval for each QTL, and the mark within bars indicates the most likely location (cM). LOD scores and percentage of the phenotypic variance explained for all detected QTLs are shown in Supporting Information Table S1.
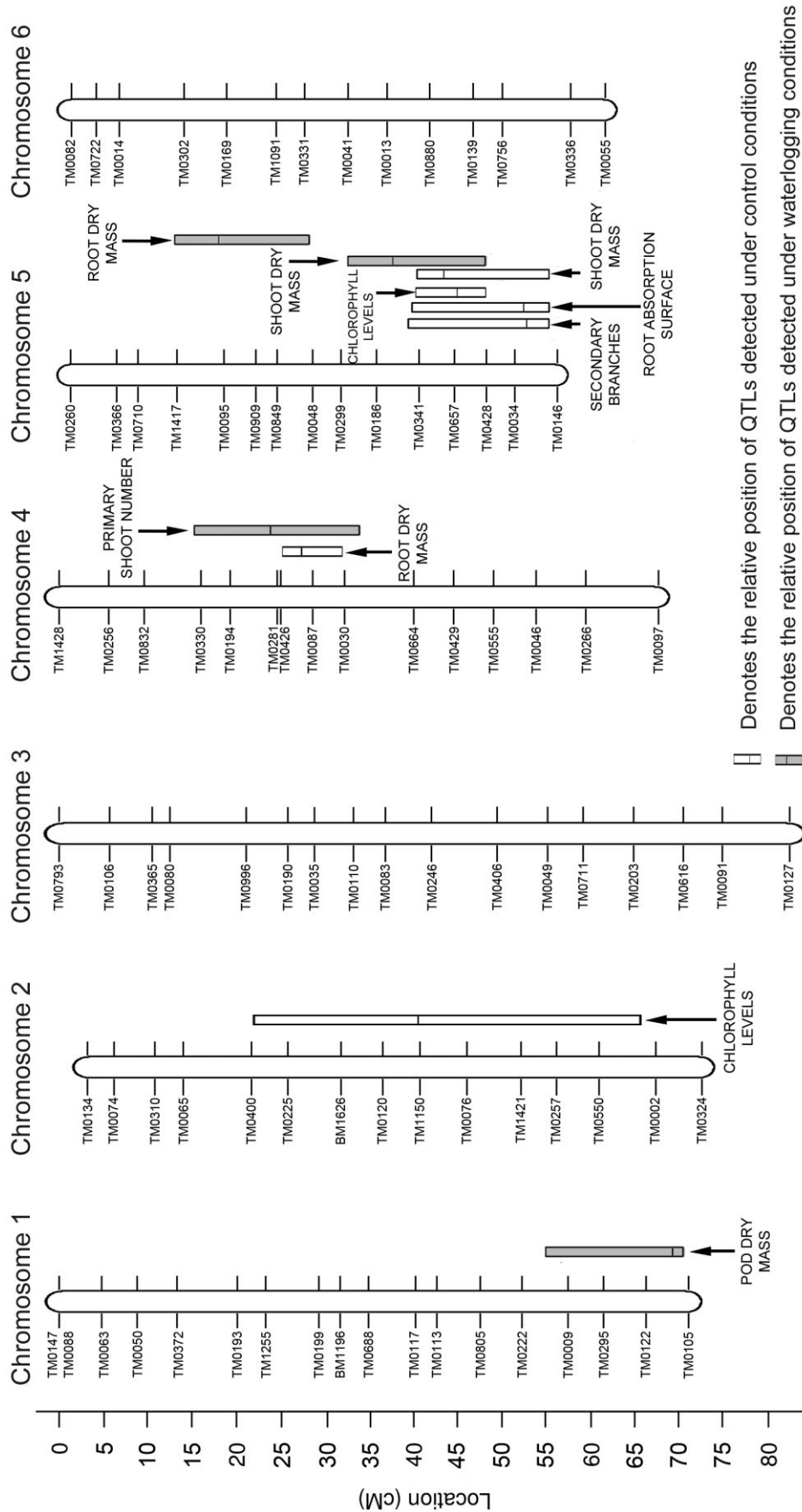
Figure 3. Molecular linkage map of *Lotus japonicus* showing quantitative trait loci (QTLs) specific for control and waterlogging conditions. Bars indicate the confidence interval for each QTL, and the mark within bars indicates the most likely location (cM). LOD scores and percentage of the phenotypic variance explained for all detected QTLs are shown in Supporting Information Table S1.
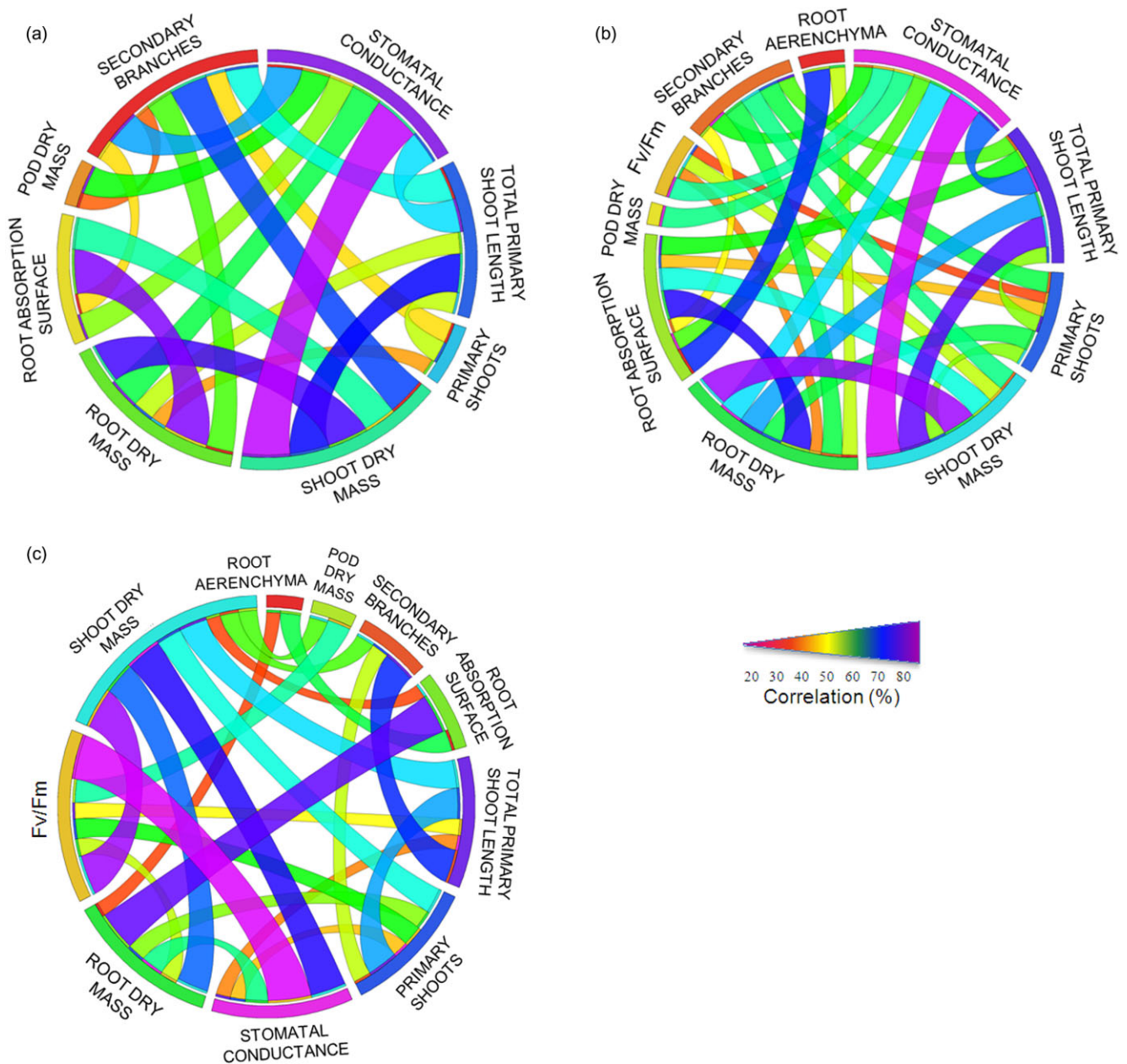
Figure 4. Phenomic network of *Lotus japonicus* incorporates root aerenchyma and Fv/Fm in response to waterlogging. (a) control. (b) waterlogging. (c) waterlogging/control. Traits are represented in the outer ring. The inner ribbons represent correlations among traits based on Pearson-ranked data (at *P* = 0.05).

QTLs for root traits (at chromosomes 1 and 3) and for shoot traits (at chromosomes 2, 5 and 6).

Similarities and diversities in the phenomic network under control and waterlogging

A circular layout showing the network of correlations for the traits of control and waterlogged plants is presented in Fig. 4a,b. The most distinctive differential feature between both conditions is that root aerenchyma and dark-adapted Fv/Fm are part of the network only for waterlogged plants. These two variables incorporated six links not present under

control conditions. It is worthy of note that the proportion of aerenchyma, which is considered a key trait favouring acclimation to waterlogging only correlated with root traits (total root length, root absorption surface and root dry mass) and not with shoot traits (Supporting Information Table S3).

Under both conditions, the shoot dry mass strongly correlated to stomatal conductance, total primary shoot length, number of secondary branches and root dry mass, and it was also correlated to total root length and root absorption surface. Pod dry mass was correlated to number of secondary branches and stomatal conductance. Root dry mass correlated to total root length and root absorption surface, and to

a lesser extent to shoot traits like total primary shoot length, number of secondary branches and stomatal conductance. Shoot morphological traits shared most of the correlations between conditions: primary shoot number, total primary shoot length and number of secondary branches were correlated among them, as well as with stomatal conductance. In both conditions, root morphological traits correlated to similar shoot traits: root absorption surface correlated with shoot dry mass, root dry mass, number of secondary branches and stomatal conductance (Fig. 4a,b; see values for correlations in Supporting Information Table S3).

In waterlogged plants, the additional links were mainly related to the incorporation of dark-adapted Fv/Fm and root aerenchyma in the network. Dark-adapted Fv/Fm, as indicative of the functioning of PSII, strongly correlated to stomatal conductance, and it also correlated to shoot dry mass, primary shoot number and root dry mass. Root aerenchyma, as a trait related to the potential for root aeration, strongly correlated to all root traits in waterlogged plants: root dry mass, total root length and root absorption surface (Fig. 4a,b). Constitutive aerenchyma (i.e. that observed under control conditions) did not correlate with any shoot trait of waterlogged plants, not even with root dry mass ($r = 0.11$; $P = 0.26$). Total root length strongly correlated to root absorption surface in both growing conditions ($r > 0.97$), but this was expected because they are not independent variables. The key features of the network did not change if the W/C ratio was used instead of the absolute values under each condition: Aerenchyma correlated only with root traits (root length, absorption surface and root dry mass) while stomatal conductance and dark-adapted Fv/Fm correlated with shoot traits (shoot dry mass, primary shoot number and total primary shoot length) and root dry mass (Fig. 4c and Supporting Information Table S4).

Comparative analysis of RILs with strong and weak response to waterlogging

Linear regression analyses of the trait values of each RIL under waterlogging conditions plotted against the values of that RIL under control conditions revealed positive relationships for traits related to dry mass, shoot and root morphology (Supporting Information Fig. S3), which at least to some extent is likely to reflect the fact that the plants were grown under common (well-drained) conditions for 2 months before the application of the treatment. This was not the case for physiological traits, as only stomatal conductance showed a general positive relationship between waterlogging and control conditions across RILs. No consistent relationships were found for dark-adapted Fv/Fm and chlorophyll levels between conditions across RILs (Supporting Information Fig S3).

A more detailed examination of the regression lines revealed considerable data dispersion under waterlogging conditions for lines having similar values under control conditions in the case of proportion of aerenchyma, stomatal conductance, dark-adapted Fv/Fm and levels of leaf chlorophyll (coefficient of variation: 18–48%). This variation

reflects the differential capacity of the lines to adjust the proportion of aerenchyma, stomatal conductance, Fv/Fm and leaf chlorophyll in response to waterlogging (Supporting Information Fig. S1). To investigate to what extent the differential adjustment of these traits impacted on other traits, the lines of the upper and lower quartiles of the proportion of aerenchyma, stomatal conductance, dark-adapted Fv/Fm and leaf chlorophyll were separated and identified within the waterlogging – control relationship of the others morphological and growth-related traits (Figs 5, 6 and 7). We then analysed the slopes and intercepts of all the waterlogging – control relationships separately for the RILs in the upper and lower quartiles to evaluate the impact of differential aerenchyma, stomatal conductance, dark-adapted Fv/Fm and leaf chlorophyll responses on other traits.

The RILs in the upper quartile for stomatal conductance tended to be in the upper quartile for Fv/Fm and vice versa (see Fig. 6). In turn, the RILs in the upper quartiles for leaf conductance and Fv/Fm also tended to be in the upper quartiles for shoot dry mass, pod dry mass and total primary shoot length (Figs 5 and 6). The presence of a RIL in the upper or lower quartiles for conductance or Fv/Fm did not relate to its position in the waterlogging – control relationships of root-related traits. On other hand, the RILs present in the upper quartile for root aerenchyma tended to be in the upper quartiles for root length, root biomass and root absorption surface (Fig. 7). However, these RILs showed no significant trend for shoot traits. Finally, analyses of the genotypes of the upper and lower quartiles of chlorophyll levels did not reveal clear indication of differences either for shoots or root traits (Supporting Information Fig. S4).

DISCUSSION

Waterlogging strongly modified the body form and function in plants of a RIL population of *L. japonicus* by impacting more on the roots, suffering the direct impact of oxygen deprivation, than on the shoots (Smethurst & Shabala 2003; Visser & Voesenek 2004; Colmer & Voesenek 2009; Rocha *et al.* 2010). In fact, traits related to the root system such as dry mass, total length and absorption surface were in average reduced 61–76% whilst traits related to organs such as shoot dry mass, total primary shoot length, primary shoot number and number of secondary branches were in average reduced 18–32% (Fig. 1). The oxygen deprivation suffered by roots along with potential elemental toxicity (i.e. accumulation of Fe^{2+} and Mn^{2+}) due to progressive decrease of soil redox potential are predicted to directly affect their growth and physiology (Armstrong 1979; Colmer 2003; Shabala 2011; Sauter 2013). In turn, the impaired function of waterlogged roots would reduce the supply of water for the shoot, indirectly impacting on its growth and physiology via hydraulic signals (Birner & Steudle 1993; Tiekstra *et al.* 2000). In addition, the stressed root generates hormonal signals (*e.g.* ABA, ACC as precursor of ethylene) that reach the shoot (Else *et al.* 1996; Jackson *et al.* 1996). These root-generated signals would trigger stomatal closure to prevent shoot dehydration (Jackson 2002; Arru *et al.* 2013; Voesenek & Bailey-Serres
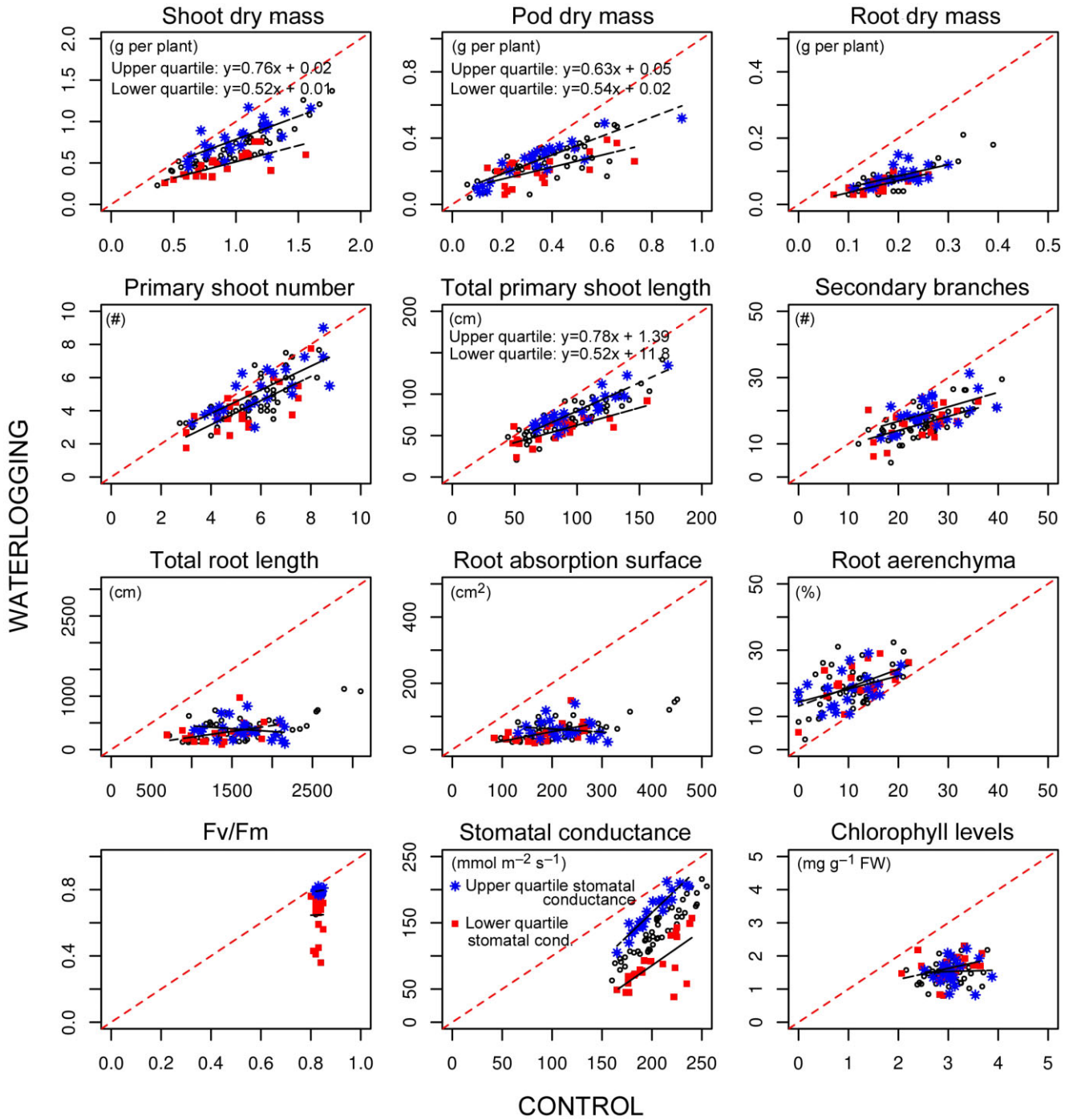
Figure 5. Relationships for values of 12 traits between waterlogged and control conditions in a population of *Lotus japonicus* recombinant inbred lines (RILs) derived from a cross between Miyakojima MG-20 and Gifu B-129. The RILs that maintain high stomatal conductance under waterlogging conditions also maintain higher shoot dry mass, pod dry mass, total primary shoot length and Fv/Fm, than RILs with low stomatal conductance. The RILs distribution of the upper (blue asterisks) and lower (red squares) quartiles of stomatal conductance in all traits was analysed through linear regressions (see Supporting Information Fig. S1). The equations are indicated only if the slope and/or intercept of the fitted regressions were significantly different between RILs of the upper and lower quartiles of stomatal conductance. Each symbol represents the average per RIL ($n = 4$ for dry mass responses, total primary shoot length, primary shoots and secondary branches; $n = 2$ for total root length, root absorption area and root aerenchyma, chlorophyll levels and dark-adapted Fv/Fm). Stomatal conductance had one replicate per RIL and condition.
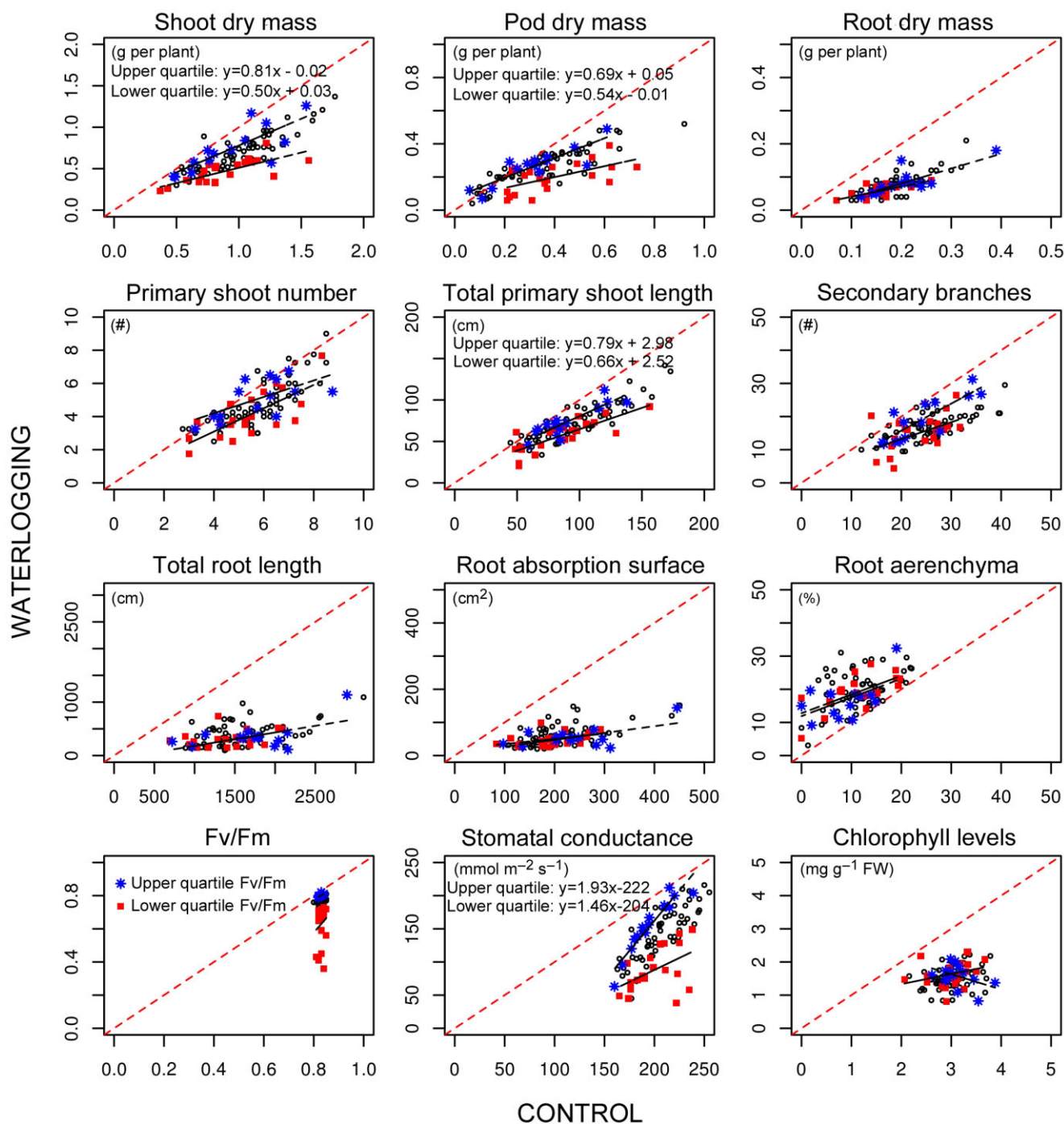
Figure 6. Relationships for values of 12 traits between waterlogged and control conditions in a population of *Lotus japonicus* recombinant inbred lines (RILs) derived from a cross between Miyakojima MG-20 and Gifu B-129. The RILs that maintain high dark-adapted Fv/Fm under waterlogging conditions also maintain higher shoot dry mass, pod dry mass, total primary shoot length and stomatal conductance, than RILs with low-dark-adapted Fv/Fm. The RILs distribution of the upper (blue asterisks) and lower (red squares) quartiles of dark-adapted Fv/Fm in all traits was analysed through linear regressions (see Supporting Information Fig. S1). The equations are indicated only if the slope and/or intercept of the fitted regressions were significantly different between RILs of the upper and lower quartiles of Fv/Fm. Each symbol represents the average per RIL (*n* = 4 for dry mass responses, total primary shoot length, primary shoots and secondary branches; *n* = 2 for total root length, root absorption area and root aerenchyma, chlorophyll levels and dark-adapted Fv/Fm). Stomatal conductance had one replicate per RIL and condition.
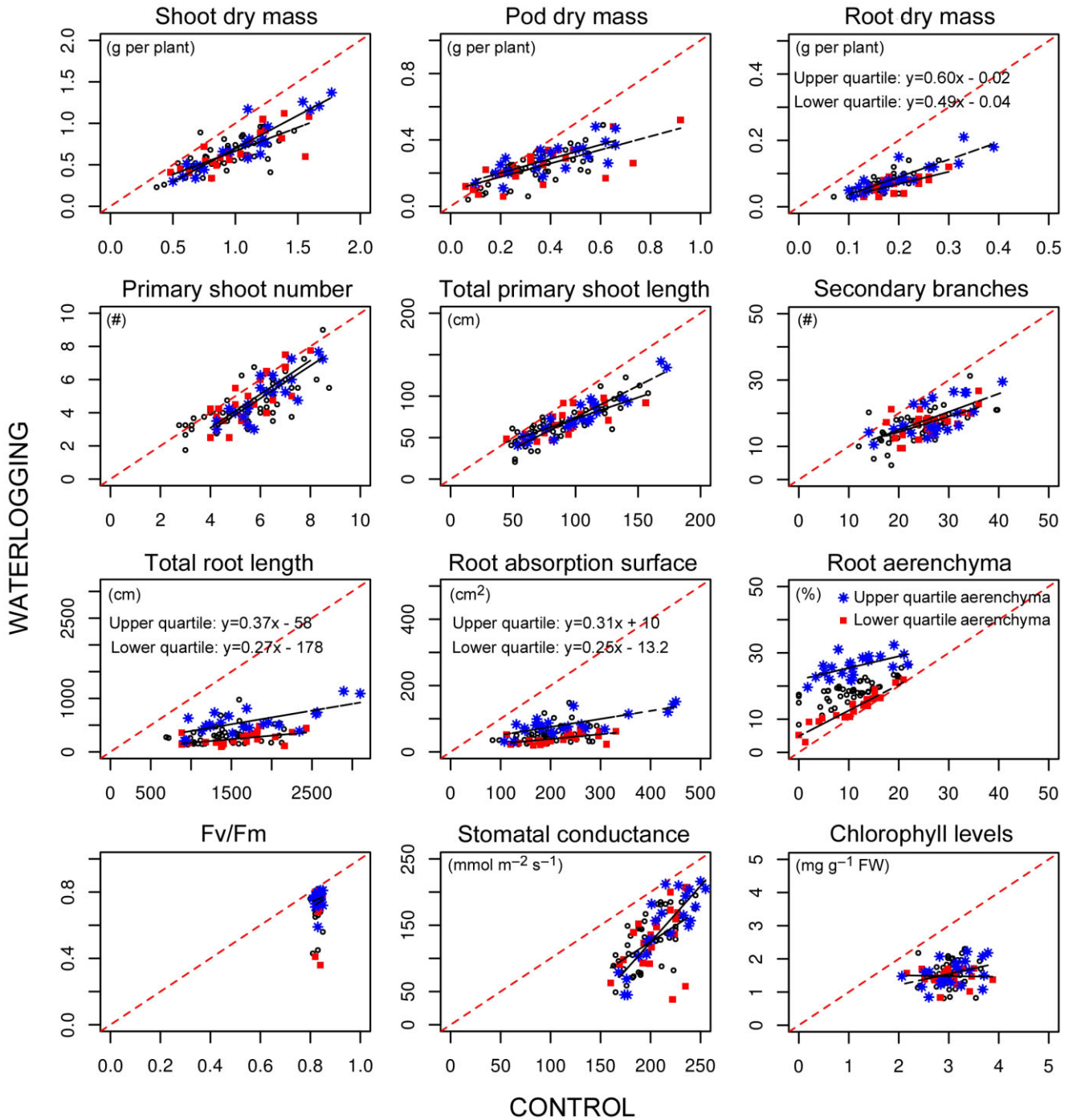
Figure 7. Relationships for values of 12 traits between waterlogged and control conditions in a population of *Lotus japonicus* recombinant inbred lines (RILs) derived from a cross between Miyakojima MG-20 and Gifu B-129. The RILs with high-induced root aerenchyma under waterlogging conditions maintain higher root length, absorption surface and root dry mass, than RILs with low root aerenchyma. The RILs distribution of the upper (blue asterisks) and lower (red squares) quartiles of root aerenchyma in all traits was analysed through linear regressions (see Supporting Information Fig. S1). The equations are indicated only if the slope and/or intercept of the fitted regressions were significantly different between RILs of the upper and lower quartiles of root aerenchyma. Each symbol represents the average per RIL (*n* = 4 for dry mass responses, total primary shoot length, primary shoots and secondary branches; *n* = 2 for total root length, root absorption area and root aerenchyma, chlorophyll levels and dark-adapted Fv/Fm). Stomatal conductance had one replicate per RIL and condition.

2013). Another mechanism by which roots impacted by waterlogging might have consequences affecting the functioning of the shoots is the disruption of nutrient uptake and, thereby a constrained delivery of nutrients to the shoot (Pang *et al*. 2007; Colmer & Voesenek 2009; Zeng *et al*. 2013). Despite these clear connections between roots and shoots where plant behaviour under root oxygen deficiency is predicted to be coordinated across the whole organism (see Arru *et al*. 2013), the results reported here argue in favour of a loose connection of the mechanisms of adjustment of the root and those of the shoot.

We detected significant variability in the traits investigated here and, when mapping QTLs for traits under each condition separately, we identified 14 QTLs shared/co-located between control and waterlogged plants (shoot dry mass, root dry mass, total primary shoot length, primary shoot number, secondary branches and stomatal conductance) (Fig. 2). These QTLs for total primary shoot length, (chromosome 3) and pod dry mass (chromosome 5) overlap with QTLs reported in previous studies for plant height and number of seeds per pod (Gondo *et al*. 2007), respectively. We also identified four QTLs specific for waterlogged plants (shoot dry mass, pod dry mass, root dry mass and primary shoot number) and six QTLs specific for control plants (shoot dry mass, root dry mass, secondary branches, chlorophyll levels, root absorption surface) (Fig. 3). In addition, we detected nine QTLs for the ratio between waterlogging and control conditions, five for root traits (root length, root surface and root dry mass) and four for shoot traits (number of secondary branches, pod dry mass and chlorophyll levels). Although the detected regions need to be more precisely mapped by incorporating more RILs and molecular markers into future experiments, these identified QTLs might provide useful information for marker-assisted selection and further genetic studies on waterlogging tolerance of *L. japonicus*. On the whole, these observations support the strong genetic basis of the variability observed here. The analysis of this variability argues against a tight association between the fate of the root and that of the shoot when the plant experiences the stress imposed by waterlogging.

Firstly, root aerenchyma correlated with root performance but not with shoot performance. Waterlogged plants showed a higher proportion of root aerenchyma, a key adaptive root response given the importance of this specialized tissue in enhancing potential for root aeration (Justin & Armstrong 1987; Colmer 2003; Colmer & Voesenek 2009). The analysis of phenomic networks revealed strong waterlogging-specific links between root aerenchyma and root dry mass or root-absorption surface, but no links between aerenchyma and shoot traits (Fig. 4). A pioneer study by Justin & Armstrong (1987) showed a positive correlation between root porosity and root maximum length in waterlogged (but not in control) plants in an exhaustive survey of 91 species that included non-wetland, intermediate and wetland species. Recently, Niones *et al*. (2013) showed significant correlation between root porosity and root length in a screening of 60 rice genotypes when looking for tolerance to transient flooding. We reasoned that the lack of correlation between aerenchyma

and shoot traits could be the result of a dilution of the impact of the root response to waterlogging on the shoot response due to constitutive (waterlogging-independent) differences in aerenchyma and shoot growth among lines. To focus on the response, we identified for each level of background aerenchyma levels the lines with stronger enhancement in response to waterlogging and the lines with the weakest positive aerenchyma response to waterlogging and analysed the performance of these lines in terms of each one of the other traits. The lines developing more aerenchymatic tissue in their root cortex, when waterlogged, showed less severe reductions in their root length, absorption surface and dry mass than the lines with poor induction of aerenchyma (Fig. 7). Importantly, the better root functioning of these genotypes was not transmitted into a better shoot functioning, as the lines with stronger induction of aerenchyma in response to waterlogging suffered average impact on shoot traits. Clearly, the presence of aerenchyma is not the only feature that defines oxygen levels in the root. Other variables such as root respiration rates and radial oxygen loss are important (Colmer *et al*. 1998; Colmer 2003; Winkel *et al*. 2013). However, the point is that aerenchyma on its own correlated with other root traits, providing a positive control for the lack of correlation with shoot traits.

Secondly, the analysis of shoot responses yielded a complementary picture as a better shoot performance was not reflected in a better root functioning. The lines with reduced impact of waterlogging on shoot traits showed average impact on root traits (Figs 5 & 6). We observed significant variability in the responses of stomatal conductance and PSII functionality (i.e. dark-adapted F_v/F_m) to waterlogging along the range of values of these traits under control conditions (Figs 5 & 6). The lines maintaining high stomatal conductance under soil hypoxia also maintained unaltered functioning of PSII, a positive correlation also observed in tomato plants over different days of exposure to waterlogging (Else *et al*. 2009). Moreover, the lines that retained higher stomatal conductance and F_v/F_m under waterlogging also retained more shoot dry mass, pod dry mass and total primary shoot length. A similar correlation can be observed when comparing species, as stomatal conductance and shoot dry mass were less reduced by waterlogging in *L. tenuis* than in *L. corniculatus* (Striker *et al*. 2005).

The link between stomatal conductance and dark-adapted F_v/F_m is interesting in itself. At least two non-mutually exclusive reasons could account for this pattern. Firstly, it is possible that K^+ concentration in leaves may be a common denominator of this response. Potassium is known to be crucial for stomatal control, as well as for the activity of key photosynthetic enzymes, including Rubisco (Dreyer & Uozumi 2011). High dark-adapted F_v/F_m values are indicative of a larger number of fully functional PSII units per leaf surface area, which might also be strongly dependent on cytosolic K^+ concentration, as a drop in cytosolic K^+ can trigger the activity of caspase-like proteases and endonucleases, resulting in elimination of some cells via programmed cell death process (Shabala 2009). Thus, maintaining high K^+ concentration in leaves might be essential to

confer both higher Fv/Fm values and higher stomatal conductance. Secondly, this pattern could also be accounted for by the enhanced diffusion of CO_2 into the leaves of the lines with higher stomatal conductance favouring the balance between carboxylation and electron transport, which would prevent the accumulation of significant intracellular levels of reactive oxygen species (ROS) and the consequent damage of PSII under waterlogging stress (Blokhina *et al.* 2003; Blokhina & Fagerstedt 2010).

The adequate balance between carbon and water/mineral nutrients, coupled to coordinate between shoots (sinks of water/mineral nutrients, sources of carbon) and roots (sinks of carbon, sources of water/mineral nutrients) (Neuman 1993; Arru *et al.* 2013), and understanding the controls that integrate root and shoot resource gathering has been one of the primary goals in plant physiology (Reynolds & Chen 1996; Chen & Reynolds 1997). In favour of this integration, we observed strong constitutive connectivity among root and shoot traits. For instance, we observed strong links between stomatal conductance and root growth or root absorption surface, secondary branches and root dry mass, total primary shoot length and root dry mass, and shoot dry mass and root absorption surface, which are largely unaffected by waterlogging (Fig. 4). Similarly, a positive correlation between stomatal conductance and root length density has been observed across five wheat cultivars irrespective of control or waterlogging conditions (Hayashi *et al.* 2013). However, despite this constitutive connectivity, we observed poor waterlogging-specific connectivity among root and shoot traits. Therefore, although it can be predicted that the lines with more efficient root acclimation to hypoxia might presumably provide a less intense stress signal for the shoot (see Jackson 2002), waterlogging appears to alter the wiring that coordinates the gathering ability of roots and shoots.

ACKNOWLEDGMENTS

REFERENCES

Abiko T., Kotula L., Shiono K., Malik A.I., Colmer T.D. & Nakazono M. (2012) Enhanced formation of aerenchyma and induction of a barrier to radial oxygen loss in adventitious roots of *Zea nicaraguensis* contribute to its waterlogging tolerance as compared with maize (*Zea mays* ssp. *mays*). *Plant, Cell and Environment* **35,** 1618–1630.

Armstrong W. (1979) Aeration in higher plants. In *Advances in Botanical Research* (ed. H.W. Woolhouse) **Vol. 7,** pp. 225–332. Academic Press, London.

Arnell N. & Liu C. (2001). Climatic change 2001: hydrology and water resources. Report from the Intergovernmental Panel on Climate Change. http://www.ipcc.ch/.

Arru L., Fornaciari S. & Mancuso S. (2013) Oxygen deficiency-induced root-to-shoot communication. In *Long-Distance Systemic Signaling and Communication in Plants*, **Vol. 19,** pp. 125–147. Springer Berlin Heidelberg, Germany.

Bai C., Liang Y. & Hawkesford M.J. (2013) Identification of QTLs associated with seedling root traits and their correlation with plant height in wheat. *Journal of Experimental Botany* **64,** 1745–1753.

Bailey-Serres J. & Voesenek L.A.C.J. (2008) Flooding stress: acclimations and genetic diversity. *Annual Review of Plant Biology* **59,** 313–339.

Birner T.P. & Steudle E. (1993) Effects of anaerobic conditions on water and solute relations, and on active transport in roots of maize (*Zea mays* L. *Planta* **190,** 474–483.

Blokhina O. & Fagerstedt K.V. (2010) Oxidative metabolism, ROS and NO under oxygen deprivation. *Plant Physiology and Biochemistry* **48,** 359–373.

Blokhina O., Virolainen E. & Fagerstedt K.V. (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Annals of Botany* **91,** 179–194.

Broman K.W., Wu H., Sen S. & Churchill G.A. (2003) R/qtl: QTL mapping in experimental crosses. *Bioinformatics (Oxford, England)* **19,** 889–890.

Chen J.L. & Reynolds J.F. (1997) GePSi: a generic plant simulator based on object-oriented principles. *Ecological Modelling* **94,** 53–66.

Colmer T.D. (2003) Long-distance transport of gases in plants: a perspective on internal aeration and radial oxygen loss from roots. *Plant, Cell and Environment* **26,** 17–36.

Colmer T.D. & Voesenek L.A.C.J. (2009) Flooding tolerance: suites of plant traits in variable environments. *Functional Plant Biology* **36,** 665–681.

Colmer T.D., Gibberd M.R., Wiengweera A. & Tinh T.K. (1998) The barrier to radial oxygen loss from roots of rice (*Oryza sativa* L.) is induced by growth in stagnant solution. *Journal of Experimental Botany* **49,** 1431–1436.

Cornelious B., Chen P., Chen Y., de Leon N., Shannon J.G. & Wang D. (2005) Identification of QTLs underlying waterlogging tolerance in soybean. *Molecular Breeding* **16,** 103–112.

Dear B.S., Moore G.A. & Hughes S.J. (2003) Adaptation and potential contribution of temperate perennial legumes to the southern Australian wheatbelt: a review. *Australian Journal of Experimental Agriculture* **43,** 1–18.

Dhondt S., Wuyts N. & Inzé D. (2013) Cell to whole-plant phenotyping: the best is yet to come. *Trends in Plant Science* **18,** 428–439.

Dreyer I. & Uozumi N. (2011) Potassium channels in plant cells. *The FEBS Journal* **278,** 4293–4303.

Else M.A., Tiekstra A.E., Croker S.J., Davies W.J. & Jackson M.B. (1996) Stomatal closure in flooded tomato plants involves abscisic acid and a chemically unidentified anti–transpirant in xylem sap. *Plant Physiology* **112,** 239–247.

Else M.A., Janowiak F., Atkinson C.J. & Jackson M.B. (2009) Root signals and stomatal closure in relation to photosynthesis, chlorophyll a fluorescence and adventitious rooting of flooded tomato plants. *Annals of Botany* **103,** 313–323.

Gondo T., Sato S., Okumura K., Tabata S., Akashi R. & Isobe S. (2007) Quantitative trait locus analysis of multiple agronomic traits in the model legume *Lotus japonicus*. *Genome* **50,** 627–637.

Hayashi T., Yoshida T., Fujii K., Mitsuya S., Tsuji T., Okada Y.,. . . Yamauchi A. (2013) Maintained root length density contributes to the waterlogging tolerance in common wheat (*Triticum aestivum* L. *Field Crops Research* **152,** 27–35.

Jackson M.B. (2002) Long–distance signalling from roots to shoots assessed: the flooding story. *Journal of Experimental Botany* **53,** 175–181.

Jackson M.B., Davies W.J. & Else M.A. (1996) Pressure–flow relationships, xylem sap solutes and root hydraulic conductance in flooded tomato plants. *Annals of Botany* **77,** 17–24.

James E.K. & Crawford R.M.M. (1998) Effect of oxygen availability on nitrogen fixation by two *Lotus* species under flooded conditions. *Journal of Experimental Botany* **49,** 599–609.

Justin S.H.F.W. & Armstrong W. (1987) The anatomical characteristics of roots and plant responses to soil flooding. *The New Phytologist* **106,** 465–495.

Kawaguchi M., Motomura T., Imaizumi-Anraku H., Akao S. & Kawasaki S. (2001) Providing the basis for genomics in *Lotus japonicus*: the accessions Miyakojima and Gifu are appropriate crossing partners for genetic analyses. *Molecular Genetics and Genomics* **266,** 157–166.

Krzywinski M., Schein J., Birol I., Connors J., Gascoyne R., Horsman D., . . . Marra M.A. (2009) Circos: an information aesthetic for comparative genomics. *Genome Research* **19,** 1639–1645.

Letey J. & Stolzy L.H. (1964) Measurement of oxygen diffusion rates with a platinum microelectrode. I. Theory and equipment. *Hilgardia* **35**, 54–55.

Malik A.I., Colmer T.D., Lambers H., Setter T.L. & Schortemeyer M. (2002) Short-term waterlogging has long-term effects on the growth and physiology of wheat. *The New Phytologist* **153**, 225–236.

Mangin B., Goffinet B. & Rebai A. (1994) Constructing confidence intervals for QTL location. *Genetics* **138**, 1301–1308.

Manzur M.E., Grimoldi A.A., Insausti P. & Striker G.G. (2009) Escape from water or remain quiescent? *Lotus tenuis* changes its strategy depending on depth of submergence. *Annals of Botany* **104**, 1163–1169.

Maxwell K. & Johnson G.N. (2000) Chlorophyll fluorescence – a practical guide. *Journal of Experimental Botany* **51**, 659–668.

Moran R. & Porath D. (1980) Chlorophyll determination in intact tissues using N,N-dimethylformamide. *Plant Physiology* **65**, 478–479.

Neuman G.S. (1993) Shoot responses to root stress – a resource gathering point of view. *Journal of Arboriculture* **19**, 118–123.

Niones J.M., Rodriguez Suralta R., Inukai Y. & Yamauchi A. (2013) Roles of root aerenchyma development and its associated QTL in dry matter production under transient moisture stress in rice. *Plant Production Science* **16**, 205–216.

Pajuelo E. & Stougaard J. (2005) Lotus japonicus as model system. In The *Lotus Japonicus* Handbook (ed. A.J. Márquez) pp. 3–24. Springer-Verlag, Dordrecht, The Netherlands.

Pang J., Ross J., Zhou M., Mendham N. & Shabala S. (2007) Amelioration of detrimental effects of waterlogging by foliar nutrient sprays in barley. *Functional Plant Biology* **34**, 221–227.

Qiu F., Zheng Y., Zhang Z. & Xu S. (2007) Mapping of QTL associated with waterlogging tolerance during the seedling stage in maize. *Annals of Botany* **99**, 1067–1081.

Reynolds J.F. & Chen J. (1996) Modelling whole-plant allocation in relation to carbon and nitrogen supply: coordination versus optimization. *Plant and Soil* **185**, 65–74.

Rocha M., Licausi F., Araujo W.L., Nunes-Nesi A., Sodek L., Fernie A.R. & van Dongen J.T. (2010) Glycolysis and the tricarboxylic acid cycle are linked by alanine aminotransferase during hypoxia induced by waterlogging of *Lotus japonicus*. *Plant Physiology* **152**, 1501–1513.

Sanchez D.H., Szymanski J., Erban A., Udvardi M.K. & Kopka J. (2012) Mining for robust transcriptional and metabolic responses to long-term salt stress: a case study on the model legume *Lotus japonicus*. *Plant, Cell and Environment* **33**, 468–480.

Sauter M. (2013) Root responses to flooding. *Current Opinion in Plant Biology* **16**, 282–286.

Shabala S. (2009) Salinity and programmed cell death: unravelling mechanisms for ion specific signalling. *Journal of Experimental Botany* **60**, 709–712.

Shabala S. (2011) Physiological and cellular aspects of phytotoxicity tolerance in plants: the role of membrane transporters and implications for crop breeding for waterlogging tolerance. *New Phytologist* **190**, 289–298.

Smethurst C.F. & Shabala S. (2003) Screening methods for waterlogging tolerance in Lucerne: comparative analysis of waterlogging effects on chlorophyll fluorescence, photosynthesis, biomass and chlorophyll content. *Functional Plant Biology* **30**, 335–343.

Soriano A. (1991) Río de la Plata grasslands. In *Ecosystems of the World 8A. Natural Grasslands. Introduction and Western Hemisphere* (ed. R.T. Coupland) pp. 367–407. Elsevier, Amsterdam, The Netherlands.

Striker G.G., Insausti P., Grimoldi A.A., Ploschuk E.L. & Vasellati V. (2005) Physiological and anatomical basis of differential tolerance to soil flooding of *Lotus corniculatus* L and *Lotus glaber* Mill. *Plant and Soil* **276**, 301–311.

Striker G.G., Insausti P., Grimoldi A.A. & Vega A.S. (2007) Trade-off between root porosity and mechanical strength in species with different types of aerenchyma. *Plant, Cell and Environment* **30**, 580–589.

Striker G.G., Izaguirre R.F., Manzur M.E. & Grimoldi A.A. (2012) Different strategies of *Lotus japonicus*, *L. corniculatus* and *L. tenuis* to deal with complete submergence at seedling stage. *Plant Biology* **14**, 50–55.

Teakle N.L., Real D. & Colmer T.D. (2006) Growth and ion relations in response to combined salinity and waterlogging in the perennial forage legumes *Lotus corniculatus* and *Lotus tenuis*. *Plant and Soil* **289**, 369–383.

Tiekstra A.E., Else M.A. & Jackson M.B. (2000) External pressures based on leaf water potentials do not induce xylem sap to flow at rates of whole plant transpiration from roots of flooded or well-drained tomato and maize plants. Impact of shoot hydraulic resistances. *Annals of Botany* **86**, 665–674.

van Ooijen J.W. (1999) LOD significance thresholds for QTL analysis in experimental populations of diploid species. *Heredity* **83**, 613–624.

Visser E.J.W. & Bögemann G.M. (2003) Measurement of porosity in very small samples of plant tissue. *Plant and Soil* **253**, 81–90.

Visser E.J.W. & Voesenek L.A.C.J. (2004) Acclimation to soil flooding – sensing and signal-transduction. *Plant and Soil* **254**, 197–214.

Visser E.J.W., Colmer T.D., Blom C.W.P.M. & Voesenek L.A.C.J. (2000) Changes in growth, porosity and radial oxygen loss from adventitious roots of selected mono- and dicotyledonous wetland species with contrasting types of aerenchyma. *Plant, Cell and Environment* **23**, 1237–1245.

Voesenek L.A.C.J. & Bailey-Serres J. (2013) Flooding tolerance: O_2 sensing and survival strategies. *Current Opinion in Plant Biology* **16**, 647–653.

Winkel A., Colmer T.D., Ismail A.M. & Pedersen O. (2013) Internal aeration of paddy field rice (*Oryza sativa* L.) during complete submergence – importance of light and floodwater O_2. *New Phytologist* **197**, 1193–1203.

Xu K., Xu X., Fukao T., Canlas P., Marghirang–Rodriguez R., Heuer S., . . . Mackill D.J. (2006) Sub1A is an ethylene-response-factor-like gene that confers submergence tolerance to rice. *Nature* **442**, 705–708.

Young N.D., Mudge J. & Ellis T.H.N. (2003) Legume genomes: more than peas in a pod. *Current Opinion in Plant Biology* **6**, 199–204.

Zeng F., Shabala L., Zhou M., Zhang G. & Shabala S. (2013) Barley responses to combined waterlogging and salinity stress: separating effects of oxygen deprivation and elemental toxicity. *Frontiers in Plant Science* **4**, 1–13.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Schematic diagram showing the comparison of lines with strong or weak response to waterlogging across the range of values under control conditions. The response to waterlogging can be seen as a deviation from the 1:1 line (i.e. when plants under control and waterlogging conditions have the same values). The more and less responsive genotypes are defined according to their strong or weak deviation from the 1:1 line.

Figure S2. Frequency distribution of the proportion of root length depending on the diameter class (<0.2, 0.2–0.4, 0.4–0.8 and >0.8 mm) of a population of *Lotus japonicus* recombinant inbred lines (RILs derived from a cross between Miyakojima MG-20 and Gifu B-129).

Figure S3. Relationships for values of 12 traits between waterlogged and control conditions in a population of *Lotus japonicus* RILs derived from a cross between Miyakojima MG-20 and Gifu B-129. The slope, R^2 and *P*-value of the linear adjustment are shown for each trait. Each symbol represents the average per RIL ($n = 4$ for dry mass responses, total primary shoot length, primary shoots and secondary branches; $n = 2$ for total root length, root absorption area and root aerenchyma, chlorophyll levels and dark-adapted Fv/Fm). Stomatal conductance had one replicate per RIL and condition.

Figure S4. Relationships for values of 12 traits between waterlogged and control conditions in a population of *Lotus japonicus* RILs derived from a cross between Miyakojima MG-20 and Gifu B-129. The distribution of RILs of the upper and lower quartiles of chlorophyll levels on the rest of traits did not reveal clear indication of differences either for shoots or root traits. The RILs distribution of the upper (blue asterisks) and lower (red squares) quartiles of chlorophyll levels in all traits was analysed through linear regressions (see Supporting Information Fig. S1). The equations are indi-

cated only if the slope and/or intercept of the fitted regressions were significantly different between RILs of the upper and lower quartiles of chlorophyll levels. Each symbol represents the average per RIL ($n = 4$ for dry mass responses, total primary shoot length, primary shoots and secondary branches; $n = 2$ for total root length, root absorption area and root aerenchyma, chlorophyll levels and dark-adapted Fv/Fm). Stomatal conductance had one replicate per RIL and condition.

Table S1. Summary of detected QTL for 12 traits analysed in a population of *Lotus japonicus* RILs derived from a cross between Gifu B-129 and Miyakojima MG-20, under control or waterlogging conditions. For each QTL detected, the chromosome (Chr), QTL position (cM), LOD score, PEV and *P*-value are presented. PEV correspond to the percentage of the phenotypic variance explained at QTL peak.

Table S2. Summary of detected QTL for the relative values (waterlogging/control) of 12 traits analysed in a population of *Lotus japonicus* RILs derived from a cross between Gifu B-129 and Miyakojima MG-20. For each QTL detected, the chromosome (Chr), QTL position (cM), LOD score, PEV, *P*-value and confidence interval are presented. PEV correspond to the percentage of the phenotypic variance explained at QTL peak.

Table S3. Pearson correlation coefficients for 12 traits of a population of *Lotus japonicus* RILs derived from a cross between Gifu B-129 and Miyakojima MG-20, and subjected to control (above the diagonal) or waterlogging (below the diagonal) conditions for 21 d. Abbreviations are: SDM (shoot dry mass), PDM (pod dry mass), RDM (root dry mass), PSN (primary shoot number), TPSL (total primary shoot length), SB (number of secondary branches), TRL (total root length), RAS (root absorption surface), AER (root aerenchyma), Fv/Fm (dark-adapted Fv/Fm), g_s (stomatal conductance) and Chl (Chlorophyll levels). Significant differences: $*P < 0.05$; $**P < 0.001$; ns, $P > 0.05$.

Table S4. Pearson correlation coefficients for the relative values (waterlogging/control) of 12 traits of a population of *Lotus japonicus* RILs derived from a cross between Gifu B-129 and Miyakojima MG-20, and subjected to control or waterlogging conditions for 21 d. Abbreviations are: SDM (shoot dry mass), PDM (pod dry mass), RDM (root dry mass), PSN (primary shoot number), TPSL (total primary shoot length), SB (number of secondary branches), TRL (total root length), RAS (root absorption surface), AER (root aerenchyma), Fv/Fm (dark-adapted Fv/Fm), g_s (stomatal conductance) and Chl (Chlorophyll levels). Significant differences: $*P < 0.05$; $**P < 0.001$; ns, $P > 0.05$.