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Comparative ionomics and metabolomics in extremophile and glycophytic *Lotus* species under salt stress challenge the metabolic pre-adaptation hypothesis

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ABSTRACT

The legume genus *Lotus* includes glycophytic forage crops and other species adapted to extreme environments, such as saline soils. Understanding salt tolerance mechanisms will contribute to the discovery of new traits which may enhance the breeding efforts towards improved performance of legumes in marginal agricultural environments. Here, we used a combination of ionomic and gas chromatography-mass spectrometry (GC-MS)-based metabolite profilings of complete shoots (pooling leaves, petioles and stems) to compare the extremophile *Lotus creticus*, adapted to highly saline coastal regions, and two cultivated glycophytic grassland forage species, *Lotus corniculatus* and *Lotus tenuis*. *L. creticus* exhibited better survival after exposure to long-term lethal salinity and was more efficient at excluding Cl^- from the shoots than the glycophytes. In contrast, Na^+ levels were higher in the extremophile under both control and salt stress, a trait often observed in halophytes. Ionomics demonstrated a differential rearrangement of shoot nutrient levels in the extremophile upon salt exposure. Metabolite profiling showed that responses to NaCl in *L. creticus* shoots were globally similar to those of the glycophytes, providing little evidence for metabolic pre-adaptation to salinity. This study is the first comparing salt acclimation responses between extremophile and non-extremophile legumes, and challenges the generalization of the metabolic salt pre-adaptation hypothesis.

Key-words: *Lotus creticus*; halophyte; ionome; legume; metabolome; salt acclimation; salt stress.

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INTRODUCTION

Saline soils generate at least two types of stress for plants: osmotic stress caused by the reduction of water potential and reduced water availability, and ionic stress linked to the accumulation of toxic ions (Munns 2002). Plants exposed to salts face several interdependent constraints on growth and survival including not only dehydration, ion toxicity and oxidative stress, but also metabolic and nutrient misbalance, which together result in a complex physiological syndrome (Tester & Davenport 2003). Plant strategies to cope with saline environments include salt exclusion and sequestration, tissue tolerance to accumulated ions and limitation of K^+ loss, osmotic adjustment and control of water homeostasis, biochemical and molecular responses, and changes in growth and development (Tester & Davenport 2003; Munns 2005; Shabala & Cuin 2007; Munns & Tester 2008; Sanchez *et al*. 2008a). Considering the complexity of plant responses to salinity, it is not surprising that salt tolerance is a quantitative trait determined by multiple genetic interactions involving changes in the activity of thousands of genes (Monforte, Asins & Carbonell 1997; Foolad 2004; Sanchez *et al*. 2008a, 2010). A distinction can be made between plant species that have evolved salt tolerance strategies to thrive in natural salty habitats (halophytes) and those that have not (glycophytes). Although the physiological mechanisms separating both groups are not always clear-cut, it is generally accepted that the differential control of salt homeostasis is involved (Orcutt & Nilsen 2000). Halophytes generally accumulate high concentrations of ions within their tissues upon salinization, whereas glycophytes do not, and differential salt tolerance in the latter is more based on efficient exclusion of ions from the shoots (Flowers, Troke & Yeo 1977; Greenway & Munns 1980; Munns & Tester 2008). Exceptions exist, since some halophytic plants such as *Thellungiella halophila* are known to be highly efficient excluders (Gong *et al*. 2005), whereas some tolerant glycophytes tend to accumulate salts to different degrees, depending on the differential capacity to interchange Na^+ for K^+ (Marschner 1995).

Legumes are second only to grasses in their importance for agriculture, and provide a valuable source of protein, oil, carbohydrate, minerals and secondary compounds for human and animal nutrition (Graham & Vance 2003). In arid climates of the Mediterranean basin where salinity is a problem, extremophiles such as the legume *Lotus creticus* serve as pastoral and forage plants (Rejili *et al*. 2007). *L. creticus* thrives on the beaches of the Mediterranean coast, usually on sandy soils, sometimes cohabiting with halophytic species such as *Limonium* spp., *Crithmum maritimum* and *Artemisia gallica* (Monserrat 1959). *L. creticus* is considered to exhibit strong salt tolerance, not only because of the ecological conditions it endures, but also because of its ability to grow for months at 70 mM NaCl without noticeable decrease in biomass (Sanchez-Blanco *et al*. 1998). In fact, high salinity has even been reported to stimulate root growth in this species (Morales *et al*. 2000). It is worth noting that *L. creticus* does not possess specialized organs such as salt glands or bladders which are adaptive anatomical structures employed by some halophytes (Orcutt & Nilsen 2000).

The characteristics of *L. creticus* render it an interesting subject for comparative studies of legume responses to salinity, especially as it is related to *Lotus japonicus*, a model for genetic and genomic research (Udvardi *et al*. 2005), and to several important forage legumes including *Lotus corniculatus* and *Lotus tenuis* (Diaz, Borsani & Monza 2005). Moreover, *L. creticus* and other *Lotus* species native to extreme environments are a potential source of tolerance genes that could be transferred to cultivated *Lotus* species. In this work, we used state-of-the-art ′omics technologies to compare the nutritional and metabolic responses of complete shoots (pooled leaves, petioles and stems) to non-lethal long-term salt exposure in *L. creticus* and two of its glycophytic relatives, *L. corniculatus* and *L. tenuis*. *L. creticus* under salinity displayed a remarkable capacity to exclude Cl^- anions from the shoots and a differential rearrangement of nutrient levels. However, its global shoot metabolic responses to the salt treatment were remarkably similar to those of the glycophytic species, indicating that *L. creticus* does not possess obvious global pre-adaptive metabolic traits. To our knowledge, this is the first time that ionomic and metabolomic comparisons have been made between phylogenetically close members of the same legume genus involving an extremophile and non-extremophiles, which challenge the generalization of the metabolic pre-adaptation hypothesis.

MATERIALS AND METHODS

Plant material and growth conditions

L. creticus seeds were kindly provided by the Servicio Devesa-Albufera Ayuntamiento de Valencia, Viveros Municipales de El Saler, CV-500, Km 8.5, El Saler, Valencia (Spain). *L. corniculatus* var. San Gabriel and *L. tenius* (*Lotus glaber*) var. La Esmeralda were obtained from the LOTASSA consortium (http://www.lotassa.com). Seeds were germinated in plates containing half-strength BD agar (Broughton & Dilworth 1971) plus 2 mM KNO_3 and 2 mM NH_4NO_3. For the evaluation of survival to long-term lethal NaCl levels, seedlings (at least 40 of each species) were transplanted to pots containing a mixture of sand and perlite (1:1) irrigated with nutrient solution, and grown in the greenhouse under a 16/8 h day/night, 23 ± 2 °C and 55–65% relative humidity regime. Eight days post-imbibition, the salt concentration in the nutrient solution was increased in 25 mM NaCl steps every three days to reach 300 mM NaCl. This concentration was used to continue irrigation, until all the plants died. The number of dead plants as a function of time was recorded. Plants were scored dead when the whole plant or all the leaves were wilted or chlorotic. For the gradual salt stress acclimation experiments, seedlings were transplanted to soil (type Null Einheitserde, Wandorf, Germany) and irrigated with nutrient solution. The stress treatment was started 8 days post-imbibition. The salt concentration in the nutrient solution was increased in three steps of 4 day intervals from 50, 100 to 150 mM NaCl (Sanchez *et al*. 2008a). In two successive independent experiments, *L. creticus* was tested parallel to the other *Lotus* species. Each independent experiment included sample sets of control and salt-treated plants. Each sample set had at least five independent biological replicates and each replicate was a pool of at least five plants. The total duration of the cultivation was 32 d. Complete shoots, pools of leaves, petioles and stems omitting cotyledons were harvested *in situ* in the middle of the light period and flash frozen in liquid nitrogen. The experimental design induced neither senescence nor was lethal within the cultivation period. At harvest, all plants were still in the vegetative stage and non-nodulated. Biomass was estimated by the mean fresh weight of the pooled shoot samples.

Ionome and metabolome profiling

Elements and relative metabolites levels were determined in each biological replicate. For ionomic profiling, 100 mg plant material was digested with 2 mL HNO_3 at 140 °C until completion. Also, 100 μL of a 100 g L^{-1} LiCl solution was added as a carrier and the final volume adjusted with ultra pure water to 10 mL. Element concentrations were determined with inductively coupled plasma-atomic emission spectrometry (ICP-AES) using an IRIS Advantage Duo ER/S (Thermo Fisher Scientific GmbH, Schwerte, Germany). Elemental quantification was validated using IC-CTA-VTL2 Virginia tobacco leaves as a certified reference material. Chloride was profiled in the same plant material using an ion chromatography method established on a Dionex ICS-2000 system (Dionex GmbH, Idstein, Germany). For metabolomic profiling, 60 mg of frozen plant tissue was extracted with methanol/chloroform, and the polar fraction was prepared by liquid partitioning into water, dried and derivatized (Desbrosses, Kopka & Udvardi 2005). Gas chromatography coupled to electron impact ionization-time of flight-mass spectrometry (GC/EI-TOF-MS) was performed using an Agilent Technologies (Waldbronn, Germany) 6890N gas chromatograph with split or splitless

injection mounted to a Pegasus III time-of-flight mass spectrometer (LECO) (Wagner *et al*. 2003). Analytes were quantified after mass spectral deconvolution (ChromaTOF software 1.00, Pegasus driver 1.61, LECO). The chemical identification was manually supervised using the NIST05 software (http://www.nist.gov/srd/nist1.cfm) and the mass spectral and retention time index collection of the Golm Metabolome Database (Hummel *et al*. 2010). The validity of this analytical approach to perform relative quantification of metabolites in plant tissues has been previously demonstrated (Allwood *et al*. 2009).

Data handling, mining and statistics

The element content was statistically analysed using Student's *t*-testing, performed with the GraphPad Prism 4.0 software. Metabolomic profiles were processed using the TagFinder software (Luedemann *et al*. 2008). Data were filtered within each independent experiment for those metabolic features which were represented by at least three intercorrelated GC/EI-TOF-MS mass fragments (Sanchez *et al*. 2008b). Resulting profiles were normalized to internal standard and fresh weight of each sample. Then, each metabolic feature was normalized to the median measurement across all samples of the three analysed species within each independent experiment and $\log_{10}$ transformed prior to statistical analysis [two-way analysis of variance (ANOVA) and Student's *t*-test]. These analyses were performed with the The Institute for Genomic Research multiple experiment viewer software (TMEV_3.1). Probabilistic principal component analysis (PPCA) was applied as non-supervised clustering and data reduction algorithm, through the MetaGeneAlyse webpage (http://metagenealyse.mpimp-golm.mpg.de). Linear and non-linear correlations were calculated with the GraphPad Prism 4.0 software. LD50 was calculated by fitting a Boltzmann sigmoidal function to the data.

RESULTS

Salt tolerance of *L. creticus* compared to the glycophytes *L. corniculatus* and *L. tenuis*

In pre-experiments, the salt tolerance of six *Lotus* species was evaluated after an initial acclimation period on the basis of plant survival in the presence of a lethal NaCl stress-dose. *L. corniculatus* and *L. tenuis* were shown to be the most salt sensitive and salt tolerant of the glycophytic species tested (Rogers, Noble & Pederick 1997; Teakle *et al*. 2007; Sanchez *et al*., unpublished results). The ability of the extremophile *L. creticus* to survive long-term salinity was compared to these two glycophytes, using gradual increases of the NaCl concentration in the irrigation solution up to a final concentration of 300 mM. Plant mortality was evaluated as a function of time using a 'lethal-dose fifty' (LD50), defined as the number of days after reaching 300 mM NaCl at which 50% of the tested plants in each genotype had died. LD50 was 47 d for *L. corniculatus*, 57 d for *L. tenuis* and 77 d for *L. creticus* (Fig. 1a). Thus, *L. creticus* was far more salt tolerant than the previously studied glycophytes. Differential anatomical changes between the species were also observed. *L. corniculatus* plants had a primary shoot and one or two basal branches at the time of salinity-induced senescence. The basal leaves in the primary shoot senesced first, a process that progressed from the basal to the apical leaves and finally to the shoot apex. Once the primary shoot had completed senescence, the basal branches followed within a few days. Salinity-induced senescence in the basal leaves of primary shoots in *L. tenuis* plants started earlier than in *L. corniculatus*, and senescent leaves of *L. tenuis* were shed from the plant more quickly, resulting in a green appearance of the remaining shoot. Apical leaves and the apex of the primary shoot completed senescence next, with symptoms similar to dehydration, whereas the basal branches died later. Salt-induced senescence of *L. creticus* resembled that of *L. tenuis*. However, after the primary shoot had died, the remaining secondary shoots continued to grow until a substantially delayed onset of senescence beginning at the basal leaves. Senescence of the basal branches began later and progressed at a slower rate than observed in *L. tenuis* and *L. corniculatus*. Whole-plant senescence of *L. creticus* occurred weeks after the first loss of basal leaves from the primary shoot, and thus much later than in the other two species.

These observations demonstrated that *L. creticus* has a higher level of salt tolerance than the two glycophytic species, *L. corniculatus* and *L. tenuis*, not only in terms of the survival time under a lethal NaCl stress-dose, but also in its ability to sustain growth after onset of deleterious salt effects on the plant.

Physiological changes in *L. creticus* during salt acclimation compared to the glycophytes, *L. corniculatus* and *L. tenuis*

In order to investigate the physiological responses of the three *Lotus* species during salt stress, two consecutive and independent experiments were performed, each comprising control and salt-treated plants acclimated to a long-term sub-lethal level of salt, that is 28 d of treatment with a final 150 mM NaCl concentration in the nutrient solution (Sanchez *et al*. 2008a). Under control conditions, biomass production was notably lower in *L. creticus* than in *L. corniculatus* and *L. tenuis*, as was expected for an extremophile (Fig. 1b, left panel). Shoot biomass decreased in all species during salt acclimation, with the relative decrease in *L. creticus* being similar to that of *L. tenuis* but significantly higher than that of *L. corniculatus*, the most salt sensitive of the three species (Fig. 1b, right panel). These results showed that the NaCl stress-dose used in these experiments elicited stress also in *L. creticus*. This stress was not lethal to any of the species, and therefore allowed us to compare their physiological responses during salt acclimation (see below). Note that under this regime, the extremophile *L. creticus* shared some morphological and growth responses with the two glycophytes, including a decrease of foliar area due to a

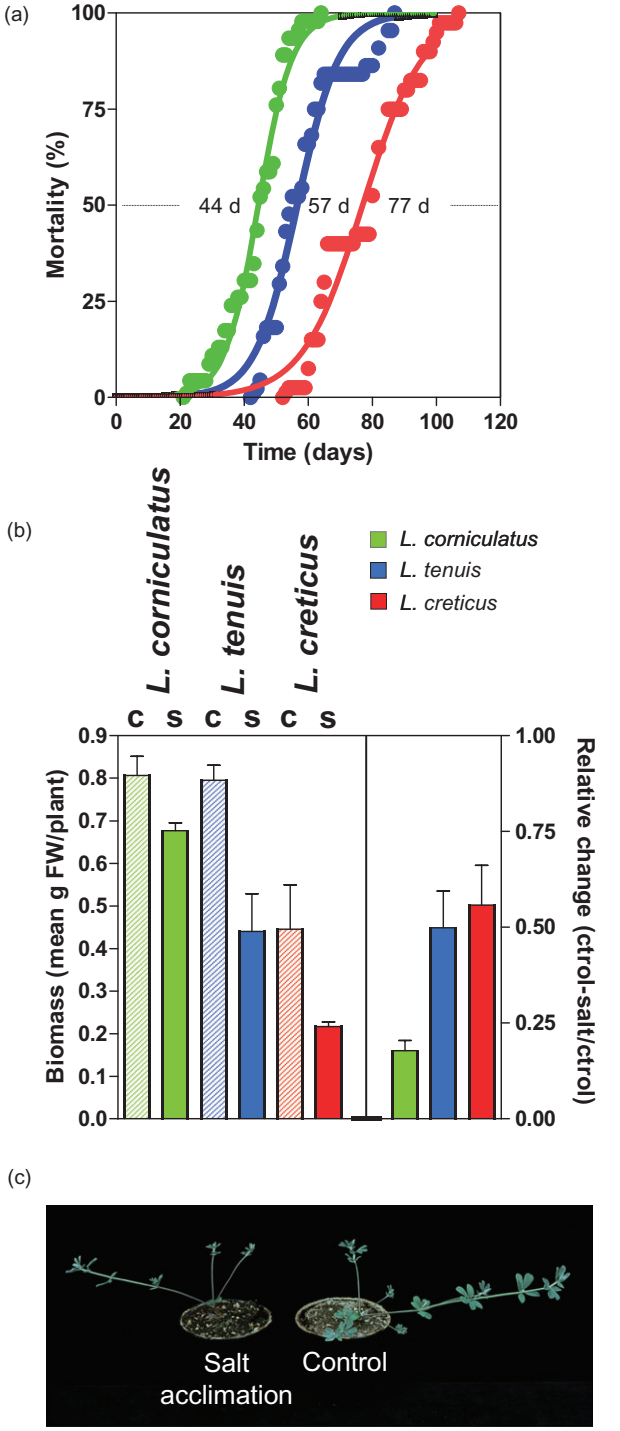

Figure 1. Survival and growth of salt-stressed *Lotus creticus* plants compared to *Lotus corniculatus* and *Lotus tenuis*. (a) Plant survival following exposure to a lethal treatment of NaCl, expressed as the percentage of mortality of at least 40 plants of each genotype. (b) Shoot biomass under control or non-lethal salt treatment conditions (left panel), and the relative change of biomass upon exposure to salt (right panel). Data represent the mean ± SD of at least five independent biologically replicated pools from at least five plants in two independent experiments. C = control, S = salt stress, FW = fresh weight. (c) Picture of *L. creticus* plants at the end of the non-lethal salt acclimation experiment.

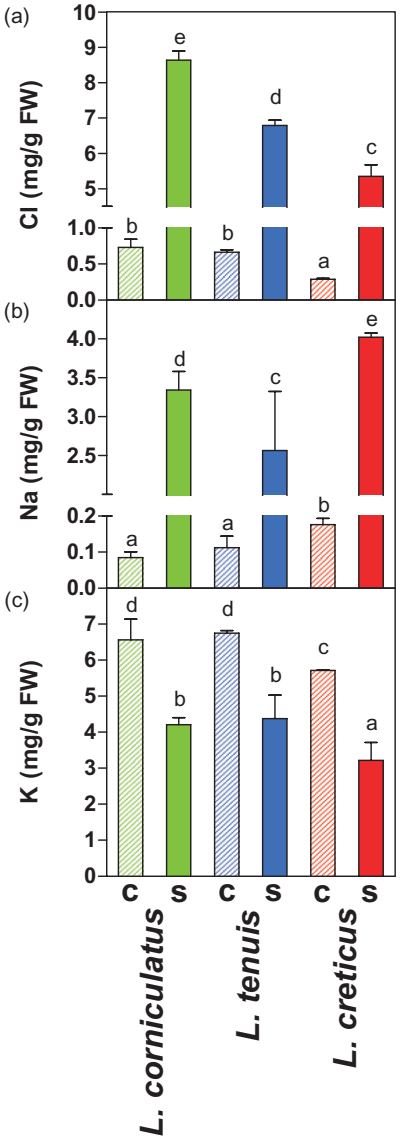

Figure 2. Cl, Na and K content in *Lotus creticus* compared to *Lotus corniculatus* and *Lotus tenuis*. Data represent the mean ± SD of at least five independent biologically replicated pools from at least five plants in two independent experiments. Different letters above bars indicate the statistical significance groupings according to the Student's *t*-test ($P < 0.05$). C = control, S = salt stress, FW = fresh weight. Colour code is as in Fig. 1.

reduction in the size and number of leaves, and suppression of branching which led to a decrease in the number of stems (Fig. 1c).

Even though the shoot Cl^- levels increased dramatically under salinity acclimation in all species, Cl^- was significantly lower in *L. creticus* than in the two glycophytes. In control *L. creticus* plants, the shoot Cl^- levels were also lower (Fig. 2a). These results are in agreement with the concept that salt-tolerant legumes accumulate lower amounts of Cl^- than sensitive relatives (Marschner 1995; Teakle, Real & Colmer 2006; Teakle *et al.* 2007; Teakle & Tyerman 2010). Shoot Na^+ concentrations increased under salt stress in all

three species but, in contrast to Cl^-, Na^+ was significantly higher in *L. creticus*. The same was true for Na^+ levels in control plants (Fig. 2b). Na^+ accumulation is known to occur in halophytic species, albeit to a much higher level than in *L. creticus* (Flowers *et al*. 1977; Flowers & Colmer 2008). Shoot K^+ content followed an inverse trend compared to Na^+, and were significantly lower in *L. creticus* than in the two glycophytes, both under control and salt-treated conditions (Fig. 2c). The differential content of ions in *L. creticus* prompted us to perform a comparison of the K^+/Na^+ and Cl^-/Na^+ ratios under salt stress with several other phylogenetically close *Lotus* glycophytes: *L. japonicus* (var. Gifu and MG20), *Lotus filicaulis*, *Lotus burttii*, *L. corniculatus*, *L. tenuis* and *Lotus uliginosus* (Degtjareva *et al*. 2006; Sanchez *et al*., unpublished results). Although *L. creticus* displayed a low K^+/Na^+ ratio, it was in the range of the other *Lotus* species (Fig. 3a). However, salt-exposed *L. creticus* plants differed substantially from the glycophytes with respect to the Cl^-/Na^+ ratio (Fig. 3b). As a whole, these data indicate that, in spite of accumulating higher Na^+ levels, *L. creticus* is able to more efficiently exclude Cl^- from shoot tissue.

Nutritional changes in *L. creticus* during salt acclimation compared to the glycophytes, *L. corniculatus* and *L. tenuis*

The nutrient composition of whole shoots (including leaves, petioles and stems) from salt-acclimated and control plants were determined using ICP-AES technology. Most changes in the levels of nutrients followed two types of trends: (1) Nutrient content increased or decreased in the three species under salinity, but the relative magnitude of change was more pronounced for *L. creticus*. Such trends were observed for P, Mn, Zn and B (Fig. 4), and (2) The response to salts in *L. creticus* was distinct from *L. corniculatus* and *L. tenuis*. Specifically the Mg and Fe levels increased in *L. creticus* but not the other species after salt treatment. In addition, Ca increased significantly only in *L. corniculatus*, the most salt sensitive of the three species, whereas S increased in both *L. creticus* and *L. tenuis*, but not in *L. corniculatus* (Fig. 4). Taken together, these results demonstrate that the distinct homeostasis of Cl^-, Na^+ and K^+ in *L. creticus* (Figs 2 & 3) is accompanied by differential nutritional changes compared to glycophytes of the same genus.

Metabolic responses of *L. creticus* during salt acclimation compared to the glycophytes, *L. corniculatus* and *L. tenuis*

Changes in the levels of soluble metabolites within the shoots of salt-acclimated plants were measured using gas chromatography-mass spectrometry technology (GC/EI-TOF-MS). For a targeted analysis of metabolites, we compiled a total of 161 mass spectral tags (MSTs, i.e. manually identified analytes Desbrosses *et al*. 2005), including known and as yet unknown compounds which were present in all the species under investigation. Of these, 44 MSTs represented unknown metabolites which were so far found only in *Lotus* species (Supporting Information Table S1). Non-supervised PPCA of the metabolite data clearly clustered the samples of the three species by PC1 and PC2 (Fig. 5a). According to PC1, both salt-treated and non-treated *L. creticus* samples were separated from the two glycophytic species. The loadings (eigenvectors) analysis of

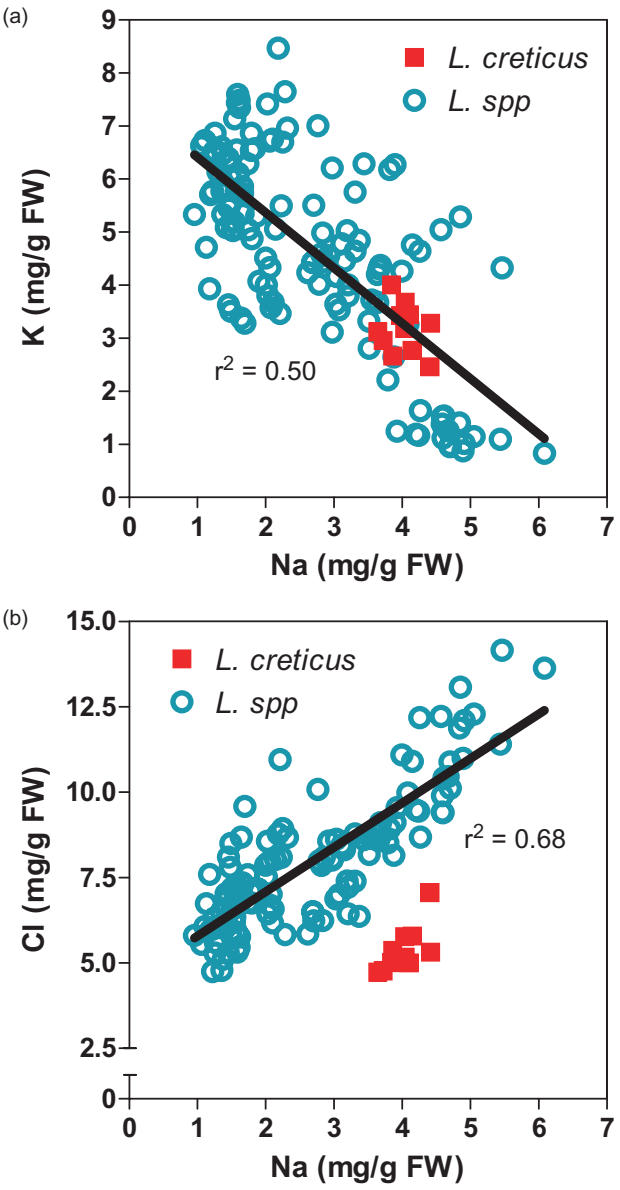

Figure 3. K/Na and Cl/Na biplots under salt stress in *Lotus creticus* compared to glycophytic species of the *Lotus* genus. (a) K/Na and (b) Cl/Na shoot ratios in *Lotus* ssp. exposed to identical non-lethal long-term salinity. Data of *L. creticus* (red symbols) were as given in Fig. 1 legend and represent two independent experiments. Data of the other *Lotus* species, *L. japonicus* (var Gifu and MG20), *L. filicaulis*, *L. burttii*, *L. corniculatus*, *L. tenuis* and *L. uliginosus* (blue symbols), were from three independent experiments with the same sampling design (Sanchez *et al*., unpublished results). Data of *Lotus spp*. without *L. creticus* were used to calculate the linear regressions.

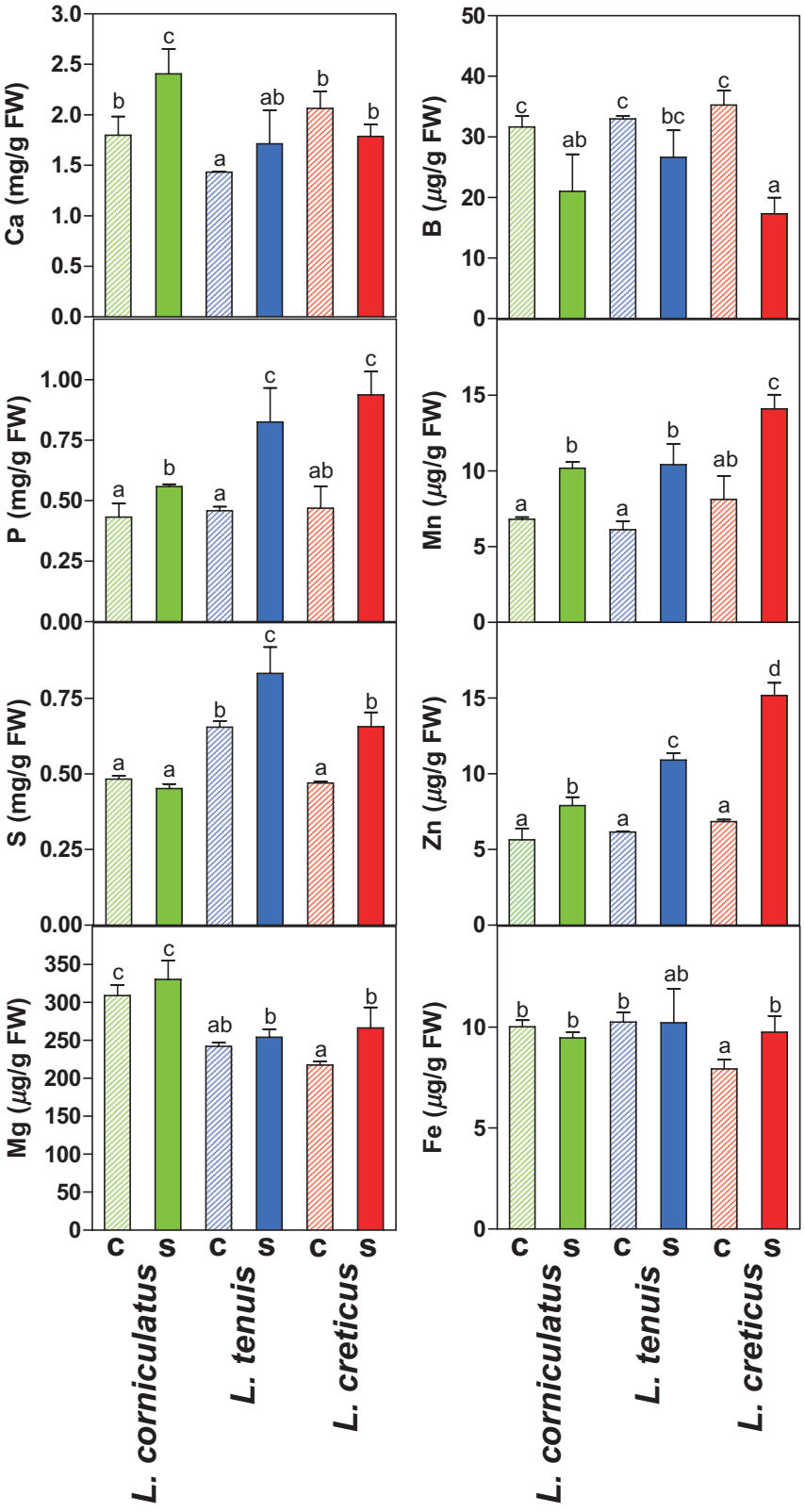

Figure 4. Elemental composition of *Lotus creticus* compared to *Lotus corniculatus* and *Lotus tenuis*. Data represent the mean ± SD of at least five independent biologically replicated pools from at least five plants in two independent experiments. Different letters indicate statistically significant differences between treatments and genotypes, according to the Student's *t*-test ($P < 0.05$). C = control, S = salt stress, FW = fresh weight. Colour code is as in Fig. 1.

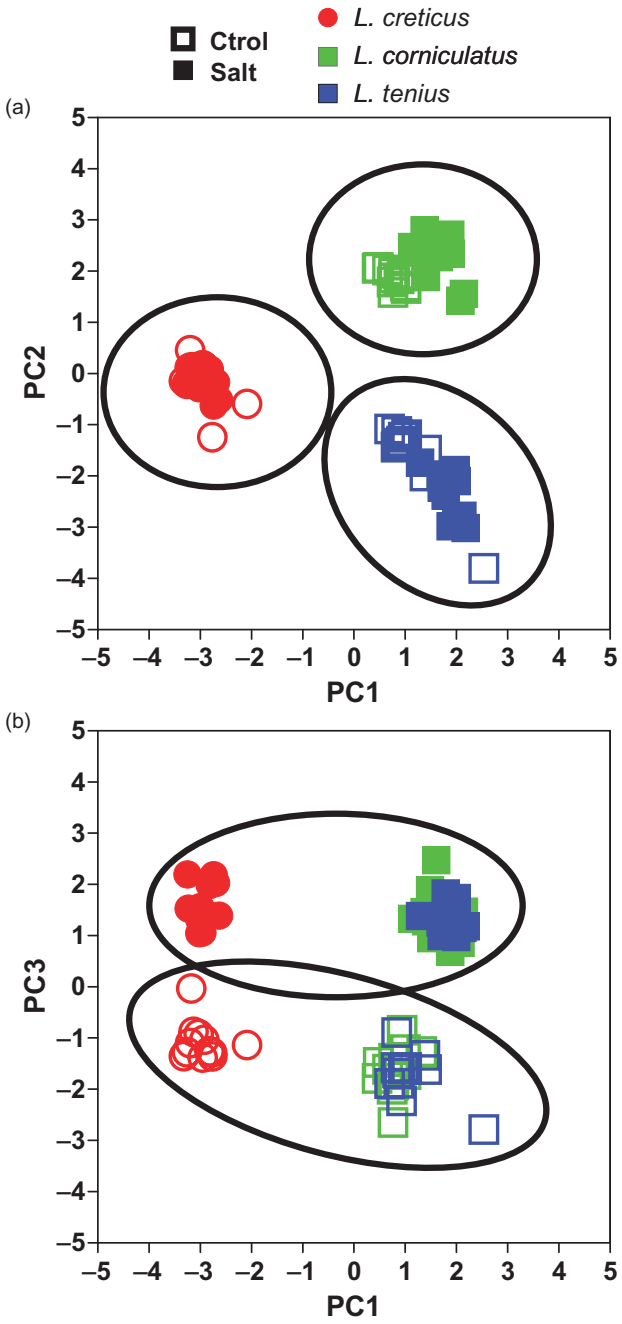

Figure 5. Non-supervised analysis of the metabolite profiles of *Lotus creticus, Lotus corniculatus* and *Lotus tenuis*. Probabilistic principal component analysis of the metabolite profiles from control (closed symbols) and salt-acclimated samples (open symbols) showing (a) PC1 versus PC2, and (b) PC1 versus PC3. Colour code is as in Fig. 1.

PC1 revealed that 2-methyl-malic acid and malonic acid were among the highly discriminatory metabolites, and their levels were found to be lower and higher in *L. creticus*, respectively. Several unknown compounds also ranked highly (Supporting Information Table S2). PC3 separated control from salt-treated samples of all three species, suggesting general similarity in their metabolic responses to NaCl (Fig. 5b). Analysis of the loadings from PC3 revealed that some known salt stress-elicited metabolites of *Lotus* were responsible for the differential clustering of salt-acclimated samples (Sanchez *et al*. 2008a,c). Exposure to salinity increased proline and glycerophosphoglycerol levels in all three *Lotus* species, while the concentrations of several organic acids decreased, for example, citric acid and members of the malic acid family of metabolites (Supporting Information Table S2).

To identify the general responses to salinity, the metabolite profiles were analysed using supervised, significance-based statistical tools, namely two-factorial ANOVA (Supporting Information Table S2), with 'treatment' and 'species' as factors at a stringent statistical threshold ($P < E^{-4}$). Metabolites corresponding to 75 MSTs were found to differ between species. Forty-one of these were lower in control, non-stressed plants of *L. creticus* than in the respective samples from the glycophytes. In contrast, only 10 MSTs were higher in *L. creticus* under control conditions. *L. tenuis* showed a contrasting pattern: 43 MSTs were higher in the controls of this species, only eight were lower. *L. corniculatus* exhibited a more equally weighted differential pattern under control conditions with 22 lower and 25 MSTs higher than in the other two species. As a whole, these analyses suggested that under control conditions, each species had a preformed distinctive metabolic pattern. However, no association of these patterns to the relative salt tolerance was observed.

On the other hand, ANOVA revealed that 56 MSTs changed significantly in at least one species in response to the salt treatment. Most of these salt-responsive analytes (48 MSTs) showed similar changes in all species, either increasing or decreasing. The 25 MSTs which increased represented metabolites which had been previously identified as salt responsive in *Lotus*, such as the amino acids proline, serine, threonine, glycine and phenylalanine; the sugars sucrose and fructose; *myo*-inositol; and compounds corresponding to unidentified MSTs (Fig. 6, Supporting Information Table S2, Sanchez *et al*. 2008a,c, 2010). Analytes that decreased in response to salt stress, in total 23 MSTs, included organic acids such as citric, succinic, fumaric, erythronic, glycolic and aconitic acid, as was described previously (Fig. 7, Sanchez *et al*. 2008c). This class also included the amines ethanolamine and putrescine (Sanchez *et al*. 2005) and some unidentified MSTs (Fig. 7, Supporting Information Table S2).

In order to identify the specific differences among the metabolic responses to NaCl, the metabolite profiles of *L. creticus* and the two glycophytes were evaluated for differential salt-responsive metabolites using Student's *t*-test ($P < 0.05$ after Bonferroni correction). More than half of the MSTs which were affected by salt treatment were common between species (Fig. 8a, Supporting Information Table S2). Only one-third of the statistically significant salt-responsive MSTs in *L. creticus* were not shared with those of the two glycophytes, and in most cases, the changes in MSTs pool sizes observed were marginal (Supporting Information Table S2). Thus, we demonstrate that only few of the NaCl-elicited changes seem to be extremophile-specific

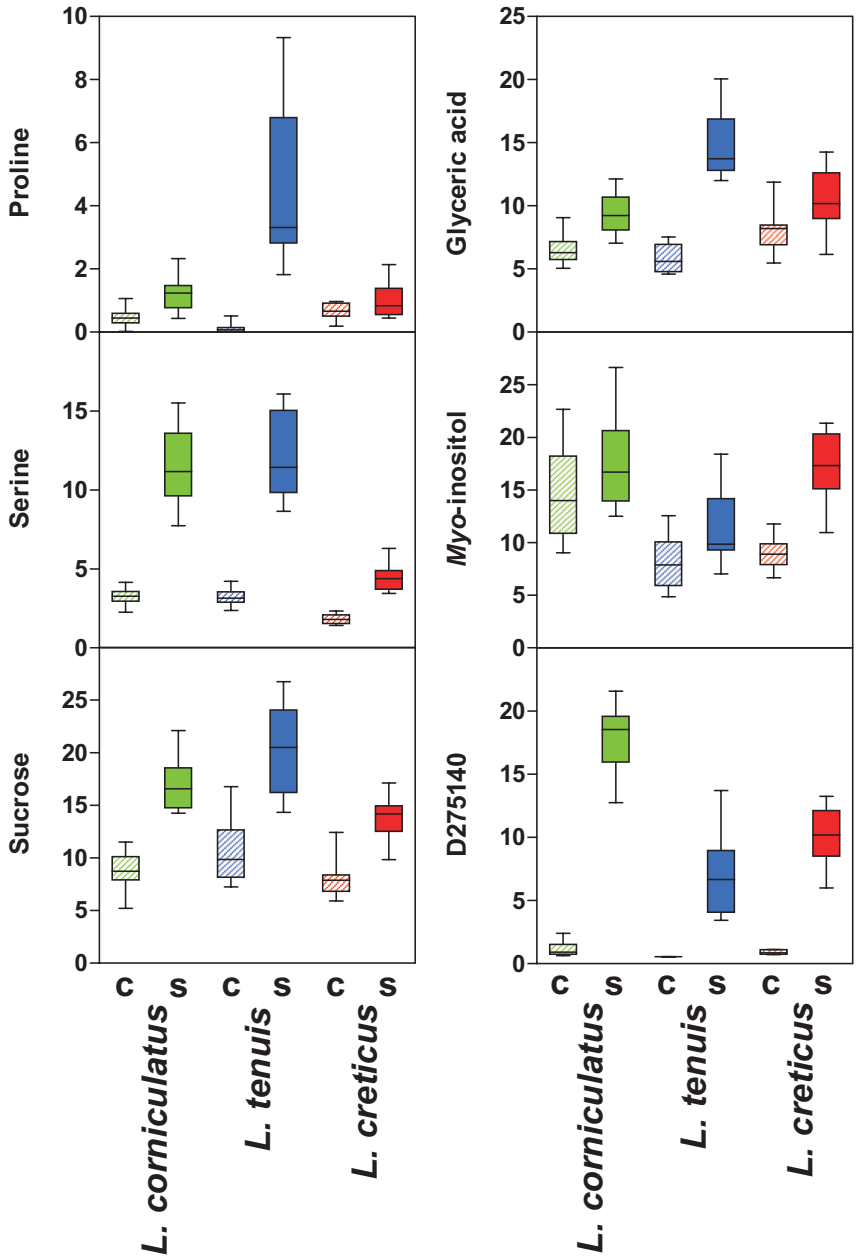

Figure 6. Exemplary metabolites which accumulate under non-lethal long-term salt stress in *Lotus creticus*, *Lotus corniculatus* and *Lotus tenuis*. Data represent normalized responses of metabolite pool measures, that is, detector signals in arbitrary units normalized to internal standard and sample fresh weight, of at least five independent biologically replicated pools of at least five plants in two independent experiments. Data are represented as box plots featuring the maxima, 75 quartiles, medians, 25 quartiles and minima of biological replicate measurements from the control (dashed plots) and salt (filled plots) treatments. Colour code is as in Fig. 1. The metabolite indicated by a D code is one of the *Lotus* ssp. specific but yet non-identified metabolites.

metabolic traits (Fig. 8a, Supporting Information Table S2). For example, the analytes A147001 and A159003 showed a salt response only in *L. creticus* (Fig. 8b), whereas other metabolites increased (ononitol) or decreased (malic acid, threonic acid, and D141181) in the glycophytes but not in *L. creticus* (Fig. 8c).

Taken together, the supervised statistical analyses confirmed and refined the observations of the non-supervised data analysis. Both suggested a preformed species-specific difference at the metabolome level, and conservation with few exceptions (e.g. Fig. 8) of the metabolic responses to salinity between *L. creticus* and the glycophytes *L. corniculatus* and *L. tenuis*.

DISCUSSION

Halophytes are generally defined as organisms which complete their life cycle and successfully propagate under

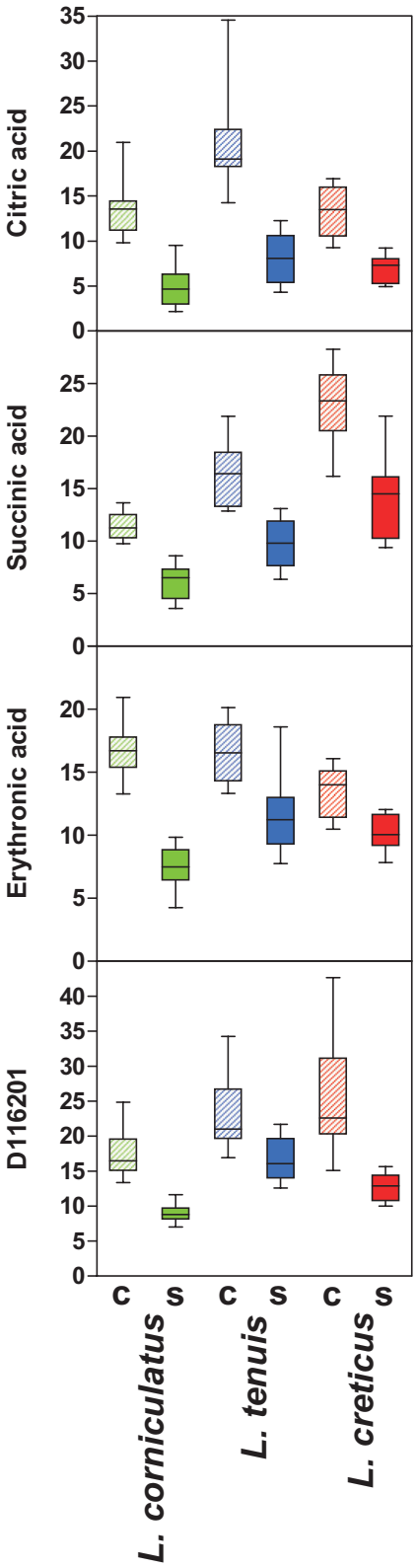

Figure 7. Exemplary metabolites which decrease under non-lethal long-term salt stress in *Lotus creticus*, *Lotus corniculatus* and *Lotus tenuis*. Data represent the normalized responses of metabolite pool measures (cf. Fig. 6) from at least five independent biological replicated pools of at least five plants in two independent experiments. Data are depicted as in Fig. 6.

extremely saline conditions (Flowers *et al*. 1977; Flowers & Colmer 2008). In this sense, *L. creticus* is an extremophile legume as it inhabits the saline environment of Mediterranean coastal regions (Monserrat 1959; Rejili *et al*. 2007). In the present study, *L. creticus* was found to accumulate higher levels of Na^+ than its glycophytic relatives, *L. corniculatus* and *L. tenuis* (Fig. 2). Accumulation of Na^+ is a typical feature of traditional halophytes, although these often accumulate even higher Na^+ levels than measured in *L. creticus* under our experimental conditions (Flowers *et al*. 1977). In contrast to traditional halophytes, however, *L. creticus* efficiently excluded Cl^- from the shoots. In legumes, salt tolerance is strongly associated with Cl^- exclusion (Marschner 1995; Teakle & Tyerman 2010). This is also the case within the *Lotus* genus which comprises sensitive genotypes that accumulate increased Cl^- levels (Teakle *et al*. 2006; Sanchez *et al*. 2010). The observations of this study and the previously reported range of salt tolerance exhibited by *L. creticus* (Sanchez-Blanco *et al*. 1998; Rejili *et al*. 2007) indicate that this extremophile is a rather weak halophyte compared to more extreme examples (Flowers & Colmer 2008). Nevertheless, *L. creticus* represents an extreme case of salt tolerance within the *Lotus* genus. Salt tolerance of *L. creticus* appears to be related to its ability to exclude Cl^- from the shoot tissue (Figs 2 & 3). The understanding of how Cl^- exclusion and alternative ion balancing is achieved in this species and the future identification of the genes involved in this process will help the breeding efforts towards improved salt tolerance in legumes.

Differential accumulation of Na^+ and Cl^- in *L. creticus* during salt acclimation was accompanied by a distinct pattern in nutrient levels. Salt-induced changes in the ionome of the extremophile *L. creticus* were more pronounced than the two glycophytes or resembled more closely those of the *L. tenuis* rather than those of the more sensitive *L. corniculatus* (Fig. 4). Thus, the relative salt tolerance of the species was reflected. The distinct ionomic responses to salt stress of *L. creticus* and *L. tenuis* were not caused by differential effects of salinity on growth, as the two species exhibited essentially similar relative decreases in biomass following salt treatment (Fig. 1). Therefore, NaCl-elicited changes in nutrient homeostasis in these legumes likely represent an acclimation response which potentially contributes to improved physiological tolerance against salt stress.

Global metabolic changes upon salt acclimation included increases in the levels of specific amino acids, sugars and polyols, which had been previously identified as stress-responsive metabolites and are usually described as compatible solutes (Sanchez *et al*. 2008c). Since in our experiments the osmotic stress imposed at root level was the same for *L. creticus*, *L. tenuis* and *L. corniculatus*, but these legumes differed in the amount of salts accumulated in shoots, it is tempting to speculate that the shared metabolic changes represent a 'hard-wired' response to the osmotic elicitation (Sanchez *et al*. 2010). Unfortunately, current metabolomic methods can only quantify a small portion of the estimated plant metabolic component (Bino

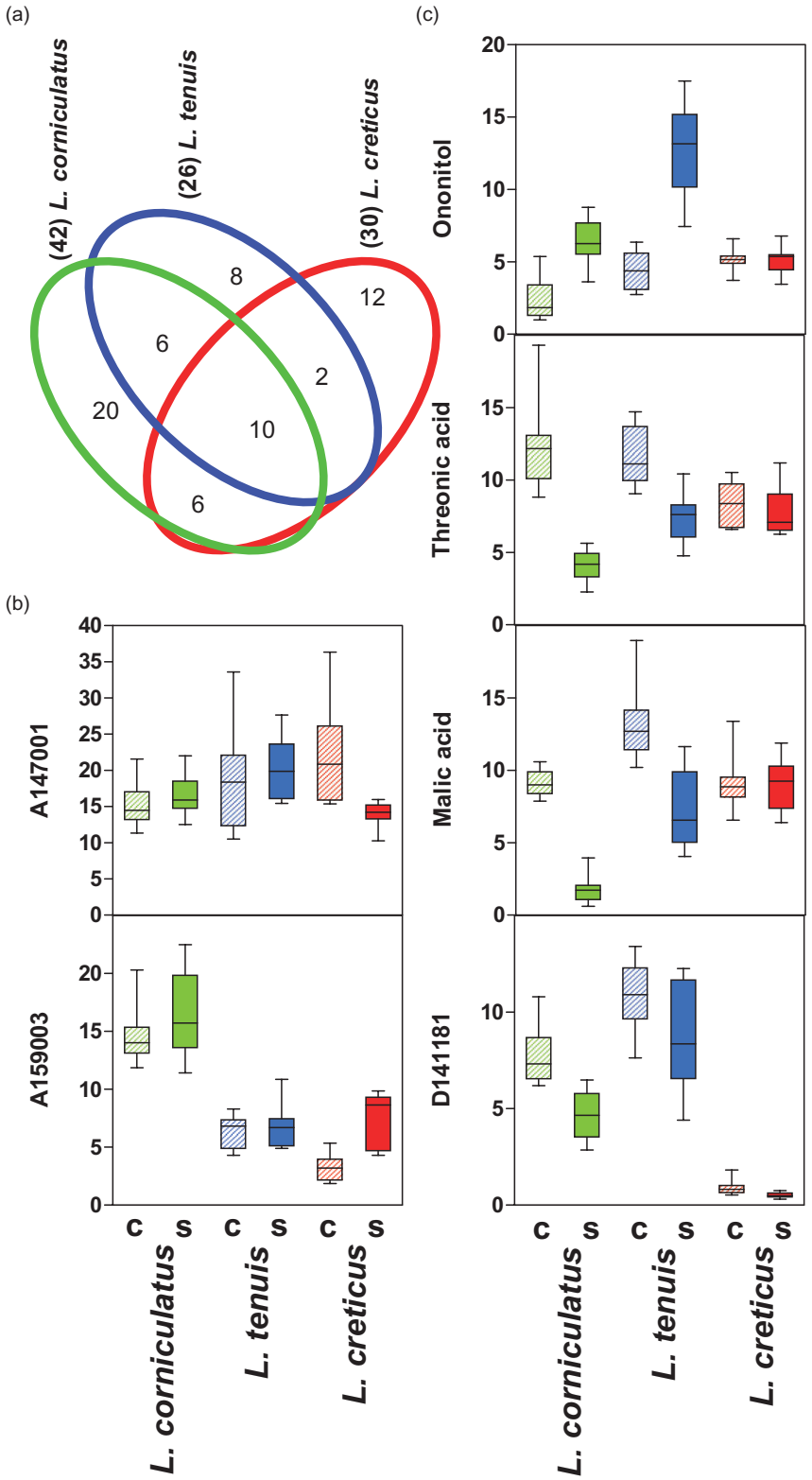

Figure 8. Differential metabolic changes and exemplary metabolites under non-lethal long-term salt stress in *Lotus creticus*, *Lotus corniculatus* and *Lotus tenuis*. (a) Venn diagram comparing analytes which were responsive to salt stress in *L. creticus*, *L. corniculatus* and *L. tenuis* (Student's *t*-test adjusted by Bonferroni correction, $P < 0.05$). Exemplary metabolites show the differential responses of *L. creticus*, that is salt-responsive (b) and non-responsive only in *L. creticus* (c). Data represent the normalized responses of metabolite pool measures (cf. Fig. 6) from at least five independent biologically replicated pools of at least five plants in two independent experiments. Data are depicted as in Fig. 6. The metabolites indicated by an A code are also found in other plant genera than legumes.

et al. 2004), and therefore, it is not possible to recognize if the concentrations of the sum of accumulated small molecules are high enough to account for the osmotic adjustment under salinity. Alternatively, these metabolites may rather be involved in other physiological roles such as protection of membranes and proteins, radical scavenging, signalling or they may act as repository of carbon and nitrogen (Smirnoff & Cumbes 1989; Delauney & Verma 1993; Hare, Cress & Van Standen 1998; Szabados & Savouré 2010). In addition, NaCl stress also elicited a decrease in many organic acids which may compensate for an ionic imbalance (Sanchez *et al*. 2008c). For both the accumulated and the decreased metabolites, the quantitative changes were rather unique to individual species without apparent link to the relative tolerance or salt accumulation (Supporting Information Table S2), implying the existence of differential metabolic rearrangements which depend on the genetic background. This may suggest distinct species-specific mechanisms aimed at improving water retention, radical scavenging or control over the activity of ion transporters mediating cell ionic homeostasis (Smirnoff & Cumbes 1989; Delauney & Verma 1993; Hare *et al*. 1998; Cuin & Shabala 2007a,b; Szabados & Savouré 2010).

Recently, it was suggested that species adapted to saline environments may be metabolically pre-adapted to salinity (e.g. Gong *et al*. 2005; Sanchez *et al*. 2008c). Assuming that the evolutionary adaptation process involves 'hard-wired' changes in metabolism, the hypothesis of metabolic pre-adaptation predicts that an extremophile species should have elevated levels of salt-induced metabolites and likewise reduced levels of salt-reduced metabolites under non-saline growth conditions. In the case of *L. creticus*, however, we found little evidence of preformed metabolic salt acclimation responses. Both the non-supervised and supervised analyses of metabolite profiles demonstrated that salt-elicited changes in metabolism were globally similar in *L. creticus* and the two glycophytes (Figs 5b & 6–8). Supervised analysis of the metabolite data indicated that the general pattern of metabolite pools under control conditions did not reflect relative salt tolerance. Finally, even though the non-supervised and supervised analyses showed some differences in metabolite levels between *L. creticus* and the two glycophytes (Fig. 5a, Supporting Information Table S2), most of these metabolites were not salt responsive or were only marginally elicited by salt in the three species, and therefore behaved contrary to our expectations for general salt protective agents (Supporting Information Table S2). The unique features of the *L. creticus* metabolome, however, could still be related to its relatively slow growth rate (Fig. 1b) or to other physiological features, such as the previously described enhanced drought tolerance (Banon *et al*. 2004).

The physiological role of those few salt-responsive metabolites which are specifically changed in *L. creticus* but not in *L. corniculatus* and *L. tenuis* remains to be investigated (Fig. 8a,b, Supporting Information Table S2). Bearing in mind that the magnitude of metabolic changes within a genotype reflects the stress-dose experienced by the plant (Sanchez *et al*. 2008a,b, 2010), it is likely that the metabolites which changed less or not at all in *L. creticus* (e.g. ononitol, malic acid and threonic acid, Fig. 8c) may simply reflect the lower cellular stress in a more tolerant species compared to the glycophytes. In agreement with this interpretation, these metabolites were found to be stress responsive in other *Lotus* glycophytes (Sanchez *et al*. 2008a).

In conclusion, our data do not support the existence of global metabolic pre-adaptation in *L. creticus* in the sense of a preformed metabolic acclimation to saline environments. However, because shoot tissues were pooled, we cannot rule out the possibility of tissue- or even cell type-specific pre-adaptations. For these questions, the adequate metabolomic tools, namely those for small sample metabolite profiling, are still under development. Of course, our study also does not rule out the existence of metabolic pre-adaptation for other halophytes. The concept of metabolic pre-adaptation may apply to the relative differences between any two extreme glycophyte–halophyte pairs, such as *Arabidopsis thaliana* and *T. halophila* (Gong *et al*. 2005), but it may not apply to the differences between less extreme cases like those present in the *Lotus* phylum. It is reasonable to speculate that the conservation of metabolic responses to salinity in *L. creticus* compared to glycophytes of the same genus may arise from a similar wiring of the response architecture and primary metabolism control which both should largely be conserve during their short phylogenetic divergence (Degtjareva *et al*. 2006). It will be interesting to shed more light on this question, and to investigate the relevance of the basic ionomic and metabolic differences by comparison of the short-term and long-term kinetics of halophyte and glycophyte responses within this and other plant genera.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Mass spectral tags (MSTs) of analytes from this study with an assigend 'D'-code (Save as text file for an upload into the NIST mass spectral search and comparison software).

Table S2. Analytes present in GC-MS profiles from *L. creticus*, *L. corniculatus* and *L. tenuis*.

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