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## SUMMARY

***Thellungiella salsuginea*, a *Brassicaceae* species closely related to *Arabidopsis thaliana*, is tolerant to high salinity. The two species were compared under conditions of osmotic stress to assess the relationships between stress tolerance, the metabolome, water homeostasis and growth performance. A broad range of metabolites were analysed by metabolic fingerprinting and profiling, and the results showed that, despite a few notable differences in raffinose and secondary metabolites, the same metabolic pathways were regulated by salt stress in both species. The main difference was quantitative: *Thellungiella* had much higher levels of most metabolites than Arabidopsis whatever the treatment. Comprehensive quantification of organic and mineral solutes showed a relative stability of the total solute content regardless of the species or treatment, meaning that little or no osmotic adjustment occurred under stress. The reduction in osmotic potential observed in plants under stress was found to result from a passive loss of water. *Thellungiella* shoots contain less water than Arabidopsis shoots, and have the ability to lose more water, which could contribute to maintain a water potential gradient between soil and plant. Significant differences between *Thellungiella* and Arabidopsis were also observed in terms of the physicochemical properties of their metabolomes, such as water solubility and polarity. On the whole, the *Thellungiella* metabolome appears to be more compatible with dehydration. Osmotic stress was also found to impact the metabolome properties in both species, increasing the overall polarity. Together, the results suggest that *Thellungiella* copes with osmotic stress by tolerating dehydration, with its metabolic configuration lending itself to osmoprotective strategies rather than osmo-adjustment.**

Keywords: Arabidopsis relative halophyte, osmotic stress tolerance, osmo-adaptation, plant metabolomics, metabolic profiling, compatible solutes.

## INTRODUCTION

Salt stress is a combination of ionic stress due to the chaotropic effects of incoming $Na^+$ and $Cl^-$, and osmotic stress resulting from a decrease in water potential. Cell homeostasis is challenged by several consequences of these primary stresses. Macromolecules and membranes suffer damage and nutritional disorders arise because the influx of soil minerals is restricted and $CO_2$ assimilation is impaired as the stomatal conductance decreases (Bohnert *et al.*,

1995). If the energy from excited electrons cannot be dissipated, the ensuing photoinhibition can lead to oxidative stress. At the whole-plant scale, salt stress leads to loss of turgor, bleaching and senescence, which have a negative impact on biomass production. In natural environments, halophytic species have developed protective or compensatory mechanisms to resist salt stress, based on the concepts of resistance, tolerance and avoidance defined by

Levitt (1972). Known strategies include tighter control of ion fluxes and compartmentalization, structural modifications of cell-wall and membrane composition, ROS scavenging and osmo-adjustment (Bray, 1993; Wang *et al.*, 2003). Adaptation to stress leads to a wide range of morphological, anatomical and biochemical traits, such as succulence in Cactaceae, salt excretion in Plumbaginaceae, a xerophytic habit such as in *Craterostigma plantagineum*, and $C_4$ or crassulacean acid metabolism in many species (Faraday and Thomson, 1986; Luttge, 1993; Cushman, 2001).

Over the past few years, *Thellungiella salsuginea* has come to the fore in research into abiotic stress resistance as it is a Brassicaceae halophyte species that is related to the model plant Arabidopsis (Bressan *et al.*, 2001; Amtmann *et al.*, 2005; Amtmann, 2009). *T. salsuginea* grows naturally in harsh environments and is very resistant to high salinity, but its genetic make-up, morphology and development are similar to those of Arabidopsis, a typical glycophyte (Inan *et al.*, 2004; Wang *et al.*, 2004). This makes it amenable to functional genomics approaches designed to identify the genes and molecular mechanisms involved in stress resistance, and so it has become a laboratory model species in its own right.

The physiologies of *Thellungiella salsuginea* and *Arabidopsis thaliana* (hereafter referred to as *Thellungiella* and Arabidopsis) have been compared in some detail. Many traits of *Thellungiella* indicate that it is better able to control water and ion fluxes, for example it has reinforced root endodermis and leaf palisade cells, high stomatal density, shows more efficient use of water, has higher levels of epicuticular wax biosynthesis and expresses water channel proteins (Inan *et al.*, 2004; Gong *et al.*, 2005). *Thellungiella* efficiently restricts $Na^+$ influx and controls its subcellular compartmentalization, protecting cells against ion toxicity (Vera-Estrella *et al.*, 2005; Wang *et al.*, 2006). The high transport rates and selectivity of *Thellungiella* ion channels and transporters also allow it to discriminate which minerals are taken up, which protects *Thellungiella* from secondary deficiencies in $K^+$ or $NO_3^-$ (Volkov *et al.*, 2004; Volkov and Amtmann, 2006; Kant *et al.*, 2008). Furthermore, efficient mechanisms to counteract the formation of reactive oxygen species (ROS) and photoinhibition have been described (Inan *et al.*, 2004; M'rah *et al.*, 2006; Stepien and Johnson, 2009).

Several studies have taken advantage of the close genetic resemblance between *Thellungiella* and Arabidopsis in order to explore the *Thellungiella* transcriptome to determine which genes are expressed in a halophyte and how they are regulated. When the known or annotated functions of differentially expressed genes were analysed, recycling of membranes, changes in the cell wall and overall protein metabolism, and modulation of central metabolism emerged as key transcriptionally controlled processes in *Thellungiella* stress resistance (Wang *et al.*, 2004; Gong

*et al.*, 2005). The transcriptome reflects important regulatory processes of gene expression, but does not necessarily mirror the final phenotype, which is the outcome of many downstream molecular events. However, the metabolome reflects function more closely, providing an indication of how re-routing metabolic fluxes modulates photosynthesis, photorespiration, nitrogen assimilation and photoassimilate partitioning. The specific *Thellungiella* metabolome has yet to be described in detail. Gong *et al.* (2005) reported constitutively higher levels of proline, inositols, sugars and organic acids than found in Arabidopsis, which were interpreted as metabolic stress anticipation in *Thellungiella*; however, no direct contribution of these traits to either osmo-adjustment or osmoprotection has been demonstrated.

Here, the occurrence of metabolic attributes of osmo-adaptation was investigated in the shoots of Arabidopsis and *Thellungiella* under selected stress conditions using a range of metabolomics techniques (Roessner *et al.*, 2001). Metabolic fingerprinting and semi-quantitative analysis were used to comprehensively explore the differences between species. These data provide a framework within which metabolic phenotypes may be analysed in functional terms, by correlating metabolic profiles with growth rate and stress intensity to distinguish pathways that are active in stress tolerance. Both organic and mineral solutes were quantified to understand how pools of metabolites may be involved in osmoregulation. Finally, we propose a new virtual way of describing the metabolome in biophysical terms to evaluate a species' overall metabolic strategies to maintain cell function under stress.

## RESULTS

### Metabolic fingerprints revealed no qualitative difference between the Arabidopsis and *Thellungiella* metabolomes

As a first approach, shoot polar metabolomes of the two species were compared, both under normal growth conditions and after 4 days of exposure to 100 mM NaCl. This stress intensity was chosen because it induces a significant stress response in Arabidopsis, at physiological levels such as the transcriptome, growth, ion management and the metabolome, without inducing major damage such as chlorosis. It was also expected to provoke a response in *Thellungiella* (Kreps *et al.*, 2002; Kant *et al.*, 2006; Ghars *et al.*, 2008). In our study, non-targeted analysis techniques were chosen to cover as many metabolites as possible.

Comparison of $^1$H-NMR spectra revealed no major qualitative differences between Arabidopsis and *Thellungiella*, with or without salt treatment, except in the aromatic region, where some rare compounds were detected (Figure 1). Signal intensities, on the other hand, were much higher for all metabolites in *Thellungiella* samples, with the notable exception of fumarate. After salt treatment, the metabolite
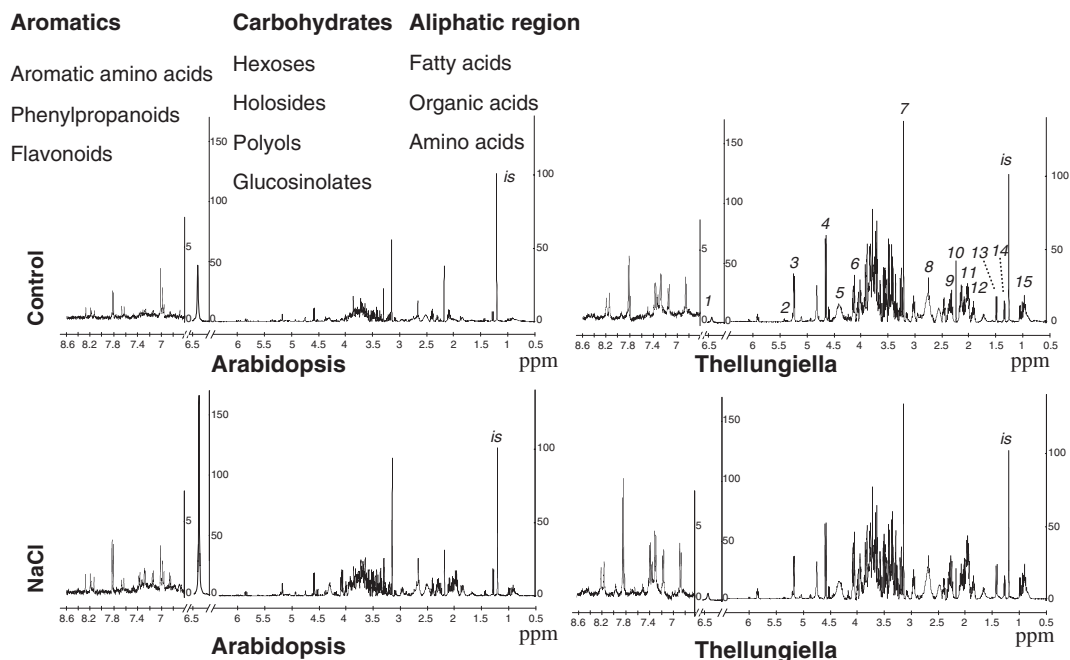
**Figure 1.** ¹H-NMR fingerprints of Arabidopsis and *Thellungiella* shoot extracts (signals normalized to internal standard) show that Arabidopsis and *Thellungiella* mainly differ in the abundance of metabolites (1, fumarate; 2, sucrose; 3 & 4, glucose; 5, malate; 6, proline; 7, choline; 8, malate and citrate; 9, proline; 10, glutamine; 11, glutamate and quinate; 12, GABA; 13, alanine; 14, threonine; 15, fatty acids, valine, leucine and isoleucine; is, internal standard).

content (per gram of dry weight) had globally increased in Arabidopsis, but did not reach the levels found in *Thellungiella*. By contrast, the *Thellungiella* spectrum barely changed in response to salt, and a notable increase occurred for only a few compounds, such as proline, GABA and aliphatic and aromatic compounds. These results were confirmed by GC-MS (Table S1). A principal component analysis (PCA) indicated that genetic background is the main contributor to the metabolome variations. This unsupervised multivariate statistic extracts the internal framework of the dataset by transferring the data into a new coordinate system of uncorrelated variables, i.e. the principal components (PCs), each accounting for a decreasing amount of data variability. Plotting of data on the first PCs generally reveals the initial variables that discriminate between the samples, accounting for the main sources of variation, such as genotype or treatments. Here, the first principal component (PC1), which accounted for 43% of the total variance, clearly discriminates between the two species, while the effect of salt treatment is associated with PC3, accounting only for 11% of data variance (Figure 2). Loading plots also show that, of 103 metabolites, 54 explain 80% of the PC1 variance, two-thirds of which have positive coordinates that indicate higher abundance in *Thellungiella* (Table S2). This result is consistent with the greater metabolite content in this species. On the other hand, sample scores show a much higher contribution of Arabidopsis to the variance of PC3 (>80%), indicating that NaCl has a greater impact on this species. It

is worth noting that samples of salt-treated *Thellungiella* contribute 32.4% to the PC1 variance, whereas control samples only contribute 16.4%. This means that part of the *Thellungiella* response to salt is represented by PC1, as its metabolome becomes more distinct from that of Arabidopsis. This is even more obvious when plotting logarithmic ratios of the 92 metabolites that discriminate between samples (Figure 3). Inter-specific differences involve abiotic stress markers (Lugan *et al.*, 2009): proline, GABA and myoinositol-1-phosphate are more abundant in *Thellungiella*, whereas levels of galactinol, raffinose, spermidine, putrescine and β-alanine are higher in Arabidopsis (Figure 3). Other central pathways also show different ratios for pools of energy-related compounds (i.e. hexoses and malate), nucleosides (i.e. guanosine and adenosine) and secondary metabolites or precursors (i.e. caffeoylquinate and phenylalanine). These were all more abundant in *Thellungiella*, while Arabidopsis instead accumulated sucrose and fumarate, urate and the phenylpropanoid sinapate. Of the 58 products that were more abundant in *Thellungiella*, the levels of 42 increased in Arabidopsis after salt treatment (e.g. proline, GABA and fructose), whereas the levels of 19 of the 34 compounds that were less abundant in *Thellungiella* decreased in Arabidopsis under stress (e.g. spermidine). The metabolic response to salt in Arabidopsis thus partially mimics the constitutive difference normally present between the two species (Figure 3). Some noticeable exceptions were sinapate, urate, galactinol, raffinose
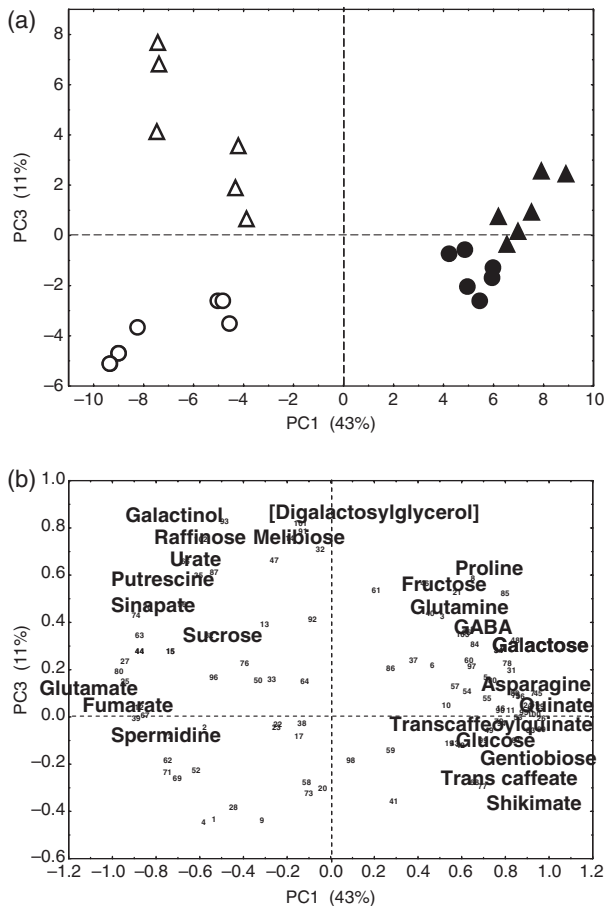
**Figure 2.** Principal component analysis score (a) and loading (b) plots for GC-MS of 103 analytes in shoots of Arabidopsis (white) and *Thellungiella* (black) after 4 days on 100 mM NaCl (triangles) or control medium (circles).
The most discriminatory metabolites are shown; other variables are identified by a number according to their ID in Table S1. Arabidopsis and *Thellungiella* are discriminated along PC1,while the effect of NaCl is apparent along PC3.

360 mM, but expanded to the whole rosette at 480 mM. The shoot growth (measured as the percentage of the FW at day 9 relative to the FW at day 0 just before starting treatment) decreased at concentrations up to 120 mM NaCl, and then became negative, indicating net loss of water during the treatment. *Thellungiella* showed no visible injury up to 240 mM, and even at a concentration of 480 mM, bleaching was restricted to fully mature leaves. Under standard conditions, shoot growth was 3.3 times lower than that of Arabidopsis, but it remained stable at concentrations up to 75 mM and decreased slowly at concentrations up to 360 mM. FW loss was visible only with 480 mM NaCl (Figure 4b).

Metabolomic data and shoot growth were expressed as logarithmic ratios over the Arabidopsis standard, and both samples and variables were then sorted by hierarchical cluster analysis (HCA) (Figure 5a). Samples mainly clustered according to species. The effect of salt was evident within each group, as samples are sorted by increasing NaCl concentration. This indicates that a progressive metabolic shift occurred as stress intensified. In addition, samples at the highest NaCl concentrations were differentiated: the set of Arabidopsis samples treated with concentrations above 240 mM formed a distinct cluster, as does the sample of *Thellungiella* treated with 480 mM NaCl. The metabolic profiles thus clearly reflect a different physiological status below and beyond those thresholds. High stress intensity in Arabidopsis induced accumulation of 60% of the detected metabolites, which is twice that observed under low stress intensity. Interestingly, raffinose and galactinol accumulated in Arabidopsis but their levels did not change in *Thellungiella* in response to stress. In both species, metabolic breakpoints were associated with a characteristic accumulation of lysine, arginine and histidine, as well as a reduction in the amounts of glutamate, glutamine and aspartate. For any stress intensity, GABA, maltose, sucrose, galactose, fructose, leucine, isoleucine, glycine and β-alanine were more abundant in the sensitive than in the resistant species. In contrast, two metabolites were highly accumulated by *Thellungiella*: hydroxyproline and proline.

Correlations between metabolite levels and NaCl concentration further highlight the link between metabolites and stress intensity (Figure 5b). Metabolites positively correlated with NaCl concentrations are hydroxyproline, arginine, proline, sucrose, glycine, malate and histidine, whereas aspartate, spermidine, glutamine and glutamate are negatively correlated. These variables follow the same trend in Arabidopsis and *Thellungiella*. The main differences are found for galactose, fructose, maltose, lysine, leucine, tyrosine, isoleucine and valine, which are positively correlated with NaCl concentration in the medium in Arabidopsis but not in *Thellungiella*, and fumarate, ammonium, asparagine and alanine, which are negatively correlated with NaCl concentration in the medium in *Thellungiella* but not in

and X11, which increased under stress in Arabidopsis but were not constitutively expressed at high levels in *Thellungiella*. Many stress markers responded similarly in *Thellungiella* and Arabidopsis, but the response was of lower magnitude in *Thellungiella* (i.e. raffinose, galactinol, proline, maltose and spermidine). *Thellungiella*-specific responses were limited to the accumulation of ribose, guanosine and phosphoric acid, and the decrease in fumarate and caffeate levels (Figure 3).
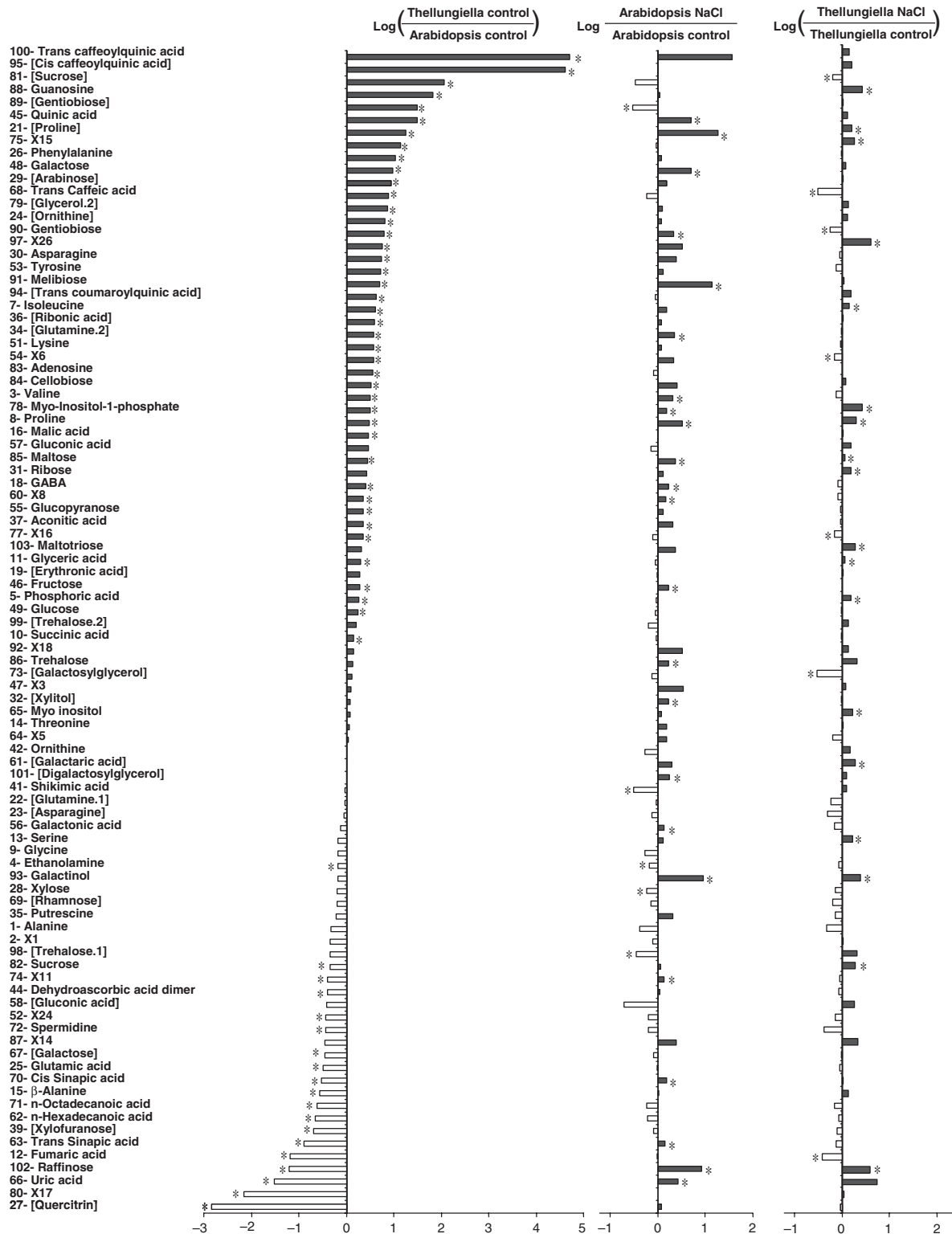
### Both species experienced NaCl dose-dependent growth reduction and comparable metabolome shifts

In a second experiment, plants were grown on various NaCl concentrations ranging from 25 to 480 mM (Table S3). The experiment was stopped after 9 days, the time at which maximum shoot damage had occurred in Arabidopsis treated with 480 mM NaCl (Figure 4a). Bleaching of older leaves occurred in Arabidopsis at concentrations of 240 and

**Figure 3.** Logarithmic ratios of averaged metabolite content in shoots.

The selected metabolites have variations >50% and/or significant differences according to the Mann–Whitney *U* test (*$P$ < 0.05, $n$ = 6). Black bars indicate metabolites that are more abundant in the numerators. The numbers in front of the metabolite names refer to their ID in Table S1. The metabolic differences between Arabidopsis and *Thellungiella* appear to be greater than changes induced by NaCl in either species, although NaCl stress in Arabidopsis brings the abundance of many metabolites closer to that observed in *Thellungiella*. Compounds labelled in parenthesis had the best match of mass spectrum from the library, but had different retention time that of the actual analysis detected.
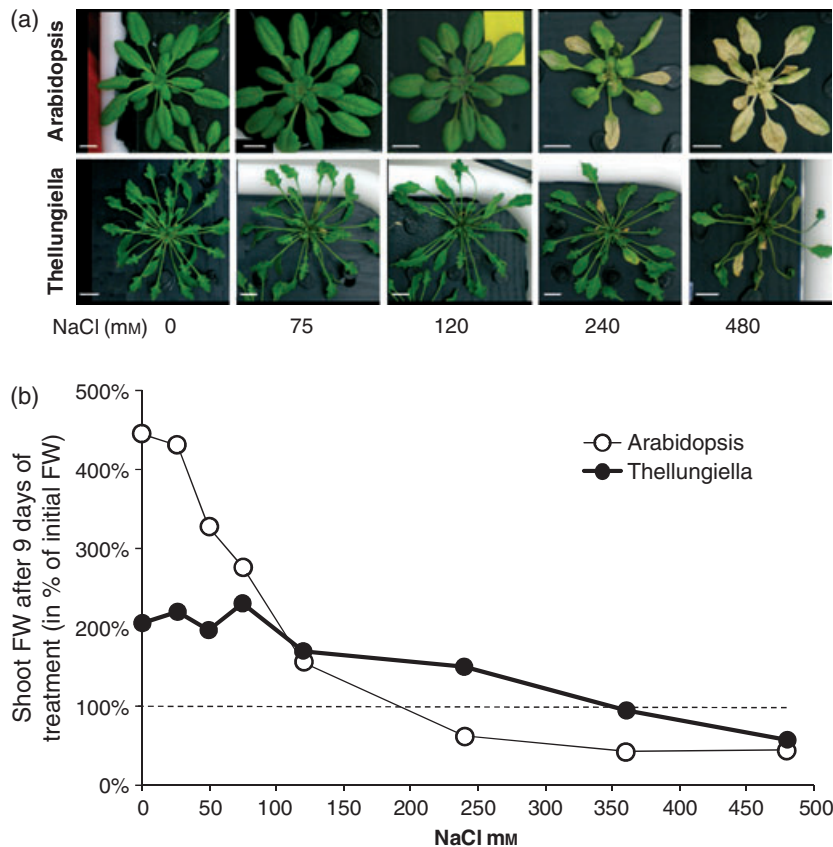
**Figure 4.** Shoot phenotype of Arabidopsis and *Thellungiella* after 9 days of treatment on various NaCl concentrations.
(a) View of rosettes. The white bars represent one unit of length.
(b) Shoot fresh weight after treatment. Each symbol represents a pool of three rosettes.

Arabidopsis. Serine is correlated in the opposite manner for the two species.

### Quantitative differences in solute and water content between species are associated with the higher osmo-compatibility of the *Thellungiella* metabolome

The previous experiment allowed us to empirically determine the stress intensities that have an equivalent impact on both species. The NaCl concentration causing 50% inhibition of growth is 75 mM for Arabidopsis and 240 mM for *Thellungiella* (Figure 4b). This model in no way assumes that both species had identical growth kinetics but simply indicates with good confidence that plants were in the same general state after 9 days of treatment at these concentrations. To compare the effects of salinity and water stress, plants were treated for 9 days with these respective NaCl concentrations or with osmotically equivalent PEG 8000 solutions. Polar metabolites, soluble inorganic ions, water content and osmotic potentials were then quantified in the shoots in order to calculate osmotic balances (Figures 6 and 7 and Table S4). At least 75% of the osmotically active solutes in the extracts were identified and quantified.

Under standard conditions, the higher level of organic solutes in *Thellungiella* roughly reflects the greater abundance of four metabolites: malate, proline, glucose and fructose. The mineral profiles were different as Arabidopsis accumulated more $NO_3^-$ and *Thellungiella* accumulated more $PO_4^{3-}$ and $SO_4^{2-}$ (Figure 6). Taken together, the higher organic solute levels in *Thellungiella* are compensated for by lower inorganic solute levels, so that the total solute content per gram dry weight almost equals that of Arabidopsis. The lower osmotic potential of *Thellungiella* shoots appears to be primarily due to its lower water content (Figure 7). Under salt stress, $Na^+$ and $Cl^-$ accumulated to comparable levels in both species at the expense of $K^+$ and $NO_3^-$. The total solute content was significantly higher under salt stress in both species, but the magnitude of the increase in organic solute levels in *Thellungiella* was much greater. However, the changes in total solutes were too weak to explain the differences in shoot osmotic potential of −0.07 MPa in Arabidopsis and −0.18 MPa in *Thellungiella* when that of the exogenous medium had changed by −0.36 and −1.17 MPa, respectively. The significant water loss incurred by both species was in fact the main factor in
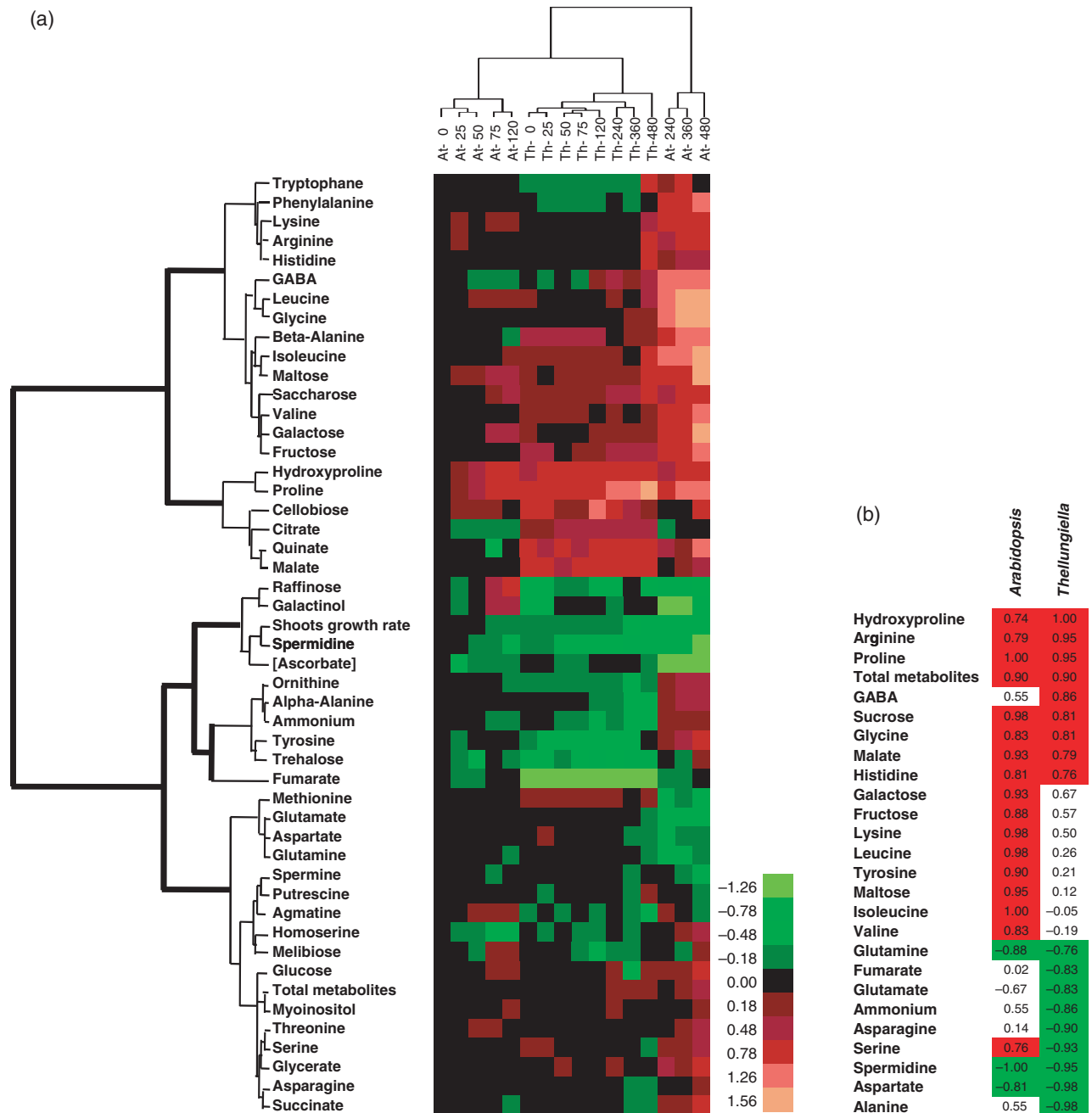
**Figure 5.** Metabolite changes in plants grown for 9 days on various concentrations of NaCl (0, 25, 50, 75, 120, 240, 360 and 480 mm).
(a) Hierarchical clustering analysis of metabolites in samples obtained from rosettes of Arabidopsis (At) and *Thellungiella* (Th). Data are expressed as logarithmic ratios over Arabidopsis control values (At-0, first column).
(b) Spearman correlation coefficients between shoot metabolite contents and NaCl concentrations in growth medium, sorted by decreasing order in *Thellungiella*. Red highlighting indicates positive correlations and green highlighting indicates negative correlations ($P < 0.05$, $n = 8$).

balancing osmotic potentials between the shoot and the environment (−0.17 MPa in Arabidopsis and −0.46 in *Thellungiella*). Water stress caused a further decrease in water content, in contrast with the trend for total solute content: which decreased in Arabidopsis and increased in *Thellungiella* because of an increase in hexose pools. Details of the differential responses to either NaCl or water stress are shown in Figure 8. In both species, water stress was associated with higher amounts of K+ and sucrose, and lower amounts of spermidine and serine. In Arabidopsis, water stress induced a greater accumulation of proline, GABA, malate, citrate, alanine, $NO_3^-$ and $SO_4^{2-}$. The response of *Thellungiella* was specifically targeted towards accumulation of non-structural carbohydrates and a decrease in
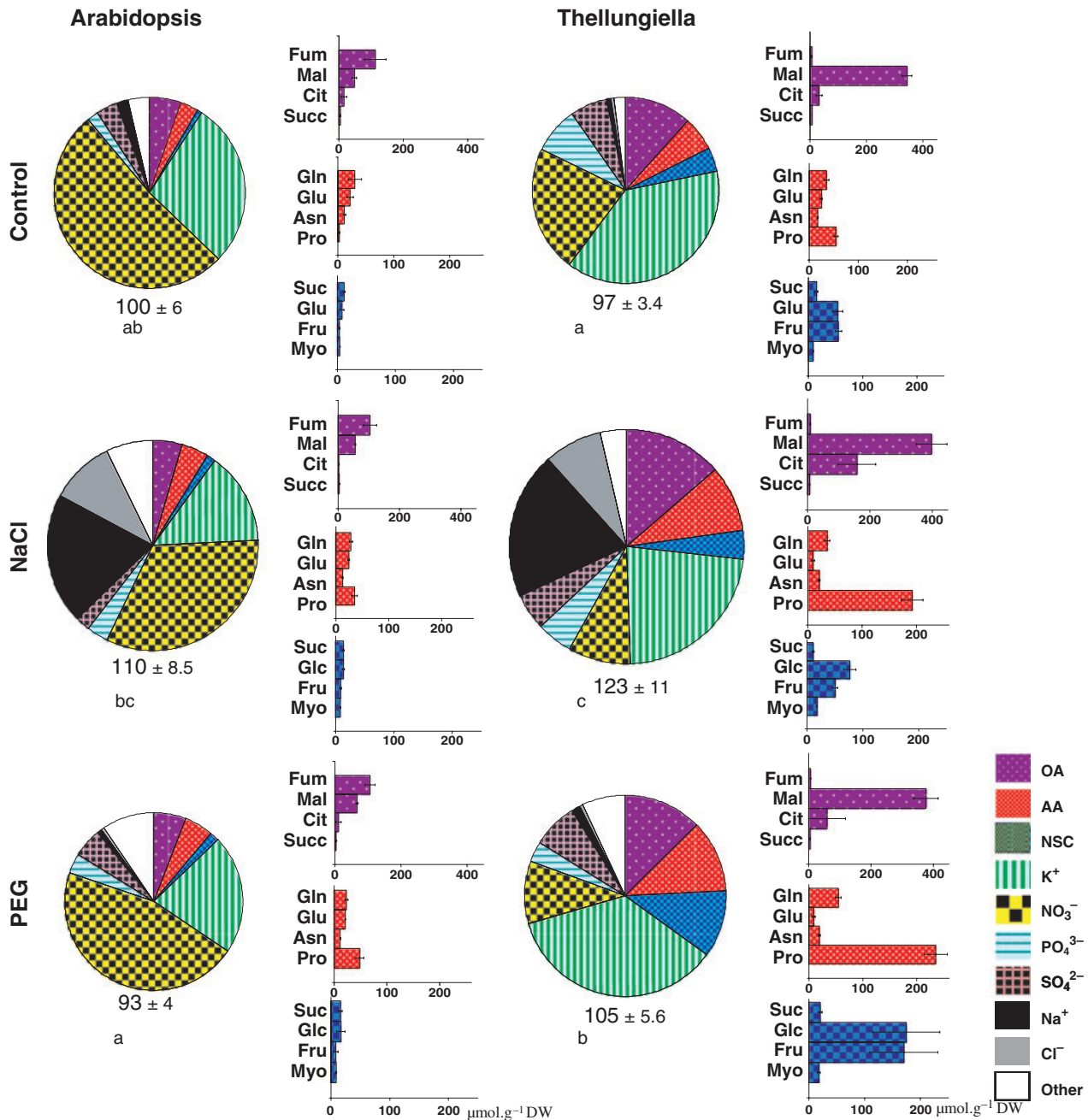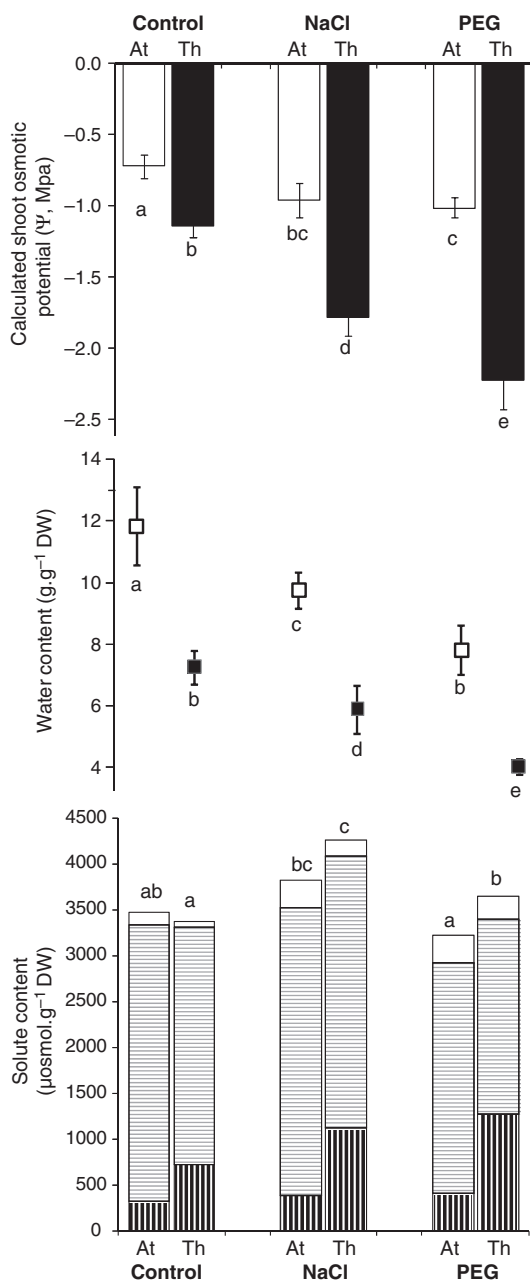
**Figure 6.** Relative composition of solutes in shoots.

Arabidopsis and *Thellungiella* seedlings were treated for 9 days with NaCl concentrations inducing 50% reduction of shoot growth, or an osmotically equivalent PEG8000 solution. Total solute contents ($\mu$osmol.g$^{-1}$ DW) are expressed below each diagram relative to that of the Arabidopsis control (100). Significant differences are indicated by letters (Mann–Whitney *U* test, $P < 0.05$, $n = 3$). The contributions of the various mineral and organic fractions are shown in the diagrams. The most important contributors for the organic function are plotted ($\mu$mol g$^{-1}$ DW $\pm$ SD). OA, organic acids; AA, amino acids; NSC, non-structural carbohydrates; Fum, fumarate; Mal, malate; Cit, citrate; Succ, succinate; Gln, glutamine; Glu, glutamate; Asn, asparagine; Pro, proline; Suc, sucrose; Glc, glucose; Fru, fructose; Myo, myoinositol.

fumarate, tyrosine, succinate, ascorbate and $PO_4^{3-}$. The polarity of the metabolites, estimated by their octanol/water partition coefficient and topological polar surface area, showed that the organic compounds that increase in *Thellungiella* under water stress, as compared to NaCl stress, tend to be more polar, while those that decrease are less polar.

To extend these observations, the metabolome of each species was considered as a single 'virtual molecule', whose physicochemical properties are the weighted averages of the properties of individual metabolites (Figure 9). In the context of osmo-adaptation, the most relevant properties are those linked to polarity, such as the octanol to water

**Figure 7.** Shoot solute content (black area represents organic solutes, grey represents minerals and white represents non-identified solutes), water content and osmotic potentials (calculated from the osmolality measured in extracts and from water content).
Arabidopsis and *Thellungiella* seedlings were grown for 9 days on NaCl concentrations inducing 50% reduction of shoot relative growth, or an osmotically equivalent PEG8000 solution. Letters indicate differences according to a Mann–Whitney *U* test ($P < 0.05$, $n = 3$). At, Arabidopsis; Th, *Thellungiella*. The osmotic potential differences appear to be associated with a reduction in water content rather than an increase in solutes.

partition coefficient (logP) and solubility. Other readily available values were also included in this analysis: molar mass, N to C ratio, carbon oxidation number, and the free

energy of formation $\Delta_f G^{\circ\prime}$. There are significant differences between the two species. Under standard conditions, the *Thellungiella* metabolome was more soluble, polar and reduced than the Arabidopsis metabolome. This may be explained by the presence in *Thellungiella* of higher proportions of non-structural carbohydrates, and reduced and soluble molecules with very negative free energies of formation, and the fact that proline (a reduced compound with a high N to C ratio and reasonable solubility) accumulates in preference to other amino acids, such as glutamine and glutamate. The difference may also be due to the greater proportions of malate compared to fumarate, the latter being a smaller molecule, with very poor solubility and a less negative free energy of formation.

Osmotic stresses changed the properties of metabolomes. Under salt stress, the Arabidopsis metabolome showed increases in polarity, carbon reduction, the N to C ratio, molar mass and $\Delta_f G^{\circ}$. In *Thellungiella*, water stress induced more dramatic changes than salt stress, but still tending towards a more reduced, polar, massive and soluble metabolome.

## DISCUSSION

### Arabidopsis and *Thellungiella* have both similar and divergent metabolic responses to osmotic stress

Here we undertook a comprehensive and unbiased survey of the metabolomes of two Brassicaceae species, one that is sensitive to salt stress, Arabidopsis, and the other that is tolerant of salt stress, *Thellungiella*. The levels of metabolites changed in both species in response to salt stress. Metabolites positively correlating with salt stress in this study are known to participate in the underlying mechanisms of cell-wall remodelling (hydroxyproline), osmo-protection and storage (proline and sucrose) and photorespiration (glycine and serine). Hydroxyproline is a major component of hydroxyproline-rich proteins, whose expression is transcriptionally regulated under abiotic stress (Gong *et al.*, 2005). Its presence as a free compound indicates that cell wall catabolism is enhanced, probably related to major reconfiguration of the cell wall structure and physical properties. Photorespiratory activity under abiotic stress replenishes the Calvin cycle and oxidized co-factors (Bohnert and Jensen, 1996; Wingler *et al.*, 2000). Serine levels, which are positively correlated with salt stress in Arabidopsis and negatively in *Thellungiella*, may indicate that *Thellungiella* has a better ability to replenish the Calvin cycle through serine degradation. Metabolites that are negatively correlated with salt stress in both species are the abundant amino acids involved in nitrogen assimilation and transport (glutamine, aspartate and glutamate), as well as a growth regulator (spermidine). Both species also showed similar NaCl dose-dependent patterns, including a gradual shifting of the metabolome with increasing concentrations
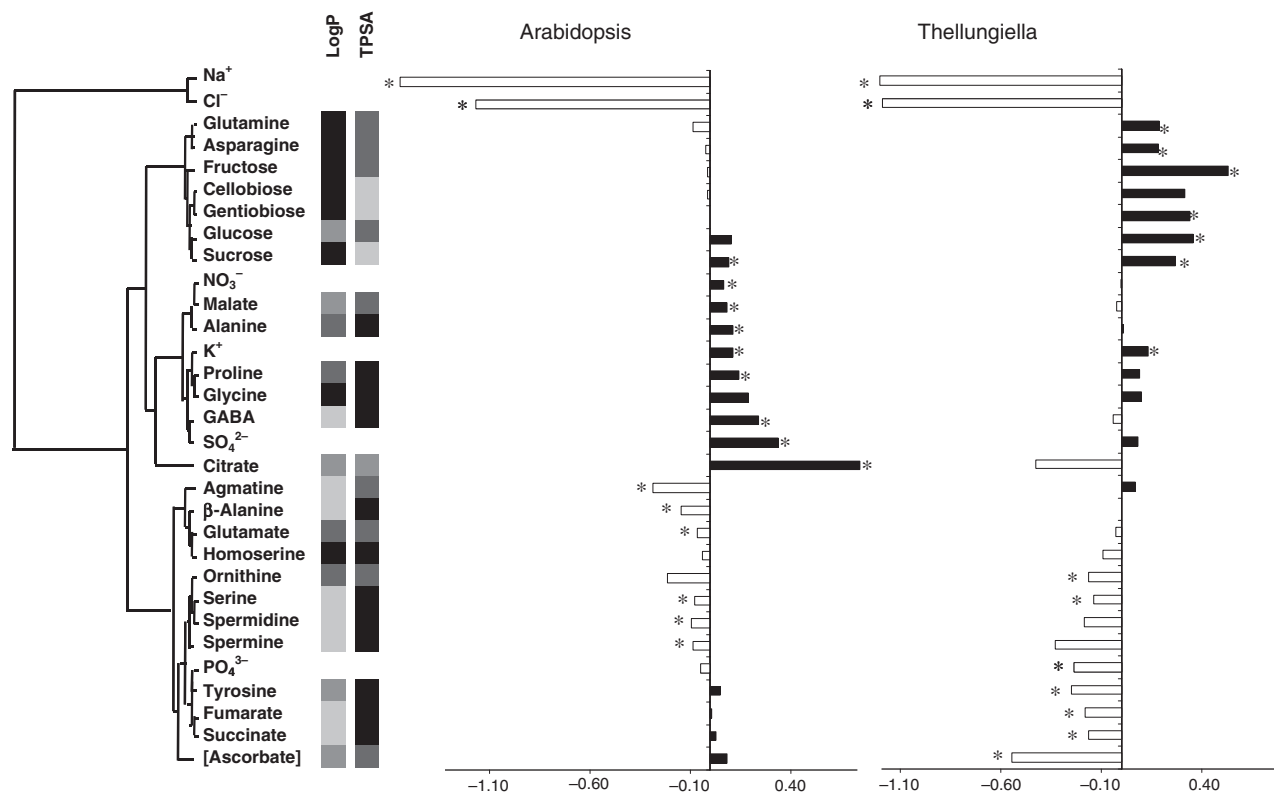
**Figure 8.** Hierarchical clustering analysis of logarithmic ratios of solute content in shoots induced by PEG800 treatment compared with NaCl treatment. Arabidopsis and *Thellungiella* seedlings were treated for 9 days with NaCl concentrations inducing 50% reduction of shoot growth, or an osmotically equivalent PEG8000 solution. The selected metabolites show variations of at least 50% and/or significant differences according to the Mann–Whitney $U$ test (*$P < 0.05$, $n = 3$). Black bars indicate metabolites that are more abundant with PEG8000 treatment than with NaCl treatment. The octanol to water partition coefficient (LogP) of metabolites and their topological polar surface area (TPSA) are indicated by the grey scale, from low (dark) to high (light).

of salt until a breakpoint was reached at higher stress intensities, resulting in a more dramatic metabolic reconfiguration. Comparable observations have already been reported in several other species (Sanchez *et al.*, 2008a,b).

Our results indicate the metabolic responses associated with the loss of water homeostasis and bleaching. Basically, low stress intensity responses are probably related to the mechanisms implicated in homeostasis, whereas high stress intensity symptoms reflect the plant's attempts to deal with more chaotic processes, such as damaged structures and premature senescence (Munns, 2002). Taken together, there were only marginal differences between the metabolic pathways activated by stress in Arabidospsis and *Thellungiella*. However, these differences suggest that *Thellungiella* was more efficient in detoxifying (ammonium) and mobilized fewer starch reserves (maltose), possibly because photosynthesis persisted longer. The most striking difference was that raffinose and its respective precursor and product, galactinol and melibiose, were stress-responsive mainly in Arabidopsis. Although not all species accumulate raffinose and related oligosaccharides under osmotic stress, these molecules are considered as important osmoprotectants in xerophytes (Peters *et al.*, 2007; Sanchez

*et al.*, 2008b). The transcriptional activation of the raffinose pathway reported in *Thellungiella* (Taji *et al.*, 2004; Wong *et al.*, 2006) may counter this, but it is possible that raffinose accumulation in itself only weakly contributes to resistance, while an increased flux through the pathway and a high turnover could participate in stress acclimation, perhaps via radical scavenging (Nishizawa *et al.*, 2008). Also, differences observed in the aromatic region of the $^1$H-NMR spectra and in GC-MS data suggest that secondary compounds from the phenylpropanoid and flavonoid pathways are discriminatory, and these specific pathways may be involved in stress resistance.

### NaCl-induced solute concentration is not sufficient for osmo-adjustment

A surprising observation characterizes the global stress response strategy of the *Thellungiella* metabolome: the most discriminatory traits between species were the absolute contents of organic compounds and water (per gram dry weight). These two parameters are of prime importance in osmotic stress resistance. Salt and drought disturb the gradient of water potential between soil and shoots, leading to dehydration and loss of turgor (Verslues

**Figure 9.** Virtual physicochemical properties of the metabolome in Arabidopsis (dotted line) and *Thellungiella* shoots.

Plants were treated for 9 days with NaCl concentrations inducing 50% reduction of shoot growth, or an osmotically equivalent PEG8000 solutions. Significant differences between the species are indicated (Mann–Whitney *U* test, *P < 0.05, n = 3). n°(C), carbon oxidation number; N/C, nitrogen to carbon ratio; $\Delta_f G^{\circ\prime}$: standard free energy of formation; logP, octanol to water partition coefficient.
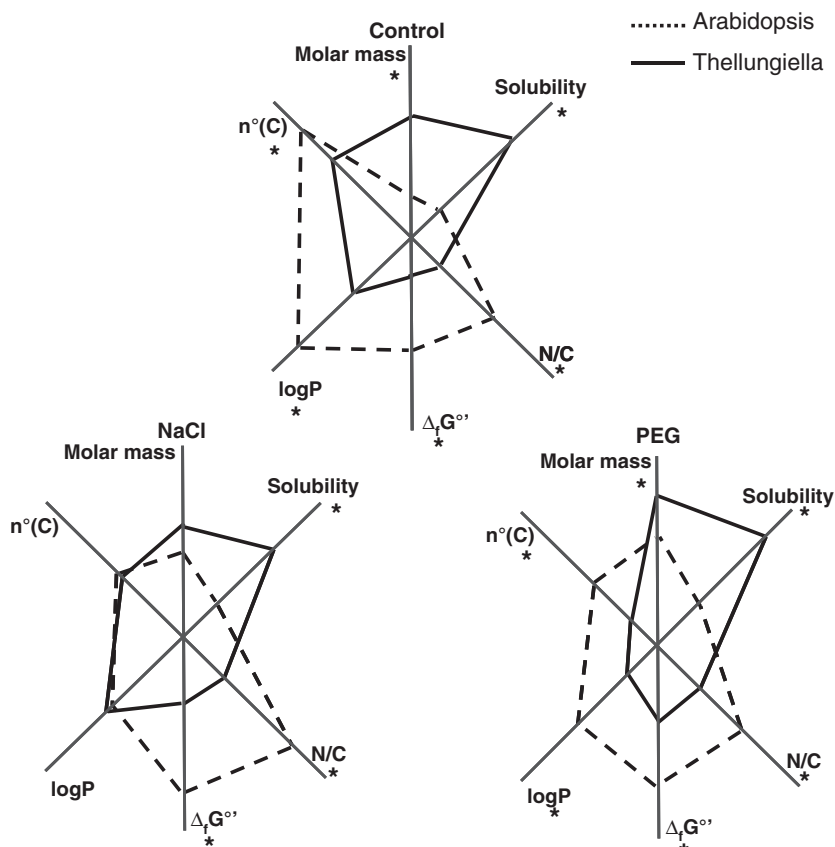


et al., 2006). Dehydration can be avoided by reducing the leaf water potential. This is achieved by osmo-adjustment, a process that reduces the osmotic potential of cells by influx of inorganic ions into the vacuole and accumulation of organic compounds in other compartments (Morgan, 1984; Bray, 1993; Gagneul *et al.*, 2007). In our experiment, salt stress significantly decreased the osmotic potential of shoots, increased total solute levels and induced accumulation of $Na^+$ and $Cl^-$. However, there was little variation in total solutes in all samples tested, because the increase in some solutes was balanced by the decrease in others, and consequently no osmotic adjustment occurred. Moreover, the leaves of *Thellungiella* have a constitutively lower water content than those of Arabidopsis, and are also able to lose more water under osmotic stress without losing turgor. Thus *Thellungiella* appears to be prepared to cope with low water potentials by sustaining a water potential gradient with the environment, due to a passive reduction in water content. Consequently, this species is not avoiding dehydration, and therefore its homeostatic target under osmotic stress is not water content.

### The metabolome is configured for high compatibility in *Thellungiella*

*Thellungiella* thus appears to be truly tolerant of dehydration according to the definition by Levitt (1972). This possi-

bility has not been raised before in relation to *Thellungiella* because water status is usually expressed as the water content relative to the water content at full turgor. A comparison of absolute water content (water mass relative to dry mass) highlights better the difference between Arabidopsis and *Thellungiella*. The higher levels of protection and repair mechanisms observed in *Thellungiella*, including production of ROS scavengers, dehydrins and late embryogenesis abundant proteins (Gong *et al.*, 2005; M'rah *et al.*, 2006; Wong *et al.*, 2006; Kartashov *et al.*, 2008), are consistent with a dehydration tolerance strategy. Compatible solutes are also often described as important osmoprotectants in a wide range of species (Schobert, 1977; Gilles, 1997, Rontein *et al.*, 2002; Hincha and Hagemann, 2004, Yancey, 2005). Nevertheless, *Thellungiella* did not accumulate typical plant osmolytes such as betaines or polyols, which derive from the primary metabolism and are found at high concentrations in many halophytes (Bohnert *et al.*, 1995; Hare *et al.*, 1998; Tipirdamaz *et al.*, 2006). By contrast, *Thellungiella* accumulates proline, just like Arabidopsis, but with enhanced regulation of its biosynthesis and degradation (Ghars *et al.*, 2008). Our quantitative study revealed that *Thellungiella*'s resistance advantage is not restricted to this single compound. This was particularly clear when comparing the impacts of water stress and NaCl stress. Water stress triggered a specific increase in non-structural carbo-

hydrates in *Thellungiella*, while Arabidopsis only further increased its proline and malate contents. Reconfiguration of the metabolome under water stress increased the overall polarity in both species, as solutes with a high polarity tended to increase, whereas those with low polarity tended to decrease, and these differences were greater in *Thellungiella*. Determination of the global physicochemical properties of the metabolomes reinforced this observation. In both species, osmotic treatment led to relatively more soluble and polar metabolomes. Interestingly, other properties were also found to be very discriminatory, even though the physiological explanations for them are not known. The changes in molar mass, N to C ratio, carbon oxidation number and free energy of formation could be indicative of functional adjustments for maintenance of carbohydrate reserves, redox and energy balances, or nitrogen nutrition. Similar global changes relating to the N to C ratio in terms of amino acid levels and charge balance have been observed in response to potassium deficiency (Armengaud *et al.*, 2009), hence some of the effects observed here may result from altered potassium assimilation. To date, most studies of osmocompatibility have focused on particular metabolites or classes of metabolite, but our results suggest that full remodelling of metabolite pools should be considered.

### The *Thellungiella* metabolome may reflect slow growth

Increasing levels of organic compounds under osmotic stress are usually thought to affect growth because of the cost associated with their synthesis (Munns, 2002). More generally, it is accepted that there is antagonism between stress tolerance and productivity (Richards, 1996, Oliver *et al.*, 2000; Flowers, 2004). *Thellungiella* appears to follow this rule, but it is worth noting that its method of stress acclimation may be less expensive than a strategy of osmo-adjustment, because the osmotic balance that it maintains with the environment is due to a passive reduction in leaf water content rather than solute accumulation. A recent study of an Arabidopsis recombinant inbred line showed an interesting relationship between biomass and metabolic composition (Meyer *et al.*, 2007). Specifically, a large majority of the central metabolic pathway intermediates that provide the building blocks for growth were found to be negatively correlated with growth rate, while the levels of growth and stress regulators such as spermidine were positively correlated with growth rate. A model was proposed to explain these results, in which signal molecules control the growth rate, which in turn controls the size of major metabolite pools. The parallel with *Thellungiella* is striking. Polyamines participate in growth programming, and are also known to regulate gene expression and to interfere with stress acclimation mechanisms (Bouchereau *et al.*, 1999a; Hanzawa *et al.*, 2000; Cuevas *et al.*, 2008). In this study, we showed that the level of free spermidine decreases

when stress intensifies, and is negatively correlated with growth. Under standard conditions, Arabidopsis had a higher polyamine content than *Thellungiella*, especially with respect to spermidine, consistent with a global orientation towards fast growth. Conversely, *Thellungiella* exhibited lower polyamine levels and slower growth. It is tempting to infer that the high metabolite levels in *Thellungiella* are partly the result of a constitutively slow growth rate. In addition, slower growth fits well with the lower carbon assimilation seen when constitutively small stomatal apertures exert tight control over water fluxes (Inan *et al.*, 2004).

## EXPERIMENTAL PROCEDURES

### Plant material, growth conditions, treatments and sampling

Arabidopsis seeds, accession Col-0, were purchased from the Nottingham Arabidopsis Stock Centre (http://nasc.nott.ac.uk) and *Thellungiella* seeds, ecotype Shandong, were kindly supplied by Dr Ray Bressa (Center for Plant Environmental Stress Physiology, Purdue University, West Lafayette, IN; http://thellungiella.org/) (Bressan *et al.*, 2001). *Thellungiella* seeds were sown 2 weeks before those of Arabidopsis, in order to reach an equivalent shoot dry biomass at the time of treatment. All treatments were applied at Arabidopsis vegetative stage 1.12 (Boyes *et al.*, 2001). Salt and osmotic stress treatments consisted of addition of NaCl or polyethylene glycol 8000 (PEG8000), respectively, to the growth medium, in a hydroponic system under constant bubbling. Details of the growth conditions, treatments and sampling procedures are given in Table S5 for each of the three experiments reported. In brief, in the first experiment, the plants were treated with the same NaCl concentration (100 mM), in the second experiment, they were treated with various NaCl concentrations (0, 25, 50; 75, 120, 240, 360 or 480 mM), and in the third experiment, plants of each species were treated with NaCl or PEG8000 medium at an osmotic potential inducing 50% growth inhibition. PEG8000 concentrations were determined as described by Michel (1983) in order to exert osmotic potentials equivalent to those exerted by the NaCl concentration used in this experiment. After treatment, plant rosettes were harvested: at least three samples (biological replicates) were collected in the first and third experiments, and one sample in the second experiment. Each sample comprised at least three rosettes. Fresh material was immediately flash frozen and lyophilized at 12 Pa over 48 h. Dry-frozen plant material was ground in a mortar, then powdered using an MM200 oscillating ball mill (Retsch GmbH, http://www.retsch.com/) and stored at $-80°C$ before subsequent analysis.

### Water content and osmotic parameters

Shoot water status was estimated by weighing three rosettes immediately after harvest (FW) and after drying for 48 h at 60°C (DW). The water content (WC) was calculated as (FW − DW)/DW. Shoot growth was determined as the percentage fresh weight after the 9 day treatment period relative to the initial fresh weight: $FW_{day\ 9} \times 100/FW_{day\ 0}$. The total solute levels in shoots, expressed in osmol $g^{-1}$ DW, were estimated by measuring the osmolality of 100 μl polar extracts using a digital cryo-osmometer (Hermann Roebling, Messtechnik, Berlin), where the total solute level = osmolality of the extract × extraction volume/extracted DW. The osmotic potential (MPa) of shoot cells was then calculated according to the van't Hoff equation: $\psi = (-R \times T \times \text{total solute level})/WC$, where $T$ is the absolute temperature and $R$ is the gas constant. All $\psi$ values are expressed relative to that of Arabidopsis under standard

conditions (At-std). The osmotic potential measured for a given sample was then broken down into a term relating to total solutes (TS) and a term relating to water loss: $\psi_{sample} = \psi_{TS} + \psi_{water\ loss}$, where $\psi_{TS} = (-R \times T \times TS_{sample})/WC_{At-std}$ and $\psi_{water\ loss} = \psi_{sample} - \psi_{TS}$.

## Metabolomics

All chemical products were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com/). Detailed protocols are given in Table S6. The results are reported according to the requirements of the Metabolomics Standard Initiative (Fiehn *et al.*, 2007; Sumner *et al.*, 2007). Full datasets are also available in Tables S1, S3 and S4.

### Semi-quantitative analysis

^{1}H-NMR fingerprinting was performed as described by Gagneul *et al.* (2007). Main peaks were identified on the basis of chemical shifts and spin–spin coupling, in comparison with published spectra (Le Gall *et al.*, 2005; Hendrawati *et al.*, 2006; Verpoorte *et al.*, 2007).

GC-MS profiling analysis was performed essentially as described by Fiehn *et al.* (2000), and refined by Colebatch *et al.* (2004) and Desbrosses *et al.* (2005). Identification was performed by comparison of mass spectra against a public database (Kopka *et al.*, 2005). It was possible to detect and identify 59 polar metabolites. In addition, the mass spectra of 27 compounds matched well with those of known metabolites but differed in retention time, and are therefore labelled in parentheses with the name of the best matching compound from the library, followed by a number if several analytes matched the same compound. Another 17 analytes matched no known structure and are labelled $X_n$. All information on identification and quantification is provided in Table S1.

### Quantitative analysis

Carbohydrates and organic acids were extracted from 20 mg of homogenized freeze-dried powder derived from the shoot, and derivatized as described by Roessner *et al.* (2000). Gas chromatography-flame ionization detector (GC-FID) analysis was performed as described by Lugan *et al.* (2009), adapted from Adams *et al.* (1999). Amino acids were extracted from 20 mg of homogenized freeze-dried powder derived from the shoot, derivatized as described by Cohen and Michaud (1993), as modified by Bouchereau *et al.* (1999b). UPLC[TM]-DAD analysis was performed as described by Jubault *et al.* (2008). Free polyamines were extracted from 10 mg of homogenized freeze-dried powder derived from the shoot, as described by Bouchereau *et al.* (1999b), derivatized using dansyl chloride (Smith and Davies, 1985), and analyzed by liquid chromatography (adapted from the method described by Hayman *et al.*, 1985). A total of 48 metabolites were reliably identified by comparison of sample chromatograms with standard mixtures of known concentration, and then quantified after normalization against internal standards and plant material dry weight. The smallest quantity measured by gas chromatography was 0.011 $\mu$mol g$^{-1}$ DW (gentiobiose) and the highest was 489 $\mu$mol g$^{-1}$ DW (malate). For liquid chromatography, the smallest quantity measured was 0.017 $\mu$mol g$^{-1}$ DW (hydroxyproline), and the greatest was 462 $\mu$mol g$^{-1}$ DW (proline).

### Inorganic solutes

Nitrate, sulfate, phosphate, chloride, sodium and potassium were quantified as described by Lugan *et al.* (2009).

## Data mining

Statistical analysis was performed using Excel (Microsoft, http://www.microsoft.com/) and Statistica version 7.1 (Statsoft, http://www.statsoft.com/). Differences between samples were validated by the non-parametric Mann–Whitney *U* test. Signals below the detection limit were replaced by an arbitrary small value (0.001) for subsequent multivariate analysis by hierarchical cluster analysis (HCA) and principal component analysis (PCA). Correlations were established by calculating non-parametric Spearman coefficients.

## Calculation of the virtual physicochemical properties of the metabolome

The polarity of metabolites was evaluated on the basis of their octanol:water partition coefficient (logP) and their topological polar surface area (TPSA). These data were obtained from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/.). Values for water solubility (mg ml$^{-1}$), molar mass (g mol$^{-1}$) and nitrogen to carbon ratios (N to C) were obtained from The Merck Index (1996). Carbon oxidation numbers were calculated as described by Bentley *et al.* (2002). Gibbs free energies of formation ($\Delta_f G^{\circ\prime}$) were obtained from the Arabidopsis Information Resource (http://www.arabidopsis.org), completed by Amend and Helgeson (1997) and Alberty (2001). In a given sample, described by *i* metabolites, the value of the physicochemical parameter for a metabolite, $X_i$, was weighted by the relative abundance of this metabolite $A_i$ (percentage of the molecule relative to the sum of all molecules in the sample), and then the weighted average was calculated as $\Sigma(A_i X_i)$, to obtain the value of the parameter *X* for a virtual molecule representing the whole metabolome. To facilitate graphic plotting, values were expressed in relative units (see Table S7). Taken together, the virtual parameters characterized the averaged physicochemical properties of the metabolome in Arabidopsis and *Thellungiella* grown under various experimental conditions.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:
**Table S1.** Relative contents of metabolites detected by GC-MS in shoot polar extracts of Arabidopsis and *Thellungiella*.
**Table S2.** Coordinates and loadings of metabolites on PCs 1 and 3 of a principal component analysis.
**Table S3.** Absolute metabolite contents ($\mu$mol g$^{-1}$ DW) in Arabidopsis and *Thellungiella* shoots, after 9 days of treatment with various NaCl concentrations.
**Table S4.** Metabolites, inorganic solutes and water contents in shoots of Arabidopsis and *Thellungiella* plants grown for 9 days on an NaCl concentration inducing 50% growth inhibition or an osmotically equivalent PEG8000 solution.
**Table S5.** Growth conditions, treatments and sampling procedures.
**Table S6.** Detailed analytical procedures.

**Table S7.** Virtual physicochemical properties of the metabolome and *P* values for significant differences.

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