Characterization of Ion Contents and Metabolic Responses to Salt Stress of Different Arabidopsis AtHKT1;1 Genotypes and Their Parental Strains

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ABSTRACT Plants employ several strategies to maintain cellular ion homeostasis under salinity stress, including mediating ion fluxes by transmembrane transport proteins and adjusting osmotic pressure by accumulating osmolytes. The HKT (high-affinity potassium transporter) gene family comprises Na+ and Na+/K+ transporters in diverse plant species, with HKT1;1 as the only member in Arabidopsis thaliana. Cell-type-specific overexpression of AtHKT1;1 has been shown to prevent shoot Na+ overaccumulation under salinity stress. Here, we analyzed a broad range of metabolites and elements in shoots and roots of different AtHKT1;1 genotypes and their parental strains before and after salinity stress, revealing a reciprocal relationship of metabolite differences between an AtHKT1;1 knockout line (hkt1;1) and the AtHKT1;1 overexpressing lines (E2586 UASGAL4:HKT1;1 and J2731* UAS GAL4:HKT1;1). Although levels of root sugars were increased after salt stress in both AtHKT1;1 overexpressing lines, E2586 UASGAL4:HKT1;1 showed higher accumulation of the osmoprotectants trehalose, gentiobiose, and melibiose, whereas J2731* UASGAL4:HKT1;1 showed higher levels of sucrose and raffinose, compared with their parental lines, respectively. In contrast, the knockout line hkt1;1 showed strong increases in the levels of the tricarboxylic acid (TCA) cycle intermediates in the shoots after salt treatment. This coincided with a significant depletion of sugars, suggesting that there is an increased rate of carbon influx into the TCA cycle at a constant rate of C-efflux from the cycle, which might be needed to support plant survival during salt stress. Using correlation analysis, we identified associations between the Na+ content and several sugars, suggesting that regulation of sugar metabolism is important in plant responses to salinity stress.

Key words: abiotic stress; salinity; salt tolerance; HKT; sodium transporter, metabolomics; enhancer trap system.

INTRODUCTION

Increasing soil salinity is one of the main environmental constraints on crop productivity worldwide (Flowers, 1999; Zhu, 2001; Horie and Schroeder, 2004). Both osmotic stress and ion toxicity are imposed on plants as a response to salt stress (Munns and Tester, 2008) and can be described using the two-phase model introduced by Munns (2002, 2005). According to this model, the first so-called ‘osmotic phase’ affects the growth rates of shoots and roots immediately upon addition of salt, independently of the accumulation of Na+ in the shoot. It may be caused by a reduction in turgor resulting from a reduced water potential and water availability. The second ‘ionic phase’ begins when concentrations of ions (Na+ and/or Cl−) build up to toxic levels within the leaves, and leads to metabolic toxicity and an attenuated acquisition of nutrients (Grattan and Grieve, 1992).

Multiple strategies must be developed to make the plants more adaptable to salinity stress. For example, it is well known that Na+ toxicity of many crop plants is often inversely related to the extent of Na+ accumulation in the shoot (Tester and Davenport, 2003; Möller and Tester, 2007; Cotsaftis et al., 2011). One of the mechanisms a plant can use to reduce shoot Na+ accumulation is to limit the amount of Na+ that is transported from the roots to the shoots. Other common plant
strategies to maintain ion homeostasis within the cell under salinity stress include subcellular Na\(^+\) compartmentalization, Na\(^+\) tissue tolerance, and mediation of ion fluxes by transmembrane transporter proteins (Hasegawa et al., 2000; Tester and Davenport, 2003; Munns, 2005; Munns and Tester 2008).

In Arabidopsis thaliana, the SOS signaling pathway is a key regulator of ion homeostasis, and overexpression of several genes controlled by this pathway including SOS1, SOS3, and AtNHX1 has proved to be a valuable strategy to improve salt stress tolerance in transgenic plants (Zhang and Blumwald, 2001; Shi et al., 2003; Yang et al., 2009).

The HKT (high-affinity potassium (K\(^+\)) transporter) gene family has been a recent focus of research regarding plant salinity tolerance. It comprises Na\(^+\) and Na\(^+\)/K\(^+\) transporters of diverse plant species including rice (Horie et al., 2007), wheat (Davenport et al., 2005), and Arabidopsis (Rus et al., 2004; Sunarpi et al., 2005; Horie et al., 2006; Davenport et al., 2007). HKT1;1 is the only member of this gene family in Arabidopsis (Ouzumi et al., 2000; Platt et al., 2006) and is expressed mainly in root stelar cells (Mäser et al., 2002; Berthomieu et al., 2003; Rus et al., 2006) where it is hypothesized to act by removing Na\(^+\) from the transpiration stream (Sunarpi et al., 2005; Davenport et al., 2007).

Møller et al. (2009) employed a GAL4 UAS\(_{gale}\) enhancer trap expression system (Hasegoff, 1999) to overexpress HKT1;1 specifically in the root stele of Arabidopsis. As a result, two different enhancer trap lines have been developed, designated as E2586\(_{UAS}\_{gale}\)HKT1;1 (E2586 \(_{UAS}\_HKT1;1\)) and J2731\(_{UAS}\_HKT1;1\) (J2731\(_{UAS}\_HKT1;1\)). Each of them drives HKT1;1 gene expression in different cell types of the root. Line J2731\(_{UAS}\_HKT1;1\) overexpresses HKT1;1 specifically in the root pericycle and is in a C24 background, which has been shown to have a higher steady-state level of Na\(^+\) in shoots than in roots compared to Col-0 due to a higher root-to-shoot transfer of Na\(^+\) in C24 (Jha et al., 2010). Line E2586 \(_{UAS}\_HKT1;1\) overexpresses HKT1;1 specifically in root xylem parenchyma cells and is in a Col-0 background, which retains more Na\(^+\) in the root than in the shoot compared to the C24 strain under normal conditions.

Cell-type-specific overexpression of HKT1;1 in the lines E2586 UAS\(_{HKT1;1}\) and J2731* UAS\(_{HKT1;1}\) has been shown to both significantly decrease Na\(^+\) accumulation in the shoot by up to 64% and increase salinity tolerance (Møller et al., 2009). Plants that overexpressed HKT1;1 in a cell-type-specific manner were not affected by 100 mM NaCl, and showed no decrease in total biomass upon salinity treatment compared to the parental lines, which had a reduced biomass of up to 37%. By contrast, hkt1;1 loss-of-function mutants over-accumulate Na\(^+\) in the shoots and are more sensitive to salt than wild-type plants in saline conditions (Mäser et al., 2002; Berthomieu et al., 2003; Rus et al., 2004; Sunarpi et al., 2005). The hkt1;1 parental strain is the Col-0 trichome mutant gl1 (glabrous 1).

However, recent studies indicate that shoot Na\(^+\) accumulation and plant salinity tolerance may not be correlated in many Arabidopsis ecotypes, suggesting that other mechanisms may be responsible for the differing salinity tolerances (Jha et al., 2010). One possible strategy for the increased osmotic potential could be to increase the levels of osmotically active metabolites. Several studies have applied metabolite profiling to dissect salt stress tolerance response pathways in plants, including studies in barley (Widodo et al., 2009), grape vines (Cramer et al., 2007), tomatoes (Johnson et al., 2003), the halophyte Thellungiella (Gong et al., 2005; Lugan et al., 2010), and Arabidopsis cell cultures (Kim et al., 2007). These studies suggest a link between increased levels of osmolytes (e.g. proline inositol, glycinebetaine, sugars), induction of metabolic pathways (e.g. glycolysis, sucrose metabolism), and salinity tolerance.

Salinity is expected to change the content of elements as well as metabolite levels in a predictable and reproducible way, according to the amount of salt accumulation in the plant (Sanchez et al., 2008a). Essential elements for plant growth are macronutrients such as calcium (Ca), potassium (K), and phosphorus (P), and micronutrients such as boron (B), iron (Fe), and zinc (Zn) (Welch and Shuman, 1995). Some elements are essential components of plant molecules (e.g. amino acids and vitamins, which require sulfur), serve as enzyme cofactors (e.g. chlorophyll, which requires Mg\(^{2+}\)), and play a role in biosynthesis of other plant molecules (e.g. Mn\(^{2+}\), which is essential for building of chloroplasts). Only a few studies have combined metabolite data with data from elemental analysis (Sanchez et al., 2011), even though this approach has a high potential to reveal currently unknown interactions and interdependent relationships between genes, metabolites, and elements in plants.

Cell-specific overexpression of HKT1;1 has the potential to increase salinity tolerance in crop plants but, for this strategy to be useful under field conditions, it is important to understand the side effects of the genetic manipulation on both the primary metabolism and levels of important elements in the plant. To address this question, we build on the Møller et al. (2009) study using a metabolomics approach, and analyzed shoot and root samples of the different plant lines that were grown under the same environmental conditions (grown hydroponically for 5 weeks and then stressed with 100 mM NaCl for 5 d). Here, we describe the profiling of important plant nutrients Fe, Mn, B, Zn, Ca, Mg, Na, K, P, and S in shoots and roots of Col-0 wild-type Arabidopsis plants as well as in the selected transgenic lines E2586 (an enhancer trap line with GFP expression in the xylem parenchyma), E2586 \(_{UAS}\_HKT1;1\) (an E2586 enhancer trap line with AthKT1;1 overexpression in the xylem parenchyma), and hkt1;1 (a AthKT1;1 knockout mutant) before and after salinity stress. Additionally, the effect of salt on the primary metabolism of two different AthKT1;1 genotypes (both cell-type-specific overexpression and loss-of-function mutants) and their parental strains was investigated (Arabidopsis vegetative stage 1.12; Boyes et al., 2001) using gas chromatography–mass spectrometry (GC–MS). These data are then used to suggest sites of metabolic
regulation that can be attributed to long-term salinity stress using correlation analysis.

RESULTS

Shoot Elemental Changes of Different AtHKT1;1 Genotypes Compared to Their Parental Strains Before and After Salinity Stress

To validate the experimental system used in Møller et al. (2009) and Rus et al. (2004) for our metallomics studies, we repeated the biomass, Na⁺, K⁺, and Ca²⁺ measurements and extended this to a range of others. The application of 100 mM NaCl for 5 d induces a significant stress response in wild-type Arabidopsis without inducing major damage such as chlorosis or necrosis (data not shown). Biomass data of E2586 UAS:HKT, I2731* UAS:HKT and hkt1;1 plants has been previously published by Møller et al. (2009) and Rus et al. (2004) for hkt1;1 plants.

We measured a range of ions (and metabolites—see below) in shoots and roots before and after salinity treatment of Col-0, E2586, E2586 UAS:HKT, and hkt1;1 to investigate the effect of the UAS₆₃₈₄::HKT1;1 transgene and its knockout hkt1;1. Levels of Fe, Mn, B, Zn, Ca, Mg, Na, K, P, and S in shoots and roots of control and salt-treated Arabidopsis plants were determined and quantified using ICP–OES (see Table 1 (shoots) and Table 2 (roots)). As seen from Table 1, hkt1;1 knockout had elevated Na⁺ concentrations in the shoots prior to salt treatment compared to the other control lines. Salt-treated Col-0 and E2586 accumulated 50-fold and 44-fold more Na⁺ in the shoots after salt treatment, respectively. Furthermore, exposure of all plants to 100 mM NaCl for 5 d resulted in significant increases in shoot Na⁺ in all AtHKT1;1 genotypes compared to their parental lines (Table 1). hkt1;1 knockout plants accumulated the highest concentration of shoot Na⁺—almost a 40-fold increase compared to control conditions. Additionally, hkt1;1 knockout plants had significantly lower levels of Ca²⁺ and K⁺, and significantly higher levels of B and S compared to their control line in shoots. E2586 UAS:HKT plants accumulated significantly less Na⁺ and B, but more K⁺ than its parental line E2586 in the shoots. Since the measurement of B was close to the detection limit level, this result should be interpreted carefully and is most likely to be negligible.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Col-0 (C)</th>
<th>hkt1;1 (T)</th>
<th>E2586</th>
<th>E UAS:HKT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>7.7 ± 0.4</td>
<td>10.3 ± 1.1</td>
<td>8.2 ± 0.5</td>
<td>11 ± 0.4</td>
</tr>
<tr>
<td>Mn</td>
<td>20.7 ± 0.9</td>
<td>16.8 ± 0.9</td>
<td>23 ± 0.3</td>
<td>16 ± 1.3</td>
</tr>
<tr>
<td>B</td>
<td>5.9 ± 0.3</td>
<td>7.1 ± 0.3</td>
<td>6.3 ± 0.2</td>
<td>9 ± 0.8</td>
</tr>
<tr>
<td>Zn</td>
<td>5.9 ± 0.2</td>
<td>7.7 ± 0.4</td>
<td>6.4 ± 0.2</td>
<td>7 ± 0.4</td>
</tr>
<tr>
<td>Ca</td>
<td>3642.9 ± 119.2</td>
<td>3414.3 ± 159.5</td>
<td>4000 ± 114</td>
<td>2840 ± 186</td>
</tr>
<tr>
<td>Mg</td>
<td>917.1 ± 26</td>
<td>771.4 ± 34</td>
<td>986 ± 26.6</td>
<td>738 ± 46.9</td>
</tr>
<tr>
<td>Na</td>
<td>77.5 ± 5.6</td>
<td>3885.7 ± 771.3</td>
<td>211 ± 13.8</td>
<td>8380 ± 297.3</td>
</tr>
<tr>
<td>K</td>
<td>4185.7 ± 131.7</td>
<td>3257.1 ± 215.9</td>
<td>4220 ± 102</td>
<td>2396 ± 220</td>
</tr>
<tr>
<td>P</td>
<td>871.4 ± 26.9</td>
<td>1032.9 ± 38.2</td>
<td>876 ± 24.2</td>
<td>1126 ± 79.6</td>
</tr>
<tr>
<td>S</td>
<td>951.4 ± 16.5</td>
<td>887.1 ± 23.1</td>
<td>966 ± 34</td>
<td>1218 ± 90.6</td>
</tr>
</tbody>
</table>

Data point represent mean ± SEM, n = 7 (Col-0), n = 5 (hkt1;1, E2586, and E2586 UAS:HKT1;1). The content in mg kg⁻¹ DW of 10 elements was measured in shoot tissue from 5-week-old plants using ICP–OES. The data were log transformed. Values that were found to be significantly different using the described Student’s t-test with P < 0.05 are indicated by shading. DW, dry weight.

Root Element Changes of Different AtHKT1;1 Genotypes Compared to Their Parental Strains Before and After Salinity Stress

In the roots, E2586 UAS:HKT plants accumulated more Na⁺ than its parental line E2586, and hkt1;1 plants accumulated more sulfur than its control line Col-0 (Table 2). No other significant differences were observed between the lines grown under control conditions. After 5 d of salt exposure, the roots (Table 2) of hkt1;1 plants accumulated only 11-fold more Na⁺ compared to the non-treated plants, and showed overall the lowest amount of root Na⁺ among all investigated lines after salt treatment. Additionally, hkt1;1 plants accumulated significantly less Ca²⁺, but more K⁺, Mn²⁺, and S than the control line after salt treatment. However, as was the case for the B measurements, the measurement of Mn²⁺ was close to the detection limit level, this result should be interpreted carefully. Roots of salt-treated Col-0 and E2586 accumulated much less Na⁺ compared to the shoots: 35-fold and 19-fold above the Na⁺-level under control conditions, respectively. Stelar-specific overexpression of HKT1;1 in E2586 showed an almost 34-fold increase in shoot Na⁺, but only a 14-fold increase in root Na⁺ after salt treatment.

In summary, salt-treated hkt1;1 plants accumulated up to 2.5 times more shoot Na⁺ than similarly treated plants from the Col-0 and E2586 lines, respectively (Table 1). Compared to the stelar-specific overexpression of HKT1;1 in E2586 after salt exposure, hkt1;1 knockout lines accumulated more than threefold higher Na⁺ levels in the shoots, but accumulated
only half of the amount of Na+ in the roots. This is consistent with previous findings (Moller et al., 2009).

These Arabidopsis mutants and wild-type plants also showed an inverse relationship between the Ca2+ content and the Na+ content in shoots, whereas Ca2+ levels show only minor increases in the roots of all lines (Tables 1 and 2).

The Enhanced AtHKT1;1 Transporter-Mediated Na+ Exclusion from the Xylem of the Arabidopsis Mutant E UAS Maintains a High K+/Na+ Ratio in the Shoots during Salinity Stress

Maintaining low Na+ and high K+ levels in the shoots during salt stress is known to stabilize the high cytosolic K+/Na+ ratio, particularly in the leaves, and has been found to be positively correlated with salt tolerance (Apse et al., 1999; Serrano and Rodríguez-Navarro, 2001; Shi et al., 2002; Ren et al., 2005). In shoots of hkt1;1 mutants, K+ accumulation was reduced and Na+ accumulation was greatly increased, suggesting that K+ transport may be indirectly affected (see also Sunarpi et al., 2005 (Table 1)). A similar effect was observed in the roots of hkt1;1 mutants (Table 2). This can be seen in the other Arabidopsis mutants (E2586 and E2586 UAS:HKT) and wild-type plants (Col-0), which show an inverse relationship on the K+ content compared to the Na+ content in shoots and roots: K+ levels in salt-treated Col-0, hkt1;1, and E2586 shoots were 0.8, 0.6, and 0.9 times lower than those of control plants, respectively (Table 1), whereas only E2586 UAS:HKT shoot K+ levels remained unchanged after salt treatment. K+ levels in salt-treated Col-0, hkt1;1, E2586, and E2586 UAS:HKT roots were 0.5, 0.9, 0.4, and 0.9 times lower than those of control plants, respectively (Table 2).

The increase in Na+ and depletion of K+ in the salt-treated plants dramatically lowered the shoot K+/Na+ ratio (Table 3). hkt1;1 plants had the lowest shoot K+/Na+ ratio (0.28 ± 0.04) and the highest root K+/Na+ ratio (5.51 ± 0.69) after salt stress. In contrast, E2586 UAS:HKT plants had the highest shoot K+/Na+ ratio (1.66 ± 0.26), but the second-lowest root K+/Na+ ratio (1.28 ± 0.16) after salt stress. While most changes in the root K+ and Na+ levels were similar to those seen in shoots, the absolute amounts of K+ and Na+ in roots were generally lower than those in shoots (Table 1 and 2).

Comparisons of the Metabolic Profiles of Shoots of Wild-Type and Transgenic Arabidopsis Plants Before and After Salt Stress

Following the ICP–OES analysis, a GC–MS-based strategy was applied to identify the specific changes in the metabolic

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**Table 2.** The Elemental Composition of the Roots of UAS::AtHKT1;1 Plants Before (C) and After Salt Treatment (T) with 100 mM NaCl.

<table>
<thead>
<tr>
<th>Element</th>
<th>Col-0 C</th>
<th>T</th>
<th>hkt1;1 C</th>
<th>T</th>
<th>E2586 C</th>
<th>T</th>
<th>E UAS:HKT C</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>301 ± 43.3</td>
<td>460 ± 63.1</td>
<td>292 ± 7.65</td>
<td>408 ± 29.7</td>
<td>256 ± 31.2</td>
<td>390 ± 63.4</td>
<td>208 ± 8.5</td>
<td>404 ± 30.4</td>
</tr>
<tr>
<td>Mn</td>
<td>45 ± 8.3</td>
<td>4 ± 0.3</td>
<td>54 ± 3.9</td>
<td>8 ± 0.9</td>
<td>46 ± 7</td>
<td>5 ± 0.5</td>
<td>49 ± 3.3</td>
<td>6 ± 1.7</td>
</tr>
<tr>
<td>B</td>
<td>4 ± 1.1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Zn</td>
<td>8 ± 0.4</td>
<td>9 ± 1.9</td>
<td>8 ± 1.4</td>
<td>8 ± 0.4</td>
<td>8 ± 0.7</td>
<td>7 ± 1.1</td>
<td>7 ± 0.3</td>
<td>10 ± 0.9</td>
</tr>
<tr>
<td>Ca</td>
<td>299 ± 11.2</td>
<td>370 ± 18.3</td>
<td>308 ± 4.7</td>
<td>346 ± 5.1</td>
<td>296 ± 9.3</td>
<td>354 ± 53.3</td>
<td>290 ± 8.4</td>
<td>372 ± 24.8</td>
</tr>
<tr>
<td>Mg</td>
<td>115 ± 2.5</td>
<td>92 ± 4.8</td>
<td>125 ± 3.3</td>
<td>93 ± 2.1</td>
<td>119 ± 4.4</td>
<td>78 ± 11.5</td>
<td>114 ± 2.4</td>
<td>88 ± 6.4</td>
</tr>
<tr>
<td>Na</td>
<td>39 ± 2.2</td>
<td>1381 ± 95.6</td>
<td>50 ± 3.3</td>
<td>574 ± 39.8</td>
<td>52 ± 3.4</td>
<td>1030 ± 149.8</td>
<td>83 ± 6.6</td>
<td>1152 ± 62.2</td>
</tr>
<tr>
<td>K</td>
<td>3200 ± 141.4</td>
<td>1557 ± 95.6</td>
<td>3420 ± 12.2</td>
<td>3120 ± 97</td>
<td>3260 ± 112.2</td>
<td>1388 ± 206.7</td>
<td>3280 ± 128.1</td>
<td>1480 ± 74</td>
</tr>
<tr>
<td>P</td>
<td>760 ± 19.1</td>
<td>719 ± 50.6</td>
<td>840 ± 9.5</td>
<td>682 ± 19.6</td>
<td>794 ± 43</td>
<td>614 ± 79.7</td>
<td>742 ± 11.6</td>
<td>656 ± 42.8</td>
</tr>
<tr>
<td>S</td>
<td>1016 ± 20.5</td>
<td>756 ± 37.3</td>
<td>1146 ± 7.2</td>
<td>890 ± 25.9</td>
<td>1082 ± 30.1</td>
<td>702 ± 94.8</td>
<td>1082 ± 34.4</td>
<td>768 ± 48.4</td>
</tr>
</tbody>
</table>

Data points represent mean ± SEM, n = 7 (Col-0), n = 5 (hkt1;1, E2586, and E2586 UAS:HKT;1). The content is expressed in mg kg⁻¹ DW of 10 elements measured in root tissue from 5-week-old plants using ICP–OES. The data were log transformed. Values that were found to be significantly different using the described Student’s t-test with P < 0.05 are indicated by shading. DW, dry weight.

**Table 3.** K+/Na+ Ratios of the Shoots and Roots in mg kg DW⁻¹ under Control (C) and Salt-Treated (T) Conditions in the Mutant and Parental Lines as Determined by ICP–OES.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Col-0</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot</td>
<td>C</td>
<td>55.51 ± 10.19</td>
<td>T</td>
<td>0.99 ± 0.43</td>
<td>Root</td>
<td>C</td>
<td>82.56 ± 9.7</td>
<td>T</td>
</tr>
<tr>
<td>hkt1;1</td>
<td>C</td>
<td>20.25 ± 2.21</td>
<td>T</td>
<td>0.28 ± 0.04</td>
<td>Root</td>
<td>C</td>
<td>71.11 ± 15.51</td>
<td>T</td>
</tr>
<tr>
<td>E2586</td>
<td>C</td>
<td>57.57 ± 10.51</td>
<td>T</td>
<td>1.18 ± 0.17</td>
<td>Root</td>
<td>C</td>
<td>62.99 ± 4.89</td>
<td>T</td>
</tr>
<tr>
<td>E UAS:HKT</td>
<td>Shoot</td>
<td>C</td>
<td>56.18 ± 13.67</td>
<td>T</td>
<td>1.66 ± 0.26</td>
<td>Root</td>
<td>C</td>
<td>39.52 ± 6.26</td>
</tr>
</tbody>
</table>
responses in the Arabidopsis lines following treatment with 100 mM NaCl for 5 d. The objective of this study was to understand how salt imposition affects the shoot and root metabolite profile of the different mutant lines compared to their parental (control) lines. Therefore, pair-wise comparisons between the metabolite profiles of E2586 UAS:HKT1;1 and its empty vector control E2586 (Figures 1 and 2), J2731 UAS:HKT1;1 and its empty vector control J2731* (Figures 1 and 2), and hkt1;1 and its parental strain gl1 (Figures 3 and 4) were performed (see also Supplemental Tables 1 and 2). For completeness, Supplemental Tables 3 and 4 show pair-wise comparisons between the metabolite profiles of samples of the same line under control and treated conditions.

In total, 64 shoot metabolites, of which 58 (91%) could be identified, and 76 root metabolites, of which 61 (80%) could be identified, formed the basis of the comparative metabolite profiling. The identified metabolites were categorized into amino acids, amines, organic acids, sugars, sugar alcohols, sugar acids, organic compounds, fatty acids, phosphorylated compounds, and unknowns to facilitate the biological interpretation of the data. Profiles of the metabolites in Arabidopsis roots identified a similar range of compounds to those in shoots, but with a lower number of identified sugars, sugar acids, and sugar alcohols and a higher number of phosphorylated compounds, organic acids, and fatty acids. Unless otherwise stated, only metabolite changes which are considered as statistically significant (Student’s t-test p-value <0.05) will be discussed.

Pair-Wise Comparisons of the Metabolite Concentrations of Arabidopsis Lines E2586 and E2586 UAS:HKT1;1 Grown under Control (3 μM NaCl) and Salt-Treated (100 mM NaCl) Conditions

Initially, the shoot and root metabolite profiles of E2586 and E2586 UAS:HKT1;1 were compared (Figures 1 and 2). Under control conditions, E2586 UAS:HKT1;1 shoots had marginally lower levels of several metabolites, particularly amino acids, sugar acids, and organic acids compared to its parental line E2586. Although γ-aminobutyrate (GABA) was present at a ninefold higher level due to the large variation across the biological replicates, it is considered as not statistically significant. Sugar acids, including galactonate, gluconate, and ribonate, as well as the organic acid saccharate, were higher in shoots of E2586 than in the more salt-tolerant E2586 UAS:HKT1;1. All significant differences (except of unknown compound 1) were a result of marginally higher metabolite levels of between 1.3 and 2.4-fold.

More differences in metabolite levels were noted in E2586 UAS:HKT1;1 roots compared to E2586 roots under control conditions, with Val, galactonate, and urea showing small but significant increases of between 1.4 and 2.4-fold, and beta-alanine, fructose, aconitate, and unknown compounds 4 and 7 showing decreases of between –1.3 and –9.9-fold.

After 5 d in 100 mM NaCl, only a few (six) shoot metabolites significantly changed their concentrations in E2586 UAS:HKT1;1 compared to the empty vector control E2586: two sugars (fructose and galactose), two sugar alcohols (erythritol and xylitol), sinapinate, and urea. All significant changes were a result of a decrease in metabolite concentration of between –1.3 and –7.3-fold, except for galactose, which increased marginally. By contrast, more than 50% of the measured root metabolites (39 out of 76) changed after salt treatment (Figure 2). Notably, the magnitude of these changes was also generally larger, with 20 out of 39 changes greater than either –2-fold or +2-fold.
Figure 2. Logarithmic Ratios of Metabolite Contents in Roots of EUAS compared to E2586 (Columns 2 and 3, Respectively) and J2731* and J2731* UAS:HKT1;1 (Columns 4 and 5, Respectively) Before (Control) and After Treatment with 100 mM NaCl (NaCl).

Values that are significantly (P < 0.05) higher are indicated with one asterisk. The threshold of a ±2-fold change is indicated as a dashed line.
In E2586 *UAS:HKT1;1* roots, the amino acids Thr, β-alanine, 5-oxoPro, Glu, Asn, and Gin increased at 1.9–3.2-fold higher levels, whereas Ile and Pro decreased at ~1.2 to ~2.1-fold lower levels. Further, most sugars, sugar acids, and sugar alcohols showed increased metabolite levels, including sucrose, trehalose, gentiobiose, melibiose, xylitol, galactitol, galactonate, and threonate. By contrast, organic acid levels were depleted, including succinate, citrate, aconitate, fumarate, and malate. This suggests that either a down-regulation of the tricarboxylic acid (TCA) cycle has occurred after salt treatment or these metabolites have been increasingly used as precursors for amino acid synthesis. The organic acids glycerate, propanoate, and butyrate showed decreased levels of between ~1.7 and ~2.5-fold in E2586 *UAS:HKT1;1* after salt treatment compared to E2586 roots. Moreover, these changes were accompanied by increases in several phosphorylated compounds including glycerol-3-phosphate, a glycerophospholipid component; myo-inositol-1-phosphate, a second messenger that is known to play a role in cell signaling; and 3-phosphoglyceric acid (3-PGA), a metabolic intermediate of the Calvin cycle and glycolysis. Further, 13 out of the 16 measured unknown metabolites showed slightly increased metabolite concentrations of up to 3.4-fold.

**Pair-Wise Comparisons of the Metabolite Concentrations of *Arabidopsis* Lines J2731* and J2731* UAS:HKT1;1 Grown under Control (3 μM NaCl) and Salt-Treated (100 mM NaCl) Conditions**

A different set of metabolic responses was observed in the pericycle-specific expression of HKT1;1 in J2731* UAS:HKT1;1 plants compared to the parental line J2731* (Figures 1 and 2). Under control conditions, only a few differences in the shoot and root metabolite concentrations are detected, but these differences are generally of larger magnitude than described previously in E2586 compared to E2586 *UAS:HKT1;1* plants. Amino acids such as Pro in the shoots and homoSer in the roots, as well as organic acids including oxalate in the shoots, and aconitate and saccharate in the roots, were present at lower levels in the more salt-tolerant J2731* UAS:HKT1;1 plants than in J2731*. In contrast, Gly, melibiose, fructose, shikimate, and sinapinate were present at higher levels in the shoots of J2731* UAS:HKT1;1 than in J2731* plants (Figure 1).

Comparisons of metabolite responses of shoots between salt-treated plants and their respective controls showed reductions, particularly in the concentrations of amino acids. Amino acids present at lower levels in J2731* UAS:HKT1;1 than in J2731* were Gly, Ile, Tyr, Val, Lys, Asn, and Glu, whereas only Asp increased marginally. Galactose, glucose, and gluconate were the only sugars or sugar acids that showed an increase, whereas glycerate, glycerol, glycerol-3-phosphate, and urea decreased. In summary, most metabolite changes in the shoots resulted in significant increases compared to the parental line.

Strikingly, the opposite trend was observed when comparing the concentration of root metabolites after salt stress (Figure 2). Similarly to the results described previously for E2586 compared to E2586 *UAS:HKT1;1* plants, more than 50% of the measured root metabolites (42 out of 76) changed significantly in J2731* UAS:HKT1;1 compared to the parental line after salt treatment, with many increases in amino acid levels. These increases are of a similar magnitude to the decreases of amino acids observed in the shoots of J2731* UAS:HKT1;1 under salt stress. This includes changes in almost all the amino acid levels (Ala, Val, Ile, Gly, Ser, Thr, β-Ala, homoSer, Asp, 5-oxoPro, Phe, Asn, Gin, and Lys) of up to 4.8-fold higher levels in J2731* than in J2731* UAS:HKT1;1. Notably, Pro and Tyr are the only amino acids that decreased. These strong increases of most amino acids were accompanied by a depletion of glucose and all measured sugar alcohols (xylitol, myo-inositol, pinitol, galactitol). Galactinol showed the biggest change, with a ~26 3-fold decrease in J2731* UAS:HKT1;1 after salt treatment compared to J2731* roots. Furthermore, sucrose and raffinose, gluconate, as well as several organic acids (glycerate, saccharate, and citrate, but not fumarate) and urea and putrescine increased up to 6.7-fold. Only a few other metabolites exhibited decreases, with the strongest being guanine (~4.9-fold).

**Pair-Wise Comparisons of the Metabolite Concentrations of *Arabidopsis* Lines *gl1* and *hkt1;1* Grown under Control (3 μM NaCl) and Salt-Treated (100 mM NaCl) Conditions**

Compared to its parental line *gl1*, *hkt1;1* shoots had lower levels of some amino acids under control conditions including Ile, Leu, Glu, and Tyr, as well as slightly higher levels of Asp, fumarate, and raffinose (Figure 3). Similarly, metabolite comparisons between *gl1* and *hkt1;1* roots showed that, in control conditions, there were only minor differences between the two genotypes (Figure 4); *hkt1;1* accumulated fewer sugars (sucrose, melibiose), amino acids (β-Ala, GABA), and organic acids (glutarate, aconitate, oxalate) than *gl1*.

Far more changes were seen in roots and shoots following salt treatment of *gl1* and *hkt1;1* compared to the AtHKT1;1 overexpression lines (Figures 3 and 4). In contrast to the results of both overexpression lines, which showed more changes in the root than in the shoot metabolites after salt treatment (Figures 1 and 2), there are similar numbers of metabolite changes in both *hkt1;1* shoots and roots (Figures 3 and 4). In *hkt1;1* shoots, levels of most amino acids and several organic acids and sugar acids were higher, and levels of sugars and sugar alcohols were lower than in *gl1* plants (Figure 3). Amino acids that increased by more than twofold included β-Ala, Tyr, and Asn, and amino acids that increased up to twofold included Ala, GABA, homoSer, Ser, and Lys. Organic acids such as sinapinate and several TCA cycle intermediates (isocitrate, malate, and succinate) showed higher changes of up to 6.1-fold.

By contrast, root levels of several amino acids, organic acids, sugars, and sugar alcohols were lower in *hkt1;1* than in *gl1* after salt treatment (Figure 4). Metabolite changes include Asp, GABA, and Phe, but none of them changed more
Figure 3. Logarithmic Ratios of Metabolite Contents in Shoots of hkt1;1 compared to gl1 Before (Control; Column 2) and After (NaCl; Column 3) Treatment with 100 mM NaCl. Values that are significantly ($P < 0.05$) higher are indicated with one asterisk. The threshold of a ±2-fold change is indicated by a dashed line.
than –2-fold. In contrast, Thr and β-Ala increased marginally, but also not more than twofold. Ethanolamine, the sugars fructose and sucrose, the TCA cycle intermediates aconitate, fumarate, and oxalate, as well as the sugar alcohols myo-inositol and galactinol decreased slightly. With the exception of one unknown compound (U7, +1.4-fold), only xylitol and urea increased slightly in hkt1;1 compared to gl1 roots after salt stress.

Integration of Metabolic and Elemental Traits using Correlation Analysis

To study the effects of Na⁺ accumulation upon salinity exposure on the metabolism and to determine possible metabolic regulation sites, we compared the metabolites of shoots and roots of selected salt-treated lines with the measured elemental traits by correlating Na⁺ concentration with the concentration of metabolites and the other elements (Figures 5 and 6, and Tables 4–6).

Na⁺ has strong significant negative correlations with Ca²⁺ and K⁺ and a weak positive correlation with B and Fe in shoots of salt-treated plants (see Figure 5 and Table 5). Salinity treatment led to increases in the levels of most of the amino acids, particularly derived from Asp, pyruvate, Glu, or Ser. These metabolites, including Thr, beta-alanine, Ser, ethanolamine, Ile, Ala, Tyr, and GABA, were highly positively correlated with Na⁺ (Figure 5 and Table 4). There is further a strong negative correlation between Na⁺ and several sugars and sugar alcohols including xylose, galactose, and erythritol. The levels of citrate and isocitrate increased slightly but not significantly in hkt1;1 when compared to gl1, whereas the levels of metabolites further downstream in the TCA cycle such as succinate and malate (but not fumarate) increased significantly in hkt1;1. Strong positive correlations were observed between these TCA cycle intermediates and Na⁺.

In roots, after salinity treatment, fewer correlations of ions and metabolites with Na⁺ were observed than in shoots (Figure 6 and Table 6). No significant correlations between Na⁺ and any of the ions were detected. Urea was the only metabolite that showed a significant negative correlation with Na⁺ (–0.65) (Figure 6 and Table 6). Significant positive correlations were detected between Na⁺ and gluconate (0.66) fructose, ribonate, saccharate, and glycerate. With the exception of glycerate, these sugar metabolites show the opposite trend when looking at correlations between Na⁺ in the shoots and roots of these three lines. Thus, in shoots, fructose, ribonate, and saccharate show negative correlations with Na⁺ (although not statistically significant) whereas, in roots, they are positively correlated with Na⁺.

DISCUSSION

We used metabolite and elemental profiling of both transgenic and wild-type Arabidopsis lines to examine the impact of salinity tolerance on their primary metabolism and mineral ion content. The transgenic lines were altered in the expression of the HKT transporter, AthHKT1;1, which is located in the plasma membrane of xylem parenchyma cells in roots and mediates the removal of Na⁺ from xylem vessels into xylem parenchyma cells to reduce shoot Na⁺ accumulation.
Figure 5. Combined Element and Metabolite Profile of Shoot Tissue of Selected Lines. Metabolites (GC–MS) and elements (ICP–OES) of the shoots of selected Arabidopsis lines after treatment with 100 mM NaCl are mapped on the central metabolism using VANTED (Junker et al., 2006). Metabolites shown in the bottom left frame could not be mapped onto this metabolic pathway. The bars represent the following lines: E2586, red; E UAS:HKT, blue; hkt1-1, black (n = 4–7). Metabolites (using metabolite response ratios) and elements (using absolute elemental concentrations) showing significant Spearman’s rank correlation with Na⁺ concentration as determined by an unpaired t-test (P < 0.05) are marked with a bold frame. Negative correlations to Na⁺ (yellow) are visualized with red backgrounds, whereas positive correlations are with blue background. The intensities of the colors represent the intensity of the correlation.
Table 4. Shoot Metabolites with Significant Spearman Correlation to Na+ after Salt Treatment for 5 d.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$p$</th>
<th>$p$-value</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA</td>
<td>0.82</td>
<td>&lt;0.001</td>
<td>14</td>
</tr>
<tr>
<td>Ile</td>
<td>0.79</td>
<td>&lt;0.001</td>
<td>14</td>
</tr>
<tr>
<td>Erythritol</td>
<td>0.73</td>
<td>0.003</td>
<td>14</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.73</td>
<td>0.003</td>
<td>14</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>0.72</td>
<td>0.004</td>
<td>14</td>
</tr>
<tr>
<td>Ala</td>
<td>0.67</td>
<td>0.009</td>
<td>14</td>
</tr>
<tr>
<td>Urea</td>
<td>0.66</td>
<td>0.01</td>
<td>14</td>
</tr>
<tr>
<td>Ser</td>
<td>0.6</td>
<td>0.02</td>
<td>14</td>
</tr>
<tr>
<td>Thr</td>
<td>0.59</td>
<td>0.03</td>
<td>14</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.59</td>
<td>0.03</td>
<td>14</td>
</tr>
<tr>
<td>beta-Alanine</td>
<td>0.55</td>
<td>0.04</td>
<td>14</td>
</tr>
<tr>
<td>Malate</td>
<td>0.54</td>
<td>0.05</td>
<td>14</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>0.54</td>
<td>0.05</td>
<td>14</td>
</tr>
<tr>
<td>Guanine</td>
<td>0.54</td>
<td>0.05</td>
<td>14</td>
</tr>
<tr>
<td>Galactose</td>
<td>−0.9</td>
<td>&lt;0.0001</td>
<td>14</td>
</tr>
<tr>
<td>Xylose</td>
<td>−0.69</td>
<td>0.006</td>
<td>14</td>
</tr>
<tr>
<td>Oxalate</td>
<td>−0.67</td>
<td>&lt;0.01</td>
<td>14</td>
</tr>
</tbody>
</table>

$p$, Spearman’s rank correlation coefficient; $p$, probability approximated to t-distribution; $n$, independent biological samples.

Table 5. Shoot Ions with Significant Spearman Correlation to Na+ after Salt Treatment for 100 mM NaCl for 5 d.

<table>
<thead>
<tr>
<th>Ion</th>
<th>$p$</th>
<th>$p$-value</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boron</td>
<td>0.68</td>
<td>0.007</td>
<td>14</td>
</tr>
<tr>
<td>Iron</td>
<td>0.56</td>
<td>0.04</td>
<td>14</td>
</tr>
<tr>
<td>Calcium</td>
<td>−0.57</td>
<td>0.03</td>
<td>14</td>
</tr>
<tr>
<td>Potassium</td>
<td>−0.93</td>
<td>&lt;0.0001</td>
<td>14</td>
</tr>
</tbody>
</table>

Other details are the same as in the legend to Table 4.

These results were expected, since the loss-of-function of AtHKT1;1 prevents the plant retrieving Na+ from the xylem vessels of the root. In contrast, prior to salt treatment, E2586 UAS:AtHKT1;1 plant roots had elevated Na+ concentrations—a result of the overexpression of AtHKT1;1, which enhances Na+ retrieval from the xylem vessels and shows a small effect even under low-salt conditions compared to its parental line E2586 (Table 2). Consequently, we also found decreased Na+ levels in the shoots of E2586 UAS:AtHKT1;1 plants (Table 1). This is consistent with the results from Möller et al. (2009), who found that E2586 UAS:AtHKT1;1 had a decreased shoot Na+ accumulation by up to 47%.

The activity of the sulfur assimilation pathway is highly regulated and responds dynamically to environmental changes, depending on the actual need for reduced sulfur (Leustek et al., 2000). Sulfur is used to synthesize Cys and Met, critical components of proteins, glutathione, phytochelatins, as well as sulfur-containing defense compounds, and numerous essential and secondary metabolites derived from these amino acids (Rausch and Wachter, 2005). Therefore, the higher accumulation of $S$ in shoots after salt treatment as determined particularly in hkt1;1 knockout plants, but also to a lesser degree in the AtHKT1;1 overexpressing lines, is possibly caused by an increase in the activity of the sulfur assimilation pathway (Table 2).

The increased accumulation in shoots of Na+ and $S$ in hkt1;1 knockout plants during salt treatment was accompanied by a significant depletion of Ca2+ and K+ compared to control conditions (Table 1). By contrast, only K+ shows a decrease after salt treatment in roots (Table 2). Although some members of the HKT family of other plant species were found to have an affinity for both Na+ and K+ (Horie et al., 2008), Möller et al. (2009) noted that the strong reciprocal relationship between Na+ and K+ shoot concentrations rules this option out for Arabidopsis HKT1;1. The importance of maintaining adequate K+ concentrations and K+/Na+ ratios in the cell to guarantee normal cellular function under saline conditions has long been recognized. Calcium is known to be important for maintaining adequate K+ transport, and to play an important role in signaling in response to osmotic and ionic stress (Epstein, 1961; Bartels and Sunkar, 2005; Hepler, 2005; Franz et al., 2011). Cramer et al. (1987) found that high Na+ concentrations,...
Figure 6. Combined Element and Metabolite Profile of Root Tissue of Selected Lines. Metabolites (GC–MS) and elements (ICP–OES) of the roots of Arabidopsis after treatment with 100 mM NaCl are mapped on the central metabolism using VANTED (Junker et al., 2006). Other details are the same as in the legend to Figure 5.
concentrations disrupt K\(^+\) and Ca\(^{2+}\) transport and interfere with growth and development of cotton (Gossypium hirsutum L.). Additionally, they demonstrated that Ca\(^{2+}\) enhances K\(^+\) uptake while decreasing the Na\(^+\) uptake, which leads to a higher K\(^+\)/Na\(^+\) ratio in plants and to an enhanced Na\(^+\) tolerance. The levels of Ca\(^{2+}\) in E2586 UAS:HKT1;1 in response to salt remained unchanged, which is most likely due to lower shoot Na\(^+\) concentrations (Tables 1 and 2). Compared to its parent E2586, E2586 UAS:HKT1;1 plants had elevated levels of K\(^+\) in the shoots after salt treatment, resulting in the highest shoot K\(^+\)/Na\(^+\) ratio among the investigated lines (Table 3). By contrast, the increase in Na\(^+\) and the depletion of K\(^+\) in the salt-sensitive hkt1;1 plants led to a dramatically lowered shoot K\(^+\)/Na\(^+\) ratio. Therefore, the disruption of both K\(^+\) and Ca\(^{2+}\) transport in hkt1;1 knockout plants may further indicate an important role of HKT1;1 not only in Na\(^+\) retrieval in xylem vessels of the roots, but also in maintaining ion homeostasis in shoots under salt stress conditions. Ion transporters may not only be targets, but rather integral components acting as sensors of signaling pathways to trigger plant responses which are designed to minimize the deleterious effects of environmental stresses (Williams and Salt, 2009).

A Global Perspective of Metabolite Changes After Salt Treatment Reveals a Reciprocal Relationship of Metabolite Differences between the AtHKT1;1 Knockout Line and the AtHKT1;1 Overexpressing Lines

The primary site of Na\(^+\) toxicity in plants is in the leaves and shoots rather than in the roots (Munns, 2002). Na\(^+\) accumulates in the shoots due to a restricted recirculation back to the root, and increases osmotic stress to cells which can ultimately lead to a disruption of metabolic processes. However, recent studies reveal that shoot Na\(^+\) levels are not always correlated with tolerance in many Arabidopsis ecotypes. This is either due to tolerance despite the fact that Na\(^+\) is present at high levels in the shoots or due to intolerance despite a restriction of Na\(^+\) accumulation in the shoots. The first case motivates research into alternative mechanisms such as enhanced tissue tolerance and cannot be addressed in this study because none of the lines shows high shoot Na\(^+\) and high salinity tolerance. The second case raises the question of whether parallel mechanisms are needed to translate low shoot Na\(^+\) into salt tolerance, and can be addressed in this study using a comparative analysis between the different Arabidopsis AtHKT1;1 genotypes and their parental strains. A response observed in the overexpressing but not in the wild-type or knockout lines could be important for salt tolerance, and may include mechanisms that are responsible for coping with the increased osmotic potential, such as increasing the levels of osmotically active metabolites.

Shoot Na\(^+\) exclusion as engineered in E2586 UAS:HKT1;1 and J2731* UAS:HKT1;1 plants led to high Na\(^+\) in the roots after salt stress; by contrast, the AtHKT1;1 knockout mutant hkt1;1 had high Na\(^+\) in the shoots after salt stress (Møller et al., 2009; Tables 1 and 2). Thus, salt-induced changes in the ionome and metabolome of the AtHKT1;1 overexpressing lines (E2586 UAS:HKT1;1 and J2731* UAS:HKT1;1) were more pronounced in the roots than in the shoots (Figures 1 and 2, and Supplemental Tables 1 and 2), whereas salt-induced changes in the ionome and metabolome of the AtHKT1;1 knockout line hkt1;1 were more pronounced in the shoots than in the roots after salt treatment (Figure 4 and Supplemental Tables 1 and 2). The osmotic stress imposed was the same for E2586 UAS:HKT1;1 and J2731* UAS:HKT1;1 and, although these lines have a different basal AtHKT1;1 expression in shoots and roots, they differ only slightly in the amount of accumulated Na\(^+\) in these tissues (Møller et al., 2009). This is why we can assume that the shared metabolic changes represent a conserved response to salt-induced stress in lines overexpressing AtHKT1;1.

Increases of Amino Acids and Urea Levels after Salt Stress May Be Related to Tissue Damage rather than a Plant Response Associated with Tolerance

After 5 d of salt imposition, both E2586 UAS:HKT1;1 (although not statistically significant) and J2731* UAS:HKT1;1 plants accumulated lower levels of most amino acids including Lys, Tyr, and Gly in the shoots, compared to their parental lines (Figure 1). However, we found the opposite effect in the roots of both E2586 UAS:HKT1;1 and J2731* UAS:HKT1;1 plants, where most amino acids increased after salt treatment (Figure 2). In contrast, the AtHKT1;1 knockout line hkt1;1 showed significant increases of most amino acids, including Asn, Tyr, and Lys in the shoots after salt treatment, but significant decreases in some amino acids and ethanolamine, important for synthesis of the osmoprotectant glycinebetaine, in the roots compared to its parental line (Figure 3). Hence, it is striking that, independently of tissue type (roots or shoots) or plant line (AtHKT1;1 knockout or overexpressor), higher Na\(^+\) led to an increase in the levels of most amino acids. Apart from the AtHKT1;1 knockout, which accumulated urea in response to salt stress in both shoot and root tissue, accumulation of urea was also positively correlated with the accumulation of Na\(^+\) specifically in the roots of both E2586 UAS:HKT1;1 and J2731* UAS:HKT1;1 plants after salt treatment. It has been proposed that increased levels of amino acids might be related to an increased tissue damage and senescence in the non-tolerant lines (Roessner et al., 2006; Sanchez et al., 2008b; Widodo et al., 2009), an increase of protein degradation (Dubido et al., 1990), and an inhibition of protein synthesis (Silveira et al., 2001). Additionally, Hoai et al. (2003) found a positive correlation between ammonium, amino acids, and urea in salt-stressed rice seedlings, and suggested that accumulation of free amino acids and urea might be a result of ammonium assimilation induced by salinity stress. Since most amino acid levels were also significantly increased in both shoots (Supplemental Table 3) and roots (Supplemental Table 4) after salt treatment when looking at the differences in the metabolite fold changes between control and salt-treated samples of the same line, these findings...
suggest that elevated amino acid levels are a reaction to salt stress rather than a plant response associated with tolerance. In this study, the E2586 and J2731* parental (non-tolerant) lines exhibited higher amounts of Na\(^+\) and higher amino acid levels in their shoots than the AtHKT1;1 overexpressing (salt-tolerant) lines, and were more clearly affected by salt stress which resulted in a significant decrease (up to 37\%) in biomass (Møller et al., 2009). In contrast, the hkt1;1 knockout (salt-intolerant) line exhibited reduced shoot growth and tip senescence of mature leaves as well as higher shoot amino acid levels compared to its parental line gl1 (Rus et al., 2004). Furthermore, we found higher levels of Gin and Glu, known potential senescence markers (Cabello et al., 2006). These metabolites increased in both E2586 UAS:HKT1;1 and J2731* UAS:HKT1;1 roots, and in hkt1;1 shoots after salt stress. Although enhanced accumulation of Pro in plants under abiotic stresses, including salinity, is well documented, Pro changed to the same extend in the these lines after salt treatment when compared to their parental lines (Figure 2). In J2731* UAS:HKT1;1 roots, Pro was at lower levels than in the less salt-tolerant parental line J2731* after salt stress. In a salinity study in Limonium latifolium, Gagneul et al. (2007) reach a similar conclusion and found the contribution of Pro to osmotic pressure being relatively small, whereas free sugars, cyclitols, and organic acids appeared to be the major osmotically active compounds.

**Altering Shoot and Root Na\(^+\) Accumulation Alters Metabolites Involved in the TCA Cycle and Sugar Metabolism in Plants Growing under High-Salt Conditions**

There was a considerable difference in the levels of organic acids within the two AtHKT1;1 overexpressers and between the AtHKT1;1 overexpressers and the AtHKT1;1 knockout following salt stress. In E2586 UAS:HKT1;1 roots after 5 d of salt stress, the levels of several metabolites of the TCA cycle including succinate, citrate, aconitate, fumarate, 2-oxoglutarate, and malate decreased markedly; however, J2731* UAS:HKT1;1 showed marginal increases in citrate, malate, shikimate, 2-oxoglutarate, and succinate, along with significant decreases in citrate and fumarate. In contrast, hkt1;1 showed strong increases in the levels of TCA cycle intermediates in the shoots after salt treatment. Citrate, fumarate, and malate, however, remained unchanged. This coincided with a significant depletion of several sugars, including glucose and fructose, in hkt1;1 shoots after salt treatment, suggesting that there is an increased rate of carbon influx into the TCA cycle (e.g. glycolysis) at a constant rate of C-efflux from the cycle (e.g. as CO\(_2\) or usage of organic acids in other reactions, such as amino acid synthesis), which might be needed to support plant survival during salt stress.

The metabolite profile also reflects differences in the expression AtHKT1;1 levels of the two AtHKT1;1 overexpressing lines E2586 UAS:HKT1;1 and J2731* UAS:HKT1;1. As mentioned, line E2586 is in Col-0 background, and HKT1;1 overexpression resulted in a smaller reduction in shoot Na\(^+\) than in line J2731*, which is in C24 background, as previously observed by Jha et al. (2010). This is most likely due to the higher native expression level of AtHKT1;1 in Col-0 roots (+13-fold; Jha et al., 2010) compared to C24, which compensates the effect of the AtHKT1;1 overexpression. Although root sugars levels were increased after salt stress in both lines, E2586 UAS_gal-HKT1;1 showed higher accumulation of trehalose, gentiobiose, and melibiose in roots after salt stress compared to its parental control, whereas J2731* UAS:HKT1;1 showed higher levels of sucrose and raffinose than its parental line J2731*. When looking at the root metabolite ratio between control and salt-treated samples within the same line (E2586 UAS:HKT1;1 and J2731* UAS:HKT1;1, respectively), we find that almost all measured sugars increased more strongly in the AtHKT1;1 overexpressors than in their respective parental lines in response to salt stress. By contrast, most sugar levels increased less in the hkt1;1 knockout compared to control conditions than in gl1 after salt stress. These observed changes indicate that this could be derived from the role of AtHKT1;1 and is not just a symptom of sodium accumulation (Supplemental Table 3).

Trehalose, sucrose, raffinose, gentiobiose, and melibiose are known osmoprotectants and stress markers and are the focus of many publications related to drought, salinity, and cold stresses (Gouffii et al., 1999; for reviews, see Bartels and Sunkar, 2005; Vinocur and Altman, 2005). As an example, a fivefold increased trehalose level was found to be linked to an increase in biomass (20%) in salt-treated Lotus japonicus plants (López et al., 2006). Furthermore, myo-inositol, galactinol, melibiose, and raffinose are members of the raffinose oligosaccharide family known to be involved in stress responses and, as expected, accumulated only in the salt-tolerant AtHKT1;1 overexpressing lines, but not in the salt-sensitive AtHKT1;1 knockout after salt stress (Sanchez et al., 2008b; Lugan et al., 2009, 2010).

Strong correlations between sugar accumulation and osmotic stress tolerance have been reported in a variety of species including Lotus japonicus (Sanchez et al., 2008a), Arabidopsis and Thellungiella halophila (Gong et al., 2005; Kim et al., 2007), in the grasses Setaria sphacelata (Silva and Arrabac, 2004) and Hordeum vulgare (Widodo et al., 2009), in the shrub Limonium latifolium (Gagneul et al., 2007), and in the tree Populus euphratica (Brosche et al., 2005). Dubey and Singh (1999) have further demonstrated that salt-tolerant and sensitive genotypes of rice differ in the activities of the sugar metabolizing enzymes starch phosphorylase, sucrose phosphate synthase, and invertase. In this study, correlation analysis suggests a negative correlation between galactose and xylose with Na\(^+\) content in the shoots after salt treatment (Figure 5 and Table 4), which reflects the strong decrease of these sugars in the high shoot Na\(^+\) accumulating line hkt1;1, whereas they are increased in the low shoot Na\(^+\) accumulating lines E2586 UAS:HKT1;1 and J2731* UAS:HKT1;1. By contrast, we find a strong positive correlation of fructose in the roots,
since the fructose levels were significantly higher in E2586 and E2586 UAS:HKT1;1 than in hkt1;1 roots, which accumulated less Na\(^+\) in the roots after salt stress than the AtHKT1;1 overexpressing lines. This indicates that the AtHKT1;1 overexpressing lines had the ability to accumulate sugars in response to salt stress, in contrast to the AtHKT1;1 knockout line. This lack of important osmolytes may have contributed to the salt sensitivity of the hkt1;1 plants.

We found several stress-responsive unknown metabolites accumulating, particularly in E2586 UAS:HKT1;1 roots after salt stress. However, none of the unknown metabolites showed significant correlation with Na\(^+\). Further work is required to determine the structures of the unknown metabolites found in this study, possibly using complementary analytical technologies such as LC–MS or NMR.

The two AtHKT1;1 overexpressing lines, E2586 UAS:HKT1;1 and J2731* UAS:HKT1;1, displayed tolerance to high Na\(^+\) concentrations without apparent reduction in biomass when grown in 100 mM NaCl (Møller et al., 2009). Associated with high root Na\(^+\) levels present in these lines after salinity stress was an increase in sugars, sugar acids, and, for J2731* UAS:HKT1;1, also organic acids, which may either act as osmoprotectants or provide carbon sources to support plant survival during salt stress. Furthermore, high levels of amino acids found to accumulate after salt stress in the roots of these lines are possibly related to tissue damage. By contrast, the AtHKT1;1 knockout hkt1;1, which showed symptoms of necrosis and a reduction in biomass after salt stress (Rus et al., 2004), showed higher levels of amino acids in the shoot, but reduced levels of sugars and sugar alcohols. A large increase in organic acid levels in hkt1;1 shoots may reflect an increased rate of metabolism in response to the high amount of Na\(^+\) accumulating in these plants. Correlation analyses suggest strong correlations between Na\(^+\) and many sugars, sugar alcohols, and sugar acids. However, we were unable to find any evidence that Pro, the most highly studied osmoprotectant, was affected by salinity when comparing the different Arabidopsis AtHKT1;1 genotypes directly with their parental strains. Therefore, we conclude that metabolic differences between AtHKT1;1 overexpressors and knockout lines following salt stress involve metabolites involved in carbon metabolism and in the TCA cycle, pointing to these pathways as potential targets for engineering of salt-tolerant plants.

**METHODS**

**Plant Material and Growth Conditions**

Seeds of the Arabidopsis thaliana ecotypes C24 and Col-0 were obtained from the Arabidopsis Stock Centre (Nottingham, UK). As described by Møller et al. (2009), J2731* was a single-insert plant with mGFP5-ER expression in the root pericycle and isolated from a collection of J2731* GAL4-VP16 mGFP5-ER enhancer trap lines generated by root transformation of C24 with the enhancer trap plasmid pET-15 (Haseloff, 1999). E2586 was obtained from Scott Poethig (University of Pennsylvania, Philadelphia, USA) from a collection of GAL4-VP16 mGFP5-ER enhancer trap lines generated by floral dip transformation of Col-0 with pET-15.

Plants were grown in a hydroponics solution as described by Møller et al. (2009); in short, sterilized seeds were sown on 0.8% (w/v) agar in 1.5-ml centrifuge tubes and vernalized for 2 d at 4°C. The plants were grown randomly in aerated solution consisting of 1.25 mM KNO\(_3\), 0.625 mM KH\(_2\)PO\(_4\), 0.5 mM MgSO\(_4\), 0.5 mM Ca(NO\(_3\))\(_2\), and 0.045 mM FeNaEDTA with the following micronutrients: 0.16 \(\mu\)M CuSO\(_4\), 0.38 \(\mu\)M ZnSO\(_4\) 1.8 \(\mu\)M MnSO\(_4\), 45 \(\mu\)M H\(_2\)BO\(_3\), 0.015 \(\mu\)M (NH\(_4\))\(_2\)MoO\(_4\), and 0.01 \(\mu\)M CoCl\(_2\), with a 10-h light/14-h dark photoperiod, a constant temperature of 21°C, and an irradiance of 75 \(\mu\)mol m\(^{-2}\) s\(^{-1}\). The pH of the hydroponic solution was monitored and maintained at pH 5.7. Salt stress was applied 5 weeks after seed germination by the addition of 100 mM NaCl in 12 hourly increments of 25 mM. Calcium activity in the growth medium was maintained at each salt application by addition of the correct amount of calcium, as calculated using Visual Minteq Version 2.3 (US Environmental Protection Agency, USA).

**Ionomne Profiling Using ICP–OES Analysis**

Plants were harvested after 5 d of salt treatment. Whole roots and shoots of control and salt-treated plants were excised separately. Plant material was dried at 65°C for 2 d and dry weight was recorded. Samples were digested in 70% nitric acid and 30% hydrogen peroxide (both GR Grade; Merck) for 2.5 h at 120°C in a hot block (Environmental Express, Mt Pleasant, South Carolina, USA). The elemental profile of plant samples was measured using an inductively coupled plasma–optical emission spectrometer (ICP–OES; ARL 3580 B, Appl. Res Lab. SA, Ecublens, Switzerland). Acid digestion and ICP–OES analysis were performed by Waite Analytical Services (University of Adelaide, Urrbrae, South Australia).

**Chemicals for Metabolite Profiling**

N-methyl-N-(trimethylsilyl)-trifluoroacetamide and N-methyl-N-(tert-butylmethylsilyl)-trifluoroacetamide were purchased from Grace. HPLC-grade methanol was obtained from Scharlau. All other chemicals were purchased from Sigma-Aldrich.

**Extraction and Derivatization**

A modified method for the preparation of plant extracts has been developed based on the method described by Jacobs et al. (2007). For each line, 60 mg of root and 30 mg of shoot tissue were weighted into cryo mill tubes (PrecelIytes lising kit, Bertin Technologies), and 0.5 ml 100% methanol was added to the plant sample. Homogenization was performed for 30 s at 6000 rpm using a cryo mill (PrecelIyces 24, Bertin Technologies). After addition of 20 \(\mu\)l internal standard solution (20 \(\mu\)l per sample from a stock solution containing 1 mg ml\(^{-1}\) 13C ribitol, 1 mg ml\(^{-1}\) 13C norleucine in water), samples were extracted for 15 min at 70°C in a thermomixer at 750 rpm. Subsequently, the sample was mixed vigorously with 1 vol. of water, and
then centrifuged for 10 min at 13 000 rpm. The supernatant was transferred into a new reaction tube, and 70-μl aliquots were transferred into glass vial inserts and dried in vacuo for further TBS (tri-tert-butyldimethylsilyl) derivatization. The remaining supernatant was purged of nonpolar metabolites by adding 100 μl chloroform, and the polar and nonpolar phases were separated by centrifugation for 10 min at 13 000 rpm. The upper polar phase was transferred into a new tube and additional 100 μl chloroform was added to the sample, which was then centrifuged again for 10 min at 13 000 rpm. For further TMS (trimethylsilyl) derivatization, 90-μl aliquots of the upper polar phase were transferred into glass vial inserts and dried in vacuo.

**GC–MS Analysis**

The GC–MS system comprised a 7890A Agilent gas chromatograph and a 5975C Agilent Triple-Axis, quadrupole, mass selective detector (Agilent, Santa Clara, USA). A Gerstel MPS2XL GC–MS autosampler performed the derivatization procedure immediately prior to injection. The samples and the derivatization reagents were added to a glass vial and then placed in the autosampler tray. The autosampler then mixed sample with derivatization reagents automatically using the following program: plant extracts were derivatized for 120 min at 37°C using 10 μl methoxyamine hydrochloride (30 μl of 30 mg ml⁻¹ in pyridine) per sample. This was followed by trimethylsilylation with 20 μl N-Methyl-N-(trimethylsilyl)-trifluoroacetamide (TMS reagent) or 20 μl N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (TBS reagent) per sample for 30 min. Finally, 2 μl retention time standard mixture (0.029% (v/v) n-dodecane, n-pentadecane, n-nona-decane, n-docosane, n-octacosane, n-dotriacontane, n-hexatriacontane dissolved in pyridine) was added per sample prior to injection onto the GC column.

One μl of either TMS or TBS derivatized sample was injected onto the GC column using a hot needle technique. The injector was operated in splitless mode, isothermally at 250°C. Helium was used as the carrier gas with a flow rate of 0.8 ml min⁻¹. Chromatographic separation was performed on a 30-m VF-5MS column (with 10-m Integra guard column, i.d. 0.25 mm, 0.25-nm film thickness (Varian, Inc., Victoria, Australia)). The MS transfer line to the quadrupole was fixed at 280°C, the EI ion source at 250°C, and the MS quadrupole at 150°C. The mass spectrometer was tuned according to the manufacturer’s protocols using tris-(perfluorobutyl)-amine (CF43). The analysis was performed under the following oven temperature program: the injection temperature was set at 70°C for TMS and 100°C for TBS-derivatized samples, respectively, followed by a 12.5°C min⁻¹ oven temperature gradient to a final 325°C, and then held for 3.6 min at 325°C. The GC–MS system was then temperature-equilibrated for 1 min at 70°C prior to injection of the next sample. Ions were generated by a 70-eV electron beam at an ionization current of 2.0 mA and spectra were recorded at 2.91 scans per second with a mass-to-charge ratio of 50–550 atomic mass units (amu) scanning range. Retention time locking of the chromatographic peak of mannitol prior to the sample run ensured repeatable retention times across systems regardless of operator, detector type, and column maintenance.

**Data Handling, Mining, and Statistics**

Analytes were semi-quantified after mass spectral deconvolution (AnalyzerPro, SpectralWorks, Runcorn, UK). The chemical identification was manually supervised using the public domain mass spectra library of Max-Planck-Institute for Molecular Plant Physiology, Golm, Germany (http://csdb.mpimp-golm.mpg.de/csdb/gmd/gmd.html) and the in-house Metabolomics Australia mass spectral library. All matching mass spectra were additionally verified by analysis of reference standard compounds. Relative response ratios were calculated using the metabolite peak area divided by both the peak area of the internal standard (ribitol for TMS derivatization, norleucine for TBS derivatization) and the sample dry weight (g), as described by Roessner et al. (2001). The response ratio for each metabolite after salt stress (100 mM NaCl for 5 d) was first centered on the control (3 mM NaCl) of the same line. Then, fold changes were calculated by dividing the response ratios of the mutant lines (E2586 UAS:HKT1;1, J2731* UAS:HKT1;1, and hkt1;1) by the response ratios of the parental strains (E2586, J2731*, and gl1, respectively). Statistical analysis was performed using Excel (Microsoft, www.microsoft.com) and the R statistical software package (R version 2.13, www.r-project.org) after log₁₀ transformation. Differences between samples were validated by the Student’s t-test. The experimental data were mapped on an author-created metabolite network of the primary metabolism via the built-in graph editor in VANTED (Junker et al., 2006). For the *Arabidopsis* lines Col-0, E2586, E2586 UAS:HKT1;1, and hkt1;1, a non-parametric Spearman’s rank correlation analysis between the Na⁺ content and (1) the metabolite response ratios and (2) the other elemental absolute concentrations was performed in VANTED to estimate their statistical dependence. We use Spearman’s rank correlation as a non-parametric alternative to correlation, since the metabolite data do not meet the assumptions about normality, homoscedasticity, and linearity. The Spearman’s rank correlation coefficient (ρ) was calculated from the ranked metabolite response rations and element concentrations as the Pearson correlation coefficient between the ranked variables. Values are ranging from +1 to −1, with ρ > 0 indicating a positive correlation, ρ < 0 indicating a negative correlation, and ρ = 0 indicating no correlation. The significance of the correlation is checked with an approximation to the Student t-test distribution (P < 0.05).

**SUPPLEMENTARY DATA**

Supplementary Data are available at Molecular Plant Online.
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